Towards sustainable bioplastic production in resource limited environments using the photoferroautotrophic and photoelectroautotrophic bacterium *Rhodopseudomonas palustris* TIE-1

Tahina Onina Ranaivoarisoa, Karthikeyan Rengasamy, Michael S. Guzman, Rajesh Singh, and Arpita Bose*

Department of Biology, Washington University in St. Louis, One Brookings Drive, St. Louis, 63130

*Corresponding author: Arpita Bose

Department of Biology, Washington University in St. Louis,

One Brookings Drive, St. Louis, 63130

Email: abose@wustl.edu

Phone: (314) 935-6236

Fax: (314) 935-4432

Keywords: Polyhydroxybutyrate, *Rhodopseudomonas palustris* TIE-1, PHB, Photoferroautotrophy, Photoelectroautotrophy, Iron oxidation

ABSTRACT

Bioplastics are an attractive alternative to petroleum-derived plastics because of the harmful environmental effects of conventional plastics and the impending fossil fuel crisis. Polyhydroxybutyrate (PHB) is a well-known bioplastic that is produced by several microbes using organic carbon sources. Autotrophic (using carbon dioxide or CO_2) PHB production is reported for only a few organisms. Sustainable PHB bioproduction using other autotrophic microbes needs to be explored. *Rhodopseudomonas palustris*, a metabolically versatile purple non sulfur bacterium is known to produce PHBs under photoheterotrophic conditions. *Rhodopseudomonas palustris* strain TIE-1 demonstrates even more metabolic versatility by using electron sources such as ferrous iron and poised electrodes for photoautotrophy. Here we report that TIE-1 is able to produce PHB under photoferroautotrophic (light - energy source, ferrous iron - electron source and CO_2 - carbon source) and photoelectroautotrophic (light - energy source, poised electrodes - electron source and CO_2 - carbon source) growth conditions. PHB accumulation was observed both under nitrogen (N_2) fixing (where the nitrogen source was N_2 gas) and non-N_2 fixing (with ammonium chloride) conditions. For comparison, we determined PHB production under chemo-, photoheterotrophic and photoautotrophic conditions using hydrogen as the electron donor. Photoferroautotrophic and photoelectroautotrophic PHB production was on par with that observed from organic carbon substrates such as butyrate. Under photoheterotrophic conditions, forcing the cells to fix N_2 increased PHB production. However, N_2 fixation did not enhance PHB production during photoautotrophic growth. RNASeq and RT-qPCR suggests that expression of the PHB cycle genes remains unchanged under the growth conditions tested.

IMPORTANCE

PHB bioproduction was reported nearly a century ago. Despite its astonishing properties, PHB's market competitiveness is affected by high production costs. Use of waste products such as molasses and industrial food waste for microbial PHB production can lower costs. An alternative cost-effective strategy is to employ microbes that use abundant and renewable resources. Toward that end, we report the ability of *Rhodopseudomonas palustris* TIE-1 to produce PHB under many growth conditions including photoautotrophic growth using electrons from ferrous iron and poised electrodes. Photoferroautotrophic PHB production is a sustainable and low-cost strategy because of the abundance of iron, carbon dioxide and light on Earth. Photoelectroautotrophic PHB production can also be a useful approach in areas that produce electricity sustainably. Overall, our observations open new doors for sustainable bioplastic production not only in resource-limited environments on Earth, but also during space exploration and for *in situ* resource utilization (ISRU) on other planets.

Polyhydroxybutyrate (PHB), a member of the polyhydroxyalkanoate (PHA) family is the most common and well-studied biopolymer produced by bacteria (1). PHB is a potent substitute for conventional petroleum-based plastics because of many desirable properties. These include thermoresistance, moldability and biodegradability (2). PHB is also useful in many medical applications such as drug delivery, reconstructive surgery and bone tissue scaffolding (3). PHB production is currently not cost-effective (4). In order to reduce production costs, researchers have used many different carbon sources, which fall into two categories: 1) food wastes

including sugar beet, soy and palm oil molasses or 2) cheap pure substrates as feedstock for PHB bioproduction. In some cases, less cost-effective substrates such as glucose that compete as food sources have also been tested for PHB production (1).

