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ORIGINAL ARTICLE

Activation of the AtoSC two-component system in the absence of the AtoC N-terminal receiver domain in *E. coli*

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Abstract The AtoSC two-component system in *E. coli* consists of the AtoS sensor kinase and the AtoC response regulator. It regulates positively the transcriptional activation of *atoDAEB* operon, encoding enzymes involved in short-chain fatty acid catabolism upon acetoacetate-mediated induction. AtoSC acting on *atoDAEB* operon, regulates the biosynthesis and the intracellular distribution of short-chain poly-(R)-3-hydroxybutyrate (cPHB). A phosphorylation-incompetent AtoC form was constructed lacking its N-terminal receiver domain, trAtoC, to study the effects of AtoC domains on cPHB biosynthesis and *atoDAEB* operon regulation. Both cPHB biosynthesis and *atoDAEB* gene expression were regulated positively by trAtoC in the absence of any inducer in *E. coli* of both *atoSC*$^+$ and Δ*atoSC* genotypes. The presence of acetoacetate or spermidine further promoted these trAtoC actions. Competitive regulatory functions between the full length AtoC and trAtoC were observed referring to *atoDAEB* and cPHB targets as well as growth of trAtoC-overproducing *atoSC*$^+$ cells on butyrate as the sole carbon source. trAtoC in contrast to the wild-type AtoC presented different modes of cPHB and *atoDAEB* regulation in the presence of compounds involved in fatty acid metabolism including CoA-SH, acetyl-CoA, sodium acetate or 3-hydroxybutyryl-CoA. These data provide evidence for a role of the AtoC N-terminal receiver domain in regulating the biological activities of AtoSC as well as additional mechanisms of interactions between the AtoSC constituents including their established inducers or new effectors towards the accomplishment of the AtoSC TCS signal transduction.

Keywords AtoSC · Truncated AtoC · *atoDAEB* · Two-component system · Poly-(R)-3-hydroxybutyrate

Abbreviations

atoSC$^+$	Genetic locus encoding the AtoS and AtoC proteins
AcAc	Acetoacetate
cPHB	Complexed poly-(R)-3-hydroxybutyrate
C48/80	Compound C48/80
HK	Histidine kinase
sPHB	Storage polyhydroxybutyrate
RR	Response regulator
TCS	Two-component system
trAtoC	N-terminal domain-truncated AtoC
URR	Upstream regulatory region
σ^{54}-RNAP	σ^{54}-RNA polymerase

Introduction

AtoSC two-component system (TCS), consisting of the AtoS sensor histidine kinase (HK) and the AtoC response

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regulator (RR), activates the expression of the *atoDAEB* operon, encoding for proteins involved in short-chain fatty acids catabolism (Clark and Cronan 1996; Filippou et al. 2008). AtoSC plays a pivotal role in many regulatory networks in *E. coli*, including, amongst others (Oshima et al. 2002; Yamamoto et al. 2005; Arifuzzaman et al. 2006), biosynthesis and intracellular distribution of the complexed short-chain poly-(R)-3-hydroxybutyrate (cPHB) through its direct effects on *atoDAEB* operon, in the presence of acetoacetate (Theodorou et al. 2006), spermidine (Theodorou et al. 2007) and other modulators as the biogenic amine histamine and the mast cell degranulating calmodulin inhibitor C48/80 (compound 48/80) (Kyriakidis et al. 2008), possibly in an extracellular Ca^{2+}-mediated manner (Theodorou et al. 2009). The abundant naturally occurring cPHB is an ubiquitous constituent of prokaryotic and eukaryotic cells. Many valuable physiological functions of *E. coli* have been attributed to the cPHB, such as Ca^{2+} homeostasis, competence for genetic transformation, protection of the complexed proteins from proteolysis, participation in DNA organization and modification of the sorting signal of the *E. coli* OmpA protein (Huang and Reusch 1996; Xian et al. 2007).

The contribution of the AtoSC TCS in multiple regulatory interactions together with the dual properties of AtoC as a member of the NtrC-NifA family of σ^{54}-RNAP activators (Canellakis et al. 1993; Grigoroudis et al. 2007) as well as the antizyme (Az) of ornithine decarboxylase (Heller et al. 1976) led the investigation of AtoC involvement in the interplay between networking structures and sensing mechanisms in *E. coli* (Yang and Liao 2005; Galperin 2006; Balaji et al. 2007).

Despite common structures and functions within individual RRs domains, interactions between receiver and effector domains confer diversity in regulatory strategies, optimizing individual RRs for specific regulatory needs of different signaling systems (Gao et al. 2008). It has long been noticed that modifications in the receiver (Lee et al. 1994; Flashner et al. 1995; Schirmer et al. 1997; Self et al. 2001; Lejona et al. 2004) or output domains (Yang et al. 2004) result in inactive proteins in some RRs and in constitutively or partially active ones in others. The conformationally dynamic nature of receiver domains is reflected by the fact that the active conformation is not restricted to phosphorylated proteins but it is also accessible to unphosphorylated forms (Lejona et al. 2004; Schirmer et al. 1997). Thus, different mechanisms have been proposed to account for the positive and/or negative regulatory roles of the receiver domain in individual RRs. In the case of AtoC, the truncated trAtoC form, lacking the N-terminal receiver domain retains AtoC ATPase activity without phosphorylation-dependent activation (Grigoroudis et al. 2007).

