Cyanobacteria are considered good models for biohydrogen production because they are relatively simple organisms with a demonstrable ability to generate H₂ under certain physiological conditions. However, most produce only little H₂, revert readily to H₂ consumption, and suffer from hydrogenase sensitivity to O₂. Strains of the cyanobacterium *Lyngbya aestuarii* and *Microcoleus chthonoplastes* obtained from marine intertidal cyanobacterial mats were recently found to display much better H₂ production potential. Because of their ecological origin in environments that become quickly anoxic in the dark, we hypothesized that this differential ability may have evolved to serve a role in the fermentation of the photosynthate. Here we show that, when forced to ferment internal substrate, these cyanobacteria display desirable characteristics of physiological H₂ production. Among them, the strain *L. aestuarii* BL J had the fastest specific rates and attained the highest H₂ concentrations during fermentation of photosynthate, which proceeded via a mixed acid fermentation pathway to yield acetate, ethanol, lactate, H₂, CO₂, and pyruvate. Contrary to expectations, the H₂ yield per mole of glucose was only average compared to that of other cyanobacteria. Thermodynamic analyses point to the use of electron donors more electronegative than NAD(P)H in *Lyngbya* hydrogenases as the basis for its strong H₂ production ability. In any event, the high specific rates and H₂ concentrations coupled with the lack of reversibility of the enzyme, at the expense of internal, photosynthetically generated reductants, makes *L. aestuarii* BL J and/or its enzymes, a potentially feasible platform for large-scale H₂ production.

**Keywords:** bidirectional hydrogenase, cyanobacteria, *Lyngbya aestuarii*, *Microcoleus chthonoplastes*, *Synechocystis*, hydrogen, fermentation
et al., 1996). The mixed acid fermentation pathway is known to produce acetate, lactate, ethanol, formate and/or CO₂, and H₂ (Van der Oost et al., 1989; Moezelaar et al., 1996; Aoyama et al., 1997; Troshina et al., 2002). Thus, the mixed acid and, to a certain extent, the homoacetate pathways result in H₂ production.

Cyanobacteria are not known to respire external electron acceptors other than O₂, and thus, when subjected to nighttime anoxia must resort to fermentation in order to maintain ATP production and regenerate excess reduction equivalents. A classic example of an environment conducive to this is cyanobacterial benthic mats (Walter, 1976; Bauld, 1981; Javor and Castenholz, 1981). In these mats, oxygenic photosynthetic activity causes the top mat layers to become supersaturated with O₂ during the daytime, but strong respiration rates overwhelm diffusive O₂ import in the dark, establishing strong anoxia (Revsbech et al., 1983) for the fermentation assays. In the first set, filaments were forced to ferment their daytime photosynthate. Fermentation products have been directly detected in hot spring microbial mats (Anderson et al., 1987; Nold and Ward, 1996). Amongst the mat inhabiting cyanobacteria, fermentation has been reported in *Oscillatoria terebriformis* (Richardson and Castenholz, 1987) and *Synechococcus* sp. strains OS-A and OS-B (Steunou et al., 2006) from hot springs. Fermentation has also been studied in marine microbial mat-building *Lyngbya aestuarii* CCY 9616 (= PCC 8106, also known as *Oscillatoria limosa* in the early literature) and *Oscillatoria* sp. SAG 3192 (Garcia-Pichel et al., 1996) [also referred to as *Microcoleus chthonoplastes* 11 or *M. chthonoplastes* SAG 3192 before (Stal and Krumbine, 1985)]. *L. aestuarii* CCY 9616 follows a homoacetate–heterolactate pathway (Heyer et al., 1989) whereas *Oscillatoria* sp. SAG 3192 ferments via a mixed acid fermentation pathway (Moezelaar et al., 1996).

Owing to the presence, multiplicity, and avidity of potential H₂ consumers in the complex microbial communities where H₂ is being produced, steady-state concentrations of H₂ tend to remain very low, usually undetectable in natural systems (Ebert and Brune, 1997; Schink, 1997). This general rule finds a clear exception in some intertidal microbial mats, where net H₂ accumulation and export has been reported (Skyring et al., 1989; Hoehler et al., 2001). Similarly, it was observed that intertidal microbial mats from Baja California, maintained in a greenhouse setting for more than 3 years under an artificial intertidal regime, continue to produce H₂ at night, exporting significant amounts to the overlying waters (Hoffmann and Maldonado, personal communication). The organisms fermenting under such conditions must thus be able to produce H₂ even against high partial pressures in the mat.

In an earlier report surveying a set of cyanobacterial strains for H₂ production in presence of excess reductants, two different patterns were observed. Pattern 1, known from fresh water strains such as *Synechocystis* sp. PCC 6803, exhibited low rates and steady-state H₂ concentrations followed by uptake of most of the produced H₂ whereas the novel Pattern 2, found only in *L. aestuarii* and *M. chthonoplastes* strains from the marine intertidal mats, exhibited much higher rates, steady-state H₂ concentrations, and a lack of H₂ uptake throughout the assay (Kothari et al., 2012). Indeed, the cyanobacterial strains isolated from these mats displayed an extraordinary potential to produce/sustain H₂ under the unusually high concentrations of H₂ prevailing in their micro-environment. However, all of this was done using standard assays (Kothari et al., 2012) that externally provide excess reductant and anaerobic conditions. Now these studies are extended to include the innate H₂ evolving capacity under fermentative conditions, using microbiological and genomic evidence.

**MATERIALS AND METHODS**

**STRAINS, MEDIA, AND GROWTH CONDITIONS**

Five strains of cyanobacteria were used for this work. *L. aestuarii* BL J, *L. aestuarii* BL AA, and *M. chthonoplastes* BM 003 were isolated from marine intertidal microbial mats in Baja California (Kothari et al., 2012). *M. chthonoplastes* PCC 7420 was originally isolated from a microbial mat in a salt marsh, Woods Hole, Massachusetts.

