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Long-term cultivation of a stable *Methylocystis*-dominated methanotrophic enrichment enabling tailored production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate)

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HIGHLIGHTS

- A *Methylocystis*-dominated CH_4-fed enrichment enabled stable PHBV synthesis.
- Harvested cells incubated with both methane and valerate produced PHBV.
- Mol% 3HV in PHBV was controllable.
- CH_4 oxidation drives valerate uptake.
- The fraction of CH_4 oxidized increases with increased mol% 3HV.

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ABSTRACT

Methane (CH_4) is a readily available feedstock for production of polyhydroxyalkanoates (PHAs). The structure and PHA production capacity of a *Methylocystis*-dominated methanotrophic enrichment was stable in long-term operation (>175 days) when grown exponentially under non-aseptic conditions in fill-and-draw batch cultures with ammonium as nitrogen source. Cells harvested in the draw step were incubated in the absence of nitrogen with various combinations of CH_4 and valerate to assess capacity for synthesis of poly(3-hydroxybutyrate) (P3HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). When fed CH_4 alone, only P3HB was produced. When fed CH_4 plus valerate, PHBV was synthesized. The mol% of 3-hydroxyvalerate (3HV) increased with added valerate. Oxidation of CH_4 was required for valerate assimilation, and the fraction of CH_4 oxidized increased with increased mol% 3HV. By separating PHA accumulation from cell replication, tailored PHA-rich biomass can be generated by addition of co-substrate, while retaining a large inoculum for the next cycle of cell division.

1. Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable, biocompatible, and renewable bioplastics that could substitute for fossil carbon plastics in many applications. They are produced by a wide variety of microorganisms under nutrient-limiting conditions. They can also be chemically recycled, by thermal depolymerization to alkenoates and biotic repolymerization (Myung et al., 2014; Sato et al., 2012). One obstacle to their more widespread use is the high cost of harvested feedstock, such as sugars and vegetable oils (Castilho et al., 2009). Harvested feedstock also presents life cycle obstacles because significant land, water, and energy are required for cultivation, processing, and purification.

Use of methane (CH_4) as a feedstock for PHA production can significantly decrease costs and environmental impacts (Rostkowski et al., 2012). CH_4 is currently widely available as the major component of natural gas and biogas obtained from the anaerobic degradation of organic waste. Production of PHAs from CH_4 appears limited to Type II methanotrophs (Pieja et al., 2011a). Researchers

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in both Germany and the US have developed CH_4-based PHA production processes (Pfluger et al., 2011; Pieja et al., 2012; Wendlandt et al., 2001). When CH_4 is the sole feedstock, poly(3-hydroxybutyrate) (P3HB) of high molecular weight is the sole PHA synthesized. Production of P3HB can be achieved in two steps: in step 1, cells replicate under conditions of nutrient-sufficient growth (i.e., sufficient CH_4, oxygen (O_2), major nutrients, and minor nutrients for cell division); in step 2, cells accumulate P3HB inclusion granules under conditions of nutrient-limited growth (i.e., sufficient CH_4 and O_2, but lacking one or more major or minor nutrients needed for cell division).

The consistent methanotrophic production of P3HB ensures product quality over time, but limits flexibility in responding to market demands. Applications for P3HB are limited by its narrow melt processing windows, low thermal stability, slow crystallization, and lack of flexibility (Choi et al., 1999). The ability to incorporate 3-hydroxyvalerate (3HV) or other monomers into the PHA polymer would increase the value of CH_4 as a PHA feedstock. Increasing 3HV content decreases melting temperature (T_m), glass transition temperature (T_g) (Amass et al., 1998), crystallinity and water permeability (Shogren, 1997), and increases impact strength and flexibility (Kotnis et al., 1995).

Many heterotrophic bacteria have non-specific enzyme activity enabling production of the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) when fatty acids containing an odd number of carbon atoms are present during the PHA production step (Byrom, 1992; Doi et al., 1987; Myung et al., 2014; Yu et al., 2005). The PHA granules are stored and used as a carbon source when the nutrients needed for cell division become available. Obligate methanotrophs are limited to C1 metabolism (Chistoserdova and Lidstrom, 2013), and consumption of stored PHA granules occurs when both CH_4 and the nutrients needed for cell division are available (Pieja et al., 2011b). Obligate methanotrophy raises questions about the feasibility of producing polymers other than P3HB. Some have reported that a few species in the *Methylocystis* genera can utilize multi-carbon substrates for growth (without CH_4) but not all *Methylocystis* strains have this capacity (Belova et al., 2011; Im et al., 2011). Also, CH_4-dependent copolymer production has never been reported. This study first demonstrates that a stable *Methylocystis*-dominated enrichment produces only P3HB when fed CH_4 alone, but produces PHBV when fed CH_4 plus valerate in the PHA production step.

2. Methods

2.1. Culture conditions

Unless otherwise specified, all cultures were grown in medium JM2, which is a modified version of ammonium mineral salts (AMS) medium (Whittenbury et al., 1970). Medium JM2 contained the following chemicals per L of solution: 2.4 mM $MgSO_4{\cdot}7H_2O$, 0.26 mM $CaCl_2$, 36 mM $NaHCO_3$, 4.8 mM KH_2PO_4, 6.8 mM K_2HPO_4, 10.5 μM $Na_2MoO_4 \cdot 2H_2O$, 7 μM $CuSO_4{\cdot}5H_2O$, 200 μM Fe-EDTA, 530 μM Ca-EDTA, 5 mL trace metal solution, and 20 mL vitamin solution. The trace stock solution contained the following chemicals per L of solution: 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 and 250 mg EDTA. The vitamin stock solution contained the following chemicals per L of solution: 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 nicotinamide.

