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CONE-FORMING CHLOROFLEXI MATS AS ANALOGS OF CONICAL STROMATOLITE FORMATION WITHOUT CYANOBACTERIA

Lewis M. Ward, Woodward W. Fischer, Katsumi Matsuura, and Shawn E. McGlynn. In preparation.

Abstract

Modern microbial mats provide useful process analogs for understanding the mechanics behind the production of ancient stromatolites. However, studies to date have focused on mats composed predominantly of oxygenic Cyanobacteria (Oxyphotobacteria) and algae, which makes it difficult to assess a unique role of oxygenic photosynthesis in stromatolite morphogenesis, versus different mechanics such as phototaxis and filamentous growth.

Here, we characterize Chloroflexi-rich hot spring microbial mats from Nakabusa Onsen, Nagano Prefecture, Japan. This spring supports cone-forming microbial mats in both upstream high-temperature, sulfidic regions dominated by filamentous anoxygenic phototrophic Chloroflexi, as well as downstream Cyanobacteria-dominated mats. These mats produce similar morphologies analogous to conical stromatolites despite metabolically and taxonomically divergent microbial communities as revealed by 16S and shotgun metagenomic sequencing and microscopy. These data illustrate that anoxygenic filamentous microorganisms appear to be capable of producing similar mat morphologies as those seen in Oxyphotobacteria-dominated systems and commonly associated with

conical Precambrian stromatolites, and that the processes leading to the development of these features is more closely related with characteristics such as hydrology and cell morphology and motility.

Introduction

Stromatolites are "attached, lithified sedimentary growth structures, accretionary away from a point or limited surface of initiation" (Grotzinger and Knoll 1999). Behind this description lies a wealth of sedimentary structures with a record dating back over 3.7 billion years that may be one of the earliest indicators of life on Earth (Awramik 1992, Nutman et al. 2016). While some stromatolites form by abiotic processes (Grotzinger and Rothman 1996), many are constructed by microbial mats (e.g. Batchelor et al. 2004). Modern analog environments provide an opportunity to investigate key processes in stromatolite formation. These occur in environments such as hot springs (Berelson et al. 2011) and alkaline lakes (Petryshyn and Corsetti 2011) where microbial mats and sediments interact in the absence of eukaryotic grazing. The modern stromatolite analogs studied to date are dominated by Cyanobacteria (e.g. Pepe-Ranney et al. 2012, Reid et al. 2000), leading to the interpretation of ancient stromatolites as trace fossils of Cyanobacteria (e.g. Awramik 1992, Bosak et al. 2013). Cyanobacteria perform oxygenic photosynthesis, using light to fix carbon with water as an electron donor, producing oxygen as a byproduct. This process evolved from anoxygenic photosynthesis, which uses alternative electron donors and does not produce oxygen (Xiong et al. 2000). It is therefore reasonable to suspect that anoxygenic photosynthetic organisms supported the biosphere early in Earth history (Kharecha et al. 2005, Fischer and Knoll 2009, Ward et al. 2017b). Though it has

been proposed that anoxygenic microbes may have produced early stromatolites (Brock 1978, Bosask et al. 2007, Ward 2016), modern analogs have never been described without Cyanobacteria or filamentous algae contributing the mat fabrics. It remains to be demonstrated, therefore, that anoxygenic photosynthetic microbes—or even nonphotosynthetic microbes—can also produce stromatolites. If they can, it is possible that some or all of the oldest stromatolites record the activities of microbes other than Cyanobacteria. This would be consistent with the observation that oxygen did not accumulate in Earth's atmosphere until ~2.35 Gya (Bekker et al. 2004, Ward et al. 2016), more than a billion years after the earliest known biogenic stromatolites (Allwood et al. 2006).

An excellent candidate for testing whether anoxygenic microbes can contribute to the production of characteristic stromatolite morphologies are the Chloroflexi. Chloroflexi (i.e. Green Nonsulfur Bacteria) are a phylum of gliding, filamentous bacteria possessing a wide diversity of metabolisms and ecological roles, but are best known as photoheterotrophs (Overmann 2008). Chloroflexi have been shown to be diverse and abundant in a range of environments including soil (Costello and Schmidt 2006, Will et al. 2010), groundwater (Hug et al. 2013), marine sediment (Inagaki et al. 2003), wastewater (Miura et al. 2007, Kragelund et al. 2007), and even the human microbiome (Campbell et al. 2014). Despite their environmental richness revealed by culture-independent surveys, most described Chloroflexi belong to a few subclades isolated from hot springs (Yamada and Sekiguchi 2009). The best-described members of the Chloroflexi belong to the genus *Chloroflexus*, including *C. aggregans* (Hanada et al. 1995) and *C. aurantiacus* (Pierson and Castenholz