*Synechocystis* sp. PCC 6803, originally a freshwater isolate, has been used in this study since it is a popular model cyanobacterium for biohydrogen research. The latter two strains were obtained from the Pasteur Culture Collection (http://www.pasteur.fr/ip/easy/site/pasteur/en/institut-pasteur). *L. aestuarii* strains were grown in IMR medium with 3% salinity (Epplsey et al., 1968), modified to incorporate commercially available Instant Ocean salt mixture instead of natural seawater. *M. chthonoplastes* strains were grown in a 1:1 mixture of IMR and ASN III media (Rippka et al., 1979) with 3% salinity. *Synechocystis* sp. PCC 6803 was grown in BG11 medium (Rippka et al., 1979). All media were supplemented with 0.5 mM (final concentration) NiSO₄ to ensure adequate supply of nickel for the working of Ni–Fe hydrogenases.

The strains *L. aestuarii* BL J, *L. aestuarii* BL AA, and *M. chthonoplastes* BM 003 were clonal and monocyanobacterial, but not always axenic. Therefore, phase contrast microscopy was used to confirm that the level of contaminating bacteria was less than 0.01% of the cyanobacterial biomass (assessed as bio-volume) for the physiological experiments. *M. chthonoplastes* PCC 7420 and *Synechocystis* sp. PCC 6803 were always used in axenic form.

For the purpose of whole genome sequencing, an axenic culture of *L. aestuarii* BL J was established by picking up the motile hormogonia developing on IMR medium—1% nobel agar plates (Rippka, 1988). These hormogonia were allowed to grow on IMR–PGY medium 1% nobel agar plates (0.25% peptone, 0.25% yeast extract, 0.25% glucose, 1.5% agar), and axenicity was determined by lack of heterotrophic bacterial growth, and through direct microscopic observation. All strains were maintained in 250 ml Erlenmeyer flasks, with 100 ml medium, starting with similar amounts of inoculum in presence of light at an intensity of 100 μmol photon m⁻² s⁻¹.

**FERMENTATIVE H₂ PRODUCTION ASSAY**

All strains were subjected to two different sets of growth conditions for the fermentation assays. In the first set, filaments were grown in continuous light without any bubbling (CL). In the second set, the strains were grown in 12-h light and 12-h dark cycle. The cultures were bubbled with air in the light period and with N₂ in the dark period to establish anoxia, forcing cells to ferment. These conditions are referred to as “Light Oxic Dark Anoxic” (LODA) conditions. All cultures were incubated for a minimum of two weeks before making any measurements. The
assay itself was carried out in the dark using whole cells (in vivo) without the addition of any external reductants. Particularly, for the cultures growing in LODA conditions, the assay was commenced at the beginning of the dark period. Small pea size pellets of biomass from log phase cultures were placed in a custom-made, 2.5 ml volume chamber with continuous stirring. Fresh medium was added to completely fill the chamber, which was sealed with no headspace. The chamber was endowed with two miniature Clark-type electrodes to monitor \( \text{H}_2 \) and \( \text{O}_2 \) partial pressure. The electrodes were connected to a pico-ammeter set at a voltage of 0.8 V for \( \text{H}_2 \) and -0.8 V for \( \text{O}_2 \). An A/D converter allowed the current signal data to be read on a computer using Sensor Trace Basic software. All electrodes and peripherals were from Unisense, Aarhus, Denmark. Before each measurement, the \( \text{H}_2 \) electrode was subject to a two-point calibration in culture medium bubbled with either air (0% \( \text{H}_2 \)) or with a custom gas mixture (10% \( \text{H}_2 \) in \( \text{N}_2 \)). The \( \text{O}_2 \) electrode was also subjected to a two-point calibration system wherein culture medium was bubbled with either air (21% \( \text{O}_2 \)) or with 100% \( \text{N}_2 \) (0% \( \text{O}_2 \)). During calibration the sealed chamber showed negligible leakage over a period of 2–3 h.

Each strain was measured in independent triplicate experiments. From the electrode traces, the following parameters were derived: the initial specific rate of fermentative \( \text{H}_2 \) production, \( R_{\text{H}_2} \); the maximum steady-state \( \text{H}_2 \) concentration reached, \( [\text{H}_2]_M \); and the time after which \( \text{H}_2 \) production stopped and reverted to consumption, \( T_{\text{R}} \). The measurements lasted for 24 h. At the end of the assay, chlorophyll was extracted from the biomass with 100% methanol and measured spectrophotometrically (MacKinney, 1941). This was done to ensure that all assays had roughly comparable biomass and to obtain specific rates of initial \( \text{H}_2 \) production (i.e., per unit biomass).

**ANALYSIS OF FERMENTATION METABOLISM IN L. AESTUARII BL J**

*L. aestuarii* BL J that had been grown in LODA conditions from three different flasks (replicates) were used in this assay. A couple of hours before the onset of a dark anaerobic period, biomass was harvested by centrifugation, acclimatized to fresh medium, and washed twice with fresh medium to get rid of any potentially existing fermentation products. The filaments form tight clumps, and hence, attempts were made to break the clumps using forceps and mild sonicadion at the lowest speed setting for 4 s to get a homogeneous cell suspension. This was required to split the biomass into two aliquots with approximately equal amounts of biomass to conduct the initial and final analyses quantifying the fermentation substrates and products. Since a non-destructive procedure that does not impart any kind of stress to the cells was necessary for quantifying the biomass, wet weights were used. Optical density cannot be employed for biomass estimation given the filamentous and clumpy nature of this strain. As described below, since only the wet weights from the two halves of the same filter were compared to each other, the errors in biomass estimation were minimized.

