Extracellular Ca^{2+} transients affect poly-(*R*)-3-hydroxybutyrate regulation by the AtoS-AtoC system in *Escherichia coli*

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Escherichia coli is exposed to wide extracellular concentrations of Ca^{2+}, whereas the cytosolic levels of the ion are subject to stringent control and are implicated in many physiological functions. The present study shows that extracellular Ca^{2+} controls cPHB [complexed poly-(*R*)-3-hydroxybutyrate] biosynthesis through the AtoS-AtoC two-component system. Maximal cPHB accumulation was observed at higher $[Ca^{2+}]_e$ (extracellular Ca^{2+} concentration) in AtoS-AtoC-expressing *E. coli* compared with their Δ*atoSC* counterparts, in both cytosolic and membrane fractions. The reversal of EGTA-mediated down-regulation of cPHB biosynthesis by the addition of Ca^{2+} and Mg^{2+} was under the control of the AtoS-AtoC system. Moreover, the Ca^{2+}-channel blocker verapamil reduced total and membrane-bound cPHB levels, the inhibitory effect being circumvented by Ca^{2+} addition only in *atoSC*$^+$ bacteria. Histamine and compound 48/80 affected cPHB accumulation in a $[Ca^{2+}]_e$-dependent manner directed by the AtoS-AtoC system. In conclusion, these data provide evidence for the involvement of external Ca^{2+} on cPHB synthesis regulated by the AtoS-AtoC two-component system, thus linking Ca^{2+} with a signal transduction system, most probably through a transporter.

Key words: AtoS-AtoC two-component system, *atoDAEB*, calcium, calcium channel blocker, histamine, poly-(R)-3-hydroxybutyrate.

INTRODUCTION

Ca^{2+} is implicated in the regulation of a wide variety of cellular processes, including signal transduction in bacteria, where cytosolic Ca^{2+} levels are tightly controlled by mechanisms involving plasma membrane transporters [1–4]. In contrast with eukaryotic cells, the role of prokaryotic Ca^{2+} transients is less well understood due to the poorly defined regulation of Ca^{2+} influx and efflux systems [3].

In *Escherichia coli*, Ca^{2+} influx arrangements include the non-proteinaceous complexes of short-chain poly-(*R*)-3-hydroxybutyrate-polyphosphate–Ca^{2+} [5,6]. cPHB [complexed poly-(*R*)-3-hydroxybutyrate] is widely found in both prokaryotes and eukaryotes [7] and possesses a number of physiological roles in *E. coli* [6,8,9]. Its biosynthesis is unresolved in *E. coli*, where a periplasmic protein, YdcS, has been reported to exhibit PHB synthase activity only very recently [10]. The non-proteinaceous complexes act as voltage-gated Ca^{2+} channels with complex gating kinetics, their activity being implicated in the phase of growth-dependent control of cytosolic Ca^{2+} levels [6,11,12]. Moreover, cPHB channels share some characteristics with eukaryotic L-type voltage-activated Ca^{2+} channels, which are targeted selectively by the Ca^{2+}-channel blockers verapamil, diltiazem and nifedipine, despite their differences in pharmacological properties [13–15].

The biosynthesis and the intracellular distribution of cPHB are regulated by the AtoS-AtoC TCS (two-component system) via its direct action on *atoDAEB* operon transcription upon acetoacetate- or spermidine-mediated induction [16,17]. In addition, AtoS-AtoC TCS-mediated regulation of cPHB biosynthesis has been reported in the presence of the biogenic amine histamine and the mast cell degranulating synthetic polyamine C48/80 (compound 48/80) [18], which has been shown to induce rapid Ca^{2+} efflux, thus altering the permeability of the outer membrane in *E. coli* [19].

The AtoS-AtoC TCS consists of the AtoS histidine kinase and the AtoC response regulator [20] of the NtrC-NifA family of σ^{54}-RNA polymerase transcriptional activators [21] and regulates the expression of the *atoDAEB* operon [21] that encodes for proteins involved in short-chain fatty acid catabolism [22]. The AtoS-AtoC TCS plays a fundamental role in *E. coli* physiology and in addition to its participation in a number of interacting regulatory networks [23–26], it may contribute to Ca^{2+} homoeostasis through cPHB regulation [16,17].

