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# Synergistic metabolism of a broad range of C1 compounds in the marine methylotrophic bacterium HTCC2181

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## Summary

**The 1.3 Mbp genome of HTCC2181, a member of the abundant OM43 clade of coastal bacterioplankton, suggested it is an obligate methylotroph. Preliminary experiments demonstrated that methanol and formaldehyde, but not other common C1 compounds such as methylamine, could support growth. Methanol concentrations in seawater are reportedly < 100 nM, suggesting either that the flux of methanol through plankton pools is very rapid, or that methanol may not be the primary growth substrate for HTCC2181. Therefore, we investigated the apparent extreme substrate range restriction of HTCC2181 in greater detail. Growth rate and maximum cell density of HTCC2181 increased with methanol concentration, yielding a $K_s$ value of 19 μM. In contrast, no growth was observed in the presence of the methylated (C1) compounds, methyl chloride, trimethylamine-oxide (TMAO) or dimethylsulfoniopropionate (DMSP) when they were the sole substrates. However, growth rate, maximum cell density and cellular ATP content were significantly enhanced when any of these methylated compounds were provided in the presence of a limiting concentration of methanol. These observations fit a model in which the metabolic intermediate formaldehyde is required for net carbon assimilation, allowing C1 substrates that do not produce a formaldehyde intermediate to be oxidized for energy, but not assimilated into biomass. Rates of methanol and TMAO oxidation and assimilation were measured with $^{14}$C-radiolabelled compounds in cultures of HTCC2181 and seawater microbial communities collected off the Oregon coast. The results indicated that in nature as well as in culture, C1 substrates are partitioned between those that are mainly oxidized to produce energy and those that are assimilated. These findings indicate that the combined fluxes of C1 compounds in coastal systems are sufficient to support significant populations of obligate methyltrophs by a metabolic strategy that involves the synergistic metabolism of multiple C1 compounds.**

## Introduction

Methylotrophy is a metabolic strategy used by bacteria to derive energy and carbon for growth from reduced one-carbon (C1) compounds. C1 compounds are defined as molecules with no C–C bonds. C1 compounds present in the marine environment include methane, methanol and methyl halides, as well as methylated amines and methylated sulfur compounds that are breakdown products of compatible solutes, such as dimethylsulfoniopropionate (DMSP) and trimethylamine-*N*-oxide (TMAO). Methylotrophs have commonly been found associated with sediments in both fresh and marine ecosystems. Obligate methylotrophic bacteria representing the genera *Methylomonas, Methylobacter, Methylocystis, Methyloversatilis*, and *Methylotenera* have been documented in culture and genomic-based approaches (Costello and Lidstrom, 1999; Auman *et al*., 2000; Miller *et al*., 2005; Nercessian *et al*., 2005; Kalyuzhnaya *et al*., 2005a,b; 2006). Pelagic enrichment studies using C1 compounds have led to the isolation of *Methylophaga* spp. that grow on dimethyl sulfide (DMS) (deZwart *et al*., 1996; Schafer, 2007), methyl bromide (Schaefer *et al*., 2002) and monomethylamine (MMA) (Kimura *et al*., 1990). Stable isotope probing (SIP) experiments, which typically involve a 4- to 7-day incubation in the presence of relatively high concentrations of a $^{13}$C-labelled compound, have shown methylotrophic activity during a phytoplankton bloom on methyl bromide, MMA and methanol by *Methylophaga* (Neufeld *et al*., 2007) and on MMA and dimethylamine (DMA) by other members of *Gammaproteobacteria* (Neufeld *et al*., 2008).

Evidence for methanol oxidation in marine ecosystems suggests this metabolic process is particularly important in coastal regions. Proteomic analysis of the microbial community from surface waters during a late summer

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upwelling off the Oregon coast revealed that 1% of protein-coding sequences identified were closely matched to OM43 (Sowell *et al*., 2010). Furthermore, 2.3% of all peptide spectra identified in that study were best matched to the predicted methanol dehydrogenase, XoxF, of the OM43 clade. Methanol utilization using $^{14}CH_3OH$ showed that methanol oxidation rates were about 1.5-fold higher in coastal waters as compared with off-shelf north-east Atlantic surface waters (Dixon *et al*., 2011). In that study, higher rates of methanol oxidation were also correlated with higher chlorophyll *a* concentrations.

Bacterial strain HTCC2181 was isolated from seawater collected from the Oregon coast and is a member of the OM43 clade of *Betaproteobacteria*. OM43 can constitute 2% of bacterial cells in near shore microbial communities from globally diverse locations (Rappé *et al*., 2000; Sekar *et al*., 2004; Galand *et al*., 2008; Song *et al*., 2009) and is commonly associated with diatom blooms (Morris *et al*., 2006). OM43 is related to the methylotrophs of the *Methylophilaceae* family which can utilize methanol, formaldehyde and formate as sole growth substrates (Anthony, 1983). The 1.3 Mbp genome of HTCC2181 remains the smallest reported for a free-living cell. The full genome sequence revealed that HTCC2181 is an obligate methylotroph with scant C1 metabolic pathways (Giovannoni *et al*., 2008). It cannot oxidize methane, but the genes encoding the RuMP pathway for methanol oxidation were identified. In that pathway, methanol is initially oxidized to formaldehyde by a methanol dehydrogenase encoded by an *xox* cluster that is homologous to the well-studied *mxa/mox* cluster. Formaldehyde is subsequently incorporated into biomass via the RuMP cycle for carbon fixation or oxidized to $CO_2$ via the dissimilatory RuMP cycle yielding one NAD(P)H (Chistoserdova, 2011). Although formaldehyde can spontaneously condense with the C1-group carrier, tetrahydrofolate (THF), forming methylene-THF, this is a slow reaction (Vorholt *et al*., 2000; Crowther *et al*., 2008) and is thus unlikely to be significant in HTCC2181. Nevertheless, the THF-dependent pathway for C1-group oxidation was identified in the HTCC2181 genome and likely operates to oxidize methylene-THF yielding $CO_2$ and energy in the form of two reduced nucleotides and one ATP. There is no genomic evidence in HTCC2181 for tetrahydromethanopterin ($H_4$MPT)-linked formaldehyde oxidation to formate yielding $CO_2$, or the serine cycle (Giovannoni *et al*., 2008).

