Involvement of the AtoSCDAEB regulon in the high molecular weight poly-(R)-3-hydroxybutyrate biosynthesis in phaCAB(+) Escherichia coli

ARTICLE *in* METABOLIC ENGINEERING · MARCH 2012

Impact Factor: 6.77 · DOI: 10.1016/j.ymben.2012.03.010 · Source: PubMed

3 AUTHORS:

Evaggelos C Theodorou
Aristotle University of Thessaloniki
6 PUBLICATIONS **53** CITATIONS
SEE PROFILE

Marina C Theodorou
Harvard University
12 PUBLICATIONS **125** CITATIONS
SEE PROFILE

Dimitrios A Kyriakidis
Aristotle University of Thessaloniki
141 PUBLICATIONS **2,360** CITATIONS
SEE PROFILE

Metabolic Engineering 14 (2012) 354–365

Contents lists available at SciVerse ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Involvement of the AtoSCDAEB regulon in the high molecular weight poly-(R)-3-hydroxybutyrate biosynthesis in *phaCAB*$^+$ *Escherichia coli*

Evaggelos C. Theodorou, Marina C. Theodorou, Dimitrios A. Kyriakidis *

Laboratory of Biochemistry, Department of Chemistry, Aristotle University of Thessaloniki, Thessaloniki GR-54124, Greece

ARTICLE INFO

Article history:
Received 24 November 2011
Received in revised form
14 March 2012
Accepted 19 March 2012
Available online 30 March 2012

Keywords:
AtoSC
atoDAEB
Poly-(*R*)-3-hydroxybutyrate
Two-component system
β-Oxidation
Fatty acid biosynthesis

ABSTRACT

AtoSC two-component system plays a pivotal role in many regulatory indispensable *Escherichia coli* processes. AtoSCDAEB regulon, comprising the AtoSC system and the *atoDAEB* operon, regulates the short-chain fatty acids catabolism. We report here, that AtoSC up-regulates the high-molecular weight PHB biosynthesis, in recombinant *phaCAB*$^+$ *E. coli*, with the *Cupriavidus necator phaCAB* operon. PHB accumulation was maximized upon the acetoacetate-mediated induction of AtoSC, under glucose 1% w/v, resulting in a yield of 1.73 g/l with a biopolymer content of 64.5% w/w. The deletion of the *atoSC* locus, in the Δ*atoSC* strains, resulted in a 5 fold reduction of PHB accumulation, which was restored by the extrachromosomal introduction of the AtoSC system. The deletion of the *atoDAEB* operon triggered a significant decrease in PHB synthesis in Δ*atoDAEB* strains. However, the acetoacetate-induced AtoSC system in those strains increased PHB to 1.55 g/l, while AtoC expression increased PHB to 1.4 g/l upon acetoacetate. The complementation of the Δ*atoDAEB* phenotype was achieved by the extrachromosomal introduction of the atoSCDAEB regulon. The individual inhibition of β-oxidation and mainly fatty-acid biosynthesis pathways by acrylic acid or cerulenin respectively, reduced PHB biosynthesis. Under those conditions the introduction of the *atoSC* locus or the atoSCDAEB regulon was capable to up-regulate the biopolymer accumulation. The concurrent inhibition of both the fatty acids metabolic pathways eliminated PHB production. PHB up-regulation in *phaCAB*$^+$ *E. coli*, by AtoSC signaling through *atoDAEB* operon and its participation in the fatty acids metabolism interplay, provide additional perceptions of AtoSC critical involvement in *E. coli* regulatory processes towards the biotechnologically improved polyhydroxyalkanoates biosynthesis.

1. Introduction

High-molecular-weight poly-(R)-3-hydroxyalkanoates (PHA) are natural polyesters that accumulate in numerous microorganisms as intracellular carbon- and energy-storage materials under nutrient-limiting conditions with excess in a carbon source (Jung et al., 2011) and are extensively used in biotechnological and medical applications (Grage et al., 2009). Many naturally PHA producing bacteria, including *Cupriavidus necator* (Schubert et al., 1988; Peplinski et al., 2010) can synthesize a wide PHA range depending on their inherent PHA biosynthetic enzymes, the carbon source, the cultivation conditions or the employed metabolic engineering (Rehm, 2003; Li et al., 2010; Jung et al., 2011). Metabolic engineering has been extensively used to manipulate natural PHA pathways in order to increase product yields from metabolic networks and improve phenotypic responses to environmental cues, as well as to introduce non-native *pha* pathways into host cells were the regulatory interactions of *pha* and native systems are considered as part of the design principles (Tsao et al., 2010; Tyo et al., 2010; Jung et al., 2011).

E. coli, is a non-PHA-accumulating bacterium (Madison and Huisman, 1999), proved however, as an advantageous host for biotechnologically valuable PHA production upon recombination with the *pha* genetic locus encoding the PHA biosynthetic enzymes from the natural producers using related or unrelated carbon sources (Klinke et al., 1999; Park et al., 2004; Li et al., 2007, 2010; Zhou et al., 2011). High molecular weight poly-(R)-3-hydroxybutyrate (PHB), the abundantly studied member of PHA, has widely been produced in recombinant *phaCAB*$^+$ *E. coli* harboring the *C. necator phaCAB* operon (Schubert et al., 1988; Fidler and Dennis, 1992). The biosynthetic pathway is initiated by the condensation of two acetyl-CoA molecules to produce acetoacetyl-CoA, which is catalyzed by the enzyme β-ketothiolase (PhaA). Acetoacetyl-CoA is then reduced to (R)-3-hydroxybutyryl-CoA by the NADPH-dependent acetoacetyl-CoA reductase (PhaB). Finally, PHB is synthesized by the function of PHB synthase (PhaC) (Schubert et al., 1988; Fidler and Dennis, 1992). *E. coli* [*fadR*

Abbreviations: atoSC$^+$, genetic locus encoding the AtoS and AtoC proteins; *fadR*, genetic locus encoding FadR protein; HK, histidine kinase; CDW, dry cell weight; cPHB, complexed poly-(R)-3-hydroxybutyrate; PHA, high-molecular-weight poly-(R)-3-hydroxyalkanoates; PHB, high molecular weight storage poly-(R)-3-hydroxybutyrate; RCM, residual cell mass; RR, response regulator; TCS, two-component system; A_{600}, optical absorption at 600 nm

* Corresponding author. Fax: +30 2310997689.
E-mail addresses: evatheod@physics.auth.gr (E.C. Theodorou), mtheod80@yahoo.com (M.C. Theodorou), kyr@chem.auth.gr (D.A. Kyriakidis).

1096-7176/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ymben.2012.03.010

atoC(Con)], expressing the *atoC*(*Con*)-encoded active AtoC form, have been extensively used for PHA biosynthesis, including PHB (Slater et al., 1992; Rhie and Dennis, 1995; Dennis et al., 1998; Fukui et al., 1999; Liu and Steinbüchel, 2000; Park et al., 2001; Matsumoto et al., 2009).

AtoC comprises the response regulator of the AtoSC two-component signal transduction system in *E. coli* (Kyriakidis and Tiligada, 2009). Adaptive microbial signal transduction involves a multifaceted regulated phosphotransfer mechanism that comprises structural rearrangements of sensor histidine kinases (HK) upon ligand-binding and phosphorylation-induced conformational changes in response regulators (RR) of versatile two-component systems (TCS). Multilayered TCSs networks play a key role in bacterial biochemistry (Kyriakidis and Tiligada, 2009; García Vésconi et al., 2010). AtoSC TCS in *E. coli*, consisting of the AtoS HK and the AtoC RR plays a pivotal role in many regulatory indispensable processes. It constitutes together with *atoDAEB* operon, the atoSCDAEB regulon, and regulates growth on short-chain fatty acids through the transcriptional activation of the *atoDAEB* operon (Kyriakidis and Tiligada, 2009). AtoSC signaling also regulates a number of indispensable *E. coli* processes, including motility and chemotaxis regulation (Theodorou et al., 2011c), as well as the biosynthesis and intracellular distribution regulation of the complexed short-chain poly-(R)-3-hydroxybutyrate (cPHB) (Kyriakidis and Tiligada, 2009).

cPHB, is an inherent *E. coli* macromolecule of low molecular weight, synthesized by native *E. coli* enzymes and is considered as a member of the biodegradable high-molecular weight PHAs family (Reusch et al., 2002). However, only many physiological roles have been attributed to *E. coli* cPHB, such as Ca^{2+} homeostasis through the non-proteinaceous complexes of PHB-polyphosphate-Ca^{2+}, acting as voltage-gated Ca^{2+} channels, competence for genetic transformation, protection of the complexed proteins from proteolysis, participation in DNA organization, and modification of the sorting signal of the OmpA protein (Reusch et al., 2002; Xian et al., 2007). Its regulation by AtoSC TCS is achieved through its direct effects on *atoDAEB* operon (Theodorou et al., 2006) as well as according to recent studies through its involvement in fatty acids metabolism (Theodorou et al., 2011a). AtoSC-mediated signal transduction has been associated with acetoacetate (Theodorou et al., 2006), spermidine (Theodorou et al., 2007), or intermediate metabolic compounds of the short-chain fatty acid pathway (Theodorou et al., 2011b). It has been also correlated with the biogenic amine histamine and the mast cell degranulating calmodulin inhibitor compound 48/80 (C48/80) (Kyriakidis et al., 2008), possibly in an extracellular Ca^{2+}-mediated manner (Theodorou et al., 2009).

cPHB regulation by the AtoSC system in *E. coli*, in combination with the use of *pha*$^+$ *E. coli atoC*(*Con*) strains for PHA production, led this investigation towards the elucidation of AtoSC TCS involvement in the PHB biosynthesis regulation in recombinant *pha*$^+$ *E. coli* with the *C. necator phaCAB* operon. The acetoacetate-induced AtoSC maximized PHB production. PHB biosynthesis in *pha*$^+$ *E. coli* is regulated by the interplay of AtoSCDAEB regulon with β-oxidation and fatty-acid biosynthesis.

2. Materials and methods

2.1. Bacterial strains and plasmids

The genotypes of *E. coli* strains used are listed in Table 1. *E. coli* strains K-12 BW25113 (*atoSC*$^+$) (Oshima et al., 2002) and BW28878 (Δ*atoSC*) (Oshima et al., 2002) were a kind gift from Hirofumi Aiba (Nagoya University, Japan). *E. coli* K-12 LJ111 [Δ(*atoC-atoB*)] (K-12 strain lacking the *atoC* gene and *atoDAEB* operon locus), were obtained from the Yale *E. coli* Stock Center. *E. coli* K-12 N99 strain was a kind gift from M. Gottesman (Columbia University Medical Center, New York, USA) (Griffo et al., 1989). Plasmid pBHR68 (Spiekermann et al., 1999) was a kind gift from Bernd H.A. Rehm (Institute of Molecular Biosciences, Massey University, Palmerstron North, New Zealand).

