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ORIGINAL ARTICLE

# Nutrient requirements for growth of the extreme oligotroph '*Candidatus* Pelagibacter ubique' HTCC1062 on a defined medium

Paul Carini, Laura Steindler[1], Sara Beszteri[2] and Stephen J Giovannoni
*Department of Microbiology, Oregon State University, Corvallis, OR, USA*

**Chemoheterotrophic marine bacteria of the SAR11 clade are Earth's most abundant organisms. Following the first cultivation of a SAR11 bacterium, '*Candidatus* Pelagibacter ubique' strain HTCC1062 (*Ca.* P. ubique) in 2002, unusual nutritional requirements were identified for reduced sulfur compounds and glycine or serine. These requirements were linked to genome streamlining resulting from selection for efficient resource utilization in nutrient-limited ocean habitats. Here we report the first successful cultivation of *Ca.* P. ubique on a defined artificial seawater medium (AMS1), and an additional requirement for pyruvate or pyruvate precursors. Optimal growth was observed with the collective addition of inorganic macro- and micronutrients, vitamins, methionine, glycine and pyruvate. Methionine served as the sole sulfur source but methionine and glycine were not sufficient to support growth. Optimal cell yields were obtained when the stoichiometry between glycine and pyruvate was 1:4, and incomplete cell division was observed in cultures starved for pyruvate. Glucose and oxaloacetate could fully replace pyruvate, but not acetate, taurine or a variety of tricarboxylic acid cycle intermediates. Moreover, both glycine betaine and serine could substitute for glycine. Interestingly, glycolate partially restored growth in the absence of glycine. We propose that this is the result of the use of glycolate, a product of phytoplankton metabolism, as both a carbon source for respiration and as a precursor to glycine. These findings are important because they provide support for the hypothesis that some micro-organisms are challenging to cultivate because of unusual nutrient requirements caused by streamlining selection and gene loss. Our findings also illustrate unusual metabolic rearrangements that adapt these cells to extreme oligotrophy, and underscore the challenge of reconstructing metabolism from genome sequences in organisms that have non-canonical metabolic pathways.**
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## Introduction

Laboratory studies of ecologically important organisms are important for understanding the principles and mechanisms that govern ecosystem behavior (Giovannoni and Stingl, 2007). For example, several studies have reported high connectedness in marine plankton communities (Fuhrman *et al.*, 2006; Steele *et al.*, 2011). 'Connectance' is a general measure of the degree to which populations within a community display correlated behavior, and, in terrestrial ecosystems, has been linked to ecosystem stability (Dunne *et al.*, 2002). The SAR11 clade of α-proteobacteria are the most abundant heterotrophs in marine euphotic zones worldwide (Morris *et al.*, 2002), and have been shown to contribute significantly to the overall connectedness of marine microbial plankton communities (Steele *et al.*, 2011). Specific mechanisms that might explain SAR11 connectedness are slowly emerging from studies of genomes and the metabolism of cells in culture (Tripp *et al.*, 2008; 2009; Schwalbach *et al.*, 2010; Sun *et al.*, 2011).

'*Candidatus* Pelagibacter ubique' HTCC1062 (*Ca.* P. ubique), a member of the SAR11 clade, has a small genome and displays characteristic signatures of streamlining selection (Giovannoni *et al.*, 2005). According to the genome streamlining theory, when effective population sizes are large, extreme selection for the efficient use of resources in nutrient-poor environments can result in genome reduction (Dufresne *et al.*, 2003; Lynch and Conery, 2003; Giovannoni *et al.*, 2005). The metabolic consequences of genome reduction in *Ca.* P. ubique

Correspondence: SJ Giovannoni, Department of Microbiology, Oregon State University, 220 Nash Hall, Corvallis, OR 97331, USA.
E-mail: steve.giovannoni@oregonstate.edu
[1]Current address: Department of Marine Biology, The Leon H Charney School of Marine Science, University of Haifa, Haifa, Israel
[2]Current address: Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, Germany
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have been the subject of several reports (Tripp *et al.*, 2008, 2009; Schwalbach *et al.*, 2010).

Metabolic reconstruction from the *Ca.* P. ubique genome highlighted the conspicuous absence of genes common to aerobic chemoorganoheterotrophs (Figure 1). Subsequent batch culture experiments, using a natural seawater medium, showed that *Ca.* P. ubique required an unusual combination of nutrients for growth (Tripp *et al.*, 2008, 2009; Schwalbach *et al.*, 2010). Specifically, *Ca.* P. ubique lacks genes necessary for assimilatory sulfate reduction, and as a result, requires reduced sulfur compounds such as methionine or 3-dimethylsulphoniopropionate for growth (Tripp *et al.*, 2008). Similarly, the unusual absence of common genes for serine and glycine biosynthesis resulted in a conditional requirement for either of these amino acids (Tripp *et al.*, 2009). Concomitantly, an uncommon arrangement of two glycine-activated riboswitches was reported; one

