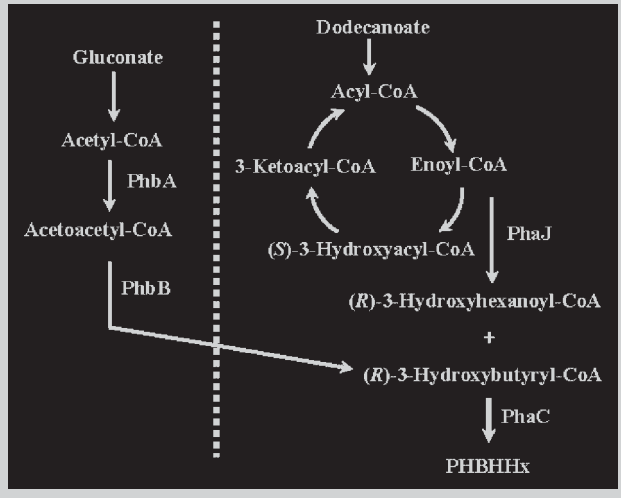
Summary: *Aeromonas hydrophila* 4AK4 was able to synthesize copolyesters consisting of 3-hydroxybutyrate (3HB) and about 15 mol-% 3-hydroxyhexanoate (3HHx) (PHBHHx) when grown in long chain fatty acids such as dodecanoate regardless of growth conditions. To regulate the unit fraction in PHBHHx, *phbA* and *phbB* genes encoding β-ketothiolase and acetoacetyl-CoA reductase in *Ralstonia eutropha*, were introduced into *A. hydrophila* 4AK4. When gluconate was used as cosubstrate of dodecanoate, the recombinant produced PHBHHx containing 3–12 mol-% 3HHx, depending on the gluconate concentration in media. *Vitreoscilla* hemoglobin gene, *vgb*, was also introduced into the above recombinant, resulting in improved PHBHHx content from 38 to 48 wt.-% in shake flask study. Fermentor studies also showed that increased gluconate concentration in medium containing dodecanoate promoted the recombinant strain harboring *phbA* and *phbB* genes to incorporate more 3HB unit into PHBHHx, resulting in reduced 3HHx fraction. Recombinant *A. hydrophila* harboring *phbA*, *phbB* and *vgb* genes demonstrated better PHBHHx productivity and higher conversion efficiency from dodecanoate to PHBHHx than those of the recombinant without *vgb* in fermentation study. Combined with the robust growth property and simple growth requirement, *A. hydrophila* 4AK4 appeared to be a useful organism for metabolic engineering.



Metabolic pathways of PHBHHx biosynthesis in recombinant *A. hydrophila* 4AK4 (pTG01) from cosubstrates.

Metabolic Engineering for the Production of Copolyesters Consisting of 3-Hydroxybutyrate and 3-Hydroxyhexanoate by *Aeromonas hydrophila*

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Introduction

Polyhydroxyalkanoates (PHA) are a family of intracellular biopolyesters which are accumulated by various microorganisms as carbon and energy storage materials.[1–3] PHA has material properties similar to thermoplastics, yet it can be produced from renewable carbon sources;[4] therefore, PHAs have been called Green Plastics.[5] Poly(3-hydroxybutyrate) (PHB) is the best characterized PHA, and its production in *Ralstonia eutropha* and other wild-type or recombinant microorganisms has been well-studied.[6–9] The PHB biosynthesis genes of *R. eutropha* form a single operon known as $phbCAB_{Re}$, which encodes PHB synthase (PhbC), β-ketothiolase (PhbA) and NADPH-dependent acetoacetyl-CoA reductase (PhbB).[10–13] A similar operon was also found in other bacteria.[14] Three types of PHA were reported to be produced in industrial scale, namely, poly-β-hydroxybutyrate (PHB),[15] the copolyester of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) (PHBV),[16,17] and the copolyester of 3-hydroxybutyrate and 3-hydroxyhexanoate (3HHx) (PHBHHx).[8] PHBHHx was found to have better physical properties[18] and biocompatibility than those of PHB and polylactic acid (PLA).[19–21]

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Many bacteria can synthesize PHB; however, only a few bacteria were reported to be able to synthesize PHBHHx. Among those bacteria, *Aeromonas hydrophila* strain 4AK4[8,22] and *Aeromonas caviae*[18,23] were the representative strains. *A. hydrophila* 4AK4 produced PHBHHx with 3HHx unit fraction over 12 mol-% (typically 12∼18 mol-%). The strain was unable to produce PHBHHx with 3HHx fraction below 10 mol-% regardless of growth conditions. However, PHBHHx containing less than 10 mol-% 3HHx was more suitable as melt-processible thermoplastic[24] and for some applications in areas of compostable single-layer films and compostable nonwoven fabrics.[25] Therefore, it would be highly desirable to produce PHBHHx with a controllable 3HHx fraction in the range lower than 10 mol-%.

