SAR11 lipid renovation in response to phosphate starvation

Paul Carini*,1, Benjamin A. S. Van Mooyb, J. Cameron Thrashc, Angelique Whiteb, Yanlin Zhaoa, Emily O. Cambella,2, Helen F. Fredricksb, and Stephen J. Giovanninib,3

*Department of Microbiology, Oregon State University, Corvallis, OR 97331; †Department of Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, Woods Hole, MA 02543; ‡Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803; and §College of Earth, Ocean, and Atmospheric Sciences, Oregon State University, Corvallis, OR 97331

Phytoplankton inhabiting oligotrophic ocean gyres actively reduce their phosphorus demand by replacing polar membrane phospholipids with those lacking phosphorus. Although the synthesis of nonphosphorus lipids is well documented in some heterotrophic bacterial lineages, phosphorus-free lipid synthesis in oligotrophic marine chemoheterotrophs has not been directly demonstrated, implying that they are disadvantaged in phosphate-deplete ecosystems, relative to phytoplankton. Here, we show the SAR11 clade chemoheterotroph *Pelagibacter* sp. str. HTCC7211 renovates membrane lipids when phosphate starved by replacing a portion of its phospholipids with monoglycosyl- and glucuronosyl-diacylglycerol and by synthesizing new ornithine lipids. Lipid profiles of cells grown with excess phosphate consisted entirely of phospholipids. Conversely, up to 40% of the total lipids were converted to nonphosphorus lipids when cells were starved for phosphate, or when growing on methylphosphonate. Cells sequentially limited by phosphate and methylphosphonate transformed >75% of their lipids to phosphorus-free analogs. During phosphate starvation, a four-gene cluster was significantly up-regulated that likely encodes the enzymes responsible for lipid renovation. These genes were found in *Pelagibacter* strains isolated from a phosphate-deficient ocean gyre, but not in other strains from coastal environments, suggesting alternate lipid synthesis is a specific adaptation to phosphate scarcity. Similar gene clusters are found in the genomes of other marine α-proteobacteria, implying lipid renovation is a common strategy used by heterotrophic cells to reduce their requirement for phosphorus in oligotrophic habitats.

Microbes primarily assimilate phosphorus (P) in its +5 valence state (phosphate; P\textsubscript{i}), which comprises ~3% of total cellular mass as a structural constituent of nucleic acids and phospholipids, and is intimately involved in energy metabolism and some transport functions (via ATP hydrolysis) (1). In oligotrophic ocean gyres, P\textsubscript{i} concentrations are extremely low (0.2–1.0 nM in the Sargasso Sea; ref. 2) and the availability of P\textsubscript{i} can limit bacterial and primary production (2–5). Microbes inhabiting these low P\textsubscript{i} environments have evolved numerous strategies to maintain growth and enhance their competitiveness for trace amounts of P\textsubscript{i}. These mechanisms are commonly induced by P\textsubscript{i} starvation and include one or more of the following: (i) expression of high affinity P\textsubscript{i} transporters (6); (ii) reduction of cellular P\textsubscript{i} quotas (7, 8); (iii) utilization of alternate phosphorus sources (9, 10); and (iv) polyphosphate storage and breakdown (11, 12). Such strategies facilitate survival in the face of P\textsubscript{i} insufficiency. Polar membrane lipids are a substantial cellular sink for phosphate in bacteria. Structural lipids consist of glycerol esterified to phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and diphosphatidylglycerol (13). However, when P\textsubscript{i} is low or limiting, many microbes replace phospholipids with those that lack phosphorus (14–16). Non-phosphorus polar head groups are structurally diverse and include sulfoquinovose (sulfolipids) (17), various monosaccharides and disaccharides (glycolipids; reviewed in ref. 18), ornithine, or other amino acids (reviewed in ref. 19). When P\textsubscript{i} stressed, the marine cyanobacterium *Prochlorococcus* and *Synechococcus* reduce their P\textsubscript{i} demand by 0.5–8.6 attomoles P per cell by substituting phosphorus-containing lipids with sulfolipids; depending on the strain, this reduction equates to 10–86% of the P bound in their nucleic acids (7). There is indirect evidence natural populations of marine chemoheterotrophic bacteria also use nonphosphorous lipids in response to P\textsubscript{i} deprivation. Bacterioplankton collected in the Sargasso Sea had greater concentrations of nonphosphorous lipids than those from adjacent regions of the North Atlantic where P\textsubscript{i} was relatively abundant (20).

