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Effects of cascaded *vgb* promoters on poly(hydroxybutyrate) (PHB) synthesis by recombinant *Escherichia coli* grown micro-aerobically

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Abstract Micro-aeration is a situation that will be encountered in bacterial cell growth especially when the saturated dissolved oxygen level cannot match the demand from cells grown to a high density. Therefore, it is desirable to separate aerobic growth and micro-aerobic product formation into two stages using methods including anaerobic or micro-aerobic promoters that are inducible under low aeration intensity. Eleven potential low aeration-inducible promoters were cloned and studied for their induction strengths under micro-aerobic conditions. Of them, *Vitreoscilla* hemoglobin promoter (P_{vgb}) was found to be the strongest among all 11 promoters. At the same time, six *E. coli* hosts harboring poly(R-3-hydroxybutyrate) (PHB) synthesis operon *phaCAB* were compared for their ability to accumulate poly(hydroxyalkanoates) (PHA). *E. coli* S17-1 was demonstrated to be the best host achieving a 70 % (mass fraction) PHB in the cell dry weigh (CDW) after 48 h under micro-aerobic growth. Cascaded P_{vgb} repeats (P_{nvgb}) were investigated for enhanced expression level under micro-aerobic growth. The highest PHA production was obtained when a promoter containing eight cascaded P_{vgb} repeats (P_{8vgb}) was used, 5.37 g/l CDW containing 90 % PHB was obtained from recombinant in *E. coli* S17-1. Cells grew further to 6.30 g/l CDW containing 91 % PHB when oxygen-responsive transcription factor ArcA (*arcA*) was deleted in the same recombinant *E. coli* S17-1. This study revealed that *vgb* promoter containing cascaded P_{vgb} repeats (P_{8vgb}) is useful for product formation under low aeration intensity.

Keywords PHB · Polyhydroxyalkanoates · Micro-aeration · *Vitreoscilla* · *vgb* · *arcA* · *Escherichia coli* · Synthetic biology

Introduction

Microbial poly(hydroxyalkanoates) (PHA), as a family of biodegradable and biocompatible thermal polyesters with diverse structures (Matsumoto and Taguchi 2013; Steinbuchel and Valentin 1995; Tian et al. 2001), have the potentials to be developed as environmentally friendly bioplastics with many applications (Chen 2009; Chen and Patel 2012; Gao et al. 2011; Laycock et al. 2014; Park et al. 2012). However, the high production cost, due to low substrate to PHA conversion, high-energy consumption, non-continuous processes, etc. (Koller et al. 2011; Wang et al. 2014), impedes the wide applications of PHA as environmentally friendly materials (Meng et al. 2014; Keshavarz and Roy 2010; Khosravi-Darani et al. 2013). Many attempts have been made to reduce PHA production cost (Wu et al. 2001; Obruca et al. 2010; Passanha et al. 2014; Quillaguaman et al. 2010; Wang et al. 2014; Yue et al. 2014). Cheap carbon sources, such as cheese whey (Pais et al. 2014), digested liquors (Passanha et al. 2013), soy waste (Hong et al. 2000), starch (Ali and Jamil 2014), kitchen wastes (Yue et al. 2014), and crude glycerol, a by-product from biodiesel production (Mothes et al. 2007; Ibrahim and Steinbüchel 2010; Hermann-Krauss et al. 2013; Song et al. 2008), were used also as substrates for PHA production, respectively. Antibiotics-free plasmid expression systems were constructed to increase the biomass and PHA content avoiding the use of expensive antibiotics (Akiyama

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et al. 2011; Fleige et al. 2011). An unsterile and continuous fermentation process was developed based on a halophile bacterium named *Halomonas* sp. TD01 (Fu et al. 2014; Tan et al. 2011); 80 g/l cell dry weigh (CDW) and 80 % poly(R-3-hydroxybutyrate) (PHB) content was achieved on glucose salt medium during a 56-h open fed-batch process. This unsterile and continuous fermentation process opens a new area for reducing the cost in PHA production (Wang et al. 2014). So far, all those successful processes for PHA production are aerobic, requiring energy-intensive aerations. In fact, cells must go through micro-aerobic conditions during the late growth phase in both shake flasks and fermenters due to inefficient oxygen supply in aqueous cultures, especially when cell density is high (Lee et al. 1994, 2000).

