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## Extracellular $\text{Ca}^{2+}$ transients affect poly-(R)-3-hydroxybutyrate regulation by the AtoS-AtoC system in *E. coli*

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**Short title:**  $\text{Ca}^{2+}$  affects cPHB regulation by the AtoS-AtoC system

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## Abstract

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

## Key words

AtoS-AtoC two-component system, *atoDAEB*, calcium, calcium channel blockers, histamine, poly-(R)-3-hydroxybutyrate

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

## INTRODUCTION

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

In *Escherichia coli*,  $\text{Ca}^{2+}$  influx arrangements include the non-proteinaceous complexes of short-chain poly-(R)-3-hydroxybutyrate-polyphosphate- $\text{Ca}^{2+}$  [5, 6]. Complexed poly-(R)-3-hydroxybutyrate (cPHB) 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* whilst a periplasmic protein, YdcS, has been reported to exhibit PHB synthase activity only very recently [10]. The non-proteinaceous complexes act as voltage-gated  $\text{Ca}^{2+}$  channels with complex gating kinetics, their activity being implicated in the phase of growth-dependent control of cytosolic  $\text{Ca}^{2+}$  levels [6, 11, 12]. Moreover, cPHB channels share some characteristics with eukaryotic L-type voltage-activated  $\text{Ca}^{2+}$  channels, which are targeted selectively by the  $\text{Ca}^{2+}$  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 two-component system (TCS) 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 compound 48/80 (C48/80) [18], which has been shown to induce rapid  $\text{Ca}^{2+}$  efflux, thus altering the permeability of the outer membrane in *E. coli* [19].

AtoS-AtoC TCS consists of the AtoS histidine kinase and the AtoC response regulator [20] of the NtrC-NifA family of  $\sigma^{54}$ -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]. 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  $\text{Ca}^{2+}$  homeostasis through cPHB regulation [16, 17].

*E. coli* adapt to a wide range of  $[\text{Ca}^{2+}]_e$ , 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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  in regulating pro-inflammatory signals and for the cross-talk between  $\text{Ca}^{2+}$  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, this study aimed to investigate the effects of extracellular  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ -mediated homeostatic mechanisms, leading to new insights on the interaction of signal transduction systems and extracellular  $\text{Ca}^{2+}$  levels, with potential implications in the symbiotic behavior of bacteria.

## MATERIALS AND METHODS

### Materials

Histamine dichloride was obtained from SERVA (Heidelberg, Germany), C48/80 from Sigma Chem. Co. (St. Louis, MO, USA) and verapamil hydrochloride 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*<sup>+</sup>) [33] and BW28878 ( $\Delta$ *atoSC*) [23] were a gift from Hirofumi Aiba (Nagoya University, Japan). Plasmid pUC-Az, containing the *atoS*, *atoC* genes and a part of the *atoDAEB* operon [34], as well as plasmids pEMZ-patoD1 (*atoDAEB* URR-*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* were grown in LB or in modified M9 mineral medium [16, 18]. AtoS-AtoC TCS was induced by the addition of 10 mM acetoacetate. CaCl<sub>2</sub> or other compounds were added in the culture medium when A<sub>600</sub> 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.

### $\beta$ -Galactosidase assay

$\beta$ -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<sub>2</sub>.

### Cell fractionation

*E. coli* cells were harvested, washed twice with PBS and suspended in 50 mM Tris-HCl, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride. 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  $\mu$ g/ml) were added and the lysates were incubated for 30 min at 4 °C prior to centrifugation at 10,000 $\times$ g for 20 min at 4 °C. The supernatants were centrifuged at 140,000 $\times$ 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 170,000 $\times$ 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 *et al.* [39], based on the acid-catalyzed  $\beta$ -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<sub>2</sub>SO<sub>4</sub> 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 4 times with 3 ml of dichloromethane. Sodium hydroxide (100  $\mu$ l of 1 N) was added to the dichloromethane extracts to convert crotonic acid to sodium crotonate and the dichloromethane was evaporated. The residual was acidified to pH 2 with 3 N 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. Quantitation 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].

### 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 orthoacetone [41]. The dehydrated *E. coli* cells or the lyophilized membrane fractions were extracted by incubation in 5 ml 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<sub>2</sub>SO<sub>4</sub> at 92 °C for 20 min [41].

