Involvement of the AtoS-AtoC signal transduction system in poly-(R)-3-hydroxybutyrate biosynthesis in *Escherichia coli*

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**Abstract**

The AtoS–AtoC signal transduction system in *E. coli*, which induces the atoDAEB operon for the growth of *E. coli* in short-chain fatty acids, can positively modulate the levels of poly-(R)-3-hydroxybutyrate (cPHB) biosynthesis, a biopolymer with many physiological roles in *E. coli*. Increased amounts of cPHB were synthesized in *E. coli* upon exposure of the cells to acetoacetate, the inducer of the AtoS–AtoC two-component system. While *E. coli* that overproduce both components of the signal transduction system synthesize higher quantities of cPHB (1.5–4.5 fold), those that overproduce either AtoS or AtoC alone do not display such a phenotype. Lack of enhanced cPHB production was also observed in cells overexpressing AtoS and phosphorylation-impaired AtoC mutants. The results were not affected by the nature of the carbon source used, i.e., glucose, acetate or acetoacetate. *E. coli* strain with a deletion in the atoS–atoC locus (ΔatoSC) synthesized lower amounts of cPHB compared to wild-type cells. When the ΔatoSC strain was transformed with a plasmid carrying a 6.4-kb fragment encoding the AtoS–AtoC system, cPHB biosynthesis was restored to the level of the atoSC+ cells. Introduction of a multicopy plasmid carrying a functional atoDAEB operon, but not one with a promoterless operon, resulted in increased cPHB synthesis only in atoSC+ cells in the presence of acetoacetate. These results indicate that the presence of both a functional AtoS–AtoC two-component signal transduction system and a functional atoDAEB operon is critical for the enhanced cPHB biosynthesis in *E. coli*.

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1. **Introduction**

Short-chain poly-(R)-3-hydroxybutyrate (PHB), a member of the polyhydroxyalkanoate family (PHAs), is an ubiquitous constituent of prokaryotic and eukaryotic cells where it has been found to form complexes with other macromolecules and is referred to as complexed PHB (cPHB) [1–7]. cPHB also complexes to calcium polyphosphate in bacterial membranes and participates in the formation of Ca2+ channels [8]. It has been reported, as well, that cPHB binds DNA during the uptake of exogenous DNA by genetically competent *E. coli* [2]. Storage polyhydroxyalkanoates (sPHAs) are accumulated as intracellular storage polymers by a wide variety of bacteria in response to metabolic stress [9–12]. *E. coli*, however, only synthesizes the complexed PHB and does not accumulate storage PHAs. A pathway of cPHB biosynthesis in *E. coli* has been proposed [2], although the cPHB biosynthetic enzymes have not yet been found and bioinformatic analyses have failed to demonstrate homologies at the gene level between *E. coli* and storage-PHB producing microorganisms [11].

Antizyme (Az) was discovered by the Canellakis group as a polyamine-inducible, non-competitive inhibitor of ornithine decarboxylase (ODC) [13]. Az plays a role in the control of the polyamine biosynthesis pathway in *E. coli* [14] and is encoded by the *atoC* gene in the AtoS–AtoC system [15]. The AtoS–AtoC system is a two-component signal transduction system that regulates the transcription of the atoDAEB operon, which encodes the enzymes responsible for the synthesis of poly-(R)-3-hydroxybutyrate (cPHB) in *E. coli*. The AtoS–AtoC system is activated by acetoacetate, an intermediate of cellular lipid metabolism, and induces the expression of the atoDAEB operon, which is involved in the biosynthesis of cPHB.

The AtoS–AtoC system is composed of two proteins, AtoS (histidine kinase) and AtoC (response regulator), which are located on the chromosome of *E. coli*. AtoS functions as a sensor kinase, responding to changes in the cellular environment, particularly to the presence of acetoacetate. AtoC, on the other hand, acts as a transcriptional activator, regulating the expression of the atoDAEB operon. The interaction between AtoS and AtoC is mediated by phosphorylation and dephosphorylation, with AtoS acting as a kinase and AtoC acting as a phosphatase. The phosphorylation state of AtoC determines whether the atoDAEB operon is transcribed or not.

The atoDAEB operon encodes the enzymes involved in the degradative pathway of poly-(R)-3-hydroxybutyrate (cPHB) in *E. coli*. The enzymes encoded by the atoDAEB operon catalyze the conversion of cPHB into acetoacetate, which can then be catabolized through the Krebs cycle. The expression of the atoDAEB operon is induced by acetoacetate, the end product of cPHB degradation, suggesting a feedback control mechanism to maintain the balance between cPHB synthesis and degradation.

