Mixotrophic and photoheterotrophic metabolism in *Cyanothece* sp. ATCC 51142 under continuous light

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The unicellular diazotrophic cyanobacterium *Cyanothece* sp. ATCC 51142 (*Cyanothece* 51142) is able to grow aerobically under nitrogen-fixing conditions with alternating light–dark cycles or continuous illumination. This study investigated the effects of carbon and nitrogen sources on *Cyanothece* 51142 metabolism via ¹³C-assisted metabolite analysis and biochemical measurements. Under continuous light (50 µmol photons m⁻² s⁻¹) and nitrogen-fixing conditions, we found that glycerol addition promoted aerobic biomass growth (by twofold) and nitrogenase-dependent hydrogen production [up to 25 µmol H₂ (mg chlorophyll)⁻¹ h⁻¹], but strongly reduced phototrophic CO₂ utilization. Under nitrogen-sufficient conditions, *Cyanothece* 51142 was able to metabolize glycerol photoheterotrophically, and the activity of light-dependent reactions (e.g. oxygen evolution) was not significantly reduced. In contrast, *Synechocystis* sp. PCC 6803 showed apparent mixotrophic metabolism under similar growth conditions. Isotopomer analysis also detected that *Cyanothece* 51142 was able to fix CO₂ via anaplerotic pathways, and to take up glucose and pyruvate for mixotrophic biomass synthesis.

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INTRODUCTION

Rising concerns about global warming due to the greenhouse effect have renewed research focused on the biological capture of CO₂. Cyanobacteria have versatile metabolic capabilities, which allow them to grow under autotrophic, heterotrophic and mixotrophic conditions (Bottomley & Van Baalen, 1978; Eiler, 2006; Yang *et al.*, 2002). More importantly, some cyanobacteria can capture solar energy to fix nitrogen and generate H₂, thereby serving as a source of biofertilizer and biofuel, while simultaneously consuming atmospheric CO₂ (Bernat *et al.*, 2009; Dutta *et al.*, 2005; Fay, 1992; Madamwar *et al.*, 2000; Tamagnini *et al.*, 2007; Tuli *et al.*, 1996). *Cyanothece* sp. ATCC 51142 (*Cyanothece* 51142), a unicellular diazotrophic cyanobacterium, is able to grow aerobically under nitrogen-fixing conditions and has been recognized as contributing to the marine nitrogen cycle. The recent sequencing of the *Cyanothece* 51142 genome and its

transcriptional analysis have uncovered the diurnally oscillatory metabolism of the bacterium in alternating light–dark cycles (photosynthesis during the day and nitrogen fixation at night) (Stöckel *et al.*, 2008; Toepel *et al.*, 2008; Welsh *et al.*, 2008). In general, cyanobacteria use spatial or temporal separation of oxygen-sensitive nitrogen fixation and oxygen-evolving photosynthesis as a strategy for diazotrophic growth (Benemann & Weare, 1974; Fay, 1992). Interestingly, *Cyanothece* 51142 demonstrates simultaneous N₂ fixation and O₂ evolution under continuous-light conditions, though it appears to be unicellular (Colon-Lopez *et al.*, 1997; Huang & Chow, 1986). For example, a recent study of the transcriptional and translational regulation of continuously illuminated *Cyanothece* has revealed a strong synthesis capability for nitrogenase and circadian expression of 10% of its genes (Toepel *et al.*, 2008). Furthermore, *Cyanothece* strains usually utilize exogenous carbon substrates for mixotrophic growth under light conditions and for heterotrophic growth under dark conditions (Reddy *et al.*, 1993). Carbon substrates are key factors controlling the efficiency of cyanobacterial aerobic growth and hydrogen production (Berman-Frank *et al.*, 2003; Reddy *et al.*, 1993; Tamagnini *et al.*, 2007). Genome analysis studies have revealed that *Cyanothece* 51142 has a unique gene cluster on its linear chromosome that contains key genes involved in glucose

Abbreviations: PSII, photosystem II; RuBisCO, ribulose-1,5-bisphosphate carboxylase oxygenase; THF, tetrahydrofuran.

Three supplementary figures, showing growth of *Cyanothece* 51142 in the presence of different carbon and nitrogen substrates under continuous light, hydrogen production under mixotrophic conditions, and growth of *Synechocystis* 6803 in the presence of glucose under continuous light, are available with the online version of this paper.

and pyruvate metabolism (Welsh *et al.*, 2008). However, the ability of this strain to metabolize glucose or pyruvate remains unknown.