## RESULTS

### Involvement of extracellular Ca<sup>2+</sup> in AtoS-AtoC TCS-mediated cPHB biosynthesis

Exposure of BW25113 (*atoSC*<sup>+</sup>) or BW28878 ( $\Delta$ *atoSC*) *E. coli* cells to various [Ca<sup>2+</sup>]<sub>e</sub> 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<sup>2+</sup> addition (Figure 1). The patterns of cPHB levels in the cytosol and membranes were comparable during *E. coli* growth in both strains, with only about 10% being obtained in the membrane fractions compared to the amounts present in the cytosol (Figure 2).

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

### Effect of extracellular Ca<sup>2+</sup> on the *ato* operon

Addition of extracellular Ca<sup>2+</sup> exerted no effect on the minimal *atoDAEB* URR in either *atoSC*<sup>+</sup> or  $\Delta$ *atoSC* cells, even when they 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*<sup>+</sup> cells transformed with plasmid pCPG5 (Figure 4). Furthermore, extracellular Ca<sup>2+</sup> induced *atoC* transcription in BW25113/pCPG6 cells but exerted no effect on *atoS* transcription in pCPG4-transformed bacteria of both *atoSC*<sup>+</sup> and  $\Delta$ *atoSC* genotypes (Figure 4).

### Effect of verapamil on cPHB biosynthesis

Verapamil elicited an inhibitory effect on cPHB biosynthesis in both  $\Delta$ *atoSC* and AtoS-AtoC overproducing cells, cPHB accumulation decreasing dramatically in the membrane fractions of both strains (Figure 5). Addition of [Ca<sup>2+</sup>]<sub>e</sub> increased cPHB levels in verapamil-treated cells. Interestingly, this phenotype was observed in the membranes of *atoSC*<sup>+</sup> but not of  $\Delta$ *atoSC* cells

(Figure 5). Extracellular  $\text{Ca}^{2+}$  was also able to reverse the diltiazem-mediated cPHB decreases in cells of both genetic backgrounds, this phenotype being circumvented with higher  $[\text{Ca}^{2+}]_e$  in  $\Delta\text{atoSC}$  cells compared to  $\text{atoSC}^+$  bacteria (data not shown). Nifedipine caused a more pronounced cPHB reduction that was not reversed by  $[\text{Ca}^{2+}]_e$  in cells of both genotypes (data not shown).

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

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

## DISCUSSION

Growth of *E. coli* in the presence of increased  $[\text{Ca}^{2+}]_e$  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  $[\text{Ca}^{2+}]_e$  in *E. coli* cells overproducing the AtoS-AtoC TCS than in their  $\Delta\text{atoSC}$  counterparts, which is indicative of a threshold in the  $\text{Ca}^{2+}$  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  $[\text{Ca}^{2+}]_e$  and  $\text{Mg}^{2+}$  addition.

*E. coli* has no known significant internal  $\text{Ca}^{2+}$  sequestration machinery, similar for instance to the eukaryotic endoplasmic reticulum. However,  $\text{Ca}^{2+}$  gradients exist and cPHB seems to control  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  levels [5], which may be associated with the inhibitory action of EGTA on cPHB biosynthesis in both  $\text{atoSC}^+$  and  $\Delta\text{atoSC}$  cells. Moreover, although verapamil may elicit effects additional to its putative  $\text{Ca}^{2+}$ -transporter actions [13], the notable decrease in cPHB accumulation in the membrane fractions of both strains, which was reversed by  $\text{Ca}^{2+}$  addition only in the  $\text{atoSC}^+$  cells, signified that AtoS-AtoC TCS may regulate predominantly channel cPHB. The significance of the differential effects of the  $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$  was observed when an extensive region of the *atoDAEB* sequence was introduced in *E. coli*. Furthermore, the effects of  $\text{Ca}^{2+}$  on *atoC* but not on *atoS* expression in  $\text{atoSC}^+$  cells pointed to different or supplementary mechanisms underlying the action of  $\text{Ca}^{2+}$  in AtoS-AtoC signaling 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  $\text{Ca}^{2+}$  in eliciting most of its physiological functions may have immense physiological significance in host-bacteria symbiotic behavior, particularly in the gastrointestinal tract [27-29]. The  $[\text{Ca}^{2+}]_e$ -dependent

induction of cPHB biosynthesis by histamine and the functionally related polyamine C48/80 in AtoS-AtoC overproducing but not in  $\Delta$ atoSC cells implied the participation of the AtoS-AtoC TCS. These results provide initial indications for a possible  $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$  transients, beyond the commonly investigated histamine receptor-mediated effects on host (patho)physiology.

