SUPPLEMENTARY MATERIAL for Dwulit-Smith *et al.*

acI Actinobacteria Assemble a Functional Actinorhodopsin with Natively-Synthesized Retinal

TABLE S1 Metadata of acI genomes referred to in this study

Classification	Genome Name	IMG Taxon ID	Recovered Genome Size (MB)	Contigs	Genes	Completeness (%)[i]	Contamination (%)[i]
acI-A	AAA024-D14[a]	2264265190	0.78	82	892	58.4	0.98
	AAA023-J06[a]	2236661001	0.70	98	818	43.7	0.00
	MMS-IA-53[b]	2775506795	1.33	1	1386	100	0.00
	AAA028-I14[c]	2545555832	0.78	54	848	50.8	0.00
	AAA028-E20[c]	2602042079	0.88	51	953	60.2	0.00
	AAA278-O22[a]	2236661007	1.14	43	1238	78.2	0.42
acI-B	AAA028-A23[a]	2236661004	0.83	64	913	67.7	0.19
	AAA027-L06[d]	2505679121	1.16	75	1282	87.4	4.37
	MMS-21-122[b]	2775506799	1.24	1	1281	100	0.00
	AAA027-J17[a]	2236661002	0.97	81	1094	73.1	0.84
	AAA278-I18[a]	2236661006	0.94	54	1037	69.0	0.17
	AAA044-D11[c]	2524023189	1.15	30	1214	75.2	0.84
acI-C	IMCC26077[e]	2602042021	1.52	1	1572	100.	0.00
	ME885[c]	2582580608	0.87	78	906	55.7	4.76
	ME578[c]	2556921504	0.36	32	390	25.9	3.45
	BIN_10[g]	NA[h]	0.98	95	1171	75.7	0.00
	ME3864[c]	2582580572	0.79	70	839	53.0	3.78

[a] Ghylin, T. W., Garcia, S. L., Moya, F., Oyserman, B. O., Schwientek, P., Forest, K. T., … McMahon, K. D. (2014). Comparative single-cell genomics reveals potential ecological niches for the freshwater acI Actinobacteria lineage. The ISME Journal, 8(12), 2503–2516. These genomes are single-cell amplified genomes (SAGs) recovered from one of three lakes including Lake Mendota, using fluorescence activated cell sorting, subsequent multiple displacement amplification (MDA) followed by sequencing and assembly. Additional details are available in the original publication of Ghylin et al 2014.

[b] Neuenschwander S.M., Ghai R., Pernthaler J., Salcher M. M. (2018) Microdiversification in genome-streamlined ubiquitous freshwater Actinobacteria. The ISME Journal, 12(1), 185-198. This genome is a MAG derived a dilution culture enriched in acI-B cells. The DNA was obtained using MDA products that were sequenced as a metagenome, assembled, and binned manually with Sanger sequencing to close gaps. Additional details are available in the original publication of Neuenschwander et al 2018.

[c] Hamilton J. J., Garcia S. L., Brown B. S., Oyserman B. O., Moya-Flores F., Bertilsson S., Malmstrom R. R., Forest K. T., McMahon K. D. 2017. Metabolic network analysis and metatranscriptomics reveal auxotrophies and nutrient sources of the cosmopolitan freshwater microbial lineage acI. mSystems 2:e00091-17. Note that the names in the IMG record are actually MEint.metabat.885, MEint.metabat.578, and MEint.3864, respectively. Briefly, they are MAGs recovered from combined assemblies (i.e. bioinformatically pooled metagenomes from 94 samples collected from the top 12 m of Lake Mendota at the deep hole). MAGs were binned using differential coverage based on metagenomes mapped to the combined assembly, and based on k-mer frequencies. Methodological details can be found in Hamilton et al 2017 and S. Roux, L-K.Chan, R. Egan, R. R. Malmstrom, K. D. McMahon, M. B. Sullivan (2017) "Ecogenomics of freshwater virophages and their giant virus hosts assessed through time-series metagenomics". *Nature Communications*. 8: article 858.

[d] Garcia, S. L., McMahon, K. D., Martinez-Garcia, M., Srivastava, A., Sczyrba, A., Stepanauskas, R., … Warnecke, F. (2013). Metabolic potential of a single cell belonging to one of the most abundant lineages in freshwater bacterioplankton. The ISME Journal, 7(1), 137–147. This genome was a SAG recovered from Lake Stechlin in Germany, using fluorescence activated cell sorting, subsequent multiple displacement amplification (MDA) followed by sequencing and assembly. Additional details are available in the original publication of Garcia et al 2013.