For obtaining two aliquots with approximately equal amounts of biomass using wet weights, the following procedure was adopted. The biomass was vacuum filtered onto a 0.4 \( \mu \)m poly-carbonate filter to establish a homogenous layer on it. The filter was cut into half, biomass scrapped off, and the wet weight of the cells on each half was measured. The biomass from each half of the filter was then introduced into a 10 ml serum bottle (one for initial and one for final analyses). To each bottle 5 ml of fresh medium was added and the bottles were sealed. To confirm the initial absence of fermentation products, 1 ml of medium was drawn out from the “initial” serum bottle for later High Pressure Liquid Chromatography (HPLC) analysis. The rest was immediately frozen in liquid \( \text{N}_2 \) and stored at -80°C to be used eventually in determining the initial fermentable glycogen content in the cells. The “final” serum bottle was bubbled with nitrogen for 30 min to establish anoxia. Gas Chromatography (GC) confirmed the absence of \( \text{O}_2 \), and the serum bottle was incubated in the dark on a rocking bench for 24 h at 25°C.

After incubation, 1 ml of medium was withdrawn for HPLC analyses of organic acids and ethanol. Hydrochloric acid was then added to the serum bottle to lower the pH of the solution and ensure that all the inorganic carbon was present as \( \text{CO}_2 \). The gases in the headspace (\( \text{CO}_2 \), \( \text{H}_2 \) and/or \( \text{O}_2 \)) were sampled by syringe and quantified by GC equipped with a thermal conductivity detector. GC was performed with Helium as the carrier gas and the concentrations of \( \text{H}_2 \) and \( \text{CO}_2 \) in the headspace were estimated as described before (Parameswaran et al., 2009). Total masses of gases were back-calculated according to volumetric partitioning. The contents of the serum bottle were then frozen in liquid \( \text{N}_2 \) and kept at -80°C for eventual glycogen content quantification.

Glycogen was extracted as per Ernst et al. (1984) and quantified using a BioAssay Systems glycogen assay kit. To determine organic acids and ethanol, HPLC was employed. All the liquid samples for HPLC were filtered through a 0.2 mm PVDF filter and the filtrate used. The HPLC was performed with Aminex HPX–87H column at 50°C with 2.5 mM sulfuric acid as eluent at a flow rate of 0.6 ml/min using a photodiode array and refractive index indicator (Parameswaran et al., 2009). Most of the common products of bacterial fermentation can be detected under these settings.

**GENOMIC AND BIOINFORMATICS ANALYSES**

The genomic DNA preparation of *L. aestuarii* BL J was subjected to MiSeq 250 Illumina sequencing, assembly, and annotation (Kothari et al., 2013). The genomic sequence of *L. aestuarii* BL J was checked for the presence of orthologs of genes potentially coding for key enzymes involved in fermentation. Protein sequences coding for cyanobacterial fermentation enzymes from NCBI database were used as query and Psi BLAST was performed against the entire *L. aestuarii* BL J genome. Given that the genome is not closed, the absence of any one gene does not necessarily imply its absence from the genome, as there is a small probability that it is found in unsequenced regions. This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession AUZM00000000. The version described in this paper is version AUZM01000000.

**RESULTS**

**FERMENTATIVE \( \text{H}_2 \) PRODUCTION**

The strains *L. aestuarii* BL J, *L. aestuarii* BL AA, *M. chthonoplastes* BM 003, *M. chthonoplastes* PCC 7420, and *Synechocystis* sp. PCC...
6803 were all capable of fermentative H$_2$ production. All strains reached anoxic conditions solely by dark respiration without the addition of any external reductants, anoxia-inducing compounds, or fermentable substrates. As soon as anoxia was established, H$_2$ production commenced without any measurable lag time in all strains.

Some variation in the parameters of fermentative H$_2$ production could be detected. These main parameters are initial specific rate, $R_H$, the maximum H$_2$ steady-state concentration, [$H_2$]$_{M}$, and the time after which the enzyme reverts in direction, $T_R$ (Figure 1). Table 1 gathers information on these parameters for all the five tested strains. In general, Lyngbya and Microcoleus strains from microbial mats produced H$_2$ faster and could reach higher equilibrium concentrations of H$_2$ than the standard strain Synechocystis sp. PCC 6803. Lyngbya and Microcoleus strains did not consume the H$_2$ produced during the assay (for up to 24 h) unlike Synechocystis sp. PCC 6803. The highest specific rate of H$_2$ production was seen in L. aestuarii BL AA and the highest steady-state H$_2$ concentration was seen in M. chthonoplastes BM 003.

**OPTIMIZATION OF FERMENTATIVE H$_2$ PRODUCTION**

Attempts were made to optimize the H$_2$ produced by acclimatizing the cells to 12-h light/dark cycles wherein the cells were exposed to anoxia in the dark (LODA). Synechocystis sp. PCC 6803 showed no significant improvements by this preconditioning in any of the parameters. The specific rates and steady-state concentrations of fermentative H$_2$ production attained in Lyngbya strains, but not those of Microcoleus strains, could be enhanced when cultures were pre-acclimated to recurrent nighttime anaerobiosis during growth. On subjecting the strains to LODA pre-conditioning all strains retained their characteristic feature of reversibility of reaction direction (or the lack of it). L. aestuarii BL J reached the highest specific rates and steady-state concentrations of H$_2$. The $R_H$ of L. aestuarii BL J grown in LODA condition doubled compared to that of cells grown in continuous light conditions; its [$H_2$]$_{M}$ increased 28-fold (Figure 2). In LODA conditions, the strain BL J performed exceptionally better than the standard Synechocystis sp. PCC 6803, its $R_H$ was 20-fold faster and [$H_2$]$_{M}$ 45-fold higher (Figure 2). While calculating the average $R_H$ of L. aestuarii BL J grown in LODA one abnormally high specific rate of 44.2 nmol (µg chl. a)$^{-1}$h$^{-1}$ was removed from the tally. Had this been incorporated, the $R_H$ value would have been 13.1 ± 17.5 nmol (µg chl. a)$^{-1}$h$^{-1}$.