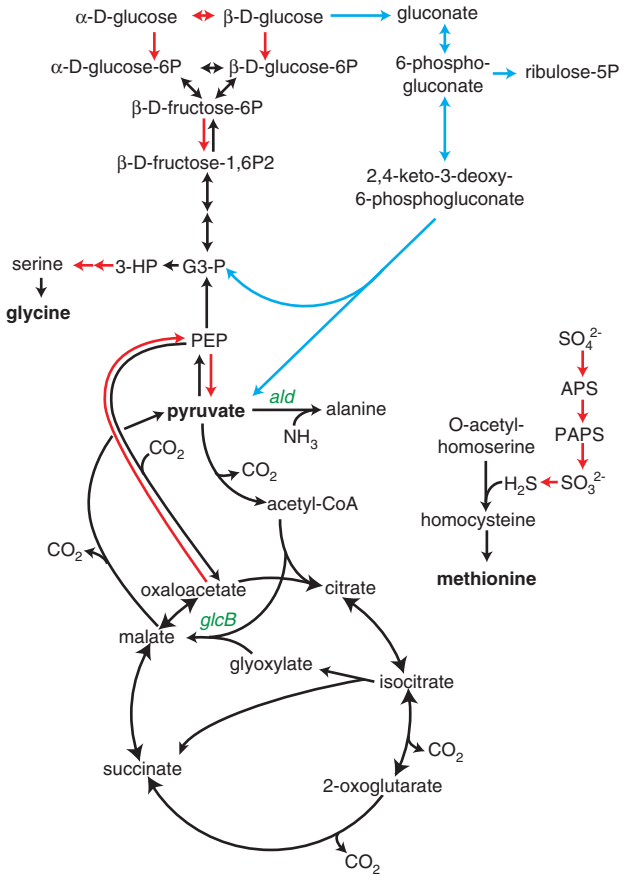


**Figure 1** Simplified illustration of central metabolism in *Ca.* P. ubique. Black lines: reactions predicted to occur in *Ca.* P. ubique based on genome content. Red lines: reactions predicted to occur in *E. coli*, but missing from *Ca.* P. ubique. Blue lines: putative glucose oxidation pathway (see Schwalbach *et al.*, 2010). Gene names in green are discussed further in the article. Bolded compounds were previously identified as growth substrates for *Ca.* P. ubique. *ald*, alanine dehydrogenase; *glcB*, malate synthase; APS, adenosine 5′-phosphosulfate; G3-P, glyceraldehyde-3-phosphate; 3-HP, 3-hydroxypyruvate; PAPS: 3′-phosphoadenylyl sulfate, PEP: phosphoenolpyruvate.

located upstream of the gene encoding for the glycine cleavage system T-protein (*gcvT*) and the other upstream of the malate synthase gene (*glcB*) (Tripp *et al.*, 2009). The first of these is a common riboswitch involved in glycine cleavage to $CO_2$ and $NH_4^+$, but the second is a rare riboswitch configuration that was implicated in glyoxylate metabolism (Tripp *et al.*, 2009). Although *Ca.* P. ubique is missing the complete Embden–Meyerhof–Parnas glycolytic pathway (genes encoding pyruvate kinase and phosphofructokinase are absent, Figure 1), a putative operon encoding genes involved in a predicted variant of the Entner–Doudoroff glycolytic pathway is present, as is a complete gluconeogenic pathway (Figure 1; Schwalbach *et al.*, 2010). Experiments showed that low molecular weight organic acids were important carbon sources for multiple SAR11 isolates and that *Ca.* P. ubique, but not all SAR11 isolates, oxidized glucose (Schwalbach *et al.*, 2010). Moreover, genes coding for proteins involved in carbohydrate metabolism are not consistently conserved across the SAR11 clade, suggesting that the metabolism of sugars and other carbohydrates may be involved in niche partitioning or ecotype variation (Grote *et al.*, 2012).

Previously, *Ca.* P. ubique was grown exclusively in natural seawater-based media. However, seawater contains an undefined mixture of naturally occurring organic compounds that vary in composition and quantity between seasons, locations and depths. The consequences of this nutrient variability on *Ca.* P. ubique's physiology was initially observed in the form of 'batch effects', in which seawater collected at different times yielded different cell densities or growth rates independent of nutrient additions (Rappé *et al.*, 2002). The three main limitations of conducting experiments with *Ca.* P. ubique cells on natural seawater-based media are: (i) the precise composition and concentrations of organic matter in seawater is unknown and variable; (ii) the finite supply of any single seawater batch limits comparisons between experiments conducted with different batches; and (iii) native concentrations of nutrients (for example, nitrogen, sulfur and carbon) cannot be excluded.

We developed a defined medium to use as a tool for studying the nutrient requirements and metabolism of *Ca.* P. ubique. Ultimately, without a defined medium, it was impossible to rule out that unknown compounds in natural organic matter were essential for growth of *Ca.* P. ubique. A simple approach to solve this problem is the addition of a diverse mixture of compounds, under the assumption that the organism will use only what it needs. This approach is commonly implemented by adding mixtures of nutrients such as yeast extract or digests of protein to growth media. However, complex, high-nutrient mixtures inhibit the growth of many oligotrophic chemoheterotrophs, making this strategy impractical (Rappé *et al.*, 2002; Tripp *et al.*, 2008, 2009). Building on previous metabolic models for

*Ca.* P. ubique, we hypothesized that the minimum nutrient requirements for growth on a defined medium would include pyruvate in addition to a source of reduced sulfur and glycine. We report here the propagation of *Ca.* P. ubique to high cell density on a defined artificial seawater medium containing inorganic micro- and macronutrients, vitamins, methionine (for reduced sulfur), glycine and pyruvate. Pyruvate, or its precursors, was identified as the last essential macronutrient needed by *Ca.* P. ubique for growth. We also demonstrate that several environmentally relevant compounds can be used by *Ca.* P. ubique in place of pyruvate and glycine, and describe unusual rearrangements of central metabolic pathways that confer these properties.

## Materials and methods

*Organism source*
*Ca.* P. ubique HTCC1062 was originally isolated from the northeast Pacific Ocean as described elsewhere (Rappé *et al.*, 2002). Frozen glycerol stocks from the original isolation of *Ca.* P. ubique were used as the source inoculum for all experiments.

*Media preparation*
Artificial seawater medium AMS1 (Table 1) was derived from the artificial seawater medium AMP1 (Moore *et al.*, 2007). Organic buffers (for example, HEPES and EDTA) were excluded to avoid potential toxic effects (Zigler *et al.*, 1985) and the possibility that they might be used as substrates for growth (Nörtemann, 1992). After autoclaving, AMS1 was sparged with 0.1 μm-filtered $CO_2$ for 5 h followed by sparging with air for 10 h to establish a bicarbonate-based buffer system (Connon and Giovannoni, 2002). All vitamins and organics were added after autoclaving and sparging. The pH of the resulting AMS1 typically ranged from 7.5 to 7.7.

*Cultivation details*
All cultures were grown in acid-washed and autoclaved polycarbonate flasks at 20 °C with shaking at 60 r.p.m. under a 12-h/12-h light (140–180 μmol photons $m^{-2} s^{-1}$)/dark cycle.

*Measurement of growth*
Cells were stained with SYBR Green I (Molecular Probes, Inc., Eugene, OR, USA) and counted with a Guava Technologies flow cytometer (Millipore, Billerica, MA, USA) at 48- to 72-h intervals as described elsewhere (Stingl *et al.*, 2007; Tripp *et al.*, 2008).