E. coli adapts to a wide range of $[Ca^{2+}]_e$ (extracellular Ca^{2+} concentration), such as in fresh or sea water [5], and in the gut where histamine plays prominent physiological roles in gastric acid secretion and inflammation [27], often through TCS-mediated signal transduction [28]. Interestingly, the involvement of Ca^{2+} in histamine release and in the action of the histamine releaser C48/80 is well documented [29], while a limited number of studies argue for the functional implication of Ca^{2+} in regulating pro-inflammatory signals and for the cross-talk between Ca^{2+}-channel blockers, histamine and bacteria–host interactions [30–32].

Since the cPHB biosynthetic pathway in *E. coli* has not been established and taking into consideration the involvement of the *ato* regulon, the present study aimed to investigate the effects of extracellular Ca^{2+} in the AtoS-AtoC TCS-regulated cPHB biosynthesis. The reported data provide evidence for an underlying modulatory role of the AtoS-AtoC TCS along with Ca^{2+}-mediated homoeostatic mechanisms, leading to new insights on the interaction of signal transduction systems and extracellular Ca^{2+} levels, with potential implications in the symbiotic behaviour of bacteria.

Abbreviations used: C48/80, compound 48/80; $[Ca^{2+}]_e$, extracellular Ca^{2+} concentration; cPHB, complexed poly-(*R*)-3-hydroxybutyrate; TCS, two-component system; URR, upstream regulatory region.

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MATERIALS AND METHODS

Materials

Histamine dichloride was obtained from SERVA (Heidelberg, Germany), C48/80 from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and verapamil hydrochloride was from Knoll AG (Ludwigshafen, Germany). All chemical reagents were of analytical grade.

Bacterial strains, plasmids and growth conditions

E. coli K-12 strains BW25113 (*atoSC*$^+$) [33] and BW28878 (Δ*atoSC*) [23] were a gift from Hirofumi Aiba (Nagoya University, Japan). Plasmid pUC-Az, containing the *atoS* and *atoC* genes and a part of the *atoDAEB* operon [34], as well as plasmids pEMZ-patoD1 [*atoDAEB* URR (upstream regulatory region)-*lacZ* in pDP8], pCPG5 (*atoDAEB* URR-*atoA*-*lacZ*), pCPG4 (*atoS URR*-*lacZ*) and pCPG6 (*atoC* URR-*lacZ*), were constructed as described previously [20,35].

E. coli was grown in LB (Luria–Bertani) broth or in modified M9 mineral medium [16,18]. The AtoS-AtoC TCS was induced by the addition of 10 mM acetoacetate. $CaCl_2$ or other compounds were added in the culture medium when A_{600} reached 0.3, at concentrations that did not affect cell growth. Cell culture samples were collected at the indicated time points and processed for crotonic acid/cPHB determination.

β-Galactosidase assay

The β-Galactosidase assay was performed using *E. coli* cells carrying the appropriate plasmids [36] and grown in modified M9 mineral medium [16] in the presence of 10 mM acetoacetate or 2.5 mM $CaCl_2$.

Cell fractionation

E. coli cells were harvested, washed twice with PBS and suspended in 50 mM Tris/HCl, pH 7.5, containing 1 mM PMSF. The cell suspensions were incubated with lysozyme (1 mg/ml) for 30 min at 4 °C and subsequently lysed by sonication. DNase I (20 units/ml) and RNase A (20 μg/ml) were added and the lysates were incubated for 30 min at 4 °C prior to centrifugation at 10000 ***g*** for 20 min at 4 °C. The supernatants were centrifuged at 140000 ***g*** for 1 h at 4 °C to pellet the membranes and separate them from the cytosolic fraction. The membranes were treated in TGS buffer [50 mM Tris/HCl, pH 7.5, 10 % (v/v) glycerol and 250 mM sucrose] at 4 °C for 1 h and recovered by centrifugation at 170000 ***g*** for 30 min [37].

Determination of cPHB

cPHB determination was carried out by the method of Karr et al. [38], as modified by Huang and Reusch [39], based on the acid-catalysed β-elimination of cPHB to crotonic acid. Dried *E. coli* cell pellets or cytosolic fractions derived from a 10 ml culture sample were incubated with 0.5 ml concentrated H_2SO_4 at 92 °C for 8 h [16,17]. 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 analysed 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 [40].