All cultures were incubated in 160-mL serum bottles (Wheaton, Mealville, NJ, USA) capped with butyl-rubber stoppers and crimp-sealed under a CH_4:O_2 headspace (molar ratio 1:1.5; >99% purity; Praxair Technology, Inc., Danbury, CT, USA). Liquid volume was 50 mL, and the headspace volume was 110 mL. Cultures were incubated horizontally on orbital shaker tables at 150 rpm. The incubation temperature was 30 °C.

2.2. Methane-fed microbial enrichment cultures

Fresh activated sludge was obtained from the aeration basin at the Palo Alto Regional Water Quality Control Plant (Palo Alto, CA, USA). Large material was removed by filtering through a 100-μm cell strainer (BD Falcon Biosciences, Lexington, TN, USA). The dispersed cells were centrifuged (3000*g*) for 15 min to create a pellet. The pellet was resuspended in medium JM2 and shaken to obtain a dispersed cell suspension. Aliquots (15 mL) of the suspension were added to two serum vials containing 35 mL of medium JM2. Every 24 h for two weeks, the headspace of each bottle was flushed with a CH_4:O_2 mixture (molar ratio of 1:1.5) and amended with 0.5 mL of ammonium stock solution (1.35 M ammonium chloride; >99.8% purity; Mallinckrodt Inc., St Louis, MO, USA). When the culture reached a final optical density (OD_{600}) of 1.2, it was centrifuged (3000*g*) for 15 min, and the pellet resuspended in 15 mL of medium JM2. The suspension was divided into 5-mL aliquots for inoculation of three fed-batch serum bottle cultures. Each fed-batch culture initially contained 5 mL of inoculum, 44.5 mL of medium JM2, and 0.5 mL of ammonium stock (total volume 50 mL). After a 24-h incubation period, each of the three enrichments was subjected to a long-term cyclic feeding and wasting regime, with alternating pulses of CH_4 and ammonium. A repeating 48-h fed-batch cycle was established enabling nearly continuous exponential growth (Fig. 1). In *Step 1*, all cultures with 10 mL of carry-over culture from the previous cycle received 40 mL of fresh medium (39.5 mL of medium JM2 plus 0.5 mL of ammonium stock) and were flushed for 5 min with a CH_4:O_2 mixture (molar ratio of 1:1.5). In *Step 2*, all cultures were incubated at 30 °C with exponential growth over a 24-h period. In *Step 3*, all cultures received a second 5 min headspace flush with a CH_4:O_2 mixture (molar ratio of 1:1.5). In *Step 4*, all cultures were incubated at 30 °C with exponential growth over a second 24-h period. Finally, in *Step 5*, 40 mL of liquid was quickly removed (5 min) from all cultures, completing a cycle. This fed-batch cycling was repeated more than 80 times with reproducible growth patterns.

Fig. 1 also illustrates production of PHA under nitrogen-limited growth conditions. A portion of the samples removed in *Step 5* was centrifuged (3000*g*) for 15 min then suspended in fresh medium without nitrogen. The headspace of each bottle was filled with a CH_4:O_2 gas mixture (molar ratio of 1:1.5) at $t = 0$ h and again at t = 24 h. Some samples were amended with sodium valerate (>99.0% purity; Sigma–Aldrich, St. Louis, MO, USA) to assess PHBV production. Other samples were also amended with sodium formate (60 mM; Sigma–Aldrich, St. Louis, MO, USA) to examine effect of added electron equivalents during PHA production step. After 48 h of incubation, cells were harvested from the triplicate samples by centrifugation (3000*g*) and freeze-dried. Preserved samples were assayed for PHA content.

2.3. Bacterial community analysis

Bacterial community was analyzed per the protocol described in Myung et al. (2015). After establishing a repeating cycle of operation, 100-μL samples were removed from each enrichment culture, and the genomic DNA (gDNA) extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA), as per the manufacture's protocol. The quality of DNA was evaluated using spectrophotometry (NanoDrop 1000, Thermo Scientific Inc., Wilmington, DE, USA). DNA samples with A260/A280 >1.7 were analyzed.

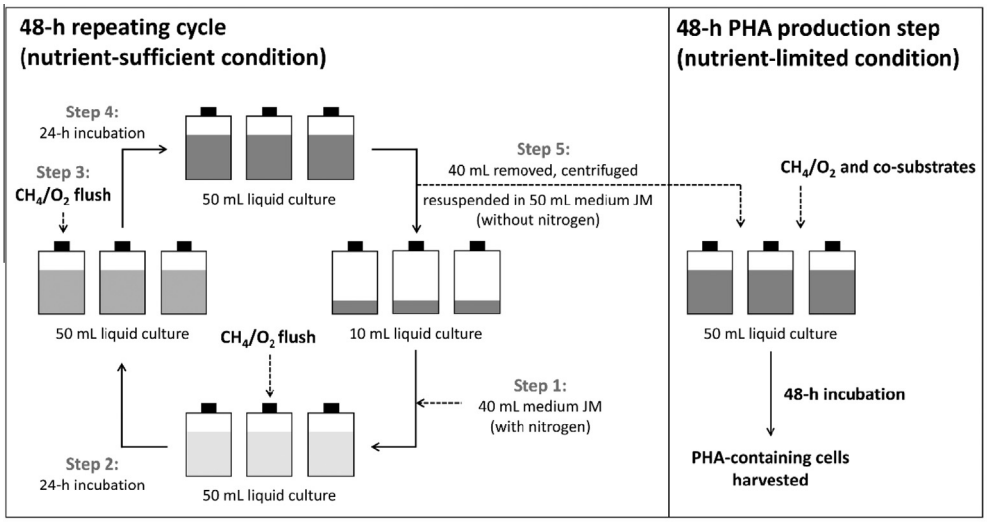

Fig. 1. Overall scheme of PHA copolymer production using CH_4 as primary substrate.