Zhang et al.[26] studied the PHBHHx synthesis genetics in *A. hydrophila* 4AK4. It was found that the PHA synthase (PhaC) and (*R*)-specific enoyl-CoA hydratase (PhaJ) of *A. hydrophila* 4AK4 were very similar to the two corresponding enzymes in *A. caviae*.[27,28] This suggested that *A. hydrophila* 4AK4 and *A. caviae* may have similar PHBHHx biosynthesis pathways.

Due to its potential pathogenesis, *A. hydrophila* has not been reported on much for metabolic engineering purposes. This study aimed to change the PHBHHx metabolic pathway of *A. hydrophila* 4AK4 for synthesis of PHBHHx with less than 10 mol-% 3HHx. At the same time, *Vitreoscilla* hemoglobin gene *vgb*, which was able to promote PHB synthesis in recombinant *Escherichia coli*,[29] was also studied for its effect on PHBHHx production by the recombinant *A. hydrophila*.

Experimental Part

Bacterial Strains, Plasmids, and Growth Conditions

The following strains were used in this study: nonpathogenic *A. hydrophila* strain 4AK4[8,22] and *E. coli* S17-1 (*recA*; harbors the *tra* genes of plasmid RP4 in the chromosome; *proA*, *thi*-1).[30] *A. hydrophila* 4AK4 and *E. coli* were cultivated in Luria-Bertani (LB) medium or on LB agar plate at 30 and 37 °C, respectively. When necessary, kanamycin (50 μg/mL) was added. Plasmid pTG01 harboring *phbA* and *phbB* genes from *R. eutropha* H16[31] was derived from pBBR1MCS-2.[32] For the conjugational transfer of pTG01 into *A. hydrophila*, the plasmid was first introduced into *E. coli* S17-1 by electroporation; conjugation was then carried out in a spot mating as described by Friedrich et al.,[33] resulting in recombinant *A. hydrophila* 4AK4 (pTG01). Plasmid pVGAB containing *phbA*, *phbB* and *vgb* genes was constructed from pTG01 as described in Figure 1 and transformed into *A. hydrophila* 4AK4 by the above mentioned conjugation process.

Culture Conditions for PHA Production

For PHBHHx synthesis in *A. hydrophila* strains, overnight-cultivated inocula in LB medium were transferred into phosphorus-limited mineral salt (MS) medium (pH 7.2) containing 4 g/L dodecanoate as carbon source and supplemented with 1 g/L yeast extract. The cells were subsequently cultivated at 30 °C for 48 h on a rotating shaker (NBS Series 25D, New Brunswick, NJ, USA) at 200 rpm. The phosphorus-limited MS medium contained 3.5 g/L $Na_2HPO_4 \cdot 12\ H_2O$, 0.70 g/L KH_2PO_4, 2.0 g/L $(NH_4)_2SO_4$, 0.8 g/L $MgSO_4 \cdot 7\ H_2O$, 0.5 g/L KCl, 1.0 g/L NaCl, and 1 vol.-% trace element solution. The trace element solution (per liter of 1 mol/L HCl) consisted of 5 g Fe^{III}–NH_4–citrate, 2 g $CaCl_2 \cdot 2\ H_2O$, 10 mg $ZnSO_4 \cdot 7\ H_2O$, 3 mg $MnCl_2 \cdot 4\ H_2O$, 30 mg H_3BO_3, 20 mg $CoCl_2 \cdot 6\ H_2O$, 1 mg $CuSO_4 \cdot 5\ H_2O$, 2 mg $NiCl_2 \cdot 6\ H_2O$, and 3 mg $NaMoO_4 \cdot 2\ H_2O$. To maintain the stability of the plasmids in those recombinant strains, kanamycin (50 μg/mL) was added to both the inoculum culture and the phosphorus-limited MS medium. Wherever indicated, 0.2 g/L isopropyl-β-D-thiogalactopyranoside (IPTG) was added at 12 h, and sodium gluconate (0–12 g/L, referred to simply as gluconate) was added as the cosubstrate at 12 and 24 h during the cultivation.