Plasmids pUC-Az, containing the *atoS*, *atoC* genes and a part of the *atoDAEB* operon (*atoD*, *atoA* and two-thirds of *atoE*) or pCPC-Az, containing the *atoC* gene and the same part of the *atoDAEB* operon as pUC-Az, have been described previously (Canellakis et al., 1993). Plasmid pUC-Az(AtoC$^-$) is a pUC-Az derivative that do not express the AtoC protein but only the AtoS (Theodorou et al., 2006). Plasmid pUC-Az(AtoDAEB$^-$) is a pUC-Az derivative lacking the *atoDAEB* operon (Theodorou et al., 2006). Plasmid pUC-AtoC is a pUC-Az(AtoDAEB$^-$) derivative which lacks the *atoDAEB* operon and does not express AtoS protein but only the

Table 1
Escherichia coli strains and plasmids.

Strain or plasmid	Genotype	SSource or reference
***E. coli* strains**		
N99	F$^-$, *galK2*(*Oc*), *lambda*$^-$, *recB21*, *rpsL200*(*strR*), *sup*^{0}	Griffo et al. (1989)
BW25113	*lacI*^{q} *rrn*B3 Δ*lac*Z4787 *hsd*R514 Δ(*ara*BAD)567 Δ(*rha*BAD)568 *rph*-1	Oshima et al. (2002)
BW28878	*lacI*^{q} *rrn*B3 Δ*lac*Z4787 *hsd*R514 Δ(*ara*BAD)567 Δ(*rha*BAD)568 *rph*-1 Δ(*ato*SC)569	Oshima et al. (2002)
LJ111	F$^-$, *lacY*1 or Δ(*codB-lacI*)3, *gln*V44(AS), *galK*2(Oc), *galT*22, *lambda*$^-$, *tyrT*58(AS), Δ(*atoC-atoB*)540, *metB*1, *hsdR*514, *trpR*55	Yale *E. coli* stock Center
Plasmids		
pUCAz	pUC19 containing the *atoS*, *atoC* genes and a part of the *atoDAEB* operon (*atoD*, *ato*A and two-thirds of *atoE*)	Canellakis et al. (1993)
pUCAz(AtoC$^-$)	pUC-Az derivative with a frameshift mutation in codon 28 of *atoC*	Theodorou et al. (2006)
pCPCAz	pIC20H with a *Sal*I/*Bam*HI fragment containing the *atoC* gene and the part of the *atoDAEB* operon	Canellakis et al. (1993)
pUCAz(AtoDAEB$^-$)	pUC-Az derivative which lacks part of *atoDAEB* operon	Theodorou et al. (2006)
pUCAtoC	pUC-Az derivative which contains the *atoC* gene but lacks *atoS* and part of *atoDAEB* operon	Theodorou et al. (2011b)
pUCAtoS	pUC-Az(AtoC$^-$) derivative which contains the *atoS* gene but lacks part of *atoDAEB* operon	Theodorou et al. (2011a)
pZatop	pZErO 2.1 containing the complete *atoDAEB* operon including its upstream regulatory sequence	Theodorou et al. (2006)
pBHR68	pBluescriptSK$^-$ containing *phaCAB* operon and its upstream regulatory region from *C. necator*	Spiekermann et al. (1999)
pLyCAB	pLysE carrying the *Sma*I/*Xho*I fragment from plasmid pBHR68 containing the *phaCAB* operon and its upstream regulatory region from *C. necator*	This study

AtoC protein (Theodorou et al., 2011b). Plasmid pUC-AtoS is a pUC-Az(AtoC$^-$) derivative which lacks the *atoDAEB* operon and does not express AtoC protein but only the AtoS protein (Theodorou et al., 2011a). Plasmid pZatop carrying the complete *atoDAEB* operon including its upstream regulatory sequences has been described (Theodorou et al., 2006).

To construct plasmid pLyCAB, the *C. necator* genomic region containing the *phaCAB* operon was removed from plasmid pBHR68 by digestion with *Sma*I and *Xho*I and religated into the *Eco*RV/*Sal*I digested pLysE vector (Novagen) (after the removal of the T7 lysozyme gene), to yield plasmid pLyCAB.

2.2. Growth conditions

E. coli cells were grown under two different conditions:

1. In M9 mineral medium supplemented with glucose (Theodorou et al., 2006). A 5 ml portion of grown cells in M9 medium was inoculated in 250 ml M9 and the cultures were grown at 37 °C. Glucose of 1% or 2% w/v for PHB synthesis was added at the cultures when $A_{600}=0.8$.
2. In LB medium during the first growth stages followed by growth in M9 medium. Cells from a 5 ml portion of the grown cultures in LB were collected, washed and inoculated into 250 ml M9 medium and the cultures were grown at 37 °C. Glucose of 1% or 2% w/v for PHB synthesis was added at the cultures when $A_{600}=0.8$.

Cell culture samples in both cases were collected at the indicated time points and processed for crotonic acid/PHB or glucose determination.

DL-proline 80 μg/ml was also added in M9 medium for *E. coli* BW25113 or BW28878 strains (Theodorou et al., 2006, 2007). Casein amino acids 0.1% w/v and DL-tryptophan 80 μg/ml were also added in the M9 medium for *E. coli* LJ111 (Theodorou et al., 2011a). Acetoacetate-mediated induction of the AtoSC TCS was initiated by the addition of 10 mM acetoacetate in the form of its sodium salt, when cultures reached A_{600} of 0.2–0.3, and it was maintained by subsequent additions of 2 mM acetoacetate every 4 h to ensure sustained induction (Theodorou et al., 2006). Acrylic acid or cerulenin were added when indicated at the final concentration of 60 μg/ml when A_{600} reached 0.2–0.3.

2.3. DNA isolation, manipulation and cell transformation

Genomic and plasmid DNA isolation, restriction enzyme digestions, ligations, transformations, were carried out with standard methodologies (Sambrook et al., 1989). Plasmids were purified using commercially available kits (Qiagen or Nucleobond) following the manufacturer's instructions.

2.4. Determination of glucose concentration

The determination of glucose concentration in the culture medium was performed according to the method based on the reducing sugars reaction with 3,5-dinitrosalycilic acid (DNS) and the photometric determination of the product on 575 nm (Miller, 1959).

2.5. Determination of PHB

The procedure is a variation of the method of Karr et al. (1983) and is based on the acid-catalyzed β-elimination of PHB to crotonic acid, followed by HPLC analysis of the produced crotonic acid on an Aminex HPX-87H ion exclusion organic acid analysis column (Bio-Rad) using a Shimadzu (Tokyo, Japan) HPLC Chromatography System. The procedure was followed as described (Theodorou et al., 2006; Karr et al., 1983) with the difference that the lyophilized dry *E. coli* cell pellets (from 10 ml of culture) were incubated with the concentrated H_2SO_4 at 92 °C for 2 h, as this was found to be the most efficient procedure for PHB extraction and conversion to crotonic acid followed by extraction with $CHCl_3$ (data not shown). The final filtrate was analyzed by HPLC with the standard conditions (Theodorou et al., 2006). Quantitation was done following comparison of peak absorbance with those of crotonic acid standards (Fluka, Buchs, Switzerland).

2.6. Polymer characterization by GC and GC/MS

The method is based on the methanolysis of the PHA esters to their constituent β-hydroxycarboxylic acid methyl esters followed by gas chromatography (GC) and GC/mass spectrometry (GC/MS) analysis of the β-hydroxycarboxylic acid methyl esters (Brandl et al., 1988).

Samples of 10 mg lyophilized cells were subjected to methanolysis in a small screw-cap test tube with a solution containing 1 ml of chloroform, 0.85 ml of methanol, and 0.15 ml of sulfuric acid for at 100 °C in a thermostat-equipped oil bath, for 3 h as this was found to be the most efficient procedure to obtain the corresponding 3-hydroxybutyric methyl esters (data not shown). The mixtures were cooled on ice and 1 ml of H_2O was added and the tubes were shaken vigorously for 1 min. Phase separation was achieved by centrifugation of the mixtures at 15,000*g* for 5 min and the organic phase (bottom layer) was removed and transferred to a small screw-cap glass vial.

GC/MS analysis was performed on Agilent Technologies (5975C Series GC/MSD) (California, USA) system equipped with an Agilent 19091F-102 (HP-FFAP) (0.33 μm, 25 m × 0.20) column (Agilent Technologies, California, USA) and a flame ionization detector. Nitrogen (1 ml/min) was used as the carrier gas. The temperatures of the injector and detector were 220 and 250 °C, respectively. A temperature program was used for efficient separation of the esters [50 °C for 3 min, temperature increase 9 °C/min until 250 °C (250 °C for 1 min)]. Under these conditions, the retention times (in minutes) of the different 3-hydroxyalkanoic acid methyl ester standards were as follows: C-4, 11.98; C-5, 13.50; C-6, 14.51; C-8, 17.33; C-9, 18.67; C-10, 19.94; C-11, 21.14; C-12, 22.29 (C–X represents the 3-hydroxyalkanoic acid methyl ester with a chain length of X carbon atoms).

2.7. Isolation of PHB from E. coli cells

E. coli cells were harvested, washed twice with PBS and resuspended in 2.5 volumes of 50 mM Tris–HCl, pH 7.5. The cell suspensions were incubated with lysozyme (1 mg/ml) for 1 h at 4 °C and subsequently lysed by sonication (4 × 30 s with intervals of 1 min). The lysates were centrifuged at 10,000*g* for 20 min at 4 °C. The biopolymer was extracted from the supernatants by 50 volumes of $CHCl_3$ at 30 °C for 48 h under high stirring. Clear chloroform phase containing the PHB was obtained by filtration (Whatman International Ltd., England). The biopolymer was recovered by nonsolvent precipitation in 10 volumes of well-stirred ice-cold methanol followed by incubation at −20 °C for 48 h. PHB was finally recovered by centrifugation at 12,000*g* for 20 min at 4 °C, dried and their molecular weight was detected.

2.8. Analytical procedures

Molecular weight and molecular weight distribution were determined by Gel Permeation Chromatography (GPC) at 30 °C using a Polymer Labs GPC210 and a Viscotek Model 210R Viscometer system, equipped by a 2× PLgel 10 μm mixed B 300 × 7.5 mm column and a PLgel 5 μm 50 × 7.5 mm guard

column. A mixture of chloroform/methanol: 95/5 was used as the eluent at flow rate of 1 ml/min and sample concentration of 2 mg/ml was applied. Polystyrene standards of low polydispersity were used to construct the calibration curve.

The melting point (T_m), glass transition temperature (T_g), enthalpy of fusion (ΔH_m) and % crystallinity were determined by differential scanning calorimetry (DSC) recorded in a temperature range between −60 and 230 °C on a TA Instruments (model DSC-Q100) equipped with a Refrigerated Cooling System (RCS) under a nitrogen flow of 20 ml/min. PHA samples were encapsulated in aluminum pan and heated at a rate of 10 °C/min.

The residual cell mass (RCM) was defined as the lyophilized dry cell weight (CDW) minus the PHB concentration. The PHB content (wt%) was defined as the percentage of the ratio of PHB concentration to CDW.

3. Results

3.1. PHB biosynthesis in E. coli strains transformed with plasmid pBHR68

The isogenic pair of *E. coli* K-12 strains BW25113 (*atoSC*$^+$) and BW28878 (Δ*atoSC*) were transformed with plasmid pBHR68 carrying the *phaCAB* operon from *C. necator*, and the biosynthesis of the PHB was studied.