In this study, trAtoC was used to elucidate the role of the AtoC N-terminal receiver domain in eliciting its biological activities. Thus, the possible interactions between trAtoC and AtoC inducers or compounds involved in fatty acid metabolism as well as its cognate AtoS HK were investigated by examining the regulation of cPHB biosynthesis and *atoDAEB* transcription in *E. coli.*

Materials and methods

Materials

All chemicals were obtained from Sigma-Aldrich (Chemie GmbH, Steinheim, Germany) unless otherwise stated. Restriction endonucleases, T4 DNA ligase, T4 phage DNA polymerase and Taq polymerase were purchased from New England BioLabs, Hertfordshire, UK. Molecular weight DNA and protein markers were obtained from Gibco-BRL (Karlsruhe, Germany). PCR primers were obtained from MWG, Germany.

Bacterial strains, plasmids

The genotypes of *E. coli* strains used are listed in Table 1. *E. coli* strains K-12 BW25113 (*atoSC*$^{+}$) (Haldimann and Wanner 2001) and BW28878 (Δ*atoSC*) (Oshima et al. 2002) were a kind gift from Hirofumi Aiba (Nagoya University, Japan).

To construct plasmid ptratoC, the *atoC* sequence encoding amino-acids 140–461 was amplified by PCR, using *E. coli* K-12 N99 genomic DNA as template. The sequences of amplification primers used were as follows: (sense) 5′-AAA CAT ATG ATC CGT CAT CTG CAC CAG GCA-3′ and (antisense) 5′-TTG GGA TCC GTG CAA ATT TCT GCA TAG CAA-3′. The 1,046 bp PCR product was blunt-ended by T4 phage DNA polymerase reaction (Sambrook et al. 1989) and then cloned into the *EcoRV* site of plasmid pZErO 2.1 (Invitrogen, San Diego, CA, USA) to yield plasmid ptratoC, which expresses the N-terminal deleted AtoC form (trAtoC), lacking its 139N-terminal amino acids. The trAtoC overexpression in *E. coli* cells (TOP10F' and BL21 [DE3]) transformed with ptratoC was verified by SDS-PAGE electrophoresis and Western blot analysis.

Plasmid pUC-AtoC was generated by digestion of pUC-Az(AtoDAEB^{-}) with *BamH*I which cleaves within *rcsC* coding sequence, 759 bp upstream of the *atoS* start codon, and with *SnaB*I, which cleaves within *atoS* coding sequences, 1,194 bp downstream of the *atoS* start codon. The ends of the linearized plasmid were filled using the Klenow fragment of the *E. coli* DNA polymerase I and the plasmid was relegated. Thus, pUC-AtoC lacks the

Table 1 *Escherichia coli* strains and plasmids

Strain or plasmid	Genotype	Source or reference
E. coli strains		
BL21 [DE3]	F$^-$ *ompT hsdS$_B$* ($r_B^- m_B^-$) *gal dcm* (DE3)	Novagen
BW25113	*lac*I^q *rrn*B3 Δ*lac*Z4787 *hsd*R514 Δ(*ara*BAD)567 Δ(*rha*BAD)568 *rph*-1	Haldimann and Wanner (2001)
BW28878	*lac*I^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)
N99	Wild-type, *λ*$^-$, *F*$^-$, *sup0, galK2*	Griffo et al. (1989)
Plasmids		
pUC-Az	pUC19 containing the *atoS*, *atoC* genes and a part of the *atoDAEB* operon (*atoD*, *ato*A and two-thirds of *atoE*)-transformed cells overproduce AtoS, AtoC and AtoDA	Canellakis et al. (1993)
pUC-Az(AtoDAEB$^-$)	pUC-Az derivative which lacks the part of *atoDAEB* operon-transformed cells overproduce AtoS and AtoC	Theodorou et al. (2006)
pHis10-trAtoC	pET16b containing the His_{10} recombinant form of *tratoC* sequence-transformed cells overexpress His_{10}-N-terminal truncated AtoC	Grigoroudis et al. (2007)
ptratoC	pZEro2.1 (InVitrogen) containing the *tratoC* sequence-transformed cells overproduce truncated AtoC	This study
pUC-AtoC	pUC-Az derivative which lacks *atoS* and the part of *atoDAEB* operon-transformed cells overproduce AtoC	This study
pEMZ	Promoterless *lacZ*, also lacking the first four codons, in pDP8	Lioliou et al. (2005)
pEMZ-patoD1	*AtoDAEB* promoter and *atoD* coding sequences (−206 to +73) cloned in pEMZ	Lioliou et al. (2005)

atoDAEB operon due to the deletion of this locus and the two-thirds of *atoS* gene which does not lead to production of the AtoS protein but encodes the AtoC protein. The lack of AtoS overexpression in *E. coli* cells (TOP10F' and BL21 [DE3]) transformed with pUC-AtoC was verified by SDS/PAGE electrophoresis and Western blot analysis.

Plasmid pUC-Az, containing the *atoS*, *atoC* genes and a part of the *atoDAEB* operon has been described (Canellakis et al. 1993). Plasmids pUC-Az(AtoDAEB$^-$) lacking the *atoDAEB* operon and pET16b-His_{10}-trAtoC, expressing the recombinant trAtoC form (His_{10}-trAtoC), have been described (Theodorou et al. 2006; Grigoroudis et al. 2007). Plasmid pEMZ-patoD1 containing the *atoDAEB* URR fused to a promoterless *lacZ* gene (*atoD1-lacZ* in pDP8) has been described (Lioliou et al. 2005).