To quantitatively examine the effect of carbon and nitrogen sources on *Cyanothece* central metabolism, this study investigated the effect of three carbon sources (glucose, glycerol and pyruvate, as representatives of sugar, lipid derivatives and organic acids from central metabolic pathways, respectively) on *Cyanothece* 51142 growth and metabolism. Two nitrogen sources other than N₂, ammonia and nitrate, were also examined. Precise readouts of metabolic state and activity were based on ¹³C-assisted metabolite analysis integrated with biochemical assays and the gene expression patterns obtained by RT-PCR (Fong *et al.*, 2006; Pingitore *et al.*, 2007; Tang *et al.*, 2007c, 2009; Wu *et al.*, 2010). Superior to the traditional ¹⁴C method (Bottomley & Van Baalen, 1978), the non-radioactive ¹³C method can provide rich information about which carbons within a metabolite are labelled, and thus enable an in-depth understanding of carbon utilization and metabolic regulation in *Cyanothece* 51142.

METHODS

Bacterial strains and growth conditions. *Cyanothece* 51142 was first grown in 150 ml Erlenmeyer flasks fed with ASP2 medium (Reddy *et al.*, 1993) without nitrate. Ambient CO₂ provided the sole carbon source. For experiments examining the effects of nitrogen sources, 18 mM NaNO₃ or 17 mM NH₄Cl was added to the medium. Cultures were grown aerobically under continuous light (50 μmol photons m⁻² s⁻¹) on a shaker at 150 r.p.m. and 30 °C. Cells at late mid-exponential phase were subcultured into different culture media with various nitrogen and carbon sources. Isotopically labelled carbon substrates (Cambridge Isotope Laboratories) were used for mixotrophic growth, including 54 mM glycerol (2-¹³C, >98%), 26 mM glucose (U-¹³C, >98%) and 11 mM sodium pyruvate (3-¹³C, >98%). For tracer experiments, a 3% inoculum from unlabelled stock culture was used to inoculate 50 ml of medium containing labelled carbon sources. At the mid-exponential phase of growth, a 3% inoculum from the first isotopically labelled culture was used to inoculate 50 ml subcultures with the same medium to remove the effect of unlabelled carbon introduced from the initial inoculum. Cell growth was monitored by a UV-Vis spectrophotometer (GENESYS, Thermo Scientific) at 730 nm. To perform a comparative study, the glucose-tolerant *Synechocystis* strain PCC 6803 (a model cyanobacterium for studying fundamental processes of photosynthetic metabolism) was also cultured in BG11 medium (pH 7.6) under the same growth conditions (continuous light and 30 °C; Stanier *et al.*, 1971). The BG11 medium was supplemented with 6 mM glucose (U-¹³C, >98%) to support mixotrophic growth. *Synechocystis* PCC 6803 was also subcultured in the same labelled medium twice before sampling for ¹³C-labelled metabolite analysis.

Metabolite and photosynthetic activity analysis. To analyse metabolites in *Cyanothece* 51142, biomass was harvested at the mid-exponential phase of growth (~90 h) by centrifugation at 7000 r.p.m. for 15 min at 10 °C. The concentrations of pyruvate, glucose and glycerol were analysed with enzymic assay kits (R-Biopharm). To measure hydrogen produced by *Cyanothece* 51142, 20 ml culture solution was taken from the culture flask after 3 days and transferred into a 35.2 ml glass vial sealed with a rubber septum and kept under

continuous light (50 μmol photons m⁻² s⁻¹). A modified protocol was used to quantify hydrogen (Rey *et al.*, 2007). Briefly, hydrogen that accumulated in the headspace of the sealed culture vials (for 5 h) was withdrawn with a Hamilton gas-tight syringe and quantified on an Agilent 6890N gas chromatograph with a Molsieve 5A 60/80 column [inner dimensions 6' × 1/8" (1830 × 3.17 mm)] and thermal conductivity detector. Injection, oven and detector temperatures were 100, 50 and 100 °C, respectively. Argon was the carrier gas (flow rate 65 ml min⁻¹). All measurements included three biological replicates.

Photosynthesis activities were determined based on measurements of chlorophyll fluorescence and oxygen evolution. Chlorophyll fluorescence profiles of photosystem II (PSII) of *Cyanothece* 51142 under different nutrient conditions were detected by an FL100 fluorometer (Photon Systems Instruments, Brno, Czech Republic) as described previously (Roose & Pakrasi, 2004). All samples taken for measurement were diluted to OD₇₃₀ ~0.2 using cell-free ASP2 medium. The samples were first adapted for 3 min in total darkness. During the measurement (performed at room temperature), the fluorometer emitted saturating light pulses to determine the fluorescence yield of the samples. The photosynthesis activity was derived by the maximum quantum yield (F_v/F_m) according to the formula F_v/F_m = (F_m - F₀)/F_m, where F₀ is initial fluorescence and F_m is maximum fluorescence at the beginning of measurement (Krause & Weis, 1991).