BW25113/pBHR68 cells, that express the AtoSC TCS, synthesized approximately 0.72 g/l PHB with a biopolymer content of 24% w/w during growth in mineral medium with glucose 1% w/v as the carbon source (Fig. 1A and Table 2). The deletion of the *atoSC* genetic locus in BW28878/pBHR68 cells caused a 2-fold reduction of PHB synthesis, resulting in 0.38 g/l PHB with 12.3% w/w biopolymer content as well as affected and the cell dry weight and consequently the residual cell mass under the same growth conditions (Fig. 1A). When 2% w/v glucose was used, *atoSC*$^+$ cells synthesized higher PHB amounts (0.85 g/l PHB). However, when cells of both genetic backgrounds were grown in rich medium followed by growth in mineral medium, their PHB production was 25% decreased (Fig. 1B and Table 2).

3.2. AtoSC signaling up-regulates PHB biosynthesis in phaCAB$^+$ E. coli

To further study the involvement of the AtoSC TCS in PHB biosynthesis, *phaCAB* operon from pBHR68 was cloned to vector pLysE, resulting in plasmid pLyCAB. This vector was chosen to be compatible with plasmids carrying the whole or parts of the *atoSCDAEB* locus as well as being a low copy number vector. BW25113 and BW28878 strains were transformed either with pLyCAB alone to express *in trans* the PhaCAB enzymes or together with plasmid pUCAz to also overproduce AtoSC TCS and the part of the *atoDAEB* operon. Cells were grown with glucose 1% or 2% w/v,

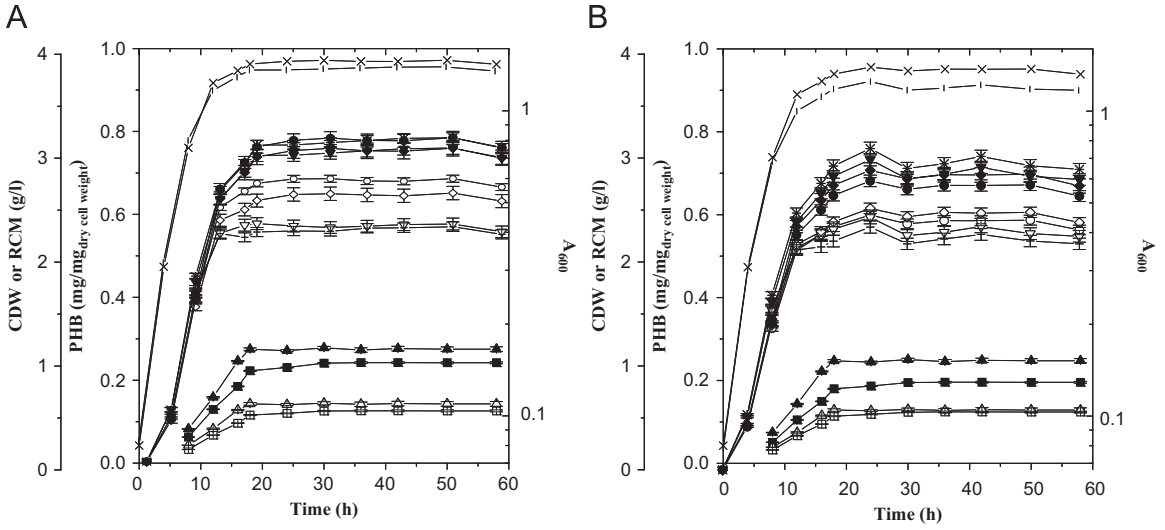

Fig. 1. AtoSC expression enhances PHB accumulation. PHB biosynthesis in pBHR68-transformed BW25113 (*atoSC*$^+$) (closed symbols) or BW28878 (Δ*atoSC*) (open symbols) strains, with glucose (■, □) 1% or (▲, △) 2% (w/v) as the carbon source for PHB production. (A) Cells were grown in M9 mineral medium during all stages. (B) Cells subcultures were grown in LB medium followed by growth in M9 mineral medium. (x,|): the absorbance at 600 nm (A_{600}) of cells with lower (□, △) or higher (■, ▲) polymer production, respectively. CDW (closed symbols) or RCM (open symbols) of BW28878/pBHR68 cells on (●, ○) 1% or (◆, ◇) 2% (w/v) glucose, or BW25113/pBHR68 cells on (▼, ▽) 1% or (+, *) 2% (w/v) glucose. The values represent the average of three independent experiments.

Table 2
PHB biosynthesis in pBHR68-transformed BW25113 (*atoSC*$^+$) and BW28878 (Δ*atoSC*) strains.

Medium of subcultures	[Glucose] (% w/v)	BW25113/pBHR68				BW28878/pBHR68			
		CDW (g/l)	PHB (g/l)	RCM (g/l)	PHB content (wt%)	CDW (g/l)	PHB (g/l)	RCM (g/l)	PHB content (wt%)
M9	1	3.0 ± 0.10	0.72 ± 0.05	2.28 ± 0.05	24.0 ± 0.80	3.1 ± 0.10	0.38 ± 0.03	2.72 ± 0.07	12.3 ± 0.40
	2	3.1 ± 0.20	0.85 ± 0.05	2.25 ± 0.05	27.3 ± 0.30	3.0 ± 0.10	0.44 ± 0.03	2.56 ± 0.07	14.6 ± 0.70
LB	1	2.8 ± 0.10	0.59 ± 0.04	2.21 ± 0.06	21.1 ± 0.50	2.7 ± 0.10	0.34 ± 0.03	2.36 ± 0.07	12.5 ± 0.40
	2	2.9 ± 0.30	0.74 ± 0.03	2.16 ± 0.27	25.0 ± 0.20	2.8 ± 0.20	0.38 ± 0.02	2.42 ± 0.18	13.1 ± 0.20

CDW: cell dry weight.
RCM: residual cell mass.

in the presence or absence of acetoacetate, the inducer of the AtoSC TCS.

AtoSC-expressing *phaCAB*$^{+}$ cells (BW25113/pLyCAB) synthesized approximately 0.43 g/l PHB in contrast to their Δ*atoSC* derivatives (BW28878/pLyCAB) that accumulated half the biopolymer amounts, in glucose 1% w/v in the absence of acetoacetate (Fig. 2A, B and Table 3). The expression of AtoSC through plasmid pUCAz in BW28878/pUCAz-pLyCAB cells, increased PHB synthesis to equal levels as in BW25113/pLyCAB cells and caused an increase of the cells dry weight (Fig. 2A, B, Table 3 and Supplement 1). AtoSC TCS overproduction enhanced PHB also in BW25113/pUCAz-pLyCAB derivatives which accumulated 0.73 g/l PHB (Fig. 2A). The induction of AtoSC by acetoacetate further enhanced significantly (3-fold) PHB accumulation in cells expressing AtoSC endo- (BW25113) or exo-chromosomally (pUCAz-transformed derivatives) (Fig. 2A and B). This acetoacetate-induced AtoSC-mediated PHB up-regulation was also followed by an increase of the cell dry weight during the whole cell growth (Supplement 1). However, acetoacetate triggered no effect in BW28878/pLyCAB cells (Fig. 2B and C). Under those conditions, the maximal PHB biosynthesis of 1.73 g/l (biopolymer content of 64.5% w/w) was achieved in AtoSC-overproducing cells (BW25113/pUCAz-pLyCAB) and was approximately 5-fold enhanced compared to the Δ*atoSC* BW28878/pLyCAB derivatives (Fig. 2A, B and Table 3). During another strategy of cell cultivation, glucose was added at the time of inoculation to the culture mediums and PHB accumulation was observed during also exponential phase of growth, which was up-regulated in acetoacetate-induced AtoSC-expressing cells (0.85 g/l with 44.7% biopolymer content), yet remaining significantly reduced compared to the maximum PHB amounts under glucose 1% added at the entry to the stationary phase of growth (data not shown).

When glucose was used at the final concentration of 2% w/v, AtoSC signaling up-regulated PHB production but those biopolymer levels remained reduced compared to the yields achieved under glucose 1% w/v, while biomass demonstrated a small decrease compared to the biomass under glucose 1% w/v (Fig. 2C, D, Table 3 and Supplement 1). Glucose consumption was enhanced in cells that accumulated higher PHB amounts with BW25113/pUCAz-pLyCAB cells to consume the higher glucose amounts in the presence of acetoacetate (Fig. 2). Analysis of the synthesized

