For consideration as a Brief Communication:

Insights on the importance of salinity from the first cultured freshwater SAR11 (LD12) representative

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SAR11 bacterioplankton dominate aquatic ecosystems but knowledge about freshwater members suffers from a lack of cultured representatives. Here, we report the first freshwater SAR11 isolate (LD12), obtained from surface waters in Lake Borgne, LA. Consistent with pervasive ecological data, strain LSUCC0530 growth was highly restricted by salinity. Comparisons with sister subclade IIIa taxa, however, suggests that niche differentiation between these groups in coastal environments results from more than just salinity.

While many environmental conditions (e.g. nutrients, temperature, pH) play an important role in structuring microbial assemblages, salinity remains one of the most important factors affecting microbial community membership [1]. This reflects the fact that evolutionary transitions between marine and freshwater environments occur rarely among members of a given phylogenetic group [2]. SAR11 is the most abundant prokaryotic marine clade, with an estimated global population size of ~10^{28} cells, and can constitute over 25% of the bacterioplankton in a given community [2,3]. The SAR11 clade, family *Pelagibacterales*, contains multiple subclades that can have unique spatiotemporal distributions [4]. However, despite the massive population size and an estimated divergence time from other *Alphaproteobacteria* roughly 1.1 billion years ago [5], only one subclade (LD12/subclade IIIb) has evolved to colonize freshwater environments [6]. Subclade IIIb was first identified in an Artic lake (LD12)[7] and represents a ubiquitous and abundant freshwater group [8,9]; like its marine counterparts, it can comprise up to 21% of freshwater bacterioplankton [10].

Our existing knowledge regarding the underlying genomic basis for the SAR11 shift to freshwater ecosystems comes from culture independent methods: metagenomics [11,12] and single-cell genomics [13,14]. These data point to changes with important cellular energetics implications, such as acquisition of the Embden-Meyerhof-Parnas glycolysis pathway, loss of the glyoxylate shunt and C1 metabolism, and a general trend towards de novo synthesis, rather than uptake, of many important amino acids, osmolytes and other compounds. While we have learned much from these efforts, the lack of cultivated representatives hampers further testing of hypotheses regarding freshwater SAR11 niche differentiation and energetics, as well as the underlying physiology that fundamentally restricts their distribution. Here we report the

first successful isolation and propagation of an LD12/subclade IIIb representative, strain LSUCC0530. Comparisons with a representative of the sister subclade IIIa, strain LSUCC0261, isolated from a nearby site in the Gulf of Mexico (GOM), revealed important differences in salinity tolerances and ecological distribution across varied environments.

Phylogenetic inference of strain LSUCC0530 placed it in the Family *Pelagibacteriales*, subclade IIIb, along with the LD12 clone (Fig. 1A). Cells of strain LSUCC0530 were curved rods, < 1µm x 0.1 µm (Fig.S1A-B), that grew to a density of 2 x 10e7 cells mL^{-1} at 24˚C in JW5 medium (Table S1) that had a salinity of 1.45 ppt. Comparatively, cells of strain LSUCC0261, from the sister subclade IIIa ("IIIa"), were also small curved rods (Fig.S1C), but that grew to a density of 1 x 10e6 cells mL^{-1} at 24˚C in JW2 with a salinity of 23.18 ppt [15]. Notably, both LSUCC0530 and LSUCC0261 matched the previous description of SAR11 strain HTCC1062 (subclade Ia)[16] demonstrating considerable morphological conservation across large evolutionary distances (LD12 vs IIIa 92%, LD12 vs Ia 89% 16S rRNA gene identity).

Ecological data from various sites around southern and Louisiana indicated that OTUs representing both taxa occurred in high abundances across varied environments. At Louisiana estuarine and coastal sites that could be classified as nearly freshwater/brackish (< 6 ppt), LD12 and IIIa shared high average rank abundances (RA) of 8.9 and 25.7, respectively (Fig. 1, Table S1). At coastal and estuarine sites with > 6 ppt, LD12 was much less abundant or not present (average RA 348.8 when present), while IIIa had slightly higher abundances (average RA 42.1) compared to those in fresh water (Fig. 1, Table S1). LSUCC0530-type organisms dominated the MSR microbiome, occupying the top 6 OTU ranks in all but two of the samples where it occurred within the top 15 ranks. On the contrary, we found no OTU representing LSUCC0261 in the MSR using the criteria of appearing with more than 2 reads in over 20% of the samples.