Oxygen evolution rates of *Cyanothece* 51142 grown under different nutrient conditions were measured with a Hansatech oxygen electrode. Assays were performed at 30 °C on whole cells in ASP2 medium with a saturating light intensity of 8250 μmol photons m⁻² s⁻¹ for 2 min in a 2.5 ml reaction volume. For each reaction, the chlorophyll concentration of each sample was diluted to ~6 μg ml⁻¹. The oxygen evolution rates [μmol O₂ (mg chlorophyll)⁻¹ h⁻¹] were then measured and normalized based on chlorophyll concentration.

RNA extraction and RT-PCR. The bacteria grown under different cultural conditions were harvested at mid-exponential phase according to the corresponding growth curves. The total RNA was extracted by using a PureLinkRNA Mini kit (Invitrogen), following the manufacturer's instructions. cDNA was synthesized from ~2 μg RNA by using a High Capacity cDNA Reverse Transcription kit (Invitrogen). The primers for RT-PCRs were designed using Primer Premier 5 software (PREMIER Biosoft) and analysed by OligoAnalyser 3.0 software (Integrated DNA Technologies). The forward primer 5'-AGCGGTGGAGTATGTGGT-3' and reverse primer 5'-GGCTGGGTTTGATGAGATT-3' were employed to amplify the 16S rRNA gene as a control. The forward primer 5'-CCGACTACACTCCGAAAG-3' and reverse primer 5'-ACGTAA-CGCCCGTAATGC-3' were used to amplify the ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) (*rbcl*) gene, and the forward primer 5'-TAATCACGAAACGGGAG-3' and reverse primer 5'-CACCACATCAGCGTATTG-3' to amplify the *prk* gene. PCRs were conducted with the following cycle conditions: 2 min of activation of the polymerase at 94 °C, followed by 30 cycles consisting of 1 min at 94 °C, 30 s at 53 °C and 2 min at 72 °C; finally, a 10 min extension was performed at 72 °C. The final PCR product was observed directly on 2% agarose gels after electrophoresis.

Isotopic analysis. The preparation and isotopic analysis of proteogenic amino acids were performed as previously described (Tang *et al.*, 2007a, b). In brief, exponentially growing biomass from ~20 ml culture was collected by centrifugation (8000 g, 10 min, 4 °C) and hydrolysed in 6 M HCl at 100 °C for 24 h. The amino acid mix was dried and derivatized in tetrahydrofuran (THF) and *N*-(*tert*-butyl dimethylsilyl)-*N*-methyl-trifluoroacetamide (Sigma-Aldrich) at 70 °C for 1 h. A gas chromatograph (Hewlett Packard model 7890A, Agilent Technologies) equipped with a DB5-MS column (J&W Scientific) and

a mass spectrometer (5975C, Agilent Technologies) were used for analysing amino acid labelling profiles. The ion $[M-57]^+$ from unfragmented amino acids was detected and mass fractions of key amino acids were calculated (Wahl *et al.*, 2004). The substrate utilization ratio R (reflecting the degree of mixotrophic metabolism) for an amino acid X was calculated from the labelling patterns of proteogenic amino acids by the following equation:

$$\frac{0.98 \times n \times V_{\text{sub}} + 0.01 \times V_{\text{CO}_2}}{m \times V_{\text{sub}} + V_{\text{CO}_2}} = \frac{\left(\sum_{i=1}^C i \times M_i\right)}{C} \Rightarrow R = \frac{V_{\text{sub}}}{V_{\text{CO}_2}} \quad (1)$$

where the ratio R indicates the utilization of labelled carbon substrate over unlabelled CO_2 for producing an amino acid X (and its precursors), M_i is the GC-MS isotopomer fraction for amino acid X (i.e. M_0 is the unlabelled fraction, M_1 is the singly labelled fraction, M_2 is the doubly labelled fraction, M_3 is the triply labelled fraction, etc.), C is the total number of carbon atoms in the amino acid molecule, V_{sub} is the carbon flux from ^{13}C -labelled substrate, V_{CO_2} is the carbon flux from CO_2 , 0.98 is the purity of the labelled carbon substrate, 0.01 is the natural abundance of ^{13}C , m is the total number of carbons in the substrate molecule and n is the total number of labelled carbons in the substrate molecule. R indicates the amount of labelled carbon that percolated through the central metabolic networks (Fig. 1).