Attempts made to further optimize the fermentative H$_2$ production from L. aestuarii BL J in LODA conditions by varying the salinity, nickel and nitrate content in the medium did not lead to any significant increase in the specific rates or steady-state H$_2$ concentrations (data not shown). On starving cells of nickel, however, a 15-fold decrease in the specific rates of fermentative H$_2$

### Table 1 | Parameters characterizing the dynamics of fermentative H$_2$ production in various cyanobacterial strains along with the effect of prior exposure to nighttime anoxia.

| Strain | Growth condition | $R_H$ [nmol (µg chl. a)$^{-1}$h$^{-1}$] | [$H_2$]$_{M}$ (µM) | $T_R$ (h) |
|--------|-----------------|---------------------------------|------------------|--------|
| L. aestuarii BL J | LODA | 5.3 ± 2.7 | 159.8 ± 30.4 | 2.24 |
| | CL | 2.8 ± 1.7 | 5.6 ± 4.0 | >24 |
| L. aestuarii BL AA | LODA | 4.0 ± 2.9 | 87.8 ± 43.0 | 2.24 |
| | CL | 8.4 ± 0.1 | 47.0 ± 2.9 | >24 |
| M. chthonoplastes | PCC 7420 | 0.4 ± 0.4 | 2.3 ± 0.6 | 2.24 |
| | BL AA | 0.4 ± 0.2 | 2.6 ± 1.2 | >24 |
| M. chthonoplastes BM 003 | LODA | 0.6 ± 0.1 | 2.7 ± 1.5 | 2.24 |
| | CL | 0.8 ± 0.1 | 35.5 ± 17.4 | >24 |
| Synechocystis sp. PCC 6803 | LODA | 0.3 ± 0.2 | 3.5 ± 2.8 | 5 ± 6.6 |
| | CL | 0.2 ± 0.1 | 2.7 ± 2.0 | 3.3 ± 3.2 |

Averages ± standard deviation with $n = 3$ independent determinations shown for each strain and condition. CL, Continuous Light; LODA, Light Oxic and Dark Anoxic, 12-h cycles.
production was observed, indicating the nickel dependency of the enzyme system involved in the process.

**FERMENTATION IN L. AESTUARII BL J**

Since *L. aestuarii* BL J displayed the highest *R*<sub>H</sub> and [H₂]<sub>M</sub>, it was chosen for further analysis. Along with H₂, fermentative production of lactate, ethanol, acetate, and CO₂ was observed. The ratio of the products of fermentation remained similar for the three independent replicate experiments. Small amounts of pyruvate were also excreted. Other common bacterial fermentation products such as formate, succinate, propionate, and butyrate were not detected. Table 2 depicts a quantitative balance analysis of the fermentation process in *L. aestuarii* BL J. Ethanol and acetate were produced in equimolar amounts. Lactate, ethanol, and acetate were seen in 1:2:2 molar ratios. One mol of H₂ was produced for every 2 moles of CO₂. In our experiments, the stoichiometry of carbon recovery and the recovery of H available was 100.07 and 100.58%, respectively.

**GENOMIC EVIDENCE**

Based on the whole genome sequence of the strain BL J (Kothari et al., 2013), orthologs of the following genes involved in glycogen metabolism were detected: Glucose-1-phosphate adenylyltransferase, glycogen synthase, ADP-glucose transglycosylase, glycogen branching enzyme (GH-57-type, archaeal), 1,4-alpha-glucan (glycogen) branching enzyme (GH-13-type), glycogen debranching enzyme, glycogen phosphorylase, and 4-alpha-glucanotransferase (amylomaltase). Orthologs of genes coding for all the enzymes involved in the pentose phosphate pathway and glycolysis, potential routes for the breakdown of glucose into pyruvate, were detected.

Genomic evidence for the presence of mixed acid fermentation pathway was clear. *L. aestuarii* BL J has orthologs coding for the enzymes pyruvate:ferredoxin oxidoreductase, ferredoxin:NADP oxidoreductase, bidirectional hydrogenase, lactate dehydrogenase, phosphotransacetylase, acetaldehyde dehydrogenase, alcohol dehydrogenase, and acetate kinase (see supplementary information for gene accession numbers). Based on the fermentation products obtained experimentally and the presence of these orthologs, the pathway proposed for fermentative degradation of glycogen is depicted in Figure 3. Notably, the gene for pyruvate formate lyase, involved in the reversible conversion of pyruvate and coenzyme-A into formate and acetyl-CoA, was not detected. This was consistent with a lack of formate amongst the fermentation products. Also worth noting is that no genomic evidence could be found for formate hydrogen lyase, involved in splitting of formate into H₂ and CO₂.

It was of interest to positively identify the enzyme responsible for the intense H₂ evolution observed in this study, as this may be the target of future studies. H₂ production in fermentative pathways can be either of *Enteric* type, with H₂ evolving from formate breakdown by formate hydrogen lyase, or of *Clostridial* type, wherein H₂ is evolved by pyruvate:ferredoxin oxidoreductase along with a hydrogenase (Hallenberg, 2009). Based on the genome, *L. aestuarii* BL J lacks both enzymes for the *Enteric* pathway, but contains a pyruvate:ferredoxin oxidoreductase and two [Ni–Fe] hydrogenases, an uptake-type hydrogenase and a bidirectional-type hydrogenase. One of these two must be involved, given that fermentative H₂ production in *L. aestuarii* BL J was found to be Ni-dependent. Because uptake hydrogenases are not known to produce H₂ under physiological conditions (Houchins and Burris, 1981; Houchins, 1984) we propose that the Ni–Fe bidirectional hydrogenase is the source of the fermentative H₂ produced in *L. aestuarii* BL J.

