Transcriptome response to different carbon sources in *Acetobacter aceti*

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The draft genome sequence of *Acetobacter aceti* NBRC 14818 was determined by whole-genome shotgun sequencing and the transcriptome profile in cells exponentially grown on ethanol, acetate or glucose was analysed by using a DNA microarray. The genes for all enzymes that constitute the complete tricarboxylic acid (TCA) cycle and glyoxylate pathway were identified in the genome. The TCA cycle genes showed higher expression levels in *A. aceti* cells grown on acetate or glucose and the glyoxylate pathway genes were significantly induced by ethanol or acetate. Many SOS-response genes were upregulated in cells grown on ethanol, indicating that ethanol provoked damage of DNA and proteins. The superoxide dismutase and catalase genes showed high expression levels in culture on glucose, indicating that oxidation of glucose induced oxidative stress. *A. aceti* NBRC 14818 was found to have a highly branched respiratory chain. The genes for two type I and one type II NADH dehydrogenase were identified. The genes for one of the type I enzymes were highly expressed when cells were grown on acetate or glucose, but were significantly downregulated in culture on ethanol, probably because ubiquinones were directly reduced by pyrroloquinoline quinone-dependent alcohol dehydrogenase. Four sets of the genes for quinol oxidases, one bo3-type (BO3), one bd-type and two cyanide-insensitive-types (CIOs), were identified in the genome. The genes for BO3, which might have proton-pumping activity, were highly expressed under the conditions tested, but were downregulated in the glucose culture. In contrast, the genes for one of the CIOs were significantly upregulated in cells grown on glucose. The two CIOs, which are expected to have lower energy-coupling efficiency, seemed to have a higher contribution in glucose-grown cells. These results indicate that energy conservation efficiency is fine-tuned by changing the respiratory components according to the growth conditions in *A. aceti* cells.

**INTRODUCTION**

Acetic acid bacteria are obligately aerobic alphaproteobacteria that have a unique ability to incompletely oxidize various alcohols and sugars to organic acids (Asai, 1968). The ability of these bacteria to incompletely oxidize ethanol to acetate has been historically utilized for vinegar production. Recently, acetic acid bacteria have come to be utilized as living biocatalysts for many biotechnological processes, such as production of L-sorbose for vitamin C synthesis and production of 6-amino-L-sorbose for synthesis of the anti-diabetic drug miglitol (Deppenmeier et al., 2002; Gupta et al., 2001). On the other hand, they are also known to cause spoilage of alcoholic beverages. Thus, the incomplete oxidation of acetic acid bacteria is industrially important.

The incomplete oxidation is catalysed by membrane-bound pyrroloquinoline quinone (PQQ)-dependent dehydrogenases, which are connected to the respiratory chain by reduction of ubiquinone to ubiquinol. Reoxidation of ubiquinol is coupled to oxygen reduction by quinol oxidases (Yakushi & Matsushita, 2010). Therefore, when alcohols and sugars are oxidized to organic acids in the periplasm by PQQ-dependent dehydrogenases, ATP is generated by oxidative phosphorylation. In many cases, the organic acid products are almost stoichiometrically

**Abbreviations:** ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; BO3, bo3-type quinol oxidase; CDSs, protein-coding sequences; CIO, cyanide-insensitive oxidase; EMP, Embden–Meyerhof–Parnas; PPP, pentose-phosphate pathway; POQ, pyrroloquinoline quinone; SCACT, succinyl-CoA : acetate CoA transferase; TCA, tricarboxylic acid.

The GenBank/EMBL/DDBJ accession numbers for the contig sequences of the draft genome of *Acetobacter aceti* NBRC 14818 are BABW01000001–BABW01001488.


A supplementary table showing the expression profiles of genes specifically regulated by different carbon sources in *A. aceti* NBRC 14818 is available with the online version of this paper.
accumulated as dead-end metabolites and are not further utilized as carbon sources for assimilation into cell materials. However, in some cases, the alcohols and sugars, such as ethanol and glucose, are simultaneously oxidized by both the membrane-bound PQQ-dependent dehydrogenases in the periplasm and the soluble dehydrogenases in the cytoplasm. In these cases, the substrates are utilized for both direct energy generation and carbon assimilation. When Acetobacter and most Gluconacetobacter species are grown on ethanol, they temporarily accumulate acetate by incomplete oxidation of ethanol. However, after ethanol is consumed, they completely oxidize the accumulated acetate. This phenomenon is called acetate overoxidation and is unfavourable for vinegar production (Saeki et al., 1997). Acetate overoxidation is reported to be caused by increased activity of the tricarboxylic acid (TCA) cycle enzymes and acetyl-CoA synthetase (Saeki et al., 1999). The mechanism of switching between incomplete oxidation and assimilatory oxidation and the control of energy and carbon metabolism in acetic acid bacteria are not fully understood. To understand the physiology and molecular biology of acetic acid bacteria better, we determined the draft genome sequence of Acetobacter aceti NBRC 14818, which is the type strain of the genus. Based on this draft genome sequence, a DNA microarray was constructed and the transcriptome profiles in A. aceti cells grown on ethanol, acetate or glucose were investigated and compared.