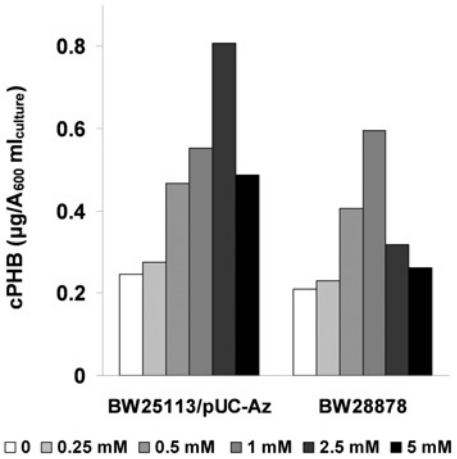

Figure 1 Dose–response of extracellular Ca^{2+} on cPHB biosynthesis

Total cPHB levels in BW25113/pUC-Az and BW28878 *E. coli* cells at 0.5 h after the addition of 0.25–5 mM $CaCl_2$ in the culture medium. Results are presented as the average of two experiments. The error for each data point is less than 5 %.

Extraction of cPHB-channels from *E. coli* cells

E. coli cultures were pelleted by centrifugation and dehydrated by two sequential washes with methanol, 1:1 methanol/acetone and acetone [41]. The dehydrated *E. coli* cells or the freeze-dried membrane fractions were extracted by incubation in 5 ml of chloroform at 4 °C for 36 h. Chloroform was subsequently evaporated with a stream of nitrogen and the amounts of cPHB-forming channels were determined as described above, with the exception that the samples were incubated with concentrated H_2SO_4 at 92 °C for 20 min [41].

RESULTS

Involvement of extracellular Ca^{2+} in AtoS-AtoC TCS-mediated cPHB biosynthesis

Exposure of BW25113 (*atoSC*$^+$) or BW28878 (Δ*atoSC*) *E. coli* cells to various $[Ca^{2+}]_e$ affected cPHB biosynthesis in a concentration-dependent manner, maximal increases being obtained with 2.5 mM and 1 mM respectively at 0.5 h after extracellular Ca^{2+} addition (Figure 1). The patterns of cPHB levels in the cytosol and membranes were comparable during *E. coli* growth in both strains, with only approx. 10 % being obtained in the membrane fractions compared with the amounts present in the cytosol (Figure 2).

This AtoS-AtoC TCS-mediated regulation of cPHB biosynthesis can be attributed to $[Ca^{2+}]_e$, since exposure of cells of both genotypes to equimolar concentrations of EGTA and Ca^{2+} failed to induce any increase in cPHB levels (Figure 3). Moreover, under higher $[Ca^{2+}]_e$ and in the presence of $MgCl_2$, 1 mM EGTA was insufficient to reverse the Ca^{2+}-induced increases in cPHB levels (Figure 3).

Effect of extracellular Ca^{2+} on the *ato* operon

Addition of extracellular Ca^{2+} exerted no effect on the minimal *atoDAEB* URR in either *atoSC*$^+$ or Δ*atoSC* cells, even when they

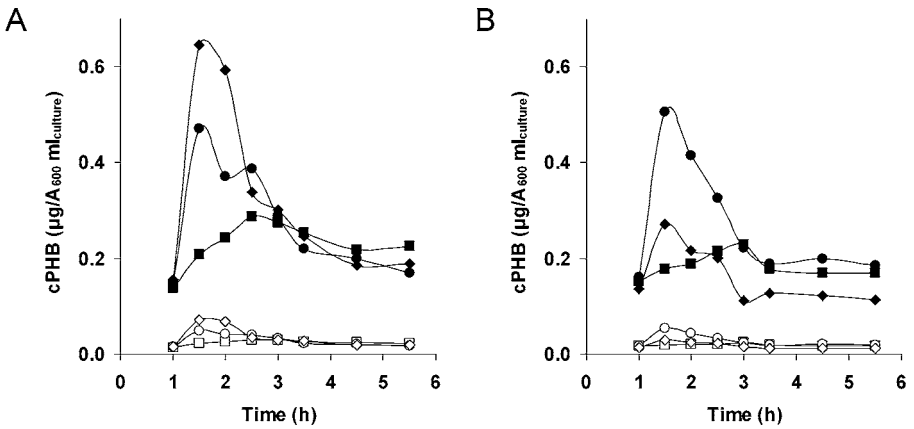

Figure 2 Effect of extracellular Ca^{2+} on cPHB levels during *E. coli* growth

Total cPHB levels in the cytosolic (closed symbols) and membrane (open symbols) fractions of (**A**) BW25113/pUC-Az and (**B**) BW28878 cells grown in the absence (■, □) or presence of 1 mM (●, ○) or 2.5 mM (◆, ◇) $CaCl_2$. Results are presented as the average of two experiments. The error for each data point is less than 5 %.