Autotrophic PHB production has been demonstrated by only a handful of organisms and remains an underexplored strategy. As carbon dioxide concentrations rise in our atmosphere, sustainable carbon-neutral bioproduction strategies need to be explored further. Several autotrophs produce PHBs including *Ideonella* sp., and *Cupriavidus necator* (5). Photoautotrophs represent an even more attractive group of organisms for bioproduction due to their ability to use solar energy for biosynthesis. To this end, researchers have shown that cyanobacteria can produce PHB while performing oxygenic photoautotrophy (6). Anoxygenic phototrophic bacteria expand the repertoire of electron donors that can be used for such bioproduction. Numerous groups have reported that purple non sulfur bacteria can produce PHBs during photoheterotrophic growth **(**7,8, 9). *Rhodopseudomonas palustris* is a biotechnologically important PNSB that can produce hydrogen under various growth conditions. This ability has sparked research on *R. palustris* strains to understand how PHB synthesis affects biohydrogen production (10, 11)**.** To the best of our knowledge, PHB bioproduction under photoautotrophic growth using inorganic electron donors has not been explored systematically in PNSB (5, 12).

To fill this knowledge gap, here we investigated the ability of the versatile photoautotroph PNS *Rhodopseudomonas palustris* TIE-1 (here on TIE-1) to accumulate PHB. We chose TIE-1 because it demonstrates extraordinary metabolic versatility even when compared to other *R. palustris* strains. For instance, similar to other *R. palustris* strains it can grow chemotrophically in rich medium and photoheterotrophically using many different organic carbon sources (13). It can use many different electron donors for photoautotrophic growth such

as hydrogen, thiosulfate, Fe(II) and also poised electrodes (13, 14). Like other *R. palustris* strains, TIE-1 can fix dinitrogen gas (13). Importantly, TIE-1 is the only genetically tractable iron oxidizing and electrotrophic photoautotroph available (13, 14). Together, these abilities make TIE-1 an ideal candidate for testing photoautotrophic PHB production using unique inorganic electron donors.

To investigate the production of PHB by TIE-1, the strain was grown under various growth conditions. These include chemoheterotrophic growth in aerobic yeast extract-peptone (YP); and photoheterotrophically (anaerobic) using different carbon sources under both non-nitrogen and nitrogen (N_2) fixing conditions. Aerobic growth on YP had the longest generation time compared to photoheterotrophic growth (Supplementary Table S3). Interestingly, YP grown cells produced the highest amount of PHB (13.94 g/L) amongst all the heterotrophic conditions tested under non-N_2 fixing conditions (Supplementary Table S3 and Figure 1 Panel A (a and b). TIE-1 produced the lowest amount of PHB (1.76 g/L) on succinate with ammonium chloride (NH_4Cl) as the nitrogen source. PHB production varied from 2.22 g/L to 3.19 g/L when cells were grown in acetate, butyrate and 3-hydroxybutyrate (Supplementary Table S3 and Figure 1 Panel A (b). These low PHB amounts might be linked to the availability of fixed nitrogen and an abundant carbon source, which could cause TIE-1 to increase cell numbers but not accumulate PHB as a carbon reserve. We tested PHB production under N_2 fixing conditions by replacing NH_4Cl in the medium with gaseous N_2. In general, the absence of NH_4Cl delayed cell growth and resulted in a longer lag (approximately 2 times higher) when compared to growth in the presence of NH_4Cl. We also observed an increase in the maximum OD_{660} under acetate and butyrate whereas no significant difference was observed under succinate and 3-hydroxybutyrate growth under N_2 fixing conditions (Supplementary Table S3). PHB accumulation increased on

all the photoheterotrophic conditions tested under N_2 fixing conditions. PHB production doubled when cells were grown on 3-hydroxybutyrate, tripled on succinate, quadrupled on acetate, and increased 10-fold to 23.12 g/L on butyrate (Supplementary Table S3 and Figure 1 Panel A (b)).