**METHODS**

**Bacterial strain and growth media.** A. aceti NBRC 14818 (formerly IFO 14818) was used for genome sequencing and microarray analysis. The bacterium was grown in basal medium, which consists of only 1% yeast extract, at 30°C. When necessary, the basal medium was supplemented with 1% ethanol (ethanol medium), 1% sodium acetate (acetate medium), 3.6% glucose (glucose medium) or 1% ethanol and 3.6% glucose (mixed medium).

**Genome sequencing and analysis.** Genomic DNA was extracted from A. aceti NBRC 14818 according to the method of Marmur (1961). The sequence of the genome was determined by using an Illumina Genome Analyser II (Illumina) as a custom service of TaKaRa Bio. A dataset of 8,463,177 reads of 36 bp sequences was obtained and 6,140,988 of these reads were assembled into 1,448 contigs with a mean length of 2,404 bp by using Edena version 2.1.1 software (Hernandez et al., 2008). The total length of the contigs was 3,577,688 bp with a mean G+C content of 57.3 mol%. Because the genome was sequenced to an average depth of 61.8-fold and the total length was comparable with those of the previously reported total genome sequences of members of the family Acetobacteraceae (Azuma et al., 2009; Bertalan et al., 2009; Greenberg et al., 2007; Prust et al., 2005), we assumed that nearly the entire sequence of the genome was obtained, and thus the sequence was used for further analysis. Protein-coding sequences (CDSs) were predicted via a combination obtained, and thus the sequence was used for further analysis. When Acetobacter and most Gluconacetobacter species are grown on ethanol, they temporarily accumulate acetate by incomplete oxidation of ethanol. However, after ethanol is consumed, they completely oxidize the accumulated acetate. This phenomenon is called acetate overoxidation and is unfavourable for vinegar production (Saeki et al., 1997). Acetate overoxidation is reported to be caused by increased activity of the tricarboxylic acid (TCA) cycle enzymes and acetyl-CoA synthetase (Saeki et al., 1999). The mechanism of switching between incomplete oxidation and assimilatory oxidation and the control of energy and carbon metabolism in acetic acid bacteria are not fully understood. To understand the physiology and molecular biology of acetic acid bacteria better, we determined the draft genome sequence of Acetobacter aceti NBRC 14818, which is the type strain of the genus. Based on this draft genome sequence, a DNA microarray was constructed and the transcriptome profiles in A. aceti cells grown on ethanol, acetate or glucose were investigated and compared.

**RNA extraction.** A. aceti NBRC 14818 was grown aerobically in 100 ml of the ethanol, acetate or mixed medium in a 300 ml Erlenmeyer flask with rotary shaking at 150 r.p.m. The experiment was performed in duplicate independent cultures. When the OD600 reached approximately 0.30–0.48, a 10–30 ml aliquot of the culture was mixed with 2 vols RNA protect (Qiagen) to stabilize total RNA. Cells were collected by centrifugation for 10 min at 3000 g. RNA was extracted from the cell pellet by a hot-phenol method as described below. The cell pellet was suspended in 800 μl RNase-free Tris/EDTA buffer (pH 8.0) containing lysosome (1 mg ml−1; Wako). The suspension was mixed with 80 μl 10% SDS and incubated at 65°C for 1–2 min. The solution was mixed with 88 μl 1 M sodium acetate (pH 5.2) and 1 ml water-saturated acidic phenol. The mixture was incubated at 65°C for 6 min with inversion every 1 min. The sample was then centrifuged at 20,400 g for 10 min at 4°C. The aqueous layer was mixed with an equal volume of phenol/chloroform/isomylalcohol (25:24:1) and centrifuged at 20,400 g for 10 min at 4°C. The aqueous layer was then mixed with an equal volume of chloroform/isomylalcohol (24:1) and centrifuged at 20,400 g for 10 min at 4°C. The aqueous layer was then mixed with a 1/10 volume of 3 M sodium acetate (pH 5.2), a 1/10 vol. of 1 mM EDTA and 2–2.5 vols of ice-cold ethanol. After incubation for more than 30 min at −80°C, the solution was centrifuged at 20,400 g for 30 min at 4°C and the ethanol was removed. The pellet was washed twice with ice-cold 80% ethanol. After centrifugation at 20,400 g for 5 min at 4°C, the ethanol was completely removed and the pellet was air-dried. The pellet was suspended in RNase-free water and subjected to RNase-free DNase treatment (RQ1 DNase; Promega). The treated sample was purified with an RNasy Mini kit (Qiagen) according to the manufacturer’s instructions. The RNase-free DNase treatment and subsequent purification step were repeated twice. All RNA samples were tested for genomic DNA contamination by 30 cycles of PCR amplification of a 98 bp partial fragment of the dnaA gene by using 5 μg of the RNA samples as templates.