Growth conditions

E. coli were grown in supplemented M9 mineral medium (Sambrook et al. 1989) with 0.5% (w/v) glucose as the carbon source. Acetoacetate-mediated induction of the AtoSC TCS was initiated by the addition of 10 mM acetoacetate in the form of 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). Spermidine was added when A_{600} reached 0.2–0.3 at the final concentrations of 5 mM (Theodorou et al. 2007). Butyrate was added at the final concentration of 50 mM when was used as the sole carbon source. The final concentrations of the compounds tested as inducers of the trAtoC were for CoA-SH 1 mM, CH_3CO-SCoA 1 mM, CH_3COONa 10 mM, 3-OH-butyryl-SCoA 10 mM and butyrate 10 mM. 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 culture samples were collected at the time points indicated and processed for crotonic acid/cPHB determination.

DNA isolation, manipulation, and cell transformation

Genomic and plasmid DNA isolation, T4 phage DNA polymerase reaction, *E. coli* Klenow fragment DNA polymerase I reaction, restriction enzyme digestions, ligations, transformations, were carried out with standard methodology (Sambrook et al. 1989). Plasmids were purified using commercially available kits (Qiagen or Nucleobond) following the manufacturer's instructions.

Protein determination and immunoblotting analyses

Protein concentrations were determined by the Bradford method (Bradford 1976) using bovine serum albumin as the reference standard. The immunoblotting analyses were performed using affinity-purified rabbit polyclonal antibodies against His_{10}-AtoC and AtoS (Lioliou et al. 2005).

β-Galactosidase assay

β-Galactosidase assay was performed using *E. coli* cells carrying the appropriate plasmids, as described previously

(Miller 1992). Strains were grown in modified M9 mineral medium as described (Theodorou et al. 2007).

Determination of cPHB

The cPHB determination was carried out by the method of Karr et al. (1983), as modified by Huang and Reusch (1996), based on the acid-catalyzed β-elimination of cPHB to crotonic acid. Dried *E. coli* cell pellets derived from a 10 ml culture sample at the indicated time points were incubated with 0.5 ml concentrated H_2SO_4 at 92°C for 8 h (Theodorou et al. 2006). The samples were placed on ice, 1 ml of saturated sodium sulfate was added, and the solution was extracted four times with 3 ml of dichloromethane. Sodium hydroxide (100 µl of 1 M) was added to the dichloromethane extracts to convert crotonic acid into sodium crotonate and the dichloromethane was evaporated. The residue was acidified to pH 2 with 1.5 M sulfuric acid and filtered. The final filtrate was analyzed by HPLC on an Aminex HPX-87H ion-exclusion organic acid analysis column (Bio-Rad), using a Shimadzu (Tokyo, Japan) HPLC chromatography system. Quantification was performed following comparison of peak absorbance with that of crotonic acid standards (Fluka Buchs, Switzerland). The cPHB content of each sample was determined from the amount of crotonic acid using the established conversion rate (Tagushi et al. 2001).

Results

trAtoC enhances cPHB biosynthesis

E. coli BL21[DE3] cells were transformed with plasmid pHis$_{10}$-trAtoC, resulting in overexpression of a His$_{10}$-tagged N-terminal truncated form of AtoC RR, His$_{10}$-trAtoC, to study its effects on cPHB regulation. trAtoC is active without phosphorylation, which requires the acetoacetate-mediated induction. These transformed cells in the absence of the inducer, accumulated enhanced cPHB (approximately 63% at 12 h of growth) compared to the control derivatives (Fig. 1). However, trAtoC was unable to enhance cPHB biosynthesis to levels observed in cells overproducing the active AtoSC TCS and the part of *atoDAEB* operon under acetoacetate (pUC-Az-transformed cells). Acetoacetate further increased cPHB (approximately 22%) in BL21[DE3]/pHis$_{10}$-trAtoC cells compared to its absence, without however to achieve the amounts produced by pUC-Az-transformed counterparts (Fig. 1). All transformed cells except the counterparts harboring pHis$_{10}$-trAtoC synthesized only control cPHB amounts in the absence of acetoacetate (data not shown) equal to those in the untransformed cells (Fig. 1).

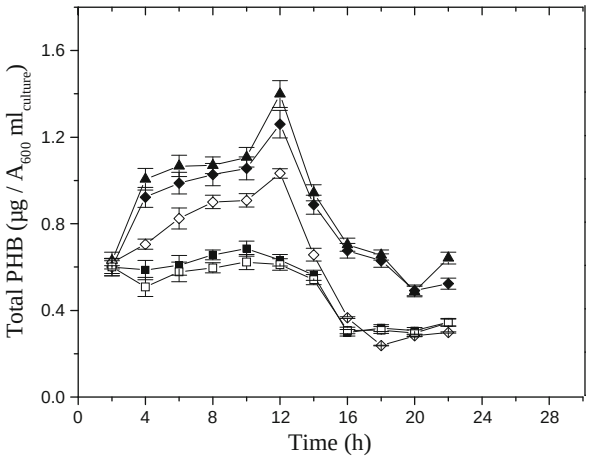

Fig. 1 Effect of trAtoC overexpression on cPHB biosynthesis. cPHB biosynthesis in BL21[DE3] cells and their transformed derivatives with the indicated plasmids in the absence (*open symbols*) or presence (*filled symbols*) of acetoacetate. Untransformed cells (*filled squares*, *open squares*), pUC-Az (*filled triangles*), pHis$_{10}$-trAtoC (*filled diamonds*, *open diamonds*). The values represent the average of two independent experiments (SD < 0.01)