*Acclimation to growth on AMS1*
Natural seawater collected from the Newport Hydroline station NH-05 (latitude: 44.65°, longitude: −124.18°) from a depth of 10 m in June 2008 was amended with glycine (1 μM), methionine (1 μM), pyruvate (50 μM), $FeCl_3$ (1 μM) and vitamins and inoculated with *Ca.* P. ubique from glycerol stocks. When exponentially growing cells in this amended natural seawater medium exceeded a density of $2.0 \times 10^6$ cells $ml^{-1}$, they were diluted (1:100) with fresh AMS1 supplemented with glycine (1 μM) (as a glycine/serine source), methionine (as a sulfur source) (1 μM), pyruvate (as a carbon source) (50 μM), $FeCl_3$ (1 μM) and vitamins. We observed no lag phase in cells that were transferred into amended AMS1 (Supplementary Figure S1). All cultures described herein are derived from this lineage and have been maintained exclusively on AMS1 for >15 consecutive batch culture transfers (approximately 150 generations).

**Table 1** Constituents of the artificial medium for SAR11 (AMS1)[a]

| *Compound* | *Final concentration* |
|---|---|
| *Base salts* | |
| NaCl | 481 mM |
| $MgCl_2 \cdot 6H_2O$ | 27 mM |
| $CaCl_2 \cdot 2H_2O$ | 10 mM |
| KCl | 9 mM |
| $NaHCO_3$ | 6 mM |
| $MgSO_4 \cdot 7H_2O$ | 2.8 mM |
| *Macronutrients* | |
| $(NH_4)_2SO_4$ | 400 μM |
| $NaH_2PO_4$ (pH 7.5) | 50 μM |
| *Trace metals* | |
| $FeCl_3 \cdot 6H_2O$ | 117 nM |
| $MnCl_2 \cdot 4H_2O$ | 9 nM |
| $ZnSO_4 \cdot 7H_2O$ | 800 pM |
| $CoCl_2 \cdot 6H_2O$ | 500 pM |
| $Na_2MoO_4 \cdot 2H_2O$ | 300 pM |
| $Na_2SeO_3$ | 1 nM |
| $NiCl_2 \cdot 6H_2O$ | 1 nM |
| *Vitamins* | |
| $B_1$ | 6 μM |
| $B_3$ | 800 nM |
| $B_5$ | 425 nM |
| $B_6$ | 500 nM |
| $B_7$ | 4 nM |
| $B_9$ | 4 nM |
| $B_{12}$ | 700 pM |
| Myo-inositol | 6 μM |
| 4-Aminobenzoic acid | 60 nM |

[a]The AMS1 medium does not include nutrients to meet the requirements for reduced sulfur (see Tripp *et al.*, 2008), glycine (see text) or pyruvate (see text).

*Pyruvate substitution experiments*
Potential pyruvate precursors (Table 2) were selected because they were either (i) 'common' sole carbon sources for chemoorganoheterotrophs or (ii) present in seawater as products of phytoplankton metabolism. Each substitute was tested at a concentration of 50 μM in AMS1 amended with 25 μM

glycine, 10 μM methionine, 1 μM $FeCl_3$ and vitamins. The positive control was amended with 50 μM pyruvate and the same concentrations of glycine, methionine, $FeCl_3$ and vitamins. The negative control contained no pyruvate, but was otherwise identical.

*Glycine substitution experiments*

Potential glycine precursors (Table 3) were selected because they were either (i) metabolic precursors of glycine in other organisms or (ii) predicted to be a precursor based on metabolic reconstruction in *Ca.* P. ubique. Each substitute was tested at a concentration of 25 μM in AMS1 with pyruvate (50 μM), methionine (10 μM), $FeCl_3$ (1 μM) and vitamins. The positive control was amended with 25 μM glycine and the negative control contained no glycine.

*Cell division experiments*

Growth media consisted of AMS1 amended with glycine (1 μM), methionine (1 μM), $FeCl_3$ (1 μM), vitamins and either 0.5 μM pyruvate (deplete conditions) or 50 μM pyruvate (replete conditions). SYBR Green I-stained cultures that exhibited relative DNA fluorescence values of 300–325 and 475–500, were independently filtered on to 0.2 μm black polycarbonate filters, and imaged using a Leica DMRB epifluorescence microscope (Wetzlar, Germany) equipped with filter sets appropriate for SYBR Green I (excitation: 450–490 nm; emission: 580 nm). Images were captured with a Hamamatsu ORCA-ER CCD digital camera (Hamamatsu City, Japan) and Scanalytics IPLab v3.5.5 scientific imaging software (Fairfax, VA, USA).

*Chemicals*

All inorganic salts were obtained from Sigma-Aldrich Co. (St Louis, MO, USA) and were of the highest available quality (typically labeled 'ultrapure'). All other compounds were obtained from Sigma-Aldrich Co. or other commercial vendors and were of reagent grade quality.

**Table 2** Potential sources of pyruvate for *Ca.* P. ubique when grown in AMS1 with 25 μM glycine and 10 μM methionine

| *Potential pyruvate source* | *Maximum density*[a] | *Growth rate*[b] |
|---|---|---|
| Glucose | 12.1 ± 3.83 | 0.27 ± 0.02 |
| Pyruvate (positive control) | 8.91 ± 0.74 | 0.33 ± 0.01 |
| Oxaloacetate | 6.92 ± 0.04 | 0.33 ± 0.04 |
| Taurine | 0.46 ± 0.14 | 0.21 ± 0.02 |
| Lactate | 0.11 ± 0.09 | 0.18 ± 0.04 |
| Ribose | 0.05 ± 0.01 | 0.23 ± 0.01 |
| Malate | 0.03 ± 0.01 | 0.14 ± 0.04 |
| Citrate | 0.03 ± 0.00 | 0.12 ± 0.01 |
| Acetate | 0.02 ± 0.01 | 0.10 ± 0.05 |
| No pyruvate (negative control) | 0.02 ± 0.01 | 0.13 ± 0.02 |
| Succinate | 0.02 ± 0.00 | 0.11 ± 0.01 |
| Alanine | 0.01 ± 0.00 | 0.01 ± 0.01 |
| Glycine | 0.01 ± 0.00 | 0.06 ± 0.01 |

[a]Presented as mean ± s.d. × $10^7$ cells $ml^{-1}$, $n = 3$.
[b]Presented as mean ± s.d., cells $day^{-1}$, $n = 3$.

