Journal of Bioscience and Bioengineering
VOL. 115 No. 1, 32–36, 2013

www.elsevier.com/locate/jbiosc

Role of the glyoxylate pathway in acetic acid production by *Acetobacter aceti*

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Received 7 June 2012; accepted 26 July 2012
Available online 16 August 2012

Wild-type *Acetobacter aceti* NBRC 14818 possesses genes encoding isocitrate lyase (*aceA*) and malate synthase (*glcB*), which constitute the glyoxylate pathway. In contrast, several acetic acid bacteria that are utilized for vinegar production lack these genes. Here, an *aceA*–*glcB* knockout mutant of NBRC 14818 was constructed and used for investigating the role of the glyoxylate pathway in acetate productivity. In medium containing ethanol as a carbon source, the mutant grew normally during ethanol oxidation to acetate, but exhibited slower growth than that of the wild-type strain as the accumulated acetate was oxidized. The mutant grew similarly to that of the wild-type strain in medium containing glucose as a carbon source, indicating that the glyoxylate pathway was not necessary for glucose utilization. However, in medium containing both ethanol and glucose, the mutant exhibited significantly poorer growth and lower glucose consumption compared to the wild-type strain. Notably, the mutant oxidized ethanol nearly stoichiometrically to acetate, which was retained in the medium for a longer period of time than the acetate produced by wild-type strain. The features of the *aceA*–*glcB* knockout mutant revealed here indicate that the lack of the glyoxylate pathway is advantageous for industrial vinegar production by *A. aceti*.

[**Key words:** Glyoxylate pathway; Acetate production; Ethanol; Incomplete oxidation; Knockout mutant; *Acetobacter aceti*]

Acetic acid bacteria are obligately aerobic α-proteobacteria that have the distinctive ability to produce organic acids by the incomplete oxidization of various alcohols and sugars (1). The incomplete oxidation reactions, which are catalyzed by membrane-bound pyrroloquinoline quinone (PQQ)-dependent dehydrogenases, are connected to the respiratory chain via quinones. The reduced quinones are reoxidized by terminal oxidases using molecular oxygen. Thus, acetic acid bacteria are able to produce energy by the incomplete oxidation of alcohols and sugars. *Acetobacter* and most *Gluconacetobacter* species produce acetate from ethanol and are predominantly utilized for vinegar production. However, after the consumption of ethanol, the produced acetate is occasionally completely oxidized. This phenomenon is termed acetate overoxidation and is unfavourable for vinegar production due to decreased yields.

Evidence suggests that acetate overoxidation is caused by the increased activities of tricarboxylic acid (TCA) cycle enzymes and acetyl-CoA synthetase (2,3). When carbon sources that are metabolized through acetyl-CoA as an obligate intermediate are utilized, the glyoxylate pathway, which consists of isocitrate lyase and malate synthase, generally functions as an anaplerotic shunt of the TCA cycle to supply oxaloacetate (4). Therefore, the glyoxylate pathway might have an important role in acetate overoxidation by acetic acid bacteria.

Genome analyses of wild-type *Acetobacter aceti* NBRC 14818 and *Gluconacetobacter diazotrophicus* Pal5 have shown that they both possess genes encoding isocitrate lyase (*aceA*) and malate synthase (*glcB*) (5,6). In contrast, these genes are missing in several acetic acid bacteria that are utilized for vinegar production, such as *Acetobacter pasteurianus* NBRC 3283 and *A. aceti* 1023 (7,8), suggesting a relationship exists between the glyoxylate pathway and acetate productivity.

In the present work, we constructed an *aceA*–*glcB* knockout mutant of *A. aceti* NBRC 14818 and examined the effect of the glyoxylate pathway deficiency on acetate production by *A. aceti*.

MATERIALS AND METHODS

Bacterial strains and growth media *A. aceti* NBRC 14818 (formerly IFO 14818) and its isogenic *aceA*–*glcB* mutant, designated strain GP, were used in this study. The strains were grown in basal medium (1% yeast extract) at 30°C with rotary shaking at 300 rpm. When necessary, the basal medium was supplemented with 200 mM ethanol (ethanol medium), 200 mM glucose (glucose medium), or 200 mM ethanol and 200 mM glucose (mixed medium).