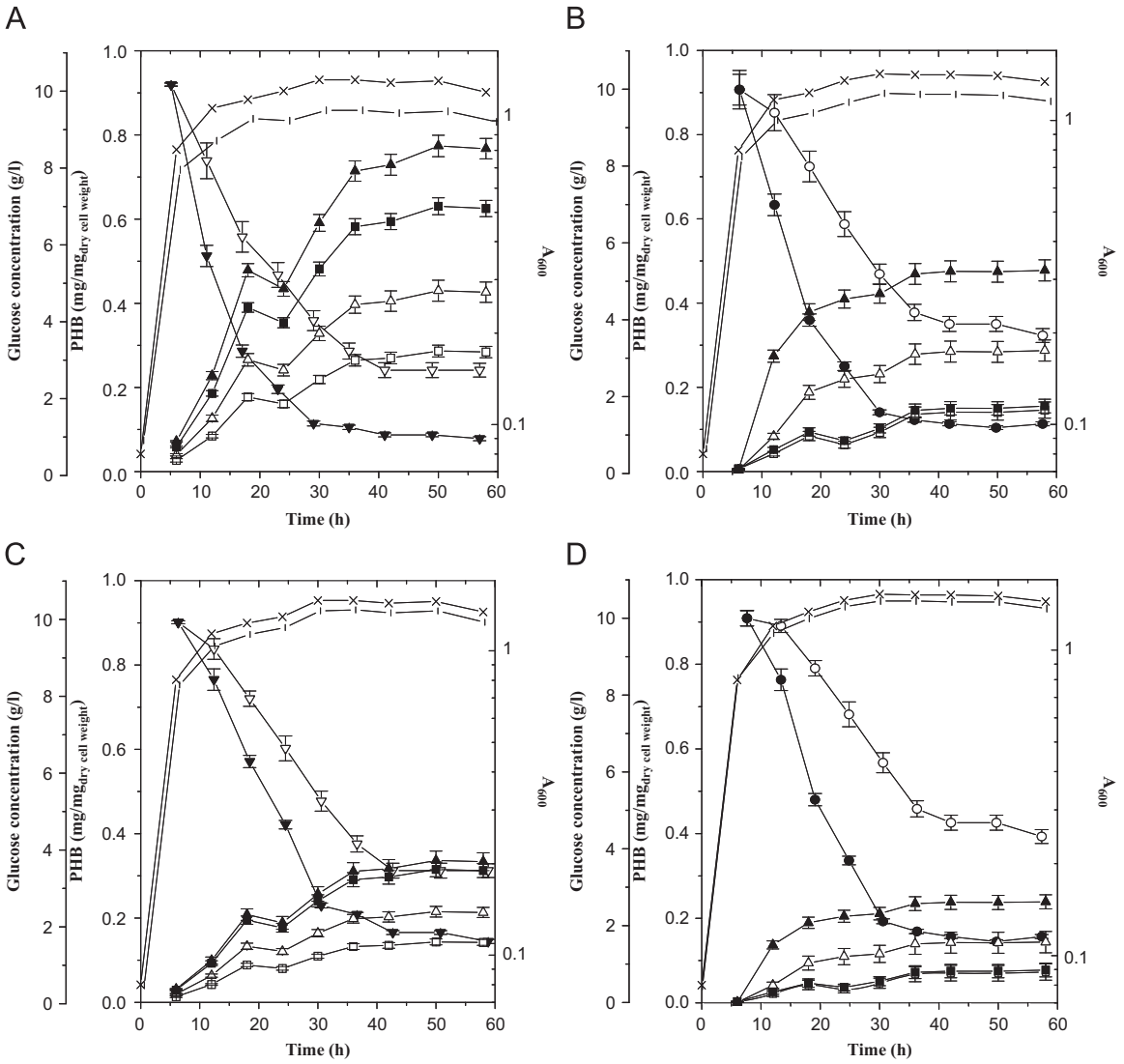

Fig. 2. Acetoacetate-induced AtoSC maximizes PHB production in *pha*$^{+}$ *E. coli*. PHB biosynthesis in (A, C) BW25113 (*atoSC*$^{+}$) or (B, D) BW28878 (Δ*atoSC*) strains transformed with plasmids (■, □) pLyCAB or (▲, △) pUCAz-pLyCAB with glucose (A, B) 1% or (C, D) 2% (w/v), in the absence (open symbols) or presence (closed symbols) of acetoacetate. Glucose concentration in the cultures of (▼, ▽) BW25113 or (●, ○) BW28878 transformed with plasmids pLyCAB (open symbols) or pUCAz-pLyCAB (closed symbols) in the presence of acetoacetate. (x,|): the absorbance at 600 nm (A_{600}) of cells with lower [A, C (□, △), B, D (■, □)] or higher [A, C (■, ▲), B, D (▲, △)] polymer production, respectively. The values represent the average of three independent experiments.

Table 3
PHB biosynthesis in pLyCAB-transformed BW25113 (*atoSC*$^+$) and BW28878 (Δ*atoSC*) strains.

[Glucose] (% w/v)	AcAc	CDW (g/l)	PHB (g/l)	RCM (g/l)	PHB content (wt%)	CDW (g/l)	PHB (g/l)	RCM (g/l)	PHB content (wt%)
		BW25113/pLyCAB				**BW28878/pLyCAB**			
1	−	1.2 ± 0.20	0.43 ± 0.04	0.77 ± 0.16	36.4 ± 0.30	0.9 ± 0.20	0.20 ± 0.03	0.7 ± 0.15	21.9 ± 0.30
	+	1.9 ± 0.30	1.07 ± 0.07	0.83 ± 0.23	55.7 ± 0.20	1.0 ± 0.20	0.22 ± 0.06	0.78 ± 0.14	23.6 ± 0.20
2	−	1.1 ± 0.20	0.23 ± 0.06	0.87 ± 0.14	22.3 ± 0.30	0.9 ± 0.10	0.11 ± 0.05	0.79 ± 0.05	12.8 ± 0.40
	+	1.6 ± 0.20	0.60 ± 0.05	1.0 ± 0.15	38.6 ± 0.50	0.9 ± 0.30	0.11 ± 0.07	0.79 ± 0.23	13.0 ± 0.30
		BW25113/pUC-Az-pLyCAB				**BW28878/pUC-Az-pLyCAB**			
1	−	1.6 ± 0.30	0.73 ± 0.06	0.87 ± 0.24	46.3 ± 0.50	1.3 ± 0.30	0.49 ± 0.04	0.81 ± 0.26	36.3 ± 0.40
	+	2.6 ± 0.40	1.73 ± 0.07	0.87 ± 0.33	64.5 ± 0.60	1.8 ± 0.50	0.90 ± 0.03	0.9 ± 0.47	48.9 ± 0.20
2	−	1.4 ± 0.20	0.43 ± 0.03	0.97 ± 0.17	30.1 ± 0.30	1.3 ± 0.40	0.21 ± 0.06	1.09 ± 0.34	16.7 ± 0.60
	+	2.2 ± 0.30	1.10 ± 0.08	1.1 ± 0.22	50.0 ± 0.70	1.9 ± 0.20	0.60 ± 0.02	1.3 ± 0.18	32.3 ± 0.50

CDW: cell dry weight.
RCM: residual cell mass.
AcAc: acetoacetate.

biopolymer showed that it was the high molecular weight PHB of 1,320,000 (M_w) and 1,260,000 (M_n) with polydispersity (PDI) 1.048. The above prove that the acetoacetate-induced AtoSC signaling maximizes the biosynthesis of PHB in *phaCAB*$^+$ *E. coli*.

3.3. AtoSC constituents separately enhance PHB synthesis

The participation of AtoS or AtoC constituents in PHB up-regulation was verified introducing in BW28878/pLyCAB cells plasmids that lead to expression of the whole AtoSC TCS or its constituents separately. The overproduction of AtoSC in BW28878/pLyCAB-pUCAz(AtoDAEB$^-$) cells, caused a PHB accumulation of 0.75 g/l (2.5-fold enhancement than control levels) (Fig. 3A). However, those PHB amounts remained lower than the optimal biopolymer accumulation in the pUCAz-transformed derivatives that overproduced the AtoSC TCS and the part of the *atoDAEB* operon (Fig. 3A). AtoC expression through pUCAtoC triggered a PHB synthesis of 0.48 g/l (2.2-fold increased PHB) in BW28878/pLyCAB-pUCAtoC derivatives, while the AtoS overproduction [through plasmids pUCAtoS or pUCAz(AtoC$^-$)], caused only a limited enhancement. Comparison of the dry cell weights as well as the residual cell mass, revealed only small differences of the cell growth between strains expressing the AtoSCDAEB regulon, AtoC and *atoDAEB* operon or AtoC, while more distinct cell mass variations were observed between the Δ*atoSC pha*$^+$ (BW28878/pLyCAB) and *atoSC*$^+$ *pha*$^+$ (BW28878/pLyCAB-pUCAz) strains (Fig. 3B). Glucose consumption followed an analogous profile to PHB production, being enhanced in cells that accumulated higher PHB, while higher amounts of the carbon source remained in the culture medium of cells that synthesized reduced biopolymer (Fig. 3C). These data suggest that the constituents of AtoSC and most efficiently AtoC, are able to up-regulate PHB synthesis, yet the whole AtoSC TCS is required for the more efficient PHB enhancement.

3.4. AtoDAEB operon participates in PHB up-regulation by AtoSC

To prove the involvement of *atoDAEB* operon in the AtoSC-mediated enhancement of PHB biosynthesis, plasmid pLyCAB was introduced in LJ111 [Δ(*atoC-atoB*)] strain, which synthesized half the PHB amounts compared to wild type N99/pLyCAB cells (Fig. 4A). Acetoacetate had no effect on PHB synthesis as well as the cell dry weight of LJ111/pLyCAB cells (Fig. 4A and Supplement 2). The expression of AtoSC and *atoDAEB* operon through pUCAz, in LJ111/pLyCAB-pUCAz cells, increased PHB levels, yet the maximal polymer synthesis (approximately 5-fold compared to the control levels) was achieved upon acetoacetate followed by a modulation of the cell dry weight (Fig. 4B and Supplement 2). Moreover, AtoC overproduction together with *atoDAEB* operon (LJ111/pLyCAB-pCPCAz derivatives) was capable to enhance PHB, but those biopolymer levels were lower than those in the pUCAz-transformed derivatives (Fig. 4B). The transformation of LJ111/pLyCAB with pZatop- or pUCAz(AtoC$^-$) carrying the *atoDAEB* operon maintained PHB and biomass to the control levels (Fig. 4B and Supplement 2). The above data prove that, the *atoDAEB* operon participates in the AtoSC-mediated up-regulation of PHB biosynthesis in *phaCAB*$^+$ *E. coli*.

To further study the possibility that AtoSC TCS regulates PHB biosynthesis through additional processes except *atoDAEB* operon, LJ111 strains were transformed with plasmids carrying the *atoSC* locus or the *atoS* or *atoC* genes separately, but lacking *atoDAEB* operon. Indeed, AtoSC up-regulated PHB (1.9-fold) in LJ111/pLyCAB-pUCAz(AtoDAEB$^-$) bacteria, a phenotype that was further 2-fold sustained upon acetoacetate reaching a PHB production of 1.55 g/l (Fig. 4C). AtoC overproduction in LJ111/pLyCAB-pUCAtoC derivatives resulted in 3.6-fold higher PHB levels upon acetoacetate (PHB yield of 1.4 g/l) (Fig. 4C). AtoS overproduction (LJ111/pLyCAB-pUCAtoS cells) caused a lesser PHB enhancement, while acetoacetate had an additional limited (1-fold) effect on PHB (Fig. 4C). Yet, AtoSC TCS caused the higher PHB accumulation compared to its components separately. Strains expressing AtoS, AtoC or the AtoSC system demonstrated no significant differences on their cell dry weight (Supplement 2). Conclusively, the AtoSCDAEB regulon is a fundamental for the biosynthesis of PHB however, AtoSC TCS is able to up-regulate the biopolymer synthesis through additional mechanisms in which its constituents can be involved separately.

3.5. Involvement of β-oxidation and fatty acid metabolism in PHB biosynthesis

The involvement of β-oxidation in PHB synthesis was studied using acrylic acid that blocks β-oxidation. Inhibition of β-oxidation resulted in a 2.5-fold reduction of PHB in LJ111/pLyCAB cells (Fig. 5A), verifying the involvement of the pathway in PHB synthesis, while the biomass in the presence of the inhibitor demonstrated only a minor decrease compared to its absence (Fig. 5C). However, expression of the AtoSC TCS or AtoC and the part of *atoDAEB* operon were capable to enhance (3.7 or 3-fold respectively) PHB upon acetoacetate, despite the β-oxidation blockage (Fig. 5A). AtoSC expression in LJ111/pLyCAB-pUCAz(AtoDAEB$^-$) bacteria lacking the *atoDAEB* locus, also increased PHB production (approximately 4-fold) (Fig. 5A). This phenotype could be attributed to its constituents individually, since AtoS or AtoC separate expression, triggered a