RESULTS

Cell growth with different carbon and nitrogen sources

Fig. 2 and Supplementary Fig. S1 show the effects of carbon and nitrogen substrates on the growth of *Cyanothece* 51142 under continuous light. Biomass growth was significantly enhanced by the addition of glycerol to ASP2 medium. For example, glycerol addition doubled the specific growth rate from 0.28 to 0.63 day^{-1} under N_2 -fixing conditions. These results are consistent with an earlier report on two *Cyanothece* strains (Reddy *et al.*, 1993). On the other hand, *Cyanothece* growth was apparently not enhanced by either glucose or pyruvate (Supplementary Fig. S1), and a high concentration of pyruvate (64 mM) inhibited *Cyanothece* growth. Compared with nitrogen-fixing cultures, the presence of nitrate salts in the growth media increased *Cyanothece* autotrophic growth rates from 0.28 day^{-1} (N_2 -fixation condition) to 0.37 day^{-1} (nitrate-sufficient condition). Similarly, the presence of glycerol enhanced the growth rate by approximately twofold (from 0.60 to 1.02 day^{-1}). As expected, high concentrations of

Fig. 1. Central metabolic pathways of *Cyanothece* 51142 with glucose, glycerol and pyruvate as carbon substrates. The dashed lines show the metabolic pathway with glycerol as carbon substrate; the heavy solid line indicates glucose; the solid line shows the common pathway for all carbon conditions. Abbreviations: ACCOA, acetyl-CoA; E4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; GAP, glyceraldehyde 3-phosphate; GLY, glycerol; GLU, glucose; ICIT, citrate/isocitrate; MAL, malate; OAA, oxaloacetate; OXO, 2-oxoglutarate; PEP, phosphoenolpyruvate; 3PG, 3-phosphoglycerate; PYR, pyruvate; R5P, ribose 5-phosphate (or ribulose 5-phosphate); R15P, ribulose 1,5-bisphosphate; S7P, sedoheptulose 7-phosphate; Xu5P, xylulose 5-phosphate.

Fig. 2. *Cyanothece* 51142 growth curves with different nitrogen and carbon sources ($n=3$ biological replicates). ◇, Glycerol + nitrate; □, Glycerol + N₂; △, CO₂ + nitrate; ○, CO₂ + N₂. To measure the labelling patterns of amino acids, the glycerol-growing samples were taken at day four when the remaining glycerol in the culture medium was still sufficient for biomass growth. The error bars are smaller than the symbols.

ammonium salts (17 mM) fully inhibited growth (data not shown) because of their well-known deleterious effect on the photosystems of cyanobacteria (Drath *et al.*, 2008; Dai *et al.*, 2008).

Isotopic analysis of amino acids

¹³C-enrichment patterns in key metabolites were used to estimate the relative utilization of labelled carbon substrates (i.e. glucose, pyruvate and glycerol) and CO₂ for metabolite synthesis under mixotrophic growth. Fig. 1 shows the central metabolic pathways in *Cyanothece* 51142 (<http://www.genome.jp/kegg/>). The labelling of five amino acids was analysed: histidine (precursors: ribose 5-phosphate and 5,10-methyl-THF), synthesized from the Calvin cycle and pentose phosphate pathway; serine (precursor: 3-phosphoglycerate, a product from the Calvin cycle); alanine (precursor: pyruvate, originating from carbon substrates or CO₂ fixation); and aspartate and glutamate (precursors: oxaloacetate and 2-oxoglutarate, respectively; synthesized from the citric acid cycle). Under nitrate-sufficient conditions, glycerol could be used as the sole carbon source for the synthesis of alanine, serine and histidine (as indicated by R values approaching infinity). This indicates that the cell was undergoing completely heterotrophic metabolism. R values of some key amino acids in glucose and pyruvate cultures were positive, and thus these two carbon sources were actually utilized for biomass synthesis (Table 1). However, their measured R values were between 0 and 0.3, which indicated that CO₂ was the main carbon source for metabolite synthesis. This result was consistent with the fact that glucose and

pyruvate did not apparently improve the biomass growth. Compared with nitrogen-sufficient conditions, nitrogen-fixing conditions further limited glucose and glycerol utilization, as shown by the decreased labelling fractions of three key amino acids (i.e. alanine, serine and histidine) (Table 1).