Phytoplankton and atmospheric deposition are considered the primary sources of C1 compounds in surface seawater (Heikes *et al*., 2002; Sinha *et al*., 2007), but little information is available about their concentrations or seasonal trends that may lead to their availability as substrates for bacterioplankton. Methanol is the most abundant volatile organic compound in the lower atmosphere; however, processes influencing the atmospheric: marine flux are not well understood (Singh *et al*., 2000). Analagous to leaf growth, pectin demethylation during phytoplankton growth is postulated to be a source of methanol.

Measurements of methanol concentrations in surface waters are few and range between 70 and 118 nM (Williams *et al*., 2004; Dixon *et al*., 2011). These values are two orders of magnitude lower than concentrations used in initial growth studies with HTCC2181 (Giovannoni *et al*., 2008) and raised questions about whether methanol is its primary growth substrate (Chistoserdova *et al*., 2009). Methyl chloride is the predominant organochlorine in the marine ecosystem and despite extremely low measured concentrations (< 10 nM; Yang *et al*., 2010), methyl chloride-utilizing bacteria have been readily isolated from seawater (McAnulla *et al*., 2001). Methylamines are the by-products of protein decomposition and major contributors to greenhouse gas. DMSP is an important player in the marine sulfur cycle because it can be synthesized by phytoplankton for use as an osmolyte, is metabolized by bacterioplankton for C and S assimilation, and its breakdown product, dimethylsulfide, contributes to cloud formation (Malin, 2006). In coastal regions, measured DMSP concentrations range from < 5 to 600 nM (Fredrickson and Strom, 2009). Measured concentrations of TMAO, another osmolyte, averaged 15 nM and were similar to DMSP concentrations in Antarctic coastal waters (Gibb and Hatton, 2004). Together, these compounds contribute to the pool of dissolved organic carbon available for substrate utilization.

HTCC2181 was first isolated on a sterile seawater medium with no added organic nutrients and in culture shows characteristics associated with cells that are adapted to oligotrophic (low nutrient) conditions. Oligotrophic conditions are thought to prevail in most parts of the oceans. Cells adapted to this environment typically are a challenge to study because of low growth rates, low cell yields and intolerance to high nutrient concentrations. The genome streamlining hypothesis (Giovannoni *et al*., 2005) has been proposed to explain the very small genomes that are found in some highly successful marine oligotrophs. This theory postulates that small cell size and small genome size are adaptations that maximize cell surface to volume ratios and minimize the nutrients required for replication.

The objective of this study was to investigate the substrate range of HTCC2181 and broaden understanding of the functional significance of methylotrophy in coastal ecosystems. The experimental design we chose was compatible with the low growth rates and cell yields that are typical of oligotrophic cells. Using pure cultures, we undertook growth studies and made measurements of

cellular respiration and ATP content. Although methanol is required for growth, we found that a surprisingly broad range of substrates served as energy sources for HTCC2181. Growth kinetics and labelling studies support the conclusion that despite extreme genome reduction, HTCC2181 has maintained the capacity to exploit an environment rich in C1 compounds. This metabolic strategy is particularly well suited to coastal regions where the environment may be enriched with a diverse array of C1 substrates.

## Results

Growth responses of HTCC2181 to methanol and other potential growth substrates were initially investigated in seawater-based media (LHNM, see *Experimental procedures*) with addition of varying concentrations of methanol or TMAO. Similar to a previous study (Giovannoni *et al*., 2008), maximum cell density increased stoichiometrically with methanol concentration (Fig. 1 left). It was previously reported that there was no difference in growth rate or maximum cell density in the presence of 100 μM–3 mM methanol (Giovannoni *et al*., 2008). This lack of growth rate dependence on methanol concentration caused speculation that methanol is not the primary growth substrate for HTCC2181 (Chistoserdova *et al*., 2009). In this study, we tested methanol concentrations < 100 μM. The growth rate dependence of HTCC2181 on methanol is shown in Fig. 1, and data fitted to the Monod growth kinetic model. The resultant $K_s$ value was 19.2 ± 3.2 μM, and the maximal growth rate ($\mu_{max}$) was estimated to be 1.52 ± 0.09 $day^{-1}$. In contrast to methanol, TMAO added to LHNM did not support growth of HTCC2181. However, addition of low concentrations of TMAO in the presence of 10 μM methanol (well below the $K_s$ for growth on methanol), enhanced the growth rate and maximum cell density of HTCC2181 above levels measured in the presence of 10 μM methanol alone (Fig. 2). The addition of 5 μM TMAO to 10 μM methanol allowed HTCC2181 to grow at an equivalent rate and to the same final cell density as cells grown on 50 μM methanol.