Three photoautotrophic growth conditions using hydrogen, Fe(II) or poised electrodes as electron donors were tested for PHB production. Photoautotrophic growth on hydrogen served as the starting point where we adapted TIE-1 by passaging cells grown on YP to hydrogen growth, followed by another hydrogen transfer with or without NH_4Cl. Under N_2 fixing conditions, we observed that PHB production was as high as that observed during aerobic growth on YP (Figure 1 panel A (c) and Supplementary Table S3). Using hydrogen photoautotrophically, TIE-1 produced 11.69 g/L PHB under N_2 fixing conditions and 8.4 g/L in the presence of NH_4Cl (Figure 1 panel A (c) and Supplementary Table S3). Growth on hydrogen under non-N_2 fixing conditions was found to favor a higher maximal OD_{660nm} of 1.16 and a shorter generation time of 34 hours when compared to N_2 fixing conditions which showed a maximal OD_{660nm} of 0.54 and a generation time of 41 hours (Supplementary Table S3). Photoferroautotrophic growth in the presence of NH_4Cl supported PHB production levels higher than those observed under YP growth, and close to those noted under the most productive photoheterotrophic condition i.e. butyrate with NH_4Cl (Figure 1 panel A (c)). The presence of PHB intracellular granules was confirmed by Scanning transmission electron microscopy-electron energy loss spectroscopy (STEM-EELS; shown for the most productive conditions with NH_4Cl i.e. butyrate, photoferroautotrophy and photoelectroautotrophy in Figure 2 panel B). Under photoelectroautotrophic conditions the generation time in the presence of NH_4Cl was 76 hours and 82 hours under N_2 fixing conditions. The control TIE-1 grown in open circuit condition (no poised electrode) did not show any growth under N_2 fixing or non-fixing growth condition

(Supplementary Table S3 and Figure 1 panel C (a)). After 96 hours of growth, the electron uptake was almost half under N_2 fixing conditions compared to growth in presence of NH_4Cl: 0.93 versus 1.92 μA/cm^2 (Figure 1 panel C (b) and Supplementary Table S4). The PHB concentration was 9.1 g/L under N_2 fixing conditions and 9.6 g/L in the presence of NH_4Cl. STEM-EELS images revealed presence of multiple PHB granules compared to a few large granules under photoferroautotrophy and butyrate growth conditions (Figure 2 panel B (c)). Slightly lower maximum planktonic OD_{660} was obtained under N_2 fixing conditions compared to growth with NH_4Cl (Figure 1 panel A (c), Panel C (b) and Supplementary Table S3). When grown under open circuit, no PHB was detected from the culture control both under N_2 fixing and with NH_4Cl (Supplementary Table S3) suggesting that PHB was used by the cells in the absence of an electron donor. In addition to carbon storage, PHB is suggested to act as an electron sink for bacteria especially under N_2 fixing conditions (15). Redox imbalance might occur during photoferroautotrophic and photoelectroautotrophic growth, where TIE-1 cells produce more NADPH than can be utilized by other processes in the cells such as carbon fixation. Under these conditions PHB synthesis might provide a much-needed electron sink, thus explaining the high level of PHB accumulation. McKinlay et al. have suggested a similar role for PHB synthesis in *R. palustris* CGA009 when it is grown under N_2 deplete conditions and supplemented with acetate (11). To determine whether the expression of the genes involved in PHB synthesis and degradation change with changes in PHB levels, we performed transcriptomic analysis using RNASeq and reverse transcription quantitative PCR (RT-qPCR). These include genes encoding the first enzyme β-ketothiolase (acetyl-CoA acetyltransferase - PhaA that condenses two acetyl CoA molecules into acetoaetyl CoA; the second enzyme, an acetoacetyl CoA reductase that catalyzes the formation of the 3-hydroxybutyrate monomer from