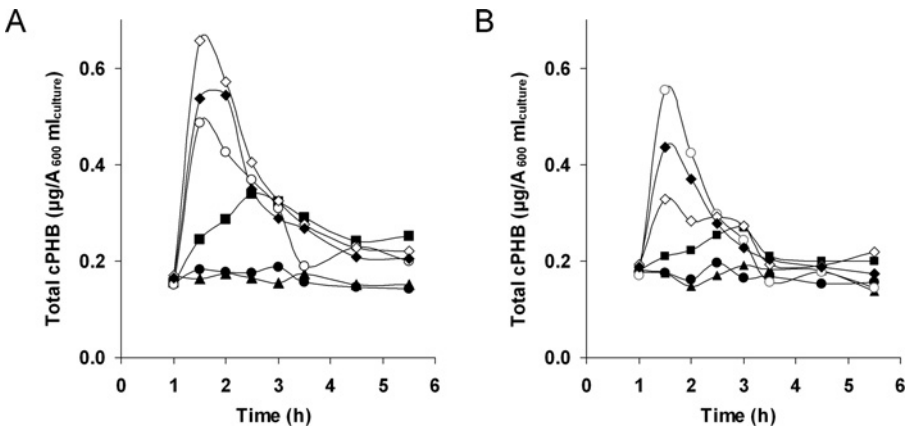

Figure 3 Effect of EGTA and $MgCl_2$ on Ca^{2+}-induced cPHB biosynthesis

(**A**) BW25113/pUC-Az and (**B**) BW28878 *E. coli* cells were grown in the absence (■) or presence of 1 mM EGTA, either alone (▲) or in combination with 1 mM (●, ○) or 2.5 mM (◆, ◇) $CaCl_2$, without (closed symbols) or with (open symbols) the addition of 1 mM $MgCl_2$. Results are presented as the average of two experiments. The error for each data point is less than 5 %.

carried the multicopy pUC-Az plasmid (Figure 4). However, it induced *atoDAEB* transcriptional activation acting on an extensive region containing *atoDAEB* URR, *atoD* and a part of *atoA* genes in *atoSC*$^+$ cells transformed with plasmid pCPG5 (Figure 4). Furthermore, extracellular Ca^{2+} induced *atoC* transcription in BW25113/pCPG6 cells, but exerted no effect on *atoS* transcription in pCPG4-transformed bacteria of both *atoSC*$^+$ and Δ*atoSC* genotypes (Figure 4).

Effect of verapamil on cPHB biosynthesis

Verapamil elicited an inhibitory effect on cPHB biosynthesis in both Δ*atoSC* and AtoS-AtoC overproducing cells, cPHB accumulation decreasing dramatically in the membrane fractions of both strains (Figure 5). Addition of Ca^{2+} increased cPHB levels in verapamil-treated cells. Interestingly, this phenotype was observed in the membranes of *atoSC*$^+$, but not of Δ*atoSC*, cells (Figure 5). Extracellular Ca^{2+} was also able to reverse the diltiazem-mediated cPHB decreases in cells of both genetic backgrounds, this phenotype being circumvented with higher $[Ca^{2+}]_e$ in Δ*atoSC* cells compared with *atoSC*$^+$ bacteria (results not shown). Nifedipine caused a more pronounced cPHB reduction that was not reversed by $[Ca^{2+}]_e$ in cells of both genotypes (results not shown).

Effect of $[Ca^{2+}]_e$ on histamine- and C48/80-regulated cPHB biosynthesis

In *atoSC*-overexpressing *E. coli*, addition of histamine or C48/80 enhanced the induction of cPHB biosynthesis by 1 mM Ca^{2+}, in contrast with the Δ*atoSC* cells (Table 1). However, both agents reduced the inductive action of 2.5 mM Ca^{2+} in the *atoSC*$^+$ strain and induced cPHB production in Δ*atoSC* cells (Table 1). At a higher $[Ca^{2+}]_e$ of 5 mM, histamine elicited a minor inductive effect in *atoSC*$^+$, but not in Δ*atoSC*, cells (Table 1).

DISCUSSION

Growth of *E. coli* in the presence of increased $[Ca^{2+}]_e$ resulted in concentration-dependent induction of cPHB biosynthesis, in both cytosolic and membrane fractions, under an apparent control of the AtoS-AtoC TCS. Maximal cPHB levels accumulated at higher $[Ca^{2+}]_e$ in *E. coli* cells overproducing the AtoS-AtoC TCS than in their Δ*atoSC* counterparts, which is indicative of a threshold in the Ca^{2+} action in the absence of *atoSC* genes. The specificity of the response was evident by its reversal by the chelating agent EGTA and the restored inductive phenotype upon higher $[Ca^{2+}]_e$ and Mg^{2+} addition.