1974). *C. aggregans* and other members of the class Chloroflexia are metabolically versatile, capable of growth either as aerobic heterotrophs or anoxygenic phototrophs (Gupta et al. 2013). Phylogenetic analysis of the phototrophy genes of Chloroflexi suggests that anoxygenic photosynthesis in this group predates the evolution of oxygenic photosynthesis in Cyanobacteria (Xiong et al. 2000), implying that this group is ancient and therefore a good candidate for investigating questions of early Earth history. Perhaps most importantly, Chloroflexi possess a filamentous morphology and the ability for gliding motility. Although known modern stromatolite analogs are formed by Cyanobacteria, recent studies have suggested that oxygenic photosynthesis is not the driving factor in the formation of these structures, but instead motility and a filamentous morphology might be the most important factors (Shepard and Sumner 2010, Ward et al. 2014, Gong et al. 2014, Frantz et al. 2015). These preliminary studies suggest that Chloroflexi or other filamentous bacteria might be capable of forming stromatolites. If this is the case, the fact that modern stromatolites are formed by Cyanobacteria might represent ecological drivers that make Cyanobacteria more competitive in these environments than anoxygenic or nonphototrophic groups. Demonstrating the ability of Chloroflexi to develop stromatolite-like morphologies might help to resolve the paradox of stromatolites appearing in the rock record long before evidence for atmospheric oxygen by relaxing constraints on the timing of evolution of Cyanobacteria.

Here, we characterize cone-forming microbial mats from Nakabusa Onsen, a sulfidic hot spring located in Nagano Prefecture, Japan. At this locality, cone-forming microbial mats develop similar morphologies in both downstream Cyanobacteria-rich regions as well

as upstream regions in which high temperatures and sulfidic conditions select against oxygenic Cyanobacteria. We show here that upstream mat fabrics are developed by filamentous Chloroflexi, with no structural support by Cyanobacteria. These mats nonetheless retain similar morphologies to downstream mats composed of filamentous Cyanobacteria, demonstrating that conical morphologies can be developed by filamentous anoxygenic phototrophic bacteria. This suggests that the mechanisms responsible for development of cones are unrelated to oxygenic photosynthesis, and are instead controlled by factors like hydrology and fluid flow or by motility of filamentous microbes.

Materials and methods:

Geological context:

Nakabusa Onsen is located in the Japanese Alps near Azumino, Nagano, Prefecture, Japan. The hot spring outflow sampled here is located at 36.392429N, 137.748038E. Nakabusa Onsen is a sulfidic, moderately alkaline hot spring with source waters near 70°C (Kubo et al. 2011). Source waters of the spring are slightly alkaline (pH 8.5–9.0) and sulfidic (0.046–0.123 mM, Nakagawa et al. 2002, Nakagawa et al. 2003).

Sample collection:

Samples were collected and experiments were collected on 17 September 2015. Samples of two cone-forming microbial mats, CP1 and CP2, were collected for 16S and metagenomic sequencing as well as for microscopy. CP1 was located ~2 meters from the main outflow of the hot spring, was 48 °C. CP2 was located ~3 meters further downstream and was 32 °C.

Samples were collected using sterile forceps and spatulas (~0.25 cm^3 of material). Samples for sequencing were collected in triplicate and processed immediately in the field. Cells were lysed and DNA preserved in the field using Zymo Terralyzer BashingBead Matrix and Xpedition Lysis Buffer (Zymo Research, Irvine, CA). Cells were disrupted immediately by attaching tubes to the blade of a cordless reciprocating saw (Black & Decker, Towson, MD) and operating for 1 minute. Samples for microscopy were fixed immediately upon collection in 2% paraformaldehyde in pH 8 HEPES buffer.

