Microb Ecol (2011) 62:564–573
DOI 10.1007/s00248-011-9873-0

ENVIRONMENTAL MICROBIOLOGY

Distribution and Selection of Poly-3-Hydroxybutyrate Production Capacity in Methanotrophic Proteobacteria

Allison J. Pieja · Katherine H. Rostkowski · Craig S. Criddle

Received: 10 March 2011 / Accepted: 3 May 2011 / Published online: 19 May 2011

Abstract Methanotrophs are known to produce poly-3-hydroxybutyrate (PHB), but there is conflicting evidence in the literature as to which genera produce the polymer. We screened type I and II proteobacterial methanotrophs that use the ribulose monophosphate and serine pathways for carbon assimilation, respectively, for both *phaC*, which encodes for PHB synthase, and the ability to produce PHB under nitrogen-limited conditions. Twelve strains from six different genera were evaluated. All type I strains tested negative for *phaC* and PHB production; all Type II strains tested positive for *phaC* and PHB production. In order to identify conditions that favor PHB production, we also evaluated a range of selection conditions using a diverse activated sludge inoculum. Use of medium typically recommended for methanotroph enrichment led to enrichments dominated by type I methanotrophs. Conditions that were selected for enrichments dominated by PHB-producing Type II methanotrophs were: (1) use of nitrogen gas as the sole nitrogen source in the absence of copper, (2) use of a dilute mineral salts media in the absence of copper, and (3) use of media prepared at pH values of 4–5.

Introduction

Polyhydroxyalkanoates (PHAs), such as poly-3-hydroxybutyrate (PHB), are polymers stored by many bacteria that can be harvested and used commercially as biodegradable substitutes for conventional petroleum-based plastics [2]. To date, the relatively high cost of PHB compared to conventional plastic resins such as polyethylene and polypropylene has limited its use [36]. A major reason for the high cost of PHB is the use of sugar from corn or sugar cane as feedstock. Up to 30% of total production cost is attributed to feedstock cost [15].

Use of an organic wastes feedstock could decrease PHB production and environmental costs [4, 10, 20, 34, 45]. Waste methane from landfills and wastewater treatment plants is often flared or simply released to the atmosphere. In 2009,142 Tg of CO_2 equivalents, or about 15 billion pounds, were released as methane to the atmosphere in the U.S.A. [49]. PHB production from waste methane sequesters a potent green house gas. At end-of-life, PHB-based materials biodegrade to methane in controlled anaerobic environments [12, 39], thus completing a cradle-to-cradle lifecycle.

Another factor that contributes to the cost of PHB production is the need to cultivate and maintain xenic cultures that are often genetically modified. This need may be eliminated if appropriate environmental pressures could be identified and applied to select and maintain enrichments capable of high levels of PHB production.

Methanotrophs are a subset of the methylotrophs, microorganisms that can grow on one-carbon compounds [37]. The proteobacteria methanotrophs are divided into two groups with distinctive carbon assimilation pathways: the type Iγ-proteobacteria that use the ribulose monophosphate (RuMP) pathway and the type IIα-proteobacteria that use the serine pathway. Type X methanotrophs, a subset of type I methanotrophs, are γ-proteobacteria that use the RuMP pathway for carbonassimilation, but also possess low levels of serine-pathway enzymes [26]. The recently discovered acidiphilic *Verrucomicrobia* methanotrophs of the genus *Methylacidiphilum* [22, 32, 44] possess

A. J. Pieja · K. H. Rostkowski · C. S. Criddle (✉)
Environmental Engineering and Science, Department of Civil and Environmental Engineering, Stanford University,
Stanford, CA 94305, USA
e-mail: ccriddle@stanford.edu

serine-pathway genes and apparently lack RuMP-pathway genes [43].

The literature contains conflicting evidence as to which methanotrophs produce PHB and which do not. Several studies have surveyed pure and mixed cultures of methanotrophs for PHB production [3, 46, 54, 58]. To date, quantitative production of PHB has only been documented in Type II methanotrophs of the genera *Methylocystis* and *Methylosinus* [3, 28, 29, 46, 50, 53, 54, 56, 58]. But there are also reports of PHB production by Type I methanotrophs [3, 7, 9, 30, 50, 54, 58]. These reports are often based on more qualitative evidence. Bowman et al. [9], for example, assayed PHB production using a presence/absence test and scored several type I genera (including *Methylococcus* and *Methylomonas* species) as positive for PHB production. One report [54] cites non-peer-reviewed literature; another [50] asserts PHB production by Type I methanotrophs, but provides no evidence. Still others [3, 31, 58] cite a study [33] in which *Pseudomonas methanica* reportedly produced PHB, but this "Type I methanotroph" [27] was later identified as *Vibrio extorquens* [48] and is now known as *Methylobacterium extorquens*, a facultative methylotroph that uses the serine cycle for carbon assimilation. Clearly, these reports of PHB production by type I methanotrophs are dubious.

Genetic data provide yet another reason to doubt reports of PHB production by type I methanotrophs. At least three genes, *phaCAB,* are considered crucial for PHB synthesis [38]. These genes encode condensation of two acetyl-CoA molecules to acetoacetyl-CoA (*phaA*), reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA (*phaB*), and polymerizationof(R)-3-hydroxybutyryl-CoA monomer units into PHB (*phaC*) [38]. These genes are fairly well conserved and have been used to screen for PHB production capacity [47]. No PHB synthesis genes have been detected in type I methanotrophs or in *Methylacidiphilum* strains [22, 32, 44].Moreover, the published genome of *Methylococcus capsulatus* str. Bath [52] does not reference any of the *phaCAB* genes, nor does it show significant similarities with any of the *phaCAB* genes sequenced from *Methylosinus trichosporium* OB3b.