Table 1 Interaction of extracellular Ca^{2+} and histamine or C48/80 in the regulation of cPHB biosynthesis

The results are expressed as μg of cPHB/A_{600} $ml_{culture}$ and presented as the means ± S.D. of two experiments in *E. coli* BW25113/pUC-Az and BW28878.

	BW25113/pUC-Az			BW28878		
$CaCl_2$ (mM)	Control	+5 mM histamine	+10 μg/ml C48/80	Control	+5 mM histamine	+10 μg/ml C48/80
0	0.24 ± 0.01	0.16 ± 0.01	0.39 ± 0.01	0.21 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
1	0.55 ± 0.03	0.65 ± 0.03	0.84 ± 0.04	0.60 ± 0.02	0.37 ± 0.02	0.51 ± 0.02
2.5	0.81 ± 0.04	0.55 ± 0.02	0.23 ± 0.01	0.20 ± 0.01	0.30 ± 0.01	0.32 ± 0.01
5	0.39 ± 0.02	0.47 ± 0.02	0.22 ± 0.01	0.28 ± 0.01	0.29 ± 0.01	0.31 ± 0.01

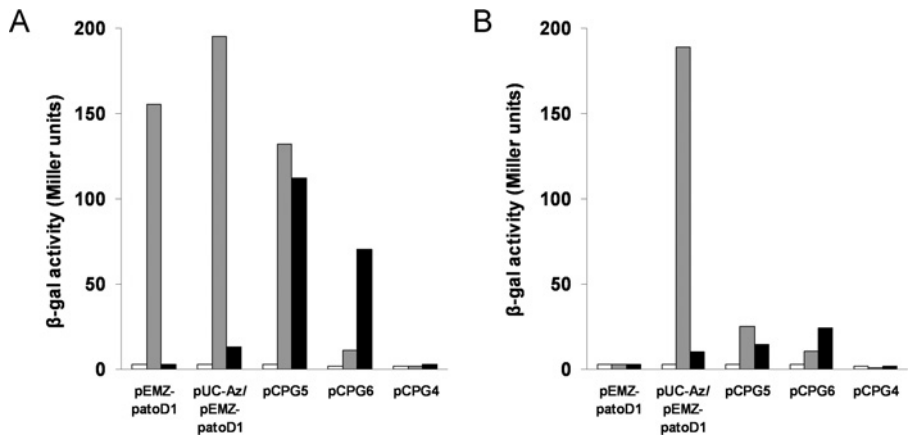

Figure 4 Effect of Ca^{2+} on *atoDAEB*, *atoC* and *atoS* transcription

Activation of *atoDAEB*, *atoC* and *atoS* promoters in (**A**) BW25113 and (**B**) BW28878 *E. coli* cells transformed with plasmids *atoDAEB* URR-*lacZ* without (pEMZ-patoD1) or with pUC-Az (pUC-Az/pEMZ-patoD1), *atoDAEB* URR-*atoD-atoA-lacZ* (pCPG5), *atoC* URR-*lacZ* (pCPG6) and *atoS URR-lacZ* (pCPG4), in the absence (open columns) or presence of 10 mM acetoacetate (shaded columns) or 2.5 mM $CaCl_2$ (filled colums). Results are presented as the average of two experiments. The error for each data point is less than 5 %.