To examine the physiological basis for our ecological observations, we tested the hypothesis that strains LSUCC0530 and LSUCC0261 would have unique salinity tolerances and optima for growth. Our experiments showed that the LD12 strain, LSUCC0530, could not grow at added NaCl of 1% or above, while subclade IIIa strain, LSUCC0261, grew at a range of salinities from 0-4% NaCl (Fig. 2). LSUCC0530 grew

optimally at 0% added NaCl at an average rate of ~ 0.04 divisions hr^{-1} and a maximum cell density of 2 x 10e7 cells mL^{-1} (Fig. 2 and S3). We note that salinity ranges between 0-1% added NaCl were not tested so LSUCC0530's true optimum may have been missed. LSUCC0261 grew optimally at 3% added NaCl with an average rate of ~0.043 divisions hr^{-1} and a maximum cell density of 5 x 10e6 cells mL^{-1} (Fig. 2 and S4). Comparatively, at 0% salinity LSUCC0261 had a growth rate of ~ 0.014 divisions hr^{-1} (Fig. 2), roughly 3x lower than LSUCC0530.

While our physiological results clearly show unique growth relationships with salinity for each strain, these data do not completely explain their ecological distribution. Inland sites appear to be dominated by LD12 taxa, but coastal sites with a dynamic interface of marine, brackish, and fresh waters do not show complete competitive exclusion (Fig. 1). The ability of LSUCC0261 to grow in 0% added NaCl suggests that salinity alone does not prevent colonization of freshwater environments by subclade IIIa taxa. Concordantly, while rank abundance had a nearly linear correlation with salinity for LD12 organisms (R^2= 0.73), consistent with its lack of growth in 1% or greater NaCl, we observed no pattern between subclade IIIa OTU abundances and salinity (Fig. S2). Furthermore, attempts to grow LSUCC0261 in JW5 medium failed (data not shown), even though it could grow in JW2 modified to a similar salinity (Table S1). This narrows the list of additional limiting factors. The two media contained identical carbon, nitrogen, iron, vitamin, and trace metal constituents (Table S1). Though phosphate was lower in JW5, it remained greater than environmental concentrations measured throughout the coastal GOM [15]. Therefore, the nutrient that prevented LSUCC0261 growth in JW5, and potentially plays a role its distribution in aquatic systems, likely was one or some combination of boron, bromine, strontium, calcium, magnesium, fluorine, or sulfur (as sulfate), many of which could serve as metabolic co-factors (Table S1). Future work will examine this hypothesis.

The first cultured representative of freshwater SAR11, strain LSUCC0530, has facilitated direct testing of the importance of salinity in differentiating the LD12 subclade from its sister group subclade IIIb. Ecological data indicates partial overlap in habitat and suggests that the mechanisms underlying niche differentiation between these clades may constitute complex traits not solely related to salinity tolerance. Future

125 research should more deeply examine the role cellular energetics (as proposed by [11,14])
126 plays in their relative success across these dynamic coastal environments.

127 For the first cultured representative of the LD12 clade, we propose the provisional
128 taxonomic assignment for strain LSUCC0530 as '*Candidatus* Fonsibacter ubiquis',

129 *Fonsibacter* gen. nov.

130 *Fonsibacter* ubiquis sp. nov.