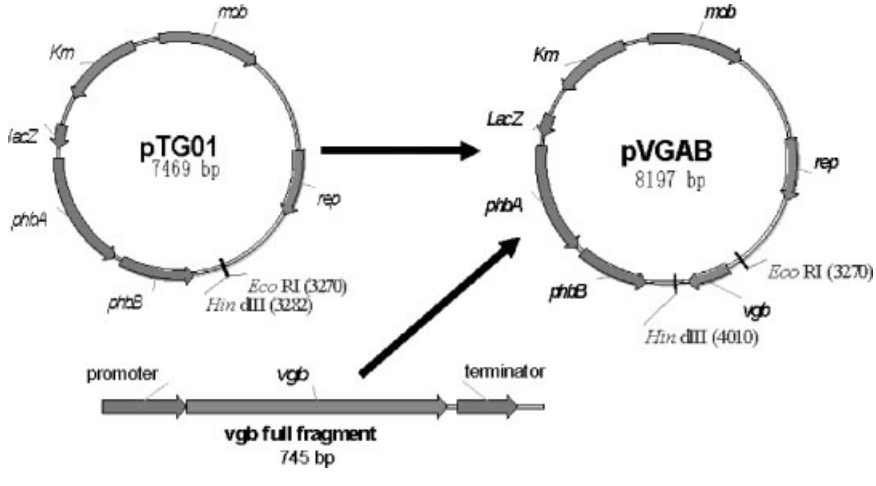

Figure 1. Construction of plasmid pVGAB.

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Analysis of PHBHHx

The cells were harvested by centrifugation, washed with ethanol and distilled water, and then lyophilized. The cellular PHBHHx and its unit composition were qualitatively and quantitatively analyzed by gas chromatography (GC) after methanolysis of lyophilized cells in chloroform.[34] GC analysis was carried out using Hewlett-Packard 6890 equipped with 30 m HP-5 capillary column.

Fermentor Study

Media for fermentor studies contained in g/L: 2.0 $Na_2HPO_4 \cdot 12\ H_2O$, 1.85 KH_2PO_4, 4 $(NH_4)_2SO_4$, 0.5 $MgSO_4 \cdot 7\ H_2O$, 1 yeast extract, and 1 vol.-% trace element solution. A 5 L NBS computer controlled fermentor (NBS Bioflo 3000, NJ, USA) containing 3 L culture was used for all fermentor studies. Fermentation was carried out at 30 °C and pH 6.5 in a dissolved oxygen concentration (DO) 15% of air saturation. Inoculum (5 vol.-%) was added at the beginning of fermentation. The inoculum culture was incubated for 12 h in LB medium.

Control studies (Figure 2A and B) were run with initial dodecanoate concentration of 10 g/L. Additional dodecanoate (80 g/L) and $(NH_4)_2SO_4$ (18 g/L) were fed into the fermentor vessel after 12 h cultivation.

To investigate the effect of gluconate and expression of *vgb* gene on PHBHHx production, the three following fermentations were carried out under the same fermentation conditions as described above except:

Fermentation C and E (Figure 2C and 2E): 10 g/L dodecanoate and 10 g/L gluconate were used as cosubstrates at the beginning. During fermentation, an additional 60 g/L dodecanoate, 60 g/L gluconate, and 18 g/L $(NH_4)_2SO_4$ were fed into the fermentor vessel after 12 h cultivation.

Fermentation D (Figure 2D): 20 g/L gluconate and 10 g/L dodecanoate were used as cosubstrates at the beginning. During fermentation, additional 30 g/L dodecanoate, 60 g/L gluconate, and 18 g/L $(NH_4)_2SO_4$ were fed into the fermentor vessel after 12 h cultivation.

Results and Discussion

Growth of Wild-Type and Recombinant A. hydrophila 4AK4 Harboring phbA and phbB Genes in Shake Flasks

Wild-type *A. hydrophila* 4AK4 produced 36 wt.-% PHBHHx consisting of 17 mol-% 3HHx when grown in dodecanoate. When gluconate was used as a cosubstrate of dodecanoate, the cell reduced PHBHHx and 3HHx fraction to 21 wt.-% and 14 mol-%, respectively (Table 1). In comparison, the recombinant synthesized 47 wt.-% PHBHHx consisting of 12 mol-% 3HHx from dodecanoate, 51 wt.-% PHBHHx, and 6 mol-% 3HHx from mixed substrates of dodecanoate and gluconate when no IPTG was added. Increased gluconate concentration in the growth culture resulted in a decreased 3HHx fraction in PHBHHx which was reduced from 12 mol-% in the absence of gluconate to 8, 6, and 3 mol-% in the presence of 4, 8, and 12 g/L gluconate, respectively.

The *phbA* and *phbB* genes in pTG01 were under the control of *lac* promoter; therefore, the effect of IPTG induction on the PHBHHx composition was studied. It was interesting to note that IPTG addition did not seem to affect the 3HHx fraction much. Regardless of the presence or absence of IPTG, the recombinant strain produced 12 mol-% 3HHx when no gluconate was added, and 6 mol-% 3HHx when 8 g/L gluconate was added.