Oligotrophic bacteria belonging to the SAR11 clade (*Pelagibacterales*) of α-proteobacteria are numerically dominant chemoheterotrophs in marine euphotic zones worldwide (21). *Pelagibacterales* cells are small (volume of 0.01 μm\textsuperscript{3}; ref. 22) and contain streamlined genomes (23, 24). The reduced cell and genome size likely stem from natural selection to reduce the overhead cost of the marine phosphorus cycle | lipids | glucuronic acid | cyanobacteria | methylphosphonate

**Significance**

Nonphosphorus lipids produced by heterotrophic bacteria have been measured in marine ecosystems without an understanding of their origins or role. This work shows SAR11 chemoheterotrophic bacteria synthesize multiple nonphosphorus lipids in response to phosphate depletion. Because this process results in a reduced cellular P:C ratio, it impacts our understanding of ocean processes related to cellular elemental stoichiometry by showing how different environmental parameters alter P:C ratios in heterotrophs. Also, SAR11 grown with excess organophosphonate synthesized phosphorus-free lipids. This finding contrasts the contemporary view of organophosphorous utilization because organophosphorate-derived phosphorus did not equally substitute for inorganic phosphate in lipids. Considering lipid phosphorus content was lower in cells using organophosphate, phosphorous-based productivity estimates may vary as a function of phosphorus source.

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1Present address: Cooperative Institute for Research in Environmental Sciences, University of Colorado, Boulder, CO 80309.

2Present address: Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, OH 45237.

3To whom correspondence should be addressed. Email: steve.giavaninni@oregonstate.edu.

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replication in oligotrophic ocean gyres where P and N may periodically limit growth (25, 26). Despite their abundance in low P environments, it remains unknown whether *Pelagibacter* synthesizes phosphorus-free lipids to reduce their P quota. The genome of *Pelagibacter ubique* str. HTCC1062 lacks genes predicted to encode proteins used in sulfolipid, betaine, or ornithine lipid biosynthesis, suggesting this strain is unable to modulate lipid composition in response to P availability (7, 9). Previous laboratory experiments partially supported this prediction by showing that *P. ubique* lacks nonphosphorus lipids when grown under P-replete conditions; however, lipids from P-limited cells were not examined in that research (7). Relative to *P. ubique*, a Sargasso Sea isolate, *Pelagibacter* sp. str. HTCC7211 (str. HTCC7211) contains extended genetic inventory associated with P acquisition, storage, and metabolism (10). When P-limited, str. HTCC7211 induces a suite of these genes, including both inorganic (pslSCAB) and organophosphate (phnCDEE₃) ABC transporters and the C–P lyase complex (phnGHJKLNM), required for phosphodegradation (10). Laboratory experiments have distinctly linked the expression of these genes to the utilization of both phosphate esters and phosphonates (including methylphosphonate; MPn) (10). This finding indicates organophosphate utilization is one strategy str. HTCC7211 employs to evade P limitation. 

While examining gene expression profiles of P-starved str. HTCC7211 cultures, we observed up-regulated and a four-gene cluster proximal to the collection of P uptake genes on the str. HTCC7211 chromosome. Two of these genes were annotated as “putative hemolysins,” one as a “glycosyltransferase,” and a “metallophosphatase.” Comparative genomic examination of these genes led us to hypothesize that the four genes might be involved in the restructuring of lipid polar head groups and the synthesis of nonphosphorus lipids. Herein, we present the results of laboratory experiments designed to test the potential for synthesis of nonphosphorus lipids in response to P-limited growth by *Pelagibacter*.