131 Etymology. *fons* (L. noun): fresh water, spring water, -bacter (Gr. Adj.): "rod,
132 bacterium". ubiquis (L. adv.): everywhere. The Genus name refers to the isolation
133 source and recognized habitat in fresh water, and its shape. The species name refers to
134 the fact that LD12/subclade lllb is ubiquitous in freshwater ecosystems.
135
136 **Methods**
137
138 Isolation, propagation, and phylogenetic identification of strain LSUCC0530
139 (subclade IIIb) followed the previously reported methodology in Henson et al.[15] with
140 additional phylogenetic inference using RAxML (Supplementary Information). Strain
141 LSUCC0530 was isolated from Lake Borgne (Shell Beach, Louisiana) (29.872035 N, -
142 89.672819 W) on July 1st, 2016 with medium JW5 (Table S1). Isolation of strain
143 LSUCC0261 (subclade IIIa) was previously reported in Henson et al. (2016b). Salinity
144 tolerances were assessed with quintuplet cultures of LSUCC0530 and LSUCC0261 in
145 JW5 and JW2 media, respectively, with altered NaCl concentrations of 0, 1, 2, 3, 4, and
146 5%. Cultures were enumerated using the Guava EasyCyte (Millipore) flow cytometer
147 roughly every 24 hours [17].Growth rates were calculated in the R software package
148 Growthcurver [18]. Calculations excluded individual replicates that grew at much lower
149 rates (Figs. S3, S4), but in all cases averaged at least quadruplicate data.
150 Ecological data from 16S rRNA gene amplicons was amassed from the
151 Louisiana coast [15], the Mississippi River[9], with additional data from Lake Martin, Sabine
152 Wetlands, and Bay of Batiste analyzed using the protocol from Henson et al.[9]. To
153 determine which OTUs represented LSUCC isolates, sequences from the OTU

representative fasta files, provided by mothur using get.oturep(), were used to create a blast database (formatdb) against which the LSUCC isolate 16S rRNA genes could be searched via blastn (BLAST v 2.2.26). All best hits ranked at 100% identity. All salinity data was recorded on site using a YSI 556 MPS (YSI, Solon, OH, USA)[15] with the exception of Mississippi river salinity data, which was inferred from USGS gauge information or previously collected data (Table S1).

For microscopy, LSUCC0530 and LSUCC0261 cells were grown to max density in their respective medium, JW5 and JW2. LSUCC0530 cells for transmission electron microscopy were prepped following Rappé *et al*.[16] with one minor change. Spun cells were concentrated in JW5 medium with no fixative added. Cells were visualized under a JEOL JSM-2011 High Resolution transmission electron microscope at the Socolofsky Microscopy Center, Baton Rouge, LA. For scanning electron microscopy, cultured cells were fixed for four hours in 4% Glutaraldehyde buffered with 0.2M Cacodylate buffer and filtered onto a 0.2 µm filter. Samples were dehydrated in an ethanol series (30%, 50%, 70%, 96 and 100%) for 15 minutes each, followed by critical point drying. Cells were visualized under a JEOL JSM-6610 scanning electron microscope at the Socolofsky Microscopy Center, Baton Rouge, LA.

Data Availability

Newly generated 16S rRNA gene sequence fastq files are available at the NCBI Sequence Read Archive under the accession numbers: SRR5082252-SRR5082264. All other accession numbers can be found in their respective publications. The strain LSUCC0530 16S rRNA gene sequence is available at NCBI under the accession number: KY290650.

Code Availability

All scripts for figure generation can be found on the Thrash Lab website (http://thethrashlab.com/publications) with the reference to this manuscript linked to “Supplementary Information”.

Author Contributions

MWH conceived and designed the experiments, performed the experiments, analyzed the data, and wrote the paper. VCL performed the experiments. JCT conceived and designed the experiments, assisted in analysis, contributed reagents/materials/analysis tools, and helped write the paper.

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Competing Financial Interests

The authors declare there are no competing financial interests.