Growth of Wild-Type Strain and Recombinant A. hydrophila 4AK4 Harboring phbA, phbB and vgb Genes in Shake Flasks

Wild-type *A. hydrophila* 4AK4, its recombinant harboring *phbA* and *phbB* genes, and recombinant carrying *phbA*, *phbB* and *vgb* genes produced 33, 38, and 48 wt.-% PHBHHx in their cell dry weight (CDW), with the two recombinants accumulating approximately 9 mol-% 3HHx compared with 13 mol-% 3HHx in PHBHHx synthesized by the wild-type strain (Table 2). It appeared that the recombinant *A. hydrophila* 4AK4 (pVGAB) accumulated PHBHHx more effectively than the wild-type strain and its recombinant harboring pTG01 did. This is indicated by the at least 27% increase in PHBHHx content produced by the *vgb* containing recombinant compared with those without *vgb* (Table 2).

Growth of Wild-Type, Recombinant A. hydrophila 4AK4 Harboring phbA and phbB and Recombinant A. hydrophila 4AK4 Harboring phbA, phbB and vgb Genes in a Fermentor

Wild-type *A. hydrophila* 4AK4 synthesized about 55 wt.-% PHBHHx consisting of 14 mol-% 3HHx in its 40 g/L CDW after 42 h growth in dodecanoate (Figure 2A). Within 42 h, recombinant *A. hydrophila* 4AK4 (pTG01) produced about 51 wt.-% PHBHHx containing 9 mol-% 3HHx in its 33 g/L CDW when grown in dodecanoate in the absence of gluconate (Figure 2B). The 3HHx fraction in PHBHHx produced by the recombinant was further reduced to about 7 and 5 mol-% when the weight ratio of dodecanoate to gluconate was 1:1 and 1:2, respectively (Figure 2C and 2D). Although the 3HB unit fraction in PHBHHx was increased by the presence of cosubstrate gluconate, CDW was reduced to 33 g/L when the weight ratio of dodecanoate to gluconate was 1:1, and 21 g/L and the weight ratio was 1:2 (Table 3).

Under similar conditions, recombinant *A. hydrophila* 4AK4 (pVGAB) produced similar amounts of PHBHHx, the 3HHx fraction, and CDW to those in *A. hydrophila* 4AK4 (pTG01). However, it appeared that the recombinant *A. hydrophila* 4AK4 harboring *phbA*, *phbB* and *vgb* genes increased copolymer productivity and conversion efficiency from dodecanoate to PHBHHx (Figure 2E and Table 3).

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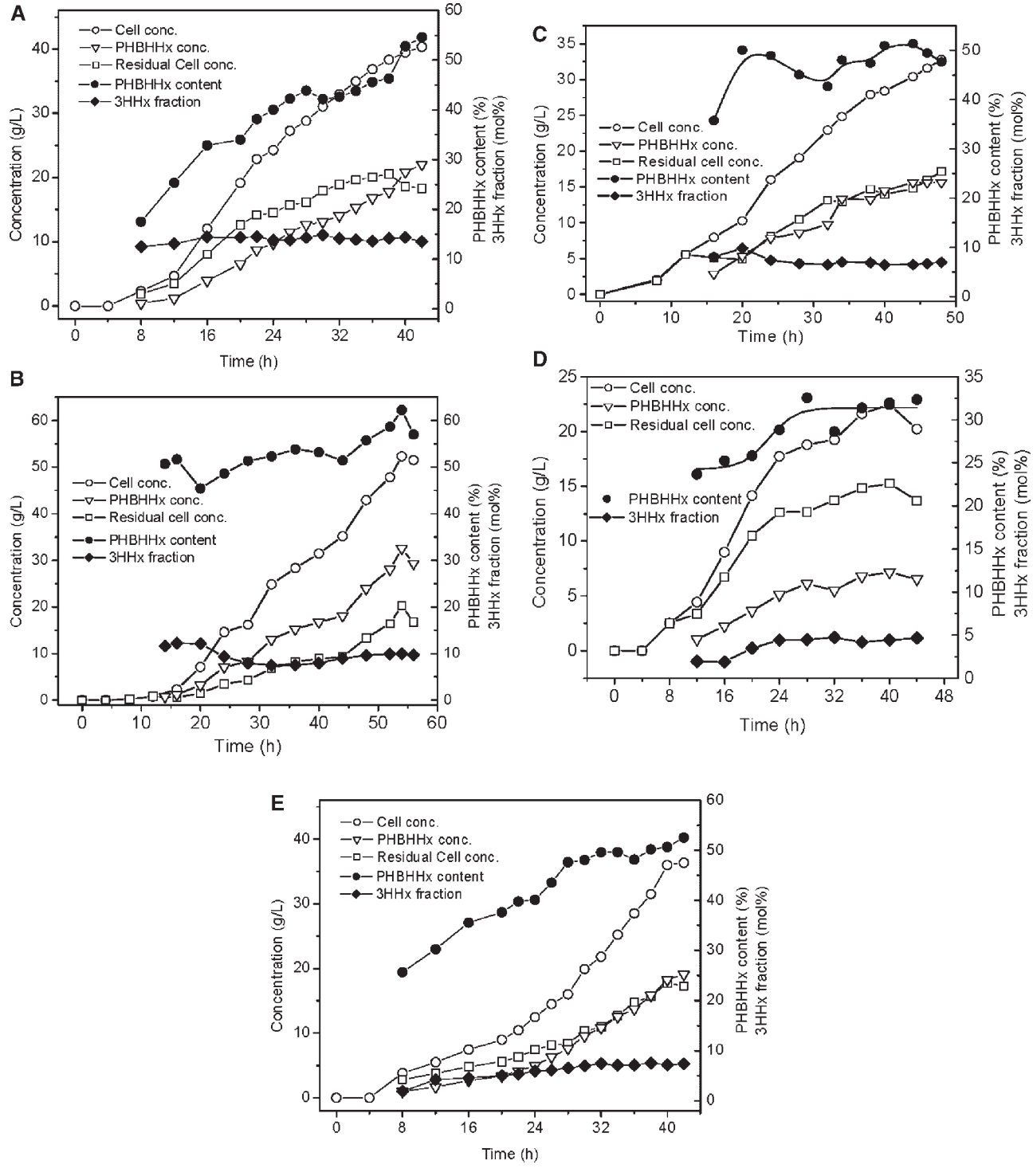