The strain BL J contains homologs of genes coding for nitrogenase, an enzyme that could in theory contribute to H₂ production in this strain. However, since the fermentation assays were performed in presence of nitrate, a condition in which the nitrogenase is known to be inactive (Ferreira, 2009), the role of the enzyme in production of H₂ is ruled out. The homologs of genes coding for uptake hydrogenase are also present in the strain BL J. However, the uptake hydrogenase is not capable of H₂ production in physiological conditions; in fact, it has to be knocked out to attain considerable H₂ production via the nitrogenase (Lindberg et al., 2002; Lindblad et al., 2002; Masukawa et al., 2002; Yoshino et al., 2007) and also via the fermentation pathway (Kim et al., 2006; Zhao et al., 2009).

**DISCUSSION**

**THE PHYSIOLOGICAL BASIS FOR STRONG FERMENTATIVE HYDROGEN PRODUCTION**

We had previously demonstrated that in standard H₂ production assays, strains of *Lyngbya* and *Microcoleus* displayed optimal H₂ evolution characteristics compared to a large number of other strains from diverse environments (Kothari et al., 2012). Since excess reductants were externally provided during that assay, the results likely are maximal potential specific rates and do not actually reflect physiologically realistic conditions. This prompted us to study the actual H₂ production capacity of these strains. Here we demonstrate that all the four strains studied had the capacity to produce fermentative H₂ naturally, at the expense of photo-synthetically fixed carbon, as did the standard strain *Synechocystis* sp. PCC 6803, which is included for reference. As reported earlier (Cournac et al., 2002), the fermentative H₂ evolution in

### Table 2 | Stoichiometry of fermentation of endogenous polyglucose and the fermentation mass balance of *L. aestuarii* strain BL J, after 24 h of dark incubation.

| Products      | mol | mol/100 mol glucose | mol C/100 mol glucose | H available | H available mol/100 mol glucose |
|---------------|-----|---------------------|-----------------------|-------------|--------------------------------|
| Glucose       | 13.27 | 100.00              | 600.00                | 24.00       | 2400.00                        |
| Pyruvate      | 0.46 | 3.48                | 10.44                 | 10.00       | 34.81                           |
| Lactate       | 5.91 | 44.54               | 133.61                | 12.00       | 534.44                          |
| Acetate       | 11.44 | 86.21               | 172.41                | 8.00        | 689.66                          |
| Ethanol       | 11.34 | 85.48               | 170.95                | 12.00       | 1025.72                         |
| H₂            | 8.37 | 63.07               | 0.00                  | 2.00        | 126.15                          |
| CO₂           | 113.04 | 113.04              | 113.04                | 0.00        | 0.00                            |

Recovery (%) | 100.07 | 100.58 |

H available is determined by oxidizing a compound to carbon dioxide with water. For example, C₆H₁₂O₆ + 6H₂O → 24H + 6CO₂. Thus, the H available value for glucose is 24 (Gottschalk, 1979).
**Synechocystis** sp. PCC 6803 commenced without any lag time, as was the case in **Microcoleus** and Lyngbya strains, and unlike what was observed in **Microcystis aeruginosa** M-176 (Asada and Kawamura, 1984). In general, the specific H$_2$ production rates and the steady-state concentrations under fermentative conditions were about an order of magnitude lower than the potential seen in standard assays in presence of excess reductant (Kothari et al., 2012). The **Microcoleus** and Lyngbya strains from the marine intertidal mats are capable of sustained fermentative H$_2$ production for at least 24 h. This was in accordance to the Pattern 2 H$_2$ production earlier reported in these strains via the hydrogenase activity assay wherein sustained H$_2$ production was also measured for up to 24 h. In comparison, the H$_2$ production phase did not last more than about 3 h in the standard strain **Synechocystis** sp. PCC 6803 (in agreement with Pattern 1 H$_2$ production earlier reported in this strain). This is consistent with the notion that cyanobacteria isolated from environments experiencing recurring nighttime anoxia (marine microbial mats) may be innately better H$_2$ producers, thus validating a general approach of bioprospecting in nature for biotechnologically useful properties of extant but little known microbes.

That H$_2$ production metabolism in intertidal mat harboring Lyngbya strains was enhanced by prior exposure to recurrent dark anaerobic growth conditions was expected under the premise that this type of fermentative metabolism would be regulated and thus subject to induction. This was clearly not the case in **Microcoleus** strains, where the capacity for fermentative H$_2$ generation, while high, seemed to be constitutive. Lyngbya typically colonizes microbial mats that desiccate frequently and are not exposed to nighttime anoxia as frequently as **Microcoleus**, which tends to dominate mats lower in the tidal gradient, with more recurrent flooding or always flooded (Javor and Castenholz, 1981; Rothrock and Garcia-Pichel, 2005). Perhaps the different responsiveness of the fermentative H$_2$ physiology has to do with this differential ecology. **Synechocystis** sp. PCC 6803, which has been in culture since 1968 does presumably not see many periods of dark anoxia during cultivation, and displayed a low-yield, non-inducible H$_2$ physiology.

When forced to ferment on a diel cycle, the highest specific rate and steady-state H$_2$ concentration was exhibited by **L. aestuarii** BL J. In comparison, to **Synechocystis** sp. PCC 6803, the initial rates of H$_2$ production were 17-fold higher in **L. aestuarii** BL J in the optimized fermentation assays. Most likely, this is due to increased content of bidirectional hydrogenase in the strain BL J or because **Synechocystis** sp. PCC 6803 employed alternative strategies in dark anaerobic conditions to regenerate NAD(P)$^+$ or because the Michaelis constant ($K_M$) of the bidirectional hydrogenase in the strain BL J is more favorable for H$_2$ production.