The literature also suggests that PHB production may be linked to the serine cycle and that type I RuMP-pathway methanotrophs may not be capable of PHB production. Several studies have examined PHB production in obligate and facultative methanol-utilizing methylotrophs. Babel hypothesized that serine-pathway methylotrophs produce PHB as a carbon storage polymer where as RuMP-pathway methylotrophs instead produce exopolysaccharides [6] under unbalanced growth conditions. The PHB cycle may play a crucial role in the serine cycle of the methylotroph *M. extorquens* AM1 by facilitating the previously undetailed step of glyoxylate regeneration [35], thus linking the PHB cycle to the serine cycle. In a separate experiment, several RuMP-pathway, methanol-utilizing methylotrophs were assayed for PHB production, and none produced a measurable amount of PHB [23]. Additionally, none possessed key enzymes required for PHB production, and none had DNA that hybridized with PHA-biosynthesis gene probes designed for *M. extorquens*, a serine-pathway methylotroph, or *Alcaligenes eutrophus* [23]. Interestingly, tranconjugants of these RuMP-pathway methylotrophs containing PHA-biosynthesis genes were able to synthesize PHB, suggesting that metabolic limitations do not prevent RuMP-pathway methylotrophs from synthesizing PHB [23]. Thus, while there is indirect evidence suggesting that RuMP-pathway methanotrophs may not produce PHB, there is confusion in the literature due to reports of PHB production in type I methanotrophs. One aim of the present study was to screen the β-(type I, RuMP-pathway) and α-(type II, serine-pathway) proteobacteria methanotrophs to determine whether PHB production is restricted to one type.

Competition between type I and type II methanotrophs may control whether a methane-utilizing community is capable of PHB production. The level of copper in the growth media is one factor that may favor type II over type I methanotrophs. All known methanotrophs, with the exception of some strains of *Methylocella* [21], possess particulate methane monooxygenase (pMMO), a copper-requiring enzyme [41, 57]. Many type II methanotrophs (all known *Methylosinus* and some *Methylocystis* strains), and some type I methanotrophs (all *Methylococcus* and some *Methylomonas* and *Methylomicrobium* strains) possess an additional, soluble methane monooxygenase (sMMO) that does not require copper [37]. Eliminating copper from the growth media may thus favor type II methanotrophs [8]. In a chemostat, Graham et al. (1993) showed that low copper levels favored a type II methanotroph, *M. trichosporium* OB3b, a species that possessess methane monooxygenase (MMO), indirect competition with the type I methanotroph, *Methylomonas albus* BG8 (now known as *Methylomicrobium album* BG8) [24], a species that lacks sMMO.

The ability to fix nitrogen is present in all type II and some type I genera [5, 8, 26, 40, 42]. Some researchers have reported that nitrogen-fixing type I methanotrophs grow more slowly on nitrogen gas than do type II methanotrophs [40]. Nitrogen fixation may thus be useful in selecting type II over type I methanotrophs. Graham et al. found that the provision of nitrogen gas as the sole source of nitrogen favored the type II methanotroph, *M. trichosporium* OB3b, over *M. albus* BG8 [24], a species later found to lack the ability to fix nitrogen [5].

Another factor that may influence type I vs. type II selection is nutrient concentration: Wise et al. (1999) found

that lower nutrient concentrations favored type II over type I methanotrophs in dilution series prepared from land fill soils [55]. In a separate study, an enrichment dominated by type II methanotrophs was obtained from a wetted soil sample, but a mixed type I/type II enrichment resulted when the same sample was a mended with nutrients [14].

Medium pH may also affect type I or type II selection. Low pH and high in organic carbon concentration shave high concentrations of dissolved carbon dioxide. The serine pathway requires 1 mol of carbon dioxide for every 2 mol of methane, so higher levels of dissolved carbon dioxide could favor type II methanotrophs. The known acidophilic and acidotolerant methanotrophic proteobacteria are type II methanotrophs [17].

This study seeks to clarify which methanotrophs are capable of PHB production by screening for *phaC* and directly assaying PHB production under nitrogen-limited conditions. Copper concentration, nitrogen source, media concentration, and pH were evaluated for their ability to select methane-utilizing cultures that produce high levels of PHB from a diverse inoculum.

Materials and Methods

Culture Conditions

Unless otherwise specified, all cultures were grown in media WI (containing 0.8 mM $MgSO_4 \cdot 7H_2O$,10 mM $NaNO_3$, 0.14 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM $NaHCO_3$, 2.35 mM KH_2PO_4, 3.4 mM K_2HPO_4, 20.7 μM $Na_2MoO_4 \cdot 2H_2O$, 1 μM ol$CuSO_4 \cdot 5H_2O$, 10 μM FeEDTA), 1 mL trace metal solution (containing, per liter: 500 mg $FeSO_4 \cdot 7H_2O$, 400 mg $ZnSO_4 \cdot 7H_2O$,20 mg $MnCl_2 \cdot 7H_2O$,50 mg $CoCl_2 \cdot 6H_2O$,10 mg $NiCl_2 \cdot 6H_2O$,15 mg H_3BO_3, 250 mg EDTA), and 10 mL vitamin solution (containing,per L:2.0 mg biotin, 2.0 mg folic acid,5.0 mg thiamine·HCl, 5.0 mg calcium pantothenate, 0.1 mg vitamin B12, 5.0 mg riboflavin, and 5.0 mg nicotiamide) under a 1:1 methane:oxygen headspace and were incubated horizontally on orbital shake tables at 150 rpm. *Methylocapsa acidiphila* was grown in medium M2 at pH 4.5 as previously described [18]. Toin-duce PHB production, all cultures were incubated in medium W1 without nitrate (nitrate-free W1 medium).Glass ware was acid-washed with 10% HCl for ≥1 h and triple-rinsed in Milli-Q water before use to remove trace metal contamination. *M. trichosporium* OB3b, *Methylocystis parvus* OBBP, *Methylocystis* strain M, *Methylocystis rosea* SV97, *Methylocystis hirsuta* CSC1, *M. capsulatus* str. Bath, and *M. album* BG8 were obtained from J. Semrau (University of Michigan); *Methylomonas* LW13 and *Methylosinus* LW3 were obtained from M. Kalyuzhnaya (Lidstromlab, University of Washington); and *Methylocaldum* O11a, *Methylosinus sporium*, *Methylocystis* 42/22, *Methylocystis* SC2, and *M. acidiphila* were obtained from P. Dunfield (University of Calgary). *M. capsulatus* str. Bath and *Methylocaldum* O11a were incubated at 37°C. *M. acidiphila* was incubated at room temperature (20–25°C).