The AtoS–AtoC system has been extensively studied in *E. coli* due to its role in the regulation of cPHB biosynthesis. However, the exact mechanism by which the AtoS–AtoC system modulates cPHB biosynthesis is still not fully understood. Recent studies have suggested that the AtoS–AtoC system may also play a role in the regulation of other processes, such as the synthesis of poly[(3-hydroxybutyrate-co-3-hydroxyvalerate)] (poly[3HB-co-3HV]), a PHA with different physical and mechanical properties compared to cPHB. Further research is needed to elucidate the role of the AtoS–AtoC system in these processes and to understand the complex regulatory mechanisms that govern the synthesis and degradation of polyhydroxyalkanoates in *E. coli*.
decarboxylase, the key enzyme of polyamine biosynthesis [13–15]. Further efforts, by the Canellakis group, to isolate the ODC-antizyme (Az) gene of E. coli led to the cloning of a 6.4 kb genomic fragment containing the gene of Az, which appeared to be the response regulator (RR) of a putative two-component system (TCS) [16]. TCSs usually comprise an inner membrane sensor histidine kinase (HK) and a cognate response regulator (RR), which most often is a transcriptional activator [17,18]. Thus, it appeared that Az has a dual function as both a post-translational regulator of polyamine biosynthesis and a σ54 RNA polymerase transcriptional activator of the NtrC–NifA family [16]. The genomic location of the cloned fragment coincides with that of the atoSC locus, which encodes a putative TCS that activates the expression of the atoDAEB operon upon acetoacetate induction [19,20]. The structural genes within the atoDAEB operon encode proteins involved in short-chain fatty acid (SCFA) metabolism, i.e., the acetyl-CoA:acetoacetyl-CoA transferase (a AtoD–AtoA heterodimer), the SCFA inner membrane transporter (AtoE) and thiolase II (AtoB) [21–24]. Recent work from our laboratory has shown that AtoS and AtoC indeed constitute a two-component system [25–27]. Specifically, it was shown that AtoS is a membrane-bound sensor HK that phosphorylates AtoC (antizyme) and that AtoS-catalyzed AtoC phosphorylation is induced by acetoacetate [25,27], the only known inducer of the atoDAEB operon [21–24]. The ability of a recombinant cytosolic region of AtoS to autophosphorylate, albeit at a very low rate, was also demonstrated in a recent global analysis of E. coli TCSs [28]. This phosphorylation of AtoC is essential for the transcriptional activation of the atoDAEB operon since mutations that alter its putative phosphorylation sites render the system nonresponsive to acetoacetate induction [27].

Recent global analyses of the E. coli TCSs [29,30] have revealed that the role(s) of the AtoS–AtoC TCS might not be limited to atoDAEB operon’s regulation but that it might be involved in a number of other processes, including flagellar synthesis and chemotaxis [29], sodium (but not potassium) sensitivity [30] and cross-regulation with the EnvZ-OmpR TCS [29].

Mutations in fadR and atoC(Con), that inactivate FadR and render AtoC constitutively active, respectively, have been employed in efforts to boost the production of precursors of biodegradable plastics in E. coli [31,32]. The involvement of atoC in PHA production led us to investigate the putative role of AtoS–AtoC TCS in the biosynthesis of endogenous E. coli ePHB. Our experiments indicate that ePHB production is indeed affected by this TCS, since alterations in the expression levels of its components affected intracellular ePHB accumulation, irrespective of the carbon source used. Furthermore, atoC phosphorylation site mutants accumulated significantly less ePHB than their wild-type counterparts. Overall, our results indicate that the AtoS–AtoC TCS, which induces transcription from the atoDAEB operon, to permit E. coli growth in short-chain fatty acids, also enhances ePHB biosynthesis in E. coli.

2. Materials and methods

2.1. Bacterial strains and plasmids

The genotypes of the E. coli strains used are listed in Table 1. E. coli K-12 BW25113 (atpSC) [33] and BW28878 (ΔatpSC) [29] were a kind gift from Hirofumi Aiba (Laboratory of Molecular Microbiology, School of Agriculture, Nagoya University, Chikusa-ku, Nagoya, 464–8601, Japan).

Plasmids pUC-Az (Fig. 1a), containing the atoSC, atoD genes and a part of the atoDAEB operon (atoD, atoA, and two thirds of atoE) and pPCP-Az (Fig. 1d), containing the atoC gene and the same part of the atoDAEB operon as pUC-Az, have been described previously [16]. Plasmid pUC-Az was used for the construction of pUC-Az(ΔatoC) and pUC-Az(ΔatoDAEB) plasmids. It must be noted that the original published sequence of the pUC-Az insert [16] contained three sequencing errors due to compressions in GC-rich regions and not due to mutations introduced during the cloning and plasmid propagation steps.

Plasmid pHis$_{i0}$-AtoC (Fig. 1e), expressing AtoC fused to an N-terminal His-10 tag, was constructed by cloning the 3.8 kb Asl–BamHI fragment of plasmid pCPC-Az into the NdeI and BamHI sites of the pET-16b expression vector (Novagen, Darmstadt, Germany). The insert also contains part of the atoDAEB operon downstream of atoC, i.e., atoD, atoA and the truncated atoE gene. The cloning junction was sequenced to verify the in-frame fusion of the His$_{i0}$-tag with AtoC (AtoC lacks the first four aminoacids). His$_{i0}$-AtoC can be expressed, upon IPTG induction, when the pHis$_{i0}$-AtoC plasmid is introduced into E. coli cells expressing the T7 phage RNA polymerase. The presence and identity of the His$_{i0}$-AtoC fusion protein was verified by western blot analyses using either a mouse monoclonal antibody against the polyhistidine tag (mAb His-1, Sigma-Aldrich H11029) or a rabbit polyclonal antibody raised against antizyme, now known as AtoC [16].