**Results**

Previously we reported P-limited growth in str. HTCC7211 significantly induced the expression of 31 genes, including those encoding P- and organophosphorus transport and catabolism proteins (10). Proximal to the genes encoding a high-affinity P transporter (pslSCAB), a four-gene cluster (HTCC7211_00011000–HTCC7211_00011030) was significantly up-regulated in P-starved cultures, relative to P-replete cultures (2.7- to 8.2-fold; reported in ref. 10). Two of these genes (HTCC7211_00011000 and HTCC7211_00011010) are oriented in a probable operon (+ strand; Fig. 1) and annotated as “putative hemolysins” belonging to COG3176. The other two genes (HTCC7211_00011020 and HTCC7211_00011030) are located downstream of HTCC7211_00011010 in a second probable operon (- strand; Fig. 1). HTCC7211_00011020 is annotated as a group 1 glycosyltransferase belonging to COG438 (RfaG). HTCC7211_00011030 is annotated as a metallophosphoesterase belonging to COG2908. Protein domain signatures in each predicted amino acid sequence were identified with InterProScan5 (27). HTCC7211_00011000 contains an acyl-CoA N-acetyltransferase domain (IPR016181; residues 26–174). HTCC7211_00011010 contains a phospholipid/glycerol acetyltransferase domain (IPR002123; residues 49–180). HTCC7211_00011020 contains a glycosyltransferase subfamily 4, N-terminal domain (IPR028098; residues 5–164), and a glycosyltransferase, family 1 domain (IPR012926; residues 177–285). HTCC7211_00011030 contains a calcium-sensing phosphoesterase domain, ipxH type domain (IPR024654; residues 10–249).

We searched for orthologs to HTCC7211_00011000–HTCC7211_00011030 in α-proteobacterial genomes by using the Hal pipeline (28) and found all four genes differentially distributed across the clade (Fig. 1 and Dataset S1). HTCC7211_00011000 clustered with amino acid sequences of characterized enzymes from *Ensifer (Sinorhizobium) melloti* 1021 (OlsB) and *Rhodobacter capsulatus* SB 1003 (OlsB) (Fig. 1). OlsB catalyzes the first step in ornithine lipid formation (29, 30) (Fig. S1). The second step of ornithine lipid biosynthesis is catalyzed by the protein product of *olsA* (Fig. S1), which is frequently found downstream of *olsB* (30, 31). Unexpectedly, the amino acid sequence of HTCC7211_00011010 did not cluster with *OlsA* sequences from characterized enzymes, despite all three having phospholipid/glycerol acetyltransferase domain (IPR002123) signatures. HTCC7211_00011020 clustered with an *Agrobacterium fabrum* C58 gene (Fig. 1) recently demonstrated to encode a bifunctional glycosyltransferase that forms both monoglucosyl- and glucuronosyl-diacylglycerols (MGDG and GADG, respectively) by the addition of glucose or glucuronic acid moieties to diacylglycerols (32) (Fig. S2). HTCC7211_00011030 clustered with an *E. melloti* gene shown to encode a phospholipase C (PlcP) (Fig. 1) that initiates lipid renovation during P starvation by cleaving phosphorus-containing polar head groups from diacylglycerols (8) (Fig. S2).

We hypothesized that HTCC7211_00011000–HTCC7211_00011030 encode proteins involved in the renovation of membrane lipids during P stress. Renovation is defined here as the combined result of: (i) the synthesis of new ornithine lipids; and (ii) the exchange of phospholipids for MGDG and/or GADG. We based this hypothesis on the increased expression of these genes under P-limiting conditions (10), and the characterized functions of orthologous genes in other *Pelagibacter* isolates (Fig. 1). We tested this hypothesis by analyzing the lipid polar head group composition over time in P-replete and P-deplete growth conditions (Fig. 2). Total lipids extracted per sample ranged from 2.8 to 9.7 attomoles-cell⁻¹ and varied as a function of growth state and P source (Dataset S2 and Fig. S3).

Strain HTCC7211 cells growing exponentially with excess P, (Fig. 2A) exclusively contained phospholipids (PG and PE) (Fig. 2B). In carbon-limited stationary phase, a low proportion of GADG lipids were detected (<1%; Fig. 2B). Cells incubated in growth medium without added P did not grow appreciably (Fig. 2C), but renovated 26–38% of total cellular phospholipids with multiple P-free lipids (GADG > MGDG > ornithine lipid; Fig. 2D). Although cells renovated membrane lipids in the absence of P, total cellular lipid content decreased, relative to P-replete cells, suggesting cell size was slightly reduced (Fig. S3).

Lipid profiles of cells resuspended into growth medium with MPn as the sole P source were measured. Exponentially growing cells using MPn as the sole P source (Fig. 2E) replaced phospholipids with P-free lipids (Fig. 2F). In this growth condition, up to 40% of total cellular phospholipids were renovated to P-free analogs. Similar to P-replete profiles, GADG was the most abundant P-free lipid (Fig. 2F). However, after an initial drop in abundance, the total percent of phospholipids increased to 87% as the cells entered carbon-limited stationary phase. Total cellular lipid contents in MPn-replete cells were comparable to those of P-replete cultures, suggesting cell size was conserved during renovation (Fig. S3).