A PCR product library was prepared using a two-step PCR amplification protocol that avoids PCR bias due to Illumina adapter and other added components. Standard primers 515F (5′-GTGCCA GCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) targeting the V4 region of both bacterial and archaeal 16S rDNA were used in the first step PCR without added components.

To increase the nucleotide base diversity in sequences of sample libraries within V4 region, phasing primers were designed and used in the second step of the two-step PCR. Spacers of different length (0–7 bases) were added between the sequencing primer and the target gene primer in each of the 8 forward and reverse primer sets. To ensure that the total length of the amplified sequences do not vary with the primer set used, the forward and reverse primers were used in a complementary fashion so that all of the extended primer sets have exactly 7 extra bases as the spacer for the sequencing phase shift. Barcodes were added to the reverse primers between the sequencing primer and the adaptor. The reverse phasing primers contained (5′–3′) an Illumina adapter for reverse PCR (24 bases), unique barcodes (12 bases), the Illumina reverse read sequencing primer (35 bases), spacers (0–7 bases), and the target reverse primer 806R (20 bases). The forward phasing primers included (from 5′ to 3′) an Illumina adapter for forward PCR (25 bases), the Illumina forward read sequencing primer (33 bases), spacers (0–7 bases), and the target forward primer 515F (19 bases).

In the first step PCR, reactions were carried out in a 50-μL reaction: consisting of: 5 μL 10 × PCR buffer II (including dNTPs), 0.5 U high fidelity AccuPrime™ Taq DNA polymerase (Life Technologies, Gaithersburg, MD, USA), 0.4 μM of both forward and reverse target only primers, 10 ng sample DNA. Samples were amplified using the following program: denaturation at 94 °C for 1 min, and 10 cycles of 94 °C for 20 s, 53 °C for 25 s, and 68 °C for 45 s, with a final extension at 68 °C for 10 min.

The triplicate products of each sample from the first round PCR were combined, purified with an Agencourt® AMPure XP kit (Beckman Coulter, Beverly, MA, USA), eluted in 50 μL water, and aliquoted into three new PCR tubes (15 μL each). The second round PCR used a 25-μL reaction (2.5 μL 10 × PCR buffer II (including dNTPs), 0.25 U high fidelity AccuPrime™ Taq DNA polymerase (Life Technologies, Gaithersburg, MD, USA), forward and reverse phasing primers (0.4 μM) and a 15 μL aliquot of the first-round purified PCR product). Samples were amplified through 20 cycles using the same temperature program specified above. Positive PCR products were confirmed by agarose gel electrophoresis. PCR products from triplicate reactions were combined and quantified with PicoGreen.

PCR products from samples to be sequenced in the same MiSeq run (generally 3 × 96 = 288 samples) were pooled at equal molality. The pooled mixture was purified with a QIAquick Gel Extraction Kit (QIAGEN Sciences, Germantown, MD, USA) and re-quantified with PicoGreen.

Sample libraries were prepared for sequencing according to the MiSeq™ Reagent Kit Preparation Guide (Illumina, San Diego, CA, USA): the combined sample library was diluted to 2 nM; samples were denatured by mixing 10 μL of the diluted library and 10 μL of 0.2 N fresh NaOH and incubation for 5 min at room temperature; 980 μL of chilled Illumina HT1 buffer was then added and mixed to create a 20 pM library and the library was adjusted to the desired concentration for sequencing. As an example, 800 μL of the 20 pM library was mixed with 200 μL of chilled Illumina HT1 buffer to make a 16 pM library to achieve about 700 paired ends reads. The 16S rDNA library for sequencing was mixed with about 10% Phix library (final concentration).

A 500-cycle v1 or v2 MiSeq reagent cartridge (Illumina, San Diego, CA, USA) was thawed for 1 h in a water bath, inverted ten times to mix the thawed reagents, and stored at 4 °C for a short time until use. Sequencing was performed for 251, 12, and 251 cycles for forward, index, and reverse reads, respectively on Miseq.

2.4. Analytical methods

To analyze concentrations of CH_4, O_2 and CO_2, 0.5 mL of gas phase from each enrichment culture was injected onto GOW-MAC gas chromatograph with an Altech CTR 1 column and a thermal conductivity detector. The following method parameters were used: injector, 120 °C; column, 60 °C; detector, 120 °C; and current, 150 mV. Peak areas of CH_4, O_2 and CO_2 were compared to standards and quantified using the software ChromPerfect (Justice Laboratory Software, Denville, NJ, USA).

Concentrations of valerate were assayed using a Dionex DX-500 ion chromatograph (Dionex, Sunnyvale, CA, USA) equipped with a GP50 gradient pump, CD25 conductivity detector, AS40 Automated Sampler, an AS11-HC ion-exchange column, and supplied a sodium hydroxide eluent. Peaks area of valerate were compared to standards and quantified using the software Chromeleon (Dionex, Sunnyvale, CA, USA).

To analyze total suspended solids (TSS), 0.5–5.0 mL of cell suspension was filtered through pre-washed, dried, and pre-weighted 0.2-μm membrane filters (Pall, Port Washington, NY, USA). The filtered cells and membrane filters were dried at 105 °C for 24 h, then weighed on an AD-6 autobalance (Perkin Elmer, Norwalk, CT, USA).