**Microarray experiments and data analysis.** A customized gene expression array with a 4 × 72 k format for A. aceti NBRC 14818 was designed and manufactured by Roche NimbleGen based on the draft genome sequence. Each quadrant of the array contained two replicates of 3643 probe sets, which consisted of 3149 CDSs predicted by CRITICA and Glimmer, 26 connected ORFs and 468 contig sequences that were more than 100 bp in length but did not carry any predicted CDSs. Each probe set contained approximately nine probes of about 60 nt in length. Control probes were also included to ensure that there was no intra-quadrant contamination during the hybridization process.

Biological duplicate RNA samples isolated from cells grown in ethanol, acetate, glucose and mixed media were used for the microarray analysis. Double-stranded (ds)-cDNA synthesis and labelling were performed according to the instructions in the NimbleGen arrays user’s guide for gene expression analysis (Roche NimbleGen). ds-cDNA was synthesized from 10 μg total RNA, which was primed with random hexamer primer (Invitrogen) by using a superscript double-stranded cDNA synthesis kit (Invitrogen). The ds-cDNA pellet was dissolved in 20 μl RNase-free water. It was confirmed that the ds-cDNA samples had a concentration ≥100 ng μl−1 and an A260/A280 ratio ≥1.8. A one-colour DNA labelling kit (Roche NimbleGen) was used for Cy3 labelling of the ds-cDNA. A hybridization solution containing 2 μg Cy3-labelled ds-cDNA, sample tracking control solution, 2 × hybridization buffer, hybridization component A and alignment oligo was prepared with a hybridization kit (Roche NimbleGen) according to the protocol supplied by the
RESULTS AND DISCUSSION

Central carbon metabolic pathway

Genome sequencing and annotation analysis of *A. aceti* NBRC 14818 revealed the central carbon metabolic pathway of this bacterium (Fig. 1). The genome carried all genes for the enzymes catalysing the eight steps of the TCA cycle. The gene for malate dehydrogenase was not identified in the draft genome, but oxidation of malate to oxaloacetate could be catalysed by malate:quinone oxidoreductase encoded by *mpq*, as previously reported in *A. aceti* 1023 (Mullins et al., 2008). The *aceA* and *gbc* genes encoding isocitrate lyase and malate synthase, respectively, which constitute the glyoxylate pathway, were found to be clustered in the genome. These gene organizations for the TCA cycle and the glyoxylate pathway enzymes were in contrast with those of the closely related bacteria *Acetobacter pasteurianus* NBRC 3283 (formerly *A. IFO* 3283) and *A. aceti* 1023 (Azuma et al., 2009; Mullins et al., 2008). These bacteria were found not to carry the genes for succinyl-CoA synthetase and the glyoxylate pathway enzymes. Succinyl-CoA synthetase activity was reported to be bypassed by succinyl-CoA:acetate CoA transferase (SCACT) encoded by *aarC* in *A. aceti* 1023 (Mullins et al., 2008). The *aarC* gene was also found in the genome of *A. aceti* NBRC 14818.

The genes for the gluconeogenetic enzymes of the Embden–Meyerhof–Parnas (EMP) pathway were identified. However, the gene for phosphofructokinase was not found. All genes for the pentose-phosphate pathway (PPP) enzymes were identified in the genome, suggesting that degradation of glucose proceeds via PPP in *A. aceti* NBRC 14818. Glucose was also expected to be oxidized to gluconate by both soluble and membrane-bound dehydrogenases (Fig. 1). It is not certain whether the Entner–Doudoroff pathway is functioning, because the gene for 2-keto-3-deoxyglucose phosphate aldolase was not identified in this study.

The genes for the three-subunit quinoprotein (PQQ) alcohol dehydrogenase (ADH) were identified in the genome. The largest subunit gene (*adhA*) and the cytochrome *c* subunit gene (*adhB*) were clustered, but the smallest subunit gene (*adhC*) was separated as in the cases of other acetic acid bacteria (Kondo & Horinouchi, 1997; Tamaki et al., 1991). The *adh1* and *adh2* genes for soluble NAD*- dependent ADHs corresponding to ADH I and ADH II of *A. pasteurianus* SKU1108, respectively (Chinnawirotpisarn et al., 2003), were also identified. They were expected to be involved in oxidation of ethanol in the cytoplasm. Two putative acetyl-CoA synthetase genes (*acs1* and *acs2*) were identified. The genes for pyruvate oxidase, phosphate acetyltransferase and acetate kinase, which were identified in the *A. pasteurianus* NBRC 3283 genome (Azuma et al., 2009), were not found in the draft genome. The acetyl-CoA synthetase and SCACT described above are likely to be the initial routes for acetate assimilation.