PHA production under micro-aerobic conditions has been considered as a more economical process as micro-aeration can effectively reduce energy for air compressors and agitation (Carlson et al. 2005; Chen and Page 1997). PHB, the well-known member of the PHA family, was studied for production under micro- or anaerobic condition by recombinant *Escherichia coli* (Carlson et al. 2005; Wei et al. 2009). Two major oxygen-responsive regulators in *E. coli* coordinate the metabolic synthesis pathways: these are the fumarate nitrate reduction regulator (FNR) and aerobic respiration regulator *arcAB* (Compan and Touati 1994). The FNR dimer, with a sequence-specific domain to target promoters, acts as an activator of anaerobic genes and a repressor of aerobic genes (Bettenbrock et al. 2014), while *arcA* regulates gene expression in response to deprivation of oxygen. Together with its cognate sensory kinase *arcB*, *arcAB* regulate the expressions of hundreds of genes either negatively or positively based on intracellular redox states (Perrenoud and Sauer 2005; Shalel-Levanon et al. 2005a, b). Nikel et al. (2006, 2008) showed that *arcA* mutant increased cell growth and PHB content by 24 % in *E. coli* when glucose was replaced with glycerol as the substrate.

Usually, PHA synthesis genes are constructed and expressed in high copy number vectors inside recombinants. However, PHA native promoters are characterized by their low activities under micro-aerobic conditions. It is therefore important to find anaerobic or micro-aerobic promoters for induction of micro- or anaerobic PHA production. Wei et al. (2009) reported the use of a promoter of alcohol dehydrogenase (P_{adhE}) that was very effective to increase 18 % PHB accumulation compared with PHB synthesis native promoter in plasmid pBHR68. Overexpression of *Vitreoscilla* hemoglobin (VHb) on plasmids or on the genome was revealed to improve oxygen uptakes by recombinants, therefore enhancing PHB and biomass accumulation under hypoxic conditions (Roos et al. 2004; Wei and Chen 2008).

Along with steam sterilization, aeration is the most energy-consuming step in microbial fermentation processes (Kreyenschulte et al. 2014), especially when cells grown to high density. Various strategies including pumping more fresh air, the use of pure oxygen, increasing reactor air pressure, increasing agitation rate, and use of air bubble breakers have to apply so that dissolved oxygen concentration can satisfy the demand from cell growth (Pena et al. 2014). However, all of these strategies require more energy inputs, resulting in increasing cost for the final products (Kreyenschulte et al. 2014).

Therefore, attentions have been paid to manipulate the microbial cells, so that they can grow at low aeration intensity yet produce sufficient products for economic recovery (Pena et al. 2014). One of these possibilities is to grow the cells first under full aeration conditions to allow formation of enough biomass, followed by product accumulation under micro-aerobic conditions that are created automatically when cells reach high density (Moita et al. 2014). Another possibility is to increase microbial oxygen uptake efficiency using *Vitreoscilla* hemoglobin VHb (*vgb*) which can be expressed in recombinant microbial cells (Liu et al. 2005). The third way is to use a micro- or anaerobic promoter to induce product formation when oxygen demand cannot be met by all the above strategies; this was the aim of this paper.

In this study, we aimed to find strong promoter(s) that helped increase cell growth and PHB production under micro-aerobic conditions.

Materials and methods

Cloning anaerobic promoters from *E. coli*

Genomic DNA of *E. coli* was prepared according to the standard protocol (Bergthorsson and Ochman 1995). Primers used for amplifying the anaerobic promoters from the *E. coli* genomic DNA were digested while *Xba*I and *BstB*I sites were added at 5′-end of the forward and reverse primers, respectively.

A series of potential low aeration induction promoters were cloned based on the *E. coli* Gene Expression Database (http://chase.ou.edu) (Table 1). Genes encoding red fluorescent protein (RFP) and PHB synthesis operon *phaCAB* from *Ralstonia eutropha* were constructed in a plasmid to allow induction under low aeration by these promoters, respectively.

Selection of bacterial host strains

Six *E. coli* host strains were used to test the ability of PHA accumulation. Strains Trans1T-1, Trans109, Trans5α, and DMT were commonly used for genetic recombination. Strains S17-1 and EC100D are frequently employed for bacterial conjugation; they are also hosts for PHA production. The ability of these strains to grow at a low oxygen level was