2.5. PHA measurement

PHA was assayed by GC per the protocol of Braunegg et al. (1978). Between 5 and 10 mg of freeze-dried biomass were weighed then transferred to a 12-mL glass vial. Each vial was amended with 2 mL of methanol containing sulfuric acid (3%, vol/vol) and benzoic acid (0.25 mg/mL methanol), supplemented with 2 mL of chloroform, and sealed with a Teflon-lined plastic cap. All vials were shaken then heated at 95–100 °C for 3.5 h. After cooling to room temperature, 1 mL of deionized water was added to create an aqueous phase separated from the chloroform organic phase. The reaction cocktail was mixed on a vortex mixer for 30 s then allowed to partition until phase separation was complete. The organic phase was sampled by syringe and analyzed using a GC (Agilent 6890 N) equipped with an HP-5 column (containing 5% phenyl-methylpolysiloxane; Agilent Technologies, Palo Alto, CA, USA) and a flame ionization detector. The oven temperature program was as follows: 50 °C for 3 min, ramp increase to 60 °C over 9 min, 60 °C for 3 min, ramp increase to 260 °C over 9 min, ramp increase to 300 °C over 6 min, and held at 300 °C for 6 min. DL-Hydroxybutyric acid sodium salt (Sigma–Aldrich, St Louis, MO, USA) and PHBV with 3HV fractions of 5 mol%, 8 mol% and 12 mol % (Sigma–Aldrich, St Louis, MO, USA) were used to prepare external calibration curves. The PHA content (wt%, w_{PHA}/w_{CDW}) of the samples and 3HV fraction of the PHAs (mol%) were calculated by normalizing to initial dry mass.

2.6. Carbon and electron balances

Carbon balances were prepared for reactants (CH_4, O_2 and valerate) and products (biomass as TSS, CO_2 and PHA) for each serum bottle culture (in C-mmol). Biomass was assumed to have an empirical composition of $C_5H_7O_2N$ (Rittmann and McCarty, 2001). Electron balances were also calculated for each serum bottle culture (in e-mmol) for CH_4, valerate and O_2 relative to the reference oxidation states of CO_2 and water.

2.7. Material characterization of the PHA generated

PHA granules were extracted from the cells by suspending 500 mg of freeze-dried cell material in 50-mL Milli-Q water, adding 400 mg of sodium dodecyl sulfate (> 99.0% purity; Sigma–Aldrich, St. Louis, MO, USA) and 360 mg of EDTA, followed by heating to 60 °C for 60 min to induce cell lysis. The solution was centrifuged (3000g) for 15 min, and the pellet washed three times with deionized water. To purify the PHA, pellets were washed with a 50-mL sodium hypochlorite (bleach) solution (Clorox 6.15%), incubated at 30 °C with continuous stirring for 60 min, and centrifuged (3000g) for 15 min. Sample pellets were then washed three times with deionized water.

Chemical structures of PHAs were analyzed by nuclear magnetic resonance (NMR) spectrometer. Samples were prepared by adding 3 mg of the PHA to 0.7-mL deuterated chloroform ($CDCl_3$), with gentle heating until the PHA had fully dissolved. The PHA samples were dissolved in deuterated chloroform ($CDCl_3$). The proton (^1H) NMR and the carbon (^{13}C) NMR spectra were measured at room temperature on a 500-MHz NMR spectrometer.

Molecular weights of PHAs were evaluated using gel permeation chromatography (GPC). Sample pellets dissolved in chloroform at a concentration of 5 mg/mL for 90 min at 60 °C were filtered through a 0.2-μm PTFE filter, then analyzed with a Shimadzu UFLC system (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a Shimadzu RID-10A refraction index detector. The GPC was equipped with a Jordi Gel DVB guard column (500 Å, Jordi Labs, Mansfield, MA, USA) and Jordi Gel DVB analytical columns (10^5 Å, Jordi Labs, Mansfield, MA, USA). The temperature of the columns was maintained at 40 °C, and the flow rate of the mobile phase (chloroform) was 1 mL min^{-1}. Molecular weights were calibrated with polystyrene standards from Varian (Calibration Kit S-M2-10, Agilent Technologies, Palo Alto, CA, USA).

Peak melting temperatures (T_m) and onset glass transition temperatures (T_g^0) of PHAs were evaluated using TA Q2000 differential scanning calorimetry (DSC, TA Instruments, New Castle, DE, USA). Thermal data were collected under a nitrogen flow of 10 mL min^{-1}. About 5 mg of melt-quenched PHA samples encapsulated in aluminum pans were heated from −40 °C to 200 °C at a rate of 10 °C min^{-1}. The peak melting temperatures were determined from the position of the endothermic peaks.

3. Results and discussion

3.1. Fed-batch enrichments: repeating cycle

Patterns of substrate consumption and community composition were evaluated for the serum bottle enrichment cultures fed CH_4 on a repeating cycle. Fig. 2 illustrates the pattern of a typical 48-h repeating cycle (Cycle 52). The errors bars represent standard deviations for triplicate batch cultures.

After 24 days, *Methylocystis* dominated the CH_4-fed enrichment, and the resulting community structure remained stable for the remainder of the study period (Cycle 12 to Cycle 89; Table 1). The coefficients of variance of each genus were all <0.3, which confirms stable community structure. *Hydrotalea*, *Hyphomicrobium* and other minor genera including *Burkholderia*, *Rhodopseudomonas* and *Castellaniella* accounted for the remaining bacteria. Methanotrophic cultures frequently coexist with methylotrophs or heterotrophs