Cells harvested in midlogarithmic phase, grown with MPn as the sole P source for >20 generations, contained a standing stock of 11% P-free lipids (Fig. S4). P-replete cells exposed to MPn levels sufficient to sustain >1 generation, but insufficient for maximal growth (that is, cells were MPn-limited; Fig. 3A), contained 77% P-free lipids in MPn-limited stationary phase (Fig. 3B). A lesser degree of conversion was observed when P-replete stars were exposed to P sufficient for >1 generation, but insufficient for maximal growth (Fig. 3A and C).

Previous measurements of the lipid composition of *P. ubique* str. HTCC11062 growing in P-replete natural seawater medium showed that only PE and PG were present in lipid extractions (7). We conducted similar experiments with P-replete and P-starved str. HTCC11062 cells grown in synthetic medium. The lipids of str. HTCC11062 cells growing in P-replete conditions were
Fig. 1. Distribution of nonphosphorus lipid synthesis genes in α-proteobacteria. Tree inferred from concatenated 16S/23S rRNA gene sequences. Most monophyletic groups where all members had the same patterns of gene distribution were collapsed. The outgroup taxa can be found in Dataset S1. Node labels represent Shimodaira–Hasegawa confidence test values: black filled, ≥0.9; gray filled, 0.7–0.9; white filled, ≤0.7. Pelagibacter sp. str. HTCC7211 gene orientation is depicted above colored bars; ”HTCC7211_000” for each gene identifier has been omitted. The colored bars are a visual representation of Hal clustering results. Bars indicate ortholog presence (filled), absence (open), and chromosomal synteny (black line between bars), or lack of synteny (no black line between bars), for the four genes. Those taxa with adjacent glycosyltransferases and metallophosphoesterases are colored blue in the tree for ease of identification. Numbers inside of colored boxes indicate genes for which functions have been characterized, as follows: 1, SMc01116 (OlsB, E. meliloti); 2, RCAP_rcc02997 (OlsB, R. capsulatus); 3, SMc00171 (phospholipase C, E. meliloti); 4, atu2297 (GADG/MGDG glycosyltransferase, A. fabrum, in collapsed node).

Note 1: Sulfitobacter spp., O. indolifex, R. denitrificans and litoralis, Rhodobacterales HTCC2083. Note 2: Erythrobacter HTCC2594/NAP1, and C. bathyomarinum JLR354, N. aromaticivorans DSM 12444, Sphingobium spp, Sphingopyxis alaskensis RB2256, Sphingomonas SKAS8.
comprised exclusively of phospholipids (PG and PE). Nonphosphorus lipids were not detected under conditions of P starvation (Fig. S5).

Discussion

Similar to phytoplankton (7) and terrestrial α-proteobacteria (8), we show Pelagibacter sp. str. HTCC7211, but not P. ubique str. HTCC1062, modulates its phospholipid composition in response to P availability (Fig. 2). We identify the genes likely to confer the ability to renovate polar lipid head groups (Fig. 1) and link to studies of their regulation (10). Published metagenomic analysis show that three of these genes are overrepresented in the Sargasso Sea (HTCC7211_00011010–HTCC7211_00011030) relative to Pelagibacterales populations inhabiting the comparatively P-replete North Pacific subtropical gyre (11). In these studies, a metric called “multiplicity per cell” was used to infer that ∼28% of the Pelagibacterales population contained HTCC7211_00011010; ∼95% contained HTCC7211_00011020 and all cells contained HTCC7211_00011030 (~117%, indicating one or more copies in some genomes). These data suggest that the capacity to convert phospholipids to glycolipids, as conferred by orthologs to HTCC7211_00011020 (MGDG/GADG glycosyltransferase) and HTCC7211_00011030 (phospholipase C), is widespread in Pelagibacterales lineages inhabiting the Sargasso Sea and is likely a specific adaptation to P scarcity. These findings likely explain reports of MGDG production by chemoheterotrophic bacteria from the Sargasso Sea (20).

The complete four-gene cluster conferring glycolipid and ornithine lipid biosynthesis in str. HTCC7211 is not found in the same syntenic arrangement in other α-proteobacteria (Fig. 1). However, tandem genes encoding phospholipase C and the MGDG/GADG glycosyltransferase were identified in a number of α-proteobacteria, including the marine strains Pelagibacterales sp. str. HIMB59, “Candidatus Puniceispirillum marinus” str. IMCC1322 (SAR116), Erythrobacter litoralis HTCC2594, and Loktanella vestfoldensis SKA53 (Fig. 1). This finding expands our knowledge of the distribution of glycolipid synthesis genes to cosmopolitan marine bacteria and implies lipid renovation in response to P scarcity may be a relatively common feature of marine chemoheterotrophic bacteria.