**Table 3** Potential sources of glycine for *Ca.* P. ubique when grown in AMS1 with 50 μM pyruvate and 10 μM methionine

| *Potential glycine source* | *Maximum density*[a] | *Growth rate*[b] |
|---|---|---|
| Glycine betaine | 13.1 ± 0.34 | 0.18 ± 0.01 |
| Glycine (positive control) | 10.2 ± 0.28 | 0.38 ± 0.01 |
| Serine | 8.34 ± 0.15 | 0.40 ± 0.01 |
| Glycolate | 1.88 ± 0.13 | 0.35 ± 0.01 |
| Acetate | 0.66 ± 0.10 | 0.30 ± 0.01 |
| Malate | 0.51 ± 0.03 | 0.32 ± 0.03 |
| Glyoxylate | 0.50 ± 0.01 | 0.38 ± 0.01 |
| Succinate | 0.46 ± 0.06 | 0.31 ± 0.01 |
| Pyruvate | 0.44 ± 0.05 | 0.35 ± 0.01 |
| Citrate | 0.44 ± 0.01 | 0.29 ± 0.01 |
| No glycine (negative control) | 0.43 ± 0.02 | 0.34 ± 0.00 |
| 2-Oxoglutarate | 0.39 ± 0.05 | 0.33 ± 0.01 |

[a]Presented as mean ± s.d. × $10^7$ cells $ml^{-1}$, $n = 3$.
[b]Presented as mean ± s.d., cells $day^{-1}$, $n = 3$.

## Results

When grown on AMS1 with methionine, glycine and pyruvate, *Ca.* P. ubique's maximum specific growth rate was 0.41 ± 0.01 $day^{-1}$ (mean ± s.d., $n = 3$), and batch cultures reached maximum cell densities of 9.18 ± 0.02 × $10^7$ cells $ml^{-1}$ (mean ± s.d., $n = 3$) (Figure 2). Additions of methionine or carbon (as pyruvate and glycine) alone did not result in increased cell densities beyond those of the negative control (Figure 2). Cell densities responded linearly to both pyruvate ($R^2 = 0.998$) and glycine ($R^2 = 0.995$) additions in AMS1 when other nutrients were in

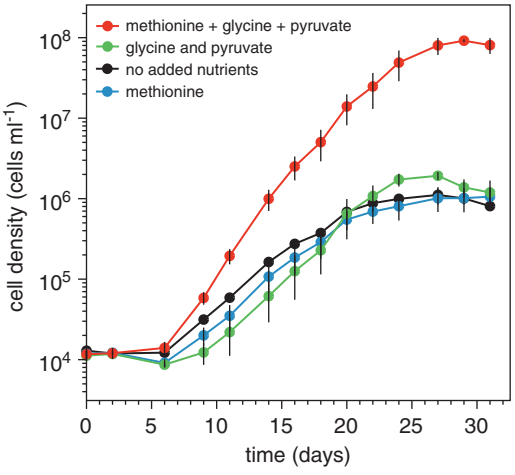


**Figure 2** Growth of *Ca.* P. ubique in AMS1 with organic carbon additions. Black: cells grown without additions of glycine, methionine or pyruvate. Red: cells amended with methionine (10 μM), glycine (50 μM) and pyruvate (50 μM). Blue: cells amended with methionine (10 μM) only. Green: cells amended with glycine (50 μM) and pyruvate (50 μM) only. Points are the average density of triplicate cultures. Error bars indicate ± 1.0 s.d. ($n = 3$). When error bars are not visible, they are smaller than the size of the symbols.

excess (Figure 3). The maximum cell density increased by $2.6 \times 10^6$ cells ml$^{-1}$ μM$^{-1}$ pyruvate (Figure 3a) and $1.0 \times 10^7$ cells ml$^{-1}$ μM$^{-1}$ glycine (Figure 3b).