Table 1 *E. coli* strains and plasmids used in this study

Names	Descriptions	References
Strains		
E. coli Trans109	*end*A1 *rec*A1 *gyr*A96 *thi*-1 *hsd*R17 (r_k^-,m_k^+) *rel*A1 *sup*E44 D (*lac-pro*AB) [F′*tra*D36 *pro*AB *laq*I^{q}ZΔM15]	Invitrogen Inc.
E. coli S17-1	*E. coli* wild type	Simon (1994) ATCC® 47055™
E. coli Trans1T-1	F-φ80 *lac*Z ΔM15 Δ*lac*X74 *hsd*R(r_k^-,m_k^+) Δ*rec*A1398 *end*A1 *ton*A	Invitrogen Inc.
E. coli Trans5α	F-φ80 *lac*Z ΔM15 Δ(*lac*ZYA-*arg*F)U169 *end*A1 *rec*A1 *hsd*R17(r_k^-, m_k^+)*sup*E44λ^- *thi*-1 *gyr*A96 *rel*A1 *pho*A	Invitrogen Inc.
E. coli EC100D	The π protein(the *pir* gene product) for replication of plasmids containing the R6Kγ origin of replication (R6Kγ ori)	TransGen Inc.
E. coli DMT	F-φ80 *lac*Z ΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+)*pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A	Invitrogen Inc.
E. coli S17-1Δ*arcA*	Carrying an *arcA* gene knock out for mutagenesis	This study
Plasmids		
pBHR68	A pBluescript II SK−derivative containing *phbCAB* operon from *Ralstonia eutropha* H16 with native promoter, Amp	Spiekermann et al. 1999
pBHR-$P_{promoter}$	*pBHR68* with a series of promoter instead of its native promoter P_{Re}	This study
PcI-RFP-$P_{promoter}$	Plasmid with *rfp* gene under the control of a series of promoters	This study
pBHR-P_{nvgb}	*pBHR68* with different repeats of promoter *vgb* instead of native promoter P_{Re}	This study
Promoters and primer sequence(5′→3′)[a]		
P_{adhE} (476 bp)	TGATCTAGAGGTTAGCTCCGAAGCAAA	AGCTTCGAAAATGCTCTCCTGATAATG
P_{hycA} (211 bp)	TGATCTAGAATCGCCTCCCATTAACTA	AGCTTCGAATGTCAGGTTACCCGTTTA
P_{slp} (247 bp)	TGATCTAGATATTGATTATTAGCACTT	AGCTTCGAAGTTACTATCCTTATCAAC
P_{hya} (310 bp)	TGATCTAGATCGAATTCCTTCTCTTTT	AGCTTCGAACACCCATATCGCACGTCT
P_{adiY} (324 bp)	TGATCTAGACCACTATTCCGCTGAAGG	AGCTTCGAAACATGTACTCCTGAGTGC
P_{dmsA} (238 bp)	TGATCTAGATACCCAATTTTTCTGAAT	AGCTTCGAAAATGGCTCACTCAAGCTT
P_{nirB} (261 bp)	TGATCTAGATTGCTCATGCCGGACGGC	AGCTTCGAATTTGCCTCGATTTCTTTTC
P_{yedY} (108 bp)	TGATCTAGATGCATAAATATGTAAAAG	AGCTTCGAAAAGCCATCACACTTTTTT
P_{narG} (515 bp)	TGATCTAGAACCGTTACTCGTCATACTT	AGCTTCGAACGGTTTTCTCCTGTGGGA
P_{hiuH} (132 bp)	TGATCTAGAGAAAATAATATGCCATAA	AGCTTCGAAGTTTATATCCTTGTCATGT
P_{vgb} (135 bp)	TGATCTAGAACAGGACGCTGGGGTTA	AGCTTCGAAGAGGGTCTTCCTTAAGTT

[a] Forward primers are listed in left column and reverse primers are listed in right column; the restriction sites are underlined

studied in order to select a suitable candidate for PHA production under low aeration intensity.

Plasmid pBHR68 containing PHB synthesis operon *phaCAB*, in which PHA synthase, 3-ketothiolase, and acetoacetyl-CoA reductase were encoded by *phaC*, *phaA*, and *phaB*, respectively, was kindly donated by Professor Alexander Steinbuchel of Münster University in Germany (Spiekermann et al. 1999). pBHR68 was transformed into the six host strains, respectively. After shake flask studies, the CDW and PHA content provided evidences for selection on the most suitable strain for PHB production under microaerobic condition.

Plasmids construction

Plasmids used in this study were all listed in Table 1. Molecular cloning standard procedures including vector isolation, DNA amplification, restriction enzyme digestion, and other DNA manipulations were employed for plasmids construction (Sambrook and Russell 2001). DNA purification and plasmids isolation kits were purchased from Biomed (Beijing, China). Restriction enzymes and ligation kits were supplied by Thermo (Beijing, China). *pfu*-DNA polymerase used in this study was from TransGen (Beijing, China). Primers were synthesized by Invitrogen (Shanghai, China).

The amplified target PCR fragments of different anaerobic promoters were digested by *Xba*I/*Bst*BI and then inserted into *Xba*I/*Bst*BI site in vector PcI-pR-RFP, resulting in a series of plasmids PcI-RFP-$P_{promoter}$ (Table 1), in which *rfp* was under the control of each anaerobic promoter (or low aeration intensity promoter). Those constructed plasmids were verified by PCR and DNA sequencing.

The construction of a series of plasmids pBHR-$P_{promoter}$ (Table 1) was similar with that of PcI-RFP-$P_{promoter}$.