As was the case for TMAO, no other C1 compounds that were tested supported growth of HTCC2181 unless methanol was also added to the media. However, a consistent growth pattern was observed for HTCC2181 grown with a range of different C1 compounds in the presence of 10 μM methanol. In general, the specific growth rate of HTCC2181 grown on 10 μM methanol with 5 μM of an added C1 compound was twice the rate of HTCC2181 grown on 10 μM methanol alone (Fig. 2). Higher concentrations of C1 compounds appeared to inhibit rather than enhance cell growth. For example, the growth rate and maximum cell density of HTCC2181 with 15 μM DMSP and 10 μM methanol were less than those observed with 1 μM DMSP and 10 μM methanol, and were similar to growth on 10 μM methanol alone (Fig. 2). Enhanced growth was not observed with addition of low concentrations of other C1 compounds that are known osmolytes, such as glycine betaine or proline betaine, to 10 μM methanol, and growth was also not enhanced with

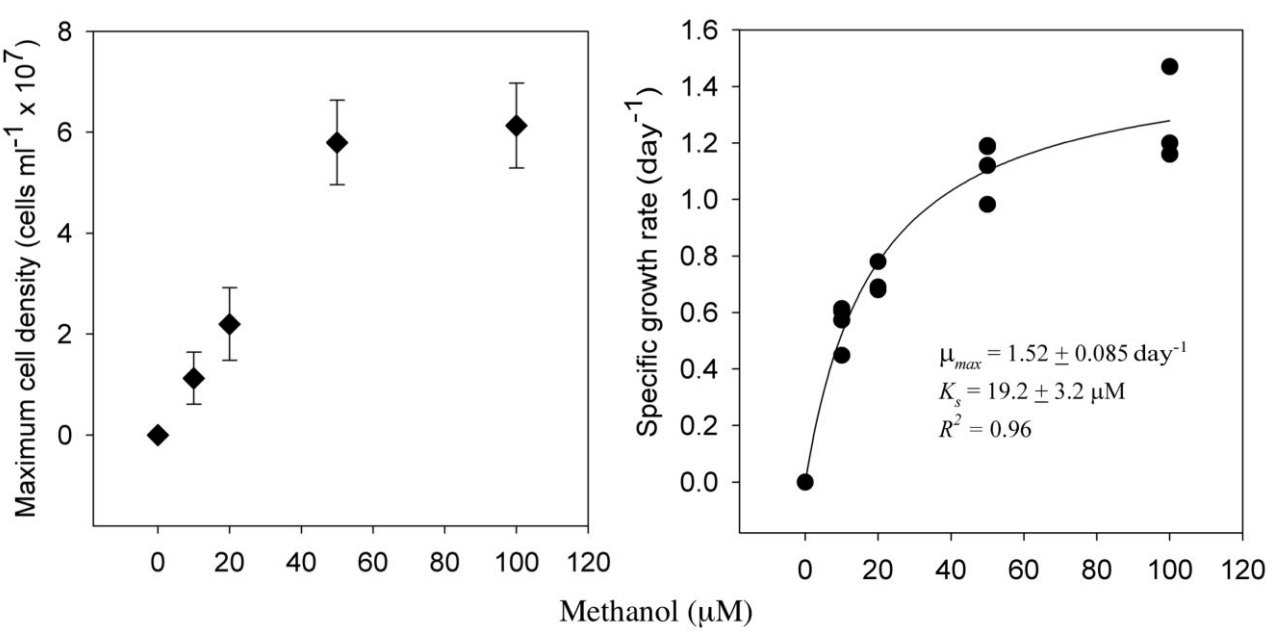


**Fig. 1.** Maximum cell density of HTCC2181 increased stoichiometrically with increasing methanol concentrations (left) Bars are SD for three independent growth experiments. Specific growth rates of HTCC2181 increased with methanol concentration (right). Data were fitted to the Monod model for bacterial growth on a limiting substrate.