Sequencing and analysis:

Following return to the lab, DNA was extracted and purified with a Zymo Soil/Fecal DNA extraction kit. The V4-V5 region of the 16S rRNA gene was amplified from each extract as well as negative controls using archaeal and bacterial primers 515F (GTGCCAGCMGCCGCGGTAA) and 926R (CCGYCAATTYMTTTRAGTTT) (Caporaso et al., 2012). DNA was quantified with a Qubit 3.0 fluorimeter (Life Technologies, Carlsbad, CA) according to manufacturer's instructions following DNA extraction and PCR steps. All samples yielded PCR amplicons when viewed on a gel after initial pre-barcoding PCR (30 cycles). Duplicate PCR reactions were pooled and reconditioned for five cycles with barcoded primers. Samples for sequencing were submitted to Laragen (Culver City, CA) for analysis on an Illumnia MiSeq platform. Sequence data were processed using QIIME version 1.8.0 (Caporaso et al., 2010). Raw sequence pairs were joined and quality-trimmed using the default parameters in QIIME. Sequences were clustered into de novo operational taxonomic units (OTUs) with 99% similarity using UCLUST open reference clustering protocol (Edgar, 2010). Then, the most

abundant sequence was chosen as representative for each de novo OTU (Wang et al., 2007). Taxonomic identification for each representative sequence was assigned using the Silva-115 database (Quast et al., 2013) clustered at 97% similarity. Singletons and contaminants (OTUs appearing in the negative control datasets) were removed. 16S sequences were aligned using MAFFT (Katoh et al. 2002) and a phylogeny constructed using FastTree (Price et al. 2010). Summary statistics were calculated using scripts in QIIME and are reported at the 97% OTU similarity levels.

Imaging

Intact cones were fixed in 2% paraformaldehyde solution and embedded in resin for microtome sectioning and observation with fluorescent microscopy using photosynthetic pigment autofluorescence and morphology to distinguish taxa.

Results and Discussion:

16S amplicon sequencing, shotgun metagenomic sequencing, and optical and fluorescent microscopy all confirmed that Cone Pool 1 contained only trace Cyanobacteria, and was instead formed primarily by *Chloroflexus*. Cone Pool 2, in contrast, was made up primarily of filamentous Cyanobacteria with only trace Chloroflexi.

Microbial community composition

Relative abundance of microbial taxa in the Cone Pool mats were estimated via 16S amplicon sequencing. Results are summarized in Figure 2, showing the difference in relative abundance of taxa between the two mats.

Samples from CP1 recovered, on average, 4106 reads and 1073 OTUs (at the 97% cutoff), while CP2 recovered an average of 35547 reads and 6756 OTUs. Despite this

difference in sequencing coverage, the Goods Coverage metric of sampling depth averaged 0.78 for CP1 and 0.86 for CP2, reflecting fairly similar recovery of rare diversity. Dominant taxa were well represented in both datasets, though rare diversity was likely unrecovered from both sites.

Both communities were dominated by just a few phyla. The CP1 community was primarily made up of anoxygenic phototrophic and nonphototrophic Chloroflexi, as well as Thermotoga. The Chloroflexi phylum alone made up approximately one third of the reads form CP1, with Thermotogae and Proteobacteria (~20 and 10%, respectively) also abundant. No other phylum made up more than 5% of total reads. The CP2 community had a more even diversity. Chlorobi were the most abundant phylum (25%), with large numbers of Cyanobacteria (22%), Chloroflexi (16%), and Bacteroidetes (12%).

In CP1, the microbial community had low OTU-level diversity, with most taxa represented by only one or two dominant OTUs. The primary exception to this was the Thermotogae, which included four OTUs at 1% relative abundance or above, and *Chloroflexus*, with two OTUs. CP2, in contrast, had much higher strain-level diversity, with most taxa present as multiple OTUs at relatively even abundance (e.g. seven Chlorobi OTUs between 0.1 and 1% relative abundance or above).

Members of the Chlorobi were the most abundant taxon in CP2; this is despite the volumetric dominance of Cyanobacteria, potentially as a result of the large cell size of filamentous Cyanobacteria relative to Chlorobi or differential DNA extraction or amplification (e.g. Trembath-Reichert et al. 2016). The Chlorobi present at Nakabusa are basal members of the phylum, 91% similar to *Chloroherpeton thalassium* 35110. Given the

oxygen content of the mats in which they are found, these strains may be aerobic, a feature only described in basal members of the phylum which may record an ancestral trait (Liu et al. 2012, Stamps et al. 2014, Fischer et al. 2016).