In conclusion, several lines of evidence indicate that  $\text{Ca}^{2+}$  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 homeostatic role in bacterial physiology and possibly pathophysiology or bacteria-host communication. This first report on the interaction of AtoS-AtoC and  $\text{Ca}^{2+}$  that govern cPHB biosynthesis provide the lead for the investigation of related signaling networks and their potential exploitation in the treatment of bacterial diseases.

## REFERENCES

- 1 Norris, V., Grant, S., Freestone, P., Canvin, J., Sheikh, F.N., Toth, I., Trinei, M., Modha, K. and Norman R. I. (1996) Calcium signaling in bacteria. *J. Bacteriol.* **178**, 3677-3682
- 2 Holland, I. B., Jones, H. E., Campbell, A. K. and Jacq, A. (1999) An assessment of the role of intracellular free  $\text{Ca}^{2+}$  in *E. coli*. *Biochimie* **81**, 901-907
- 3 Dominguez, D.C. (2004) Calcium signaling in bacteria. *Mol. Microbiol.* **54**, 291-297
- 4 Hoffer, S. M., Schoondermark, P., van Veen, H. W. and Tommassen, J. (2001) Activation of gene amplification of pitB, encoding a third phosphate transporter of *Escherichia coli* K-12. *J. Bacteriol.* **183**, 4659-4663
- 5 Jones, H. E., Holland, I. B., Baker, H. L. and Campbell, A. K. (1999) Slow changes in cytosolic free  $\text{Ca}^{2+}$  in *Escherichia coli* highlight two putative influx mechanisms in response to changes in extracellular calcium. *Cell Calcium* **25**, 265-274
- 6 Reusch, R. N., Huang, R. and Bramble, L. L. (1995) Poly-3-hydroxybutyrate/polyphosphate complexes form voltage-activated  $\text{Ca}^{2+}$  channels in the plasma membranes of *Escherichia coli*. *Biophys. J.* **69**, 754-766
- 7 Seebach, D., Brunner, A., Burger, H. M., Schneider, J. and Reusch, R. N. (1994) Isolation and  $^1\text{H-NMR}$  spectroscopic identification of poly(3-hydroxybutanoate) from prokaryotic and eukaryotic organisms; determination of the absolute configuration (R) of the monomeric unit 3-hydroxybutanoic acid from *Escherichia coli* and spinach. *Eur. J. Biochem.* **224**, 317-328
- 8 Reusch, R. N., Hiske, T. W. and Sadoff, H. L. (1986) Poly- $\beta$ -hydroxybutyrate membrane structure and its relationship to genetic transformability in *Escherichia coli*. *J. Bacteriol.* **168**, 553-562
- 9 Xian, M., Fuerst, M. M., Shabalin, Y. and Reusch, R. N. (2007) Sorting signal of *Escherichia coli* OmpA is modified by oligo-(R)-3-hydroxybutyrate. *Biochim. Biophys. Acta* **1768**, 2660-2666
- 10 Dai, D. and Reusch, R. N. (2008) Poly-3-hydroxybutyrate synthase form the periplasm of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **374**, 485-489
- 11 Campbell, A. K., Naseem, R., Holland, I. B., Matthews, S. B. and Wann, K. T. (2007) Methylglyoxal and other carbohydrate metabolites induce lanthanum-sensitive  $\text{Ca}^{2+}$  transients and inhibit growth in *E. coli*. *Arch. Biochem. Biophys.* **468**, 107-113