All other cultures were incubated at 30°C.

Screening Methanotroph Strains for PHB Production

Fifty-milliliter cultures were grown in triplicate in 125-mL serum bottles sealed with butyl-rubber stoppers and crimp seals under a1:1 methane:oxygen headspace. Cultures were grown to mid-exponential phase and transferred to nitrate-free W1 medium. Cultures were centrifuged at 4,816×*g* (4,700 rpm) for 8 min, washed once with nitrate-free W1 medium, re-centrifuged, and re-suspended in the same volume of nitrate-free W1 medium. After an additional 24 h of incubation, cultures were harvested, immediately frozen at −20°C, and freeze-dried for subsequent PHB analysis.

PHB Measurement

For each sample, approximately 3–6 mg of freeze-dried biomass was added to a 12-mL glass vial with a PTFE-lined plastic cap (Wheaton Science Products). A modified version of the protocol described by Braunegg et al. was used for the PHB assay [11]. The organic phase of the resulting mixture was analyzed using an Agilent 6890N gas chromatograph equipped with an HP-5 column (containing (5% phenyl)-methylpolysiloxane, Agilent Technologies) and FID detector. DL-β-hydroxy butyric acid sodium salt (Sigma) was used as a standard.

Selection Experiments

Activated sludge from the Palo Alto Regional Water Quality Control Plant (Palo Alto, CA, USA) was used as a diverse inoculum to test whether environmental variables could be used to selectively enrich methanotrophs that produce high levels of PHB.

Unless otherwise specified, cultures were incubate din W1 medium, under conditions typically recommended methanotroph enrichment [7, 8, 25]: neutral pH (pH=7), 10 mM nitrate, and 5 μM Cu, with a gas phase containing 50% methane and 50% oxygen. The basal medium was a modified Whitten bury medium. To test the effects of varying the concentration of mineral salt sand copper (added as $CuSO_4 \cdot 5 \cdot H_2O$), cultures were incubated in W1 medium and in a 10% dilution of W1 medium, each at final copper concentrations of 0 and 5 μM. To test the effects of nitrogen source, cultures were incubated with either nitrate

or nitrogen gas (1:1 methane:airhead space) as the sole nitrogen source, with no added copper. To test the effects of varying pH and inorganic carbon concentration, cultures were incubated at pH 4, 5, 6, 7, and 8. The pH of each enrichment medium was adjusted prior to inoculation by adding 1% and 10% hydrochloric acid and/or sodium hydroxide. Cultures at each pH were incubated at 1 and 10 mM carbonate. Elimination of copper from the media significantly slowed growth, so a trace level (0.01 μM) was used in samples testing the effects of pH and carbonate.

To prepare each enrichment microcosm, activated sludge was inoculated into 50 mL of media. A seed volume of 2.5 mL was used for evaluation of pH and nitrogen; 1 mL for evaluation of copper and mineral salts, and all conditions were tested in triplicate. All enrichments were incubated at 30 C on orbital shake tables at 150 rpm. Once turbidity was observed visually (OD_{670}≈0.2–0.4), enrichments were transferred (5% by volume) to fresh media under conditions identical to the initial inoculation. Turbidity appeared in the initial enrichments after approximately 1 week. Subsequent transfers required less time to become turbid and were accordingly transferred after approximately 1–2 days each. After a minimum of four transfers, methanotrophic genera were identified by analysis of terminal restriction fragment length polymorphisms (T-RFLP) of the *pmoA* gene, and PHB production was assayed under nitrogen-limited conditions.

Molecular Analyses

Biomass pellets from exponential phase cultures were stored at −20°C.

DNA was extracted from biomass samples using the Fast DNA®SPIN Kit for Soil (Qbiogene) according to manufacturer's protocols.

Primers were developed to amplify a section of the *phaC* gene. Previously described *phaC* primers, phaCF1 and phaCR4 [47], were designed to amplify *phaC* from a variety of genera, but they did not amplify *phaC* from methanotrophs known to harbor *phaC* (data not shown), so a new primer set was designed and tested. The *phaC* gene has been sequenced in *M. trichosporium* OB3b and *Methylocella silvestris* (carbon-one oxidation network). The BLAST function from the National Center for Biotechnology Information was used to identify closely related sequences, including representatives from *Bradyrhizobium*, *Rhodopseudomonas*, and *Methylobacterium*. These sequences were aligned with the previously described *phaC* primers,phaCF1 and phaCR4 [47]. The primers phaCF-M (forward, 5′-ATCAAYAARTTCT ACRTBCTCGAYCT-3′) and phaCR- M (reverse, 5′-ATGTAATTGTTGAYGAMRWAGGWCCA-3′) were selected and tested on *M. trichosporium* OB3b.

Reactions for *phaC* polymerase chain reaction (PCR) contained, per 30-μL reaction : ~5 ng DNA, 1× Premix D (Epicentre Biotechnologies), 0.75 μM each primer (phaCF-Mandpha CR-M),and 1 U Failsafe™ Enzyme Mix(Epicentre Biotechnologies). Amplifications were performed in a Veriti®96-Well Thermal Cycler (Applied Biosystems) with the following thermal cycling conditions: 94°C for 10 min, 51°C for 2 min, 72°C for 2 min; followed by 35 cycles of denaturation (94°C for 20 s), annealing (57°C for 45 s), and elongation (72°C for 1 min); and a final extension at 72°C for 10 min. PCR products were visualized on a 1.5% agarose gel prepared with TAE buffer and stained with ethidium bromide. DNA extracted from all methanotrophs trains was screened for *phaC*. The expected amplicon size is 481 bp, and observed bands of this length were scored positive for *phaC*. Positive PCR reactions were sequenced to verify the identity of the products.