Construction of an *aceA*–*glcB* knockout mutant of *A. aceti* NBRC 14818 The *aceA* and *glcB* genes, which are clustered in the genome of NBRC 14818 (5), were knocked out by homologous recombination using the plasmid pUC-aceAglcB, which was constructed as follows. Two 1.2-kb fragments carrying the upstream and downstream regions of *aceA* and *glcB* were amplified by PCR with the primer sets aceAglcB1 (ACCTCATGGTACCCTCCCTTCCATGTCG)-aceAglcB2 (ACCTTCGTCGACCCGGAAGAAGCCTTCC) and aceAglcB3 (ACCGGTCGACGTGATCGACGCCGCGATCC)-aceAglcB4 (GAACGCTGCAGGCTGGTCATCACAGGG), respectively, and tandemly inserted into the KpnI–SalI and SalI–PstI sites, respectively, of pUC18

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Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CFE, cell-free extract; DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; PQQ, pyrroloquinoline quinone.

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2012.07.017

(9). A tetracycline-resistance gene cassette, which was obtained as a SalI fragment from pPS854*tet* (10), was then cloned into the plasmid at the SalI site. The constructed plasmid, designated pUC-aceAglcB, was transformed into NBRC 14818 by electroporation, and *aceA–glcB* knockout mutants were then isolated on tetracycline-containing plates. One of the mutants was selected and designated strain GP. Mutation of the *aceA* and *glcB* genes was confirmed by PCR (data not shown) and by comparing the isocitrate lyase activities of strains NBRC 14818 and GP grown in mixed medium, which were 94.4 and 0.9 nmol/min/mg protein, respectively.

Preparation of cell-free extract Strains NBRC 14818 and GP were grown aerobically in 100 ml ethanol medium or mixed medium in a 300-ml Erlenmeyer flask at 30°C with rotary shaking at 150 rpm. When the optical density at 600 nm (OD_{600}) of the culture reached 0.3–0.5, bacterial cells were harvested by centrifugation at 10,200 $\times g$ for 10 min at 4°C and then washed twice with ice-cold 50 mM potassium phosphate buffer (KPB; pH 7.5). The cell pellet was resuspended in KPB at a rate of 1 g wet weight/4 ml buffer, and the cell suspension was then passed twice through a French pressure cell (American Instruments Company) at 16,000 psi. After removing intact cells by centrifugation at 5800 $\times g$ for 15 min at 4°C, the resulting supernatant was collected as cell-free extract (CFE).

Enzyme activity assays The enzymatic activities of ethanol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) were determined spectrophotometrically at 25°C, as described below (11–13). For the assay of membrane-bound PQQ-dependent dehydrogenases, phenazine methosulfate (PMS) coupled with 2,6-dichlorophenolindophenol (DCIP) was used as an artificial electron acceptor. The reaction mixture consisted of 16.67 mM Tris–HCl buffer (pH 8.75), 0.02 mM DCIP, 0.67 mM PMS, 1 mM potassium cyanide, 33.33 mM ethanol or acetaldehyde, and CFE in a total volume of 1 ml. The reaction was started by the addition of ethanol or acetaldehyde. Formation of reduced DCIP was measured by the absorbance decline at 600 nm using a millimolar extinction coefficient of 15.1. One unit of activity was defined as the amount of enzyme that reduced 1 nmol of DCIP per minute.

For the assay of cytoplasmic NAD^+ or $NADP^+$-dependent dehydrogenases, the reaction mixture consisted of 100 mM glycine–NaOH (pH 8.5), 100 mM ethanol or acetaldehyde, 0.1 mM NAD^+ or $NADP^+$, and CFE in a total volume of 1 ml. The reaction was started by the addition of CFE. Formation of NADH or NADPH was measured by the absorbance increase at 340 nm using a millimolar extinction coefficient of 6.22. One unit of activity was defined as the amount of enzyme that produced 1 nmol of NADH or NADPH per minute. The measurements were performed in duplicate using the same CFE sample. The protein concentration of the CFE was determined by the Bradford method using a protein assay kit (Bio-Rad). Bovine serum albumin was used as a standard.

RNA extraction Strains NBRC 14818 and GP were grown aerobically in 100 ml mixed medium in a 300-ml Erlenmeyer flask at 30°C with rotary shaking at 150 rpm. When the OD_{600} of the culture reached 0.3, a 30-ml aliquot of the culture was collected and used for RNA extraction, as described previously (5). The RNA extraction procedure was performed in biological duplicate using independent cultures.

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 of the strain (5). Each quadrant of the array contained 2 replicates of 3643 probe sets. Biological replicate RNA samples isolated from cells grown in mixed medium were used for the microarray experiments and data analysis, as described previously (5). All microarray data associated with this publication are available at the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE38524.