- 12 Campbell, A. K., Naseem, R., Wann, K., Holland, I. B. and Matthews, S. B. (2007) Fermentation product butane 2,3-diol induces  $\text{Ca}^{2+}$  transients in *E. coli* through activation of lanthanum-sensitive  $\text{Ca}^{2+}$  channels. *Cell Calcium* **41**, 97-106
- 13 Andersen, C. L., Holland, I. B. and Jacq, A. (2006) Verapamil, a  $\text{Ca}^{2+}$  channel inhibitor acts as a local anesthetic and induces the sigma E dependent extra-cytoplasmic stress response in *E. coli*. *Biochim. Biophys. Acta* **1758**, 1587-1595
- 14 McDonald, T. F., Pelzer, S., Trawtwein, W. and Pelszer, D. J. (1994) Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells. *Physiol. Rev.* **74**, 365-507
- 15 Ling, V. (1997) Multidrug resistance: molecular mechanisms and clinical relevance. *Cancer Chemother. Pharmacol.* **40**, S3-S8 (Suppl.)
- 16 Theodorou, M. C., Panagiotidis, C. A., Panagiotidis, C. H., Pantazaki, A. A. and Kyriakidis, D. A. (2006) Involvement of the AtoS-AtoC signal transduction system in poly-(R)-3-hydroxybutyrate biosynthesis in *Escherichia coli*. *Biochim. Biophys. Acta* **1760**, 896-906
- 17 Theodorou, M. C., Theodorou, E. C., Panagiotidis, C. A. and Kyriakidis, D. A. (2007) Spermidine triggering effect to the signal transduction through the AtoS-AtoC/Az two component system in *Escherichia coli*. *Biochim. Biophys. Acta* **1770**, 1104-1114
- 18 Kyriakidis, D. A., Theodorou, M. C., Filippou, P. S., Kyriakidis, K. D. and Tiligada, E. (2008) Effect of histamine on the signal transduction of the AtoS-AtoC two component system and involvement in poly-(R)-3-hydroxybutyrate biosynthesis in *Escherichia coli*, *Amino acids* **35**, 45-52
- 19 Katsu, T., Yoshimura, S. and Fujita, Y. (1984) Increases in permeability of *E. coli* outer membrane induced by polycations, *FEBS Lett* **166**, 175-178
- 20 Lioliou, E. E., Mimitou, E. P., Grigoroudis, A. I., Panagiotidis, C. H., Panagiotidis, C. A. and Kyriakidis, D. A. (2005) Phosphorylation activity of the response regulator of the two-component signal transduction system AtoS-AtoC/Az in *E. coli*, *Biochim. Biophys. Acta* **1725**, 257-268
- 21 Stock, A. M., Robinson, V. L. and Goudreau, P. N. (2000) Two-component signal transduction, *Annu. Rev. Biochem.* **69**, 183-215
- 22 Clark, D. P. and Cronan, Jr. J. E. (1996) Two-carbon compounds and fatty acids as carbon sources. In *Escherichia coli* and *Salmonella typhimurium* (Cellular and Molecular Biology) (Neidhardt, F. C., ed.), vol 1. pp 343-357, American Society of Microbiology, Washington, D.C.
- 23 Oshima, T., Aiba, H., Masuda, Y., Kanaya, S., Masahito, S., Wanner, B. L., Mori, H. and Mizuno, T. (2002) Transcriptome analysis of all two component regulatory system mutants of *Escherichia coli* K-12. *Mol. Microbiol.* **46**, 281-291
- 24 Zhou, L., Lei, X. H., Bochner, B. R. and Wanner, B. L. (2003) Phenotype microarray analysis of *Escherichia coli* K-12 mutants with deletions of all two-component systems. *J. Bacteriol.* **185**, 4956-4972
- 25 Yamamoto, K., Hirao, K., Oshima, T., Aiba, H., Utsumi, R. and Ishihama, A. (2005) Functional characterization *in vitro* of all two-component signal transduction systems from *Escherichia coli*, *J. Biol. Chem.* **280**, 1448-1456
- 26 Arifuzzaman, M., Maeda, M., Itoh, A., Nishikata, K., Takita, C., Saito, R., Ara, T., Nakahigashi, K., Huang, H.-C., Hirai, A., Tsuzuki, K., Nakamura, S., Altaf-Ul-Amin, M., Oshima, T., Baba, T., Yamamoto, N., Kawamura, T., Ioka-Nakamichi, T., Kitagawa, M.,