[e] Kang I., Kim S., Islam M. R., Cho J-C. (2017). The first complete genome sequences of the acI lineage, the most abundant freshwater Actinobacteria, obtained by whole-genome-amplification of dilution-to-extinction cultures. Sci Rep 7. This genome is a MAG derived from a dilution culture enriched in acI-C cells. The DNA was obtained using MDA products that were sequenced as a metagenome, assembled, and binned manually with Sanger sequencing to close gaps. Additional details are available in the original publication of Kang et al 2017.

[g] Tsementzi, D., Poretsky, R. S., Rodriguez-R, L. M., Luo, C., & Konstantinidis, K. T. (2014). Evaluation of metatranscriptomic protocols and application to the study of freshwater microbial communities. Environmental Microbiology Reports, 6(6), 640–655.

[h] Data are available at http://enve-omics.ce.gatech.edu/data/MTR

[i] Inferred using CheckM and the set of 204 Actinobacteria-specific marker genes, or set to 100% and 0% because genome is listed as complete in the original publication.

SUPPLEMENTARY FIGURE LEGENDS

FIG S1 Xantho-opsin phylogeny groupings provide clear evidence for a distinct grouping of action-opsins. A conserved proline in α4 differentiates actino-opsins (ActRs) from other xantho-opsins (XRs) in the supergroup. Bolded opsins are aligned in Fig. 1A.

FIG S2 Alignments for lycopene cyclase chains (acI-CrtYc and acI-CrtYd). Proteins from acI genome AAA278-O22 are aligned to CrtYc (A) and CrtYd (B) from *Myxococcus xanthus* (Mx), which form the functionally characterized heterodimeric β-cyclase. In this and following five figures, identical (*), highly similar (:), and slightly similar (.) residues are indicated, and transmembrane helix predictions are highlighted in gray. The catalytic PxE(E/D) motif is highlighted in orange.

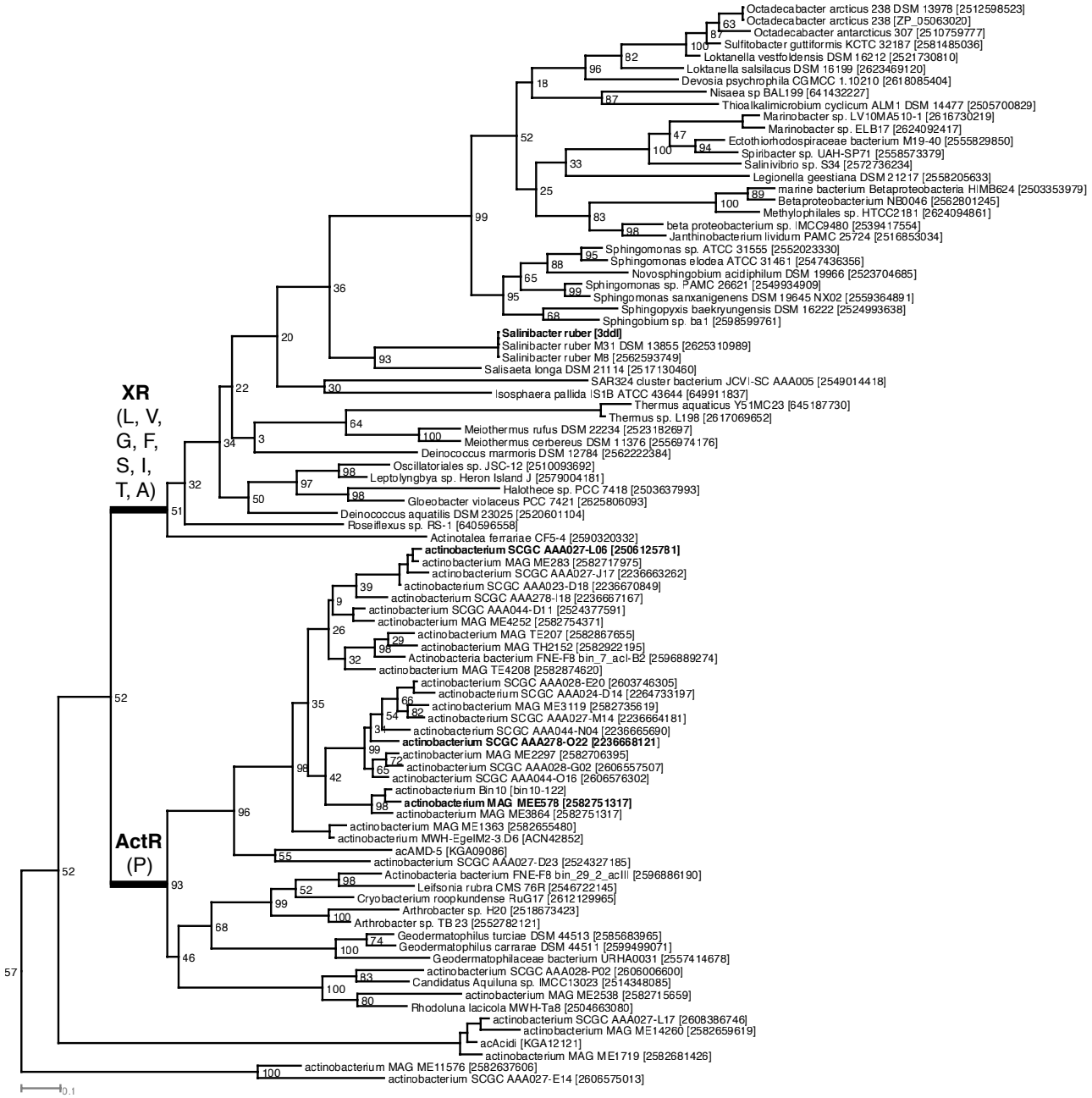
FIG S3 Alignment for 15-15'-β-carotene dioxygenase (acI-Blh). The protein from acI genome AAA278-O22 is aligned to a functionally characterized 15-15'-β-carotene dioxygenase from Uncultured Marine Bacterium 66A03 (Umb66A03). The histidines indicated for non-heme iron binding are highlighted in orange.