acetoacetyl CoA (PhaB); and finally, the polymerase (PhaC) that synthesizes the polymer PHB. When bacteria need to mobilize PHB, depolymerization of the granules is performed by the PHB depolymerase (PhaZ). These genes involved in PHB pathway are well characterized in the PHB producing model bacteria *Ralstonia eutropha* (16, 17, 18). The potential roles of analogous enzymes and proteins in TIE-1 are summarized in a simplified PHB cycle depicted in Supplementary Figure 1. Similar to *R. eutropha* (19), and *Bradyrhizobium japonicum* (20), there are multiple isozymes for the PhaA and PhaC enzymes respectively in TIE-1. In *Bradyrhizobium diazotoefficiens*, an organism closely related to *R. palustris*, PhaR regulates PHB biosynthesis by repressing the expression of $phaC_1$ and $phaC_2$. In addition, PhaR regulates PhaP, a protein that binds to the surface and controls the number and size of the PHB granules. PhaR also binds to PHB granules during PHB synthesis and dissociates from it as the granule size grows (21). RNASeq analysis showed that the genes for only a few of the isozymes for PhaA, PhaB and PhaC are significantly expressed under the growth conditions tested (Supplementary Table S5, S6 and S7). RT-qPCR analysis was performed on the isozymes that showed average RPKM values above 150. Based on this criterion, we chose the *phaA* gene with the locus tag Rpal_0532 and the *phaB* gene with the locus tag Rpal_0533 as the primary *phaA* and *phaB* genes (Supplementary Table S8). The fact that these two genes appeared to be part of an operon supported our selection (Supplemental figure S1 (b)). Moreover, the gene for *phaR* (Rpal_0531) is next to the *phaA* (Rpal_0532) gene but expressed from the opposite strand. RT-qPCR corroborated the RNASeq data, which together show that the expression of the genes in the PHB cycle does not change under different growth conditions (Figure 2. Panel A a-b). McKinlay et al. reported similar results when *R. palustris* CG009 was grown photoheterotrophically on acetate, under nitrogen deprivation when dinitrogen gas was replaced by argon (11). They observed that

although PHB accumulated under these conditions, no change in PHB biosynthesis genes was notable (11).

Implications

Here, we report PHB production by *R. palustris* TIE-1 under chemoheterotrophic (in YP); photoheterotrophic (using organic carbon sources: succinate, acetate, butyrate or 3-hydroxybutyrate); and photoautotrophic (using hydrogen, Fe(II) or poised electrodes as electron donor) conditions. Photoheterotrophic growth under N_2 fixing condition yielded higher PHB. The highest PHB production was obtained from butyrate under N_2 fixing condition: 23.12 g/L. This production is just 2-fold lower than the autotrophic organism, *Cupriavidus necator* NCIMB 11599 (41.5 g/L) when it's grown with wheat based media under nitrogen limited conditions (12). When compared to other autotrophs, the ability of TIE-1 to produce PHB under a large range of growth conditions presents a major advantage. In addition to its ability to produce PHB under heterotrophic conditions, TIE-1 can produce PHBs under unique photoautotrophic growth condition using H_2, Iron (II) or poised electrodes as electron source. Its nitrogen fixation ability makes TIE-1 even more metabolically versatile. Both under NH_4Cl and N_2 fixing conditions, PHB concentration during autotrophic growth was comparable to that produced under chemoheterotrophic growth in YP and was even higher than YP under photoferroautotrophic growth with NH_4Cl (19.3 g/L).

Our results expand the substrate range that can be used by microbes for PHB production. TIE-1's unique metabolic abilities such as photoferroautotrophy and photoelectroautotrophy can be used in novel sustainable PHB bioproduction platforms. Iron is the fourth most abundant

element on Earth, and by using iron with solar energy TIE-1 can be used to produce biochemicals such as PHB while capturing the potent and abundant greenhouse gas, carbon dioxide (22, 23). Such a strategy will be especially valuable in resource-limited environments on Earth. Electricity can be produced renewably using solar and wind energy on many parts of our planet. In combination with solar energy and carbon dioxide, TIE-1 can be used to produce PHB and also other biochemicals using renewable electricity. Because TIE-1 can produce PHBs using many organic carbon sources, the use of waste materials as substrates is conceivable. Thus, TIE-1 represents a very metabolically versatile microbe that should be explored further for biochemical production not only on Earth but also during space exploration, and for *in situ* resource utilization (ISRU) on planets like Mars rich in iron, sunlight carbon dioxide and nitrogen (24). TIE-1 based bioproduction strategies should be also be considered for waste management on Earth and “in flight” and “post arrival” during space exploration.