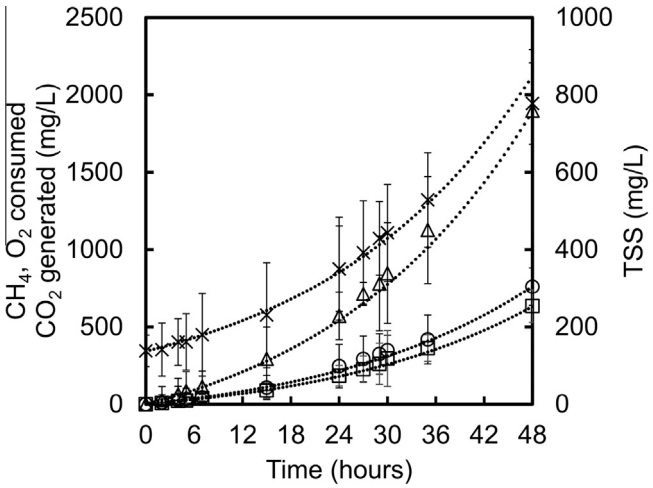

Fig. 2. Methanotrophic enrichment culture stoichiometry for a typical repeating cycle (Cycle 52) after community structure stabilized, showing cumulative consumption of CH_4 (○) and O_2 (△), and cumulative production of CO_2 (□) and biomass as TSS (×) in triplicate serum bottle cultures during nutrient-sufficient growth (average ± standard deviation). Also shown is a best-fit exponential growth model (accounting for headspace partitioning with Henry's constant, k_H = 0.0013 at 30 °C) with the following parameters: maximum specific rate of CH_4 utilization $\hat{q}_{CH_4}$ = 0.021 ± 0.004 g CH_4 g TSS^{-1} h^{-1}, maximum specific rate of O_2 utilization $\hat{q}_{CO_2}$ = 0.051 ± 0.007 g O_2 g TSS^{-1} h^{-1}, maximum specific rate of CO_2 production $\hat{q}_{CO_2}$ = 0.017 ± 0.003 g CO_2 g TSS^{-1} h^{-1}, and maximum specific growth rate $\hat{\mu}$ = 0.038 ± 0.003 h^{-1}, r^2 = 0.995.

Table 1
Most probable affiliation of the genus-level bacterial community structures based on 16S rRNA OTUs retrieved from the triplicate CH_4-utilizing microbial enrichment cultures.

Affiliation	Proportions (%)				
	Inoculum	Cycle 12	Cycle 32	Cycle 52	Cycle 89
Proteobacteria					
Alphaproteobacteria					
Rhizobiales					
Methylocystis	<0.1	65.0 ± 7.0	74.9 ± 7.5	77.2 ± 8.0	76.4 ± 4.0
Hyphomicrobium	<0.1	7.1 ± 2.8	6.5 ± 2.2	6.1 ± 1.9	8.9 ± 1.8
Rhodopseudomonas	<0.1	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.2	0.4 ± 0.2
Betaproteobacteria					
Burkholderiales					
Burkholderia	<0.1	1.2 ± 0.4	0.7 ± 0.2	0.8 ± 0.4	0.8 ± 0.3
Castellaniella	0.1	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.3 ± 0.1
Bacteroidetes					
Sphingobacteriia					
Sphingobacteriales					
Hydrotalea	<0.1	5.8 ± 2.5	8.1 ± 2.9	10.1 ± 3.5	8.8 ± 3.5
Others	99.8 ± 0.1	20.3 ± 7.3	9.1 ± 4.0	5.0 ± 3.3	4.4 ± 1.2

that survive on methanotrophic by-products such as methanol (Dedysh and Dunfield, 2011). *Hyphomicrobium* is a facultative methylotrophic genus that can grow on C1 and C2 compounds and accumulate intracellular PHA (Zhao et al., 1993). Information about *Hydrotalea* is limited, but they assimilate formate and can use carbohydrates as growth substrates (Kämpfer et al., 2011).

3.2. Fed-batch enrichments: PHA production step

Fig. 3 illustrates the patterns of consumption for CH_4, O_2 and valerate and production of CO_2, TSS and PHA for a typical 48-h PHA production step (Cycle 57). Three different conditions were evaluated: (1) PHA production without valerate (Fig. 3a), (2) PHA production with a low concentration (100 mg /L) of valerate (Fig. 3b), and (3) PHA production with a high concentration (400 mg/L) of valerate (Fig. 3c).

With fed CH_4 alone, final PHA content was ~39 wt% (Fig. 3a), and no 3HV units were incorporated into the PHA polymer (Fig. 3a). The following yields were obtained: 0.35 mg TSS mg CH_4^{-1}, 0.31 mg PHA mg CH_4^{-1}, 3.0 mg O_2 mg CH_4^{-1} (1.5 mol O_2 mol CH_4^{-1}) and 1.1 mg CO_2 mg CH_4^{-1} (0.40 mol CO_2 mol CH_4^{-1}).

PHA production required concomitant oxidation of CH_4. Addition of valerate in the absence of CH_4 did not result in PHA production, and no valerate uptake was observed. But addition of valerate plus CH_4 resulted in PHBV synthesis. In the presence of CH_4 plus low levels of valerate (100 mg/L), final PHA content was ~43 wt% (Fig. 3b). Approximately 20 mol% of 3HV was incorporated into the polymer, and this value was constant during the PHA production step (Fig. 3b). Final yield values were: 0.37 mg TSS mg CH_4^{-1}, 0.36 mg PHA mg CH_4^{-1}, 3.5 mg O_2 mg CH_4^{-1} (1.7 mol O_2 mol CH_4^{-1}), 1.4 mg CO_2 mg CH_4^{-1} (0.52 mol CO_2 mol CH_4^{-1}), and 0.27 mg valerate mg CH_4^{-1} (0.043 mol valerate mol CH_4^{-1}).