The gene *pmoA* encodes one subunit of the pMMO and is present in all methanotrophs with the exception of some strains of *Methylocella*. *pmoA* was amplified from strains screened using the primersA189f and mb661r [16]. Each 30-μL reaction contained 1× Premix F (Epicentre Biotechnologies), each primer at a concentration of 0.33 μM, and 1 U Failsafe™ Enzyme Mix (Epicentre Biotechnologies). DNA amplification was performed on a Gene Amp® PCR System 9700 (Applied Biosystems) using the following thermal cycling conditions: 2 min at 94°C; 25 cycles of denaturation (1 min at 95°C), annealing (1.5 min at 55°C), and extension (1 min at 72°C); and a final extension of 5 min at 72°C. In the rare cases where multiple bands were observed, the reaction was re-run with an annealing temperature of 57°C to eliminate the second band. PCR products were visualized on a 1.5% agarose gel prepared with TAE buffer and stained with ethidium bromide.

After PCR products were visualized on a gel to verify single-band amplification, the remaining products were purified using the Min Elute PCR Purification Kit (Qiagen), quantified on a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific),and sequenced with their respective forward and reverse PCR primers (Elim Biopharmaceuticals, Inc., Hayward, CA).

Sequences were trimmed and edited using Sequencher™ 4.10.1 (Gene Codes Corporation).

T-RFLP analysis was used to characterize communities enriched in selection experiments. PCR on the *pmoA* gene was conducted as described previously except that the primers used were labeled with the fluorescent dyes6-carboxyfluorescine (A189f) and 5-hexachlorofluorescein (mb661r). PCR products were purified using a Montage®PCR Centrifugal Filter Devices (Millipore) and quantified on a Nano Drop ND-1000 spectrophotometer. Approximately 200 ng of purified PCR products were digested with 15 U AluI (New England Biolabs, Inc.) in 1× NE Buffer 4 for

180 min at 37°C. Digestion products were purified using Montage® PCR Centrifugal Filter Devices (Millipore) and analyzed at MCLAB (South San Francisco, CA, USA). Chromatograms were analyzed using Gene Marker® (Soft Genetics LLC). An in silico analysis of known *pmoA* genes was used to identify fragments, and peak identities were confirmed by sequencing *pmoA* from the strains used in this study.

Clone libraries were prepared for *pmoA* for one sample from each enrichment experiment to verify the identity of T-RFs. *pmoA* was amplified in triplicate PCR reactions using unlabeled primers as described previously, except that a final extension step of15 minat 72°Cwasused. Triplicate PCR products for each sample were pooled and purified using the Min Elute PCR Purification Kit (Qiagen). Cloning was performed using the TOPO TA Cloning Kit for sequencing with the pCR® 4 TOPO® vector (Invitrogen) according to manufacturer's protocols. Clones were sequenced (Elim Biopharmaceuticals, Inc., Hayward, CA) and sequences were trimmed and edited using Sequencher™ 4.10.1 (Gene Codes Corporation).

Results

Screening Methanotrophs Trains for PHB Production

Four type I methanotrophs strains and eight type II methanotroph strains were screened for *phaC* and PHB production under nitrogen limitation. The results are summarized in Table 1. A 481-bp fragment was amplified from all of the type II methanotrophs screened, and no amplification was observed from any of the type I methanotrophs screened. *phaC* sequences were deposited in GenBank under the accession numbers HQ860422–HQ860430. Type II methanotroph cultures produced 7–45% PHB (mg PHB/mg total suspended solids), but none of the type I methanotrophs produced quantifiable PHB. The highest average PHB contents observed were 38%±4% in *M. trichosporium* OB3 band 36%±8% in *M. parvus* OBBP. One strain, *M. acidiphila*, did not grow in a dense enough culture to enable a direct measurement of PHB.

Selection Experiments

Table 2 summarizes results of the selection experiments. The presence of many different genera in the various enrichments indicates that the inoculum contained diverse type I and II genera. Communities enriched under control conditions (10 mM nitrate, pH 7.5 μM Cu) produced ≤1% PHB and methanotrophs present were predominantly from the genus *Methylomicrobium*.

Removing copper from the modified Whitten bury medium did not significantly change the composition of the communities enriched. Cultures enriched without copper did not produce a measurable amount of PHB and methanotrophs present were predominantly from the genus *Methylomicrobium*, although the genera *Methylosinus*/*Methylocystis* were present in all replicates.

The communities enriched in dilute medium differed from communities enriched in modified Whitten bury medium. In dilute medium with 5 μM Cu, one culture produced a significant amount of PHB (25%) and was

Table 1 Screening results for methanotrophia proteobacteria: presence of *pha*C and PHB synthesized under nitrogen-limited conditions

Strain	Type	Carbon assimilation pathway	%PHB±std. dev.[a]	Presence/absence (+/−) of *pha*C
Methylocaldum O11a	I	RuMP	0±0	−
Methylomicrobium album BG8	I	RuMP	0 (range=0)	−
Methylomonas LW13	I	RuMP	0±0	−
Methylococcus capsulatus str. Bath	X(I)	RuMP	0 (range=0)	−
Methylocapsa acidiphila	II	Serine	n/a[b]	+
Methylocystis 42/22	II	Serine	25±7	+
Methylocystis hirsuta CSC1	II	Serine	7±2	+
Methylocystis parvus OBBP	II	Serine	36±8	+
Methylocystis rosea SV99	II	Serine	9±1	+
Methylocystis SC2	II	Serine	30±13	+
Methylocystis strain M	II	Serine	14[a] (range: 7–21)	+
Methylosinus sp. LW4	II	Serine	10±2	+
Methylosinus sporium	II	Serine	9	+
Methylosinus trichosporium OB3b	II	Serine	38±4	+

[a] Range is reported for duplicates; standard deviation for triplicates

[b] Not available; culture did not grow to sufficient density

Table 2 Methanotrophic enrichment cultures: PHB synthesized under nitrogen-limited conditions and genera detected