Molecular manipulations were carried out according to the standard procedures (Sambrook and Russell 2001). Cascaded P_{vgb} repeats were obtained using Gibson Assembly (Gibson et al. 2008). To construct two cascaded *vgb* promoters (P_{2vgb}), three target PCR fragments including one short linear plasmid and two P_{vgb} with different overlapped base pairs were amplified using Q5® High-Fidelity DNA Polymerase purchased from NEB (Beijing, China). The primers were designed based on NEBuilder (http://nebuilder.neb.com). The three fragments were purified and mixed in 6:6:1 molar ratio for P_{vgb}, P_{vgb}, and linear plasmid to the Gibson Assembly Master Mix purchased from NEB. After incubation at 50 °C for 30 min, 2 μl Gibson Assembly reaction mixture was transformed into the competent *E. coli* culture. A colony harboring plasmid containing P_{2vgb} (P-P_{2vgb}) was selected via colony PCR and DNA sequencing. Based on this P-P_{2vgb} and the above-described procedure, the plasmid containing P_{4vgb} was assembled with two P_{2vgb} fragments and one short linear plasmid. Based on this approach, cascaded various P_{vgb} repeats were obtained. Six plasmids with P_{vgb} repeats ranging from 1 to 10 were constructed, and the amplified target fragments of different promoters were digested and inserted into pHBR68 using *Xba*I/*BstB*I, generating pBHR-$P_{promoter}$.

arcA knockout in *E. coli*

The *E. coli arcA* mutant was constructed by one-step disruption on the chromosome (Datsenko and Wanner 2000). To investigate the possible recombination of gene deletion fragments with *arcA* in plasmids, 39 bp homologous upstream of *arcA* gene was used, and pKD13 was used as the template for the middle part of the PCR fragments. PCR amplification of gene deletion fragment using *pfu*-DNA polymerase was carried out to generate a ~1.5-kb fragment. The fragment was separated by agarose gel electrophoresis and purified from the gel by DNA purification kits to generate a DNA concentration of 50 ng/μl.

E. coli S17-1 was grown in Luria-Bertani (LB) medium until OD_{600} reached 0.1–0.2, then 0.2 % L-arabinose was added to the culture at 37 °C. After that, the bacteria were maintained ice-cold for 30 min in order to prepare the electro-competent cells. The ice-cold cells were harvested through centrifugations to an appropriate concentration. The precipitated cells were washed by pre-cooling distilled water once followed by addition of a 10 % pre-cooled glycerol twice. Then, 10 μl of the deletion DNA fragment was added into the 100-μl competent cells; the mixture was added into an ice-cold 0.1 cm cuvette for electroporation which was conducted at 1.25 kV under 25 μF and 200 Ω (Bio-Rad Inc., USA). The resulting cells were supplemented with 600 μl LB medium. Subsequently, they were incubated for 2 h at 37 °C. Furthermore, cells were spread onto an LB agar plate with 50 μg/ml kanamycin for selection purpose. PCR verification was employed for positive colony screening. When transforming plasmid pCP20 which expresses the FLP recombinase and loses at 42 °C, the Kan resistant gene on the chromosome was eliminated. The elimination of Kan^R and loss of pCP20 were verified by negative selection on LB plates along with PCR analysis. Finally, DNA sequencing was employed to confirm the gene knockout. At the end, chromosomal *arcA* deleted strain *E. coli* S17-1 was obtained.

Culture medium and growth conditions

During DNA manipulations and construction, all strains were cultured in LB medium containing 10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl at a pH 7.2 and 37 °C. When antibiotic selection was required, the medium was supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), or chloramphenicol (30 μg/ml). For PHA production, glucose (20 g/l) was added into the medium as carbon source.

Aerobic growth was conducted using a 500-ml baffled shake flask containing 50 ml LB under vigorous agitation at 200 rpm (HNY, HONOUR, Tianjin, China), while micro-aerobic growth was conducted using a 500-ml shake flask without baffles containing 100 ml culture medium at 100 rpm on the same rotary shaker.

Fluorescence assays and real-time quantitative PCR

Fluorescence assays were carried out using microplate reader (ABI-7500, Invitrogen, USA). Red fluorescent protein was measured to observe relative expression levels of different promoters in *E. coli* under micro-aerobic condition.

The total RNA of *E. coli* was isolated by RNAprep pure Cell/Bacteria Kit. The cDNA was synthesized using Fastquant RT Kit for mRNA analysis. Real-time PCR assay for mRNA was performed with SuperReal PreMix (SYBR Green), while 16S rRNA was used as the inner standard. All manipulations were carried out according to the specifications.

The calibration of the real-time quantitative PCR (RT-PCR) was conducted using 16S rRNA of *E. coli*. The primers (16S forward: CACACTGGAACTGAGACAC; 16S reverse: CTTCTTCTGCGGGTAACG) were used to amplify the calibration fragment. The length of the calibration part was less than 300 bp. On the other hand, the product of *phaC* was also less than 300 bp. Calculation was carried out based on reported method (Schmittgen and Livak 2008).