In addition to the phototrophic *Chloroflexus* and *Roseiflexus,* both mats contained abundant nonphototrophic Chloroflexi, including Anaerolineae, Caldilineae, and *Herpetosiphon*. These taxa are typically described as filamentous heterotrophs (e.g. Yamada et al. 2006, Ward et al. 2015b). While the Anaerolineae have classically been described as obligate anaerobes (Yamada et al. 2006), genomic sequencing has revealed widespread genes for aerobic respiration in this class (e.g. Hemp et al. 2015a, Hemp et al. 2015b, Pace et al. 2015, Ward et al. 2015a), so the strains at Nakabusa may also be aerobic.

Preliminary optical and autofluorescence microscopy of embedded and sectioned intact cones from CP1 and CP2 has been performed, although rigorous analysis remains to be performed. This analysis confirmed that CP1 contained Chloroflexi as the primary filamentous component, with only unicellular Cyanobacteria, while CP2 was made up primarily of filamentous Cyanobacteria.

Filamentous microorganisms and cone formation

While overall diversity of the two mats is very distinct, focus should be drawn to the relative abundance of filamentous organisms in each mat. Filamentous organisms form cohesive fabrics in microbial mats, and are responsible for maintaining structural integrity, developing distinctive morphologies like cones, and trapping and binding sediment (Ward et al. 2014, Frantz et al. 2014). In CP1, the majority of filamentous organisms (as assessed by estimates of number and volume from microscopy as well as 16S sequencing reads)

were Chloroflexi, particularly the anoxygenic phototrophic genus *Chloroflexus*, along with lower abundances of *Roseiflexus,* Anaerolineae, and Caldilineae. Cyanobacteria were not abundant, and those present were primarily unicellular. CP2 contained much lower abundances of phototrophic Chloroflexi, and the filamentous community was instead composed predominantly of Anaerolineae and filamentous Cyanobacteria related to *Leptolyngbya* (a common member of hot spring microbial mats of similar temperatures, e.g. Roeselers et al. 2007, Bosak et al. 2012, Ward et al. 2017a). *Chloroflexus* and filamentous Cyanobacteria like *Leptolyngbya* are not closely related, having evolved filamentous morphologies and phototrophy independently. The nature of their photosynthetic pathways also differs in their reaction centers, carbon fixation pathways, and metabolic byproducts (Fischer et al. 2016, Shih et al. 2017). Despite these differences, the growth of these organisms has led to the development of similar cone morphologies in CP1 and CP2. This reflects the convergent evolution of gross mat morphology between different lineages of microbes, and that factors other than taxonomic affinity, such as hydrology and fluid flow or cell morphology or motility control overall mat morphology. By the same token, similar microbial communities can lead to different mat morphologies (e.g. Trembath-Reichert et al. 2016).

Conclusions

Demonstrating that Chloroflexi can form stromatolite-like structures has several key implications, but first and foremost it provides confirmation that stromatolites need not necessarily reflect the presence of Cyanobacteria, which will help resolve a current billion-year discordance between records of stromatolites and environmental oxygen (e.g. Bosak et

al. 2007, Ward et al. 2016).

While the cone-forming microbial mats described here are made up primarily of photoautotrophic microbes, it remains possible that stromatolites could be formed by nonphototrophic microbes, such as filamentous methanogens or sulfur oxidizers. Additional analog characterization or experiments with nonphototrophic filamentous organisms can help resolve this possibility.

More specifically, conical stromatolites are among the oldest in the rock record (e.g. Nutman et al. 2016), and no known process is known to be capable of producing conical stromatolites in the absence of microbial mats (Grotzinger and Rothman 1996, Batchelor et al. 2004). It's therefore crucial to understand the formation processes of these structures to better interpret their occurrences in deep time. Demonstrating that conical morphologies need not always involve Cyanobacteria (e.g. Bosak et al. 2009) opens the possibility that other processes control conical mat formation, such as phototaxis (Walter et al. 1976), diffusion limitation of nutrients (Petroff et al. 2010), or aggregation of motile filaments (Shepard and Sumner 2010, Ward et al 2014, Gong et al 2014).

It is worth noting that the microbial mats investigated here are not mineralized, and so have no chance of entering the rock record. However, this lack of mineralization is a result only of the geochemical details of the hot spring environment. Given the ubiquity of Chloroflexi in a range of environments (Yamada and Sekiguchi 2009), including hot springs where mineralization is occurring (Brock 1978, Ward et al. 2014), it is a reasonable assumption that appropriate environments for mineralized Chloroflexi stromatolites can be found.