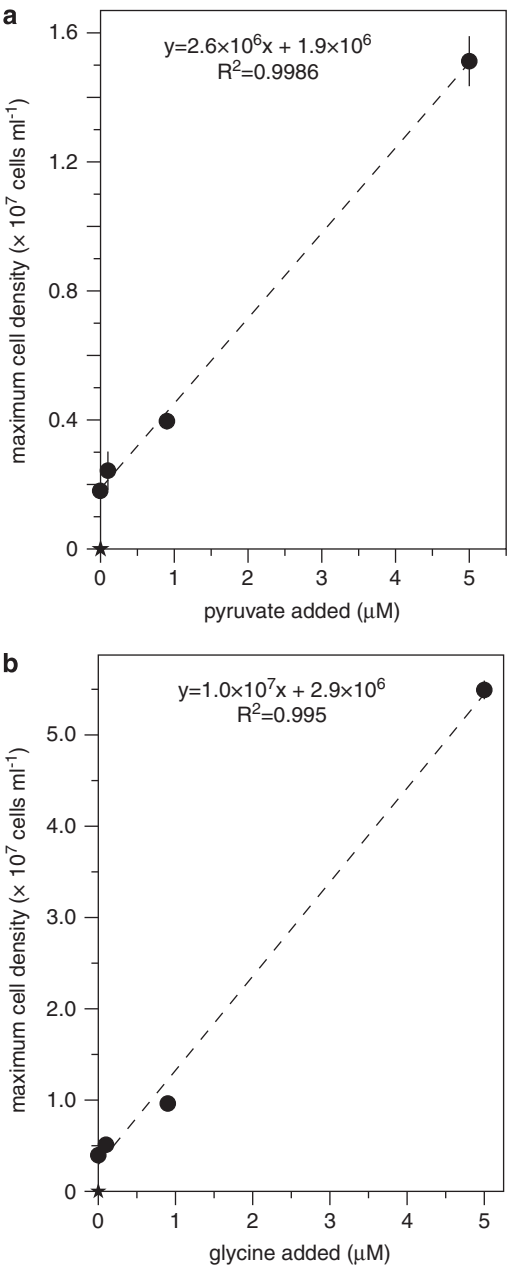


**Figure 3** Maximum cell yields of *Ca.* P. ubique in response to pyruvate and glycine additions. (**a**) Pyruvate titration in AMS1 supplemented with glycine (50 μM) and methionine (10 μM). Using the formula of the regression line, the maximum cell density achievable from pyruvate carryover with source inoculum (156 pM) was calculated to be 400 cells ml$^{-1}$ (filled star). (**b**) Glycine titration into AMS1 supplemented with pyruvate (50 μM) and methionine (10 μM). Using the formula of the regression line, the maximum cell density achievable from glycine carryover with source inoculum (500 pM) was calculated to be 5000 cells ml$^{-1}$ (filled star). Filled circles are the average maximum cell densities of triplicate batch cultures. Error bars indicate ± 1.0 s.d. ($n = 3$). When error bars are not visible, they are smaller than the size of the symbols.

*Ca.* P. ubique utilized oxaloacetate and glucose in place of pyruvate on AMS1 (Table 2). As observed previously (Schwalbach *et al.*, 2010), *Ca.* P. ubique's specific growth rate was slower with glucose as a sole pyruvate source. Addition of taurine or lactate resulted in cell densities in excess of fivefold greater than the negative control, but did not achieve the cell densities of the pyruvate treatment. Notably, in the absence of pyruvate, additions of alanine or glycine did not improve growth yield.

Glycine betaine and serine were able to fully replace glycine in AMS1 and glycolate partially substituted for glycine (Table 3). *Ca.* P. ubique grew slower but to slightly higher cell densities when glycine betaine was the sole glycine source. Glycolate led to cell density increases fourfold greater than those of the negative control. The addition of pyruvate, without glycine, did not result in higher cell densities.

While developing the AMS1 medium, unusual cell division patterns were observed under pyruvate-deplete conditions. When SYBR Green was used to stain DNA in early stationary-phase cells, fluorescence from pyruvate limited cells was about two-fold higher than from cells raised in a pyruvate-replete medium (Figures 4a and b). Microscopic images from these cultures showed that the increase in DNA fluorescence was caused by elongated cells containing two nucleoids ('doublets') (Figures 4c and d). We observed this unusual cell division pattern when cells entered pyruvate-limited stationary-phase across a range of pyruvate:glycine ratios (Supplementary Figure S2). This phenomenon was previously observed when *Ca.* P. ubique cells were grown in a natural seawater medium without added pyruvate (unpublished data). Experiments conducted in natural seawater found alanine induced the division of cell doublets (unpublished data). The effect of different alanine concentrations on *Ca.* P. ubique cell morphology in stationary-phase cultures grown in AMS1 was tested using relative DNA fluorescence as a proxy for the formation of cell doublets (Supplementary Figure S3). DNA fluorescence profiles and cell counts suggested that alanine induced cell division in pyruvate-limited cultures.

## Discussion

Similar to the goals of Neidhardt *et al.* (1974), who developed a defined medium for the growth of enterobacteria, one of our objectives was to prepare a 'physiologically optimal and experimentally useful' medium in which *Ca.* P. ubique, and other SAR11 isolates, could be propagated reproducibly. This artificial medium for the growth of *Ca.* P. ubique contains only methionine, glycine, pyruvate, vitamins and inorganic salts. The specific growth rates observed on AMS1 ($0.41 \pm 0.01$ day$^{-1}$) were comparable to those previously published for *Ca.* P.

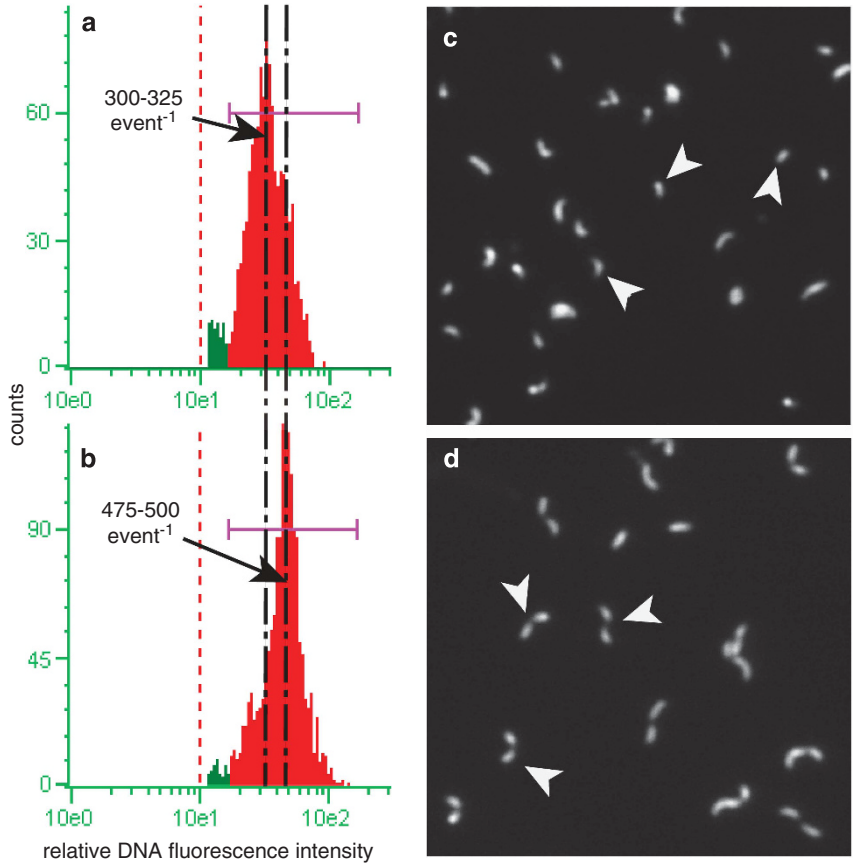


**Figure 4** DNA content and morphology of SYBR Green I-stained stationary-phase cells from pyruvate-deplete and -replete batch cultures. Red dashed line in **a** and **b** represents the minimum threshold of fluorescence detection. Black dashed lines in **a** and **b** represent relative DNA fluorescence values of 300–325 per event and 475–500 per event, as indicated with black arrows. (**a**) Relative DNA fluorescence of cells from pyruvate-replete (50 μM) stationary-phase cultures, and (**b**) pyruvate-deplete (0.5 μM) stationary-phase cultures. (**c**) Fluorescent microscopy image of cells from (**a**). Arrowheads point to single cells. (**d**) Fluorescent microscopy image of cells from (**b**). Arrowheads point to cell doublets.

ubique grown in seawater batch cultures (Rappé *et al.*, 2002; Tripp *et al.*, 2008; Schwalbach *et al.*, 2010) and to those from microcosm experiments with natural assemblages of plankton (Teira *et al.*, 2009; Ferrera *et al.*, 2011).