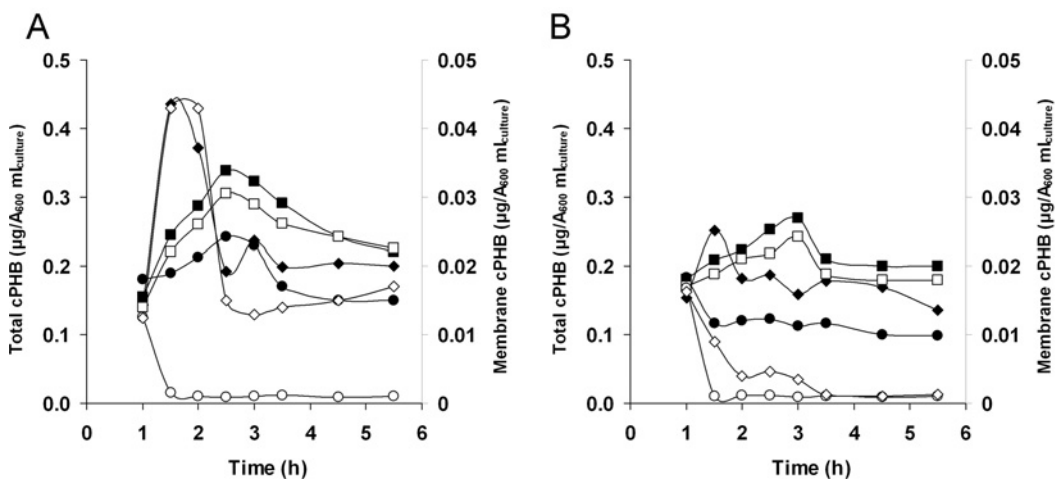

Figure 5 Effect of verapamil on AtoS-AtoC-regulated cPHB biosynthesis

Total cPHB levels in the cytosolic (closed symbols) and membrane (open symbols) fractions of (**A**) BW25113/pUC-Az and (**B**) BW28878 cells grown in the absence (■, □) or presence of 500 μM verapamil alone (●, ○) or with 5 mM $CaCl_2$ (◆, ◇). Results are presented as the average of two experiments. The error for each data point is less than 5 %.

E. coli has no known significant internal Ca^{2+} sequestration machinery, similar to, for instance, the eukaryotic endoplasmic reticulum. However, Ca^{2+} gradients exist and cPHB seems to control Ca^{2+} storage in *E. coli* cytosol and membranes [5,6,42]. Its redistribution from mostly cytosolic to membrane-bound, upon AtoS-AtoC TCS induction, particularly during the stationary phase of growth [17], may have substantial implications in its channel function. Previous reports have implicated cPHB as a possible target of the EGTA-induced rapid decrease in free intracellular Ca^{2+} levels [5], which may be associated with the inhibitory action of EGTA on cPHB biosynthesis in both *atoSC*$^+$ and Δ*atoSC* cells. Moreover, although verapamil may elicit effects additional to its putative Ca^{2+}-transporter actions [13], the notable decrease in cPHB accumulation in the membrane fractions

of both strains, which was reversed by Ca^{2+} addition only in the *atoSC*$^+$ cells, signified that the AtoS-AtoC TCS may predominantly regulate channel cPHB. The significance of the differential effects of the Ca^{2+}-channel blockers in the regulatory action of the AtoS-AtoC TCS on cPHB biosynthesis is under investigation.

cPHB induction has been attributed to the *atoDAEB* transcriptional activation by the AtoS-AtoC TCS [16,17]. Activation in the presence of extracellular Ca^{2+} was observed when an extensive region of the *atoDAEB* sequence was introduced in *E. coli*. Furthermore, the effects of Ca^{2+} on *atoC*, but not on *atoS*, expression in *atoSC*$^+$ cells pointed to different or supplementary mechanisms underlying the action of Ca^{2+} in AtoS-AtoC signalling towards its downstream targets.

The previously reported histamine-induced down-regulation of cPHB biosynthesis in AtoS-AtoC TCS-expressing bacteria [18] and the interplay of this biogenic amine with Ca^{2+} in eliciting most of its physiological functions may have immense physiological significance in host–bacteria symbiotic behaviour, particularly in the gastrointestinal tract [27–29]. The $[Ca^{2+}]_e$-dependent induction of cPHB biosynthesis by histamine and the functionally related polyamine C48/80 in AtoS-AtoC-overproducing, but not in Δ*atoSC*, cells implied the participation of the AtoS-AtoC TCS. These results provide initial indications for a possible Ca^{2+}-dependent action of histamine in essential processes of bacterial cells, such as cPHB production, and urge the exploration of yet undefined effects of this host-derived component on the symbiotic *E. coli* through Ca^{2+} transients, beyond the commonly investigated histamine receptor-mediated effects on host (patho)physiology.

In conclusion, several lines of evidence indicate that Ca^{2+} is a key mediator in the regulation of bacterial processes such as motility and chemotaxis [31], as well as in TCS induction [43], establishing its homoeostatic role in bacterial physiology and possibly pathophysiology or bacteria–host communication. This first report on the interaction of the AtoS-AtoC and Ca^{2+} that govern cPHB biosynthesis provide the lead for the investigation of related signalling networks and their potential exploitation in the treatment of bacterial diseases.

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