Figure 2. Time profiles of cell concentration, PHBHHx concentration, PHBHHx content, residual cell concentration (CDW minus PHBHHx concentration), and 3HHx molar faction: wild-type *A. hydrophila* 4AK4 (A) and recombinant *A. hydrophila* 4AK4 (pTG01) (B) grown in dodecanoate as sole substrate; *A. hydrophila* 4AK4 (pTG01) in cosubstrates dodecanoate and gluconate (1:1) (C); *A. hydrophila* 4AK4 (pTG01) in cosubstrates (1:2) (D); *A. hydrophila* 4AK4 (pVGAB) in cosubstrates (1:1) (E).

In all five fermentation studies, the 3HHx unit fraction of PHBHHx remained relatively stable after 24 h of cell growth. Unlike wild-type strain and recombinant *A. hydrophila* (pVGAB), in which PHBHHx content was in close association with CDW (Figure 2A and 2E), recombinant *A. hydrophila* (pTG01) produced relatively stable PHBHHx in CDW (Figure 2B, 2C, and 2D). During the fermentation, foaming was only a problem in dodecanoate. The presence

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Table 1. Production of PHBHHx by wild-type *A. hydrophila* 4AK4 and its recombinant harboring *phbA* and *phbB* genes.

Strains	IPTG	Gluconate	CDW	PHA/CDW	3HHx/PHA	PHBHHx
		g/L	g/L	wt.-%	mol-%	g/L
4AK4[a)]	–	0	1.94 ± 0.02	35.64 ± 0.36	16.85 ± 0.07	0.69 ± 0.01
4AK4 (pTG01)	–	0	1.92 ± 0.15	46.75 ± 5.24	12.29 ± 1.38	0.90 ± 0.17
4AK4 (pTG01)	+	0	2.20 ± 0.20	41.45 ± 2.84	12.25 ± 0.68	0.91 ± 0.14
4AK4	–	8	2.25 ± 0.96	20.61 ± 1.47	13.84 ± 0.75	0.45 ± 0.16
4AK4 (pTG01)	–	8	2.55 ± 0.53	50.65 ± 2.36	5.71 ± 0.20	1.29 ± 0.30
4AK4 (pTG01)	+	8	2.66 ± 0.32	39.71 ± 1.24	5.72 ± 0.11	1.06 ± 0.16
4AK4 (pTG01)	+	4	2.79 ± 0.54	41.09 ± 1.29	7.69 ± 0.07	1.15 ± 0.23
4AK4 (pTG01)	+	12	3.09 ± 0.29	37.52 ± 1.84	2.80 ± 0.39	1.16 ± 0.16

[a)] Wild-type *A. hydrophila* 4AK4 and its recombinant harboring *phbA* and *phbB* genes were cultivated in phosphorus-limited mineral salt medium containing 4 g/L dodecanoate and 1 g/L yeast extract for 48 h. Incubation was carried out at 30 °C and 200 rpm. IPTG (0.2 g/L) was added at 12 h when indicated.

