**INTRODUCTION**

The sun is the dominant source of energy on Earth, and many organisms have evolved ways to use light. Only recently was it suggested that some members of the candidate phyla radiation (CPR)—a highly diverse group of bacteria originally detected by 16S rRNA sequencing [1] and subsequently characterized by genome-resolved metagenomics [2, 3]—may be able to use rhodopsins for proton translocation and thus energy generation [4, 5]. However, experimental evidence supporting this function was lacking. Here, we biophysically characterize rhodopsins from putatively symbiotic Saccharibacteria (TM7 lineage of CPR) and explore their relevance for metabolism. We also consider how rhodopsins may play a role in the interactions between Saccharibacteria and their putative microbial hosts in sunlit environmental microbiomes.

**RESULTS**

Phylogenetic placement of Saccharibacteria rhodopsins (SacRs) shows that these sequences form a sibling clade to characterized light-driven inward and outward H⁺ pumps (Fig. 1a). We selected three phylogenetically diverse SacRs from freshwater lakes (Table S1) and two related, previously uncharacterized sequences from the Gammaproteobacteria (Kushneria aurantia and Halomonas sp.) for synthesis and functional characterization (highlighted in Fig. 1a). All sequences have Asp–Thr–Ser (DTS) residues at the positions of D85–T96–D96 of bacteriorhodopsin (BR) in the third transmembrane helix (Fig. S1). These residues are known as the triplet DTD motif and represent key residues for proton pumping function in BR [6].

Proton transport assays for the SacRs and Gammaproteobacteria proteins expressed in *Escherichia coli* showed marked decrease of external pH upon light illumination (Fig. 1b and Fig. S2), indicating that these proteins are light-driven outward H⁺ pumps. The pH decrease was almost eliminated after adding the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP), which dissipates the H⁺ gradient, confirming that it was indeed formed upon illumination (Fig. 1b and Fig. S2). We also characterized the absorption spectra and the photocycle of the SacRs, showing that the three rhodopsins have an absorption peak around 550 nm (Fig. S3). The photocycle of the SacRs, determined by measuring the transient absorption change after nanosecond laser pulse illumination (Fig. 1c and Fig. S4), displays a blue-shifted M intermediate that represents the deprotonated state of the retinal chromophore. This has been observed for other H⁺ pumping rhodopsins [7, 8] and indicates that the proton bound to retinal is translocated during pumping.

Given that SacRs function as outward proton pumps, we searched Saccharibacteria genomes for the *F*₁*F*₅ ATP synthase that...
would be required to harness the generated proton motive force for ATP synthesis. HMM searches showed that all genomes encoded the complete ATP synthase gene cluster and, furthermore, had c subunits with motifs consistent with H^+ binding, instead of Na^+ binding (Table S1 and Fig. S5). Together, our experimental and genomic analyses strongly suggest that some Saccharibacteria utilize rhodopsins for auxiliary energy generation in addition to their core fermentative capacities [6].

Retinal is the rhodopsin chromophore that enables function of the complex upon illumination [9]. We found no evidence for the presence of β-carotene 15,15'-dioxygenase (blh), which produces all-trans-retinal (ATR) from β-carotene, in Saccharibacteria genomes encoding rhodopsin. This absence was likely not due to genome incompleteness, as genomic bins were generally of high quality (79–98% completeness, Table S1) and rhodopsin genomic loci were well-sampled. Additionally, no conserved hypothetical proteins were present in these regions, where blh is often found [10] (Fig. 1d, Fig. S6 and Table S2). As SacRs do contain the conserved lysine for retinal binding [4], we instead hypothesized that Saccharibacteria may uptake retinal from the environment, as
has been previously observed for other microorganisms encoding rhodopsin but also lacking blh [11, 12].

We tested the ability of SacR proteins to bind ATR from an external source by performing a retinal reconstitution assay. In contrast to the proton transport assays, where rhodopsin was expressed in the presence of ATR, here ATR was dissociated from the purified complex and the visible absorbance of rhodopsin was measured upon re-addition of ATR [13]. Both Gloeobacter rhodopsin (GR), a typical Type-1 outward H\(^+\) pump, and SacRs showed an increase in absorption in the visible region with time after the addition of ATR (Fig. 2a and Fig. S7). For all SacRs, the binding of ATR by their apoprotein was saturated within 30 sec after retinal addition (Fig. 2b), indicating that SacR is able to be efficiently functionalized using externally derived ATR. The observed reconstitution rate is substantially faster than that of GR (> 20 min) and comparable to that of heliorhodopsin, which is used by other microorganisms also lacking a retinal synthetic pathway and rapidly binds ATR through a small opening in the apoprotein [12]. In the structure of SacR NC335 modeled by Alphafold2 [14, 15], a similar hole is visible in the protein moiety constructing the retinal binding pocket (Fig. S8). Hence, SacRs may also bind retinal through this hole in a similar manner to TaHeR (heliorhodopsin).