In the presence of CH_4 plus high levels of valerate (400 mg/L), final PHA content was ~30 wt% (Fig. 3c). Approximately 40 mol% of 3HV was incorporated into the polymer, and this value was constant during the PHA production step (Fig. 3c). Final yield values were: 0.36 mg TSS mg CH_4^{-1}, 0.35 mg PHA mg CH_4^{-1}, 3.8 mg O_2 mg CH_4^{-1} (1.9 mol O_2 mol CH_4^{-1}), 2.5 mg CO_2 mg CH_4^{-1} (0.91 mol CO_2 mol CH_4^{-1}), and 0.64 mg valerate mg CH_4^{-1} (0.10 mol valerate mol CH_4^{-1}).

Table 2 summarizes PHA production (average ± one standard deviation) over six cycles of stable operation (Cycles 17, 25, 43, 57, 69, and 85). Both wt% PHA and mol% 3HV values were stable, and the standard deviations were low, indicating long-term stable capacity for PHBV production.

3.3. Fed-batch enrichments: carbon and electron balances

To assess process stoichiometry, carbon and electron balances were calculated for both the repeating cycle with CH_4 as sole carbon substrate and the PHA production step with different levels of added valerate (0, 100, and 400 mg/L). As shown in Table 3, the relative errors in the carbon and electron balances for reactants and products were <6% for both the repeating cycle (no valerate) and the PHA production step (plus added valerate), and thus confirmed high accuracy of measurements.

3.4. NMR analysis of the PHA generated

To confirm the chemical structure of the PHA generated, ^1H and ^{13}C NMR spectra were obtained of the PHA harvested from Cycle 58. The peak assignments in both spectra were in close agreement with those reported previously (Han et al., 2015; Wei et al., 2014; Žagar et al., 2006). Most interestingly, the ^{13}C NMR spectrum showed three characteristic peaks at a chemical shift (δ) of 169.3–169.7 ppm, corresponding to the carbonyl carbons in the PHBV. These carbonyl peaks indicate that the PHA polymer produced with valerate is the copolymer PHBV, not a blend of P3HB and poly(3-hydroxyvalerate) (P3HV).

3.5. Molecular weight characterization of the PHA generated

Table 4 illustrates molecular weight and molecular weight distributions (PDI = M_w/M_n) of P3HB and PHBV generated in the methanotrophic enrichment cultures (Cycle 57). These values are comparable to those of heterotrophic enrichments (Myung et al., 2014) and to commercial P3HB and PHBV powders (Sigma–Aldrich, St Louis, MO, USA) (Myung et al., 2014), but are more uniform, with higher molecular weights and lower PDI values.

3.6. Melting temperatures of the PHA generated

DSC analysis on PHA polymers produced from the CH_4-fed enrichment cultures without addition of valerate had a peak melting temperature (T_m) of 178 °C and an onset glass transition temperature (T_g^0) of 8 °C, values typical of P3HB (Loo and Sudesh, 2007). The PHA polymers containing 3HV units had peak melting temperatures (T_m) of 151 °C (3HV fraction of 19 mol%) and 136 °C (3HV fraction of 39 mol%), and onset glass temperatures of (T_g^0) of −2 °C and −6 °C, values typical of PHBV (Loo and Sudesh, 2007). The presence of only one peak melting temperature suggests that