Figure 1: Photographs of cone-forming microbial mats at Nakabusa Onsen. Above: CP1, a cone-forming microbial mat growing at 48°C, whose fabric was made up of filamentous Chloroflexi. Below: CP2, a cone-forming microbial mat growing at 32°C, whose fabric was made up of filamentous Cyanobacteria.

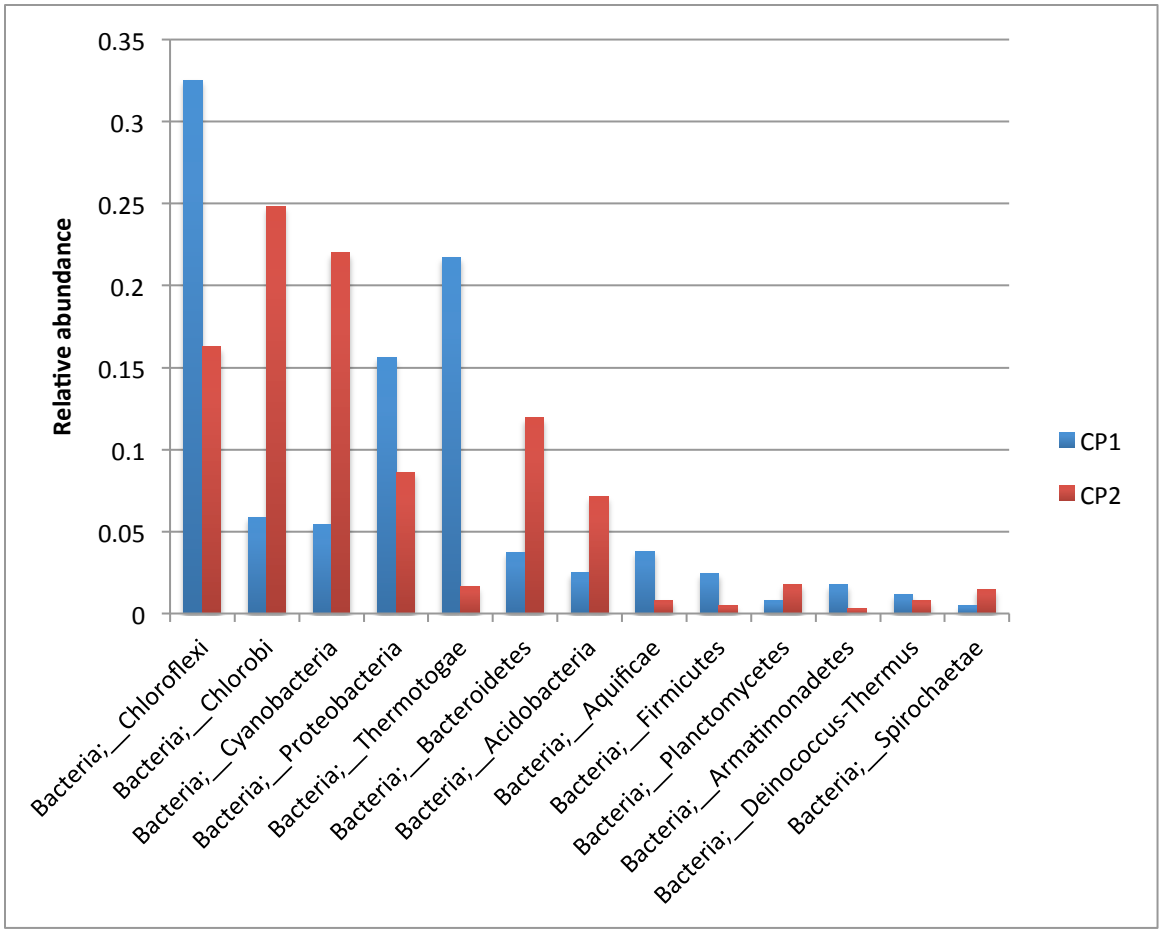

Figure 2: Relative abundance of most abundant taxa in Nakabusa samples clustered at the phylum level. Values presented are averaged across replicates within a site.