- Tomita, M., Kanaya, S., Wada, C. and Mori, H. (2006) Large-scale identification of protein-protein interaction of *Escherichia coli* K-12. *Genome Res.* **16**, 686-691
- 27 Guarner, F. and Malagelada, J. R. (2003) Gut flora in health and disease. *Lancet* **361**, 512-519
- 28 Hoch, J. A., Silhavy, T. J. (1995) In Two-component signal transduction (Hoch, J. A. and Silhavy, T. J., eds.), American Society for Microbiology, Washington D.C.
- 29 Chang, W. C. (2006) Store-operated calcium channels and pro-inflammatory signals. *Acta Pharmacol Sin.* **27**, 813-20
- 30 Hung, C. R. (2007) Protective effect of verapamil on gastric hemorrhagic ulcers in severe atherosclerotic rats. *Chin J Physiol.* **50**, 315-25
- 31 Tisa, L. S., Sekelsky, J. J. and Adler, J. (2000) Effect of organic antagonists of  $\text{Ca}^{2+}$ ,  $\text{Na}^{+}$  and  $\text{K}^{+}$  on chemotaxis and motility of *Escherichia coli*, *J. Bacteriol.* **182**, 4856-4861
- 32 Jones, H. E., Holland, B., Jacq, A., Wall, T. and Campbell, A. K. (2003) *Escherichia coli* lacking the AcrAB multidrug efflux pump also lacks nonproteinaceous, PHB-polyphosphate  $\text{Ca}^{2+}$  channels in the membrane. *Biochim. Biophys. Acta* **1612**, 90-97
- 33 Haldimann, A. and Wanner, B. L. (2001) Conditional-replication, integration, excision and retrieval plasmid-host systems for gene structure-function studies of bacteria. *J. Bacteriol.* **183**, 6384-6393
- 34 Canellakis, E. S., Paterakis, A. A., Huang, S. C., Panagiotidis, C. A. and Kyriakidis, D. A. (1993) Identification, cloning, and nucleotide sequencing of the ornithine decarboxylase antizyme gene of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7129-7133
- 35 Matta, M. K., Lioliou, E. E., Panagiotidis, C. H., Kyriakidis, D. A. and Panagiotidis, C. A. (2007) Interactions of the antizyme AtoC with regulatory elements of the *Escherichia coli* *atoDAEB* operon. *J. Bacteriol.* **189**, 6324-6332
- 36 Miller, J. H. (1992) In, A short course in bacterial genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- 37 Tokishita, S. -I., Yamada, H., Aiba, H. and Mizuno, T. (1990) Transmembrane signal transduction and osmoregulation in *Escherichia coli*: II. The osmotic sensor, EnvZ, located in the isolated cytoplasmic membrane displays its phosphorylation and dephosphorylation abilities as to the activator protein, OmpR. *J. Biochem.* **108**, 488-493
- 38 Karr, D. B., Waters, J. K. and Emerich, D. W. (1983) Analysis of poly- $\beta$ -hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exclusion high-pressure liquid chromatography and UV detection. *Appl. Environ. Microbiol.* **46**, 1339-1344
- 39 Huang, R. and Reusch, R. N. (1996) Poly(3-hydroxybutyrate) is associated with specific proteins in the cytoplasm and membranes of *Escherichia coli*. *J. Biol. Chem.* **271**, 22196-22202
- 40 Tagushi, S., Maehara, A., Takase, K., Nakahara, M., Nakamura, H. and Doi, Y. (2001) Analysis of mutational effects of a polyhydroxybutyrate (PHB) polymerase on bacterial PHB accumulation using an in vivo assay system, *FEMS Microbiol. Lett.* **198**, 65-71
- 41 Pavlov, E., Grimby, C., Diao, C. T. and French, R. J. (2005) A high-conductance mode of a poly-3-hydroxybutyrate/calcium/polyphosphate channel isolated from competent *Escherichia coli* cells, *FEBS Lett* **579**, 5187-5192
- 42 Michiels, J., Xi, C., Verhaert, J. and Vanderleyden, J. (2002) The functions of  $\text{Ca}^{2+}$  in bacteria: a role for EF-hand proteins? *Trends Microbiol.* **10**, 87-93
- 43 Rampersaud, A., Utsumi, R., Delgado, J., Forst, S. A. and Inouye, M. (1991)  $\text{Ca}^{2+}$ -enhanced phosphorylation of a chimeric protein kinase involved with bacterial signal transduction. *J. Biol. Chem.* **266**, 7633-7637

## Figure legends

### Figure 1 Dose-response of extracellular $\text{Ca}^{2+}$ on cPHB biosynthesis

Total cPHB levels in BW25113/pUC-Az and BW28878 *E. coli* cells at 0.5 h after addition of 0.25-5 mM  $\text{CaCl}_2$  in the culture medium. Results are presented as the average of two experiments. The error for each data point is less than 5 %.