Plasmids pEM-Az-D55, pEM-Az-H73 and pEM-Az-D55/H73 [27] are similar to pUC-Az but carry either single mutations in atoC codons 55 (D to G) or 73 (H to L), or mutations in both codons, respectively. Therefore, all three plasmids express wild-type AtoC together with a mutant form of AtoC [27]. Plasmid pUC-Az(ΔatoC) was generated by digestion of pUC-Az with BstBI, fill-in of the linearized plasmid with Klenow polymerase and religation. This process generates a frameshift within the atoC coding sequence 87 bp downstream of the atoC start codon. Thus, pUC-Az(ΔatoC) does not lead to production of the AtoC protein due to the frame shift mutation introduced at codon 28 (Fig. 1b). The lack of AtoC overexpression in E. coli cells (TOP10F and BL21 [DE3]) transformed with pUC-Az(ΔatoC) was verified by SDS/PAGE electrophoresis and western blot analysis.

Plasmid pUC-Az(ΔatoDAEB) was generated by digestion of pUC-Az with RsalII, which cleaves within the atoD coding sequence, 184 bp downstream of the atoD start codon, and with Xhol, which cleaves within the vector sequences. The ends of the linearized plasmid were filled using the Klenow fragment of the E. coli DNA polymerase I and the plasmid was religated. Thus, pUC-Az (ΔatoDAEB) (Fig. 1e) lacks the atoDAEB operon due to the deletion of this locus but encodes the AtoS and AtoC proteins.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Escherichia coli strains</th>
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<tbody>
<tr>
<td>Strain</td>
<td>Genotype</td>
</tr>
<tr>
<td>N99</td>
<td>Wild type, Δf17, Δf18, sup0, galK2</td>
</tr>
<tr>
<td>BW25113</td>
<td>lacY1 rruB3 ΔlacZ587 hisR514 Δ(araBAD)567 Δ(budBAD)568 rph-1</td>
</tr>
<tr>
<td>BW28878</td>
<td>lacY1 rruB3 ΔlacZ587 hisR514 Δ(araBAD)567 Δ(budBAD)568 rph-1 (ΔatoSC)569</td>
</tr>
<tr>
<td>XL1-blue</td>
<td>recA1 endA1 gyrA96 thi-1 hisD17 suppl44 relA1 lac Δ(f1) proph lacZΔ2 MA15 Tn10 (Tet$^\beta$)</td>
</tr>
<tr>
<td>TOP10F</td>
<td>F’ [lacIq1 tri1 (Tet$^\beta$) mcrA Δ(mrr-hsdRMS-merC)8]OlacZAM15 ΔlacX74 deor recA1 arad319 Δ(ara-leu)769 glnU gaaK rpsL (Str$^\beta$) endA1 mupG</td>
</tr>
<tr>
<td>DH5a</td>
<td>F’ [lacU169 delR122lacI15 (lacZΔM15-argF15) U169 deor recA1 endA1 hisD172 (rpsL2, mcr) phoA supE44 Δf17 thi-1 gyrA96 relA1 lacZAM15 (ΔlacX74) deor recA1 arad319 Δ(ara-leu)769 glnU gaaK rpsL (Str$^\beta$) endA1 mupG</td>
</tr>
<tr>
<td>BL21 [DE3]</td>
<td>F’ ompT hsdS2(80m8) gal dcm (DE3)</td>
</tr>
</tbody>
</table>
To construct plasmid pZatop (Fig. 1f), the complete atoDAEB operon sequences including its promoter were PCR amplified using *E. coli* K-12 N99 genomic DNA as template. The sequences of amplification primers used were as follows: (sense) 5′-TCG GAA TTC ATT GAT GTA TAA ACT CCA GGA A-3′ (hybridizes 253 bp upstream of the start codon of *atoD* gene) and (antisense) 5′-CGC TGATCA CCG ATG AAG GAT-3′ (hybridizes 177 bp downstream of the end of *atoB* gene). The 4277-bp product was blunt ended by T4 phage DNA polymerase reaction [34] and then cloned into the *Eco*RV site of plasmid pZErO 2.1 (Invitrogen, San Diego, CA, USA) to yield plasmid pZatop.

Similarly, to construct plasmid pZato (Fig. 1g), the atoDAEB operon coding sequence without the promoter sequence was PCR amplified from *E. coli* K-12 N99 genomic DNA, using the following amplification primers: (sense) 5′-TAC GAA TTC AAC TAA ATC CAA TAA TCT CAT T-3′ (hybridizes 93 bp upstream of the start codon of *atoD* gene) and (antisense) 5′-CGC TGA TCA CCG ATG AAG GAT-3′ (hybridizes 177 bp downstream of the end of *atoB* gene). The 4117 bp product was blunt ended by T4 phage DNA polymerase reaction [34] and then cloned into the *Eco*RV site of plasmid pZErO 2.1 (Invitrogen, San Diego, CA, USA) to yield plasmid pZato.