Transcriptional regulation of the central carbon metabolism genes

A custom gene expression microarray for *A. aceti* NBRC 14818 was designed according to the draft genome sequence and was used to determine gene expression levels in cells grown on ethanol, acetate, glucose or a mix of ethanol and glucose. Microarray experiments were performed for two biological replicates for each growth condition. The genes (probe sets) that showed twofold or greater difference in expression level with 95% confidence were considered significant. All microarray data associated with this publication are available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE24361.

Quantitative analysis of ethanol, glucose, acetate and gluconate. Ethanol, acetate, D-glucose and D-gluconate in culture medium were measured enzymically with an F-kit (Roche) according to the manufacturer’s instructions.
succinyl-CoA synthetase (sucCD), succinate dehydrogenase (sdhABCD), two types of fumarate hydratases (fumA and fumC) and malate:quinone oxidoreductase (mqo) — showed higher expression levels in cells grown on acetate or glucose than in cultures on ethanol or the mix of ethanol and glucose (Supplementary Table S1). The expression profile of acnA is shown as a representative in Fig. 3(a). Upregulation of aconitase by acetate has been reported previously and a high copy number of aconitase is considered to be involved in tolerance to acetate (Nakano et al., 2004). The aarC gene for SCACT, which is also known to be involved in tolerance to acetate (Mullins et al., 2008), was also upregulated when A. aceti was grown on acetate or glucose (Fig. 3b). These results strongly suggest that the metabolic flow through the TCA cycle is higher in cells grown on acetate or glucose. The high activities of the TCA cycle enzymes and SCACT might be important to avoid accumulation of toxic acetate in the cytoplasm. It was not certain why aarC was upregulated in the culture grown on glucose. A possible explanation is that acetate may have been synthesized in the cytoplasm during cell growth on glucose by the overflow metabolism as in the case of Escherichia coli (el-Mansi & Holms, 1989). Accumulation of acetate from glucose was recently reported in Gluconobacter oxydans mutants that were deficient in glucose dehydrogenase genes (Krajewski et al., 2010). It was proposed that acetate was formed from pyruvate via pyruvate decarboxylase and NADP+-dependent ALDH, because ALDH activity was high in these mutants. In the present study, some genes encoding NAD(P)+-ALDH family enzymes were also significantly upregulated in A. aceti NBRC 14818 when the cells were grown on glucose (Supplementary Table S1).

Expression levels of the aceA and glcB genes encoding the glyoxylate pathway enzymes, isocitrate lyase and malate synthase, respectively, were very low in the culture on glucose, but were significantly upregulated by ethanol or acetate (Supplementary Table S1). The expression profile of aceA is shown in Fig. 3(c). The glyoxylate pathway is an anaplerotic shunt of the TCA cycle as it can be used to fill the TCA cycle intermediates (Kornberg & Krebs, 1957).
Transcriptome response to carbon sources in *A. aceti*

Because the TCA cycle intermediates are utilized as precursors for synthesis of molecules such as amino acids, nucleotides and other cofactors, the supply of oxaloacetate is necessary to keep the TCA cycle going during cell culture on carbon sources that are metabolized through acetyl-CoA as an obligate intermediate. The upregulation of the glyoxylate pathway genes by ethanol or acetate suggested that the pathway was important for the supply of oxaloacetate in *A. aceti* NBRC 14818. The result also suggested that the ethanol carbon was partially assimilated even at the acetate-accumulating phase. The glyoxylate pathway genes also showed high expression levels when the cells were grown on the mix of ethanol and glucose, indicating that these genes are not subjected to catabolite repression by glucose even when oxaloacetate was provided from phosphoenolpyruvate. *A. aceti* 1023 and *A. pasteurianus* NBRC 3283 have been reported to have no glyoxylate pathway genes, although they can utilize ethanol or acetate as a carbon source (Azuma et al., 2009; Mullins et al., 2008). An alternative route to supply oxaloacetate might be functioning in these bacteria.

*A. aceti* NBRC 14818 has two putative acetyl-CoA synthetase genes (*acs1* and *acs2*). Interestingly, the *acs1* and *acs2* genes were significantly upregulated by ethanol and acetate, respectively (Fig. 3d, e), suggesting that the two isoymes share certain roles.