Study on cell growth and PHA production

Cells were harvested by centrifugation at 10,000×*g* for 10 min and washed with distilled water twice. CDW was measured after lyophilization over 10 h. Methanolysis was carried out by adding 2 ml CH_3OH/H_2SO_4 (100/3 vol) together with 1 g/l benzoic acid internal standard and 2 ml $CHCl_3$ to the samples,

and then incubated at 100 °C for 4 h. After cooling to room temperature and addition of 1 ml ddH_2O, the organic phase was analyzed by gas chromatography using a Spectra System P2000 (Thermo Separation, USA) (Sim et al. 1997) to determine the intracellular PHA content (Brandl et al. 1988). Pure PHB (Sigma, USA) was used as a standard sample.

Results

Screening for promoters inducible under micro-aerobic conditions

Ten potential low aeration induction promoters were cloned based on the *E. coli* Gene Expression Database (http://chase.ou.edu) (Table 1). As a comparison, *Vitreoscilla* hemoglobin promoter (P_{vgb}) inducible under micro-aerobic condition was used as a control. To quantitatively evaluate strengths of the 11 promoters, they were constructed upstream red fluorescent protein gene *rfp* to generate 11 plasmids termed PcI-RFP-$P_{promoter}$ (Table 1).

When *E. coli* Trans 109 was transformed with the 11 promoters, respectively, intensities of the RFP emission from the recombinant *E. coli* were in proportion to the relative expression levels (strength) of different promoters (Fig. 1). It was demonstrated that five recombinant strains harboring plasmids PcI-RFP-P_{slp}, PcI-RFP-P_{hya}, PcI-RFP-P_{dmsA}, PcI-RFP-P_{nirB}, and PcI-RFP-P_{vgb}, respectively, showed approximately twofold higher red fluorescence intensity compared with that of six other recombinants grown under micro-aerobic conditions (Fig. 1). No significant difference among the five strong strains in terms of RFP expression was observed. Thus, these five promoters, namely, P_{hya}, P_{dmsA}, P_{nirB}, P_{slp}, and P_{vgb}, were demonstrated to have similarly higher strength induced under micro-aerobic conditions.

These five strong micro-aerobic promoters, namely, P_{hya}, P_{dmsA}, P_{nirB}, P_{slp}, and P_{vgb}, were used to construct PHB production strains inducible under micro-aerobic conditions, respectively. They were inserted upstream the PHB synthesis operon *phaCAB* genes using *SmaI* and *BstB*I enzymes, respectively, into plasmid pBHR68, to generate five plasmids including pBHR-P_{hya}, pBHR-P_{dmsA}, pBHR-P_{nirB}, pBHR-P_{slp}, and pBHR-P_{vgb} (Tables 1 and 2). Subsequently, the five plasmids were transformed into *E. coli* Trans109, respectively, for evaluating PHB production under micro-aerobic conditions.

Under micro-aerobic conditions, recombinant *E. coli* harboring the native promoter P_{Re} grew to 2.3 g/l containing 61 % PHB after 48 h, better than all other recombinants containing micro-aerobic promoters except the one containing recombinant P_{vgb}, which grew to 2.5 g/l dry weight containing 68 % PHB, the highest among all recombinants (Table 2), suggesting that P_{vgb} was the strongest promoter for growth and for inducing PHB accumulation under micro-aerobic conditions. Therefore, *Vitreoscilla* hemoglobin promoter P_{vgb} was selected for further study.

Screening for the most suitable ***E. coli*** host for PHB accumulation under micro-aerobic conditions

Six commonly used *E. coli* strains containing PHB synthesis operon encoded in pBHR68 were investigated for their growth and PHB accumulation under micro-aerobic conditions (Table 3). *E. coli* S17-1 synthesized over 70 % PHB in 2.7 g/l CDW, the highest among all six tested strains, followed by *E. coli* Trans 109 with 2.6 g/l CDW containing 69 % PHB. Other four hosts accumulated 18–60 % PHB in their respective CDW. Based on this result, *E. coli* S17-1 was chosen as a host for further study.