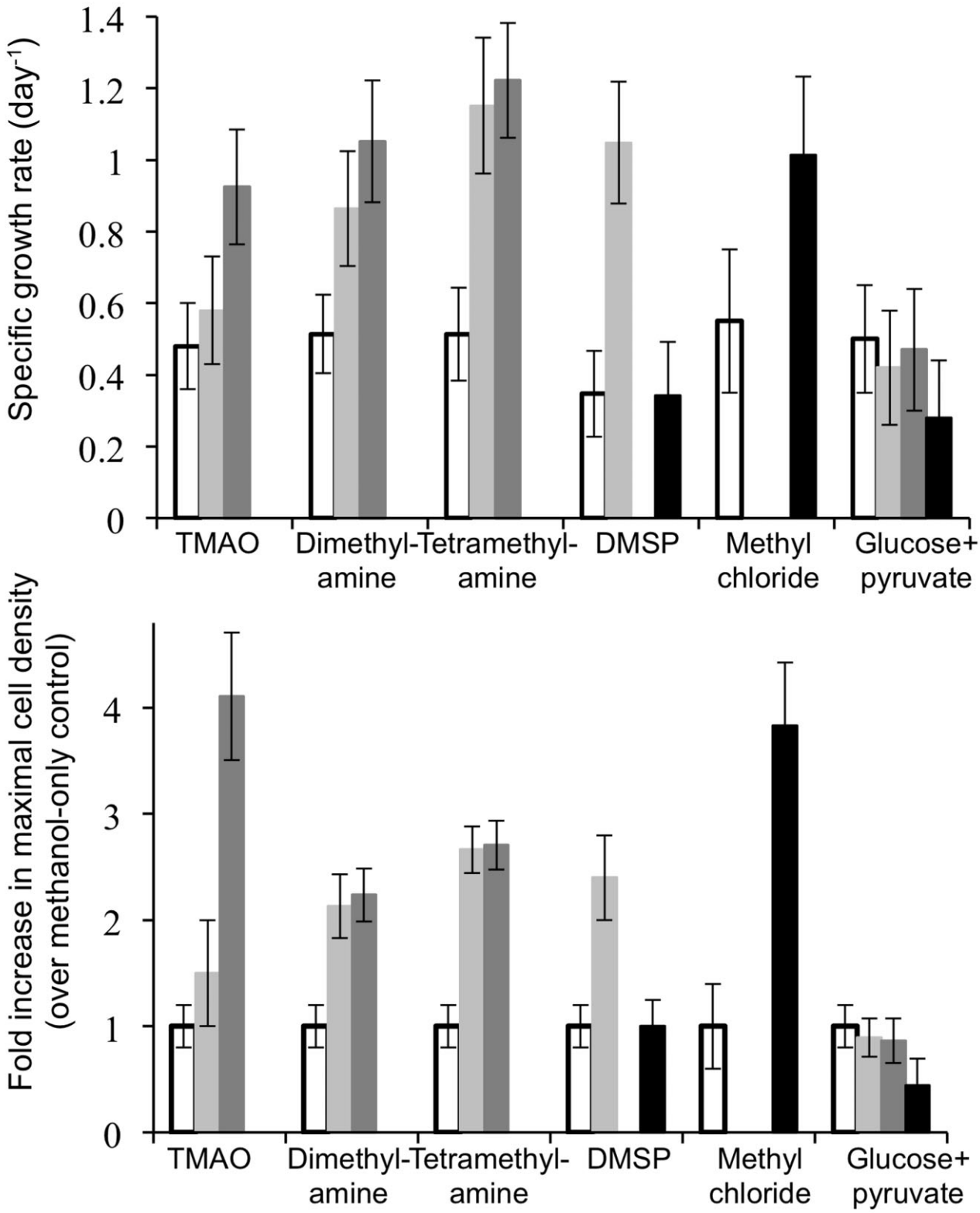


**Fig. 2.** Specific growth rates (top) and increases in maximal cell density (bottom) of HTCC2181 in response to different methylated compounds in the presence of methanol. For all experiments, bars represent different concentrations of methylated compounds added to LHNM media containing 10 μM methanol (50 μM methanol for methyl chloride experiment): white, 0 μM; light grey, 1 μM; dark grey, 5 μM; black, 10 μM (15 μM for DMSP). Glucose + pyruvate were added together at the same concentrations as non-methylated compound control.

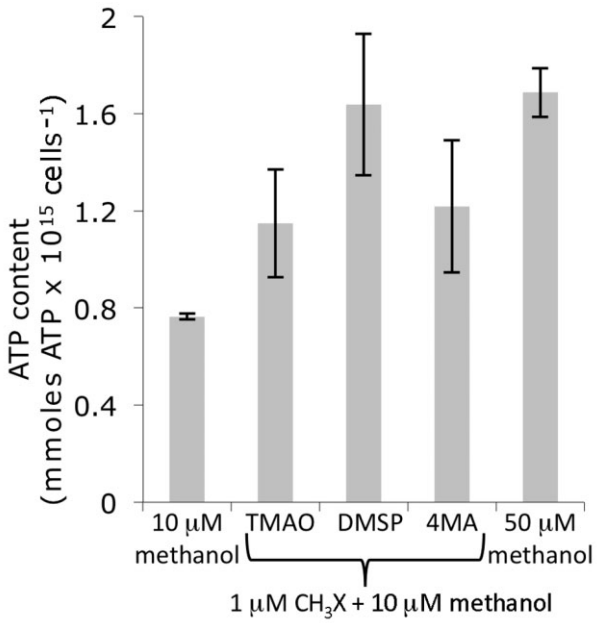


**Fig. 3.** Cellular ATP content increases with addition of methylated compounds. Cells were grown in LHNM with 10 or 50 μM methanol or 1 μM methylated compound ($CH_3X$) and 10 μM methanol, harvested at late exponential phase, and immediately assayed for ATP content. 4MA: tetramethylamine hydrochloride.

addition of MMA. Glucose and pyruvate were tested as non-C1 control compounds and stimulated no growth in the absence of methanol and no enhancement of growth in the presence of 10 μM methanol (Fig. 2).

ATP content was higher in cells growing with a C1 compound in the presence of 10 μM methanol than in cells growing with 10 μM methanol alone (Fig. 3). Cells growing with 1 μM DMSP added to 10 μM methanol had twice the ATP content as cells growing with 10 μM methanol, and matched the ATP content of cells growing with 50 μM methanol. These values are very similar to those recently measured in *Candidatus* Pelagibacter ubique, a member of the ubiquitous SAR11 clade of marine alphaproteobacteria (Steindler *et al.*, 2011) and are about twofold less than steady-state *Serratia* and *Vibrio* sp. grown in chemostats (Hamilton and Holm-Hansen, 1967). The increase in ATP content at elevated (50 μM) methanol concentrations suggests that energy production from methanol has a high threshold for saturation.

The genomic information and growth and kinetic patterns displayed by HTCC2181 grown on LHNM amended only with methanol confirmed that it can serve as the primary source of both carbon and energy for this bacterial strain. The distribution of methanol-carbon between the assimilatory RuMP pathway and formaldehyde oxidation pathways (dissimilatory RuMP and THF-dependent pathway for energy generation) was determined using $^{14}CH_3OH$. The rates of HTCC2181 methanol-carbon incorporation and oxidation to $CO_2$ were linear over an 18 h time-course, and combined, the rate of methanol-carbon utilization was 2.0 μmol $(10^{12}$ cells · h$)^{-1}$; $r^2 = 0.92$ (Fig. 4). The rate of methanol oxidation to $CO_2$ was 3.5-fold higher than the rate of methanol-carbon incorporation into cells in the pure culture experiment.