References:

1. Allwood, AC, MR Walter, BS Kamber, CP Marshall, and IW Burch. 2006. Stromatolite reef from the Early Archean era of Australia. Nature 441 pp714-718.
2. Awramik, SM. 1992. The oldest records of photosynthesis. Photosynthesis Research 33 pp75-89.
3. Batchelor, MT, RV Burne, BI Henry, and MJ Jackson. 2004. A case for biotic morphogenesis of coniform stromatolites. Physica A 337 pp319-326.
4. Bekker, A, et al. 2004. Dating the rise of atmospheric oxygen. Nature 427 pp117-120.
5. Berelson, WM, FA Corsetti, C Pepe-Ranney, DE Hammond, W Beaumont, and JR Spear. 2011. Hot spring siliceous stromatolites from Yellowstone National Park: assessing growth rate and laminae formation. Geobiology 9 pp411-424.
6. Bosak, T, et al. 2007. A Likely Role for Anoxygenic Photosynthetic Microbes in the Formation of Ancient Stromatolites. *Geobiology* 5(2) pp119–26.
7. Bosak, T, B Liang, MS Sim, and AP Petroff. 2009. Morphological record of oxygenic photosynthesis in conical stromatolites. PNAS 106(27) pp10939-10943.
8. Bosak, T, JWM Bush, MR Flynn, B Liang, S Ono, AP Petroff, and MS Sim. 2010. Formation and stability of oxygen-rich bubbles that shape photosynthetic mats. Geobiology 8 pp45-55.
9. Bosak, T. *et al*. Cyanobacterial diversity and activity in modern conical microbialites. *Geobiology* **10,** 384–401 (2012).

10. Bosak, T, AH Knoll, and AP Petroff. 2013. The meaning of stromatolites. Annu. Rev. Earth Sci. 41 pp21-44.
11. Brock TD (1978) Thermophilic microorganisms and life at high temperatures. Springer, New York
12. Campbell, AG, et al. 2014. Diversity and genomic insights into the uncultured *Chloroflexi* form the human microbiota. Environmental Microbiology 16(9) pp2635-2643.
13. Caporaso, JG, et al. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J 6 pp1621–1624.
14. Costello, EK and SK Schmidt. 2006. Microbial diversity in alpine tundra wet meadow soil: novel Chloroflexi from a cold, water-saturated environment. Environmental Microbiology 8(8) pp1471- 1486.
15. Fischer, WW and AH Knoll. 2009. An Iron Shuttle for Deepwater Silica in Late Archean and Early Paleoproterozoic Iron Formation. *Bulletin of the Geological Society of America* 121 pp222–35.
16. Fischer, W., Hemp, J. & Johnson, J. E. Evolution of Oxygenic Photosynthesis. *Annu. Rev. Earth Planet. Sci.* **44,** (2016).
17. Frantz, CM, V Petryshyn, and F Corsetti. 2015. Grain trapping by filamentous cyanobacterial and algal mats: implications for stromatolite microfabrics through time. *Geobiology* 13 pp409-423.
18. Gong, J, Z Zeng, and MM Tice. 2014. Evidence of microbial bioturbation and its unique records in clastic sediments. Geological Society of America Abstracts with

Programs. Vol. 46, No. 6, p.682.

19. Gupta, RS, P Chander, and S George. 2013. Phylogenetic framework and molecular signatures for the class Chloroflexi and its different clades; proposal for division of the class *Chloroflexi* class. Nov. into the suborder *Chloroflexineae* subord. nov., consisting of the emended family *Oschillochloridaceae* and the family *Chloroflexaceae* fam. nov., and the suborder *Roseiflexineae* subord. nov., containing the family *Roseiflexaceae* fam nov. Antonie van Leeuwenhoek 103 pp99-119.
20. Grotzinger, JP and DH Rothman. 1996. An abiotic model for stromatolite morphogenesis. Nature 383 pp423-425.
21. Grotzinger, JP and AH Knoll. 1999. Stromatolites in Precambrian carbonates: evolutionary mileposts or environmental dipstics? Annu. Rev. Earth Planet. Sci. 27 pp313-358.
22. Hanada, S, A Hiraishi, K Shimada, and K Matsuura. 1995. *Chloroflexus aggregans* sp. nov., a filamentous phototrophic bacterium which forms dense cell aggregates by active gliding movement. IJSEM 45(4) pp676-681.
23. Hemp J, **Ward LM**, Pace LA, Fischer WW. 2015a. Draft genome sequence of *Levilinea saccharolytica* KIBI-1, a member of the *Chloroflexi* class *Anaerolineae*. Genome Announc 3(6):e01357-15.
24. Hemp J, **Ward LM**, Pace LA, Fischer WW. 2015b. Draft genome sequence of *Ornatilinea apprima* P3M-1, an anaerobic member of the *Chloroflexi* class *Anaerolineae*. Genome Announc 3(6):e01353-15.