trAtoC counteracts full-length AtoC effect on *atoDAEB* operon transcription

trAtoC was further expressed in BW28878 (Δ*atoSC*) and BW25113 (*atoSC*$^+$) strains via the constructed ptratoC plasmid in order to study its ability to activate *atoDAEB* operon transcription. trAtoC activated *atoDAEB* transcription in cells of Δ*atoSC* (approximately 115-fold) and *atoSC*$^+$ (125-fold) genotypes (ptratoC/pEMZ-patoD1 transformed cells) in the absence of the inducers. Acetoacetate or spermidine presence in ptratoC/pEMZ-patoD1 transformed counterparts of both genotypes resulted in a further *atoDAEB* up-regulation compared to the pEMZ-patoD1 transformed cells in the presence of the inducers (Fig. 2). The *atoDAEB* transcription levels by trAtoC were higher in *atoSC*$^+$ cells than in the Δ*atoSC* counterparts either in the absence or presence of the inducers possibly due to the AtoS HK expression in *atoSC*$^+$ counterparts. Slightly increased *atoDAEB* activation was observed in Δ*atoSC* cells expressing trAtoC, compared to the wild-type pUC-Az-transformed derivatives in the presence of the inducers (approximately 1.23-fold) (Fig. 2). However, in trAtoC-overproducing *atoSC*$^+$ strains the presence of both the wild-type AtoC and trAtoC restricted *atoDAEB* transcription to slightly lower levels than the BW25113/pUC-Az cells expressing the wild-type AtoSC (approximately 0.9-fold). The introduction of AtoC in trans (pUC-AtoC transformed cells) resulted in weak inducer-independent activation of *atoDAEB* expression in both *atoSC*$^+$ and Δ*atoSC* genetic backgrounds. However, the levels remained significantly lower than in bacteria expressing trAtoC or AtoSC TCS.

trAtoC inhibits growth with butyrate as the carbon source

To further elucidate the above observed restriction of the *atoDAEB* transcription when both the wild-type AtoC and trAtoC were expressed, ptratoC-transformed cells were grown on the short-chain fatty acid butyrate as the sole carbon source. Butyrate cannot act as an alternative inducer of AtoSC TCS, since *atoDAEB* operon transcription was not induced by AtoSC in its presence, but is catabolized by the *atoDAEB* encoded enzymes under acetoacetate-mediated induction of AtoSC TCS. Δ*atoSC* cells were only limited grown with butyrate even in the presence of acetoacetate as the sole carbon source as a consequence of the AtoSC TCS absence. Expression of trAtoC in Δ*atoSC* cells either in the absence or presence of acetoacetate resulted in cell growth comparable to that observed in pUC-Az-transformed cells under acetoacetate (Fig. 3a). The competitive actions of the wild-type AtoC and trAtoC towards *atoDAEB* expression were observed in BW25113/ptratoC cells which demonstrated a reduced ability to grow on butyrate in the absence or presence of the inducer compared to the pUC-Az-transformed counterparts (Fig. 3b) or the pUC-AtoC-transformed cells, which were grown at the levels of the pUC-Az-transformed bacteria (data not shown). The pUC-Az-transformed counterparts of both genetic backgrounds were grown at the levels of the control cells in the absence of the inducers. To verify the specificity of this inhibitory effect on *atoDAEB* operon regulation, the growth of the above strains on glucose was also compared, where no differences were observed (data not shown).

cPHB regulation by trAtoC in the absence or presence of acetoacetate or spermidine

The modulatory action of trAtoC on cPHB regulation was further studied in Δ*atoSC* and *atoSC*$^+$ strains, to elucidate the possible interplay between trAtoC and the inducers of the AtoSC TCS as well as the competitive function between trAtoC and the wild-type AtoSC TCS. The extrachromosomal expression of trAtoC in Δ*atoSC* cells (BW28878/ptratoC) resulted to 37% enhanced cPHB amounts than in the control derivatives in the absence of any inducer (Fig. 4a). Interestingly, those cPHB amounts surpassed those accumulated to cells overproducing AtoSC TCS together with the part of *atoDAEB* operon (BW28878/pUC-Az) (approximately 11% enhancement) or only AtoSC [BW28878/pUC-Az(AtoDAEB$^-$)]. Acetoacetate (Fig. 4a) as well as spermidine (data not shown) demonstrated an additional up-regulation effect with trAtoC since they further increased the trAtoC-regulated cPHB levels. This could not be attributed to any indirect effects of metabolism since their presence on the untransformed cells had no effect on the Δ*atoSC*-mediated cPHB phenotype as well as cPHB in cells expressing only the wild-type AtoC in trans (BW28878/pUC-AtoC counterparts) remained at the control levels (data not shown).

A different phenotype of cPHB regulation was observed in trAtoC overproducing *atoSC*$^+$ (BW25113/ptratoC) cells. trAtoC in the absence of inducers up-regulated at about 21% cPHB biosynthesis compared to the untransformed *atoSC*$^+$ cells where the wild-type AtoSC was induced [Fig. 4b (acetoacetate) and data not shown (spermidine)]. cPHB levels were also higher compared to the BW28878/