Results observed with cells growing in AMS1 support the previous conclusions that *Ca.* P. ubique requires a reduced sulfur source, glycine and an organic acid for growth. Our results also confirm that methionine meets *Ca.* P. ubique's requirement for exogenous reduced sulfur compounds (Tripp *et al.*, 2008, 2009) but cannot substitute for glycine or pyruvate as a sole carbon source. The linear responses to both pyruvate and glycine additions, when other constituents were in excess, indicated that both pyruvate and glycine were necessary for optimal growth and used in the molar ratio of 4.0:1 (pyruvate:glycine). In addition, we showed that alanine is required for septation, the final step in cell division, and that in the absence of pyruvate, *Ca.* P. ubique cells were not able to synthesize alanine by other routes, thus implicating pyruvate as a primary precursor for alanine biosynthesis. In *Escherichia coli*, the rate of cell wall biosynthesis increases during septation in order to accommodate the synthesis of the new daughter cell poles (Wientjes and Nanninga, 1989). Because alanine is a major component of cell walls in Gram-negative bacteria (Mengin-Lecreulx *et al.*, 1982), we propose that in pyruvate-limiting conditions, *Ca.* P. ubique is unable to synthesize sufficient alanine to complete septation, resulting in the formation of cell doublets.

Our observations show that pyruvate or its precursors are required to synthesize alanine, but that alanine cannot replace pyruvate (Table 2). In *Ca.* P. ubique, L-alanine is putatively formed by an alanine dehydrogenase (encoded by *ald*, SAR11_0809—Figure 1). Ald catalyzes the formation of alanine from pyruvate and ammonia via the oxidation of reduced nicotinamide adenine dinucleotide, and has been shown to be involved in both the catabolism and anabolism of alanine in *Pseudomonas*, *Rhodobacter* and *Sphingopyxis* species (Bellion and Tan, 1987; Caballero *et al.*, 1989; Williams *et al.*, 2009). Although Ald catalyzes a reversible reaction in some organisms, our data suggest that in *Ca.* P. ubique, Ald does not

catalyze the formation of pyruvate from alanine under the conditions tested.

The observation that both glucose and oxaloacetate could replace pyruvate suggests that pyruvate is a metabolic intermediate formed during the catabolism of these compounds. Both compounds were previously identified as carbon sources for *Ca.* P. ubique in natural seawater (Schwalbach *et al.*, 2010). The use of oxaloacetate in place of pyruvate is consistent with the presence of a predicted malic enzyme gene (*maeB*, SAR11_0375) that may be involved in the decarboxylation of oxaloacetate to form pyruvate. Our results are also consistent with Schwalbach's proposed glycolytic pathway in *Ca.* P. ubique that predicted pyruvate was an end product of glucose metabolism (Schwalbach *et al.*, 2010). Previously, in natural seawater-based media, taurine, acetate and lactate additions resulted in cell density increases similar to those observed with pyruvate additions (Schwalbach *et al.*, 2010). However, in our experiments on artificial seawater media, maximum cell densities decreased in this order: pyruvate » taurine > lactate » acetate (Table 2). Taurine is putatively metabolized to acetyl-CoA in *Ca.* P. ubique, suggesting that taurine catabolism supplies two-carbon units for biosynthesis. In our experiments, acetate, a direct precursor to acetyl-CoA, could not replace pyruvate (Table 2). This contradicts the prediction of a metabolic pathway for acetate assimilation as a sole carbon source via the glyoxylate bypass in *Ca.* P. ubique (Figure 1). We postulate that the glyoxylate bypass has instead been recruited to function in glycine metabolism, as described below.

We show for the first time that glycine betaine, in addition to serine, can meet *Ca.* P. ubique's glycine requirement. Previously, Tripp *et al.* (2009) identified serine as the only compound able to replace glycine in natural seawater-based batch cultures of *Ca.* P. ubique. Consistent with this finding, we found that serine was an effective replacement for glycine on AMS1 (Table 3). Glycine betaine is an important osmolyte that can be degraded by marine bacteria (Kiene and Williams, 1998; Keller *et al.*, 1999; Sun *et al.*, 2011). *Ca.* P. ubique's genome encodes a full suite of genes predicted to be involved in the acquisition and stepwise oxidation of glycine betaine to form glycine (Tripp *et al.*, 2009; Sun *et al.*, 2011). Experiments also showed that *Ca.* P. ubique can oxidize methyl groups derived from glycine betaine to $CO_2$ (Sun *et al.*, 2011). Although glycine betaine fully replaced glycine, we observed a reduction in growth rate when glycine betaine was supplied as a sole glycine source. Tripp *et al.* (2009) previously reported that large amounts of glycine betaine had unpredictable or deleterious effects on the growth of *Ca.* P. ubique in natural seawater. We propose that growth rate reduction may be in part due to toxicity resulting from the production of formaldehyde (by the predicted dimethylglycine dehydrogenase, EC:1.5.99.2, SAR11_1253) and hydrogen peroxide (by the predicted sarcosine oxidase, EC:1.5.3.1; for genes, see Sun *et al.*, 2011) during glycine betaine catabolism.