HTCC7211_00011010 likely catalyzes the final step of ornithine lipid biosynthesis in str. HTCC7211. Ornithine lipids are synthesized by the N-acylation of ornithine with a hydroxy-fatty acyl group (by OlsB) (29), followed by an O-acylation of lysornithine (by OlsA) to form ornithine lipid (Fig. S1). In most ornithine lipid synthesizing bacteria, olsA and olsB genes form an operon. We identified a probable olsB gene in the str. HTCC7211 genome (Fig. 1; HTCC7211_00011000) but were unable to identify an ortholog to known olsA genes with all vs. all BLASTP and Markov clustering (MCL). To further examine this question, we searched the str. HTCC7211 genome with a hidden Markov model (HMM) trained on amino acid sequences of characterized olsAs from E. melilotii and R. capsulatus plus the best BLAST hits to each (SI Methods). The analysis returned low E-value hits to HTCC7211_0001100 and did not provide additional evidence that HTCC7211_00011010 is an olsA ortholog. Thus, the primary amino acid sequence of HTCC7211_00011010 does not likely encode an ortholog to OlsA. However, OlsA has homology to glycerol acyltransferases, the protein domain we identified in HTCC7211_00011010. These similarities, plus the syntenic arrangement akin to other olsBA pairs, suggest HTCC7211_00011010 has an activity similar to that of characterized OlsAs. Regarding the str. HTCC7211 ornithine lipid synthesis gene cluster, we did not find other α-proteobacteria with the same chromosomal arrangement, implying this arrangement may be unique to a subset of organisms in the Pelagibacterales.

The use of lipids that lack P, together with the utilization of assorted organophosphorus sources (10), may enable certain Pelagibacterales members to cope with patchiness in the dissolved organic phosphorus pool and maintain growth in low P waters. In P-replete conditions, the lipid polar head groups of str. HTCC7211 and str. HTCC1062 had a molar P:C ratio of ∼0.4 (Fig. 4). Under conditions of P deprivation or growth on MPn, the average molar P:C ratio decreased to ~0.2 in str. HTCC7211 (Fig. 4). From the change in composition of polar head groups during P starvation, we calculated that lipid renovation reduces...
Majority of lipids are phosphate-free analogs under conditions of methylphosphonate starvation. (A) Bacterial growth curves of previously P$_i$ starved str. HTCC7211 cells in growth medium with growth-limiting amounts of P$_i$ or MPn. Lipid profiles before and after reaching MPn (B) or P$_i$ (C) limitation. Red arrows in A point to P limited sample points for B and C.

Fig. 3. Majority of lipids are phosphate-free analogs under conditions of methylphosphonate starvation. (A) Bacterial growth curves of previously P$_i$ starved str. HTCC7211 cells in growth medium with growth-limiting amounts of P$_i$ or MPn. Lipid profiles before and after reaching MPn (B) or P$_i$ (C) limitation. Red arrows in A point to P limited sample points for B and C.

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Methods

Organism Source and Growth Conditions. P. ubique str. HTCC1062 and Pelagibacter sp. str. HTCC7211 were revived from frozen stocks and cultivated on AMS1 synthetic growth medium without added phosphate as described (10, 33, 34). Cell growth was monitored by flow cytometry as described (35). Phosphorus was added as Na$_2$HPO$_4$ or methylphosphonate (MPn), as indicated in the text.

Cell Harvesting for Lipid Profiles. Strain HTCC7211 cells, grown in AMS1 with excess P$_i$ (100 μM), were harvested in late-logarithmic growth-phase (approximately 1.0 × 10$^8$ cells·ml$^{-1}$) by centrifugation (17,664 × g for 1.0 h at 20 °C). Pellets were washed twice with growth medium and resuspended in one of the following conditions: (i) P$_i$ replete (100 μM); (ii) MPn-replete medium (100 μM); or (iii) P$_i$ deplete growth medium (no P$_i$ added). Suspensions were monitored for growth (reported in Fig. 2 A, C, and E) and subsampled (40 mL) by centrifugation (48,298 × g for 1.0 h at 4 °C) at t = 0, 2, 4, 6, and 8 d. The supernatant was removed and cell pellets were frozen at −80 °C until lipid extraction.