Ethanol is oxidized to acetic acid via acetaldehyde by sequential reactions of ADH and ALDH (Fig. 1). Unexpectedly, the genes encoding PQQ-ADH and two NAD+ -ADHs were downregulated in cells grown on ethanol (Fig. 3f–h), although it is known that PQQ-ADH activity is higher in cells grown on ethanol (Jucker & Ettlinger, 1985). Post-translational activation of PQQ-ADH is probably operative in the cells grown on ethanol. In contrast with the ADH genes, the genes for membrane-bound ALDH (*aldFGH*) were significantly upregulated during culture on ethanol (Fig. 3i). The activities of ADH and ALDH might be coordinately regulated in order to avoid accumulation of the toxic intermediate acetaldehyde. The membrane-bound ALDH activity was probably the rate-limiting step of ethanol oxidation in the periplasm.

Most of the genes for the EMP pathway enzymes were constitutively expressed or slightly downregulated when cells were grown on ethanol. The genes for fructose-1,6-bisphosphate aldolase (*fbAB*) and fructose-1,6-bisphosphatase (*glpX*) were found to be upregulated when cells were grown on acetate (Supplementary Table S1). The expression profile of *glpX* is shown in Fig. 3(j). The result might confirm that the EMP pathway is operative for gluconeogenesis. Most of the PPP genes were constitutively expressed or showed higher expression levels in the culture on acetate or glucose, as in the cases of the TCA cycle genes (Supplementary Table S1). The gene encoding a soluble NAD(P)+ -dependent glucose dehydrogenase (*gdh*), which might be involved in oxidation of glucose to gluconate in the cytoplasm, was upregulated more than twofold in cells grown on glucose (Fig. 3k), although the difference was not significant based on the filtering criterion due to high variation between the duplicate samples of the glucose-grown cells (Fig. 3k). The glucokinase gene (*glk*) was slightly upregulated in the culture on glucose, although the differences in expression levels were less than twofold (data not shown). The glucose 6-phosphate dehydrogenase gene (*zwf*) was significantly upregulated in cells grown on glucose (Supplementary Table S1). These results suggested that glucose was oxidized through PPP via either glucose 6-phosphate or gluconate (Fig. 1).

Two sets of the *pdh* genes encoding pyruvate dehydrogenases were identified in the genome. The *pdh* genes were significantly upregulated in the culture on glucose (Supplementary Table S1). The expression profile of one of the *pdh* genes, *pdhA1*, is shown in Fig. 3(l). The result indicates that pyruvate dehydrogenase is important for the supply of acetyl-CoA during cell culture on glucose. When cells were grown on the

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**Fig. 2.** Growth of *A. aceti* NBRC 14818 on ethanol (a), glucose (b) and the mix of ethanol and glucose (c). Data are representative of three independent cultures. •, OD$_{600}$; ■, ethanol concentration; □, acetate concentration; △, glucose concentration; ▲, gluconate concentration. The time points at which RNA samples for microarray experiments were extracted are indicated by small arrows.
mix of ethanol and glucose, acetyl-CoA might have been supplied by acetyl-CoA synthetases (Fig. 3d, e).

There are at least four putative membrane-bound PQQ-dependent sugar dehydrogenase genes in the genome. Two of them showed significant upregulation when cells were grown on glucose (Supplementary Table S1), although it is not certain which of them is involved in the accumulation of gluconate in the medium by the incomplete oxidation of glucose. When *A. aceti* NBRC 14818 was grown on glucose, glucose was almost stoichiometrically converted to gluconate, indicating that only a little of the glucose carbon was utilized for cell growth. This was in line with the low final optical density of the culture on glucose (Fig. 2b). When the strain was grown in medium containing both ethanol and glucose, the profiles of growth, ethanol consumption and acetate accumulation were similar to those when the strain was grown on ethanol (Fig. 2a, c). In contrast, glucose consumption and gluconate accumulation were significantly reduced compared with those in the culture on glucose (Fig. 2b, c). These results suggested that ethanol is preferred over glucose as a substrate of incomplete oxidation by the membrane-bound PQQ-dependent dehydrogenases. The final gluconate concentration was slightly higher in the mixed medium than in the glucose medium, suggesting that the coexistence of ethanol lowered the metabolic flow from glucose to pyruvate. This might explain why the pyruvate dehydrogenase genes and the many central carbon metabolic genes showed low expression levels when cells were grown in the mixed medium.