### 2.2. Growth conditions
*E. coli* were grown at 37 °C either in Luria–Bertani (LB) medium [34] with 0.5% (w/v) glucose or in M9 mineral medium [34], supplemented with 0.1 mM CaCl₂, 1 mM MgSO₄, 1.7 × 10⁻³ mM FeSO₄, 1 μg/ml thiamine and 80 μg/ml n-leucine or 80 μg/ml n-proline (for BW25113 and BW28878 strains) and carbon source [0.5% (w/v) glucose or 25 mM sodium acetate or 25 mM acetoacetate]. Acetoacetate induction of the AtoS–AtoC TCS was initiated by the addition of the inducer (final concentration 10 mM) in the cell cultures, when the *A₆₀₀* reached 0.2–0.3, and was maintained by repeated subsequent additions of 2 mM acetoacetate every 4 h to ensure sustained induction. In experiments where acetoacetate was used both as the carbon source and as the inducer of the AtoS–AtoC system, it was added to a final concentration of 25 mM at 4-h intervals. Stock acetoacetate solutions were prepared in the form of its sodium salt [35]. Ampicillin and kanamycin were used in the culture media, when necessary, at final concentrations of 100 and 50 μg/ml, respectively. Induction of recombinant *His₁₀*-AtoC expression in *E. coli* BL21[DE3] carrying the expression plasmid pHis₁₀-AtoC was achieved by addition of IPTG (1 mM) to the cultures. IPTG was added when the absorbance at 600nm reached 0.4. No significant variation in either growth rates or final cell densities was observed between the strains growing under the same conditions (irrespective of whether they carried an atoSC mutation or a multicopy plasmid). Cell sample collections for crotonic acid/cPHB determination were initiated at the time of the first addition of acetoacetate and continued at the specified time points indicated in the pertinent figures (Figs. 2–6).

### 2.3. DNA isolation, manipulation and transformation
Genomic and plasmid DNA isolation, T4 phage DNA polymerase reaction, restriction enzyme digestions, ligations, transformations and western blotting, were carried out with standard methodology [34,36]. Plasmids were purified using commercially available kits (Qiagen or Nucleobond) following the manufacturer’s instructions.

### 2.4. Electrophoresis and immunoblotting
SDS-polyacrylamide gel electrophoresis (SDS/PAGE) was performed using 10% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS as...
described by Laemmli et al. [37]. Gels were stained with either Coomassie Brilliant Blue R250 [38] or silver nitrate [39]. Proteins were transferred to nitrocellulose membranes following the method of Towbin et al. [40] and immunostained either with the rabbit polyclonal anti-AtoC and anti-AtoS antibodies [27] or a mouse monoclonal antibody against the polyhistidine tag (mAb His-1, Sigma-Aldrich H1029).

2.5. Nile Red viable-colony staining method

The accumulation of cPHB in E. coli was assessed qualitatively with the Nile-Red viable-colony staining method that monitors the fluorescent intensity of colonies growing on LB agar plates containing the fluorescent dye Nile Red [final concentration of 0.5 μg dye/ml of LB agar]. The Nile Red (Sigma, St. Louis, MO, USA) stock solution was prepared at a concentration of 0.25 mg/ml in DMSO, as described elsewhere [41].

2.6. Determination of cPHB

The procedure used is a variation of the method of Karr et al. [42] as described by Huang et al. [5] and is based on the acid-catalyzed β-elimination of cPHB to crotonic acid, followed by HPLC analysis of the produced crotonic acid. The procedure was followed as described [5], with the difference that the dry E. coli cell pellets (from 10 ml of culture) were incubated with the concentrated H2SO4 at 92 °C for 8 h, as this was found to be the most efficient procedure for cPHB extraction (data not shown). The final filtrate was analyzed by HPLC on an Aminex HPX-87H ion exclusion organic acid analysis column.
3. Results

3.1. Enhancement of cPHB biosynthesis in E. coli upon induction of the AtoS–AtoC two-component signal transduction system by acetoacetate

To measure cPHB accumulation in E. coli, we employed a sensitive and quantitative method [5,42] which is based on the acid-catalyzed β-elimination of cPHB to crotonic acid, followed by HPLC analysis (see Methods). While cPHB levels were roughly equivalent in both untransformed BL21[DE3] and in pUC-Az-bearing cells (Fig. 2a), upon acetoacetate addition the cPHB levels were significantly increased in the bacteria carrying the atoSC genes in the multicopy plasmid pUC-Az, compared to the untransformed controls. This increase reached its maximum (approximately 2.5 fold) at 6 h after acetoacetate addition (Fig. 2a). It should be noted that exposure of control cells to acetoacetate produced a small but measurable increase (less than 40%) in cPHB accumulation (Fig. 2a). These data are consistent with results obtained using the fast semiquantitative method of viable colony staining with the fluorescent dye Nile Red [41].

The above effects were not limited to the E. coli strain used (i.e., BL21[DE3]) since similar results were obtained with E. coli K-12 TOP10F’ harboring pUC-Az (2.5 fold increase in cPHB levels at 6 h post-induction) (data not shown) as well as with BW25113/pUC-Az cells (see below). No effect was produced when the “empty” pUC19 vector was introduced in these cells (data not shown).

A series of experiments was performed to assess whether the presence of either AtoS or AtoC alone can increase the intracellular levels of cPHB upon acetoacetate induction, or whether the presence of both components of the AtoS–AtoC TCS is required for this effect. To evaluate whether the overexpression of AtoS alone was sufficient to affect cPHB biosynthesis we introduced a frameshift mutation in atoC (codon 28) within the context of plasmid pUC-Az. Western blot analyses of extracts obtained from cells transformed either with pUC-Az or pUC-Az(AtoC\(^{-}\)), the plasmid carrying the atoC frameshift mutation (Fig. 1b), verified that pUC-Az(AtoC\(^{-}\)) fails to direct AtoC expression while producing amounts of AtoS equivalent to the parental pUC-Az plasmid (data not shown). Cells transformed with pUC-Az(AtoC\(^{-}\)) failed to accumulate increased cPHB levels, compared to pUC-Az-transformed cells, but rather they behaved like their untransformed counterparts in both the presence (Fig. 2b) and the absence of acetoacetate (data not shown).