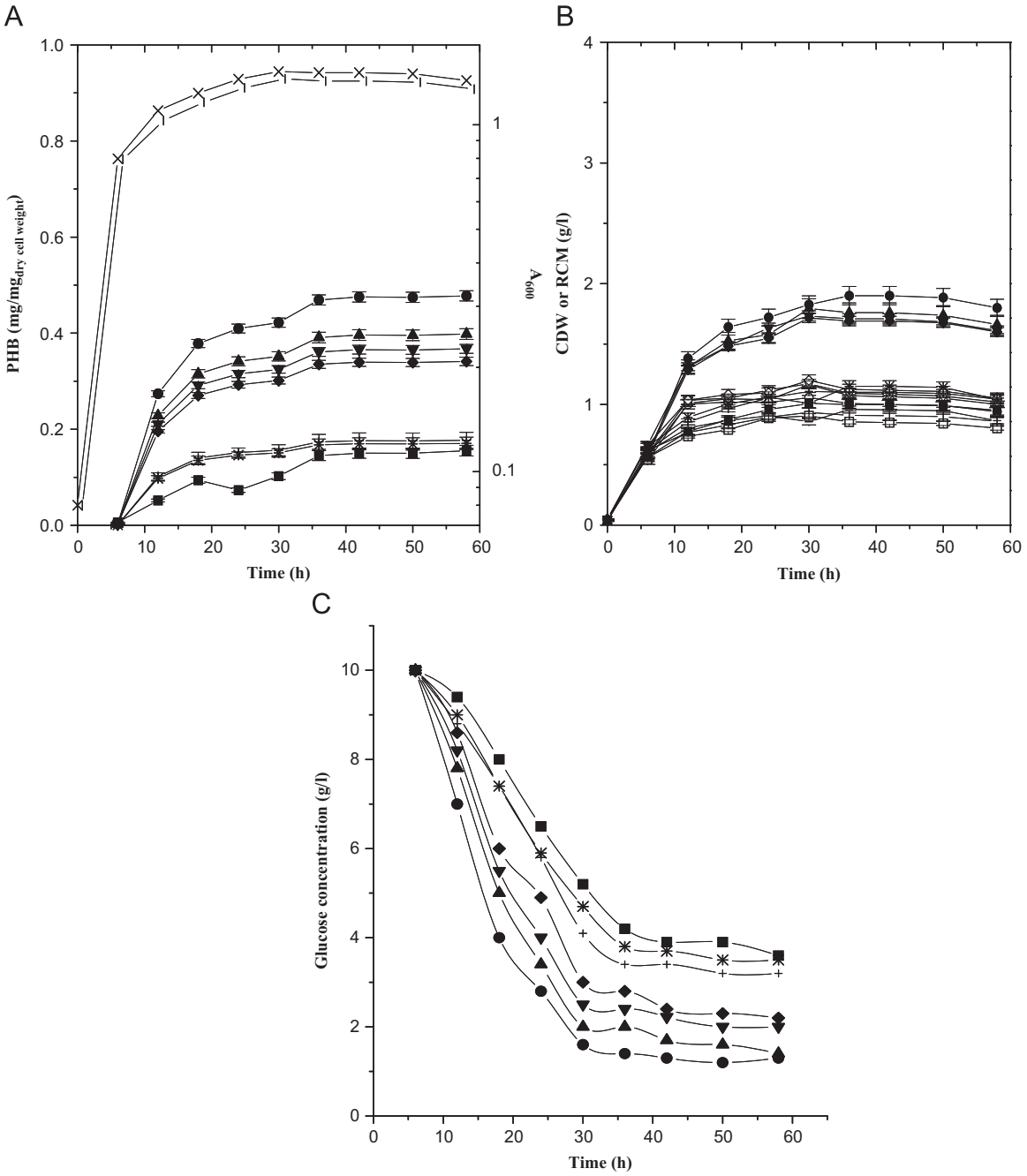

Fig. 3. PHB regulation by AtoS and/or AtoC. (A) PHB biosynthesis and (C) glucose concentration in the culture medium of (■) BW28878/pLyCAB cells or their transformed derivatives with plasmids (●) pUCAz, (▲) pCPCAz, (+) pUCAz(AtoC$^-$), (▼) pUCAz(AtoDAEB$^-$), (*) pUCAtoS and (♦) pUCAtoC upon glucose 1% w/v and acetoacetate. (x,|): the absorbance at 600 nm (A_{600}) of cells with lower (■, +, *) or higher (●, ▲, ▼, ♦) polymer production, respectively. (B) CDW (closed symbols) or RCM (open symbols) of (■, □) BW28878/pLyCAB cells or their transformed derivatives with plasmids (●, ○) pUCAz, (▲,△) pCPCAz, (+, -) pUCAz(AtoC$^-$), (▼, ▽) pUCAz(AtoDAEB$^-$), (*,I) pUCAtoS or (♦, ◇) pUCAtoC. The values represent the average of three independent experiments.

1.0 or 3.2-fold increased PHB, respectively (Fig. 5A). Yet, all these PHB phenotypes remained significantly lower than those observed in the absence of acrylic acid as well as the cell weights of all strains expressing the parts of AtoSCDAEB regulon were slightly lower under β-oxidation blockage compared to the inhibitors absence (Fig. 4).

The possible involvement of fatty acid biosynthetic pathway in PHB biosynthesis was studied using its inhibitor cerulenin. Fatty acid biosynthesis inhibition led to a 4-fold decrease of PHB amounts in LJ111/pLyCAB cells (Fig. 5B), verifying its fundamental participation in biopolymer synthesis with glucose as the carbon source. Neither the AtoSC TCS alone nor its constituents AtoS or AtoC separately, achieved to reverse the cerulenin inhibitory effect in the above counterparts, resulting in the same PHB levels to the untransformed cells (Fig. 5B). However, PHB production was 3.8-fold enhanced in LJ111/pLyCAB-pUCAz bacteria (Fig. 5B) which expressed the AtoSC TCS and the atoDAEB operon. Fatty acid biosynthesis inhibition by cerulenin triggered a biomass reduction of all strains to analogous levels as in the presence of acrylic acid (Fig. 5C and D). Moreover, the presence of both acrylic acid and cerulenin in LJ111/pLyCAB-pUCAz(AtoDAEB$^-$) cells reduced dramatically PHB compared to the presence of each inhibitor alone (Fig. 5B). Conclusively, a regulatory interplay between the fatty acid metabolic pathways takes place towards PHB biosynthesis where the AtoSCDAEB regulon also participates.

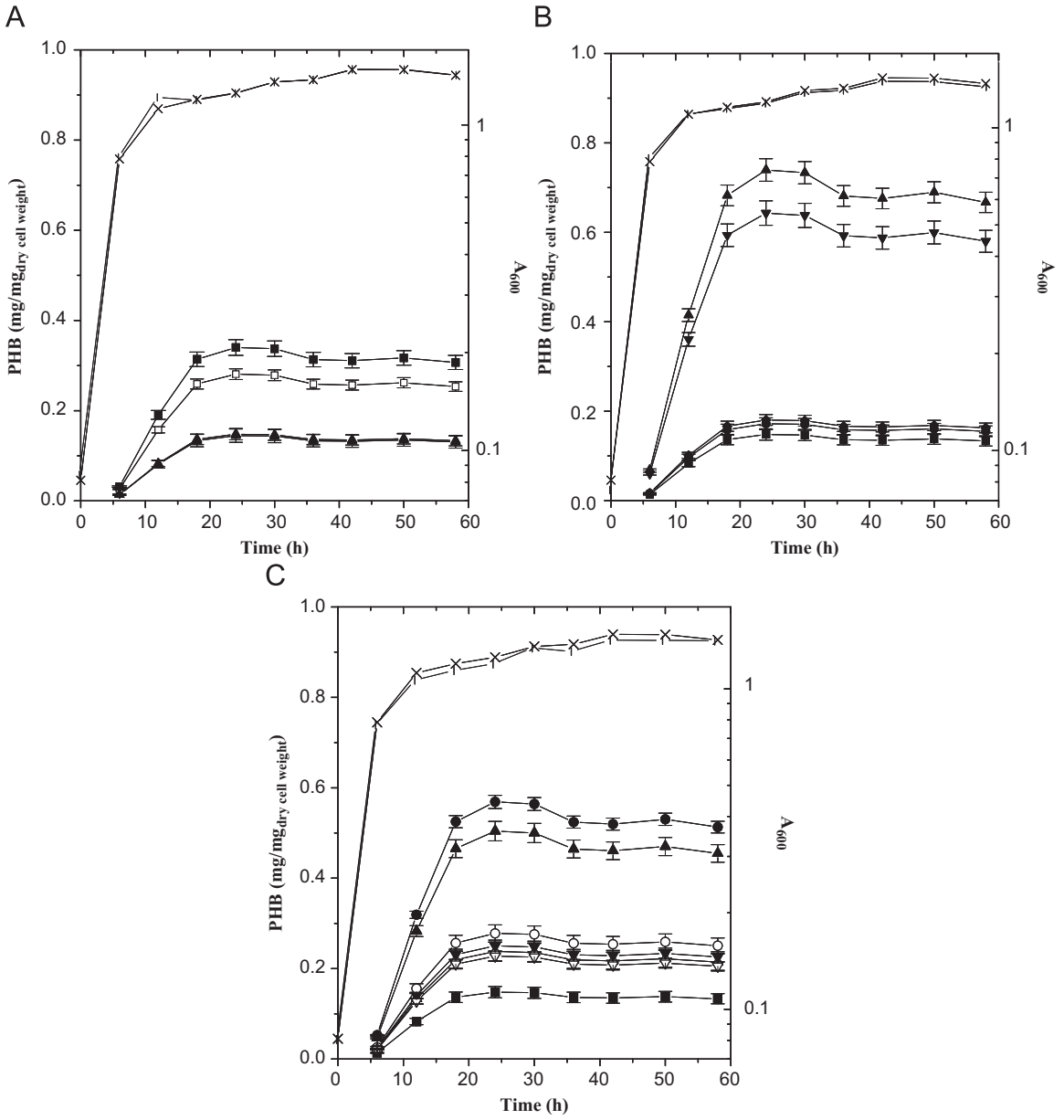

Fig. 4. AtoSC enhances PHB production in LJ111 cells. (A) PHB biosynthesis in (▲, △) LJ111/pLyCAB [Δ(*atoC-atoB*)] and (■, □) N99/pLyCAB (wild type) cells in the absence (open symbols) or presence (closed symbols) of acetoacetate with glucose 1% w/v. (x,|): the absorbance at 600 nm (A_{600}) of (|) N99/pLyCAB or (x) LJ111/pLyCAB cells. (B) PHB biosynthesis in (■) LJ111/pLyCAB and their transformed derivatives with plasmids (▲) pUCAz, (▼) pCPCAz, (♦) pUCAz(AtoC$^-$) or (●) pZatop upon acetoacetate. (C) PHB biosynthesis in (■) LJ111/pLyCAB and their transformed derivatives with plasmids (●, ○) pUCAz(AtoDAEB$^-$), (▲, △) pUCAtoC or (▼, ▽) pUCAtoS, in the absence (open symbols) or presence (closed symbols) of acetoacetate. (A, B, C) (x,|): the absorbance at 600 nm (A_{600}) of cells with lower [A (▲, △), B (■, ♦, ●) C (■, ▼, ○, △, ▽)] or higher [A (■, □), B (▲,▼), C (▲, ●)] polymer production, respectively. The values represent the average of three independent experiments.

4. Discussion

The signal transduction through the AtoSC TCS, is a key regulator of multiple *E. coli* processes (Kyriakidis and Tiligada, 2009), including the biosynthesis of the inherent low-molecular-weight complexed cPHB (Theodorou et al., 2006, 2007, 2009, 2011a, b; Kyriakidis et al., 2008), a macromolecule with many physiological actions (Reusch et al., 2002). The recombinant *pha*$^+$ *E. coli fadR atoC*(Con) strains are also abundantly used for the biosynthesis of the biodegradable storage PHA (Slater et al., 1992; Rhie and Dennis, 1995; Yim et al., 1996; Dennis et al., 1998; Fukui et al., 1999; Liu and Steinbüchel, 2000; Park et al., 2001; Kichise et al., 2002; Matsumoto et al., 2009).