25. Hug, LA, CJ Castelle, KC Wrighton, BC Thomas, I Sharon, KR Frischkorn, KH Williams, SG Tringe, and JF Banfield. 2013. Community genomic analyses constrain the distribution of metabolic traits across the Chloroflexi phylum and indicate roles in sediment carbon cycling. Microbiome 1 pp1-22.
26. Inagaki, F, M Suzuki, K Takai, H Oida, T Sakamoto, K Aoki, KH Nealson, and K Horikoshi. 2003. Microbial communities associated with geological horizons in coastal subseafloor sediments from the Sea of Okhotsk. Appl. Environ. Microbiol. 69(12) pp7224-7235.
27. Kharecha, P, J Kasting, and J Siefert. 2005. A Coupled Atmosphere–ecosystem Model of the Early Archean Earth. *Geobiology* 3 pp53–76.
28. Kragelund, C, C Levantesi, A Borger, K Thelen, D Eikelboom, V Tandoi, Y Kong, J van der Waarde, J Krooneman, S Rossetti, TR Thomsen, ad PH Nielsen. 2007. Identitiy, abundance and ecophysiology of filamentous *Chloroflexi* species present in activated sludge treatment plants. FEMS Microbiol Ecol 59 pp671-682.
29. Kubo, K, K Knittel, R Amann, M Fukui, and K Matsuura. 2011. Sulfur-metabolizing bacterial populations in microbial mats of the Nakabusa hot spring, Japan. Systematic and Applied Microbiology 34(4) pp293-302.
30. Liu, Z. *et al*. Candidatus Thermochlorobacter aerophilum: an aerobic chlorophotoheterotrophic member of the phylum Chlorobi defined by metagenomics and metatranscriptomics. *ISME J*. **6,** 1869–1882 (2012).
31. Miura, Y, Y Watanabe, and S Okabe. 2007. Significance of *Chloroflexi* in performance of submerged membrane bioreactors (MBR) treating municipal

wastewater. Environ. Sci. Technol. 41 pp7787-7794.

32. Nakagawa, T., Fukui, M. (2002) Phylogenetic characterization of microbial mats and streamers from a Japanese alkaline hot spring with a thermal gradient. J. Gen. Appl. Microbiol. 48, 211–222.
33. Nakagawa, T., Fukui, M. (2003) Molecular characterization of community structures and sulfur metabolism within microbial streamers in Japanese hot springs. Appl. Environ. Microbiol. 69, 7044–7057
34. Nutman, A. P., Bennett, V. C., Friend, C. R. L., Van Kranendonk, M. J. & Chivas, A. R. Rapid emergence of life shown by discovery of 3,700-million-year-old microbial structures. *Nature* 1–12 (2016). doi:10.1038/nature19355
35. Overmann, J. 2008. Green nonsulfur bacteria. In: Encyclopedia of Life Sciences (ELS). John Wiley & Sons, Ltd: Chichester.
36. Pace LA, Hemp J, **Ward LM**, Fischer WW. 2015. Draft genome of *Thermanaerothrix daxensis* GNS-1, a thermophilic facultative anaerobe from the *Chloroflexi* class *Anaerolineae*. Genome Announc 3(6):e01354-15.
37. Pepe-Ranney, C, WM Berelson, FA Corsetti, M Treants, and JR Spear. 2012. Cyanobacterial construction of hot spring siliceous stromatolites in Yellowstone National Park. Environmental Microbiology 14(5) pp1182-1197.
38. Petroff, AP, MS Sim, A Maslov, M Krupenin, DH Rothman, and T Bosak. 2010. Biophysical basis for the geometry of conical stromatolites. PNAS 107(22) pp9956-9961.
39. Petryshyn, V and FA Corsetti. 2011. Analysis of growth directions of columnar

stromatolites from Walker Lake, western Nevada. Geobiology 9 pp425-435.