Nitrogenase-dependent hydrogen production, photosynthesis and Calvin cycle activity

Hydrogen production under continuous light with different carbon substrates (N₂ as the sole nitrogen source) was measured in the exponential (day 4) and stationary (day 9) growth phases (Supplementary Fig. S2). In the exponential growth phase under nitrogen-fixing conditions, hydrogen production rates were as follows: glycerol, $25 \pm 6 \mu\text{mol H}_2 (\text{mg chlorophyll})^{-1} \text{ h}^{-1}$; glucose, $13 \pm 9 \mu\text{mol H}_2 (\text{mg chlorophyll})^{-1} \text{ h}^{-1}$; pyruvate, $4 \pm 2 \mu\text{mol H}_2 (\text{mg chlorophyll})^{-1} \text{ h}^{-1}$; and under photoautotrophic conditions, $5 \pm 1 \mu\text{mol H}_2 (\text{mg chlorophyll})^{-1} \text{ h}^{-1}$. Under all nitrate or ammonium chloride conditions, hydrogen production was not detected, regardless of the carbon substrate.

The measurement of photosynthetic parameters (Fig. 3) suggested that, compared with photoautotrophic conditions, addition of an exogenous carbon source (glycerol, glucose or pyruvate) did not strongly suppress the maximal quantum yield of PSII (F_v/F_m) or the oxygen evolution rate. Nitrate-sufficient conditions enhanced the oxygen evolution rates by two- to threefold compared with nitrogen-fixing conditions, while the changes of the quantum yields of PSII were much less significant (10–30 %). Gene expression in the carbon fixation pathway was also determined (Fig. 4). RT-PCR results indicated that two key enzymes in the Calvin cycle [RuBisCO (*rbcL*) and phosphoribulokinase (*prk*)] were functional under conditions of growth with glycerol or glucose. The above measurements confirmed that the light-dependent reactions were active under all culture conditions, even though carbon substrates reduced the relative contribution of CO₂ fixation to biomass synthesis.

DISCUSSION

Carbon substrate utilization and regulation

In continuous light, *Cyanothece* 51142 can efficiently utilize glycerol for aerobic growth. Based on the measurement of carbon substrates in the culture medium during the exponential growth phase, the uptake rates for glycerol were $0.22 \pm 0.05 \text{ g (g dry biomass)}^{-1} \text{ day}^{-1}$ under nitrogen-fixing and $0.35 \pm 0.06 \text{ g (g dry biomass)}^{-1} \text{ day}^{-1}$ under nitrate-sufficient conditions. Glycerol promoted *Cyanothece* 51142 growth because it provided carbon and energy sources. Under nitrate-sufficient conditions, the high values of R for the serine, alanine and histidine labelling data indicated that the 3-phosphoglycerate,

Table 1. Isotopic analysis of the labelling profiles of amino acids in *Cyanothece* 51142 and *Synechocystis* 6803 under different growth conditions (the standard error for GC-MS measurement was below 0.02, technical replicates, $n=2$)

Amino acid	[M-57] ⁺	N ₂						NaNO ₃						Synechocystis 6803 (nitrate medium)	
		Glucose	R*	Pyruvate	R	Glycerol	R	Glucose	R	Pyruvate	R	Glycerol	R	Glucose	R
Ala	M ₀	0.67	0.032	0.41	0.597	0.19	4.2	0.61	0.042	0.51	0.327	0.07	+∞	0.04	
	M ₁	0.19		0.55		0.71		0.19		0.48		0.85		0.05	0.92
	M ₂	0.11		0.03		0.10		0.17		0.01		0.07		0.28	
Ser	M ₀	0.65	0.033	0.98	0	0.20	3.7	0.58	0.046	0.97	0	0.08	+∞	0.04	0.87
	M ₁	0.22		0.02		0.72		0.22		0.03		0.81		0.06	
	M ₂	0.10		0		0.09		0.16		0		0.10		0.28	
Asp	M ₀	0.58	0.030	0.54	0.195	0.10	2.2	0.59	0.032	0.94	0.005	0.07	1.53	0.04	0.44
	M ₁	0.24		0.43		0.64		0.20		0.06		0.78		0.05	
	M ₂	0.11		0.04		0.25		0.17		0		0.15		0.19	
	M ₃	0.06		0		0.01		0.03		0		0		0.47	
Glu†	M ₀	0.43	0.041	0.15	1.25	0.02	-1.78	0.38	0.051	0.47	0.170	0.01	-2.11	0.02	0.76
	M ₁	0.26		0.44		0.14		0.22		0.49		0.15		0.02	
	M ₂	0.21		0.37		0.62		0.27		0.04		0.74		0.04	
	M ₃	0.07		0.04		0.21		0.09		0		0.10		0.07	
	M ₄	0.02		0		0.01		0.03		0		0		0.53	
His	M ₀	0.44	0.032	0.91	0	0.05	2.83	0.33	0.049	0.92	0	0.01	+∞	0.01	1.73
	M ₁	0.28		0.08		0.28		0.24		0.08		0.21		0.01	
	M ₂	0.17		0		0.50		0.22		0		0.55		0.02	
	M ₃	0.07		0		0.16		0.12		0		0.20		0.03	
	M ₄	0.03		0		0		0.07		0		0.03		0.06	
	M ₅	0		0		0		0.01		0		0		0.22	