Saccharibacteria with rhodopsin must obtain retinal from other organisms. To evaluate possible sources of ATR, we investigated the genetic potential for retinal biosynthesis in 15 subarctic and boreal lakes [16] where Saccharibacteria with rhodopsin were present (Fig. S9). Blh-encoding scaffolds were found in 14 of the 15 metagenomes profiled (~93%) and, in nearly all cases, these scaffolds derived from Actinobacteria (Fig. 2c and Table S3). This is intriguing because Actinobacteria are known to be hosts of Saccharibacteria in the human microbiome [17, 18] and potentially more generally [4, 19]. BLAST searches against genome bins from the same samples indicated that these Actinobacteria were members of the order Nanopelagicales (Table S3) and often encode a rhodopsin (phylogenetically distinct from SacRs) in close genomic proximity to blh genes (Table S4). HMM searches revealed that these genomes also harbor homologs of the **crtI**, **crtE**, **crtB**, and **crtY** genes necessary for **β**-carotene production [20].

**DISCUSSION**

Here, we add to growing evidence that DTS-motif rhodopsins can function as outward H\(^+\) pumps [21] and infer that Saccharibacteria use them to establish a proton gradient for energy generation, given a source of ATR and light. This is one of the very few known ways that any CPR organism can pump protons across the membrane. However, the source of ATR enabling the function of Saccharibacteria rhodopsins is unclear. While there is precedent for external supply of ATR to functional rhodopsins in other bacteria [11, 12], the mechanism by which this hydrophobic compound is transferred to the membrane of such bacteria is also unknown.

Experimental co-cultures of Saccharibacteria with Actinobacteria from multiple microbiome types [18, 19] suggest that a host bacterium for the Saccharibacteria studied here may be the source of ATR. We infer that these hosts are co-occurring Nanopelagicales Actinobacteria that dominate retinal production in microbial communities containing Saccharibacteria with rhodopsin. These **Nanopelagicales** bacteria are sufficiently abundant to represent plausible hosts (Fig. S10a) and have average genome sizes of
approximately 1.25 Mbp (Fig. S10b). This is substantially smaller than known hosts of Saccharibacteria from other environments (Fig. S10b) but still larger than Saccharibacteria themselves (~0.78 Mbp, on average). However, the genetic requirements to host CPR symbionts is currently unknown.

If Nanopelagicales bacteria are indeed the hosts of freshwater Saccharibacteria with rhodopsin, then retinal produced by the former from β-carotene could be transferred to the latter either by membrane contact, a common feature in imaged CPR-host interactions \([17, 22]\), or possibly via extracellular vesicles (Fig. 2d). ATR produced by Actinobacteria is required for their own rhodopsins \([11]\) (Fig. 2d), but it is conceivably that they produce ATR in excess to deliberately supply Saccharibacteria symbionts, possibly to ensure interdependence. Alternatively, Saccharibacteria scavenge ATR. Regardless of the source organism and ATR transfer mechanism, our analyses suggest a new aspect of Saccharibacteria lifestyles, in which they employ rhodopsins and externally derived retinal to produce energy via phototrophy.

**DATA AVAILABILITY**

All accession information for the genomes and metagenomic samples analyzed in this study are listed in the Supplementary Tables. Additional files (including the masked rhodopsin alignment and maximum likelihood tree), supplementary tables, and custom code for the described analyses are also available on Zenodo (https://doi.org/10.5281/zenodo.6038621).