**Differential expression of the highly branched respiratory chain**

*A. aceti* is an obligately aerobic bacterium that uses the electron transport chain, with molecular oxygen as the terminal electron acceptor. The genome sequence of *A. aceti* NBRC 14818 showed that the respiratory chain of this bacterium is highly branched (Fig. 4). Microarray analysis revealed that the components of the respiratory chain were differentially regulated according to the carbon sources.
Three sets of the NADH dehydrogenase (complex I) genes were identified in the genome. Two of them, the *nuo1* and *nuo2* gene clusters, encode multi-component type (type I) enzymes and the other one, *ndh*, encodes a type II enzyme. The type I enzyme is known to have proton-pumping activity but the type II enzyme does not (Calhoun et al., 1993; Leif et al., 1995). The expression profiles of *nuoA1*, *nuoA2* and *ndh* are shown in Fig. 5(a–c). The *nuo2* genes showed relatively high expression levels. These results suggested that one of the type I NADH dehydrogenases encoded by the *nuo2* genes was the main enzyme functioning under the conditions tested. The *nuo2* genes were significantly downregulated in cells grown on ethanol (Supplementary Table S1 and Fig. 5b). The NADH regeneration activity might have been low in the cytoplasm at the acetate-accumulating phase in the culture on ethanol (Fig. 2a) because ethanol was mainly oxidized in the periplasm by PQQ-ADH, which is directly coupled to reduction of ubiquinone to ubiquinol (Yakushi & Matsushita, 2010) (Fig. 4).

Four sets of gene clusters encoding ubiquinol oxidases, *cyo*, *cyd*, *cio1* and *cio2*, were identified in the genome (Fig. 4). The *cyoABCD* genes encode a bo3-type quinol oxidase (BO3). The *cydAB* genes encode a bd-type quinol oxidase (CYD). The *cioA1B1* and *cioA2B2* genes encode cyanide-insensitive oxidases (CIO1 and CIO2). CIO is closely related to but phylogenetically distinct from CYDs (Cunningham et al., 1997). A recent analysis of *Gluconobacter oxydans* CIO revealed that the haem content of CIO is the same as that of CYD of *E. coli*, but the spectroscopic and ligand-binding properties of CIO are unique (Mogi et al., 2009).

BO3 is known to pump protons over the membrane with an H\(^+\)/2e\(^-\) stoichiometry of 4 (Puustinen et al., 1989; Wikström et al., 1994). The proton translocation activity of CIO has not yet been reported. *E. coli* has two CYDs, cytochrome *bd*-I and cytochrome *bd*-II, and their H\(^+\)/2e\(^-\) ratios were reported to be 2 and 0, respectively (Bekker et al., 2009; Puustinen et al., 1991). Because CIO is a close relative of CYD (Mogi et al., 2009), the H\(^+\)/2e\(^-\) ratio of CIO is expected to be lower than that of BO3. The low proton translocation activity of CIO was also indicated in *Gluconobacter suboxydans*, because the H\(^+\)/2e\(^-\) ratio of the mutant cells in which CIO activity was high was lower than that of the wild-type cells in which BO3 was the major oxidase (Soemphol et al., 2008).

The microarray expression profiles of the main subunit genes of the quinol oxidases, *cyoB*, *cydA*, *cioA1* and *cioA2*, are shown in Fig. 5(d–g). The expression profiles of the other subunit genes were similar to those of the corresponding main subunit genes (data not shown). The *cyo* genes showed high expression levels under the conditions tested. Although the enzyme activities of the gene products could not be directly compared by the microarray signal intensities, the result strongly suggested that BO3 was the main enzyme functioning under these conditions. Expression levels of the *cyo* genes were downregulated when the cells were grown on glucose. The *cyd* genes were expressed at low levels when the cells were grown on ethanol, glucose or the mix of ethanol and glucose, and significantly downregulated when the cells were grown on acetate. The *cio1* genes were constitutively expressed, although their expression levels were relatively lower. In contrast with the *cyo* genes, the *cio2* genes showed significant upregulation in the culture on glucose. CIO2 probably played an important role in the cells grown on glucose. The *cyd* and *cio2* genes were significantly repressed in the culture on acetate, suggesting that CYD and CIO2 were not required under this condition. The pH of the culture medium decreased when the cells were grown on ethanol, glucose or the mix of ethanol and glucose because of the accumulation of acetate and/or gluconate by the function of the PQQ-dependent dehydrogenases. In contrast, the pH did not decrease when sodium acetate was used as a carbon source (data not shown). CYD and/or CIO2 might be involved in the respiration in low pH environments. Induction of cyanide-insensitive quinol oxidase activity at low pH has also been reported in *Gluconobacter suboxydans* (Matsushita et al., 1989).

A gene encoding a putative subunit I of cytochrome *c* oxidase (coxA) was identified. Bacterial cytochrome *c* oxidase is generally composed of three major subunits, but the genes for subunits II and III were not found in the genome. Similarly, in the genomes of *A. pasteurianus* NBRC 3283 and *Gluconobacter oxydans* 621H, the gene
corresponding to coxA was found but no genes encoding subunits II and III of cytochrome c oxidase were detected (Azuma et al., 2009; Prust et al., 2005). It seems likely that cytochrome c oxidase is not functioning in these acetic acid bacteria. Conversely, the pathogenic acetic acid bacterium Granulibacter bethesdensis has all the genes for three subunits of putative cytochrome c oxidase (Greenberg et al., 2007). However, it is suspicious that cytochrome c oxidase is functioning in Granulibacter bethesdensis, because the genes for ubiquinol-cytochrome c oxidoreductase (cytochrome bc1 complex), which provides reduced cytochrome c as an electron donor for cytochrome c oxidase, were not identified in the genome. Gluconacetobacter diazotrophicus Pal5 also has the orphan coxA-like gene. In addition, the bacterium has a complete set of the genes for another putative cytochrome c oxidase (Bertalan et al., 2009).