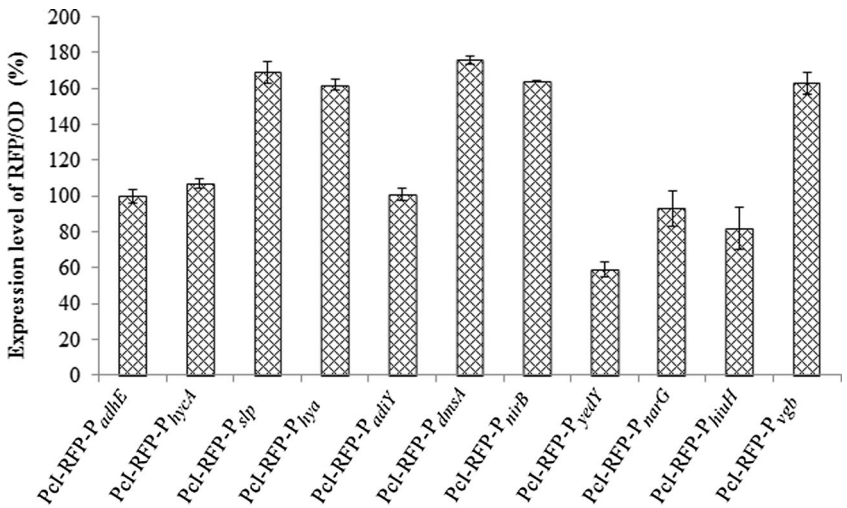

Fig. 1 RFP expression levels under different promoters in *E. coli* Trans 109 under micro-aerobic conditions. The relative expression of RFP was measured as the RFP levels/OD, and expression level of RFP under P_{adhE} was defined as 100 here. Data shown were the average values of three parallels studies. PcI-RFP-P_{adhE} represented *E. coli* Trans 109 harboring plasmid PcI-RFP-P_{adhE}, and the rest were named in the same way

Table 2 Cell growth and PHB production by *E. coli* Trans 109 harboring different plasmids under micro-aerobic conditions, respectively

Plasmids[a]	CDW (g/l)[b]	PHB content (wt%)[b]
pBHR68	2.31±0.08	60.55±0.52
pBHR-P_{hya}	1.64±0.09	36.25±0.45
pBHR-P_{dmsA}	1.02±0.06	25.20±0.71
pBHR-P_{nirB}	1.55±0.05	32.81±0.89
pBHR-P_{slp}	2.12±0.52	58.47±1.08
pBHR-P_{vgb}	2.52±0.52	68.48±1.15

[a] Cells were cultivated in LB medium containing 20 g/l glucose at 37 °C for 48 h

[b] Data are expressed as the *M*±SD, *M* refers to mean values and SD refers to standard deviations

Enhanced promoter P_{vgb} strength via cascaded P_{vgb} tandem repeats

Since P_{vgb} was found the strongest promoter under micro-aerobic conditions, and *E. coli* S17-1 the most suitable host for PHB accumulation, P_{vgb} was used to induce PHB synthesis in recombinant *E. coli* S17-1 grown under micro-aerobic conditions.

Recombinant *E. coli* S17-1 (pBHR-P_{vgb}) accumulated approximately 70 % PHB in 2.4 g/l CDW after 48 h growth under micro-aerobic conditions (Table 2). In contrast, recombinant *E. coli* S17-1 (pBHR-P_{vgb}) grew to 7.5 g/l CDW containing 62 % PHB under full aeration conditions as described in "Materials and methods." This showed that the micro-aerobic condition reduced cell growth from aerobic 7.5 g/l to micro-aerobic 2.4 g/l, even though micro-aerobic 70 % PHB was a bit higher than aerobic 62 % PHB.

To improve cell growth and PHB accumulation, plasmids harboring different tandem repeat numbers of *vgb* promoter (P_{vgb}), including 2, 4, 6, 8, and 10 P_{vgb} repeats, were constructed and named pBHR-P_{nvgb} (n=2, 4, 6, 8, and 10, respectively) (Table 1). The total length of *vgb* promoter (P_{vgb}) was less than 165 bp, allowing tandem assembly of up to 10 repeats. It was difficult to prepare repeats >10 P_{vgb} due to their self recombination effort.

When P_{vgb} tandem repeats reached eight, recombinant *E. coli* S17-1 (pBHR-P_{8vgb}) grew to 5.4 g/l CDW containing over 90 % PHB (Table 4 and Fig. 2). Obviously, increasing P_{vgb} tandem repeat number from two to eight improved PHB accumulation associated with CDW increases (Fig. 2). However, dramatic decreases on CDW and PHB accumulation were visible when P_{vgb} tandem repeats reached 10.

As the number of *vgb* promoter increases, the efficacy of gene expression level under the promoter control could also be improved. However, when the repeat number reaches a threshold value, the gene expression level could be down-regulated. As evidenced from real-time quantitative PCR, the mRNA transcription level of *phaC* was higher in the strain harboring P_{8vgb} than that having native promoter P_{Re} or P_{1vgb} (Fig. 3). During a 48-h shake flask study, the mRNA level of *phaC* in recombinant (pBHR-P_{8vgb}) was two- to fourfold higher than that of strains harboring pBHR-P_{1vgb} at least after 16 or 48 h. As the PHB production was related to the transcription level of *phaC*, this should be the reason why P_{8vgb} had the highest PHA content. When the repeats time of *vgb* promoter reached 10 (P_{10vgb}), the mRNA transcription level was reduced to the minimum value with poor cell growth and PHB accumulation (Fig. 3). Therefore, P_{8vgb} was selected for cell growth and PHA accumulation by recombinant *E. coli* S17-1 incubated under micro-aerobic condition.