Table 2. Production of PHBHHx by wild-type *A. hydrophila* 4AK4 and its recombinant strains harboring plasmids pTG01 or pVGAB in shake flask.

Strains	CDW	PHA/CDW	3HHx/PHA	PHBHHx
	g/L	wt.-%	mol-%	g/L
4AK4[a)]	3.12 ± 0.20	33.2 ± 2.0	12.5 ± 0.5	1.04 ± 0.07
4AK4 (pTG01)	3.69 ± 0.20	38.0 ± 1.6	9.4 ± 0.3	1.40 ± 0.08
4AK4 (pVGAB)	3.54 ± 0.19	48.2 ± 1.0	9.2 ± 0.5	1.71 ± 0.09

[a)] Wild-type *A. hydrophila* 4AK4, recombinant *A. hydrophila* 4AK4 (pTG01) harboring *phbA* and *phbB* genes, and recombinant *A. hydrophila* 4AK4 (pVGAB) harboring *phbA*, *phbB* and *vgb* genes were cultivated in mineral salt medium containing 15 g/L dodecanoate, 1 g/L yeast extract, and 3 g/L $(NH_4)_2SO_4$ for 48 h. Incubation was carried out at 30 °C and 200 rpm.

of gluconate helped reduce foaming over the entire fermentation period.

The unit fraction of a PHA copolyester lies on the substrate specificity of PHA synthase, monomer-supplying enzymes, carbon sources in medium, and other cultivation conditions. For a specific PHA synthase, the unit fraction will mainly be dependent on monomers available in vivo, which are dependent on both monomer-supplying enzymes and carbon sources. In view of this, the controlled synthesis of copolyester consisting of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV) in *R. eutropha* is a good example. Holmes et al.[35] described the synthesis of the copolyester PHBV, in which the 3HV fraction could be controlled by the concentration of propionate in the growth medium. By

Table 3. Production of PHBHHx by wild-type *A. hydrophila* 4AK4 and its recombinant strains harboring plasmids pTG01 or pVGAB in a 5 L NBS fermentor.

Strains	Substrate[a)]	CDW[b)]	PHA/CDW	3HHx/PHA	P_{PHBHHx}[c)]	PHA/DD[d)]
	g/L	g/L	wt.-%	mol-%	g/L/h	g/g
4AK4	DD 80	40.4	54.6	14.4	0.525	0.276
4AK4 (pTG01)	DD 80	32.7	51.0	8.7	0.397	0.208
4AK4 (pTG01)	DD 60 Glna 60	32.8	52.0	6.7	0.355	0.284
4AK4 (pTG01)	DD30 Glna 60	21.3	31.0	4.5	0.157	0.220
4AK4 (pVGAB)	DD 60 Glna 60	38.4	52.0	7.4	0.475	0.333

[a)] Abbreviation: DD: dodecanoate; Glna: sodium gluconate. DD 80 and DD 60 Glna 60 indicate that total 80 g/L dodecanoate or a mixture of 60 g/L dodecanoate and 60 g/L gluconate were fed to the fermentor after 12 h cultivation, respectively.
[b)] CDW: cell dry weight.
[c)] P_{PHBHHx}: PHBHHx productivity.
[d)] PHA/DD: conversion efficiency from dodecanoate to PHBHHx.

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varying the ratio of acetate to propionate in the cultures, *R. eutropha* synthesized PHBV containing 0–45 mol-% of the 3HV unit.[36]

To increase the 3HB unit fraction in PHBHHx, one has to increase the 3HB-CoA precursor supply for PHA synthase. However, the two key enzymes, β-ketothiolase (PhbA) and acetoacetyl-CoA reductase (PhbB) that supply 3HB-CoA precursors, were found to have low activities in wild-type *A. hydrophila* 4AK4. That restrained the synthesis of 3HB-CoA precursor from acetyl-CoA. To increase the 3HB-CoA precursor supply for PHBHHx synthesis, an additional monomer-supplying pathway containing *phbA* and *phbB* genes encoding PhbA and PhbB from *R. eutropha* was engineered into *A. hydrophila*. In the newly constructed pathway, the 3HB-CoA precursor could be supplied from both enoyl-CoA, the intermediates of β-oxidation, by the action of (*R*)-specific enoyl-CoA hydratase and acetyl-CoA by the joint action of PhbA and PhbB. Gluconate could be used as the substrate for the synthesis of additional 3HB-CoA precursor from acetyl-CoA (Figure 3). The successful construction of the 3HB monomer-supplying pathway into *A. hydrophila* 4AK4 and the presence of gluconate in medium led to an increased 3HB fraction accompanied by the reduced 3HHx fraction in PHBHHx (Table 1 and 2). In the range studied in this paper, a higher gluconate concentration clearly led to a higher 3HB fraction and a lower 3HHx fraction in PHBHHx. The result could be attributed to the fact that more gluconate produced more acetyl-CoA, which was used for the synthesis of 3HB-CoA precursor, resulting in higher 3HB fraction and lower 3HHx fraction. It appeared that gluconate concentration can be used as a parameter to control the unit fraction of PHBHHx.

