Short Communication

Complex History of Aerobic Respiration and Phototrophy in the *Chloroflexota* Class *Anaerolineae* Revealed by High-Quality Draft Genome of *Ca.* Roseilinea mizusawaensis AA3_104

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Anoxygenic phototrophs in the *Chloroflexota* (formerly *Chloroflexi* or Green Nonsulfur Bacteria) phylum include the well known genera *Chloroflexus* and *Roseiflexus* isolated and characterized from sulfidic hot springs (e.g. Thiel et al., 2018), but more recent metagenomic analyses have revealed several additional lineages of phototrophic *Chloroflexota* from environments including iron-rich hot springs (e.g. Ward et al., 2019a; Ward et al., 2019b), carbonate tidal flats (Ward et al., 2020), and soda lakes (e.g. Grouzdev et al., 2018). While some of these novel phototrophic *Chloroflexota* belong to the *Chloroflexia* class together with *Chloroflexus* and *Roseiflexus* (e.g. Grouzdev et al., 2018) (Ward et al. 2019 Genomic evidence for phototrophic oxidation of small alkanes in a member of the *Chloroflexi* class such as *Chloroflexus* and *Roseiflexus*. Here, we present a high-quality MAG of a member of the *Roseiflexus*, improving our understanding of the metabolic capacity and phylogeny of this genus, and resolving the multiple instances of horizontal gene transfer that have led to its metabolic potential. These data allow us to propose a candidate family for these organisms, *Roseilineaceae*, within the *Anaerolineae* class.

**Key words:** photosynthesis, thermophile, sulfide, phylogenetics, metagenomics, *Chloroflexi*, green nonsulfur bacteria

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Metagenomic sequencing of 4 samples from Mizusawa Hot Spring, Japan, was performed to study the microbial community. Bulk environmental DNA was extracted and purified after return to the lab with a ZymoPrep Soil/Fecal DNA extraction kit. Quantification of DNA was performed with a Qubit 3.0 fluorimeter (Life Technologies). Purified DNA was operated for 1 min. Bulk environmental DNA was extracted and purified after return to the lab with a ZymoPrep Soil/Fecal DNA extraction kit. Quantification of DNA was performed with a Qubit 3.0 fluorimeter (Life Technologies) according to manufacturer’s instructions. Purified DNA was submitted to the Integrated Microbiome Resource for library preparation and sequencing following established protocols (Comeau et al., 2017) with 2×150 bp Illumina NextSeq. Raw sequence reads were quality controlled with BBTools (Bushnell, 2014) and coassembled with MegaHit v. 1.02 (Li et al., 2016). Genome bins were constructed based on differential coverage using MetaBAT (Kang et al., 2015). Completeness and contamination/redundancy were determined with CheckM v1.1.2 (Parks et al., 2015). The genome was uploaded to RAST v2.0 for annotation and characterization (Aziz et al., 2008). Presence or absence of metabolic pathways of interest was predicted using MetaPOAP v1.0 (Ward et al., 2018c). Taxonomic assignment was determined with GTDB-Tk v1.2 (Parks et al., 2018; Chaumeil et al., 2020; Parks et al., 2020). Genomes were compared with AAI (Rodriguez-R and Konstantinidis, 2014) to verify species and genus-level divisions. Organismal phylogenies were built using concatenated ribosomal proteins following methods derived from Hug et al. (2016) using the software pipeline described below. Protein sequences were extracted from genomes using the tblastn function of BLAST+ (Camacho et al., 2009), aligned using MUSCLE (Edgar, 2004), and manually trimmed and curated using Jalview (Waterhouse et al., 2009). Trees were calculated using RAxML v8.2.12 (Stamatakis, 2014) on the Cipres science gateway (Miller et al., 2010). Transfer bootstrap support values were calculated by BOOSTER (Lemoine et al., 2018), and trees were visualized with the Interactive Tree of Life viewer (Letunic and Bork, 2016). All software was run with default parameters.

