Microbiological Research 215 (2018) 130–140

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Insights into the carbonic anhydrases and autotrophic carbon dioxide fixation pathways of high CO_2 tolerant *Rhodovulum viride* JA756

Lakshmi Venkata Naga Satya Khandavalli[a], Tushar Lodha[a,1], Maaged Abdullah[c], Lalitha Guruprasad[c], Sasikala Chintalapati[b], Venkata Ramana Chintalapati[a,*]

[a] Department of Plant Sciences, School of Life Sciences, University of Hyderabad, P.O. Central University, Hyderabad, 500 046, India
[b] Bacterial Discovery Laboratory, Centre for Environment, IST, JNT University Hyderabad, Kukatpally, Hyderabad, 500085, India
[c] School of Chemistry, University of Hyderabad, P.O. Central University, Hyderabad, 500046, India

ARTICLE INFO

Keywords:
Biofixation
Autotrophic
Carbonic anhydrase
High CO_2cells
Rhodovulum viride JA756
CO_2 fixation pathway

ABSTRACT

Biofixation of CO_2 is being extensively investigated to solve the global warming problem. Purple non-sulfur bacteria are fast growers that consume CO_2 and produce beneficial biomass. Better the growth at higher CO_2 levels, more efficient are the strains for biofixation. Nine among fifty strains that were analyzed at elevated CO_2 levels responded with better growth. Considering its enhanced growth at high CO_2 and metabolic versatility, *Rhodovulum viride* strain JA756 was chosen to make further studies. Strain JA756 tolerates up to 50% (v/v) CO_2 with its optimum between 20–40% (v/v), yielding a biomass of 3.4 g. L^{-1}. The pattern of specific enzyme activity of carbonic anhydrase corresponded well with that of its growth. To gain insights into the genomic composition and genes related to carbonic anhydrases and CO_2 fixation, draft genome sequencing of JA756 was carried out which revealed the presence of two non-homologous genes encoding for β and γ carbonic anhydrases, both of which are assumed to be implicated in maintaining intracellular inorganic carbon concentration at equilibrium. Most of the genes involved in the Calvin pathway, reductive tricarboxylic acid pathway, 3-hydroxypropionate bicycle and C_4 pathways were found in the draft genome. While the experimental determinations of active roles of two of these pathways are still underway, the expression of key genes of Calvin and C_4 pathway suggest their functional role in the organism. Owing to its metabolic versatility, JA756 can be advantageous for biological CO_2 assimilation facilities located by the coastline, inland and also at wide ranges of CO_2 concentrations.

1. Introduction

Global warming induced by increasing concentrations of CO_2, a chief greenhouse gas (Olah et al., 2009) is causing a great concern. While the upper safety limit for atmospheric CO_2 is 350 ppm (Hansen et al., 2008; Ramanathan and Feng, 2008), the present CO_2 levels have already surpassed 400 ppm (NASA Global climate change - https://climate.nasa.gov/climate_resources/24/graphic-the-relentless-rise-of-carbon-dioxide/; Earth's CO_2 home page - https://www.co2.earth). While there is no best method, several physical, chemical and biological technologies are under investigation to mitigate CO_2 levels in atmosphere (Zheng et al., 2011). Photosynthetic organisms such as plants, algae and bacteria in addition to using atmospheric CO_2 for growth, also recycle it into potentially beneficial biomass; hence can be effectively employed as agents for biological CO_2 fixation. Though much of the work is focused on the plant systems, microorganisms are considered advantageous as they are capable of fixing several folds more CO_2 per unit area than plants (Salih, 2011). Moreover, since certain environments (such as power plants) emit high concentrations of CO_2 (15–20%) into the atmosphere, identification of high CO_2 tolerant

Abbreviations: 'C', carbon; 3-HP, 3-hydroxypropionate; 4-HB, 4-hydroxybutyrate; ACL, ATP-citrate lyase; ADP, adenosine diphosphate; CA, carbonic anhydrase; CBB, Calvin-Benson-bassham; CCL, citryl-CoA lyase; CCM, carbon concentrating mechanism; CCS, citryl-CoA synthetase; CHNS, carbon, hydrogen, nitrogen and sulfur; DC, dicarboxylate; DEAE/CM, diethylaminoethyl/carboxymethyl; kDa, kilodalton; Mb, mega base; OAA, oxaloacetic acid; OD, optical density; ORF, open reading frames; PDB, Protein Data Bank; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PEPCK, PEP carboxykinase; PNSB, purple non-sulfur anoxygenic phototrophic bacteria; PSI-BLAST, position-specific iterative basic local alignment search tool; RAST, rapid annotations using subsystems technology; rTCA, reductive tricarboxylic acid; RubisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase

* Corresponding author at: Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad, 500 046, Telangana, India.
E-mail addresses: cvr449@gmail.com, cvramana449@gmail.com (V.R. Chintalapati).
[1] Current address: National Centre for Microbial Resources, NCCS, Pune-411021.

https://doi.org/10.1016/j.micres.2018.07.006
Received 9 February 2018; Received in revised form 20 June 2018; Accepted 13 July 2018

species is equally important. Microalgae sit on the top of the choices for their small size, exceptionally high efficiency in energy conversion, CO_2 removal and usefulness of their byproducts (Salih, 2011; Zheng et al., 2011). There are several reports on CO_2 tolerant species of algae, such as *Chlorella*, *Scenedesmus* and *Cyanadium* which grow under 2–100% of CO_2 (Makarevičienė et al., 2011; Salih, 2011). At enriched CO_2 conditions, significant enhancement in algal growth rates as compared to ambient air were common and the rates declined only at much higher levels (Kodama et al., 1993; Nakano et al., 1995).

Purple non-sulfur anoxygenic phototrophic bacteria (PNSB) are a metabolically versatile group of bacteria which perform anoxygenic photosynthesis (Larimer et al., 2004; Pfennig, 1978). Most PNSBs are primary producers having the capability of photolithoautotrophic growth, in both aerobic as well as anaerobic illuminated environments, using inorganic compounds as electron donors and subsequently fixing CO_2 (Inui et al., 1999). They are known to use the Calvin pathway, mediated by RubisCO, for autotrophic CO_2 fixation. PNSB can prove highly beneficial for industrial applications similar to those for which microalgae are already being used in the field of biotechnology and microbiology (Becker, 1994), however their potentials are being recognized only recently (Merugu et al., 2012). Though PNSB are CO_2 fixers, equally environment friendly and share additional advantages over microalgae in having metabolic versatility, faster proliferation rates coupled to their harboring oxic as well as anoxic habitats (Sasikala and Ramana, 1995), the response of these bacteria to varying CO_2 concentrations has not been investigated. There is only a single report so far available which indicates a preliminary study on isolation of such bacteria from soils of elevated CO_2 chambers (Kumar et al., 2011). This study thus becomes an intriguing aspect considering the lack of research on growth of PNSB at varying CO_2 levels coupled to their CO_2 fixation pathways.

Carbonic anhydrases (CAs) are Zn containing metalloenzymes which catalyse the inter conversion between CO_2 and HCO_3^-. This enzymatic conversion is a vital process which not only helps the cells to concentrate CO_2 for fixation by cellular enzymes but also helps the cells to maintain an equilibrium between CO_2 and HCO_3^- to carry out cellular process (Smith and Ferry, 2000). CAs can be found located in cytoplasm or/and periplasm and their localization in the cell helps in understanding physiology (Smith et al., 1999). Prokaryotic CA's belong to 3 different classes- α, β and γ and there are no significant sequence homologies between representatives of different classes, implying their convergent evolution (Smith and Ferry, 2000). While genes encoding for one/two or all of the three classes of CAs can be present in a given organism, why three classes have evolved and what advantage each class provides over the other is still unknown (Smith and Ferry, 2000)! The ubiquitous occurrence of CAs in photosynthetic organisms has shown that there is a direct correlation between these enzymes and CO_2 metabolism (Bundy, 1976). Later a primary role of CAs in efficient autotrophic CO_2 fixation pathways was understood (Smith et al., 1999) and CAs are now considered as efficient biochemical markers for carbon sequestration (Warrier and Jayaraj, 2015). In oxygenic phototrophs, CA and RubisCO are associated in the carboxysomes and pyrenoids, respectively (Price et al., 1992; Puskas et al., 2000), where the former accumulate CO_2 at the active site of RubisCO, thus increasing the availability of CO_2 per minute for efficient fixation of CO_2. When CAs catalyse the reversible reaction, HCO_3- accumulates in the cell aiding in the progress of C_4 pathway (Berg, 2011).