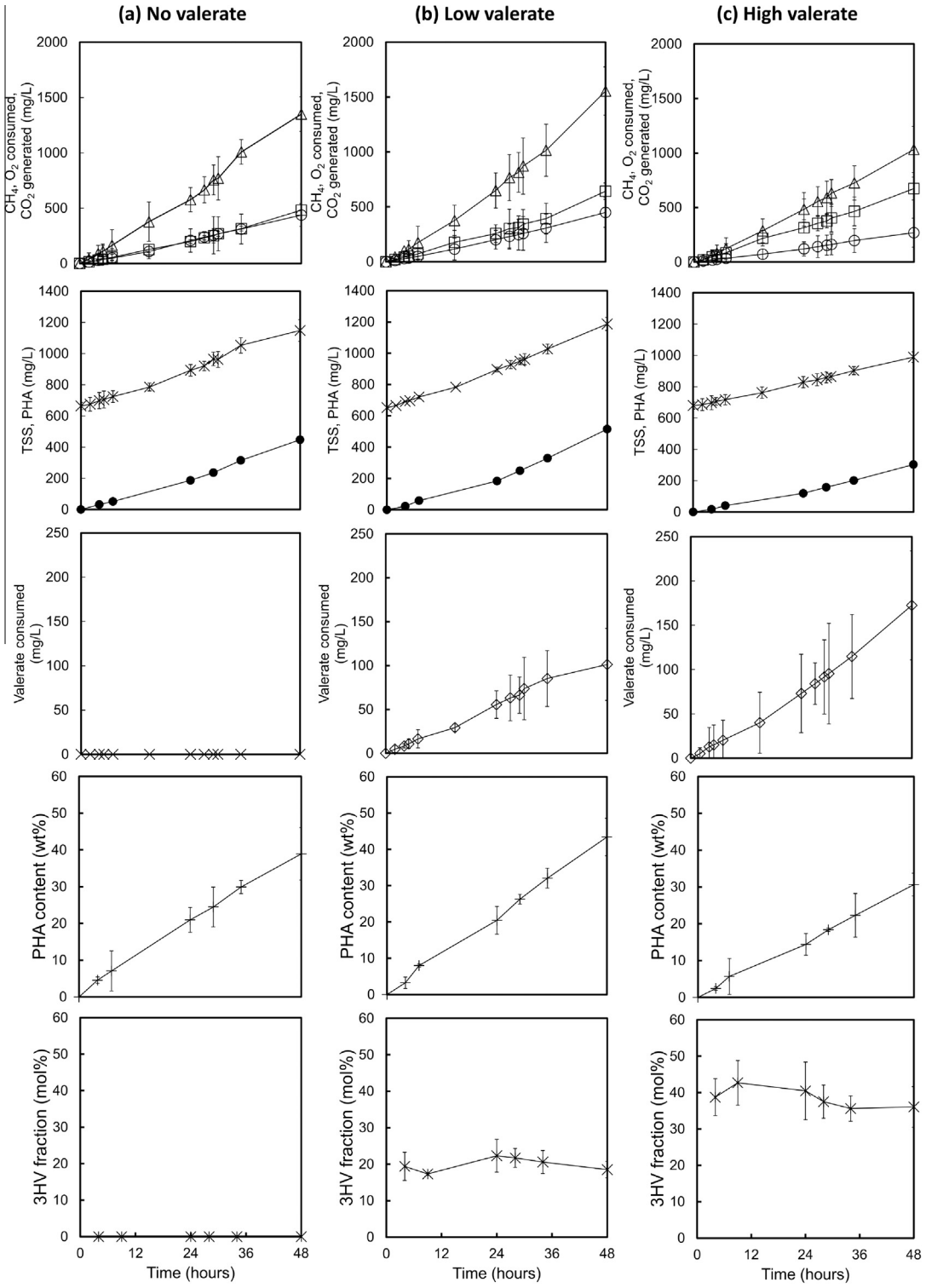

Fig. 3. Methanotrophic enrichment culture stoichiometry for a typical PHA production step (Cycle 57), in which the cells are harvested from a repeating cycle. Cumulative consumption of CH_4 (○), O_2 (△) and valerate (◇), and cumulative production of CO_2 (□), biomass as TSS (×), PHA concentration in system (mg/L) (●), PHA content in cells (w_{PHA}/w_{CDW}) (+) and mol% of 3HV in PHA produced (*) are shown. (a) No valerate addition, (b) low valerate addition (100 mg/L), and (c) high valerate addition (400 mg/L). CH_4 and O_2 additions occur at $t = 0$ and $t = 24$ h.

the PHA polymer produced in incubation with valerate is the copolymer PHBV, not a blend of P3HB and poly(3-hydroxyvalerate) (P3HV).

3.7. Kinetic and stoichiometry of growth and PHA accumulation

The triplicate cultures of exponentially growing Type II methanotrophic enrichment doubled every 18 h (Fig. 2). The maximum specific growth rate ($\hat{\mu}$) was 0.038 ± 0.003 h^{-1} (0.91 ± 0.07 d^{-1}). The maximum specific rate of CH_4 utilization ($\hat{q}_{CH_4}$) for the repeating cycle was 0.021 ± 0.004 g CH_4 g TSS^{-1} h^{-1}, with a biomass yield for all time points of 0.90 ± 0.02 g TSS g CH_4^{-1}. These values are at the lower end of the range reported for Type II methanotrophs (Rostkowski et al., 2013). A possible explanation for slower substrate utilization and growth rates is the use of elevated levels of

Table 2
Reproducible PHA production pattern observed during Cycle 17 to Cycle 85.

		(a) No valerate	(b) Low valerate (100 mg/L)	(c) High valerate (400 mg/L)
Cycle 17	Final PHA content (wt%)	39 ± 3	44 ± 4	29 ± 2
	3HV fraction (mol%)	0	18 ± 2	39 ± 4
Cycle 25	Final PHA content (wt%)	37 ± 3	45 ± 2	30 ± 3
	3HV fraction (mol%)	0	22 ± 3	40 ± 1
Cycle 43	Final PHA content (wt%)	40 ± 4	43 ± 2	32 ± 4
	3HV fraction (mol%)	0	18 ± 2	39 ± 2
Cycle 57	Final PHA content (wt%)	39 ± 6	43 ± 4	30 ± 3
	3HV fraction (mol%)	0	20 ± 3	40 ± 6
Cycle 69	Final PHA content (wt%)	38 ± 3	45 ± 2	32 ± 2
	3HV fraction (mol%)	0	18 ± 1	38 ± 1
Cycle 85	Final PHA content (wt%)	39 ± 1	45 ± 2	27 ± 3
	3HV fraction (mol%)	0	21 ± 3	37 ± 2
Average	Final PHA content (wt%)	39 ± 3	44 ± 2	30 ± 3
	3HV fraction (mol%)	0	20 ± 3	39 ± 3

ammonium (13.5 mM NH_4Cl added). Ammonium can be fortuitously oxidized to toxic hydroxylamine by methane monooxygenase (Dalton and Stirling, 1982). By lowering the ammonium level by 20%, the maximum TSS value increased from 38.9 mg TSS/bottle (798 mg TSS/L) to 65.7 mg TSS/bottle (1,314 mg TSS/L).

Rostkowski et al. (2013) described the stoichiometry for methanotrophic growth assuming that O_2 has two roles in CH_4 metabolism: it is (1) a reactant needed for oxidation of CH_4 to methanol by methane monoxygenase (an oxidation that actually requires two moles of electrons for each mole of CH_4 oxidized), and (2) the terminal electron acceptor for energy production. A fraction f_e of the CH_4 is oxidized to CO_2, and a fraction f_s of the CH_4 is assimilated into cell biomass, where $f_e + f_s = 1$. Assuming that ammonium is the nitrogen source and the empirical formula for cell biomass of $C_5H_7O_2N$, the following expression can be derived for the repeating cycle:

$$\frac{1}{4}CH_4 + \left(\frac{1}{4}+\frac{f_e}{4}\right)O_2 + \frac{f_s}{23}HCO_3^- + \frac{f_s}{23}NH_4^+ + \left(\frac{20f_s}{23}+f_e-1\right)H^+ \rightarrow \left(\frac{1}{4}-\frac{4f_s}{23}\right)CO_2 + \left(\frac{f_e}{2}+\frac{9f_s}{23}\right)H_2O + \frac{f_s}{23}C_5H_7O_2N$$

From the above stoichiometry, the molar ratio of O_2 to CH_4 is $1 + f_e$. For the repeating cycle, a molar ratio of O_2 to CH_4 of 1.27 ± 0.10 was obtained. These values correspond to an electron equivalent conversion fraction for energy f_e of 0.27 ± 0.10, with f_s values of 0.73 ± 0.10.