Having shown that increased cPHB accumulation requires the presence of a functional \(\text{atoC}\), we next asked whether AtoC could produce this effect in the absence of AtoS. To this end, E. coli BL21[DE3] cells were transformed with plasmid pHis\(_{10}^{-}\)-AtoC (Fig. 1e), which produces a recombinant form of AtoC fused to an N-terminal His-10 tag. These cells failed to accumulate higher cPHB amounts than their untransformed counterparts, either in the presence or in the absence of acetoacetate (Fig. 2b). The presence of the N-terminal His-10 tag in the recombinant AtoC protein (which also lacks the first four AtoC aminoacids) does not appear to affect its function since it retains its DNA binding ability, its DNA-dependent ATPase activity and its ability to serve as an AtoS-dependent phosphotransfer acceptor (unpublished results). However, to further exclude the possibility that this recombinant AtoC is not functional, the same experiment was performed with BL21[DE3] transformed with pCPC-Az (Fig. 1d), a plasmid that expresses native AtoC under its own (atoC) promoter [16]. The results of these experiments were similar to those obtained with pHis\(_{10}^{-}\)-AtoC (data not shown).

Taken together, the above results indicate that the overexpression of either AtoC or AtoS alone fails to yield increased cPHB levels, even in the presence of acetoacetate. Therefore, the overexpression of both AtoS and AtoC is required for the increased cPHB production in E. coli, following exposure to acetoacetate.

3.2. cPHB biosynthesis in E. coli cells under different carbon sources

It has been reported that \(\text{atoDAEB}\) operon expression can be repressed by glucose [44], which is the carbon source used in the above described experiments. To eliminate this variable, the experiments were repeated using sodium acetate as the sole carbon source instead of glucose [45,46].

The substitution of the carbon source did not appear to affect the E. coli cPHB levels, which remained at approximately 0.30 \(\mu\)g cPHB per ml of culture per \(A_{600}\) unit. In the presence of acetoacetate, E. coli BL21[DE3] pUC-Az synthesized approximately 4.5 times more cPHB over control cells during the stationary phase of growth, at 16 h post acetoacetate induction (Fig. 3a). As was the case with cells growing in the presence of glucose, cells growing in acetate and overexpressing either AtoC or AtoS singly failed to accumulate increased cPHB levels (Fig. 3a).

We also assessed the effects of AtoC and/or AtoS overproduction on cPHB accumulation when acetoacetate was used both as the inducer and sole carbon source. To serve as carbon source, acetoacetate was added to the culture medium at a final concentration of 25 mM. Furthermore, acetoacetate was being repeatedly added, during culture growth, at 4-h intervals, to prevent exhaustion of the carbon source (Fig. 3b). Under these conditions, BL21[DE3] pUC-Az synthesized significantly higher amounts of cPHB (4-fold increase) than control E. coli BL21[DE3] cells, at 28 h of growth (Fig. 3b). This increase is the greatest relative enhancement in the cPHB accumulation among the three carbon sources tested in this work. Not surprisingly, BL21[DE3] pHis\(_{10}^{-}\)-AtoC and BL21[DE3] pUC-Az(AtoC\(^{-}\)) failed to synthesize more cPHB than their untransformed counterparts.
These data suggest that the overproduction of the AtoS–AtoC TCS enhances cPHB biosynthesis in E. coli growing either in acetate or acetoacetate carbon sources.

3.3. cPHB biosynthesis in E. coli K-12 with a deletion of the atoSC genetic locus

The requirement for overproduction of both components of the AtoS–AtoC TCS for the increased cPHB accumulation in E. coli led to the postulation that deletion of atoS and/or atoC should have an adverse effect on cPHB biosynthesis. To test this notion, cPHB synthesis was measured in an isogenic pair of E. coli strains which differed only in that they were either atoSC+ (BW25113) or ΔatoSC (BW28878).

It was found that, when glucose was the sole carbon source, the atoSC deletion resulted in a consistent decrease in the basal cPHB levels (approximately 70% of atoSC+ cells), as well as in a failure of the ΔatoSC cells to produce more cPHB upon acetoacetate induction (Fig. 4a). In contrast, the atoSC+ cells displayed a 1.2 fold increase in their cPHB levels when induced with acetoacetate (Fig. 4a).

The inability of the ΔatoSC cells to accumulate higher amounts of cPHB upon acetoacetate induction could be complemented by pUC-Az, which provides the functions of the AtoS–AtoC system extrachromosomally (Fig. 4b). It is worth noting, however, that the induced cPHB amounts in ΔatoSC cells harboring pUC-Az, remained at lower levels than those measured in their pUC-Az-carrying atoSC+ counterparts both in the absence or presence of inducing acetoacetate (Fig. 4b). This might indicate that these cells (i.e., BW25113 and BW28878) might have some cryptic differences in genes involved in cPHB biosynthesis, other than their characterized atoSC+ or ΔatoSC genotypes. In agreement with the data presented above (Figs. 2 and 3), extrachromosomal introduction of either AtoS or AtoC alone, through the respective use of pUC-Az(AtoCΔ) or pCPC-Az plasmids, failed to complement the ΔatoSC phenotype both in the absence or in the presence of acetoacetate (Fig. 4b).