The major difference between **L. aestuarii** BL J and **Synechocystis** sp. PCC 6803 was that the latter exhibited a decline in the concentrations of H$_2$ leading to the consumption of almost all the H$_2$ produced. This decline was seen in presence of excess external reductants (Kothari et al., 2012) and internal reductants (**Figure 1**) implying that the concentration of reductants was not the limiting factor. The observed decline also has little to do with the loss of enzyme activity, since the bidirectional hydrogenase

---

**FIGURE 3** Proposed fermentation pathway of glycogen (polyglucose) fermentation in **L. aestuarii** BL J. Compounds in bold are fermentation products. The numbers refer to the enzymes involved: 1, pyruvate ferredoxin oxidoreductase; 2, ferredoxin NADP oxidoreductase; 3, bidirectional hydrogenase; 4, lactate dehydrogenase; 5, phosphotransacetylase; 6, acetaldehyde dehydrogenase; 7, alcohol dehydrogenase; 8, acetate kinase.
works in the direction of H$_2$ consumption and is thus still active. We must thus postulate a regulatory cause for these differences.

The optimized steady-state H$_2$ concentrations in fermentative assays in the strain BL J were only three-fold lower in magnitude than those seen in standard assays in the presence of excess reductant (Kothari et al., 2012). This is suggestive of the presence of a strong H$_2$ producing system, which, might be of particular fitness value in the uniquely H$_2$ accumulating intertidal mats. In optimized fermentation assays, the steady-state H$_2$ concentration in the strain BL J was 45-fold higher than that reached by *Synechocystis* sp. PCC 6803. Free sugar concentrations during fermentation are unlikely to be so different between the two cyanobacteria to account for such differences, and, even in the presence of excess externally provided reductants, the steady-state H$_2$ concentration in *Synechocystis* sp. PCC 6803 was 15-fold lower than the strain BL J (Kothari et al., 2012). Again here, the only possible explanation for this behavior is some sort of regulation of the hydrogenase enzyme in the strain PCC 6803.

On the basis of thermodynamics it may be theoretically possible (albeit difficult) for NAD(P)H to act as the sole electron donor to the bidirectional hydrogenase in *Synechocystis* sp. PCC 6803 during the fermentation assay. The intracellular ratio of [NAD(P)H] to [NAD(P)$^+$] required for *Synechocystis* sp. PCC 6803 to produce 3 μM H$_2$ at equilibrium is 7.63. In *Synechocystis* sp. PCC 6803, the measured [NADPH]/[NADP$^+$] is 3.03 under light anaerobic conditions (Cooley and Vermaas, 2001), but in dark anaerobic conditions the cell is even more reduced, so the ratios are expected to be higher. This is not unlike the ratios found in heterotrophic bacteria, which can go up to 6.66–0.7 (Decker et al., 1991), but in dark fermentation assay.

The optimized steady-state H$_2$ concentrations in fermentative assays in the strain BL J were only three-fold lower in magnitude than those seen in standard assays in the presence of excess reductant (Kothari et al., 2012). This is suggestive of the presence of a strong H$_2$ producing system, which, might be of particular fitness value in the uniquely H$_2$ accumulating intertidal mats. In optimized fermentation assays, the steady-state H$_2$ concentration in the strain BL J was 45-fold higher than that reached by *Synechocystis* sp. PCC 6803. Free sugar concentrations during fermentation are unlikely to be so different between the two cyanobacteria to account for such differences, and, even in the presence of excess externally provided reductants, the steady-state H$_2$ concentration in *Synechocystis* sp. PCC 6803 was 15-fold lower than the strain BL J (Kothari et al., 2012). Again here, the only possible explanation for this behavior is some sort of regulation of the hydrogenase enzyme in the strain PCC 6803.

On the basis of thermodynamics it may be theoretically possible (albeit difficult) for NAD(P)H to act as the sole electron donor to the bidirectional hydrogenase in *Synechocystis* sp. PCC 6803 during the fermentation assay. The intracellular ratio of [NAD(P)H] to [NAD(P)$^+$] required for *Synechocystis* sp. PCC 6803 to produce 3 μM H$_2$ at equilibrium is 7.63. In *Synechocystis* sp. PCC 6803, the measured [NADPH]/[NADP$^+$] is 3.03 under light anaerobic conditions (Cooley and Vermaas, 2001), but in dark anaerobic conditions the cell is even more reduced, so the ratios are expected to be higher. This is not unlike the ratios found in heterotrophic bacteria, which can go up to 6.66–0.7 (Decker et al., 1991), but in dark fermentation assay.

The intracellular pH of the strain BL J was as low as five, that would bring the required [NAD(P)H]/[NAD(P)$^+$] to 3.8, making it feasible to attain high H$_2$ concentrations using NAD(P)H as an electron donor. However, such intracellular pH would be unprecedented, particularly given the extreme sensitivity of cyanobacteria to even moderately acidic conditions.

**FERMENTATION PATHWAYS AMONG THE FILAMENTOUS NON-HETEROCYSTOUS CYANOBACTERIA**

*L. aestuaruii* BL J has all the products and the orthologs of genes coding for all enzymes of the mixed acid fermentation pathway. In view of the stoichiometric ratios of the products of fermentation (Table 2), our strain does not follow any one ideal fermentation pathway or even a combination of pathways. This is also the case for *Cyanothece* sp. PCC 7822 (Van der Oost et al., 1989), *Microcystis* sp. PCC 7806 (Moezelaar and Stal, 1994), and *Oscillatoria* sp. SAG 3192 (Moezelaar et al., 1996). The fermentation pathway is likely to be similar to that observed in *Microcystis* sp. PCC 7806 (Moezelaar and Stal, 1994) and *Gloeocapsa alpica* CALU 743 (Troshina et al., 2002).

Since *L. aestuaruii* BL J accumulates very high concentrations of H$_2$, it was of significance to characterize the molar conversion ratio of glucose to H$_2$. Theoretically 1 mole of glucose can give rise to a maximum of 4 moles of H$_2$ via fermentation (Thauer, 1977). Amongst cyanobacteria, this theoretical maximum has been observed only in *G. alpica* CALU 743 (Troshina et al., 2002). *Microcystis* sp. PCC 7806 yields 0.51 (Moezelaar and Stal, 1994) and *Cyanothece* sp. PCC 7822 yields 0.76 moles/mol (Van der Oost et al., 1989). *L. aestuaruii* BL J, at 0.6 moles/mol, was certainly not the best. The strong H$_2$ production characteristics of *L. aestuaruii* BL J cannot be attributed to a high glucose to H$_2$ molar conversion ratio, but likely reside in the bidirectional hydrogenase enzyme system.