The AtoSC TCS extends its action beyond the inherent cPHB, on the most widely used member of PHA, the PHB, which can be synthesized in recombinant *phaCAB*$^+$ *E. coli*, harboring the *phaCAB* operon from *C. necator*. AtoSC up-regulates PHB biosynthesis proved by the significant PHB reduction in Δ*atoSC* derivatives, which was restored by the expression of AtoSC TCS through plasmid pUCAz. This AtoSC-mediated PHB enhancement can be attributed to AtoSC TCS induction by intermediates of the fatty acids metabolism, as we have previously reported (Theodorou et al., 2011b). Fatty acid metabolism intermediate products such as acetyl-CoA have been reported to induce the AtoSC TCS (Theodorou et al., 2011b), while high amounts of acetyl-CoA have been proved to result in high PHB (Kessler and Witholt, 2001). Possibly, acetyl-CoA flow is implicated in PHB enhancement through the induction of the AtoSC TCS. Furthermore, the acetoacetate-induced AtoSC signaling maximizes PHB biosynthesis, proved by the higher yield of PHB accumulation and the cellular

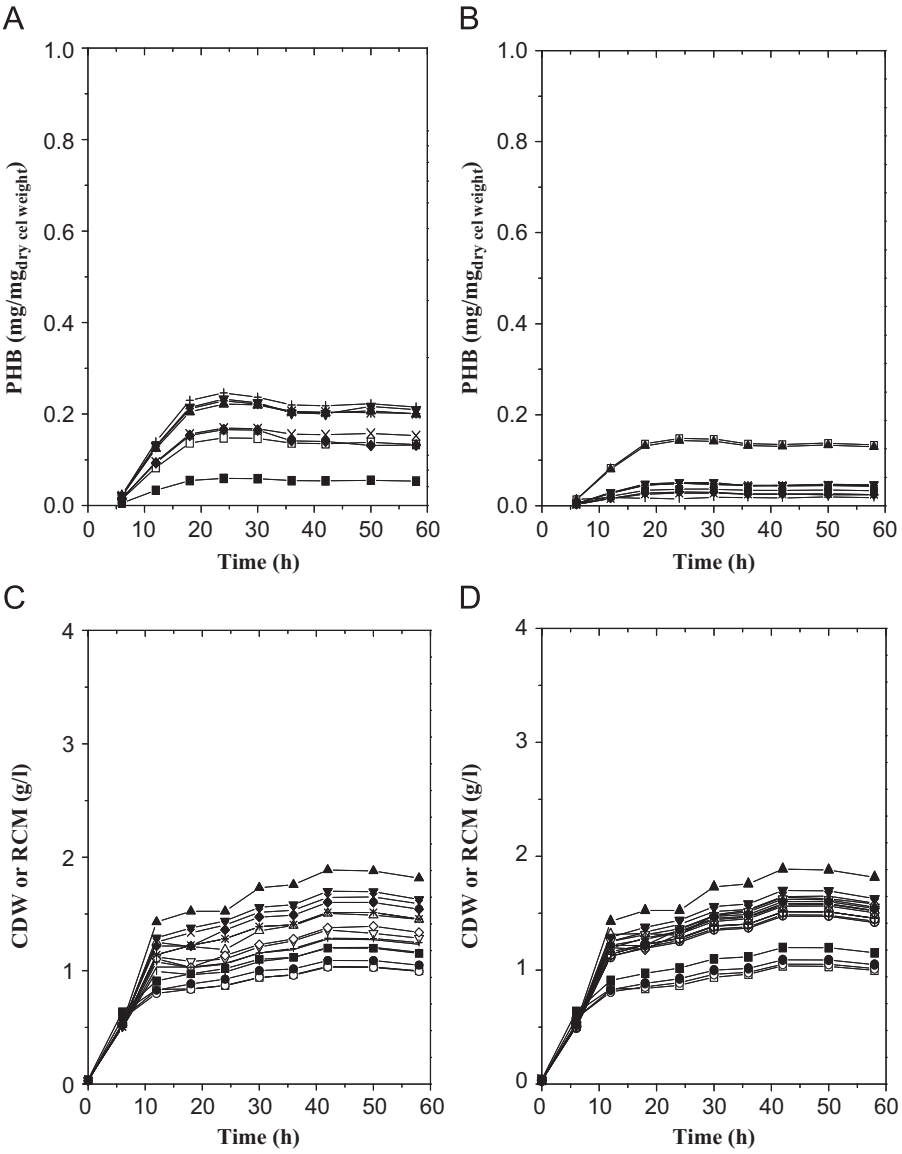

Fig. 5. PHB synthesis when β-oxidation and/or fatty acid biosynthesis are inhibited. PHB biosynthesis in LJ111/pLyCAB [Δ(*atoC-atoB*)] cells and their transformed derivatives with the indicated plasmids grown with glucose 1% w/v in the presence of (A) acrylic acid for β-oxidation inhibition or (B) cerulenin for fatty acid biosynthesis inhibition. AtoSC TCS was induced by acetoacetate. (A, B) (■) LJ111/pLyCAB, (▲) pUCAz, (♦) pUC-Az(AtoC$^-$), (+) pUCAz(AtoDAEB$^-$) (▼) pCPCAz, (x) pUCAtoS, (*) pUCAtoC double transformed LJ111/pLyCAB derivatives. (|)LJ111/pLyCAB-pUCAz(AtoDAEB$^-$) cells in the presence of both acrylic and cerulenin. (□) LJ111/pLyCAB cells in the absence of inhibitors. (C, D) CDW (closed symbols) or RCM (open symbols) during PHB biosynthesis in LJ111/pLyCAB cells in the (■, □) absence or (●, ○) presence of (C) acrylic or (D) cerulenin, or their double transformed derivatives with plasmids (▲, △) pUCAz, (▼, ▽) pCPCAz, (♦, ◇) pUCAz(AtoC$^-$), (+, x) pUCAz(AtoDAEB$^-$), (no symbol, *) pUCAtoS, (|, -) pUCAtoC. (ø, ◯) CDW or RCM of LJ111/pLyCAB-pUCAz(AtoDAEB$^-$) cells in the presence of both acrylic and cerulenin. The values represent the average of three independent experiments. The error for each data point is less than 5%.

content on biopolymer as well as the cell weights being closely related to the PHB biosynthesis in AtoSC-expressing *phaCAB*$^+$ recombinant bacteria, in the presence of acetoacetate. Other TCSs, amongst additional factors (Kessler and Witholt, 2001), have been implicated in secondary metabolites (Mendes et al., 2007) and PHA production in a number of naturally PHA-producing bacteria, including the pho regulon in *Acinetobacter* (Schembri et al., 1995), NtrB-NtrC in *Azospirillum brasilense* SP7 (Sun et al., 2000), GacS-GacA or PhbR RR in *Azotobacter vinalendii* (Castañeda et al., 2000; Peralta-Gil et al., 2002) and LemA-GacA in *P. putida* KT2442 (Madison and Huisman, 1999).

The expression of lower copies of the PhaCAB enzymes through, the constructed in this study, low copy plasmid pLyCAB elicited increased PHB production, compared to the amounts accumulated under high copies of the PhaCAB enzymes through plasmid pBHR68 (Spiekermann et al., 1999). An analogous relation between the biosynthetic enzyme copies and the expression system used in the presence or absence of inducers and the yield as well as the size of the produced PHAs, have been reported previously for homologous or heterologous PHAs synthesis (Kessler and Witholt, 2001; Prieto et al., 1999; Li et al., 2010; Tyo et al., 2010; Zhou et al., 2011). Concerning the biotechnologically optimal biopolymer accumulation, growth of *phaCAB*$^+$ *atoSC*$^+$ *E. coli* exclusively in mineral medium with 1% w/v glucose, constitutes the better growth conditions towards PHB production. Previous studies have proposed that new engineering strategies need to be employed to improve PHB accumulation during the bacterial growth phase (Tyo et al., 2010). The capability of recombinant *atoSC*$^+$ *phaCAB*$^+$ *E. coli* upon acetoacetate to synthesize PHB during the log phase of growth could suggest that the AtoSC signaling possibly participates in the flux regulation towards the PHB biosynthesis process during the active cell

growth phase. The above signify the efficient biotechnological PHB production in wild type *E. coli* strains by the acetoacetate-induced AtoSC signaling without the need for the mutant *E. coli* strains used to date.

The catabolism of short-chain fatty acids through the *atoDAEB* operon participates in PHB synthesis and its regulation by AtoSC, since its deletion from the *E. coli* genome led to a consistent reduction of PHB levels. The same mode of regulation has been also proved for cPHB of *E. coli* (Theodorou et al., 2006, 2007). The complementation of the Δ*atoDAEB* phenotype via the extrachromosomal *atoDAEB* introduction was achieved only when AtoSC was also expressed and induced by acetoacetate, signifying the AtoSC-DAEB regulon as a prerequisite for the enhanced PHB accumulation in *phaCAB*$^{+}$ *E. coli*. However, AtoSC is capable to improve PHB synthesis also in the absence of *atoDAEB* in LJ111 bacteria by additional biochemical processes. This can be attributed to either its constituents AtoS or AtoC, which enhance PHB when expressed separately. AtoC is more effective on PHB enhancement, but the entire AtoSC triggers the maximal PHB effect even without the need for acetoacetate-mediated activation. Analogous regulon engineering on the quorum sensing signaling in *E. coli* has proposed that pho regulon enables an individual bacterium metabolic state to be communicated to and ultimately control the phenotype of an emerging population even in the absence of its inducers (Tsao et al., 2010).

The biosynthesis of PHA in recombinant *pha*$^{+}$ *E. coli* has been proposed to be regulated by several inherent *E. coli* metabolic pathways and enzymes (Li et al.., 2007, 2010; Jung et al., 2011; Zhou et al., 2011). The interplay between fatty acid biosynthesis and β-oxidation plays a central role to produce the suitable intermediates which have been proposed to be supplied by several enzymes, to the biosynthetic pathway of polyhydroxyalkanoates constituted by medium-chain-length monomers (Huijberts et al., 1995; Qi et al., 1998; Klinke et al., 1999). In an effort to define the possible involvement of the central *E. coli* metabolism in the short-chain-length PHB biosynthesis, without necessarily triggering the highest biopolymer levels, acrylic acid the inhibitor of the β-oxidation 3-ketoacyl-CoA thiolase or/and cerulenin the inhibitor of the fatty acid synthetase were used. An analogous interplay between these two processes (fatty acid biosynthesis and β-oxidation) and the AtoSCDAEB regulon regulating PHB biosynthesis was observed in the present study and is proposed to the model of Fig. 6.