*Values in bold type are the carbon substrate (glycerol, pyruvate or glucose) utilization ratios (substrate/CO₂ fixation) for amino acid synthesis, calculated according to equation (1).

†The glutamate synthesis pathway involves the loss of two carbons from pyruvate to α -ketoglutarate. Such a microbial process changes the labelling enrichment, and the negative values indicate the net loss of unlabelled CO₂.

pyruvate and ribose 5-phosphate nodes in the central metabolic pathways (Fig. 1) originated completely from glycerol, while the contribution of CO₂ photofixation to these metabolite nodes was negligible. As a comparison, the glucose-tolerant strain *Synechocystis* sp. 6803 was cultured with fully labelled glucose under continuous light and nitrogen-sufficient conditions (Supplementary Fig. S3). The measured R values (Table 1) for serine (0.87), alanine (0.92) and histidine (1.73) indicated that *Synechocystis* 6803 had typical mixotrophic growth. In general, cyanobacterial heterotrophic growth has been reported only under three conditions: complete darkness, dim light and pulses of light (Anderson & McIntosh, 1991; Van Baalen *et al.*, 1971). When the light is sufficient for photoautotrophy, *Cyanothece* photoheterotrophic growth is only achieved by addition of PSII inhibitors (Reddy *et al.*, 1993). This study shows that rapidly growing *Cyanothece* 51142 cells can shift their metabolic strategy from mixotrophic or autotrophic growth to photoheterotrophic growth, possibly because maximal utilization of an energy-rich carbon substrate (glycerol) can reduce energy costs related to CO₂ fixation (fixation of one CO₂ consumes two ATP and one NADPH) and building-

block synthesis, so that maximal biomass growth can be achieved.

On the other hand, glucose was apparently not consumed by *Cyanothece* 51142 (the consumed concentrations were below 1 mM in all experiments). In the [U-¹³C]glucose experiments (Table 1), all five amino acids contained labelled carbons, which indicated that the labelled glucose had percolated through all the central metabolic pathways, thereby confirming the ability of *Cyanothece* 51142 to metabolize glucose. The R values of all key amino acids were below 0.05 for both nitrogen-fixation and nitrate-sufficient conditions, suggesting that a large fraction of the carbon in the biomass had originated from CO₂ fixation. In contrast, glucose is the most favourable carbon source for *Synechocystis* species (Yang *et al.*, 2002), and the R values (Table 1) for key amino acids were ~0.4–1.7). While both *Synechocystis* 6803 and *Cyanothece* 51142 have completely annotated central pathways for glucose metabolism, *Synechocystis* 6803 contains a glucose transporter (gene code Sll0771) that shares a sequence relationship with mammalian glucose transporters (Bottomley & Van

Fig. 3. Maximum quantum yields of PSII (a) and oxygen evolution rates (b) of *Cyanothece* 51142 under different growth conditions ($n=3$ biological replicates). All samples were taken in the exponential growth phase, based on the growth curve. Filled bars, N_2 as nitrogen source; open bars, $NaNO_3$ as nitrogen source. Error bars, SD.

Baalen, 1978; Flores & Schmetterer, 1986; Schmetterer, 1990). So far, the presence of a glucose transporter in *Cyanothece* 51142 has not been rigorously verified. From the genome database (DOE Joint Genome Institute, <http://www.jgi.doe.gov/>), a gene (*cce_3842*) has been identified as a glucose transport protein that shared weak (25 %) amino acid identity with the Sll0771 protein of *Synechocystis* PCC6803. Based on the glucose-dependent growth data, we conclude that the enzymes involved in glucose transport or utilization in *Cyanothece* 51142 may not be as efficient as those of *Synechocystis* PCC6803.