Quantitative analysis of ethanol, acetic acid, glucose, and gluconate The concentrations of ethanol, acetate, D-glucose, and D-gluconate in culture media were measured enzymatically using F-kits (Roche) according to the manufacturer's instructions.

RESULTS

Effect of glyoxylate pathway deficiency on growth and acetate productivity A knockout mutant of *A. aceti* NBRC 14818 lacking the *aceA* and *glcB* genes, encoding the glyoxylate pathway enzymes isocitrate lyase and malate synthase, respectively, was constructed and designated strain GP. The growth profiles and changes in the concentrations of ethanol, acetate, glucose, and gluconate when strains NBRC 14818 and GP were cultivated in medium containing ethanol, glucose, or both ethanol and glucose as carbon sources were investigated (Fig. 1). In the ethanol medium, strain GP initially grew at the same rate as the wild-type strain, but the growth was slower as the accumulated acetate was oxidized (Fig. 1A). The transition phase between the ethanol oxidation and acetate overoxidation phases was clearly longer for strain GP compared to NBRC 14818.

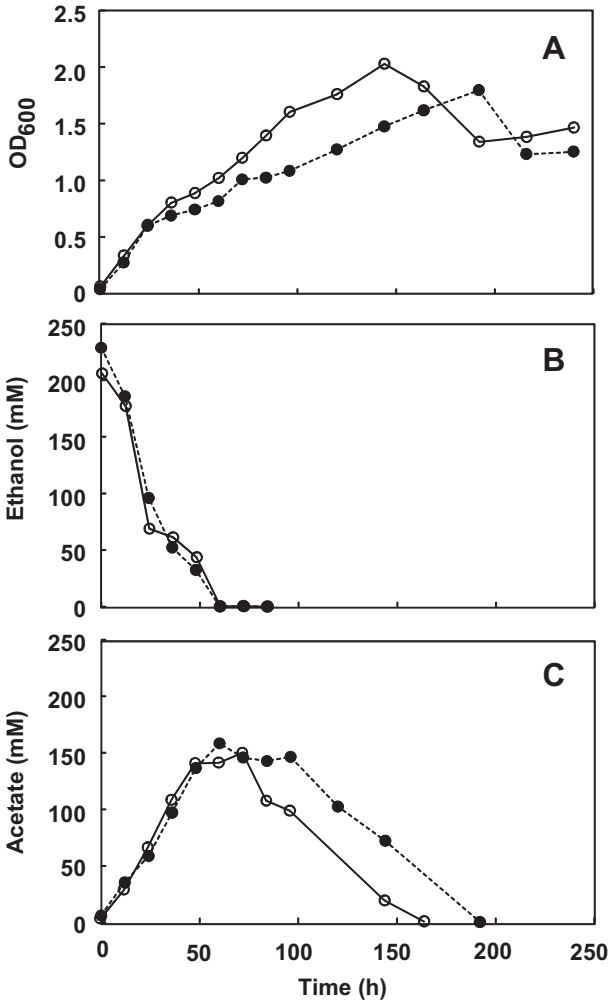

FIG. 1. Growth of *A. aceti* strains NBRC 14818 and GP in ethanol medium. (A) Growth profiles of the two strains. (B, C) Concentrations of ethanol (B) and acetate (C) in the culture medium. Data are representative of three independent experiments. Open and closed circles indicate strains NBRC 14818 and GP, respectively.

During the initial growth phase of both strains, ethanol was nearly stoichiometrically oxidized to acetate (Fig. 1B), indicating that most ethanol was utilized as a respiratory substrate for energy production and that the major carbon source was the yeast extract components in the medium. Although the growth rate of strain GP during the acetate overoxidation phase was lower than that of the wild-type strain, the two strains exhibited comparable acetate consumption rates (Fig. 1C), suggesting that the efficiency of acetate anabolism was adversely affected by the lack of the glyoxylate pathway.

In the glucose medium, disruption of the glyoxylate pathway genes had no effect on cell growth, glucose oxidation, or gluconate accumulation (Fig. 2). This result indicates that the glyoxylate pathway is unnecessary under these conditions, because TCA cycle intermediates could be provided through glycolysis via pyruvate or phosphoenolpyruvate. This finding is consistent with our previous report demonstrating that the *aceA* and *glcB* genes are expressed at low levels when NBRC 14818 is grown in glucose medium (5).

In the mixed medium containing ethanol and glucose, strain GP exhibited drastically poorer growth than that of wild-type strain (Fig. 3). Notably, the growth impairment of the mutant was more severe than that observed in the medium containing only ethanol