The AtoSC TCS and *atoDAEB* operon constituting the atoSCDAEB regulon, are induced by acetoacetate, spermidine, or other amine analogs and fatty acid metabolic intermediates (Theodorou et al., 2006, 2007, 2009, 2011a, b; Kyriakidis et al., 2008), leading to the homodimeric membrane-bound AtoS autophosphorylation and subsequently to the phosphotransfer to AtoC. The active AtoC then induces *atoDAEB* operon transcription upon binding oligomerized to

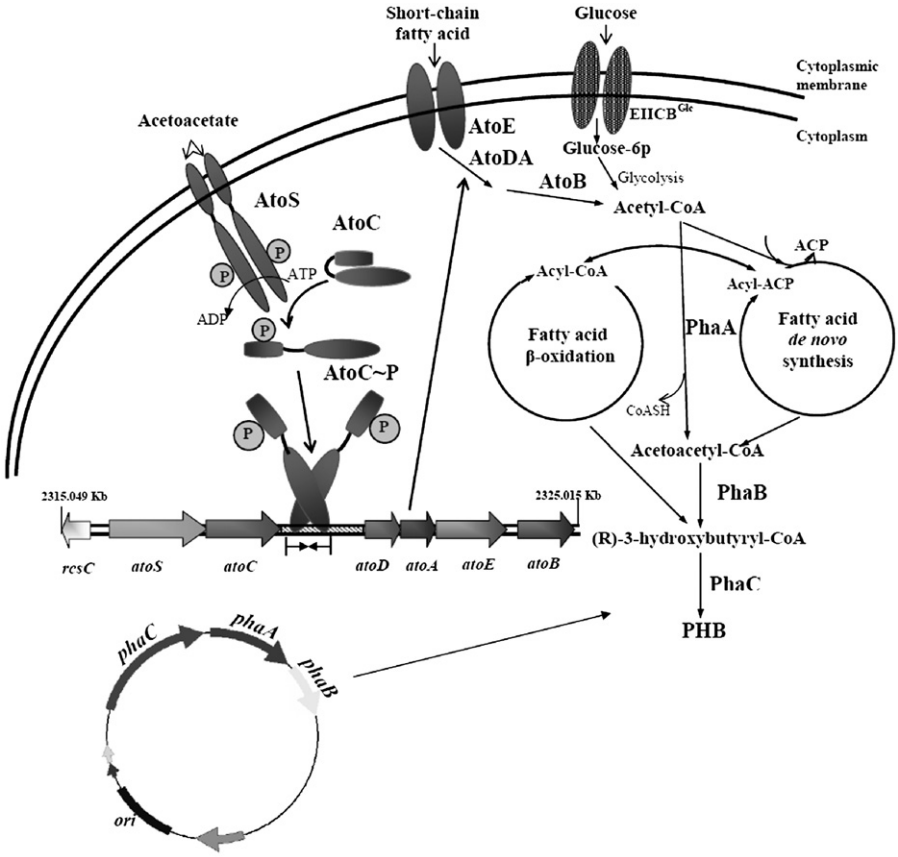

Fig. 6. A model for PHB biosynthesis regulation by the AtoSCDAEB regulon in *phaCAB*$^{+}$ *E. coli*. The AtoSC TCS and *atoDAEB* operon constituting the atoSCDAEB regulon, are induced by acetoacetate, spermidine, or other amine analogs and fatty acid metabolic intermediates, leading to the homodimeric membrane-bound AtoS kinase autophosphorylation and subsequently to the phosphotransfer to AtoC response regulator. The active AtoC then induces *atoDAEB* operon transcription upon binding oligomerized to its promoter, resulting to short-chain fatty acids catabolism and its participation in the PHB biosynthetic pathway. Acetoacetate or other short-chain fatty acids are transported through the cytoplasmic membrane by the AtoE membrane transporter and they are finally converted to acetyl-CoA by the *atoDA*-encoded acetyl-CoA: acetoacetyl-CoA transferase and the *atoB*-encoded thiolase II. Acetyl-CoA, the substrate for PHB biosynthesis, is mainly synthesized through the glycolysis pathway when glucose is the sole carbon source. β-Oxidation pathway participates while fatty acid biosynthesis is a prerequisite for PHB synthesis. AtoSC TCS is involved in the β-oxidation- or fatty acid biosynthesis-mediated PHB synthesis. Interplay between those pathways organizes PHB biosynthesis according to the current conditions while AtoSC TCS and the atoSCDAEB regulon are involved in this regulatory mechanism.

its promoter, resulting to short-chain fatty acids catabolism and its participation in the PHB biosynthetic pathway. Acetoacetate or other short-chain fatty acids are transported through the cytoplasmic membrane by the AtoE membrane transporter and they are finally converted to acetyl-CoA by the *atoDA*-encoded acetyl-CoA: acetoacetyl-CoA transferase and the *atoB*-encoded thiolase II. Acetyl-CoA is mainly synthesized through the glycolysis pathway when glucose is the sole carbon source. Acetyl-CoA serves as the substrate for PHB biosynthesis through the established pathway (Schubert et al., 1988; Fidler and Dennis, 1992). The active participation of β-oxidation to PHB synthesis is proved by the biopolymer but not the cell growth reduction when the pathway is inhibited by acrylic acid. AtoSC TCS and AtoC alone are involved in the β-oxidation-mediated PHB biosynthesis, suggested by the enhanced PHB in AtoSC- or AtoC-expressing cells despite the inhibition of β-oxidation, yet implying for additional mechanisms of AtoSC function. Depending on the current conditions, AtoB or FadA thiolases could contribute the substrates towards PHB biosynthesis. However, in Δ(*atoC-atoB*) cells, AtoB is deleted while FadA is inhibited by acrylic acid, strengthening either the participation of an additional pathway or enzyme that is necessary to provide the PHB biosynthetic mechanism with the valuable substrates or that the AtoSC TCS could be able to trigger a positive effect on the β-oxidation functions that are not inhibited. This additional process seems to be regulated by AtoSC considering the fact that AtoSC-overproducing Δ*atoDAEB* cells achieve to accumulate increased biopolymer. Inherent *E. coli* enzymes, including hydratases, have been proposed to be induced under β-oxidation-blockage and channel the intermediate products of the fatty acid metabolism towards PHAs synthesis (Fukui et al., 1998; Park and Lee, 2003).

Fatty acid biosynthesis is a prerequisite for PHB synthesis when glucose is used as the carbon source, suggested by the major PHB reduction observed in the presence of its inhibitor cerulenin (Funabashi et al., 1989), without however significant modulation in cell growth. The first step of fatty acid biosynthesis catalyzed by the acetyl-CoA carboxylase (Fujita et al., 2007) produces malonyl-CoA from the acetyl-CoA moieties and could supply the PHB intermediates, without the need of a thiolase function. AtoSC and AtoC involvement in fatty acid biosynthesis towards PHB up-regulation are signified by the TCS incapability to direct any PHB enhancement upon fatty acid biosynthesis inhibition. The severe drop of PHB when both the fatty acid metabolic pathways are inhibited in AtoSC-expressing bacteria, establish them as the fundamentals for PHB biosynthesis in *pha*$^+$ *E. coli*. Furthermore, the fact that under those conditions PHB synthesis is observed suggests that PhaA thiolase is not inhibited by acrylic acid, being still active towards PHB synthesis. An interplay between them organize PHB synthesis according to the current conditions while AtoSC TCS is involved in this mechanism either regulating the respective genes or affecting some enzymes of the fatty acid metabolism concurrently to its direct effects on the *atoDAEB* operon.

Studies on metabolic control along with improved bacterial product yield have revealed that regulon engineering, rewiring a native and prevailing signal transduction circuit exemplifies an emerging class of metabolic engineering approaches that target regulatory functions and result in the metabolically balanced coordination of the entire culture for a user-specified purpose. Amonsgt other, the cell–cell communication signaling process known as quorum sensing which is controlled by the PhoPR TCS has been proved to enable an individual bacterium's metabolic regulatory circuit to control the phenotype of an emerging population and initiate and drive product synthesis (Tsao et al., 2010). The AtoSC TCS signaling and the atoSCDAEB regulon primary involvement in optimal PHB accumulation in *phaCAB*$^+$ *E. coli* along with its interplay with additional biochemical mechanisms including fatty acids metabolism provide additional perceptions of the AtoSC contribution on *E. coli* mechanisms towards the biotechnologically optimal storage polyhydroxyalkanoates biosynthesis.

5. Conclusions

AtoSC two-component system up-regulates PHB biosynthesis, in recombinant *phaCAB*$^+$ *E. coli* strains, with the *Cupriavidus necator phaCAB* operon. The acetoacetate-mediated AtoSC induction maximizes PHB production. AtoSC regulates PHB through its direct effects on *atoDAEB* operon. Furthermore, AtoSC or its constituents and mainly AtoC separately up-regulate PHB through additional biochemical mechanisms. Interplay between β-oxidation and fatty-acid biosynthesis including the AtoSCDAEB regulon participation takes place for PHB biosynthesis in *phaCAB*$^+$ *E. coli*.

Conflict of interest

None to declare.

Acknowledgments

We thank Dr. Bernd H.A. Rehm (Institute of Molecular Biosciences, Massey University, Private Bag 11222, Palmerstron North, New Zealand) for plasmid pBHR68.

This work was supported by doctoral research scholarships to E.C.T. by the "State Scholarships Foundation" and "Onassis Foundation". We thank Dr. Hirofumi Aiba (Nagoya University, Japan) for *E. coli* K-12 strains BW25113 and BW28878. We thank Dr. S. Parouti and Professor C. Kyparrisides (CERTH/CPERI, Thessaloniki, Greece) for their help with the GPC and DSC procedures.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2012.03.010.

References

Brandl, H., Gross, R.A., Lenz, R.W., Fuller, R.C., 1988. *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential application as biodegradable polyesters. Appl. Environ. Microbiol. 54, 1977–1982.

Canellakis, E.S., Paterakis, A.A., Huang, S.C., Panagiotidis, C.A., Kyriakidis, D.A., 1993. Identification, cloning, and nucleotide sequencing of the ornithine decarboxylase antizyme gene of *Escherichia coli*. Proc. Natl. Acad. Sci. USA 90, 7129–7133.

Castañeda, M., Guzmán, G., Moreno, S., Espín, G., 2000. The GacS sensor kinase regulates alginate and poly-β-hydroxybutyrate production in *Azotobacter vinelandii*. J. Bacteriol. 182, 2624–2628.

Dennis, D., McCoy, M., Stangl, A., Valentin, H.E., Wu, Z., 1998. Formation of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) by PHA synthase from *Ralstonia eutropha*. J. Biotechnol. 64, 177–186.

Fidler, S., Dennis, D., 1992. Polyhydroxyalkanoate production in recombinant *Escherichia coli*. FEMS Microbiol. Rev. 103, 231–236.

Fujita, Y., Matsuoka, H., Hirookam, K., 2007. Regulation of fatty acid metabolism in bacteria. Mol. Microbiol. 66, 829–839.

Fukui, T., Shiomi, N., Doi, Y., 1998. Expression and characterization of (R)-specific enoyl coenzyme A hydratase involved in polyhydroxyalkanoate biosynthesis by *Aeromonas caviae*. J. Bacteriol. 180, 667–673.

Fukui, T., Yokomizo, S., Kobayashi, G., Doi, Y., 1999. Co-expression of polyhydroxyalkanoate synthase and (R)-enoyl-CoA hydratase genes from *Aeromonas caviae* establishes copolyester biosynthesis pathway in *Escherichia coli*. FEMS Microbiol. Lett. 170, 69–75.

Funabashi, H., Kawaguchi, A., Tomoda, H., Omura, S., Okuda, S., Iwasaki, S., 1989. Binding site of cerulenin in fatty acid synthetase. J. Biochem. 105, 751–755.