FIG S4 Alignment for predicted spheroidene mono-oxygenase (acI-CrtA). The protein from acI genome AAA278-O22 is aligned to functionally characterized CrtA proteins from *Rhodobacter sphaeroides* (Rs) and Marine bacterium P99-3 (MbP99-3).

FIG S5 Alignment for predicted γ-carotene 1'-hydroxylase (acI-CruF). The protein from acI genome AAA278-O22 is aligned to functionally characterized CruF proteins from *Deinococcus radiodurans* R1 (Dr) and *Deinococcus geothermalis* DSM11300 (Ds).

FIG S6 Alignment for glycosyltransferase (acI-CruG). The protein from acI genome AAA278-O22 is aligned to functionally characterized CruG proteins from *Gemmatimonas aurantiaca* (Ga) and *Synechococcus* sp. PCC7002 (SP).

FIG S7 Alignment for predicted γ carotene 3',4' desaturase (acI-CrtD). The protein from acI genome AAA278-O22 is aligned to functionally characterized CrtD proteins from Marine bacterium P99-3 (MbP99-3) and *Nonlabens dokdonensis* DSW-6 (Nd).



Dwulit-Smith et al., Suppl. F1

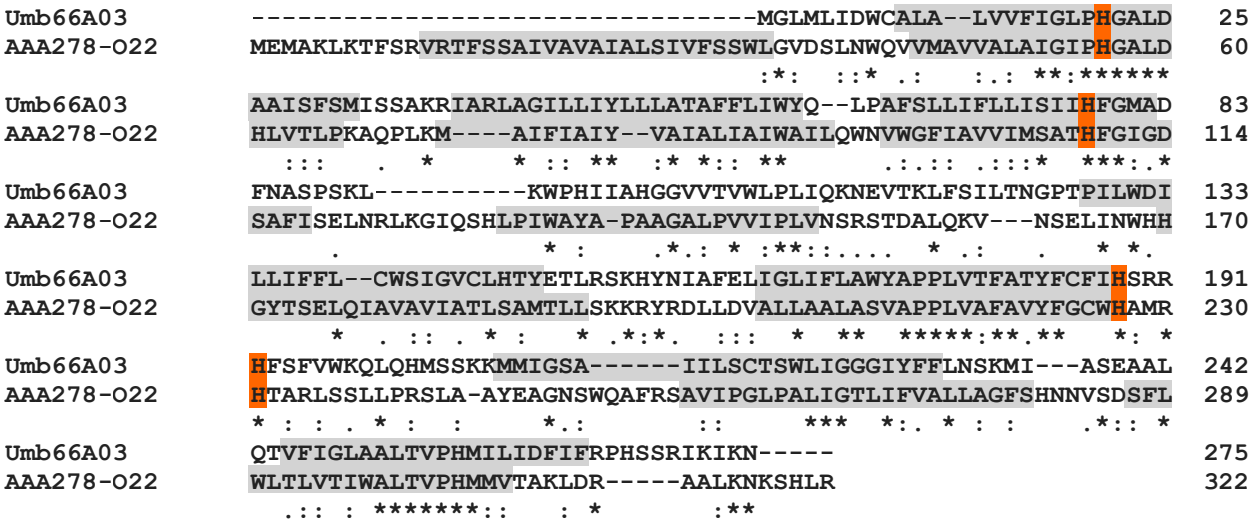
A

```
AAA278-O22      ------MGMLIFT-LCGSGWLEIVLKTGVLRRLKRAALSILPISIFFLIWDAYAIAKGHW   53
Mx              MTYARFLGLFVVVPILFLAWRYR--RTFTARSLAPMGLLLIVVYAATSPWDNLAVKWGLW   58
                      :*:::.. :   .*     :* . * *   .* :: :      **  *:  * *
AAA278-O22      FFDRQQMLGIIGPFNIPLEEYLFFIVVPLAAILTIEG---------VTTVKPHWRKGEFG  104
Mx              GFDPERIWGIKLG-YLPLEEYLFFALQTLLVGLWAQARLARALAPDAQASRPAAETGERR  117
                 ** ::: **     :******** :  * . *  :.          . : :*  ..**
AAA278-O22      E----------                                                    105
Mx              EGALTAREVAP                                                   128
                *
```