### Figure 2 Effect of extracellular $\text{Ca}^{2+}$ 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 (◆, ◇)  $\text{CaCl}_2$ . 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 $\text{MgCl}_2$ on $\text{Ca}^{2+}$ -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 (◆, ◇)  $\text{CaCl}_2$ , without (closed symbols) or with (open symbols) the addition of 1 mM  $\text{MgCl}_2$ . Results are presented as the average of two experiments. The error for each data point is less than 5 %.

### Figure 4 Effect of $\text{Ca}^{2+}$ 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  $\text{CaCl}_2$  (filled columns). 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  $\mu\text{M}$  verapamil alone (●, ○) or with 5 mM  $\text{CaCl}_2$  (◆, ◇). Results are presented as the average of two experiments. The error for each data point is less than 5 %.

**Table 1 Interaction of extracellular Ca<sup>2+</sup> and histamine or C48/80 in the regulation of cPHB biosynthesis**

The results are expressed as  $\mu\text{g cPHB}/A_{600} \text{ ml}_{\text{culture}}$  and presented as the means  $\pm$  S.D. of two experiments in *E. coli* BW25113/pUC-Az and BW28878.

CaCl <sub>2</sub> mM	BW25113/pUC-Az			BW28878	
		+ Histamine 5 mM	+ C48/80 10 $\mu\text{g}/\text{ml}$	+ Histamine 5 mM	+ C48/80 10 $\mu\text{g}/\text{ml}$
0	0.24 $\pm$ 0.01	0.16 $\pm$ 0.01	0.39 $\pm$ 0.01	0.21 $\pm$ 0.01	0.13 $\pm$ 0.01
1	0.55 $\pm$ 0.03	0.65 $\pm$ 0.03	0.84 $\pm$ 0.04	0.60 $\pm$ 0.02	0.57 $\pm$ 0.02
2.5	0.81 $\pm$ 0.04	0.55 $\pm$ 0.02	0.23 $\pm$ 0.01	0.20 $\pm$ 0.01	0.30 $\pm$ 0.01
5	0.39 $\pm$ 0.02	0.47 $\pm$ 0.02	0.22 $\pm$ 0.01	0.28 $\pm$ 0.01	0.29 $\pm$ 0.01

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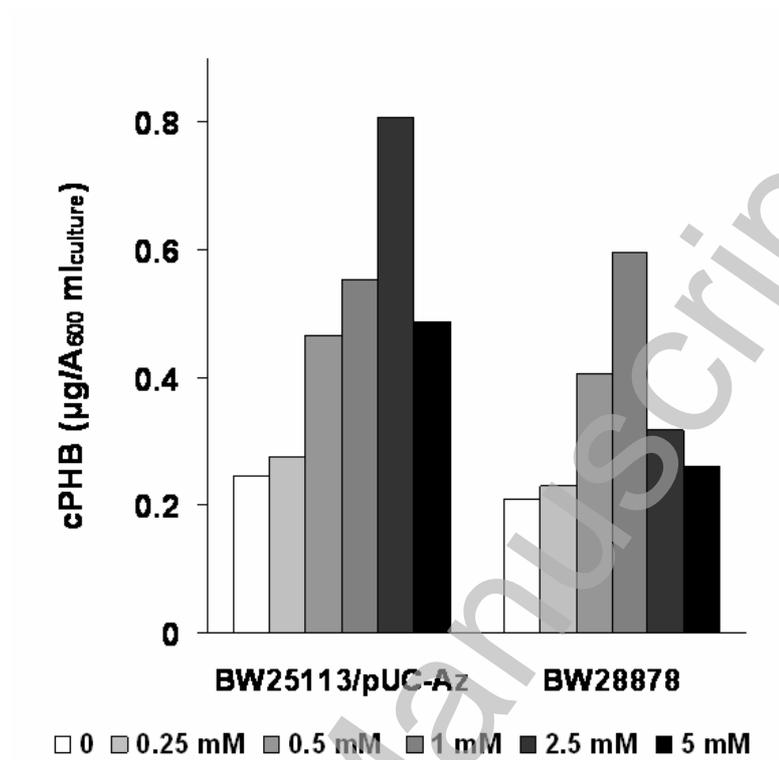