To study the effect of sequential P$_i$ and MPn limitation on lipid composition, str. HTCC7211 cells were grown with excess P$_i$ to late-logarithmic growth phase and harvested by centrifugation. Pellets were washed with growth medium, resuspended in growth medium without added P (to approximately 4 × 10$^7$ cells·ml$^{-1}$), and incubated for 4 d to induce the P$_i$ starvation response. After starvation, P$-i$ starved cells were used to inoculate growth media containing a growth-limiting amount of P$_i$ (1.2 μM) or MPn (1.5 μM). The carbon and nitrogen constituents of this medium support cell yields in excess of 5.0 × 10$^7$ cells·ml$^{-1}$. Therefore, P$_i$ or MPn were growth-limiting, as calculated from the cellular P quotas of 11 amol P·cell$^{-1}$ (P$_i$-grown) or 10 amol P·cell$^{-1}$ (MPn-grown) (10). Cell suspensions were subsampled at centrifugation at t = 0 d and after P-limited stationary phase had been reached, as determined from direct cell counts: 9 d for P$_i$-grown cells; 12 d for MPn-grown cells.

Lipid Analysis. Polar lipids were extracted from the cell pellets as described (36). Published methods were also used as a basis for polar lipid separation by normal-phase high performance liquid chromatography (20) and concomitant analysis by positive electrospray ionization ion-trap mass spectrometry (37). Additional structural elucidation of GADG and ornithine lipids was conducted by using the same HPLC method in conjunction with positive

is associated with specific genes that previously had poorly assigned functions. Because many genes in microbial genomes have incompletely assigned functions, studies like this improve the accuracy of annotations and are important to improving the long-term impact of genome data on the prediction geochemical processes. In future work, it will be important to understand why cells prefer phospholipids and to identify changes in fitness associated with the substitution of glycolipids.
electrospray ionization high mass-resolution mass spectrometry (Figs. S6 and S7 and SI Methods).

**Lipid Polar Head Group Elemental Stoichiometry.** The relative elemental ratios of polar head groups under different P regimes were calculated from the molar proportion of each membrane lipid type in each treatment and the molecular formulas of each polar head group: C18H28O7P for phosphoethanolamine; C16H20O6P for phosphoglycerol; C10H6O7P for glyceric acid; and C12H12N2O7 for ornithine (Dataset S2).

**Gene Identifiers.** All gene identifiers in this study are presented as Integrated Microbial Genomes (IMG) Gene ID numbers. In previous works (10, 11, 38), the genes HTCC7211_00001000–HTCC7211_0011030 were listed with different identifiers (listed as: previous identifier (IMG Gene ID): PB7211_1302 (HTCC7211_00011000); PB7211_635 (HTCC7211_00011010); PB7211_980 (HTCC7211_00011020); PB7211_983 (HTCC7211_00011030).

**Identification of Putative Lipid Renovation Gene Orthologs.** The Hal software package (28) was used to generate orthologous protein clusters from 272 α-proteobacteria from the IMG database (39). All vs. all BLASTP was followed by MCL at 13 inflation parameters. Clusters generated with the inflation parameter of 1.5 were used to identify orthologs of the genes predicted to be involved in lipid remodeling in str. HTCC7211 (HTCC7211_00011000–HTCC7211_0011030).

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The complete distribution for all four genes in all α-proteobacteria can be found in Dataset S1.

α-Proteobacterial Phylogeny. Concatenated 16S-23S RNA genes from almost all of the above α-proteobacteria and six outgroups (including members of the p-, y-, and s-proteobacteria) were used to manually construct a maximum likelihood tree similar to that in ref. 40. Some RNA gene sequences from organisms included in the Hal analysis were excluded from the 16–23S tree because of poor quality or truncated RNA genes (Dataset S1). All identifiers for taxa represented in the tree are provided in Dataset S1. The 16S and 23S RNA genes were aligned separately with MUSCLE (41) by using default settings and corrected with the following settings: 1 = (0.2x + 4); b2 = (n + 1) / 2 = 2; b4 = 2; h = where n = number of taxa. Alignments were normalized and concatenated with normalize_alignments.py and catPhylip.pl, respectively, included in the Hal package. The final alignment contained 261 taxa and 4,096 characters. The tree was inferred by using FastTree2 (43) with default settings.

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