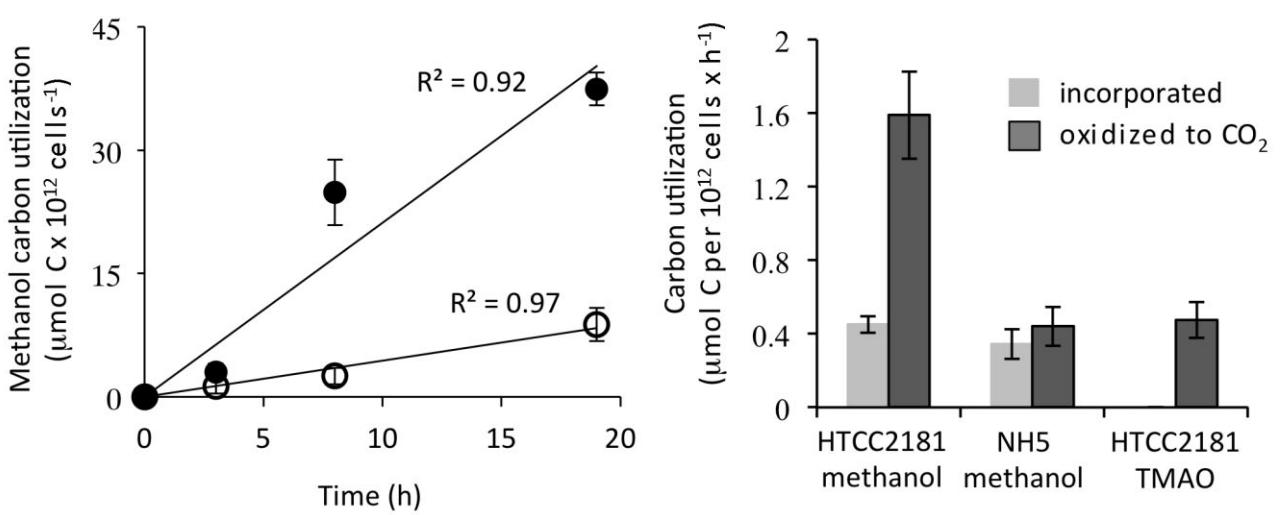


**Fig. 4.** C1 compound utilization by HTCC2181 in culture and seawater collected off the Oregon coast. Left panel: methanol carbon utilized by HTCC2181 in culture determined by 50 μM $^{14}CH_3OH$ addition to concentrated cells in sealed vials. Total carbon utilized (filled circles; methanol oxidized to $CO_2$ + incorporated) measured following addition of base, $Na_2CO_3$ and $BaCl_2$ and filtration. Carbon incorporated into biomass (open circles) measured following addition of cold TCA and filtration. Bars are SE for three samples at each time point. Right panel: rates of methanol carbon incorporation or oxidation by HTCC2181 in culture and in seawater collected at the Newport harbour station (NH5) and rate of TMAO oxidation to $CO_2$ by HTCC2181 in culture.

Methanol utilization was also assessed in natural seawater collected off the Oregon coast from the same site that HTCC2181 was originally isolated. At the time of seawater collection for this study, diatoms dominated the phytoplankton community. HTCC2181 is a dominant member of the bacterioplankton community that is typically associated with diatom blooms (Morris *et al.*, 2006). In natural seawater, the rate of methanol carbon incorporated into cells was only slightly less than the rate of incorporation into HTCC2181 during the pure culture experiment (Fig. 4, right panel). In contrast, methanol oxidation to $CO_2$ was threefold slower in the natural seawater experiment as compared with the pure culture experiment. One possible explanation for the lower rate of oxidation to $CO_2$ in the seawater experiment is that the seawater may have had relatively high concentrations of C1 compounds (such DMSP and TMAO) that were available for cellular oxidation. Osmolyte concentrations in phytoplankton are typically 100–400 mM, and can significantly increase under low $CO_2$ and solar ultraviolet radiation (Sunda *et al.*, 2002); conditions that co-occur at the peak of a phytoplankton bloom in mid-summer. The release of osmolytes from dead or dying phytoplankton (Malin, 2006) could easily contribute to a higher initial concentration of C1 compounds.

Methyl group utilization was also studied using radiolabelled TMAO in the presence of 5 μM methanol. No $^{14}C$ was detected in the TCA-precipitated fraction, but $^{14}CO_2$ was detected at a rate of 0.47 μmol C $(10^{12}$ cells · h$)^{-1}$ (Fig. 4, right panel). This result confirmed our hypothesis that strain HTCC2181 can oxidize methyl groups to $CO_2$.

HTCC2181 growth experiments and ATP content determinations suggested that this obligate methanol oxidizer can utilize other C1 compounds to supplement energy generation allowing methanol carbon to be more efficiently utilized for biomass accumulation. We tested this hypothesis using a reciprocal labelling experiment. First, increasing concentrations of TMAO were added to concentrated HTCC2181 cell suspensions in the presence of 10 μM $^{14}CH_3OH$ and incubated for 20 h. The amount of methanol-carbon oxidized to $CO_2$ decreased, while the amount of methanol-carbon assimilated into biomass increased with TMAO concentration (Fig. 5). In the reciprocal experiment, increasing concentrations of $^{14}C$-labelled TMAO were added to concentrated HTCC2181 cell suspensions in the presence of 10 μM unlabelled $CH_3OH$, resulting in increasing amounts of $^{14}CO_2$ detected (Fig. 5). When 10 μM $^{14}C$-TMAO was added in the presence of 10 μM methanol, 6.3 ± 1.2 μmol $^{14}CO_2$ was detected. This quantity is similar to the 10.0 μmol difference in $CO_2$ detected in samples incubated with 10 μM $^{14}CH_3OH$ and either 0 or 10 μM unlabelled TMAO, suggesting that, when available, TMAO is oxidized to $CO_2$ in lieu of methanol.

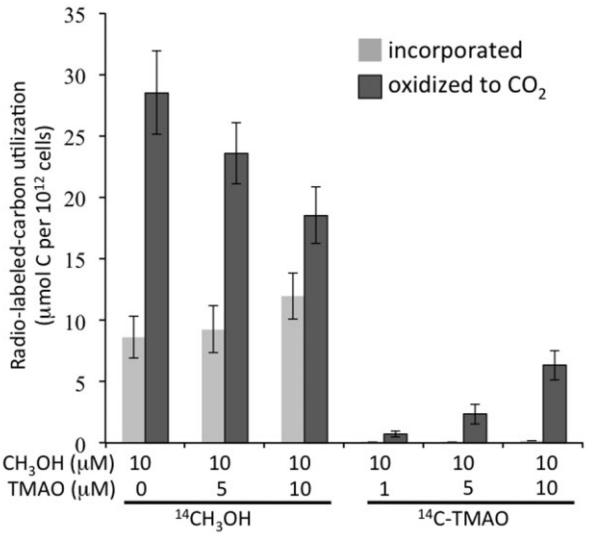


**Fig. 5.** Reciprocal labelling experiments discriminating C1 utilization into biomass (light grey bars) or oxidized to $CO_2$ (dark grey bars) by HTCC2181 in the presence of methanol and TMAO. Concentrated cell suspensions were incubated with 10 μM $^{14}CH_3OH$ and 0, 5 or 10 μM unlabelled TMAO (left three sets of bars). In the reciprocal experiment, 1, 5 or 10 μM $^{14}C$-labelled TMAO was added to concentrated cell suspensions in the presence of 10 μM unlabelled $CH_3OH$ (left three sets of bars).