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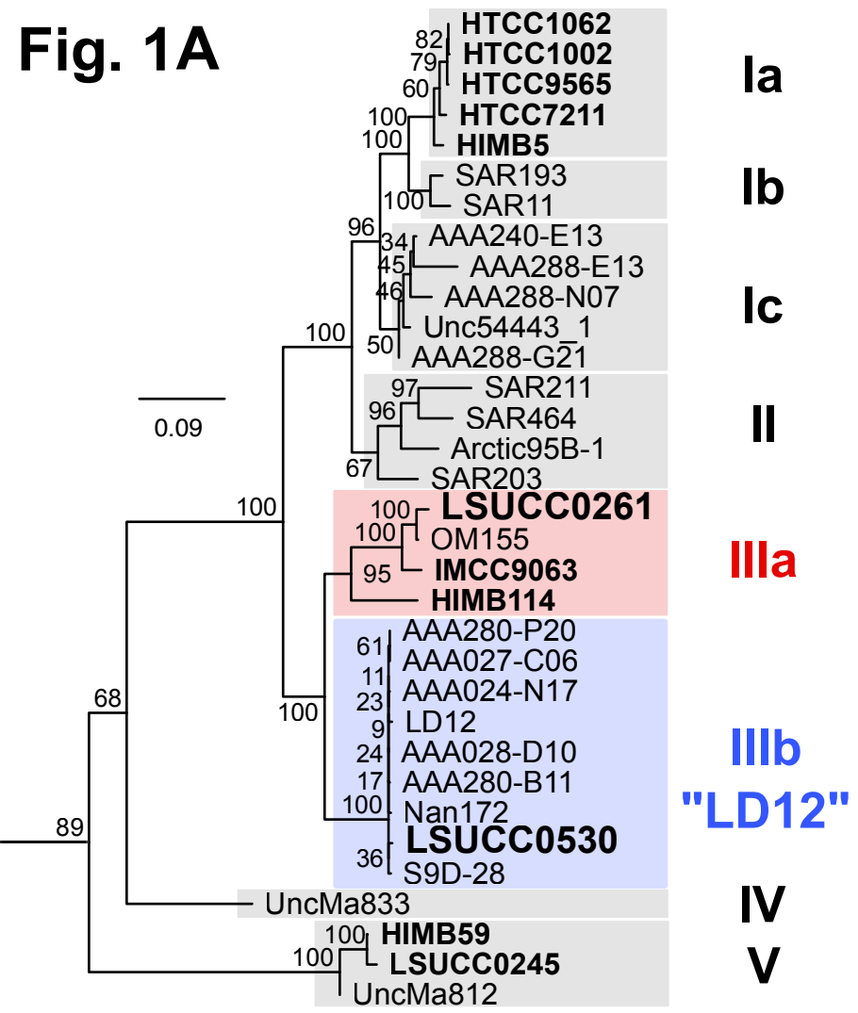
Figure Legends

Figure 1. A) Phylogenetic tree of the SAR11 clade. Nodes highlighted in blue are part of subclade IIIb, while nodes highlighted in red are part of subclade IIIa. Values at internal nodes indicate Shimodaira-Hasegawa “like” test values. B) Ecological distribution of SAR11 LSUCC0530 and LSUCC0261 along the Louisiana coast, along the Mississippi River, and at Lake Martin. LSUCC0530 and LSUCC0261 are blue and red dots, respectively. Size of the dot corresponds to the log transformed rank abundance, while shade of the dot represent the measured or inferred salinity.

Figure 2. Growth rate of LSUCC0530 and LSUCC0261 replicates as calculated in the various %NaCl concentrations added to the JW2 and JW5 media (0-5). LSUCC0530 and LSUCC0261 are blue and red dots, respectively. Non-linear regressions are provided for guidance.