ArcA deletion improved growth and PHB production of *E. coli* S17-1 (pBHR-P_{8vgb})

ArcAB and FNR systems regulate catabolic gene expressions, thus affecting cell growth under different oxygen conditions (Nikel et al. 2006). Bacteria can grow better under micro-aerobic conditions when gene *arcA* was deleted compared with its wild type (Nikel et al. 2006). The *arcA* mutant *E. coli* S17-1 Δ*arcA* (pBHR-P_{8vgb}) grew to a CDW of 6.3 g/l containing over 91 % PHB after 48-h shake flask

Table 3 Growth and PHB production by different *E. coli* strains harboring pBHR68 under micro-aerobic cultivation, respectively

E. coli strains[a]	CDW (g/l)[b]	PHB content (wt%)[b]
S17-1	2.65±0.34	70.22±1.23
Trans 1-T1	2.49±0.04	59.87±0.25
Trans 5α	2.01±0.03	54.01±1.05
Trans 109	2.57±0 .04	68.75±0.45
EC100D	1.17±0.10	28.20±1.21
DMT	1.06±0.01	18.12±0.85

[a] Cells were cultivated in LB medium containing 20 g/l glucose at 37 °C for 48 h

[b] Data are expressed as *M*±SD, *M* refers to mean values and SD refers to standard deviations

Table 4 Comparison of cell growth and PHB production between *E. coli* S17-1 and *E. coli* S17-1 Δ*arcA* under micro-aerobic conditions

E. coli strains[a]	CDW (g/l)[b]	PHB content (wt%)[b]
S17-1 (pBHR68)	2.65±0.34	70.22±1.23
S17-1Δ*arcA* (pBHR68)	3.79±0.02	75.01±3.33
S17-1 (pBHR-P_{8vgb})	5.37±0.31	90.02±1.28
S17-1Δ*arcA* (pBHR-P_{8vgb})	6.30±0.15	91.04±1.90

[a] Cells were cultivated in LB medium containing 20 g/l glucose at 37 °C for 48 h

[b] Data are expressed as *M*±SD, *M* refers to mean values and SD refers to standard deviations

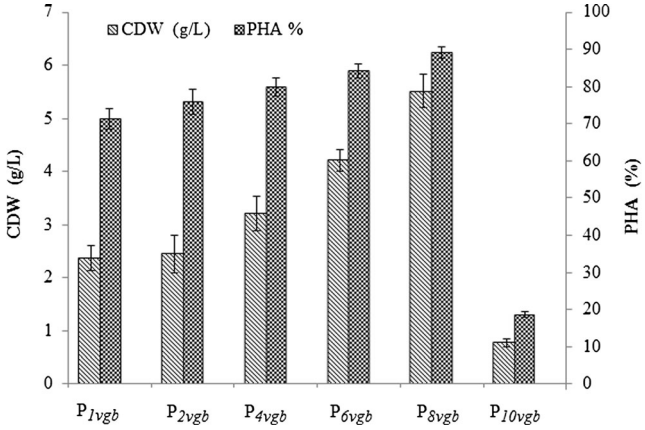

Fig. 2 Cell growth and PHA production by *E. coli* S17-1 harboring plasmid pBHR-P_{nvgb} under micro-aerobic conditions (n=1–10). Cells were cultivated in LB medium containing 20 g/l glucose at 37 °C for 48 h. Data shown were the average values of three parallels studies. P_{1vgb} represented *E. coli* S17-1 harboring plasmid pBHR-P_{1vgb} and so on

incubation under micro-aerobic conditions (Table 4). In contrast, all control strains produced significantly reduced CDW and PHB contents (Table 4). Remarkably, *E. coli* S17-1 Δ*arcA* (pBHR68) produced similar results containing 6.5 g/l CDW with 47 % PHB under full aeration conditions. These results showed a successful combination of strong micro-aerobic promoter, strain, and manipulation on aerobic respiration regulator *arcAB* can lead to better PHB formation under micro-aerobic conditions comparable or better to full aerobic growth.

Discussion

Li et al. (2012) used a uniquely designed in vitro assembling process to construct a series of *tac* promoter clusters. The transcription strength of these promoter clusters showed a stepwise enhancement with the increase of tandem repeats number until it reached the critical value of five. Application of the *tac* promoter clusters proved that it was efficient in enhancing PHB accumulation to 23.7 % PHB of the cell dry weight in batch cultivation. Therefore, it is interesting to use similar approach for enhancing oxygen uptake employing multiple *vgb* promoters.