Figure 1

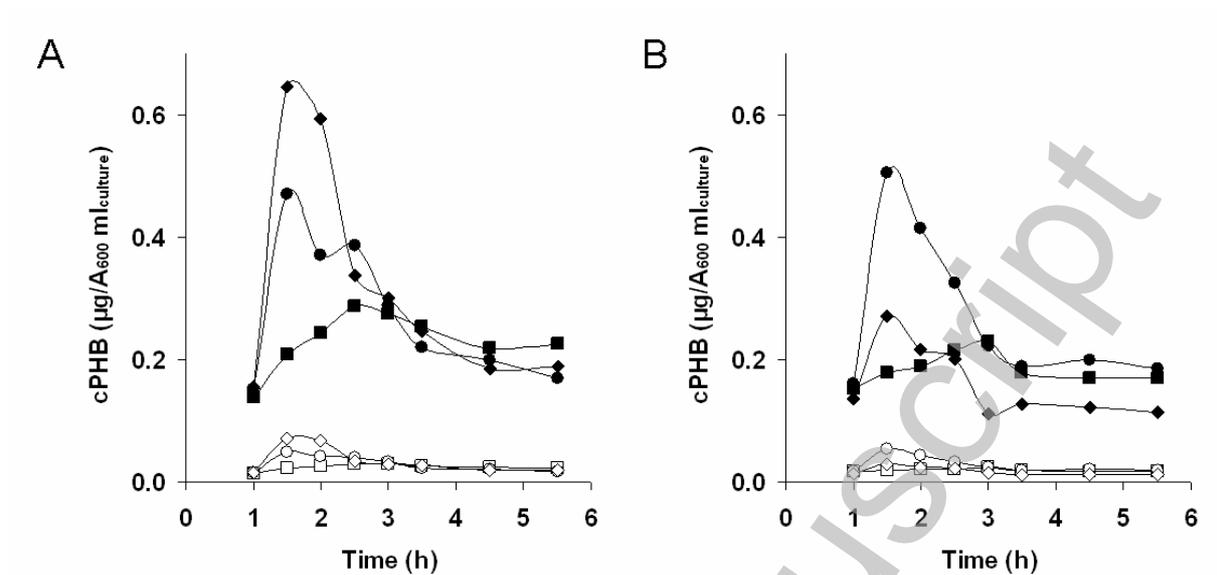


Figure 2

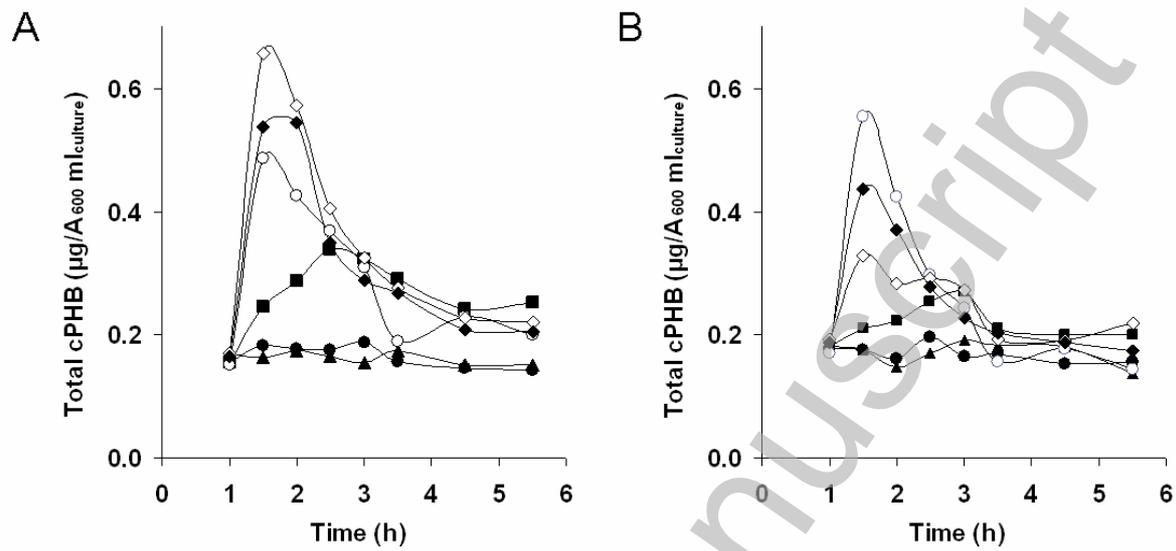


Figure 3

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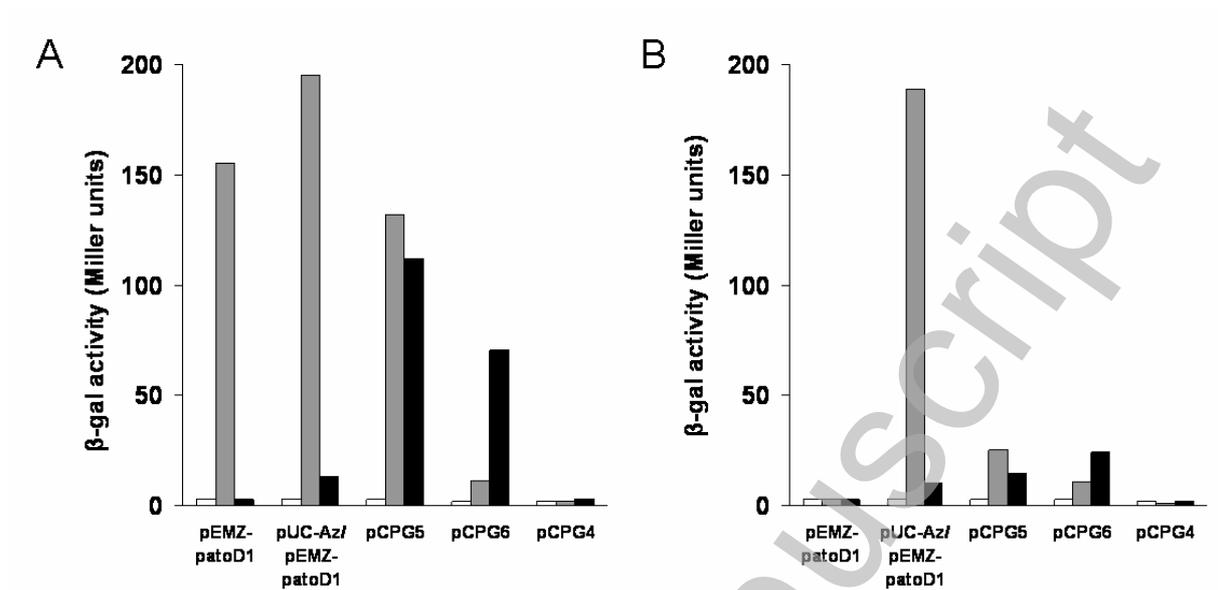