Fig. 1A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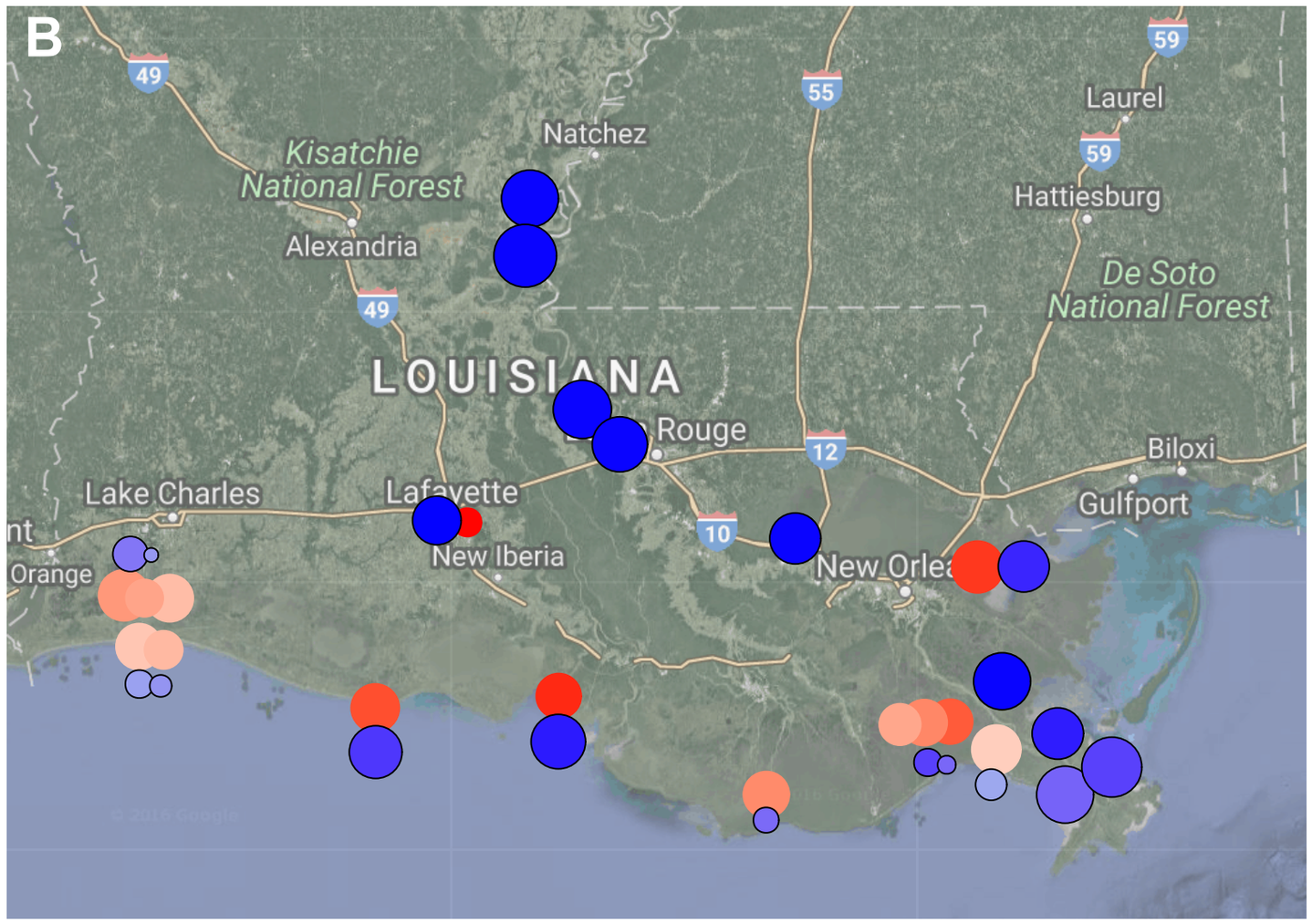
HTCC1002
HTCC9565
HTCC7211
HIMB5
SAR193
SAR11
AAA240-E13
AAA288-E13
AAA288-N07
Unc54443_1
AAA288-G21
SAR211
SAR464
Arctic95B-1
SAR203
LSUCC0261
OM155
IMCC9063
HIMB114
AAA280-P20
AAA027-C06
AAA024-N17
LD12
AAA028-D10
AAA280-B11
Nan172
LSUCC0530
S9D-28
UncMa833
HIMB59
LSUCC0245
UncMa812
0.09
Ia
Ib
Ic
II
IIIa
IIIb
"LD12"
IV
V


B
Natchez
Kisatchie
National Forest
Alexandria
LOUISIANA
Rouge
Lake Charles
Lafayette
New Iberia
Laurel
Hattiesburg
De Soto
National Forest
Biloxi
Gulfport
49
55
59
12
10