Variable	Modification of growth medium	%PHB	Type I genera detected*	Type II genera detected*
Concentrations of trace Cu and mineral salts	[Cu]=5 μM, mineral salts=100%	1, 0		*Methylomicrobium*(+,−)
	[Cu]=5 μM, mineral salts=10%	25, 0	*Methylomonas/Methylobacter* (+,−), *Methylomicrobium* (+)	*Methylocystis/Methylosinus*(+)
	[Cu]=0 μM, mineral salts=100%	0, 0, 0	*Methylomicrobium, Methylocaldum/ Methylococcus, Methylobacter*	*Methylocystis/Methylosinus*
	[Cu]=0 μM, mineral salts=10%	7, 15, 17		*Methylocystis/Methylosinus*
Nitrogen source	Nitrate	2, 2, 3	*Methylobacter, Methylomicrobium*	
	N_2	43, 43, 46	*Methylocaldum/Methylococcus*	*Methylosinus trichosporium, Methylocystis/Methylosinus, Methylosinus*
pH and added carbonate	pH 4, added carbonate=1 mM	4, 11, 12		*Methylocystis/Methylosinus*
	pH 5, added carbonate=1 mM	14, 0, 0	*Methylobacter*(+), *Methylomonas*(+,−)	*Methylocystis/Methylosinus*(+)
	pH 6, added carbonate=1 mM	0, 0, 0	*Methylomonas*	
	pH 7, added carbonate=1 mM	0, 0, 0	*Methylomonas, Methylobacter*	
	pH 8, added carbonate=10 mM	0, 0, 0	*Methylomonas, Methylobacter*	
	pH 4, added carbonate=10 mM	0, 0, 0	*Methylomonas*	
	pH 5, added carbonate=10 mM	4, 6		*Methylocystis/Methylosinus*
	pH 6, added carbonate=10 mM	0, 0, 0	*Methylomonas*	
	pH 7, added carbonate=10 mM	0, 0, 0	*Methylomonas*	*Methylocystis/Methylosinus*
	pH 8, added carbonate=10 mM	0, 0, 0	*Methylomonas*	

Unless otherwise indicated, medium was prepared at pH 7 with 10 mM nitrate and 5 μM Cu. Phylogenetic analyses were based on T-RFLP analysis of *pmo*A. A slash sign'/'indicates that the indicated genera were not distinguished by T-RFLP. When both PHB-positive and PHB-negative enrichments were present, the symbol in parentheses indicates whether the indicated genus was present in PHB-positive (+) or negative (−) enrichments

dominated by *Methylocystis*/*Methylosinus*. The other two replicate enrichments also contained representatives of *Methylocystis*/*Methylosinus* in addition to representatives of type I genera. One replicate could not be tested due to an error in the measurement process, and PHB was not detected in the other.

After removal of copper from the dilute medium, PHB was detected in all samples, and replicates produced an average of 13% PHB. Additional testing is needed to determine whether copper influenced the level of PHB production. In all enrichments in dilute media lacking copper, only the genera *Methylocystis*/*Methylosinus* were detected, indicating that these conditions selected type II methanotrophs.

When nitrogen gas was provided as the sole nitrogen source in the absence of copper, enrichments were dominated by type II methanotrophs capable of PHB production. Under nitrogen starvation conditions, the cultures produced an average of 44% PHB, and the majority of methanotrophs present were from *M. trichosporium* and other members of *Methylosinus*/*Methylocystis*. This was the highest percentage of PHB achieved under any selection conditions. Communities enriched with nitrate in the absence of copper produced an average of just 2% PHB, and the methanotrophs present were mostly from the genus *Methylomicrobium*.

When pH of the modified Whitten bury medium was varied in media with 0.01 μM Cu, only very low pH was effective at selecting for type II methanotrophs capable of PHB production. Trace copper was added (64 ppt) because growth was very slow in its absence. Medium pH was varied at both high (10 mM) and low (1 mM) concentrations of added carbonate. In medium with 10 mM added carbonate, PHB production was only observed at pH 4,

where two out of three enrichments produced an average of 5% PHB. In medium with 1 mM added carbonate, three out of three enrichments at pH 4 produced an average of 8% PHB, and one enrichment at pH 5 produced 14% PHB. In all pH 4 enrichments, only type II methanotrophs from the genera *Methylocystis* and *Methylosinus* were detected.

Concentrations of dissolved carbon dioxide, bicarbonate, and carbonate were computed at each pH level tested. DIC (the sum of carbonic acid, bicarbonate, and carbonate) did not affect the type of methanotroph enriched, but did affect PHB production, with enrichments at lower DIC levels yielding higher PHB levels at the same pH.

Discussion

Combining our results with the previously published results of others, we conclude that representatives from all four genera of type II methanotrophs-*Methylocystis*, *Methylosinus*, *Methylocapsa*, and *Methylocella*- possess *phaC* and can produce PHB under nitrogen-limited conditions. Six *Methylocystis* strains and two *Methylosinus* strains produced PHB. *Methylocapsa* did not grow to a sufficiently dense culture, preventing quantification of PHB, but PHB production was observed visually in stained cells of *M. acidiphila* [19] and *M. silvestris* [21]. We obtained *phaC* sequences from all three of the type II genera tested. Others have sequenced *phaC* from the representative of the fourth genus, *M. silvestris* [21]. By contrast, no type I methanotroph genera produced measurable PHB, and *phaC* has not been detected in any type I strain. Our data support the conclusion that serine-pathway type II methanotrophs produce PHB while RuMP-pathway type I methanotrophs do not.

Production of PHB proceeds via acetyl-CoA. Its early synthesis via the serine pathway may partially explain why PHB production is limited to type II methanotrophs and is not found in type I methanotrophs where the early metabolites of the RuMP-pathway are sugars [6]. There is also no indication that type I methylotrophs express a complete tricarboxylic acid cycle, required for extraction of energy from acetyl-CoA. Additionally, the serine cycle requires greater inputs of reducing power than the RuMP cycle [26], and PHB is a supply of reducing power for the type II methanotroph *M. parvus* OBBP (Pieja, unpublished data). Serine-cycle methanotrophs may use the reducing power supplied by PHB to facilitate carbon assimilation.