The present study aims in understanding the CO_2 tolerance and thereby CO_2 fixation capabilities of a PNSB, *Rhodovulum viride* JA756 with further establishing a correlation between CO_2 concentration and its CA activity. Purification, characterization and protein chemistry of CA is also described. Genome sequence analysis further helped in gaining insights into the possible CO_2 fixation pathways operating in this organism.

2. Materials & methods

2.1. Cultivation conditions

Pure cultures of purple bacteria were obtained from the stock cultures available in our laboratory (Supplementary Table S1 online). For initial screening of these bacteria for photolithoautotrophy, cultures were cultivated anaerobically (28 ± 2 °C; light 2,400 lx) for 2–4 days on agar slants in a selective mineral medium (Lakshmi et al., 2009) supplemented with sodium bicarbonate (0.1%, v/v) as the sole carbon source and Na_2S (1 mM) as electron donor. For further experimentations, a buffered liquid medium provided with CO_2 as the sole carbon source was employed. For comparisons at different CO_2 concentrations, buffered medium without CO_2 was maintained as a control (unless otherwise mentioned) while the test samples contained varying concentrations of CO_2 (0.1–100%, v/v of gas phase unless mentioned). Growth was monitored by measuring an increase in optical density (OD_{660nm}), dry weight or bacteriochlorophyll content. For the adaptation studies of potent strains, the cultures were acclimatized to CO_2 by maintaining them for few subcultures in a medium supplemented with desired CO_2.

2.2. Biomass, carbon content and CO_2 fixation rate of *Rhodovulum viride* JA756

Triplicate samples of strain JA756 at each of the CO_2 concentration was checked for biomass concentration ($g.L^{-1}$) at regular time intervals by measuring the bacteriochlorophyll content (Blankenship et al., 1995; Clayton, 1966), optical density (OD_{660nm}) spectrophotometrically and a calibration curve of OD_{660nm} versus dry biomass was obtained. Productivity ($g.L^{-1}.day^{-1}$) was obtained according to Schmidell et al. (2001). Biomass carbon content was determined using a Thermo Finnigan Flash EA 1112 series CHNS (carbon, hydrogen, nitrogen and sulfur) element analyzer calibrated to 100% value using a cystine standard (de Morais and Costa, 2007a). CO_2 fixation rate ($g.L^{-1}.day^{-1}$) at the respective CO_2 concentration was calculated from the CHNS biomass carbon content values (Zheng et al., 2011).

2.3. Genome sequencing and annotations

Genomic DNA was isolated from strain JA756 and sequencing was carried out using Illumina HiSeq 2500 by paired end chemistry which was outsourced from SciGenom Labs. The sequence data were *de novo* assembled using the SPAdes assembly software. The raw reads were checked for the quality and trimmed by using fastQC and fastx tools, respectively. tRNA and rRNA were predicted using RNAmmer. Annotations were performed using the Rapid Annotations using Subsystems Technology (RAST) server (Aziz et al., 2008).

2.4. RNA isolation and real time analysis

For transcription analysis of β-, γ- CA, small-, large subunits-RubisCO and PEPCK, JA756 was grown photolithoautotrophically at ambient CO_2 condition. The cells maintained as five replicates each were then treated with selective CO_2 concentrations of 0.1%, 5%, 10% and 20% during their mid-log phase. After a definite time of exposure to CO_2, the replicates from individual experiment were pooled together, to minimize biological variation and the cells were harvested by centrifugation (5000 rpm for 5 min, 4 °C), snap frozen in liquid nitrogen and stored at −80 °C until further process. Cells grown photo-organoheterotrophically (with pyruvate [22 mM] as carbon source and electron donor) were maintained as untreated control calibrators. The total RNA was isolated by the Trizol method (Rio et al., 2010) and cDNA synthesis was carried out for 2 µg RNA. Real-time PCR using gene-specific primers (Supplementary table S3 online) was carried out in triplicates for each of the above genes along with *rpoZ* as a