**REFERENCES**

1. Hugenholtz P, Pitulle C, Hershberger KL, Pace NR. Novel division level bacterial diversity in a Yellowstone hot spring. J Bacteriol. 1998;180:366–76.
2. Wrighton KC, Thomas BC, Sharon I, Miller CS, Castelle CJ, Verberkmoes NC, et al. Fermentation, hydrogen, and sulfur metabolism in multiple uncultivated bacterial phyla. Science. 2012;337:1661–5.
3. Brown CT, Hug LA, Thomas BC, Sharon I, Castelle CJ, Singh A, et al. Unusual biology across a group comprising more than 15% of domain Bacteria. Nature. 2015;523:208–11.
4. Jaffe AL, Thomas AD, He C, Keren R, Valentín-Alvarado LE, Munk P, et al. Patterns of gene content and co-occurrence constrain the evolutionary path toward animal association in Candidate Phyla Radiation Bacteria. mBio. 2021;12:e00521-21.
5. Chiriac M-C, Bulu P-A, Andre A-S, Okazaki Y, Nakano S, Haber M, et al. Ecogenicomics sheds light on diverse lifestyle strategies in freshwater CPR. Res. Square. 2021.
6. Béjà O, Landy JK. Nature’s toolkit for microbial rhodopsin ion pumps. Proc Natl Acad Sci USA. 2014;111:6338–9.
7. Chizhov I, Chemavskiv DS, Engelhard M, Mueller KH, Zubov BV, Hess B. Spectrally silent transitions in the bacteriorhodopsin photocycle. Biophys J. 1996;71:2329–45.
8. Béjà O, Spudich EN, Spudich JL, Leclerc M, DeLong EF. Proteorhodopsin photography in the ocean. Nature. 2001;411:786–9.
9. Rozenberg A, Inoue K, Kandori H, Béjà O. Microbial rhodopsins: the last two decades. Annu Rev Microbiol. 2021;75:427–47.
10. Pinhasi J, DeLong EF, Béjà O, González JM, Pedrós-Alió C. Marine bacterial and archaeal ion-pumping rhodopsins: genetic diversity, physiology, and ecology. Microbiol Mol Biol Rev. 2016;80:929–54.
11. Keffer JL, Hahn MW, Maresca JA. Characterization of an unconventional rhodopsin from the freshwater actinobacterium Rhodolana lacticola. J Bacteriol. 2015;197:2704–12.
12. Shihoya W, Inoue K, Singh M, Konno M, Hososhima S, Yamashita K, et al. Crystal structure of heliorhodopsin. Nature. 2019;574:132–6.
13. Pushkariev A, Inoue K, Laramo S, Flores-Uribe J, Singh M, Konno M, et al. A distinct abundant group of microbial rhodopsins discovered using functional metagenomics. Nature. 2018;558:595–9.
14. Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, et al. Highly accurate protein structure prediction with AlphaFold. Nature. 2021;596:583–9.
15. Mirdita M, Schütze K, Moriwaki Y, Heo L, Ovchinnikov S, Steinegger M. ColaFold - Making protein folding accessible to all. bioRxiv. 2021; Preprint at https://doi.org/10.1101/2021.08.15.456425.
16. Buck M, Garcia SL, Fernandez L, Martin G, Martinez-Rodriguez GA, Saarennehimo J, et al. Comprehensive dataset of shotgun metagenomes from oxygen stratified freshwater lakes and ponds. Sci Data. 2021;8:131.
17. He X, McLean JS, Edlund A, Yooseph S, Hall AP, Liu S-Y, et al. Cultivation of a human-associated TM7 phenotype reveals a reduced genome and epibiotic parasitic lifestyle. Proc Natl Acad Sci USA. 2015;112:244–9.
18. Utter DR, He X, Cavanaugh CM, McLean JS, Bor B. The saccharibacterium TM7x elicits differential responses across its host range. ISME J. 2020;14:3054–67.
19. Batinovic S, Rose JJA, Ratcliffe J, Seviour RJ, Petrovski S. Co-cultivation of an ultraslimm, environmental parasitic bacterium with lytic ability against bacteria associated with wastewater foams. Nat Microbiol. 2021;6:703–11.
20. Duvilit-Smith JR, Hamilton JJ, Stevenson DM, He S, Oyserman BO, Moya-Flores F, et al. acl Actinobacteria assemble a functional actinorhodopsin with natively synthesized retinal. Appl Environ Microbiol. 2018;84:e01678–18.
21. Malina N, Ohkrimenko IS, Petrovskaya LE, Alekseev AA, Kovaliev KV, Sololov DV, et al. Novel pH-sensitive microbial rhodopsin from Sphingomonas paucimobilis. Dokl Biochem Biophys. 2020;495:342–6.
22. He C, Keren R, Whittaker ML, Farag IF, Doudna J, Cate JHD, et al. Genome-resolved metagenomics reveals site-specific diversity of epiphasic CPR bacteria and DPANN archaea in groundwater ecosystems. Nat Microbiol. 2021;6:354–65.

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**AUTHOR CONTRIBUTIONS**

ALJ, OB, KI, and JFB designed the project. ALJ and KI performed bioinformatic and phylogenetic analyses. MK, YK, CK, and HK performed biophysical assays. ALJ, JFB, KI, and MK wrote the manuscript. All authors made comments on the manuscript.

**COMPETING INTERESTS**

JFB is a co-founder of Metagenomi. The other authors declare no competing interests.

**ADDITIONAL INFORMATION**

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