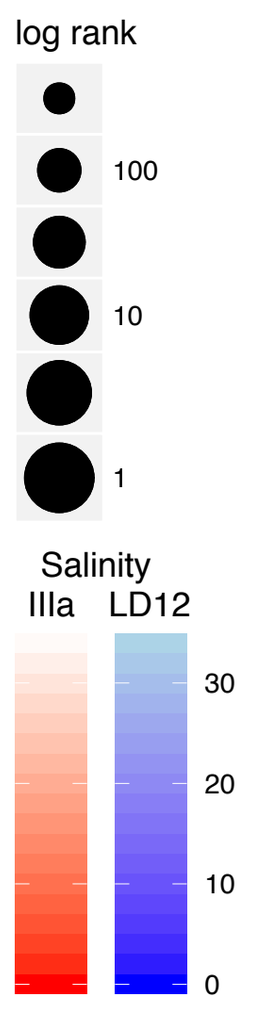
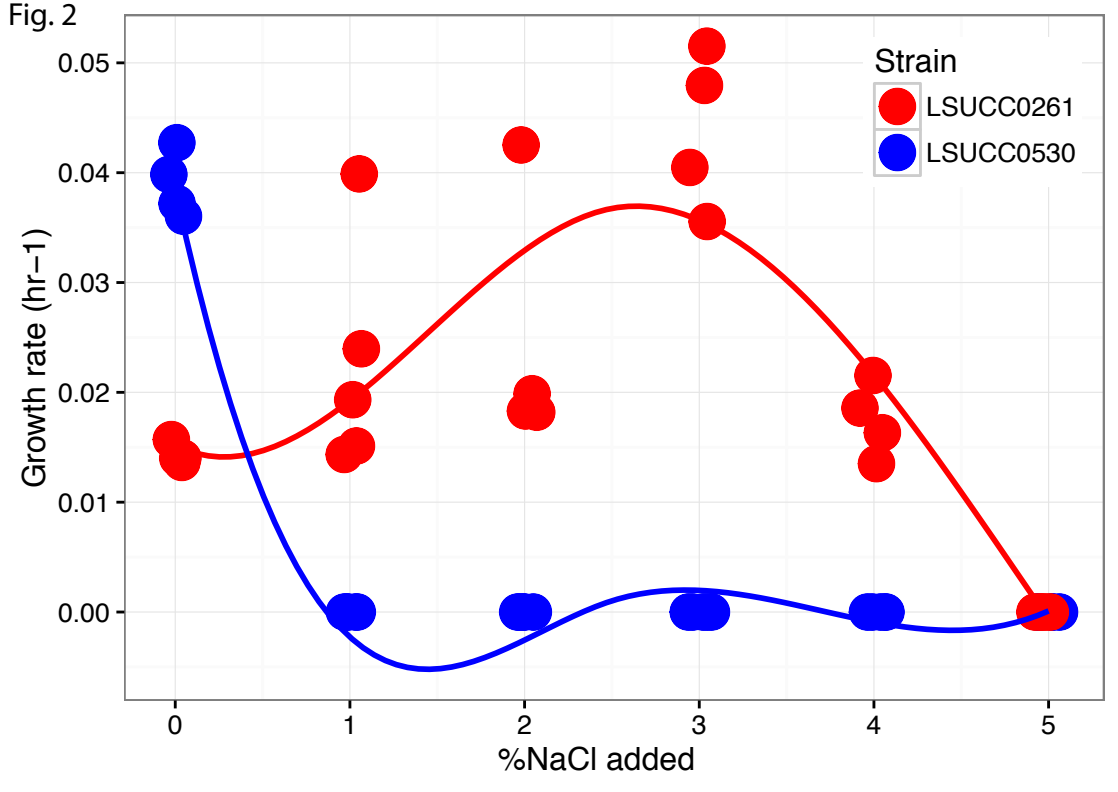

log rank
100
10
1
Salinity
IIIa
LD12
30
20
10
0

Fig. 2


Strain
LSUCC0261
LSUCC0530
Growth rate (hr−1)
0.05
0.04
0.03
0.02
0.01
0.00
0
1
2
3
4
5
%NaCl added