## Discussion

This study examined the methylotrophic growth strategy of HTCC2181, a member of the OM43 clade of *Betaproteobacteria* that is broadly distributed in coastal ecosystems. Growth experiments demonstrated stoichiometric growth on methanol, confirming earlier predictions based on genomic analysis and initial work with HTCC2181 in culture (Giovannoni *et al.*, 2008). However, results presented here expand the view of this methylotroph from one of an obligate methanol oxidizer, to one of a broad spectrum C1 oxidizer that nevertheless employs a remarkably thin set of metabolic pathways.

Growth kinetics of HTCC2181 on methanol highlight the quandary of survival by obligate methylotrophy in the carbon-dilute marine environment. While reported concentrations of methanol in seawater are in the range of 0.1 μM (Williams *et al.*, 2004; Dixon *et al.*, 2011), the $K_s$ value for HTCC2181 was 19 μM (Fig. 1B). The orders of magnitude difference between methanol concentrations in seawater and the HTCC2181 $K_s$ for methanol would lead to an unlikely growth rate of $< 0.01$ day$^{-1}$. The few available studies that estimated growth rates of marine bacteria indicate specific growth rates that are an order of magnitude higher. For example, *in situ* specific growth rates of SAR11 measured by radiolabelled leucine assimilation were 0.13–0.72 day$^{-1}$ (Malmstrom *et al.*, 2005), and the minimal and maximal steady-state growth rates of marine isolates in chemostats were 0.24–0.6 day$^{-1}$ (Kemp

*et al*., 1993). The gene encoding the methanol dehydrogenase identified in HTCC2181, *xoxF*, is highly similar to *xoxF* in *Methylobacterium extorquens* AM1 (Giovannoni *et al*., 2008). Purified XoxF from *M. extorquens* AM1 yielded a $K_m$ for methanol of 11 μM (Schmidt *et al*., 2010). Methanol affinity constants for other, more distantly related methanol dehydrogenases from *Methyloversatilis universalis* FAM5, *Methylophilus methylotrophus* W3A1 and *Burkholderiales* strain RZ18-153, were remarkably conserved, ranging from 10 to 20 μM (Kalyuzhnaya *et al*., 2008).

So, how does HTCC2181 maintain a growth rate typical of marine bacteria when its primary substrate, methanol, is available at extremely suboptimum levels? The apparent solution to this problem for HTCC2181 is to utilize methanol for biomass accumulation while simultaneously utilizing other low-molecular-weight C1 compounds strictly for energy production. An important result supporting this conclusion is suppression of HTCC2181 growth in the presence of high concentrations of DMSP and 10 μM methanol (Fig. 2). We speculate that the excess methyl groups 'hoard' THF, a required cofactor for biosynthetic processes other than THF-dependent C1-group oxidation.

Our results suggest that other C1 compounds must enter cell metabolism in a manner that does not allow methyl group assimilation, but does provide cellular energy. Formaldehyde is a key intermediate in methanol assimilation. The RuMP cycle is initiated by hexulose 6-phosphate synthase, which catalyses the formation of hexose 6-phosphate from formaldehyde and ribulose 5-phosphate (Fig. 6). This formaldehyde requirement for carbon assimilation prohibits the assimilation of methyl groups from other reduced C1 compounds in HTCC2181. Addition of 1–5 μM C1 compounds to HTCC2181 grown on a limiting concentration of methanol, increased both growth rate and maximum cell density, but no growth was observed on any of the C1 compounds alone. Thus, while methanol is metabolized via the RuMP pathway, methyl groups from other C1 compounds most likely enter the THF-dependent pathway for energy generation (Fig. 6) and allow greater efficiency of methanol assimilation (Fig. 5).

There is a precedent for such metabolic discrimination in the example of 'carboxydovores' carbon monoxide (CO) oxidizers that derive energy from CO but cannot grow autotrophically (Moran *et al*., 2004; King and Weber, 2007). *Methylocella silvestris* metabolizes methylamine by the glutamate-mediated methylamine utilization pathway into formaldehyde and ammonium for assimilation, or the formaldehyde may be oxidized to $CO_2$ for energy generation (Chen *et al*., 2010). However, as previously discussed, the THF-dependent pathway in this MMA utilization scheme is probably insignificant due to the relatively slow condensation reaction of formaldehyde