Acknowledgements

We are grateful to Josh Blodgett and Yifei Hu for their support during PHB analysis using LC-MS. We appreciate funding provided by The David and Lucile Foundation as well as the Department of Energy (grant number DESC0014613).

References

1. Verlinden RA, Hill DJ, Kenward MA, Williams CD, Radecka I. 2007. Bacterial synthesis of biodegradable polyhydroxyalkanoates. J Appl Microbiol 102:1437-49.

2. Chen G-Q. 2010. Plastics from bacteria: natural functions and applications. Springer, Heidelberg; New York.

3. Manavitehrani I, Fathi A, Badr H, Daly S, Negahi Shirazi A, Dehghani F. 2016. Biomedical applications of biodegradable polyesters. Polymers 8:20.

4. Sabbagh F, Muhamad II. 2017. Production of poly-hydroxyalkanoate as secondary metabolite with main focus on sustainable energy. Renewable & Sustainable Energy Reviews 72:95-104

5. Khosravi-Darani K, Mokhtari Z-B, Amai T, Tanaka K. 2013. Microbial production of poly(hydroxybutyrate) from C1 carbon sources. Appl Microbiol Biotechnol 97:1407–1424

6. Troschl C, Meixner K, Drosg B. 2017. Cyanobacterial PHA Production-Review of Recent Advances and a Summary of Three Years' Working Experience Running a Pilot Plant. Bioengineering (Basel)

7. Mukhopadhyay M, Patra A, Paul AK. 2005. Production of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by *Rhodopseudomonas palustris* SP5212. World Journal of Microbiology and Biotechnology. 21(5):765–769

8. Mukhopadhyay M, Patra A, Paul AK. 2013. Phototrophic Growth and Accumulation of Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by Purple Nonsulfur Bacterium *Rhodopseudomonas palustris* SP5212. J. of Polymers 2013

9. Merugu R, Girisham S., Reddy S.M. 2010. Production of PHB (Polyhydroxybutyrate) by *Rhodopseudomonas palustris* KU003 under nitrogen limitation. International Journal of Applied Biology and Pharmaceutical Technology. 676. ISSN 0976-4550

10. Wu SC, Liou SZ, Lee CM. 2012. Correlation between bio-hydrogen production and polyhydroxybutyrate (PHB) synthesis by *Rhodopseudomonas palustris* WP3-5. Bioresource Technology 113:44-50

11. McKinlay JB, Oda Y, Ruhl M, Posto AL, Sauer U, Harwood CS. 2014. Non-growing *Rhodopseudomonas palustris* increases the hydrogen gas yield from acetate by shifting from the glyoxylate shunt to the tricarboxylic acid cycle. J Biol Chem 289:1960-70.

Parida B, Iniyan S, Goic R. 2011. A review of solar photovoltaic technologies. Renewable & Sustainable Energy Reviews 15:1625-1636.

12. Pagliano G, Ventorino V, Panico A, Pepe O. 2017. Integrated systems for biopolymers and bioenergy production from organic waste and by-products: a review of microbial processes. Biotechnology for Biofuels 10

13. Jiao Y, Kappler A, Croal LR, Newman DK. 2005. Isolation and characterization of a genetically tractable photoautotrophic Fe(II)-oxidizing bacterium, *Rhodopseudomonas palustris* strain TIE-1. Appl Environ Microbiol 71:4487-96.

14. Bose A, Gardel EJ, Vidoudez C, Parra EA, Girguis PR. 2014. Electron uptake by iron-oxidizing phototrophic bacteria. Nat Commun 5:3391.

15. Dawes EA. 1988. Polyhydroxybutyrate: an intriguing biopolymer. Biosci Rep 8:537-47.

16. Peoples OP, Sinskey AJ. 1989. Poly-beta-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (phbC). J Biol Chem 264:15298-303.

17. Peoples OP, Sinskey AJ. 1989. Poly-beta-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. Characterization of the genes encoding beta-ketothiolase and acetoacetyl-CoA reductase. J Biol Chem 264:15293-7.

18. Uchino K, Saito T, Jendrossek D. 2008. Poly(3-hydroxybutyrate) (PHB) depolymerase PhaZa1 is involved in mobilization of accumulated PHB in *Ralstonia eutropha* H16. Applied and Environmental Microbiology 74:1058-1063.