We also report the new finding that *Ca.* P. ubique partially utilized glycolate to meet its glycine requirement. Glycolate is commonly formed by phytoplankton as a result of photorespiration (Leboulanger *et al.*, 1994, 1997, 1998; Schnitzler Parker *et al.*, 2004; Bertlisson *et al.*, 2005). In *E. coli*, carbon from glycolate is assimilated into biomass after it is oxidized to glyoxylate by the glycolate oxidase (*glcDEF*) (Pellicer *et al.*, 1996, 1999). Glyoxylate is then condensed with acetyl-CoA by the malate synthase (*glcB*) to form the tricarboxylic acid cycle intermediate malate (Pellicer *et al.*, 1996). In *Ca.* P. ubique, genes encoding the glycolate oxidase are found in a putative operon with a pyridoxal-phosphate-dependent aminotransferase, annotated as an aspartate aminotransferase (*aspC*), but separate from the malate synthase gene (Figure 5). Previously, Tripp *et al.* (2009) had proposed that this aminotransferase produces glycine from glyoxylate, but, working with natural seawater media, were unable to demonstrate replacement of glycine by glycolate to validate this hypothesis.

Results presented here help resolve an enigmatic arrangement of glycine-activated riboswitches associated with genes of the glyoxylate cycle and glycine catabolism. *Ca.* P. ubique has genes for the proteins required to channel glycolate into the tricarboxylic acid cycle where it can be assimilated into biomass or oxidized (*glcDEF* and *glcB*; Tripp *et al.*, 2009). In *Ca.* P. ubique, a glycine-activated riboswitch is located in a very unusual position upstream of the *glcB* coding sequence, and a second glycine-activated riboswitch is located in a common arrangement, upstream of *gcvT*, where it functions to regulate glycine cleavage to $CO_2$ and $NH_4^+$. It is now apparent that this unusual configuration of two riboswitches results in glycine concentrations regulating the fate of glyoxylate, as illustrated in Figure 5. We postulate that when intracellular glycine concentrations fall too low, the switch on *glcB* closes, shunting glycolate through glyoxylate to form glycine. When intracellular glycine concentrations are ample, the glyoxylate bypass opens to channel glycolate-derived carbon into the tricarboxylic acid cycle. We attribute the observation that glycolate did not support as high a cell yield as glycine (Table 3) to its dual role in these pathways. This model also helps explain why *Ca.* P. ubique does not respond vigorously to acetate addition; the glyoxylate bypass, which in most cells is used for acetate assimilation, has been recruited to functions that are adaptive to the ocean environment.

One of the mysteries yet to be fully explained is the growth of *Ca.* P. ubique cells to $10^6$ cells $ml^{-1}$ in the absence any additional carbon compounds except vitamins. We determined the maximum amount of methionine, glycine and pyruvate carried

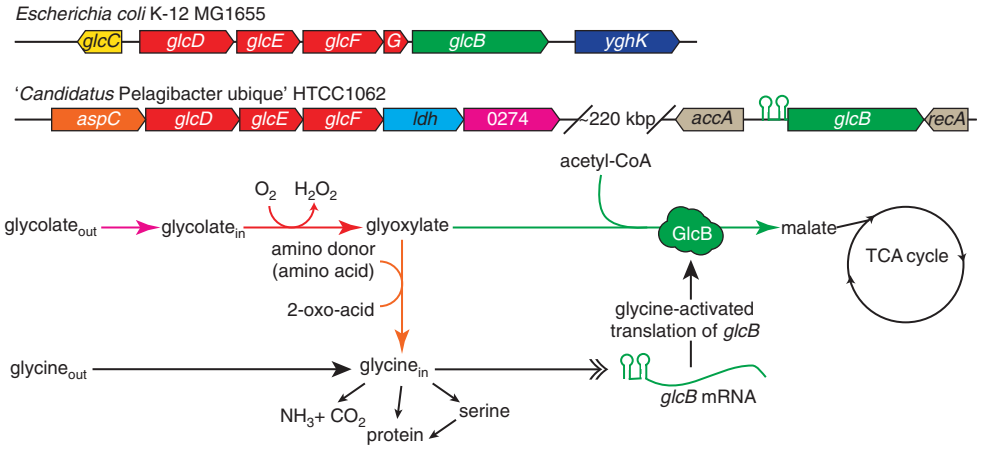


**Figure 5** Glycolate assimilation gene organizations in *E. coli* and *Ca.* P. ubique. Reaction arrows are colored by the genes predicted to catalyze the reaction. Green stem-loop images represent the glycine-activated riboswitch (Tripp *et al.*, 2009). Gene annotations are as described in NCBI, however, we predict the reaction catalyzed by AspC to be as described in the figure. *accA*, acetyl-CoA carboxylase; *aspC*, probable aspartate transaminase; *glcC*, DNA-binding transcriptional dual regulator, glycolate-binding; *glcD*, glycolate oxidase subunit, FAD-linked; *glcE*, glycolate oxidase, FAD-binding subunit; *glcF*, glycolate oxidase, iron-sulfur subunit; *G*, *glcG*; Putative *glc* operon gene, function unknown; *glcB*, malate synthase G; *recA*, recombinase A; *yghK*, glycolate transporter; *ldh*; probable 2-hydroxyacid dehydrogenase; 0274, *SAR11_0274*; major facilitator superfamily transporter, possible sugar-phosphate transporter; TCA, tricarboxylic acid.

over with the source inoculum to be 500, 500 and 156 pM, respectively. Based on the calculated per-cell glycine and pyruvate requirement (regression lines in Figure 3), and the previously published sulfur requirement (Tripp *et al.*, 2008), we conclude that the carryover of these nutrients does not sufficiently explain the growth of the negative controls observed in Figures 2 and 3. One alternative explanation for this growth is that *Ca.* P. ubique is able to utilize the carbon and sulfur originating from one or more of the vitamins, vitamin degradation products or traces of contaminating carbon in the vitamin stocks. We tested this and found that *Ca.* P. ubique responded in a dose-dependent manner to increasing amounts of freshly prepared vitamins (Supplementary Figure S4). It is not clear whether *Ca.* P. ubique can utilize the vitamins themselves for growth or if the vitamins enable more efficient use of other traces of contaminating carbon in AMS1. Regardless of the source of the contaminating nutrients, we show that maximum cell densities are two orders of magnitude greater when methionine, glycine and pyruvate are added to the medium.