García Vésconi, E., Sciara, M.I., Castelli, M.E., 2010. Two component systems in the spatial program of bacteria. Curr. Opin. Microbiol. 13, 210–218.
Grage, K., Jahns, A.C., Parlane, N., Palanisamy, R., Rasiah, I.A., Atwood, J.A., Rehm, B.H.A., 2009. Bacterial polyhydroxyalkanoate granules: biogenesis, structure, and potential use as nano-/micro-beads in biotechnological and biomedical applications. Biomacromol 10, 660–669.
Griffo, G., Oppenheim, A.B., Gottesman, M.E., 1989. Repression of the lambda *pcin* promoter by integrative host factor. J. Mol. Biol. 209, 55–64.
Huijberts, G.N.M., de Rijk, T.C., de Waard, P., Eggink, G., 1995. ^{13}C nuclear magnetic resonance studies of *Pseudomonas putida* fatty acid metabolic routes involved in poly(3-hydroxyalkanoate) synthesis. J. Bacteriol. 176, 1661–1666.
Jung, Y.K., Lee, S.Y., Tam, T.T., 2011. Plastics from bacteria: natural functions and applications, towards systems metabolic engineering of PHA producers. In: Chen, G.-Q. (Ed.), Microbiology Monographs, vol. 14. Springer-Verlag, Berlin, Heidelberg.
Karr, D.B., Waters, J.K., Emerich, D.W., 1983. Analysis of poly-β-hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exclusion high-pressure liquid chromatography and UV detection. Appl. Environ. Microbiol. 46, 1339–1344.
Kessler, B., Witholt, B., 2001. Factors involved in the regulatory network of polyhydroxyalkanoate metabolism. J. Biotechnol. 86, 97–104.
Kichise, T., Taguchi, S., Doi, Y., 2002. Enhanced accumulation and changed monomer composition in polyhydroxyalkanoate (PHA) copolyester by in vitro evolution of *Aeromonas caviae* PHA synthase. Appl. Environ. Microbiol. 68, 2411–2419.
Klinke, S., Ren, Q., Witholt, B., Kessler, B., 1999. Production of medium-chain-length poly(3-hydroxyalkanoates) from gluconate by recombinant *Escherichia coli*. Appl. Environ. Microbiol. 65, 540–548.
Kyriakidis, D.A., Theodorou, M.C., Filippou, P.S., Kyriakidis, K.D., Tiligada, E., 2008. Effect of histamine on the signal transduction of the AtoS-AtoC two component system and involvement in poly-(R)-3-hydroxybutyrate biosynthesis in *Escherichia coli*. Amino Acids 35, 45–52.
Kyriakidis, D.A., Tiligada, E., 2009. Signal transduction and adaptive regulation through bacterial two-component systems: the *Escherichia coli* AtoSC paradigm. Amino Acids 37, 443–458.
Li, R., Zhang, H., Qi, Q., 2007. The production of polyhydroxyalkanoates in recombinant *Escherichia coli*. Bioresour. Technol. 98, 2313–2320.
Li, Z.-J., Shi, Z.-Y., Jian, J., Guo, Y.-Y., Wu, Q., Chen, G.-Q., 2010. Production of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) from unrelated carbon sources by metabolically engineered *Escherichia coli*. Metab. Eng. 12, 352–359.
Liu, S.J., Steinbüchel, A., 2000. A novel genetically engineered pathway for synthesis of poly(hydroxyalkanoic acids) in *Escherichia coli*. Appl. Environ. Microbiol. 66, 739–743.
Madison, L.L., Huisman, G.S., 1999. Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. Microbiol. Mol. Biol. Rev. 63, 21–53.
Matsumoto, K., Takase, K., Yamamoto, Y., Doi, Y., Taguchi, S., 2009. Chimeric enzyme composed of polyhydroxyalkanoate (PHA) synthases from *Ralstonia eutropha* and *Aeromonas caviae* enhances production of PHAs in recombinant *Escherichia coli*. Biomacromolecules 10, 682–685.
Mendesa, M.V., Tunca, S., Antna, N., Recio, E., Sola-Landa, A., Aparicio, J.F., Martín, J.F., 2007. The two-component *phoR-phoP* system of *Streptomyces natalensis*: inactivation or deletion of *phoP* reduces the negative phosphate regulation of pimaricin biosynthesis. Metab. Eng. 9, 217–227.
Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428.
Oshima, T., Aiba, H., Masuda, Y., Kanaya, S., Masahito, S., Wanner, B.L., Mori, H., Mizuno, T., 2002. Transcriptome analysis of all two component regulatory system mutants of *Escherichia coli* K-12. Mol. Microbiol. 46, 281–291.
Park, S.J., Ahn, W.S., Green, P.R., Lee, S.Y., 2001. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate) by metabolically engineered *Escherichia coli* strains. Biotechnol. Bioeng. 74, 81–86.
Park, S.J., Choi, J.I., Lee, S.Y., 2004. Engineering of *Escherichia coli* fatty acid metabolism for the production of polyhydroxyalkanoates. Enzyme Microb. Technol. 36, 579–588.
Park, S.J., Lee, S.Y., 2003. Identification and characterization of a new enoyl coenzyme A hydratase involved in biosynthesis of medium-chain-length polyhydroxyalkanoates in recombinant *Escherichia coli*. J. Bacteriol. 185, 5391–5397.
Peplinski, K., Ehrenreich, A., Döring, C., Bömeke, M., Reinecke, F., Hutmacher, C., Steinbüchel, A., 2010. Genome-wide transcriptome analyses of the 'Knallgas' bacterium *Ralstonia eutropha* H16 with regard to polyhydroxyalkanoate metabolism. Microbiology 156, 2136–2152.
Peralta-Gil, M., Segura, D., Guzmán, J., Servin-González, L., Espín, G., 2002. Expression of the *Azotobacter vinelandii* poly-β-hydroxybutyrate biosynthetic *phbBAC* operon is driven by two overlapping promoters and is dependent on the transcriptional activator abr. J. Bacteriol. 184, 5672–5677.
Prieto, M.A., Kellerhals, M.B., Bozzato, G.B., Radnovik, D., Witholt, B., Kessler, B., 1999. Engineering of stable recombinant bacteria for production of chiral medium-chain-length poly-3-hydroxyalkanoates. Appl. Environ. Microbiol. 65, 3265–3271.
Qi, Q.S., Steinbüchel, A., Rehm, B.H.A., 1998. Metabolic routing towards polyhydroxyalkanoic acid synthesis in recombinant *Escherichia coli* (*fadR*): inhibition of fatty acid L-oxidation by acrylic acid. FEMS Microbiol. Lett. 167, 89–94.
Rehm, B.H.A., 2003. Polyester synthases: natural catalysts for plastics. Biochem. J. 376, 15–33.
Reusch, R.N., Shabalin, O., Crumbaugh, A., Wagner, R., Schröder, O., Wurm, R., 2002. Posttranslational modification of *E. coli* histone-like protein H-NS and bovine histones by short-chain poly-(R)-3-hydroxybutyrate (cPHB). FEBS Lett. 527, 319–322.
Rhie, H.G., Dennis, D., 1995. Role of *fadR* and *atoC*(Con) mutations in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) synthesis in recombinant *pha*$^+$ *Escherichia coli*. Appl. Environ. Microbiol. 61, 2487–2492.
Sambrook, J., Fitsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor.
Schembri, M.A., Bayly, R.C., Davies, J.K., 1995. Phosphate concentration regulates transcription of the *Acinetobacter* polyhydroxyalkanoic acid biosynthetic genes. J. Bacteriol. 177, 4501–4507.
Schubert, P., Steinbüchel, A., Schlegel, H.G., 1988. Cloning of the *Alcaligenes eutrophus* poly-b-hydroxybutyrate synthetic pathway and synthesis of P(3HB) in *Escherichia coli*. J. Bacteriol. 170, 5837–5847.
Slater, S., Gallaher, T., Dennis, D., 1992. Production of poly-(3-hydroxybutyrate-*co*-3-hydroxyvalerate) in a recombinant *Escherichia coli* strain. Appl. Environ. Microbiol. 58, 1089–1094.
Spiekermann, R., Rehm, B.H.A., Karlscheuer, R., Baumeister, D., Steinbüchel, A., 1999. A sensitive, viable-colony staining method using Nile Red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch. Microbiol. 171, 73–80.
Sun, J., Peng, X., Impe, J.V., Vanderleyden, J., 2000. The *ntrB* and *ntrC* genes are involved in the regulation of poly-3-hydroxybutyrate biosynthesis by ammonia in *Azospirillum brasilense* Sp7. Appl. Environ. Microbiol. 66, 113–117.
Theodorou, E.C., Theodorou, M.C., Kyriakidis, D.A., 2011a. AtoSC two-component system is involved in cPHB biosynthesis through fatty acid metabolism in *E. coli*. Biochim. Biophys. Acta 1810, 561–568.
Theodorou, E.C., Theodorou, M.C., Samali, M.N., Kyriakidis, D.A., 2011b. Activation of the AtoSC two component system in the absence of the AtoC N-terminal receiver domain in *E. coli*. Amino Acids 40, 421–430.
Theodorou, M.C., Panagiotidis, C.A., Panagiotidis, C.H., Pantazaki, A.A., Kyriakidis, D.A., 2006. Involvement of the AtoS-AtoC signal transduction system in poly-(R)-3-hydroxybutyrate biosynthesis in *Escherichia coli*. Biochim. Biophys. Acta 1760, 896–906.
Theodorou, M.C., Theodorou, E.C., Kyriakidis, D.A., 2011c. Involvement of AtoSC two-component system in *Escherichia coli* flagellar regulon. Amino Acids 10.1007/s00726-011-1140-7.
Theodorou, M.C., Theodorou, E.C., Panagiotidis, C.A., Kyriakidis, D.A., 2007. Spermidine triggering effect to the signal transduction through the AtoS-AtoC/Az two component system in *Escherichia coli*. Biochim. Biophys. Acta 1770, 1104–1114.
Theodorou, M.C., Tiligada, E., Kyriakidis, D.A., 2009. Extracellular Ca^{2+} transients affect poly-(R)-3-hydroxybutyrate regulation by the AtoS-AtoC system in *E. coli*. Biochem. J. 417, 667–672.
Tsao, C.-Y., Hooshangi, S., Wu, H.-C., Valdes, J.J., Bentley, W.E., 2010. Autonomous induction of recombinant proteins by minimally rewiring native quorum sensing regulon of *E. coli*. Metab. Eng. 12, 291–297.
Tyo, K.E.J., Fischer, C.R., Simeon, F., Stephanopoulos, G., 2010. Analysis of polyhydroxybutyrate flux limitations by systematic genetic and metabolic perturbations. Metab. Eng. 12, 187–195.
Xian, M., Fuerst, M.M., Shabalin, Y., Reusch, R.N., 2007. Sorting signal of *Escherichia coli* OmpA is modified by oligo-(R)-3-hydroxybutyrate. Biochim. Biophys. Acta 1768, 2660–2666.
Yim, K.S., Lee, S.Y., Chang, H.N., 1996. Synthesis of poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) by recombinant *Escherichia coli*. Biotechnol. Bioeng. 49, 495–503.
Zhou, Q., Shi, Z.-Y., Meng, D.-C., Wu, Q., Chen, J.-C., Chen, G.-Q., 2011. Production of 3-hydroxypropionate homopolymer and poly(3-hydroxypropionate-*co*-4-hydroxybutyrate) copolymer by recombinant *Escherichia coli*. Metab. Eng. 13, 777–785.