19. Slater S, Houmiel KL, Tran M, Mitsky TA, Taylor NB, Padgette SR, Gruys KJ. 1998. Multiple beta-ketothiolases mediate poly(beta-hydroxyalkanoate) copolymer synthesis in *Ralstonia eutropha*. J Bacteriol 180:1979-87.

20. Quelas JI, Mongiardini EJ, Perez-Gimenez J, Parisi G, Lodeiro AR. 2013. Analysis of Two Polyhydroxyalkanoate Synthases in *Bradyrhizobium japonicum* USDA 110. Journal of Bacteriology 195:3145-3155.

21. Quelas JI, Mesa S, Mongiardini EJ, Jendrossek D, Lodeiro AR. 2016. Regulation of Polyhydroxybutyrate Synthesis in the Soil Bacterium *Bradyrhizobium diazoefficiens*. Applied and Environmental Microbiology 82:4299-4308.

22. Greenwood NN, Earnshaw A. 1984. Chemistry of the elements. Pergamon Press, Oxford.

23. Parida B, Iniyan S, Goic R. 2011. A review of solar photovoltaic technologies. Renewable & Sustainable Energy Reviews 15:1625-1636

24. Menezes AA, Cumbers J, Hogan JA, Arkin AP. 2015. Towards synthetic biological approaches to resource utilization on space missions. Journal of the Royal Society Interface 12.

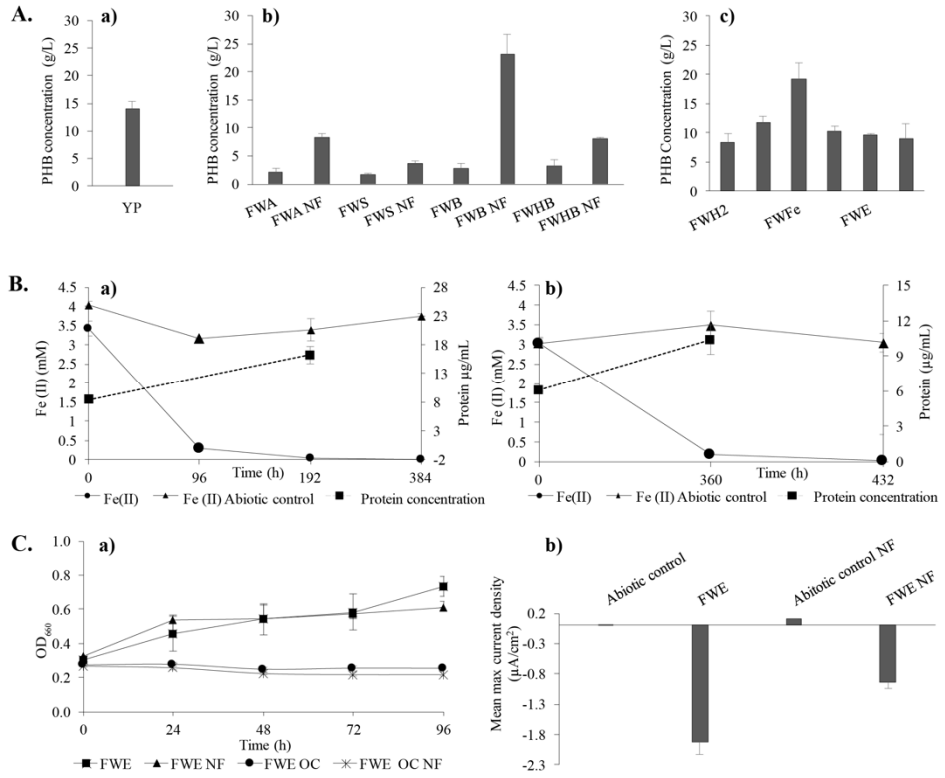
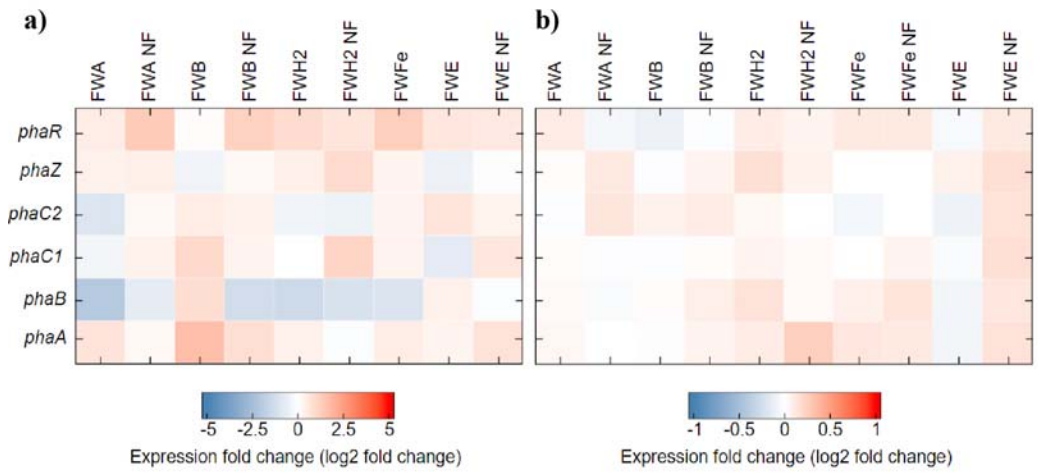