It may be of interest to discuss the fermentative metabolism of strain BL J in comparison to the few other closely related cyanobacterial species whose fermentation has been studied in any detail. *L. aestuaruii* CCY 9616, a strain phylogenetically close to *L. aestuaruii* BL J (99% identity based on 16S rRNA), behaves quite differently: it can ferment trehalose, its osmoprotectant, via the homoacetate pathway into mostly acetate with small amounts of H$_2$ and CO$_2$ (Heyer et al., 1989). It can also ferment glycoly by a heterolactic fermentation pathway producing equimolar amounts of ethanol, lactate, and CO$_2$ (Heyer et al., 1989). It is unknown if *L. aestuaruii* BL J has the same osmoprotectant, or if it can be metabolized via a similar fermentation. This is however unlikely, in that neither the enzyme trehalase, involved in trehalose breakdown, nor the carbon monoxide dehydrogenase, a key enzyme for homoacetate fermentation, could be detected in its genome. Notably, the genome had orthologs of the enzymes trehalase synthase and trehalose-6-phosphate synthetase involved in trehalose synthesis. This may be worth a direct assessment. Another closely related strain, the thermophilic *O. terebriformis*, was also fermentatively distinct; it produced lactate by anaerobic degradation of photosynthetically accumulated glycolyen, without producing acetate, butyrate, isobutyrate or n-butyrate (Richardson and Castenholz, 1987). In comparison, *L. aestuaruii* BL J produces equimolar amounts of ethanol and acetate, a characteristic feature also seen in more distantly related *Oscillatoria* sp. SAG 3192, which also uses the mixed acid fermentation pathway (Moezelaar et al., 1996). Phylogeny seems thus to be a poor predictor of fermentative pathways.
In conclusion, the H₂ production in the strain BL J is unique in accumulating high steady-state concentrations of H₂ thus simplifying harvest of the end product and making it desirable for long-term applications.

AUTHOR CONTRIBUTIONS
Concept by Ankita Kothari and Ferran Garcia-Pichel, all the experimental work and analysis done by Ankita Kothari and with assistance from Prathap Parameswaran on HPLC analysis. Writing by Ankita Kothari and editorial help by Ferran Garcia-Pichel.

ACKNOWLEDGMENTS
This research and Ankita Kothari were supported by an endowment from B. Swoffette through the ASU President’s Fusion Fund. The funding source had no involvement in experimental design, data collection, analysis, report writing or decision to submit article. We thank Dr. Willem F. J. Vermaas, Dr. Cesar Torres, and Dr. Jens Appel for assistance with thermodynamic calculations.

SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00680/abstract

REFERENCES
Akkerman, I., Janssen, M., Rocha, J., and Wijffels, R. H. (2002). Photobiological hydrogen production: photochemical efficiency and bioenergy efficiency. Int. J. Hydrogen Energy 27, 1195–1208. doi: 10.1016/S0360-3199(02)00071-X
Anderson, K. L., Tayne, T. A., and Ward, D. M. (1987). Formation and fate of fermentation products in hot spring cyanobacterial mats. Appl. Environ. Microbiol. 53, 2343–2352.
Aoyama, K., Uemura, I., Miyake, J., and Asada, Y. (1997). Fermentative metabolism to produce hydrogen and organic compounds in a cyanobacterium, Spirulina platensis. J. J. Ferment. Bioeng. 83, 17–20. doi: 10.1016/S0922-338X(97)87320-5
Appel, J., Phunpruch, S., Steinmuller, K., and Schulz, R. (2000). The bidirectional NiFe-hydrogenase in Synechocystis sp. PCC 6803 works as an electron valve during photosynthesis. Arch. Microbiol. 173, 333–338. doi: 10.1007/s002030000139
Asada, Y., and Kawamura, S. (1984). Hydrogen evolution by Microcystis aeruginosa in darkness. Agric. Biol. Chem. 48, 2595–2596. doi: 10.1271/bbb1961.48.2595
Baudt, J. (1981). “Occurrence of benthic microbial mats in saline lakes,” in Lakes, ed W. D. Williams (Dordrecht: Springer), 87–111.
Cooley, J. W., and Vermaas, W. F. J. (2001). Succinate dehydrogenase and other respiratory pathways in thylakoid membranes of Synechocystis sp. strain PCC 6803: capacity comparisons and physiological function. J. Bacteriol. 183, 4251–4258. doi: 10.1128/JB.183.14.4251-4258.2001
Cournac, L., Mus, F., Bernard, L., Guedeney, G., Vignais, P., and Peliter, G. (2002). Limiting steps of hydrogen production in Chlamydomonas reinhardtii and Synechocystis PCC 6803 as analysed by light-induced gas exchange transients. Int. J. Hydrogen Energy 27, 1229–1237. doi: 10.1016/S0360-3199(02)00105-2
De Philippis, R., Margheri, M. C., and Vincenzini, M. (1996). Fermentation in symbiotic and free-living cyanobacteria. Arch. Hydrobiol. 83, 459–468.
ebert, A., and Brune, A. (1997). Hydrogen concentration profiles at the anoxic-oxic interface: a microsensor study of the hindgut of the wood-feeding lower termite Reticulitermes flavipes (Kollar). Appl. Environ. Microbiol. 63, 4039–4046.
Eibl, H. J. (1983). Aspects of hydrogen metabolism in nitrogen-fixing legumes and other plant-microbe associations. Annu. Rev. Plant Physiol. Plant Mol. Biol. 34, 105–136. doi: 10.1146/anneurev.pp.34.060183.000541
Eppley, R. W., Holmes, R. W., and Strickland, J. D. H. (1968). Sinking rates of marine phytoplankton measured with a fluorometer. J. Exp. Mar. Biol. Ecol. 1, 191–208.
Ernst, A., Kirschchenhofer, H., Diez, J., and Boger, P. (1984). Glycogen content and nitrogenase activity in Anabaena variabilis. Arch. Microbiol. 140, 120–125. doi: 10.1007/BF00445913
Faro, M., Gómez- Moreno, C., Stankovich, M., and Medina, M. (2002). Role of critical charged residues in reduction potential modulation of ferredoxin-NADP+ reductase. Eur. J. Biochem. 269, 2656–2661. doi: 10.1046/j.1432-1033.2002.02925.x
Ferreira, D. (2009). Nitrogen Fixation and Transcription/Regulation of Genes Related to Hydrogenases in Lyngbya sp. Porto: Universidade do Porto.
Fujita, Y., and Myers, J. (1965). Hydrogenase and NADP-reduction reactions by a cell-free preparation of Anabaena cylindrica. Arch. Biochem. Biophys. 111, 619–625.
Garcia-Pichel, F., Prufert-Bebout, L., and Muyzer, G. (1996). Phenotypic and phylogenetic analyses show Microcoleus chthonogetes to be a cosmopolitan cyanobacterium. Appl. Environ. Microbiol. 62, 3284–3291.
Gottschalk, G. (1979). Bacterial Metabolism. Gottingen, Germany: Springer. doi: 10.1007/978-1-4684-0465-4
Gutekunst, K., Chen, X., Schreiber, K., Kaspar, U., Makam, S., and Appel, J. (2014). The bidirectional NiFe-hydrogenase in Synechocystis sp. PCC 6803 is reduced by flavodoxin and ferredoxin and is essential under mixotrophic, nitrate-limiting conditions. J. Biol. Chem. 289, 1930–1937. doi: 10.1074/jbc.M113.252676.
Hallenbeck, P. C. (2009). Fermentative hydrogen production: principles, progress, and prognosis. Int. J. Hydrogen Energy 34, 7379–7389. doi: 10.1016/j.ijhydene.2008.12.080
Heyer, H., Stal, L., and Krumbein, W. E. (1989). Simultaneous heterolactic and acetate fermentation in the marine cyanobacterium Oscillatoria limosa incubated anaerobically in the dark. Arch. Microbiol. 151, 558–564. doi: 10.1007/BF00454875
Hoehler, T. M., Bebout, B. M., and Des Marais, D. J. (2001). The role of microbial mats in the production of reduced gases on the early Earth. Nature 412, 324–327. doi: 10.1038/35085554
Houchins, J. P. (1984). The physiology and biochemistry of hydrogen metabolism in cyanobacteria. Biochim. Biophys. Acta 768, 227–225. doi: 10.1016/0304-4173(84)90018-1
Houchins, J. P., and Burris, R. H. (1981). Comparative characterization of two distinct hydrogenases from Anabaena sp. strain 7120. J. Bacteriol. 146, 215–221.
Javor, B. J., and Castenholz, R. W. (1981). Laminated microbial mats, Oscillatoria limosa and Microcystis aeruginosa. Geomicrobiol. J. 3, 257–273. doi: 10.1007/978-1-4684-0465-4
Kothari, A., Potratfalva, R., and Garcia-Pichel, F. (2012). Diversity in hydrogen evolution from bidirectional hydrogenases in cyanobacteria from terrestrial, freshwater and marine intertidal environments. J. Biotechnol. 162, 105–114. doi: 10.1016/j.jbiorhe.2012.04.017
Kothari, A., Vaughn, M., and Garcia-Pichel, F. (2013). Comparative genomic analyses of the cyanobacterium, Lyngbya aestuarii BL J, a powerful hydrogen producer. Front. Microbiol. 4:363. doi: 10.3389/fmicb.2013.00363
Krause, G., Lundstrom, J., Barea, I. L., De La Cuesta, C. P., and Holmgren, A. (1991). Mimicking the active site of protein disulfide-isomerase by substitution of proline 34 in Escherichia coli thioredoxin. J. Biol. Chem. 266, 9494–9500.
Lee, H. C., Kim, J. S., Kang, W., and Kim, S. Y. (2009). Thymidine production by overexpressing NAD+ kinase in an Escherichia coli recombinant strain. Biotechnol. Lett. 31, 1929–1936. doi: 10.1007/s10529-009-0997-x
Lindberg, P., Schutz, K., Harpe, T., and Lindblad, P. (2002). A hydrogen-producing, hydrogenase-free mutant strain of Nostoc punctiforme ATCC 29133. Int. J. Hydrogen Energy 27, 1291–1296. doi: 10.1016/S0360-3199(02)00212-0
Lindblad, P., Christenson, K., Lindberg, P., Federov, A., Pinto, F., and Tysga, A. (2002). Photoproduction of H₂ by wild-type Anabaena PCC 7120 and
Cyanobacterial powerful fermentative hydrogen evolution

Kothari, A., Parameswaran, P., and Garcia-Pichel, F. (2014) Powerful fermentative hydrogen evolution of photosynthate in the cyanobacterium Lyngbya aestuarii BI mediated by a bidirectional hydrogenase. Front. Microbiol. 5, 680. doi: 10.3389/fmicb.2014.00680

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 September 2014; accepted: 20 November 2014; published online: 10 December 2014.

Citation: Kothari A, Parameswaran P and Garcia-Pichel F (2014) Powerful fermentative hydrogen evolution of photosynthate in the cyanobacterium Lyngbya aestuarii BI mediated by a bidirectional hydrogenase. Front. Microbiol. 5:680. doi: 10.3389/fmicb.2014.00680

This article was submitted to Microbial Physiology and Metabolism, a section of the journal Frontiers in Microbiology.

Copyright © 2014 Kothari, Parameswaran and Garcia-Pichel. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.