Two sets of the genes for cytochrome bc1 complex (complex III) were identified in the genome. The petB1C1 genes encoding one of the cytochrome bc1 complexes were followed by the petA1 gene encoding an Fe–S protein. The petB2C2 cluster encoding another cytochrome bc1 complex was not accompanied by the Fe–S protein gene. The organization of these two pet gene clusters was the same as that in A. pasteurianus NBRC 3283 (Azuma et al., 2009). Gluconobacter oxydans 621H and Gluconacetobacter diazotrophicus Pal5 have only one set of the genes for the cytochrome bc1 complex (Bertalan et al., 2009; Prust et al., 2005). It remains unclear why A. aceti, A. pasteurianus and Gluconobacter oxydans have the genes for the cytochrome bc1 complex even though they do not have a functional cytochrome c oxidase. The coxA and pet2 genes were constitutively expressed under the conditions tested. The
pet1 genes were found to be significantly repressed in the cells grown on glucose (Fig. 5h–j).

One of the candidates that may receive electrons from cytochrome c is cytochrome c peroxidase encoded by ccpR (Fig. 4). However, the expression level of ccpR was very low under the conditions tested (Fig. 5k). The apo gene encoding quinol peroxidase was also identified. The apo gene is highly similar to ccpR, but the deduced amino acid sequence of apo has three haem c-binding motifs while that of ccpR has two motifs (Yamada et al., 2007). The expression level of apo was higher in cells grown on ethanol or the mix of ethanol and glucose, but significantly reduced in those grown on glucose (Fig. 5l). This expression profile is similar to that of pet1 (Fig. 5i).

### Other genes specifically regulated by different carbon sources

When A. aceti NBRC 14818 was grown on ethanol, some stress-responsive genes were found to be significantly upregulated (Supplementary Table S1). These genes included recA, recN, uvrABC, dinB, dnnA, dnaK, clpB, hslVU and htpG. The expression profiles of some of these genes are shown in Fig. 6(a–e). The recA gene encodes a recombinase A and the recN gene encodes a DNA repair protein. They are involved in the SOS response to DNA damage. The uvrABC genes encode an excinuclease that is also involved in DNA repair in the SOS system. The dinB gene encodes the damage-inducible, error-prone DNA polymerase IV. The dnaJ, dnaK, clpB, hslVU and htpG gene products are heat-shock proteins (molecular chaperones). The grpE gene encoding another heat-shock protein is located upstream of dnaK. The expression level of grpE in the ethanol medium was more than twofold higher than that in the acetate medium, but the difference was not significant based on the filtering criteria (data not shown). The upregulation of these stress-responsive genes when the cells were grown on ethanol indicates that the cells suffered damage to their DNA and proteins. This was probably because of the toxicity of acetaldehyde, which is an oxidation intermediate of ethanol and is known to induce DNA damage and protein denaturation. In A. pasteurianus NBRC 3283, it was reported that the grpE–dnaK–dnaJ genes were upregulated by ethanol and that the over-expression of these genes resulted in improved growth in the ethanol-containing medium (Ishikawa et al., 2010a; Okamoto-Kainuma et al., 2004). The clpB gene was also reported to be upregulated by ethanol in A. pasteurianus.

**Fig. 6.** Microarray expression profiles of the genes specifically regulated by carbon sources. The recA gene (a) encodes recombinase A; uvrA (b) encodes a subunit of excinuclease UvrABC; dinB (c) encodes DNA polymerase IV; dnaJ (d) and clpB (e) encode heat-shock proteins; sod (f) encodes superoxide dismutase; katE (g) encodes catalase; and flaC (h) and mcp1 (i) are the flagellar- and chemotaxis-related genes, respectively. White, pale grey, dark grey and black bars labelled E, A, G and M on the vertical axes indicate when the cells were grown on ethanol, acetate, glucose and the mix of ethanol and glucose, respectively. The mean signal value is the mean of the microarray expression signal values of each gene obtained from duplicate experiments. Error bars, SD.
NBRC 3283, although its role in ethanol tolerance is not certain (Ishikawa et al., 2010b).