Figure 1. PHB production by TIE-1. Panel A. (a) Under chemoheterotrophy in YP. (b) Under anaerobic photoheterotrophic conditions with ammonium chloride (NH_4Cl) or under nitrogen fixing conditions (NF). Cells were grown with Fresh Water (FW) media supplemented with 10 mM succinate (FWS), acetate (FWA), butyrate (FWB) or hydroxybutyrate (FWHB). Cells were grown to late exponential phase: OD_{660}~0.7. (c) Under anaerobic photoautotrophic conditions in H_2CO_2 (FWH2) grown to an of OD_{660}~0.7, in 5 mM Iron (II) chloride (FWFe) for 8 days of growth with NH_4Cl, 15 days of growth under nitrogen fixing condition (FWFe NF) and photoelectroautotrophy with NH_4Cl (FWE), and for 4 days under nitrogen fixing conditions (FWE NF). Panel B. Iron (II) oxidation and protein concentration during the growth of TIE-1 under photoferroautotrophic growth. Cells were grown in FW media supplemented with 5 mM Iron (II) chloride and NH_4Cl in panel (a) and under nitrogen fixation condition in panel (b). Panel C. (a) OD_{660} values of the growth of TIE-1 grown under photoelectroautotrophy in FW supplemented with NH_4Cl (FWE), under nitrogen fixing condition (FWE NF) or when the electrodes are not poised, called open circuit (OC). The two open circuit conditions shown are FWE OC and FWE OC NF. (b) Mean maximum current density ($\mu A/cm^2$) (n=3) of FWE and FWE NF conditions and the abiotic controls.

A.


a)
FWA
FWA NF
FWB
FWB NF
FWH2
FWH2 NF
FWFe
FWE
FWE NF
phaR
phaZ
phaC2
phaC1
phaB
phaA
-5 -2.5 0 2.5 5
Expression fold change (log2 fold change)
b)
FWA
FWA NF
FWB
FWB NF
FWH2
FWH2 NF
FWFe
FWFe NF
FWE
FWE NF
-1 -0.5 0 0.5 1
Expression fold change (log2 fold change)

B.