B

```
AAA278-O22      -----------MIYSDIAIAAFGISVMVDLFIFKNSMLTRAAFWTSYAIILPFQLLTNWW   49
Mx              MMETKWAYLIHLLGWTLPVIAFQLVVLVRHYKERSGAVLKA--------VLPPAFIMGLY   52
                           ::   : : ** : *:*  :  :.. : :*        :**  :: . :
AAA278-O22      -------LTSRNIVMYNPDAIIGVRICSAPVEDLLFGF-ALVLSVMGMWEFWGRRGLQRH  101
Mx              LSIADHLAISTGIWNFGEGKHLGVYLGVVPLEEVVFFLITSVLVSLGLALFTGLVALLGE  112
                         * .*  :. .  :** :  .*:*:::* : : **  :*:  * *  .*  .
AAA278-O22      ----                                                          101
Mx              ARAS                                                          116
```

Dwulit-Smith et al., Suppl. F2



Dwulit-Smith et al., Suppl. F3

```
Rs              --MQTVTLSIFRFNEFEKRLWVLGQMTANKLGMHYLPKAKFWKMFGSGTGQGFTP-KPNW   57
MbP99-3         MSKQITTLTFFKYPKLKDKIWAFWMMQFAHSALRRQSGLQSYKLLGSGKEG-FSP-WPDW   58
AAA278-O22      --MQLTVVYLFSVE-RKSIPFALISMAIDRLRTRMFTGISFSKLLGTGTGRTFTPSDADL   57
                   * ..: :*     :.  :.:  *   :   :      .  *::*:*.   *:*   :
Rs              HVWSILAVWPDEETARREVAESPIYQRWTKMADESYTVLLQPTSAWGKWDGKEPFEPVKP  117
MbP99-3         SVYGLLQVWDSHKEAQEFFDSSSLYKKYLNHSEQQLTFYMKNIKAYGQWSKKNPFEQHSD  118
AAA278-O22      TRWGMVVVID--KDRLDAFDQSSIVTNWRKRSTSEFRALLSPLSSHGLWAKKNPFDFVAP  115
                  :.:: *    :     . .* :  .: : : ..    :.  .: * *  *:**:
Rs              ASDVRP-IAALTRATVKFWKAERFWAREPAISHMIGRNKDVVFKIGVGEVPFVQQVTFSI  176
MbP99-3         MDTNNPYLTVITRATIKPHMLKKFWDYVPISQKGLKENPSLLFTAGVGERPFTHMATFSL  178
AAA278-O22      FSNSEAQIAAITRARIKWNKNFIFWKSVPPVVLDLHSNPGLIAAIGIGEAPIGLQGTFSL  175
                      .  .  ::.:*** :*      **   *:  * .::   *:** *:    ***:
Rs              WPDASSMEEFARGAGGPHGEAIKAVRAENWFKEELYARFQILGTIGKWEGKDPVGEALTA  236
MbP99-3         WDDARALKKFAYR-GNNHKQAIQQTQALQWYKEEMFSRFQPYLITGSWQGFTIPELL-SF  236
AAA278-O22      WQSASALRDFAYK-SKAHQVAIAQTESIGWYSEELFARFEVLELRGEITAHAGK------  228
                * .* ::..**   .  *  **  ..:  *:.**:::**:     *.  .
Rs              RPSEAPKPAPAPAAAQPAPAVEAPKPAPAPVAEKPALAVEMPKSAEPPKPVVEAPKPASA  296
MbP99-3         KET-----------------------------------------------------------  239
AAA278-O22      -----------------------------------------------------------  228

Rs              PVASKPMPQGGKPNFKGKPGKGGRKENA                                  324
MbP99-3         ----------------------------                                  239
AAA278-O22      ----------------------------                                  228
```

Dwulit-Smith et al., Suppl. F4

Dwulit-Smith et al., Suppl. F5

Dwulit-Smith et al., Suppl. F6

Dwulit-Smith et al., Suppl. F7