Production of PHB by serine-pathway, type II methanotrophs suggests that strategies to enhance PHB production should focus on selection of type II methanotrophs. The results indicate that it is possible to favor type I or type II methanotrophs by manipulation of enrichment conditions. As often noted, traditional media are not optimal for enrichment of communities that simulate those found in the natural environment [13, 51]. Moreover, in this study, enrichment communities derived from the same inoculum were variable and dependent upon culture conditions.

In some cases, PHB production was observed in communities in which only type I methanotrophs were detected. This may be because type II methanotrophs were undetected or because non- methanotrophs contributed low levels of PHB production in these enrichments.

Eliminating copper from the media was expected to select for type II methanotrophs because many possesss MMO inaddition to the copper-requiring pMMO that is present exclusively in some type I genera [8]. The data did not support this hypothesis: representatives from type II genera were present in all enrichments in the absence of Cu, but were not necessarily dominant. Several type I genera, including *Methylococcus*, *Methylomonas*, and *Methylomicrobium*, possesss MMO and could therefore be enriched in the absence of copper [37]. *Methylomicrobium* was the genus most commonly detected in enrichments from the modified Whitten bury medium without copper, and several strains in this genus possesss MMO. The absence of copper is thus not a sufficient criterion for selection of type II methanotrophs. A more effective enrichment for type II methanotrophs was achieved when copper removal was combined with other selection criteria, such as use of dilute media or use of nitrogen gas as the sole nitrogen source.

Use of a dilute medium was also not sufficient to select exclusively for type II methanotrophs, but, as noted above, the combination of dilute medium and the absence of Cu exclusively enriched type II methanotrophs. The results suggest that type II methanotrophs may be better adapted to nutrient acquisition at oligotrophic concentrations or they may be inhibited at higher salt concentrations [55].

Low pH and high carbonate concentrations were expected to select preferentially for type II methanotrophs because these conditions result in higher concentrations of aqueous carbon dioxide, an input to the serine cycle. Low pH conditions did favor type II methanotrophs, but the computed concentration of dissolved carbon dioxide did not affect community composition, suggesting that pH changes exerted other selective effects that were more important than carbonate speciation. It is possible that the concentration of carbonate used (1 mM) provided sufficient carbon dioxide for the serine cycle and that lower concentrations might select against type II methanotrophs, but this was not tested. In any case, imposition of a low pH did select for type II methanotrophs capable of PHB production from the genus *Methylocystis*. Among the proteobacteria, the only known acidophilic methanotrophs are type II; all members of the genera *Methylocapsa* are obligately acidophilic, and some members of the genera *Methylocystis* have been detected in samples at a pH range of 3.6–4.5 [17].

Providing nitrogen gas as the only nitrogen source was expected to favor type II methanotrophs over type I methanotrophs. All type II methanotrophs and members of the type X genus *Methylococcus* are able to fix atmospheric nitrogen, and although some type I genera are also able to fix nitrogen, nitrogen-fixing type I methanotrophs grow more slowly on nitrogen gas than type II methanotrophs [1, 8, 26, 37, 40, 42].The combination of using nitrogen gas as the sole nitrogen source and eliminating copper from the medium effectively selected type II methanotrophs that produced substantially more PHB than a community enriched with nitrate as the nitrogen source Removal of nitrate from the media also significantly decreased the salinity of the media (from 21 to 11 mM). Further testing is needed to determine whether nitrogen fixation or lower salinity is the key factor favoring selection of type II methanotrophs.

In some instances, copper concentration affected the levels of PHB produced by enrichment cultures. *M. trichosporium* IMV3011, which produced more PHB at 8 μM Cu than at 0 or 16 μM Cu [56]. Enrichment cultures in dilute media produced more PHB when copper was present at 5 μM than when it was absent. In *M. trichosporium* OB3b, however, combined copper and nitrogen limitation may cause additional accumulation of PHB [46]. The concentration of copper may thus play a crucial role both in selecting bacteria that produce PHB and in maximizing PHB accumulation, underscoring the need to assess its optimal concentration. When nitrogen gas was provided as the nitrogen source, relatively high levels of PHB were produced in the absence of copper.

Several of the environmental pressures tested in this study were successful in selecting type II methanotrophs from a diverse inoculum, and some may be appropriate for the long-term maintenance of a methane-utilizing culture capable of high levels of PHB production. Low pH selected type II methanotrophs, but both growth rates and PHB production levels were relatively low. Removal of copper from the medium was not in itself a sufficient selection criterion but was effective when used in conjunction with other selection pressures. Use of nitrogen gas as the sole nitrogen source with dilute media also selected for PHB production and type II methanotrophs.

We conclude that PHB production is likely limited to type II, serine-pathway methanotrophs, despite earlier reports of PHB production by type I methanotrophs. Additional testing is required to determine whether the *Verrucomicrobia* methanotrophs, which also possess serine-pathway enzymes, can produce PHB. It also appears that environmental selection criteria can be used to maintain enrichments capable of high levels of PHB production. Environmental conditions that selected preferentially for type II methanotrophs that produce PHB included use of dilute media in the absence of copper, use of nitrogen gas as a nitrogen source in the absence of copper, and low pH. Future work should focus on the ability of these factors and combinations thereof to maintain methane-utilizing enrichments capable of sustained PHB production.

Acknowledgments We thank Dr. Lisa Stein and the Carbon-one Oxidation Network for the *phaC* gene sequence for *M. trichosporium* OB3b. We also thank Holly Sewell for her help in the laboratory.

This work was supported by a Graduate Research Fellowship from the National Science Foundation to AJP and by the California Environmental Protection Agency, Department of Toxic Substances Control under contract 07T3451.