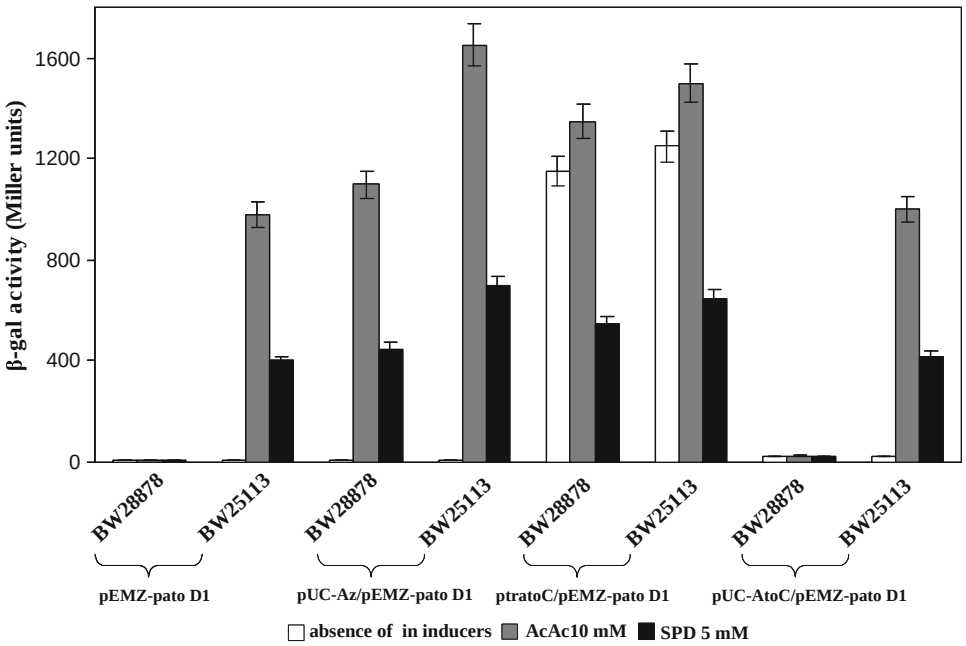

Fig. 2 Effect of trAtoC on the inducibility of *atoDAEB* promoter by acetoacetate or spermidine. BW25113 (*atoSC*$^+$) and BW28878 (Δ*atoSC*) bacteria transformed with pEMZ-patoD1 (*atoD1-lacZ* fusion) plasmid alone or in combination with ptratoC, pUC-AtoC or pUC-Az were grown in the absence or presence of AcAc or SPD. *β*-Galactosidase activity is expressed in Miller units. The values represent the average of two independent experiments

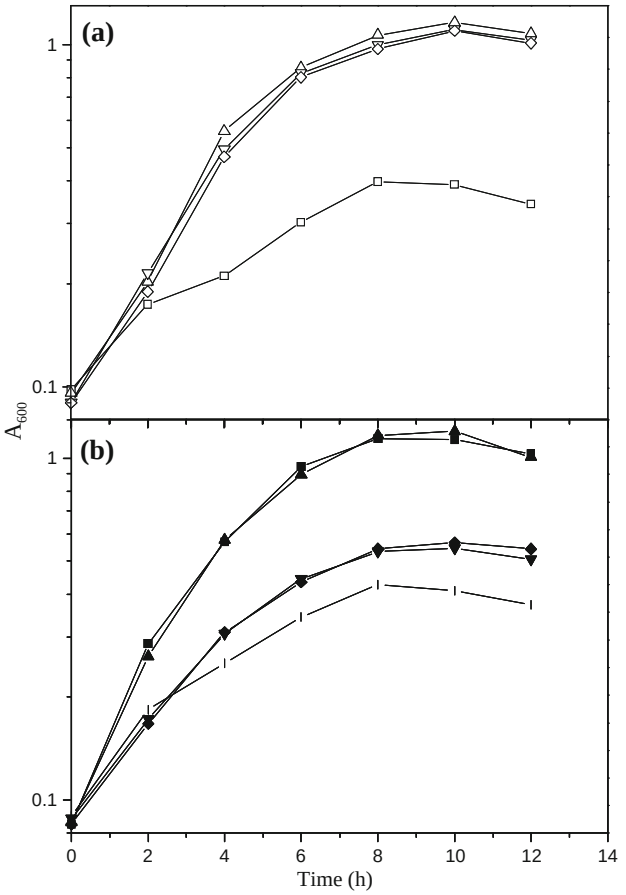

Fig. 3 Effect of trAtoC on *E. coli* growth with butyrate. Growth of BW28878 (Δ*atoSC*) and BW25113 (*atoSC*$^+$) strains and their transformed derivatives with the indicated plasmids, on butyrate as the sole carbon source, in the absence or presence of the AtoSC inducer AcAc. **a** BW28878 ± AcAc (*open squares*), pUC-Az + AcAc (*open triangles*), ptratoC-AcAc (*open diamonds*), ptratoC + AcAc (*open inverted triangles*). **b** BW25113-AcAc (*vertical lines*), BW25113 + AcAc (*closed squares*), pUC-Az + AcAc (*closed triangles*), ptratoC-AcAc (*closed diamonds*), ptratoC + AcAc (*closed inverted triangles*). The values represent the average of two independent experiments