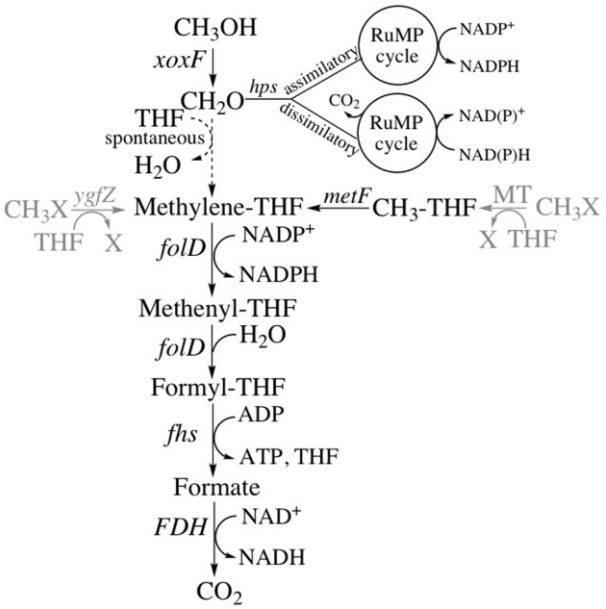


**Fig. 6.** Metabolic pathways for C1 metabolism in HTCC2181. Genes for methanol metabolism leading to carbon incorporation (assimilatory RuMP pathway) or oxidation to $CO_2$ (dissimilatory RuMP pathway) and the THF-dependent pathway are shown in black. Possible pathways for oxidation of C1 compounds such as DMSP, TMAO and methyl chloride are shown in grey and because the RuMP cycle requires a formaldehyde intermediate, can enter only the THF-oxidative pathway. THF, tetrahydrofolate; $CH_3X$, methylated compound; MT, unknown methyltransferase.

with THF (Crowther *et al*., 2008). *Methylobacterium chloromethanicum* CM4 can grow on either methyl chloride or methanol as its sole source of carbon and energy (Studer *et al*., 2002). Growth of *M. chloromethanicum* CM4 on methanol is initiated by a methanol dehydrogenase, and growth on methyl chloride requires a $B_{12}$ and THF-dependent methyltransferase, encoded by *cmuAB* genes, to transfer the methyl group to THF (Studer *et al*., 2001). In that organism, methyl-THF is oxidized by *metF* to form methylene-THF that can enter the serine cycle for carbon assimilation (not present in HTCC2181) or be further oxidized by linear THF-linked oxidation yielding $CO_2$ and energy in the form of one ATP and two reduced nucleotides. Another THF-dependent methyltransferase, DmdA, appears to be highly specific for DMSP in isolates of SAR11 and Roseobacter (Reisch *et al*., 2008). Neither CmuAB nor DmdA is present in the HTCC2181 genome. Furthermore, no genetic evidence exists in HTCC2181 for glycine betaine catabolism (*bhmT*, *sardh*, *dmgdh* genes) or methylamine utilization (γ-glutamylmethylamide/*N*-methylglutamate pathway).

The enzyme system that facilitates methyl group transfer to the THF-linked oxidation pathway in HTCC2181 is unknown. Its streamlined genome harbours few methyltransferases with functions other than those associated with biosynthetic processes typical in all cells. The range

of C1 compounds that enhanced growth of HTCC2181 in the presence of methanol suggests that this organism may utilize a single, as yet unidentified, broad substrate range methyltransferase (MT). One possibility, analagous to the CmuAB or DmdA systems, is methyl group transfer to THF by MT forming methyl-THF that is further oxidized to methylene-THF via MetF (Fig. 6; Mb2181_00485). The Mts system in the methanogenic archaea, *Methanosarcina acetovorans*, functions to facilitate CO-dependent DMS metabolism and methanogenesis. This corrinoid MT appears to be specific for methylsulfides, but is induced by a wide range of C1 compounds (Oelgeschlager and Rother, 2009). A second possible mechanism for C1 utilization in HTCC2181 is methyl group transfer that directly forms methylene-THF, as is the case for the glycine cleavage system (GCVT). Interestingly, in HTCC2181, a protein of the COG0354 family, which includes GcvT, DmdA, and other proteins that mediate C1 transfers with THF, is part of an operon encoding proteins that have known and unknown functions involved in methanol dehydrogenase activity. This protein, YgfZ (Mb2181_03355) like all COG0354 proteins, is predicted to form a folate-binding pocket. Evidence that YgfZ is involved in methyl group transfer includes functional analysis of YgfZ from *Escherichia coli* and *Klebsiella pneumonia* demonstrating methylation of the toxin plumbagin (Lin *et al*., 2010). YgfZ from *E. coli* shares 82% identity with YgfZ from *K. pneumonia*. Methylation activity of *E. coli* Δ*ygfZ* was partially restored with YgfZ from *Mycobacterium tuberculosis*, even though the homologues share only 20% identity. YgfZ from HTCC2181 shares 50% and 46% identity with homologues from *E. coli* and *K. pneumonia*. Another study establishes that folate is required as a cofactor for YgfZ and proposes that it mediates methyl group transfer from iron-sulfur cluster proteins to THF (Waller *et al*., 2010). Thus, we suggest that in HTCC2181, YgfZ may serve to transfer methyl groups from a broad range of substrates to THF (Fig. 6).

The genomic view of HTCC2181 is one of obligate methanol oxidation; however, this study revealed a much wider methylotrophic capacity than was previously recognized. The range of C1 substrates that enhanced growth of HTCC2181 are components of the dissolved organic carbon pool in seawater that are likely to be omnipresent, albeit in concentrations that may vary regionally and/or seasonally. HTCC2181 and diatom abundances are well correlated (Morris *et al*., 2006). Diatoms typically follow a 'boom and bust' growth pattern, and are likely a good source of methylotrophic growth substrates (e.g. methanol and highly methylated osmolytes) during growth and bloom demise. A number of marine heterotrophic bacteria, including HTCC2181, have been successfully isolated by dilution to extinction methods that allow genomic and physiological analysis of highly abundant, yet metabolically enigmatic organisms such as OM43 and SAR11. Physiological data, such as those presented here, provide valuable information that help identify growth substrates, estimate rates of substrate utilization and provide connections between key players of the marine microbial loop.