Vitreoscilla haemoglobin (VHb) encoded by the *vgb* gene, an oxygen-binding protein, allows the bacteria to grow aerobically even under microaerophilic conditions. It was reported that expression of the *vgb* gene in *E. coli* positively affected volumetric transfer coefficient of the recombinant organism.[29] Since PHBHHx production on dodecanoate involved β-oxidation of the substrate, the introduction of the *vgb* gene could be helpful for the copolymer synthesis. Both the shake flask and the fermentor studies showed that PHBHHx production was more efficient in recombinant *A. hydrophila* harboring *vgb* genes than those without *vgb* (Table 2 and 3).

To prove the results obtained from shake flasks, fermentor studies were conducted in a 5 L NBS computer controlled vessel. The 3HHx fraction in PHBHHx decreased from 12–18 mol-% produced by wild-type strain in dodecanoate[8,22] to 9–10 mol-% in dodecanoate, 6–7 mol-% in dodecanoate and gluconate (1:1), and 4–5 mol-% in dodecanoate and gluconate (1:2) produced by the recombinant strain harboring *phbA* and *phbB* genes. The fermentor results agreed well with the shake flask ones: increased gluconate concentration in the culture reduced the 3HHx unit fraction in PHBHHx produced by the recombinant.

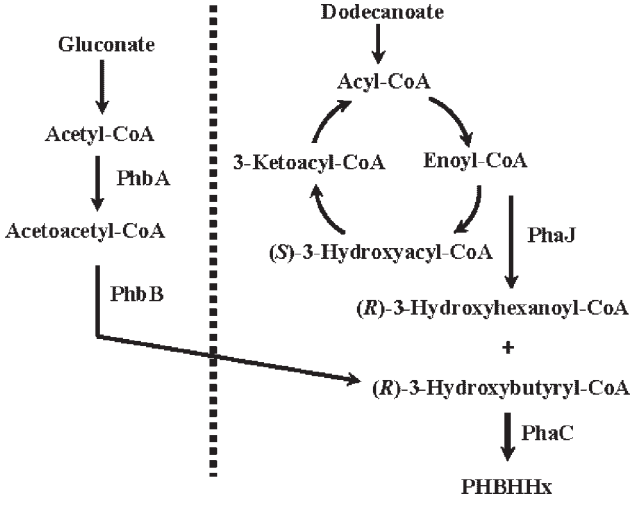

Figure 3. Metabolic pathways of PHBHHx biosynthesis in recombinant *A. hydrophila* 4AK4 (pTG01) from cosubstrates. PhbA: β-ketothiolase; PhbB: NADPH-dependent acetoacetyl-CoA reductase; PhaC: PHA synthase; PhaJ: (*R*)-specific enoyl-CoA hydratase. The right side of the dashed line was the native PHBHHx synthesis pathway in wild-type *A. hydrophila*, which was similar to that in *A. caviae* proposed by Fukui and Doi (1997). The left side of the dashed line was the newly constructed pathway for supplying additional 3HB-CoA precursor from gluconate for PHBHHx synthesis in recombinant *A. hydrophila* 4AK4 (pTG01).

Conclusion

Due to pathogenesis of many *A. hydrophila* strains, not much work has been done to investigate the pathway engineering of this organism, although the strain was used for large-scale production of PHBHHx. It was found that foreign genes could be successfully expressed in this organism without IPTG induction. Gluconate could be used as a parameter to regulate PHBHHx composition produced by *A. hydrophila* (pTG01) harboring *phbA* and *phbB* genes. The introduction of the *vgb* gene into the *A. hydrophila* improved the PHBHHx production efficiency. Combined with the robust growth property, simple growth requirement and convenience for genetic engineering, the nonpathogenic *A. hydrophila* strain 4AK4 may be developed into a very useful organism for industrial application.