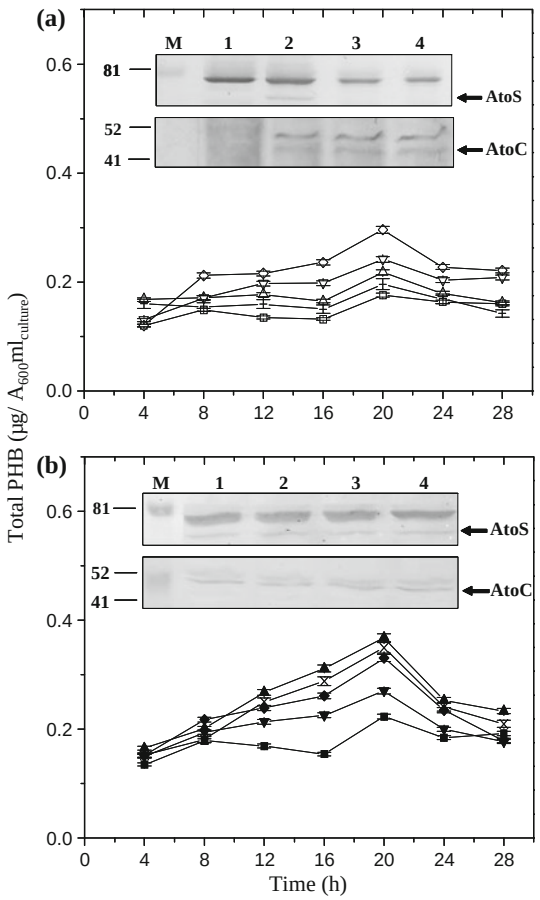

Fig. 4 cPHB biosynthesis regulation by trAtoC under acetoacetate. **a** BW28878 (Δ*atoSC*) (*open symbols*) and **b** BW25113 (*atoSC*$^+$) (*filled symbols*) cells transformed with the indicated plasmids were grown in the absence or presence of AcAc. Untransformed + AcAc (*closed squares*, *open squares*), pUC-Az + AcAc (*closed triangles*, *open triangles*), pUC-Az(AtoDAEB$^-$) + AcAc (*times*, *plus*), ptratoC + AcAc (*closed diamonds*, *open diamonds*), ptratoC-AcAc (*closed inverted triangles*, *open inverted triangles*). The values represent the average of two independent experiments (SD < 0.01). *Insets* Immunostaining of Δ*atoSC* (**a**) or *atoSC*$^+$ cells (**b**) (25 μg total protein) untransformed (lane 1) or transformed with pUC-Az (lane 2), pUC-AtoC (lane 3), ptratoC (lane 4) or with polyclonal anti-AtoS (*upper*) and anti-AtoC (*lower*) antibodies

ptratoC cells (Fig. 4a). In the presence of acetoacetate (Fig. 4b) or spermidine (data not shown), cPHB biosynthesis was more enhanced at approximately 23% in BW25113/ptratoC cells, yet remaining lower than the levels observed in pUC-Az or pUC-Az(AtoDAEB$^-$)-derivatives as a consequence of the *trans*-dominant effects between AtoC and trAtoC. Once more, cPHB biosynthesis in BW25113/pUC-AtoC cells even under inducers as well as the rest transformed counterparts of both genetic backgrounds except the ptratoC-derivatives in the absence of the inducers remained at the control levels. No effect was produced when the "empty" pUC19 or pZErO 2.1 vectors were introduced in cells of both genotypes (data not shown). All the above facts could not be attributed to effects by different protein levels, since immunoblot analysis of transformed cells of both *atoSC*$^+$ and Δ*atoSC* genotypes, growing in the absence or presence of acetoacetate (Fig. 4, inserts), indicated that AtoS and AtoC levels are equivalent.

trAtoC effects in response to different compounds involved in fatty acid metabolism

Different compounds involved in fatty acid metabolism have been suggested to act as inducers of the AtoSC TCS either interacting with the wild-type AtoC at the first step of the signal transduction or with the already active AtoC by phosphorylation at the next step. In an attempt to trace

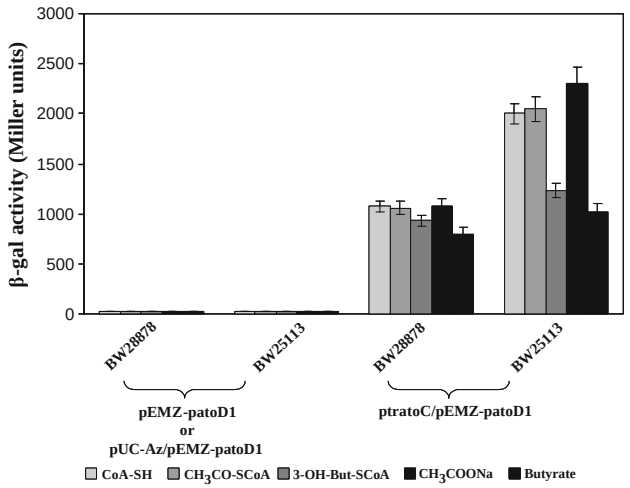

Fig. 5 Effect of trAtoC on the inducibility of *atoDAEB* promoter in the presence of compounds involved in fatty acid metabolism. BW25113 (*atoSC*$^+$) and BW28878 (Δ*atoSC*) bacteria transformed with pEMZ-patoD1 (*atoD1-lacZ* fusion) plasmid alone or in combination with ptratoC or pUC-Az were grown in the presence of CoA-SH 1 mM, CH_3CO-SCoA 1 mM, 3-hydroxybutyryl-SCoA 10 mM, CH_3COONa 10 mM or butyrate 10 mM. *β*-Galactosidase activity is expressed in Miller units. The values represent the average of two independent experiments

possible interactions between AtoC and compounds involved in fatty acid metabolism, we determined the cPHB regulation and transcriptional activation of *atoDAEB* operon by wild-type AtoC or trAtoC in response to CoA-SH, acetyl-CoA, sodium acetate, 3-hydroxybutyryl-CoA and sodium butyrate. trAtoC, but not the wild-type AtoC, demonstrated increased *atoDAEB* transcription in the presence of compounds involved in fatty acid metabolism in *E. coli* of both genotypes (Fig. 5). Sodium acetate caused cPHB elevation in trAtoC-expressing cells of both genotypes (Fig. 6a, b). HS-CoA resulted to cPHB reduction in cells of both genotypes (Fig. 6a, b), while CH_3CO-SCoA affected cPHB only in *atoSC*$^+$ bacteria (Fig. 6b). However, higher cPHB amounts were accumulated in *atoSC*$^+$ than Δ*atoSC* counterparts (Fig. 6a, b).