Analysis of labelled pyruvate-grown *Cyanothece* cells showed that serine (precursor 3-phosphoglycerate) and histidine (precursor ribose 5-phosphate) were completely unlabelled ($R=0$). Such a labelling profile suggests that CO_2 was used as the sole carbon source for synthesis of metabolites in glycolysis and the pentose phosphate pathway (i.e. there was no gluconeogenesis activity). Pyruvate was used only to synthesize alanine ($R=0.3-0.6$) and metabolites in the tricarboxylic acid cycle (pyruvate→oxaloacetate→Asp) (pyruvate→acetyl-CoA→citrate→2-oxoglutarate→glutamate), as reflected by the labelled carbon present in glutamate and aspartic acid. Interestingly, the R values for alanine (0.60) and glutamate (1.25) were higher under nitrogen-fixing conditions than under nitrate-sufficient conditions, indicating that relatively more labelled pyruvate was used for glutamate synthesis under these conditions. The nitrogen fixation was via nitrogenase: $N_2 + 6H^+ + 6e^- \rightarrow 2NH_3$, and the nitrogenase-generated ammonium was assimilated into amino acids through the glutamine synthetase/glutamate synthase pathway (Postgate, 1998). Utilization of supplemented pyruvate for glutamate synthesis could facilitate the nitrogen-fixation process.

The enzyme RuBisCO is known to be the rate-limiting factor in the Calvin cycle for capturing CO_2 to synthesize three-carbon sugars (glycerate 3-phosphate) (Atsumi *et al.*,

Fig. 4. RT-PCR investigation of RuBisCO (*rbcL*) and phosphoribulokinase (*prk*) gene expression under different mixotrophic growth conditions. (a) $CO_2 + N_2$, (b) $CO_2 + NaNO_3$, (c) glycerol + $NaNO_3$, (d) glucose + $NaNO_3$. The 16S rRNA gene (16S) was used as the internal reference; the no template control (NTC) was added under all mixotrophic growth conditions.

2009). We examined RuBisCO (*rbcL*) and phosphoribulokinase (*prk*) gene expression to reveal the metabolic regulation in the Calvin cycle at the transcriptional level. Under photoautotrophic, mixotrophic and heterotrophic growth conditions, expression of the two genes was clearly observed. Although the Calvin cycle genes were expressed, *Cyanothece* 51142 still grew heterotrophically in the presence of glycerol and nitrate, based on the isotopomer data (no apparent incorporation of CO_2 from the Calvin cycle). These inconsistencies indicate that ^{13}C -assisted metabolite analysis provides a direct readout of actual metabolic status, while gene expression results alone cannot be relied upon, as there are many points of possible post-transcriptional regulation.

Furthermore, *Cyanothece* 51142 can fix CO_2 via anaplerotic pathways (i.e. C4 carbon fixation) (Slack & Hatch, 1967). In the presence of glycerol and under nitrate-sufficient conditions (Table 1), the R ratio for aspartate synthesis was 1.53, much smaller than the R ratios ($R=\infty$) for Ala, Ser and His. This indicates that, even though phototrophic CO_2 fixation was significantly inhibited, CO_2 was utilized for the synthesis of C4 metabolites in the tricarboxylic acid cycle via anaplerotic pathways: (1) $\text{PEP} + \text{CO}_2 \rightarrow \text{oxaloacetate}$ (catalysed by phosphoenolpyruvate carboxylase or phosphoenolpyruvate carboxykinase) or (2) $\text{pyruvate} + \text{CO}_2 \rightarrow \text{malate}$ (catalysed by malic oxidoreductase). Such anaplerotic pathways synthesize key TCA cycle metabolites such as oxaloacetate and succinate (precursors of chlorophyll).

Meanwhile, CO_2 was generated by two reactions (i.e. $\text{pyruvate} \rightarrow \text{acetylCoA} + \text{CO}_2$; $\text{isocitrate} \rightarrow 2\text{-oxoglutarate} + \text{CO}_2$), which are essential steps for glutamate synthesis. These catabolic processes cause the loss of unlabelled carbon when the second-position labelled glycerol is used as the main carbon source. Therefore, the coefficients V_{CO_2} (CO_2 utilization flux) and R (carbon utilization ratio) were both negative for glutamate synthesis (equation 1) in glycerol-supplemented cultures (under both nitrogen-fixation and nitrate-sufficient conditions) (Table 1).

Photosynthesis activity

Photosynthesis activity was estimated by the F_v/F_m parameter (maximum quantum efficiency of PSII) (Pirintsos *et al.*, 2009). When glycerol or glucose was utilized, the maximum quantum yield F_v/F_m (i.e. efficiency of PSII) in *Cyanothece* 51142 was not significantly affected (changes were within $\sim 30\%$, Fig. 3a). Although chlorophyll fluorescence estimation is not an accurate method for the determination of absolute PSII activity (Schreiber *et al.*, 1995; Ting & Owens, 1992), we have used it in our study as a tool only to confirm active photon capture in the light-harvesting antenna complexes of PSII under both heterotrophic and mixotrophic conditions.