Therefore, these data further support the notion that the presence of both functional AtoS and AtoC is required for the acetoacetate induction of cPHB synthesis.

3.4. Effects of mutations in the putative AtoC phosphorylation sites on cPHB synthesis

We recently described that AtoC contains two putative phosphorylation sites, i.e., a conserved aspartic acid (D55) and a histidine residue (H73) in an unexpected “H box” consensus sequence, which is normally common to histidine kinases [25,27]. Mutations that alter these putative phosphate acceptor sites render the system unresponsive to acetoacetate induction [27]. These findings raised the question as to whether either or both AtoC phosphorylation sites are involved in the positive regulation of cPHB biosynthesis by the AtoS–AtoC TCS.

To measure the effects of the above-mentioned mutations on cPHB accumulation we transformed E. coli BL21(DE3) with pUC-Az derivative plasmids pEM-Az-D55, pEM-Az-H73 and pEM-Az-D55/H73, which express mutant forms of AtoC, i.e., AtoC with D55G, H73L or D55G/H73L mutations, and wild-type AtoS [27]. The cPHB levels of these cells, as well as of cells transformed with wild-type pUC-Az, were measured in the absence (Fig. 5a) or presence (Fig. 5b) of inducing acetoacetate.

It was found that substitution of D55 to glycine significantly reduces cPHB accumulation (approximately 40% at 6 h post-induction), compared with cells expressing wild-type AtoC, whereas the H73L mutation has an even more pronounced effect (approximately 70% reduction). Introduction of both AtoC mutations resulted in total abrogation of the AtoS–AtoC

Fig. 4. Decreased cPHB biosynthesis in E. coli K-12 cells with a deletion of the atoS–atoC genetic locus, grown in glucose in the presence or absence of acetoacetate. a) BW25113 (atoSC+) and BW28878 (ΔatoSC) cells were grown in mineral medium M9 containing 0.5% glucose in the presence (filled symbols) or absence (open symbols) of 10 mM AcAc; (■), BW25113; (□), BW28878. b) The atoSC+ and ΔatoSC cells were transformed with the indicated plasmids and grown under the same as above conditions (■), BW25113; (▲), BW25113/pUC-Az(atoS+); (●), BW28878; (+), BW28878/pUC-Az; (◇), BW28878/pUC-Az(AtoCΔ); (◇), BW28878/pCPC-Az. Samples were collected at the indicated time points and total PHB (µg A600 ml−1) was determined as in Materials and methods. The t50 of these cultures was 2 h.

The arrows correspond to the time points where AcAc was added (long arrows: 10 mM AcAc; short arrows: 2 mM AcAc). The values represent the average of two independent experiments.
overexpression effect phenotype, i.e., cells carrying pEM-Az-D55/H73 had cPHB levels similar to those found in untransformed cells.

These results indicate that only phosphorylation-competent AtoC can lead to enhanced production of cPHB, when overexpressed with AtoS. This is not surprising since AtoC phosphorylation is an integral step in the acetoacetate-induced AtoS–AtoC signal transduction pathway that leads to activation of atodaeb operon expression. It is puzzling, however, that the relative effects of the D55G and H73L on cPHB accumulation are inversed as to their effects on atodaeb operon expression, i.e., D55G has a more potent phenotype than H73L [27].

3.5. The role of atodaeb operon in cPHB biosynthesis

Having shown the requirement for the AtoS–AtoC TCS in cPHB biosynthesis, it was necessary to clarify the role of the atodaeb operon in this process. To this end two plasmids were constructed carrying the atodaeb operon with (pZatop) (Fig. 1f) or without (pZato) (Fig. 1g) a functional promoter.

Introduction of either of these two plasmids in E. coli (BW28878) had no effect on cPHB synthesis either in the absence or in the presence of acetoacetate (Fig. 6 and results not shown). In contrast, introduction of the plasmid carrying the atodaeb operon with a functional promoter in the atosc+ isogenic strain (BW25113) resulted in a significant increase (approximately 1.4 fold at 16 h after acetoacetate induction) in cPHB levels. No such increase was observed either in the absence of acetoacetate or in cells harboring the plasmid-borne promoterless atodaeb operon. This indicates that increased cPHB synthesis, upon acetoacetate induction, requires the presence of a functional atodaeb operon acting in concert with the AtoS–AtoC TCS.

It has to be stated in this context that pUC-Az, which is used to provide the cells with wild-type AtoS and AtoC in trans, also contains the regulatory elements of the atodaeb operon driving the expression of atoD, atoA and a truncated form of atoE (Fig. 1a). Therefore, it was essential to examine whether its observed effect on cPHB synthesis was solely due to AtoS and AtoC overproduction, or whether the expression of the truncated atodaeb operon products (AtoDA, truncated AtoE) affected this phenotype. To this end, we deleted atodaeb operon sequences from pUC-Az, thus generating pUC-Az(atodaeb−) (Fig. 1e and Materials and methods). Introduction of pUC-Az (atodaeb−) in cells of both atosc+ and atoscΔ genetic lines resulted in a significant decrease in cPHB levels. No such decrease was observed either in the absence of acetoacetate or in cells harboring the plasmid-borne promoterless atodaeb operon. This indicates that increased cPHB synthesis, upon acetoacetate induction, requires the presence of a functional atodaeb operon acting in concert with the AtoS–AtoC TCS.