References

1. Amaral JA, Archambault C, Richards SR, Knowles R (1995) Denitrificationassociated with groups I and II methanotrophs in a gradient enrichment system. FEMS Microbiol Ecol 18:289–298
2. Anderson AJ, Dawes EA (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev 54:450–472
3. Asenjo JA, Suk J (1986) Microbial conversion of methaneintopoly-beta-hydroxybutrate (PHB)-growth and intracellular product accumulation in a type-II methanotroph. J Ferment Technol 64:271–278
4. Ashby RD, Solaiman DKY, Foglia TA (2004) Bacterialpoly (hydroxyalkanoate) polymer production from the bio diesel co-product stream. J Polym Environ 12:105–112
5. Auman AJ, Speake CC, Lidstrom ME (2001) *nifH* sequences and nitrogen fixation in type I and type II methanotrophs. Appl Environ Microbiol 67:4009–4016
6. Babel W (1992) Pecularities of methylotrophs concerning over flow metabolism, especially the synthesis of polyhydroxyalkanoates. FEMS Microbiol Rev 103:141–148
7. Bowman JP (2001) Family I. *Methylococcaceae* and Family V. *Methylocystaceae*. In: Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore, pp 256–270, pp. 411–420
8. Bowman JP (2006) The methanotrophs-the families *Methylococcaceae* and *Methylocystaceae.* In: The prokaryotes a: handbook on the biology of bacteria. Springer, NewYork, pp 266–289
9. Bowman JP, Sly LI, Nichols PD, Hayward A (1993) Revised Taxonomy of the Methanotrophs: Description of *Methylobacter* gen.nov., emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. Int J Syst Bacteriol 43:735–753
10. Braunegg G, Lefebvre G, Genser KF (1999) Polyhydroxyalkanoates, biopolyesters from renewable resources: physiological and engineering aspects. J Biotechnol 65:127–161
11. Braunegg G, Sonnleitner B, Lafferty R (1978) Arapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Eur J Appl Microbiol Biotechnol 6:29–37
12. Budwill K, Fedorak PM, Page WJ (1992) Methanogenic degradation of poly (3- hydroxyalkanoates). Appl Environ Microbiol 58:1398–1401
13. Bussman I, Pester M, Brune A, Schink B (2004) Preferential cultivation of type II methanotrophic bacteria from littoral sediments (Lake Constance). FEMS Microbiol Ecol 47:179–189
14. Cebron A, Bodrossy L, Stralis-Pavese N, Singer AC, Thompson IP, Prosser JI, Murrell JC (2007) Nutrient amendments in soil