Metabolic reconstruction has shown *Ca.* P. ubique's unusual requirement for a balanced supply of organic matter is a consequence of streamlining selection for minimal genome size and metabolic simplicity (Figures 1 and 5; Tripp *et al.*, 2008, 2009; Schwalbach *et al.*, 2010). We speculate that the unusual arrangement of central metabolism in *Ca.* P. ubique is adaptive not only because it is small and simple, and therefore requires fewer nutrients to replicate, but also because it is suited to planktonic environments where the flux of labile dissolved organic matter is low but continuous most of the time. In addition to the potential supply of pyruvate from glycolysis in some strains, pyruvate and its precursors oxaloacetate and glyoxylate are common metabolic intermediates in other organisms and also are formed by the photooxidation of dissolved organic matter (Kieber and Mopper, 1987; Kieber *et al.*, 1989; Mopper *et al.*, 1991; Moran and Zepp, 1997; Obernosterer *et al.*, 1999). Taurine, a compound produced by both phytoplankton and animals (Huxtable, 1992), also substituted for pyruvate, but at greatly reduced efficiency. Previously it was shown that the *Ca.* P. ubique glycine requirement could be met by glycine or serine, which are found in seawater at nanomolar concentrations (Tripp *et al.*, 2009). We also found that the common osmolyte glycine betaine and the photorespiration product glycolate could serve as precursors for glycine biosynthesis. Glycolate is produced by oxygenic phototrophs when they become carbon limited—including both eukaryotic phytoplankton (Leboulanger *et al.*, 1997, 1998; Schnitzler Parker *et al.*, 2004) and marine cyanobacteria (Bertlisson *et al.*, 2005)—the dominant marine phototrophs in temperate and tropical oceans. Tripp showed previously that either methionine or 3-dimethylsulphoniopropionate, a phytoplankton osmolyte, could meet the *Ca.* P. ubique requirement for organosulfur (Gage *et al.*, 1997; Tripp *et al.*, 2008). Therefore, the results of this study suggest that *Ca.* P. ubique has evolved to efficiently use a combination of ubiquitous, low molecular weight metabolites produced by phytoplankton, or resulting from the photooxidation of dissolved organic carbon, which may partially explain its high abundance in the euphotic zone.

A number of definitions have been proposed for the term 'oligotroph' based on optimal and inhibitory nutrient concentrations (reviewed in Schut *et al.*, 1997). These definitions do not easily fit the metabolic behaviors being observed in the

experiments presented here. The observation that alanine, derived from pyruvate or its precursors, is required for cell division (Figure 4, Supplementary Figures S2 and S3), but that large amounts of alanine adversely affect growth (Table 2), is an example of a deleterious imbalance caused by the presentation of a metabolic substrate to cells at either unnatural concentrations or in unnatural combinations. Unusual nutrient requirements, together with cryptic patterns of nutrient substitution and inhibition, present the experimentalist with a complex problem. This problem can be solved by the stepwise process of metabolic reconstruction coupled with experimentation if a defined medium is available. Although this approach is time consuming (especially with slow-growing cells, such as *Ca.* P. ubique), it has been successfully applied in other systems (Renesto *et al.*, 2003). The capacity of *Ca.* P. ubique for growth with the ambient concentrations of organic matter found in autoclaved seawater (Rappé *et al.*, 2002), and in mineral salts without added carbon (Figure 2, Supplementary Figure S4) are ample evidence of the adaptation of these cells to growth at low nutrient concentrations. More importantly, members of the SAR11 clade are the most successful chemoorganoheterotrophs in oligotrophic ocean systems. Therefore, although *Pelagibacter* defies conventional definitions of the term 'oligotroph' because it can tolerate some compounds at relatively high concentrations, it clearly conforms to the concept of oligotrophy.

Metabolic reconstruction *in silico* results in metabolic models that are subject to uncertainties that can only be resolved by studying the physiology and metabolism of cells *in vitro*. The defined medium presented here for *Ca.* P. ubique, provides a platform from which such controlled experiments can occur. *Ca.* P. ubique and other cells with streamlined metabolism are particularly challenging to model because of uncertainties arising from the loss of genes that function in canonical pathways (Figure 1). For instance, the requirements for glycine and alanine were not evident from the initial genome analysis of *Ca.* P. ubique (Giovannoni *et al.*, 2005). In addition, the hypothesis that pyruvate could serve as the sole carbon precursor for both biosynthesis and energy production was not predicted by previous metabolic models. Uncommon gene arrangements also complicate the interpretation of metabolic models in *Ca.* P. ubique (Figure 5). In *E. coli*, the genes coding for proteins required for the assimilation of carbon from glycolate are found at a single locus and are transcribed to a polycistronic message. In *Ca.* P. ubique, the same genes are physically separated from one another—but their functions are linked through the metabolic intermediate glycine (Figure 5). Experiments with cultures growing on a defined medium are an important step in the refinement of metabolic models such as the one presented in Figures 1 and 5.

The original success of dilution to extinction approaches was founded on the principle that if cells could be detected and cultured at low concentrations, on low nutrient media, their physiological responses to specific nutrient additions could be studied in subsequent experiments. The results presented in Tables 2 and 3, in light of the previously reported metabolic abilities of *Ca.* P. ubique (Tripp *et al.*, 2009; Schwalbach *et al.*, 2010), highlight one of the challenges associated with cultivating specialist oligotrophic organisms. Our results suggest that perhaps the largest obstacle to overcome in the cultivation of marine oligotrophs is the specific, unusual and often combinatorial or conditional nutrient requirements. There is growing evidence that a number of abundant microbial plankton species that are not yet cultivated, or are cultivated but difficult to propagate, are similar to *Ca.* P. ubique in that they are abundant, small cells that contain relatively small genomes ($<2.0$ Mbp) (Giovannoni *et al.*, 2008; Dupont *et al.*, 2011; Santoro and Casciotti, 2011). The success of cultivating *Ca.* P. ubique on artificial media relied on a targeted, minimal combination of nutrients deduced from metabolic reconstruction from a sequenced genome. If, as is likely, such requirements are common among organisms with streamlined genomes, it is hoped that this approach may serve as a blueprint for the cultivation of other important taxa that are difficult to grow in the laboratory.

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