Fig. 5. Effects of AtoC phosphorylation site mutations on cPHB synthesis. BL21[DE3] cells, and their derivatives, were grown in mineral medium M9 containing 0.5% glucose in the presence (filled symbols) or absence (open symbols) of 10 mM AcAc. (□, ■), BL21[DE3]; (∆, ▲), BL21[DE3]pUC-Az; (○, ●), BL21[DE3]pEM-Az-D55; (○, ●), BL21[DE3]pEM-Az-H73; (●, ▼), BL21[DE3]pEM-Az-D55/H73. The construction of the pUC-Az derivative plasmids with AtoC mutations altering either the D55 (pEM-Az-D55), or H73 (pEM-Az-H73) or both (pEM-Az-D55/H73) has been described [27]. Samples were collected at the indicated times and total PHB (μg/A600 ml culture) was quantitated as in Materials and methods. The t0 of all the above cultures was approximately 2.5 h. The AcAc addition points are indicated with arrows (long arrows: 10 mM AcAc; short arrows: 2 mM AcAc). The values represent the average of two independent experiments.

Fig. 6. Enhanced cPHB biosynthesis under a functional expression of both the AtoS–AtoC system and the atodaeb operon in atoscΔ cells. BW25113 (atoscΔ) (filled symbols) and BW28878 (Δatosc) (open symbols) cells, and their derivatives, were grown in M9 mineral medium containing 0.5% glucose and AcAc was added at the indicated times (long arrows: 10 mM acetoacetate; short arrows: 2 mM acetoacetate). (□, ■), BL21[DE3]; (●, ▼), BL21[DE3]pUC-Az; (○, ●), BL21[DE3]pEM-Az-D55; (○, ●), BL21[DE3]pEM-Az-H73; (●, ▼), BL21[DE3]pEM-Az-D55/H73. The construction of the pUC-Az derivative plasmids with AtoC mutations altering either the D55 (pEM-Az-D55), or H73 (pEM-Az-H73) or both (pEM-Az-D55/H73) has been described [27]. Samples were collected at the indicated time points and total PHB (μg/A600 ml culture) was determined as in Materials and methods. The t0 of all the above cell cultures was approximately 2 h. The values represent the average of two independent experiments.
backgrounds resulted in modestly decreased cPHB levels (approximately 10–20%) compared to cells carrying the wild-type pUC-Az (Fig. 6). It has to be mentioned that all E. coli strains used contain a functional chromosomal copy of the atoDAEB operon.

Taken together, the above data suggest that AtoDAE functions that are also encoded by pUC-Az might have some influence on cPHB production (in addition to the established AtoS–AtoC effects).

4. Discussion

A number of different copolymers, that are generally referred to as storage polyhydroxyalkanoates (sPHAs) are accumulated as intracellular storage polymers by a wide variety of bacteria in response to metabolic stress [9–12]. The pathways for sPHA biosynthesis have been identified and it has been found that the pha genes, encoding 3-ketothiolase, acetoacetyl-CoA reductase, and PHA synthase, play a central role in this process [9,11]. sPHAs have recently received attention as possible precursors of biodegradable plastics [9] and efforts have been made to express them in recombinant E. coli expressing the products of pha genes, from sPHA-synthesizing bacteria, on multicopy plasmids [47–50].

E. coli normally does not accumulate storage PHAs but it only synthesizes the complexed PHB [1–7]. A pathway of cPHB biosynthesis has been proposed [2], although the biosynthetic enzymes of cPHB have not yet been identified and no homologies at the gene level have been identified between the E. coli genome and the pha genes from storage-PHB producing microorganisms [11]. The expression of these pha genes in E. coli does promote sPHA synthesis, although optimal sPHA production requires the constitutive activity of AtoC and the inactivation of the FadR repressor [47–50]. Specifically, the atoC(Con) mutation increases propionate uptake, whereas the fadR mutation enhances the subsequent conversion of propionate to 3-hydroxyvalerate (3HV), a precursor of the poly[3HB-co-3HV] copolymer [31,32]. The sPHAs accumulate within inclusion body-type structures, from which they can be isolated easily [51].

Little is known, however, about the pathways of E. coli cPHB biosynthesis and catabolism and their regulation, since no enzymes have been yet identified [2,11], with the exception of a suggestion on the possible presence of a cPHB depolymerase [12]. Another peripheral evidence on this issue is that AcrAB, the multi-drug efflux pump, is required for the assembly of the PHB-polyphosphate Ca2+ channels [52].

We measured the accumulation of cPHB following manipulation of the intracellular levels of the AtoS–AtoC TCS components and its activity (minus or plus the inducing acetoacetate). Increased levels (50–100 fold) of AtoS and AtoC by the introduction of a multicopy plasmid carrying their genes led to increased cPHB accumulation in the presence of inducing acetoacetate. The effect was not strain-specific since it could be reproduced in different strains (BL21(DE3), TOP10F′, BW25113) that carried pUC-Az.