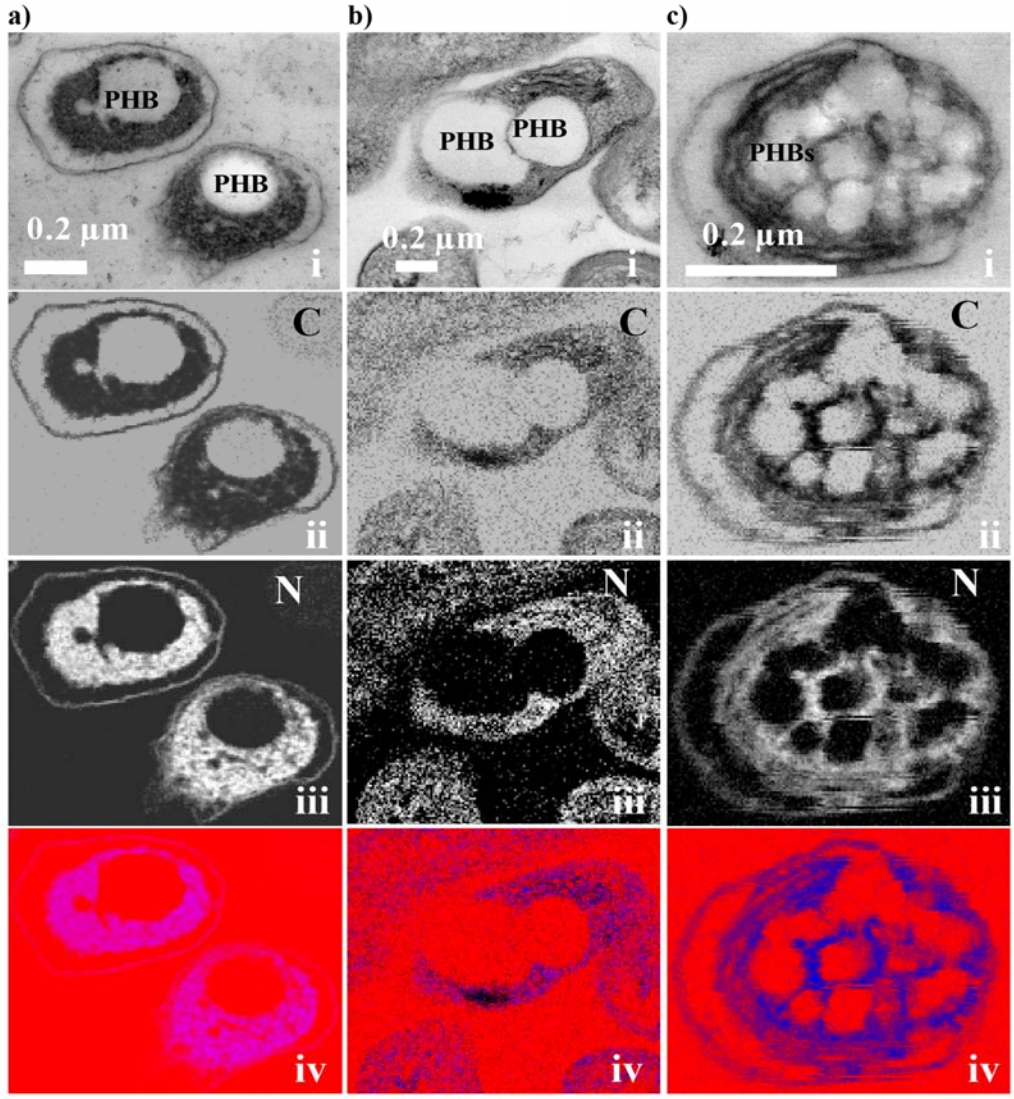

a)
b)
c)
PHB
PHB
PHB
PHB
PHBs
0.2 µm
0.2 µm
0.2 µm
i
C
ii
N
iii
iv

Figure 2. Panel (A). Heat map showing log2 fold change expression of PHB genes. (a) from RNA sequencing analysis. (b) from RT-qPCR. Results are from different growth conditions as described previously in Fresh Water (FW) medium with 10 mM succinate (FWS), acetate (FWA), butyrate (FWB), hydroxybutyrate (FWHB), or with H_2CO_2 (FWH2), with 5 mM Iron (II) chloride (FWFe), and under photoelectroautotrophy (FWE) in the presence of NH_4Cl or under nitrogen fixing condition (NF). Results are from n=3. (B) STEM-EELS images of TIE-1 with PHB granules and corresponding carbon and nitrogen maps grown under FW with 10 mM butyrate Panel (a), 5 mM Iron (II) chloride in Panel (b), and photoautoelectrophy in Panel (c) conditions. Bright areas represent the dominance of corresponding element. Each panel comprises, (i) TEM bright field image, (ii) nitrogen map (N) (iii) carbon map (C) and (iv) composite where red represents carbon, blue nitrogen.