## Experimental procedures

### *Culture conditions*

HTCC2181 was maintained in sterilized seawater-based media LNHM (Connon and Giovannoni, 2002) amended with 1 μM $FeCl_3$ and 50 μM methanol in the dark at 18°C. All growth experiments were conducted in 50 ml of culture media in 250 ml acid-washed polycarbonate flasks except for growth experiments with methyl chloride. In this case, cultures were grown in sealed (720 ml) glass serum vials that were acid washed and soaked for at least 4 h in 10% BSA. Following BSA treatment, vials were rinsed six times with nanopure water and autoclaved. This BSA treatment was deemed necessary for growth of HTCC2181 in the glass serum vials. Presumably, this treatment bound BSA to any 'active sites' on the inner vial surface, thus preventing adsorption of methanol or other substrates to the surface, allowing their full availability for growth. Vials contained 25 ml of LNHM. A dimensionless Henry's constant of 0.27 (Gossett, 1987) was used to quantify methyl chloride additions. For assays requiring greater culture volumes, cells were grown in 250 ml of culture media in 1 l polycarbonate flasks. Cell concentrations were determined using SYBR Green DNA binding dye (Invitrogen, CA) and counting using a Guava flow cytometer as described previously (Stingl *et al*., 2007).

### *ATP assays*

Cells were grown in LNHM with 10 or 50 μM methanol or 10 μM methanol with 1 μM TMAO, DMSP or tetramethylamine. Cultures were harvested by ultracentrifugation (Beckmann, 45 Ti, 50 000 *g*, 30 min) during mid-exponential phase of growth. Cells were resuspended in 3 ml of fresh LNHM to $2–6 \times 10^8$ cells $ml^{-1}$, sampled for cell counts by Guava flow cytometry (see above), and cellular ATP content was immediately measured using 0.02 ml of resuspended cells and 0.09 ml of BacTiter Glo™ reagent (Promega, Madison, WI) dispensed into white 96-well assay plates (White with Lid, Tissue Culture-Treated, BD Biosciences, San Jose, CA). Luminescence was measured after 4 min using a multifunction plate reader (Infinite M200, Tecan) with a 1 s integration and 10 ms settle time. An ATP standard curve was generated using ATP and instructions provided by the kit manufacturer and used to calculate the concentration of ATP in the samples.

### *Radiolabelled methanol and TMAO uptake and oxidation*

For $^{14}CH_3OH$ and $^{14}$C-TMAO experiments to distinguish between carbon incorporated into biomass and oxidized

to $^{14}CO_2$, a method was devised for volatile $^{14}C$ compounds (e.g. $^{14}C$-labelled methanol). First, trichloroacetic acid (TCA) precipitation, a well-established method for assessing $^{14}C$ incorporation into proteins, was used. Second, $^{14}CO_2$ was precipitated by addition of NaOH, $Na_2CO_3$ and $BaCl_2$, forming $Ba^{14}CO_3$ and BaOH. Upon filtration, unincorporated $^{14}CH_3OH$ and BaOH is removed, thus minimizing potential quenching effects of the base.

For experiments using pure cultures, HTCC2181 was harvested in mid-exponential phase by ultracentrifugation (Beckmann, 45 Ti, 30 000 r.p.m., 30 min). Cells were resuspended in 0.6 l of LHNM to approximately $10^7$ cells $ml^{-1}$. A portion of the culture was treated with 10% formaldehyde and served as the 'killed' control. $^{14}CH_3OH$ (0.5 mCi $mmol^{-1}$; 50 μM, 12 μCi or 10 μM, 2.4 μCi) or $^{14}C$-TMAO (1 μM, 0.9 μCi; 5 μM, 4.5 μCi; or 10 μM, 9 μCi) was added to the 'live' and 'killed' cultures. Samples (200 μl) were taken to determine the total activity of $^{14}CH_3OH$ added. Inoculated cultures were aliquoted into acid washed and BSA-treated glass serum vials (described above) and sealed. At each time point, reagents were added to cultures using syringes inserted through the stoppers to assay for $^{14}C$ incorporation or total $^{14}C$ utilization (incorporation + oxidation to $^{14}CO_2$). For $^{14}C$ incorporation, 10% culture volume 100% w/v cold TCA was added, and vials placed at 4°C. For total $^{14}C$ utilization, 0.05 N NaOH, 2.5 mM $Na_2CO_3$ and 50 mM $BaCl_2$ were added, and vials placed at 4°C. Within 30 h of the final time point, precipitates were collected on 0.2 μm nitrocellulose filters (Millipore, MA), washed three times with 3 ml of 100% w/v TCA ($^{14}C$ incorporation) or 3 ml of LNHM media (total $^{14}C$ utilization) and transferred to scintillation vials. Prior to counting, the thick precipitates formed by $BaCl_2$ precipitation were disrupted following addition of scintillation cocktail by $3 \times 30$ s pulses using a sonication bath. $^{14}CH_3OH$ or methyl groups from $^{14}C$-TMAO oxidized to $^{14}CO_2$ were determined by difference.

For $^{14}CH_3OH$-based experiments using natural seawater, 20 l of seawater was collected by Niskin bottle, and transferred to an acid-washed, autoclaved polycarbonate carboy at Newport hydrostation NH5 on 8 September 2010 and stored at 18°C overnight. The seawater was pre-filtered using a GF/A in-line filter followed by tangential flow filtration using stacked 100 and 30 Kd cartridges to a final volume of 0.6 l. The bacterial cell count of the concentrated seawater was $8E + 6$ $ml^{-1}$. The experiment proceeded as described above. Radiolabelled $CH_3OH$ was obtained from Sigma-Aldrich.

### *Radiolabelled TMAO uptake and oxidation*

Uniformly $^{14}C$-labelled TMAO was synthesized as previously described (Dunstan and Goulding, 1899). $^{14}C$-trimethylamine (50 μCi) was dried and resuspended in 0.1 N NaOH, 4.5% $H_2O_2$. The solution was incubated at room temperature overnight, heated at 70°C to dryness and resuspended in 1 ml of $H_2O$. The $^{14}C$-TMAO was verified by TLC analysis using a silicon plate and a methanol : chloroform : ammonia : $H_2O$ (50:50:12.5:10 v/v) solvent system. Labelled products were detected after 72 h exposure by autoradiography. The $R_f$ values obtained were 0.57 for TMAO and 0.38 for TMA.

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