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observations of cells putatively identified as Ca. Roselinea gracile do show autofluorescence consistent with bacteriochlorophyll a (Tank et al., 2017), suggesting that this pigment is in fact synthesized in these organisms. Genes such as bchK, bchU, and bchQ are absent from AA3_104, consistent with this organism not producing bacteriochlorophylls c, d, or e. Taken all together, these data are consistent with previous proposals that phototrophic Anaerolineae (both Roselinea and members of the order Aggregatilineales) utilize a novel bacteriochlorophyll a synthesis pathway that makes use of uncharacterized or multifunctional enzymes to perform steps typically performed by BchE, BchM and the BchLNb complex (e.g. a multifunctional BchXYZ complex for the conversion of both protochlorophyllide a to chlorophyllide a and the conversion of chlorophyllide a to 3-vinyl-bacteriochlorophyllide a) (Ward et al., 2018a; Ward and Shih, 2021).

Like previously described phototrophic Anaerolineae (including Roselinea and phototrophic members of Aggregatilineales), but unlike phototrophic Chloroflexia, AA3_104 lacks key marker genes for carbon fixation pathways including the Calvin cycle (e.g. rubisco) and the 3-hydroxypropionate bi-cycle (e.g. the trifunctional mali-CoA/beta-methylmalyl-CoA/citramalyl-CoA lyase). AA3_104 is therefore most likely incapable of autotrophy and instead makes a living as an anoxygenic phototroph.

AA3_104 appears to be capable of at least facultative aerobic respiration via both an A- and a B-family heme copper O₂ reductase (HCO), two bc Complex IIIis, and a bd oxidase. This complement of respiration genes is consistent with that previously described for members of Roselinea (Ward et al., 2018a; Ward et al., 2019b).

The presence of a B-family HCO in phototrophic Roselinea is a trait shared with all other known phototrophic Chloroflexota as well as phototrophic members of the closely related phylum Eremiobacterota, although closely related nonphototrophic strains lack a B-family HCO (e.g. Ward et al., 2018a; 2019a; 2020). The functional link between the B-family HCO and phototrophy in these organisms is not well understood, but may relate to the adaptation of B-family HCOs to relatively low oxygen concentrations (Han et al., 2011) and the oxygen sensitivity of proteins involved in anoxygenic phototrophy (e.g. Hamilton, 2019) linking anoxygenic phototrophy in these organisms to low-oxygen environments. Interestingly, phylogenetic relationships of B-family HCO proteins are incongruent with organismal relationships and with relationships among phototrophy proteins (Fig. 3 and 4). This suggests that B-family HCOs and phototrophy proteins have independent histories of horizontal gene transfer in the Chloroflexota despite their apparent functional link. B-family HCO genes are not collocated with phototrophy genes and do not display conserved synteny between phototrophic Chloroflexota lineages, consistent with these genes having an independent history of horizontal gene transfer.

Unlike other phototrophic Chloroflexota, AA3_104 and other Roselinea do not encode an Alternative Complex III. While the absence of genes encoding this protein from previous MAGs could have been ascribed to their low completeness, the high quality of AA3_104 makes it quite unlikely that this enzyme would not have been recovered in the MAG if it were found in the source genome (MetaPOAP false negative estimate <2.5% for AA3_104 alone, <10⁻⁴ for all three available Roselinea genomes considered together). It therefore appears that members of Roselinea, in contrast to other phototrophic Chloroflexota, use a bc Complex III in their phototrophic electron transport chain instead of an
Alternative Complex III. The evolutionary and biochemical logic for this difference is not yet apparent, but it does appear to confirm that an Alternative Complex III is not essential for phototrophy in the Chloroflexota.