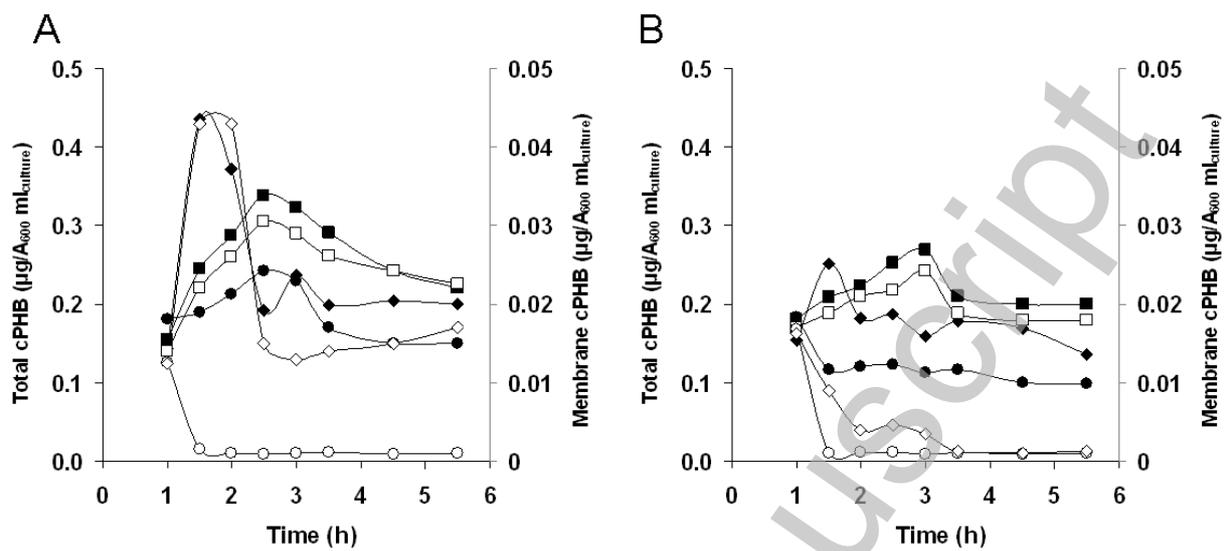


Figure 4

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**Figure 5**

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