DNA stable isotope probing experiments reduce the observed methanotroph diversity. Appl Environ Microbiol 73:798–807
15. Choi J, Lee SY (1997) Process analysis and economic evaluation for Poly (3- hydroxybutyrate) production by fermentation. Bioprocess Eng 17:335–342
16. Costello AM, Lidstrom ME (1999) Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. Appl Environ Microbiol 65:5066–5074
17. Dedysh SN (2002) Methanotrophic bacteria of acids phagnum bogs. Mikrobiologiia 71:741–754
18. Dedysh SN, Khmelenina VN, Suzina NE, Trotsenko YA, Semrau JD, Liesack W, Tiedje JM (2002) *Methylocapsa acidiphila* gen. nov., sp. nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from sphagnum bog. Int J Syst Evol Microbiol 52:251–261
19. Dedysh SN, Liesack W, Khmelenina VN, Suzina NE, Trotsenko YA, Semrau JD, Bares AM, Panikov NS, Tiedje JM (2000) Methylocellapalustrisgen.nov., sp.nov., a new methane-oxidizing acid ophilic bacterium from peat bogs, representing a novel subtype of serine-pathway methanotrophs. Int J Syst Evol Microbiol 50:955–969
20. Du G, Chen LXL, Yu J (2004) High-efficiency production of bioplastics from biodegradable organic solids. J Polym Environ 12:89–94
21. Dunfield PF, Khmelenina VN, Suzina NE, Trotsenko YA, Dedysh SN (2003) *Methylocella silvestris*sp.nov.,a novel methanotroph isolated from an acidic forest cambisol. Int J Syst Evol Microbiol 53:1231–1239
22. Dunfield PF, Yuryev A, Senin P, Smirnova AV, Stott MB, Hou S, Ly B, Saw JH, Zhou Z, Ren Y, Wang J, Mountain BW, Crowe MA, Weatherby TM, Bodelier PL, Liesack W, Feng L, Wang L, Alam M (2007) Methane oxidation by an extremely acid ophilic bacterium of the phylum *Verrucomicrobia*. Nature 450:879–882
23. Follner CG, Babel W, Valentin HE, Steinbuchel A (1993) Expression of polyhydroxy alkanoic-acid-biosynthesis genes in methylotrophic bacteria relying on the ribulose monophosphate pathway. Appl Microbiol Biotechnol 40:284–291
24. Graham DW, Chaudhary JA, Hanson RS, Arnold RG (1993) Factors affecting competition between type I and type II methanotrophs in two-organism, continuous-flowreactors. Microb Ecol 25:1–17
25. Hanson RS (1998) Ecology of methylotrophic bacteria. In: Techniques in microbial ecology. Oxford University Press, London, pp 137–162
26. Hanson RS, Hanson TE (1996) Methanotrophic bacteria. Microbiol Rev 60:439–471
27. Harrington AA, Kallio RE (1960) Oxidation of methanol and formal dehyde by *Pseudomonas methanica*. Can J Microbiol 6:1–7
28. Helm J, Wendlandt KD, Jechorek M, Stottmeister U (2008) Potassium deficiency results in accumulation of ultra-high molecular weight poly-beta-hydroxybutyrate in a methane-utilizing mixed culture. J Appl Microbiol 105:1054–1061
29. Helm J, Wendlandt KD, Rogge G, Kappelmeyer U (2006) Characterizing a stable methane-utilizing mixed culture used in the synthesis of a high-quality biopolymer in an open system. J Appl Microbiol 101:387–395
30. Heyer J, Berger U, Hardt M, Dunfield PF (2005) Methylohalobiuscrimeensisgen.nov., sp.nov., a moderately halophilic, methanotrophic bacterium isolated from hyper-saline lakes of Crimea. Int J Syst Evol Microbiol 55:1817–1826
31. Higgins IJ, Best DJ, Hammond RC, Scott D (1981) Methane-oxidizing microorganisms. Microbiol Rev 45:556–590
32. Islam T, Jensen S, Reigstad LJ, Larsen O, Birkeland NK (2008) Methane oxidation at 55 degrees C and pH2 by a thermo acid ophilic bacterium belonging to the *Verrucomicrobia* phylum. Proc Natl Acad Sci USA 105:300–304
33. Kallio RE, Harrington AA (1960) Sudanophilic granules and lipid of *Pseudomonas methanica*. J Bacteriol 80:321–324
34. Koller M, Bona R, Braunegg G, Hermann C, Horvat P, Kroutil M, Martinz J, Neto J, Pereira L, Varila P (2005) Production of polyhydroxyalkanoates from agricultural waste and surplus materials. Biomacro Molecules 6:561–565
35. Korotkova N, Lidstrom ME (2001) Connection between poly-beta-hydroxybutyrate biosynthesis and growth on C (1) and C (2) compounds in the methylotroph *Methylobacterium extorquens* AM1. J Bacteriol 183:1038–1046
36. Lee SY, Park SJ, Park JP, Lee Y, Lee SH (2005) Economic aspects of biopolymer production, vol 2. WILEY-VCH, Weinheim
37. Lidstrom ME (2006) Aerobic methylotrophic prokaryotes. In: Prokaryotes. pp. 618–634
38. Madison LL, Huisman GW (1999) Metabolic engineering of poly (3-hydroxyalkanoates): from DNA to plastic. Microbiol Mol Biol Rev 63:21–53
39. Morse M, Liao Q, Criddle CS, Frank CW (2011) An aerobic biodegradation of the microbial copolymer poly (3-hydroxybutyrate-co-3-hydroxyhexanoate): Effects of comonomer content, processing history, and semi-crystalline morphology. Polymer 52:547–555
40. Murrell J, Dalton H (1983) Nitrogen-fixation in obligate methanotrophs. J Gen Microbiol 129:3481–3486
41. Nguyen HH, Elliott SJ, Yip JH, Chan SI (1998) The particulate methane monooxygenase from *M. capsulatus* (Bath) is a novel copper-containing three-subunit enzyme. J Biol Chem 273:7957–7966
42. Oakley C, Murrell J (1988) *Nifh* genes in the obligate methane oxidizing bacteria. FEMS Microbiol Lett 49:53–57
43. HJ Op denCamp, Islam T, Stott MB, Harhangi HR, Hynes A, Schouten S, Jetten MS, Birkeland NK, Pol A, Dunfield PF (2009) Environmental, genomic and taxonomic perspectives on methanotrophic *Verrucomicrobia*. Environ Microbiol Rep 1:293–306
44. Pol A, Heijmans K, Harhangi HR, Tedesco D, Jetten MS, OpdenCamp HJ (2007) Methanotrophy below pH1 by a new *Verrucomicrobia* species. Nature 450:874–878
45. Povolo S, Casella S (2003) Bacterial production of PHA from lactose and cheese whey permeate. Macromol Symp 197:1–9
46. Shah NN, Hanna ML, Jackson KJ, Taylor RT (1996) Batch cultivation of *Methylosinus trichosporium* OB3B: IV production of hydrogen-driven soluble or particulate methane monooxygenase activity. Biotechnol Bioeng 45:229–238
47. Sheu DS, Wang YT, Lee CY (2000) Rapid detection of polyhydroxyalkanoate-accumulating bacteria isolated from the environment by colony PCR. Microbiology 146:2019–2025
48. Stocks PK, Mc Cleskey CS (1964) Identity of the pink-pigmented methanol- oxidizing bacteria as *Vibrio extorquens*. J Bacteriol 88:1065–1070
49. U.S.EnvironmentalProtectionAgency. Methane: sources and emissions. http://www.epa.gov/outreach/sources.html. Accessed April21, 2011
50. Vecherskaya M, Dijkema C, Stams AJ (2001) Intracellular PHB conversion in a type II methanotroph studied by13CNMR. J Ind Microbiol Biotechnol 26:15–21
51. Vorob'ev AV, Dedysh SN (2008) Use of enrichment cultures for assessing the structure of methanotrophic communities in peat soil: the problem of representativity of the results. Mikrobiologiia 77:566–569
52. Ward N, Larsen O, Sakwa J, Bruseth L, Khouri H, Durkin AS, Dimitrov G, Jiang L, Scanlan D, Kang KH, Lewis M, Nelson KE, Methe B, Wu M, Heidelberg JF, Paulsen IT, Fouts D, Ravel J, Tettelin H, Ren Q, Read T, DeBoy RT, Seshadri R, Salzberg SL, Jensen HB, Birkeland NK, Nelson WC, Dodson RJ, Grindhaug

SH, Holt I, Eidhammer I, Jonasen I, Vanaken S, Utterback T, Feldblyum TV, Fraser CM, Lillehaug JR, Eisen JA (2004) Genomic insights into methanotrophy: the complete genome sequence of *M. capsulatus* (Bath). PLoS Biol 2:e303

53. Wendlandt KD, Geyer W, Mirschel G, Al-HajHemidi F (2005) Possibilities for controlling a PHB accumulation process using various analytical methods. J Biotechnol 117:119–129
54. Wendlandt KD, Jechorek M, Helm J, Stottmeister U (2001) Producing poly-3-hydroxybutyrate with a high molecular mass from methane. J Biotechnol 86:127–133
55. Wise MG, McArthur JV, Shimkets LJ (1999) Methanotroph diversity in land fill soil: isolation of novel type I and type II methanotrophs whose presence was suggested by culture-independent 16S ribosomal DNA analysis. Appl Environ Microbiol 65:4887–4897
56. Xin JY, Zhang YX, Zhang S, Xia CG, Li SB (2007) Methanol production from $CO_2$by resting cells of the methanotrophic bacterium *Methylosinus trichosporium* IMV3011. J Basic Microbiol 47:426–435
57. Zahn JA, DiSpirito AA (1996) Membrane-associated methane monooxygenase from *M. capsulatus* (Bath). J Bacteriol 178:1018–1029
58. Zhang Y, Xin J, Chen L, Song H, Xia C (2008) Biosynthesis of poly-3-hydroxybutyrate with a high molecular weight by methanotroph from methane and methanol. J Nat Gas Chem 17:103–109