Deletion of the genes coding for the AtoS–AtoC system resulted in decreased cPHB levels and this difference was observed, albeit to different extents, both in the absence and presence of acetoacetate. The diminished cPHB production capacity of the ΔatoSC cells could be complemented by the reintroduction of wild-type atoSC on a multicopy plasmid (pUC-Az). However, plasmids containing either atoS or atoC singly failed to complement the phenotype, indicating that both components of the AtoS–AtoC TCS are required for the increased cPHB production. The observation that mutations altering the putative phosphate-acceptor sites in AtoC (D55G and H73L) also diminish, or abrogate, the induction of cPHB accumulation imply that AtoC phosphorylation is required in this process. While this is not surprising, since RR phosphorylation is an integral step in two-component signal transduction pathways, we have no explanation as to whether the relative effects of the D55G and H73L on cPHB accumulation are inverted as to their effects on atoDAEB operon expression, i.e., D55G has a more potent phenotype than H73L [27]. Additionally, these data propose that only limited, if any, cross-talk takes place between the AtoS–AtoC and other TCSs. Such cross-talk between UhpB, another HK, and AtoC has been implied by recent findings that this kinase is able to phosphorylate AtoC in vitro [28]. Our results indicate that such cross-talk is very limited in vivo under the conditions of our experiments. The inconsistency between in vitro and in vivo results may be due to a number of factors, such as different topologies between the UhpB HK and the AtoC RR, or the lack of activation of the HK activity of UhpB under the conditions of our experiments.

The above results do not exclude the possibility that the AtoS–AtoC TCS enhances cPHB production through a mechanism other than its established effects on atoDAEB transcriptional activation. However, despite that such indirect effects of AtoS–AtoC on other cellular processes have been reported [29,30], it seems unlikely that they affect cPHB accumulation since introduction of multiple copies of the atoDAEB operon, in the absence of AtoS–AtoC overexpression, resulted in a similar cPHB increase upon acetoacetate induction. This effect required both a functional atoDAEB promoter and the AtoS–AtoC TCS. Therefore, the AtoS–AtoC system increases cPHB production through its direct effects on the atoDAEB operon transcription.

The data in this paper, combined with those of our previous work [16,25–27], allow us to propose a model for the cPHB biosynthesis pathway in E. coli (Fig. 7). Rhie and Dennis [31] have proposed an analogous model where in recombinant pha E. coli K12 fadR atoC(Con) strain [32], the poly[3HB-co-3HV] production is facilitated by the atoC(Con) mutation. Our model refers to AtoS–AtoC TCS involvement on the cPHB biosynthesis in E. coli. Acetoacetate, acetate as well as glucose cross the outer membrane via porin channels [24]. It is thought that it is the periplasmic acetoacetate that serves as the inducer of the AtoS–AtoC TCS [25–27]. It is presumed that the signal transduction cascade initiates when acetoacetate binds to the sensing periplasmic domain of the AtoS HK and induces its dimerization, in a fashion analogous to that observed for HK.
sensors of other TCSs [17,18]. This acetoacetate binding to AtoS induces its ATP-dependent autophosphorylation on a histidine residue (Fig. 7) and the phosphorylated AtoS interacts with and phosphorylates the AtoC RR by protein–protein phosphate transfer. AtoC phosphorylation induces its oligomerization and makes it competent to activate the *atoDAEB* operon transcription, following binding to the promoter sequences. It is the binding of oligomeric AtoC to the *ato* promoter, and the subsequent activation of the *atoDAEB* operon, that induces the expression of the *atoA*, *atoB*, *atoD* and *atoE* genes, encoding functions essential for the catabolism of short-chain fatty acids [25–27].

Acetoacetate is transported through the cytoplasmic membrane by the AtoE membrane transporter [24] (Fig. 7) and it is converted to acetyl-CoA by the *atoDA*-encoded acetyl-CoA:acetoacetyl-CoA transferase. This tetrameric enzyme is mainly cytosolic, although a significant fraction (approximately 10%) has been found in the membrane fraction [53]. This membrane-bound AtoDA might participate in the regulation of acetoacetate transport by AtoE, since a acetyl-CoA:acetoacetyl-CoA transferase mutant *E. coli* displays defective short-chain fatty acid (SCFA) transport [22]. Once acetoacetate enters the cytosol, it is converted to acetoacetyl-CoA and cleaved thiolytically by thiolase II to yield two molecules of acetyl-CoA [21–23] which serves as the substrate for PHB biosynthesis [2–11]. Acetoacetyl-CoA reductase, the second PHB biosynthetic enzyme, may use the acetoacetyl-CoA as substrate for the biosynthesis of 3-hydroxybutyryl-CoA. Acetate is transported, through the inner membrane, by the ActP transporter in combination with the Acs acetyl-CoA synthetase function [54], although the presence of another, so-far unidentified, acetate transporter has been suggested [53]. Acetate kinase, a cytosolic enzyme, catalyzes the conversion of acetate to acetyl-phosphate, which is then transformed to acetyl-CoA by the PtA action [24]. Acetyl-CoA can also be synthesized through the glycolysis pathway from glucose, which is transported by the *ptsG*-encoded EIIICBGlc transporter [55,56].

Acetyl-CoA levels can be modulated by the carbon source used, i.e., they increase in the presence of glucose and decrease with acetate [57]. Fluctuations in the acetyl-CoA levels do not appear to account for the differences observed in cPHB accumulation in this study, growth in the presence of glucose or acetate yielded similar results. It is the presence of acetoacetate and the subsequent activation of the AtoS–AtoC...
TCSs that appear to be required for such an increase in cPHB production to occur. Therefore, according to our data, the AtoS–AtoC TCS acts through its direct effects on the atoDAEB operon transcription to enhance cPHB biosynthesis in E. coli.

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References