Discussion

AtoC the response regulator of AtoSC TCS (Kyriakidis and Tiligada 2009) is a member of the NtrC-NifA family of σ^{54}-RNAP transcriptional activators (Reitzer and Schneider 2001; Grigoroudis et al. 2007). They bind cooperatively to the enhancers of their downstream target genes and multimerize via phosphorylation and ATP-attachment, leading to a remodeling from close to open forms of the σ^{54}-RNAP-promoter DNA complexes towards transcriptional activation by coupling the energy of ATP hydrolysis.

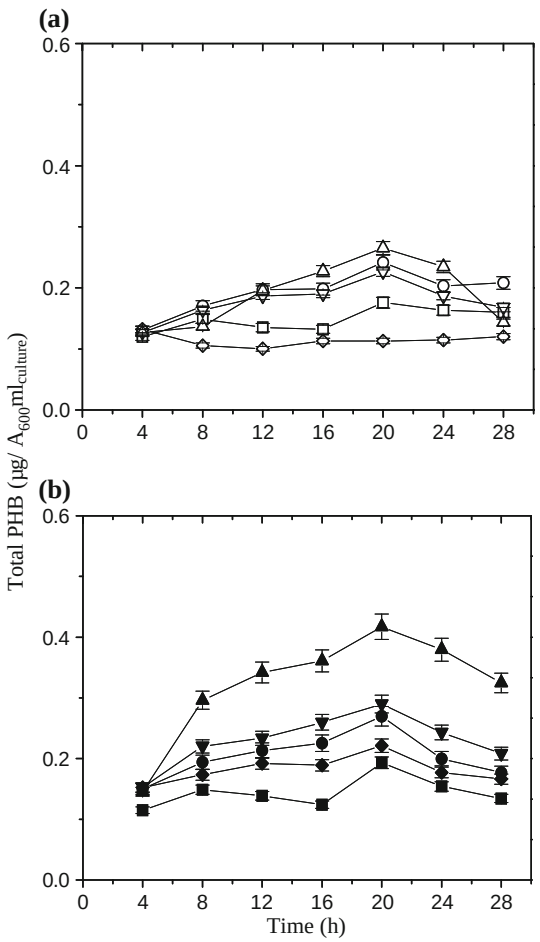

Fig. 6 trAtoC response to different compounds involved in fatty acid metabolism. cPHB biosynthesis in BW28878/ptratoC **a** or BW25113/ptratoC **b** cells grown in the absence (*closed circles*, *open circles*) or the presence of (*closed diamonds*, *open diamonds*) CoA-SH 1 mM, CH_3CO-SCoA 1 mM (*closed inverted triangles*, *open inverted triangles*), or CH_3COONa 10 mM (*closed triangles*, *open triangles*). cPHB biosynthesis in untransformed *atoSC*$^+$ and Δ*atoSC* derivatives in the absence of any compound, respectively (*closed squares*, *open squares*). The values represent the average of two independent experiments (SD < 0.02)

The ATPase activity of these RRs lies in the highly conserved central AAA$^+$-ATPases family domain and is activated by inducer-dependent phosphorylation and strongly stimulated by site-specific DNA binding (Reitzer and Schneider 2001). Studies in this family have shown that unphosphorylated forms of RRs are constitutively active retaining their ATPase activity and enable target-gene transcription in the presence of inducers, some of which may not be limited to act only as the signals for the transduction (Shingler 1996).

To further study the regulatory roles of the AtoC RR subdomains as well as any possible additional interactions between the AtoC RR and inducers and/or its cognate AtoS HK we used a N-terminal deletion mutant AtoC (trAtoC), which preserves its in vitro activities without

phosphorylation requirement (Grigoroudis et al. 2007). The enhancement of *atoDAEB* transcription and cPHB biosynthesis in cells overexpressing trAtoC in the absence of inducer, verified the in vivo effects of this constitutive active AtoC form, as an outcome of its preserving ATPase and DNA binding activities and its ability to couple these functions towards the transcriptional activation of *atoDAEB* and subsequently to cPHB up-regulation. Analogous receiver domain-deleted mutants of σ^{54}-dependent activators have been reported which exhibit constitutive ATPase activity leading to their targets induction (Austin and Dixon 1992; Lee et al. 1994; Schirmer et al. 1997; Self et al. 2001; Lejona et al. 2004; Assumpção et al. 2007), as a consequence of their ability to bypass the negative control of catalytic activity exerted by the amino-terminal domain and permitting RRs to bind to their dyad symmetry enhancer sites though their C-terminal domains and catalyze the σ^{54}-RNAP activity through their central domain.