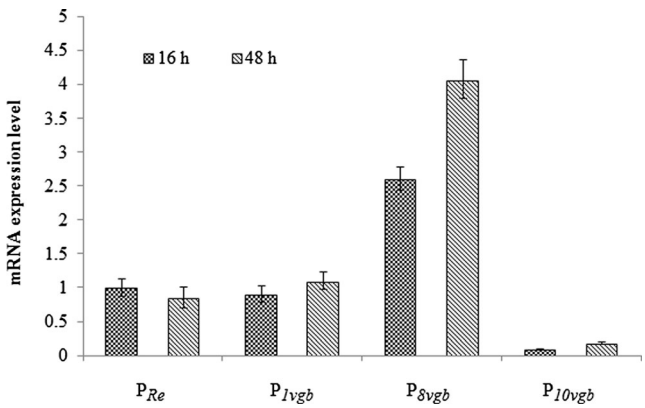

Fig. 3 mRNA transcription levels of *phaC* in *E. coli* S17-1 harboring different plasmids under micro-aerobic conditions. The total RNA of *E. coli* was isolated by RNAprep pure Cell/Bacteria Kit. The cDNA was synthesized using Fastquant RT Kit for mRNA analysis. Real-time PCR assay for mRNA was performed with SuperReal PreMix (SYBR Green), while 16S rRNA was used as the inner standard. The mRNA expression levels of *phaC* were shown in the *y*-axis. Data shown were the average values of three parallels studies

Among all the micro- or anaerobic promoters found on the *E. coli* Gene Expression Database (http://chase.ou.edu), 10 most promising ones were cloned and constructed upstream of RFP gene to allow evaluate promoter strengths based on expression of RFP in the recombinant (Table 1). Five promoters (including *vgb* promoter P_{vgb}) were found similarly strong in expressing RFP under micro-aerobic conditions (Fig. 1). They were selected to induce PHB production micro-aerobically (Table 2). All five promoters induced PHB production in different intensities, P_{vgb} was the strongest accumulating over 68 % PHB under micro-aerobic conditions compared with all other strong promoters inducing only 25–58 % PHB accumulation (Fig. 2). Therefore, P_{vgb} was chosen for further applications.

Among the six common *E. coli* strains studied, *E. coli* S17-1 stood out as the most capable one to produce PHB under micro-aerobic conditions (Table 3), as it synthesized over 70 % PHB in 2.7 g/l CDW while all other five strains accumulated less than 70 % PHB in 1.2–2.6 g/l CDW. However, *E. coli* S17-1 (pBHR-P_{vgb}) grew better under aerobic condition, producing over 7.5 g/l CDW containing 62 % PHB, significantly higher than 7.1 g/l CDW containing 47 % PHB by *E. coli* S17-1 (pBHR68). The results showed that P_{vgb} was useful both under micro-aerobic or aerobic conditions.

Efforts must be made so that both cell growth and PHB accumulation can be improved under micro-aerobic conditions compared with full aerobic ones. By assembling repeated P_{vgb} promoter in a tandem way, it was found that eight tandem repeated P_{vgb} created the strongest induction effect, leading to 5.7 g/l CDW, the highest cell dry weight, and over 90 % PHB accumulation (Fig. 2). The PHB accumulation was much high than 62 % accumulated under aerobic growth. The strongest P_{8vgb} was due to the strong mRNA transcription induced by the promoter itself (Fig. 3). More P_{vgb} assembly (such as P_{10vgb}) resulted in a dramatic reduction of induction intensity (Figs. 2 and 3).

Finally, PHB accumulation and cell growth were further improved when oxygen-responsive transcription factor ArcA (*arcA*) was deleted in the same recombinant *E. coli* S17-1 (pBHR-P_{8vgb}); the resulting strain *E. coli* S17-1Δ*arcA* (pBHR-P_{8vgb}) grew to 6.3 g/l CDW containing over 91 % PHB, very close to 6.5 g/l CDW produced by the control *E. coli* S17-1 Δ*arcA* (pBHR68) which accumulated only 47 % PHB. The results demonstrated that the combination of various possibilities could lead to similar growth between

aerobic and micro-aerobic conditions, yet PHB accumulation was always better under micro-aerobic conditions

In summary, the assembling of eight repeated *vgb* promoters formed a much stronger promoter able to improve PHB accumulation significantly. Combined with deletion of oxygen-responsive transcription factor ArcA (*arcA*), PHB accumulation improved further to over 91 % under micro-aerobic conditions. It thus becomes possible to produce PHB under less energy-demanding conditions, the micro-aerobic conditions, with more efficiency than under energy-intensive aeration conditions.

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