When A. aceti NBRC 14818 was grown on glucose, the genes for superoxide dismutase (sod) and catalase (katE) showed high expression levels (Fig. 6f and g, and Supplementary Table S1). These gene products are known to be involved in the oxidative stress response. Peroxide was probably generated during the process of glucose degradation, although disruption of the katE gene of A. pasteurianus NBRC 3283 had no effect on the growth on glucose (Okamoto-Kainuma et al., 2008). Upregulation of the katE gene for catalase was in contrast to the downregulation of the apo gene for quinol peroxidase (Fig. 5l). Some genes encoding cation/multidrug efflux pumps were significantly upregulated when the cells were grown on glucose, although the reason for this is unknown (Supplementary Table S1).

At least 29 and 10 genes for flagellar- and chemotaxis-related genes, respectively, were identified in the genome. Most of these genes were found to be significantly upregulated when the cells were grown on glucose or the mix of ethanol and glucose (Supplementary Table S1). The expression profiles of the fliC and mcp1 genes are shown in Fig. 6(h and i) as representatives. The results suggested that the glucose-grown cells were highly motile. It seems likely that the major source of energy for motility was provided by oxidation of glucose.

Conclusion

An acetic acid bacterium A. aceti NBRC 14818T was revealed to have a complete set of the genes for the TCA cycle and glyoxylate pathway (Fig. 1). The contribution of the glyoxylate pathway to the utilization of ethanol or acetate as a carbon source was suggested by the results of the microarray experiments. Gluconacetobacter diazotrophicus Pal5T also has the complete TCA cycle and glyoxylate pathway, but in A. pasteurianus NBRC 3283 and Gluconobacter oxydans 621H, the TCA cycle is incomplete and the glyoxylate pathway is missing (Azuma et al., 2009; Bertalan et al., 2009; Prust et al., 2005). Thus, there seems to be diversity in the central carbon metabolic pathway in acetic acid bacteria.

A. aceti NBRC 14818 was shown to have a highly branched respiratory chain (Fig. 4). The genes for three NADH dehydrogenases, two cytochrome bc1 complexes and four quinol oxidases were identified in the genome. A. aceti, A. pasteurianus and Gluconobacter oxydans have the cytochrome bc1 complex but have only the subunit I gene for cytochrome c oxidase (Azuma et al., 2009; Prust et al., 2005). On the other hand, Granulibacter bethesdensis does not have the genes for the cytochrome bc1 complex but has all subunit I, II and III genes for cytochrome c oxidase (Greenberg et al., 2007). The complete combination of the functional cytochrome bc1 complex and cytochrome c oxidase might be detrimental for these acetic acid bacteria.

The components of the respiratory chain were found to be differentially regulated according to the carbon sources (Figs 4 and 5). One of the type I NADH dehydrogenases encoded by the nuo2 genes, which might have proton-pumping activity, was highly expressed in cells grown on acetate or glucose, but was significantly downregulated in cells grown on ethanol. Because ubiquinone was directly reduced by PQQ-ADH at the ethanol oxidation phase, the demand for NADH dehydrogenase might be low. The cyo gene encoding BO3 was highly expressed under the conditions tested but was downregulated in cells grown on glucose. Expression levels of the genes for the other three quinol oxidases were relatively lower. However, in cells grown on glucose, the total expression level of the cio1 and cio2 genes encoding CIOs became higher. These results suggested that BO3 was the major terminal oxidase but that the contribution of CIOs increased in cells grown on glucose. BO3 is known to have proton-pumping activity and CIO is thought to have lower energy-coupling efficiency (Puustinen et al., 1989; Soemphol et al., 2008). When grown on glucose, NADH could be sufficiently provided by oxidation of glucose, so a chemiosmotic gradient sufficient for cell growth would be formed by the proton-pumping activity of NADH dehydrogenase or ATP would be sufficiently provided by substrate-level phosphorylation. Therefore, the proton-pumping by terminal oxidases would be less important. When grown on ethanol, ubiquinol was mainly reduced by PQQ-ADH at the ethanol oxidation phase. Therefore, formation of the chemiosmotic gradient by BO3 would be more important. When grown on the more oxidized substrate acetate, proton-pumping by both NADH dehydrogenase and BO3 might be necessary for the formation of a sufficient chemiosmotic gradient. When grown on the mix of ethanol and glucose, the nuo2 genes were slightly downregulated and the cyo genes showed high expression levels, despite the fact that NADH was expected to be sufficiently provided by the oxidation of glucose. There may be some functional connection between PQQ-ADH and BO3, as suggested between PQQ-dependent glycerol dehydrogenase and BO3 in Gluconobacter frateurii (Soemphol et al., 2008). Another possibility is that the expression of these respiratory genes was regulated by the redox balance, such as the NADH/NAD+ ratio or the quinol/quinone ratio, rather than the ATP level. These speculations are based only on the gene expression profiles. Further biochemical analysis will be required to elucidate the function of the respiratory components.

REFERENCES


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