Applying the method of Rostkowski et al. (2013) to the PHA production step, the following expression can be derived:

$$\frac{1}{4}CH_4 + \left(\frac{1}{4}+\frac{f_e}{4}\right)O_2 \rightarrow \left(\frac{1}{4}-\frac{2f_s}{9}\right)CO_2 + \left(\frac{f_e}{2}+\frac{f_s}{3}\right)H_2O + \frac{f_s}{18}C_4H_6O_2$$

Table 4
Peak molecular weight and polydispersity index (PDI) of extracted polymers at the end of Cycle 57 in the microbial enrichment cultures.

	Peak molecular weight	PDI
P3HB	1.20 ± 0.20 E+06	1.76 ± 0.22
PHBV with 20 mol% 3HV	1.15 ± 0.11 E+06	1.88 ± 0.18
PHBV with 40 mol% 3HV	9.34 ± 0.78 E+05	2.14 ± 0.27

For production of P3HB (empirical formula $C_4H_6O_2$) from CH_4 alone, f_e was 0.25 ± 0.04 and f_s was 0.75 ± 0.04. When valerate was added as a co-substrate with CH_4, the value f_e increased to 0.35 ± 0.05 at 100 mg/L valerate and to 0.45 ± 0.05 for 400 mg/L valerate. This increase likely reflects an increased energy requirement for uptake of valerate from solution and subsequent incorporation of 3HV into PHA. Uptake of fatty acids is energy dependent in methanotrophs (Shishkina and Trotsenko, 1982).

3.8. Addition of formate as reducing equivalents

To test the role of CH_4 in providing energy for 3HV incorporation, the effects of added formate was examined. Formate is a source of reducing equivalents for methanotrophs (Henrysson and McCarty, 1993; Pieja et al., 2011b). Addition of formate (60 mM sodium formate) decreased CH_4 requirements by 14% at 0 mg valerate/L; by 13% at 100 mg valerate/L; and by 21% at 400 mg valerate/L. This result is consistent with the hypothesis that valerate uptake is driven by oxidation of C1 units.

3.9. Comparison with a previous report

Previous phenotypic and genotypic screening of Type II methanotrophs indicated that Type II methanotrophs are capable of PHA production, but produce P3HB exclusively when supplied CH_4 alone (Pieja et al., 2011a). A recent report (Zuñiga et al., 2013) indicates that *Methylobacterium organophilum*, a facultative methanotroph, can produce PHBV when grown with citric acid, propionic acid, and CH_4 and incubated in the absence of nitrogen, but the mass of CH_4 consumed was less than 10% of the total substrate consumed. With the obligate Type II methanotrophs evaluated in this work, growth occurs on CH_4 and CH_4 fuels uptake of valerate: the copolymer PHBV is only produced in the presence of CH_4 and valerate. The results of this study indicate control over the concentration of co-substrates added during the PHA production step may enable production of high-value "tailored" copolymers.

4. Conclusions

We conclude that a repeating cycle of methanotroph cultivation can yield a stable community capable of reproducibly producing

Table 3
Carbon and electron balances for the fed-batch experiment in Fig. 2 and Fig. 3.

	Repeating cycle		PHA production step No valerate		PHA production step Low valerate (100 mg/L)		PHA production step High valerate (400 mg/L)	
	C balance (C-mmol)	e^- balance (e-mmol)	C balance (C-mmol)	e^- balance (e-mmol)	C balance (C-mmol)	e^- balance (e-mmol)	C balance (C-mmol)	e^- balance (e-mmol)
Biomass	1.5 ± 0.2	6.2 ± 0.8	0.06 ± 0.01	0.26 ± 0.04	0.03 ± 0.01	0.13 ± 0.04	0.01 ± 0.01	0.04 ± 0.02
PHA	0	0	0.67 ± 0.09	3.0 ± 0.4	0.77 ± 0.08	3.5 ± 0.4	0.45 ± 0.05	2.1 ± 0.2
CH_4	−2.4 ± 0.3	−19.0 ± 2.4	−1.4 ± 0.2	−11.0 ± 1.6	−1.3 ± 0.2	−11.2 ± 1.7	−0.84 ± 0.1	−6.7 ± 0.8
CO_2	0.72 ± 0.05	0	0.55 ± 0.07	0	0.73 ± 0.07	0	0.77 ± 0.06	0
H_2O (from O_2)	0	11.9 ± 1.0	0	7.2 ± 0.6	0	9.7 ± 0.7	0	6.5 ± 0.6
Valerate	0	0	0	0	−0.30 ± 0.04	−1.6 ± 0.2	−0.42 ± 0.07	−2.2 ± 0.4
Balance	−0.13	−0.97	−0.08	−0.47	−0.02	0.53	−0.04	−0.41
Error (%)	5.5	5.1	6.1	4.3	1.5	−4.1	2.9	4.5

PHA. By separating PHA accumulation from cell replication, tailored PHA-rich biomass can be generated by addition of co-substrate, while retaining a large inoculum for the next cycle of cell division. When fed CH_4 plus valerate, PHBV was synthesized, and the mol% of 3HV increased with increasing added valerate. Oxidation of CH_4 was required for valerate assimilation, and the fraction oxidized increased with increased mol% 3HV. The results are promising for production of tailored copolymers with CH_4 as a low-cost primary substrate.

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