In most lineages of phototrophic bacteria, it appears that the capacity for aerobic respiration was acquired before the acquisition of phototrophy (Fischer et al., 2016). This trend has been confirmed for phototrophs in the Chloroflexota orders Chloroflexales and Aggregatilineales, in which A-family HCOs and other components of electron transport chains were acquired to enable aerobic respiration before the acquisition of phototrophy and B-family HCOs (Shih et al., 2017; Ward et al., 2020). However, at present it is impossible to confirm whether this trend extends to Roseilinea. While genes for aerobic respiration are widespread in even apparently obligate anaerobic members of the Anaerolineae class of Chloroflexota (e.g. Hemp et al., 2015; Pace et al., 2015; Ward et al., 2018b), it appears that these genes were acquired independently in many lineages within this class subsequent to their divergence (e.g. Ward et al., 2020). Members of Roseilineaceae described so far consist of a single apparent genus at the end of a relatively long branch whose closest relatives appear to be a family of Thermoflexales provisionally identified by GTDB as Fen-1058 (Fig. 2). Fen-1058 does encode aerobic respiration; however, respiratory proteins in the families of Thermoflexaceae—Roseilineaceae, Fen-1058, and Thermoflexales—are not closely related (Fig. 4). This suggests that each of the known families of Thermoflexales acquired respiration independently after they diverged. Both aerobic respiration and phototrophy therefore appear to have been acquired along the long branch leading to crown group Roseilinea, in the absence of additional information it is therefore impossible to determine which of these traits was acquired first along this branch. Resolving this uncertainty will require recovering additional Roseilineaceae diversity that breaks up the long branch leading to Roseilinea. However, it is clear from phylogenetic relationships of proteins that components of the electron transport chain in Roseilineaceae were acquired through independent HGT events from different sources, suggesting that the capacity for respiration and phototrophy was not acquired in a single large HGT event.

We propose the assignment of AA3_104, described here, and J036, described in Ward et al., 2019, to the Roseilinea genus proposed by Tank et al. (2017). We propose the specific epithets Ca. Roseilinea mizusawaensis for AA3_104 in recognition of Mizusawa Onsen as the source of this organism, and Ca. Roseilinea jinataensis for J036 in recognition of its discovery in Jinata Onsen. Given the apparent divergence of Roseilinea from other members of the phylum Chloroflexota as determined by concatenated ribosomal protein phylogenies as well as analysis via GTDB-Tk, we propose the assignment of these organisms to a novel family in the Thermoflexales order of the Anaerolineae class of the Chloroflexota phylum, Roseilineaceae, fam. nov. As it is currently the highest quality MAG available for this clade, we propose Ca. Roseilinea mizusawaensis AA3_104 as the type genome for Roseilineaceae until such time as an isolate and/or a complete genome is available, following recent recommendations for candidatus taxa (Chuvchinda et al., 2019).

The family Roseilineaceae is so far known only as putatively at least facultatively aerobic phototrophs from hot springs. Members of Roseilineaceae are currently known from geographically and geochemically diverse hot springs in the United States and Japan, including iron-rich and moderately acidic intertidal hot springs in Tokyo Prefecture (Ward et al., 2019b), moderately acidic and sulfidic hot springs in Akita Prefecture (this study), and alkaline siliceous hot springs in Yellowstone National Park (Klatt et al., 2011). However, 16S rRNA gene amplicon data suggests that this clade may have a wider environmental distribution. Highly similar 16S sequences (>97%) to that of Ca. Roseilinea mizusawaensis AA3_104 have been reported from wastewater treatment systems (Karst et al., 2018) while somewhat similar (>94%) sequences have been reported from environments including contaminated aquifers (Thavamani et al., 2012) and soil (Xiao et al., 2009). Given the long phylogenetic branch between crown group Roseilinea and the divergence of Roseilineaceae from Thermoflexus and other members of Thermoflexales, together with current understanding of the diversification rates of bacterial lineages through time (Louca et al., 2018), it should be expected that much more diversity of Roseilineaceae has existed through geologic time. Ignoring possibilities of apparently rare extreme extinction events and population bottlenecks, it seems highly likely that additional
Roseilineaceae lineages exist in the environment today. Additional genome-resolved metagenomic sequencing of diverse environments is likely to yield additional diversity of this group, potentially breaking up the relatively long phylogenetic branch leading to *Roseilinea* and allowing for better resolution of the evolutionary transitions that have led to the significant divergence of *Roseilinea* from its closest known relatives.