40. Pierson, BK and RW Castenholz. 1974. A phototrophic gliding filamentous bacterium of hot springs, Chloroflexus aurantiacus, gen. and sp. nov. *Archives of Microbiology* 100 pp5-24.
41. Reid, RP, PT Visscher, AW Decho, JF Stolz, BM Bebout, C Dupraz, IG Macintyre, HW Paerl, JL Pinckney, L Prufert-Bebout, TF Steppe, and DJ DesMarais. 2000. The role of microbes in accretion, lamination and early lithification of modern marine stromatolites. Nature 406 pp989- 992.
42. Roeselers, G. et al. Diversity of phototrophic bacteria in microbial mats from Arctic hot springs (Greenland). Environ. Microbiol. 9, 26–38 (2007).
43. Shepard, RN and DY Sumner. 2010. Undirected motility of filamentous cyanobacteria produces reticulate mats. Geobiology 8 pp179-190.
44. Shih, PM, LM Ward, J Hemp, and WW Fischer. 2017. Proterozoic origin of phototrophic Chloroflexi and the 3-hydroxypropionate bicycle. In prep.
45. Stamps, B. W., Corsetti, F. A., Spear, J. R. & Stevenson, B. S. Draft Genome of a Novel Chlorobi Member Assembled by Tetranucleotide Binning of a Hot Spring Metagenome. *Genome Announc*. **2,** 4669 (2014).
46. Trembath-Reichert E, **Ward LM**, Slotznick SP, Bachtel SL, Kerans C, Grotzinger JP, Fischer WW (2016) Gene sequencing microbial community analysis of mat morphologies, Caicos Platform, British West Indies, *Journal of Sedimentary Research* 86(6) pp629-636.

47. Walter, M. R., Bauld, J., and Brock, T. D., 1976, Microbiol- ogy and morphogenesis of columnar stromatolites (Conophyton, Vacerrilla) from hot springs in Yellow- stone Park, in Walter, M. R., ed., Stromatolites: Amster- dam, Elsevier, p. 273–310. Walter,

48. Ward, LM, Z Kerrigan, H Agic, M Juarez Rivera, VA Petryshyn, BW Stamps, HA Johnson, BS Stevenson, JR Spear, and FA Corsetti. 2014. Metagenomic insights into cone formation in hot spring microbial mats. Geological Society of America Abstracts with Programs. Vol. 46, No. 6, p.472.

49. **Ward LM**, Hemp J, Pace LA, Fischer WW. 2015a. Draft genome sequence of *Leptolinea tardivitalis* YMTK-2, a mesophilic anaerobe from the *Chloroflexi* class *Anaerolineae*. Genome Announc 3(6):e01356-15.

50. **Ward LM**, Hemp J, Pace LA, Fischer WW. 2015b. Draft genome sequence of *Herpetosiphon geysericola* GC-42, a nonphototrophic member of the *Chloroflexi* class *Chloroflexia*. Genome Announc 3(6):e01352-15

51. **Ward, L. M.,** Kirschvink, J. L. & Fischer, W. W. 2016. Timescales of Oxygenation Following the Evolution of Oxygenic Photosynthesis. Orig. Life Evol. Biosph. 46(1) pp51-65.

52. **Ward, LM,** A Idei, T Kakegawa, WW Fischer, and SE McGlynn. 2017a. Microbial diversity and iron oxidation at Okuoku-hachikurou Onsen, a Japanese hot spring analog of Precambrian iron formation. Geobiology, in review.

53. Ward, Lewis M, Birger Rasmussen, and Woodward W. Fischer. 2017b. Electron donor limitation of the biosphere before oxygenic photosynthesis. In preparation.

54. Will, C, A Thuermer, A Wollherr, H Nacke, N Herold, M Schrumpf, J Gutknecht, T Wubet, F Buscot, and R Daniel. 2010. Horizon-specific bacterial community composition of German grassland soils, as revealed by pyrosequencing-based analysis of 16S rRNA genes. Appl. Environ. Microbiol. 76(20) pp6751-6759.

55. Xiong, J, WM Fischer, K Inoue, M Nakahara, and CE Bauer. 2000. Molecular evidence for the early evolution of photosynthesis. Science 289 pp1724-1730.

56. Yamada, T, H Imachi, A Ohashi, H Harada, S Hanada, Y Kamagata, and Y Sekiguchi. 2007. Bellilinea Caldifistulae Gen. Nov., Sp. Nov and Longilinea Arvoryzae Gen. Nov., Sp. Nov., Strictly Anaerobic, Filamentous Bacteria of the Phylum Chloroflexi Isolated from Methanogenic Propionate-Degrading Consortia."*International Journal of Systematic and Evolutionary Microbiology* 57 pp2299–2306.

57. Yamada, T and Y Sekiguchi. 2009. Cultivation of uncultured *Chloroflexi* subphyla: significance and ecophysiology of formerly uncultured *Chloroflexi* 'Subphylum I' with natural and biotechnological relevance. *Microbes Environ*. 24pp205–216.