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[1] Y. Doi, ''*Microbial Polyesters*'', VCH, New York 1990.
[2] A. Steinbüchel, *Macromol. Biosci.* **2001**, *1*, 1.
[3] L. L. Madison, G. W. Huisman, *Microbiol. Mol. Biol. Rev.* **1999**, *63*, 21.
[4] K. Sudesh, H. Abe, Y. Doi, *Prog. Polym. Sci.* **2000**, *25*, 1503.
[5] S. Y. Lee, *Biotechnol. Bioeng.* **1996**, *49*, 1.
[6] W. S. Ahn, S. J. Park, S. Y. Lee, *Appl. Environ. Microbiol.* **2000**, *66*, 3624.
[7] J. Kidwell, H. E. Valentin, D. Dennis, *Appl. Environ. Microbiol.* **1995**, *61*, 1391.
[8] G. Q. Chen, G. Zhang, S. J. Park, S. Y. Lee, *Appl. Microbiol. Biotechnol.* **2001**, *57*, 50.
[9] S. N. Chohan, L. Copeland, *Appl. Environ. Microbiol.* **1998**, *64*, 2859.
[10] O. P. Peoples, A. J. Sinskey, *J. Biol. Chem.* **1989**, *264*, 15298.
[11] O. P. Peoples, A. J. Sinskey, *J. Biol. Chem.* **1989**, *264*, 15293.
[12] S. C. Slater, W. H. Voige, D. E. Dennis, *J. Bacteriol.* **1988**, *170*, 4431.
[13] P. Schubert, A. Steinbüchel, H. G. Schlegel, *J. Bacteriol.* **1988**, *170*, 5837.
[14] J. I. Choi, S. Y. Lee, K. Han, *Appl. Environ. Microbiol.* **1998**, *64*, 4897.
[15] O. Hrabak, *FEMS Microbiol. Rev.* **1992**, *103*, 251.
[16] G. Q. Chen, K. H. Koenig, R. M. Lafferty, *Antonie van Leeuwenhoek* **1991**, *60*, 61.
[17] D. Byrom, *FEMS Microbiol. Rev.* **1992**, *103*, 247.
[18] Y. Doi, S. Kitamura, H. Abe, *Macromolecules* **1995**, *28*, 4822.
[19] X. S. Yang, K. Zhao, G. Q. Chen, *Biomaterials* **2002**, *23*, 1391.
[20] Y. Deng, K. Zhao, X. F. Zhang, P. Hu, G. Q. Chen, *Biomaterials* **2002**, *23*, 4049.
[21] K. Zhao, Y. Deng, J. C. Chen, G. Q. Chen, *Biomaterials* **2003**, *24*, 1041.
[22] S. H. Lee, D. H. Oh, W. S. Ahn, Y. Lee, J. Choi, S. Y. Lee, *Biotechnol. Bioeng.* **2000**, *67*, 240.
[23] E. Shimamura, K. Kasuya, G. Kobayashi, T. Shiotani, Y. Shima, Y. Doi, *Macromolecules* **1994**, *27*, 878.
[24] K. P. Caballero, S. F. Karel, R. A. Register, *Int. J. Biol. Macromol.* **1995**, *17*, 86.
[25] U.S.A. 5,502,116 (1996), The Procter & Gamble Company, inv.: I. Noda.
[26] G. Zhang, PhD Thesis, Tsinghua University, Beijing, China, 2002.
[27] T. Fukui, Y. Doi, *J. Bacteriol.* **1997**, *179*, 4821.
[28] T. Fukui, N. Shiomi, Y. Doi, *J. Bacteriol.* **1998**, *180*, 667.
[29] H. M. Yu, Y. Shi, Y. P. Zhang, S. L. Yang, Z. Y. Shen, *FEMS Microbiol. Lett.* **2002**, *214*, 223.
[30] R. Simon, U. Priefer, A. Pühler, *Bio/Technology* **1983**, *1*, 784.
[31] K. Zhao, G. Tian, Z. Zheng, J. C. Chen, G. Q. Chen, *FEMS Microbiol. Lett.* **2003**, *218*, 59.
[32] M. E. Kovach, P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop, II, K. M. Peterson, *Gene* **1995**, *166*, 175.
[33] B. Friedrich, C. Hogrefe, H. G. Schlegel, *J. Bacteriol.* **1981**, *147*, 198.
[34] W. N. He, W. D. Tian, G. Zhang, G. Q. Chen, Z. M. Zhang, *FEMS Microbiol. Lett.* **1988**, *169*, 45.
[35] U.S.A. 4,477,654 (1984), Imperial Chemical Industries PLC, invs.: P. A. Holmes, S. H. Collins, L. F. Wright.
[36] Y. Doi, M. Kunioka, Y. Nakamura, K. Soga, *Macromolecules* **1987**, *20*, 2988.

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