Even though the truncated forms of RRs lose their inducer-dependent activation by phosphorylation, the presence of acetoacetate or spermidine corroborated the trAtoC-regulated cPHB implying a possible additional role of the inducer on AtoC RR, which has not been identified yet. This observation was also clarified by the induction of *atoDAEB* transcription by trAtoC upon acetoacetate or spermidine induction. It concerns a trAtoC specific and not an indirect metabolic effect signified by the unaffected phenotypes in Δ*atoSC* strains or AtoC-overproducing cells under the inducers presence. Unlike the majority of NtrC-like regulators, the XylR-subgroup sense their respective signals by direct binding of the inducer, leading to transcriptional activation (Delgado and Ramos 1994; Ng et al. 1995; Hopper and Böck 1995; Schirmer et al. 1997). trAtoC could share characteristics of this subgroup, sensing and binding the inducer towards activation for a better accomplishment of the σ^{54}-holoenzyme prior to transcriptional activation. This could be due to either conformational change in truncated AtoC challenged as a consequence of the receiver domain deletion, which permits trAtoC to interact with the inducer in contrast to the full-length AtoC or that the receiver domain impedes inducer binding on AtoC RR which is restored after AtoC phosphorylation leading to autonomy of the rest protein to further interact with its inducers. A possible mechanism could comprise that multimerization and ATP hydrolysis by AtoC are not the only prerequisites for activating formation of the open complex by σ^{54}-holoenzyme, conformational changes in the activator during the catalytic cycle or a multicomponent complex may be required to engage the polymerase and thus couple the energy of ATP hydrolysis to DNA strand separation.

Trans dominant effects of N-terminal domains of RRs on the wild-type proteins have reported for members of σ^{54}-RNAP activators including the XylR-subgroup (Verstraete et al. 1994; Drummond et al. 1996; Schirmer et al. 1997). This is also valid for AtoC as verified by the growth inhibition with butyrate as the sole carbon source of *atoSC*$^{+}$ cells expressing also trAtoC extrachromosomally and the competitive effects between the full length and the truncated AtoC on cPHB and *atoDAEB* expression. According to the previous observations for the members of the XylR-subgroup, there may be two potential explanations of this effect: the formation of inactive heterodimers between the full-length and the truncated form or competition between the two polypeptides for binding to the upstream regulatory region of *atoDAEB*. Further elucidation however, is required for their confirmation on AtoSC TCS.

The relationship between the catalytic activity and interactions connecting the receiver domain-deleted RRs or the phosphorylation-mediated active forms of the full length RRs and their cognate HKs has not been clearly defined. The enhancement of cPHB biosynthesis and *atoDAEB* transcription by trAtoC under AtoS expression and inducers suggest possible additional interactions between AtoC or at least between the central and output domains of the RR and its cognate AtoS HK, probably concerning the correct folding or/and appropriate environment of the RR. This AtoS requirement has also been observed during our previous in vitro RR activity studies, where the presence of AtoS assisted the ATPase activity of trAtoC (Grigoroudis et al. 2007).

Intermediate metabolic compounds of the *atoDAEB* pathway accumulated intracellularly during active metabolism have been proposed to act as inducers of the AtoSC TCS (Clark and Cronan 1996). However, the full-length AtoC demonstrates specificity against the recognized inducers acetoacetate or spermidine (Kyriakidis and Tiligada 2009) since CoA-SH, acetyl-CoA, sodium acetate, butyrate and 3-hydroxybutyryl-CoA had no effect on the *atoDAEB* operon transcription regulation by AtoSC TCS. The trAtoC response against those compounds suggests that the bypass of the receiver domain inhibitory control achieved by deletion which reflects the bypass after phosphorylation during the signal transduction, results to AtoC induction by additional signals towards the same or other targets. AtoS HK participates in trAtoC response to different compounds verifying additional interactions between the full-length AtoS and the N-truncated AtoC.

The above hypothesis and the possibility for multiple interactions seem to be strengthened by the different modes of cPHB regulation by trAtoC in the presence of these effectors. It has been proved that high amounts of acetyl-CoA result in high storage PHB (sPHB) (Kessler and Witholt 2001). This coincides with our previous studies (Theodorou et al. 2006, 2007) where the transcriptional

activation of *atoDAEB* operon results in high levels of acetyl-CoA and subsequently cPHB enhancement. The high expression of *atoDAEB* operon under the trAtoC control in the presence of acetyl-CoA, could result in elevated cPHB levels as well. However, in trAtoC-expressing cells acetyl-CoA did not trigger a significant cPHB enhancement, suggesting for different downstream targets of acetyl-CoA-induced trAtoC. The observed cPHB reduction in the presence of high amounts of CoA-SH has been reported for the biosynthesis of storage PHB (sPHB) in *E. coli* were CoA-SH inhibits the 3-ketothiolase activity (Kessler and Witholt 2001). The transcriptional activation of *atoDAEB* by trAtoC was in agreement with cPHB enhancement in the presence of sodium acetate, showing that the acetyl- group but not the CoA-SH could act as a signal on trAtoC towards cPHB up-regulation. The induction of the phosphorylation-incompetent form of AtoC by these compounds in contrast to the wild-type RR provide the lead for the elucidation of yet unresolved interactions between the constituents of the AtoSC TCS.

Conclusively, trAtoC regulated positively cPHB biosynthesis and *atoDAEB* operon expression, effects that were sustained in the presence of the AtoSC inducers. trAtoC but not AtoC induced *atoDAEB* transcription and cPHB biosynthesis in the presence of compounds involved in fatty acid metabolism, signifying that the abrogation of the inhibition that the N-terminal domain displays, permits the AtoC RR to respond to additional inducers. These data provide evidence for additional yet unresolved mechanisms of interactions between the AtoSC constituents except those required for the phosphate transfer towards the accomplishment of the TCS signal transduction and their established inducers or new effectors that regulate the biological activities of AtoSC TCS.

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