*Ca.* Roseilinea mizusawaensis AA3_104 improves the available genomic diversity of anoxygenic phototrophic *Chloroflexota* outside of the *Chloroflexia* class, and provides the best available metagenome-assembled genome within the newly proposed Roseilineaceae family (Table 1). Improving sampling across the diversity of *Chloroflexota*—particularly within novel phototrophic lineages—provides valuable insight into the evolutionary trends leading to the extant diversity of phototrophs within this phylum and across the entire tree of life. In particular, the apparently extensive role of horizontal gene transfer in shaping the distribution of phototrophy and related pathways across the
tree of life (e.g. Raymond et al., 2002; Hohmann-Marriott and Blankenship, 2011; Fischer et al., 2016; Shih et al., 2017; Ward et al., 2018a; 2019a; Ward and Shih, 2019) provides an opportunity to investigate the history and evolution of functional traits even though most bacteria that have possessed these traits through geologic time are now extinct (Louca et al., 2018).

Utilizing the extant diversity and evolutionary relationships of microorganisms to understand the early evolution of metabolic traits requires adequate sampling across extant diversity. The recovery of Ca. Roseilinea mizusawaensis AA3_104 and other members of Roseilineaceae provide an excellent example of the taxonomic and metabolic novelty that exists in the environment which can be discovered via genome-resolved metagenomic sequencing and which will provide crucial data for comparative phylogenetic analyses that can help answer longstanding questions about the evolution of phototrophy, respiration, and other traits deep in the tree of life.

Data Availability: The AA3_104 genome has been uploaded to the NCBI WGS database under the submission ID SUB8655613 and will be publicly available immediately following processing.

| Genome ID | GTDB Classification | # Contigs | Size | N50 | Largest contig | GC%  | Completeness | Contamination | Strain Heterogeneity | Source                      |
|-----------|---------------------|-----------|------|-----|---------------|------|--------------|---------------|-----------------------|-----------------------------|
| AA3_104   | d_Bacteria p_Cloroflexota c_Anaerolineae o_Roseiflexales f__ g__ s__ | 59 | 3,855,811 | 137,185 | 436,788 | 0.61647 | 97.71 | 1.1 | 0 | Sulfidic hot spring |
| BEHY01    | d_Bacteria p_Cloroflexota c_Anaerolineae o_Roseiflexales f_Roseiflexus g_Roseiflexus sp002898735 | 175 | 2,931,246 | 34,479 | 127,152 | 0.66051 | 90.37 | 1.1 | 25 | Ammonia-oxidizing enrichment culture |
| FYEK01    | d_Bacteria p_Cloroflexota c_Anaerolineae o_Roseiflexales f_Roseiflexus g_Roseiflexus hugenholzii | 87 | 3,216,440 | 139,933 | 294,380 | 0.67347 | 97.25 | 0.92 | 0 | Hot spring |
| J036      | d_Bacteria p_Cloroflexota c_Anaerolineae o_Roseiflexales f__ g__ s__ | 150 | 3,068,507 | 30,577 | 104,612 | 0.63702 | 93.12 | 0.1 | 0 | Iron-rich hot spring |
| PMCF01    | d_Bacteria p_Cloroflexota c_Anaerolineae o_Roseiflexales f_Fen-1058 g_Fen-1058 s_Fen-1058 sp003154115 | 516 | 7,694,415 | 25,596 | 118,786 | 0.57589 | 95.77 | 4.59 | 0 | Permafrost |
| PMDR01    | d_Bacteria p_Cloroflexota c_Anaerolineae o_Roseiflexales f_Fen-1058 g_Fen-1058 s_Fen-1058 sp003154115 | 982 | 6,553,527 | 8,786 | 44,993 | 0.57971 | 79.93 | 3.67 | 0 | Permafrost |
| Roseilinea_gracile | d_Bacteria p_Cloroflexota c_Anaerolineae o_Roseiflexales f__ g__ s__ | 2329 | 2,103,554 | 1,147 | 4,984 | 0.63651 | 76.33 | 1.83 | 0 | Sulfidic hot spring |
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