Oxygen evolution was measured as one molecule of the pigment chlorophyll absorbs one photon and uses its

energy to generate NADPH, ATP and O_2 via the light-dependent reactions (Kaftan *et al.*, 1999). The oxygen evolution rates in *Cyanothece* 51142 rose by two- to threefold under all nitrate-sufficient conditions compared with corresponding nitrogen-fixation conditions (Fig. 3b). The significantly higher rates of oxygen evolution indicated that the photosynthetic process of water splitting was more active and provided more energy (ATP and NADPH) to support biomass growth under nitrate-sufficient conditions.

Finally, precise determination of the photosynthetic activity of *Cyanothece* 51142 is difficult, as its metabolic behaviour fluctuates under continuous light due to its circadian rhythm (Colon-Lopez *et al.*, 1997; Toepel *et al.*, 2008). The photoreaction activity data in Fig. 3 represent only qualitative (not quantitative) evidence to support the presence of active light-dependent reactions under all culture conditions.

Nitrogen utilization and nitrogenase-dependent hydrogen production

Under anaerobic conditions (using argon gas to flush the culture), hydrogen production rates of *Cyanothece* 51142 were as high as $100 \mu\text{mol (mg chlorophyll)}^{-1} \text{h}^{-1}$ (data not shown). Under aerobic conditions, the hydrogen production enzyme (hydrogenase) is completely inactivated by oxygen (Tamagnini *et al.*, 2007). *Cyanothece* 51142 uses nitrogenase for both nitrogen fixation and hydrogen production. Nitrate, ammonium and some amino acids inhibit nitrogenase activity and thus fully prohibit aerobic hydrogen production by cyanobacteria (Rawson, 1985). Furthermore, NH_4^+ is a direct nitrogen source (nitrate is reduced to NH_4^+) that can be incorporated into biomass via glutamine/glutamate synthase (Muro-Pastor *et al.*, 2005). *Cyanothece* 51142, however, only grows at low concentrations of NH_4^+ (below 1 mM) because of an inhibition effect (Galmozzi *et al.*, 2007; Rawson, 1985). Nitrogen fixation is an energy-demanding process:

The addition of glycerol reduces CO_2 fixation via the Calvin cycle, so more energy (ATP and NADH) can be directed to nitrogen fixation, thus promoting hydrogen production four- to fivefold (Dutta *et al.*, 2005; Madamwar *et al.*, 2000). Glucose and pyruvate cannot significantly promote hydrogen production because their utilization is very low and their effect on the energy economy limited. Hydrogen production rates dropped for all mixotrophic cultures of *Cyanothece* 51142 after 9 days, suggesting that inhibitory metabolites that reduced nitrogenase activity accumulated during cultivation (Atsumi *et al.*, 2009; Nyström, 2004). Finally, the coexistence of oxygen-evolving photosynthesis and oxygen-sensitive nitrogen fixation (indicated by hydrogen evolution) is an attractive characteristic in some cyanobacteria (Benemann & Weare, 1974; Huang & Chow, 1986). Unlike filamentous cyano-

bacterial species, in which nitrogen fixation and oxygenic photosynthesis are spatially segregated (Berman-Frank *et al.*, 2001), *Cyanothece* 51142 is able to maintain activities for N₂ fixation, respiration and photosynthesis within the same cell under continuous light. The strain not only has a strong ability to scavenge intracellular oxygen and synthesize nitrogenase (Colon-Lopez *et al.*, 1997; Fay, 1992), but also develops a highly circadian mechanism for nitrogen fixation (Elvitigala *et al.*, 2009).

This study improves our understanding of *Cyanothece* 51142 physiology with different carbon and nitrogen sources as well as its potential application for hydrogen production. In general, exogenous carbon substrates may improve cellular growth but have strong negative effects on CO₂ fixation. Continuously illuminated *Cyanothece* 51142 shows simultaneous oxygen evolution and nitrogenase-dependent hydrogen production, while hydrogen production can be significantly enhanced by the addition of glycerol. A comparison of metabolic status under autotrophic, mixotrophic and heterotrophic growth conditions indicated that *Cyanothece* 51142 has an inherent metabolic strategy for maximal biomass production at low energy cost. Finally, this study has further confirmed that ¹³C-assisted metabolite analysis is a high-throughput method which can provide new and precise information to understand a biological system.

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