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ORIGINAL ARTICLE

Involvement of AtoSC two-component system in *Escherichia coli* flagellar regulon

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Abstract The AtoSC two-component system in *Escherichia coli* is a key regulator of many physiological processes. We report here the contribution of AtoSC in *E. coli* motility and chemotaxis. *AtoSC* locus deletion in Δ*atoSC* cells renders cells not motile or responsive against any chemoattractant or repellent independently of the AtoSC inducer's presence. AtoSC expression through plasmid complemented the Δ*atoSC* phenotype. Cells expressing either AtoS or AtoC demonstrated analogous motility and chemotactic phenotypes as Δ*atoSC* cells, independently of AtoSC inducer's presence. Mutations of AtoC phosphate-acceptor sites diminished or abrogated *E. coli* chemotaxis. trAtoC, the AtoC constitutive active form which lacks its receiver domain, up-regulated *E. coli* motility. AtoSC enhanced the transcription of the *flhDC* and *fliAZY* operons and to a lesser extent of the *flgBCDEFGHIJKL* operon. The AtoSC-mediated regulation of motility and chemotactic response required also the expression of the CheAY system. The AtoSC inducers enhanced the AtoSC-mediated motility and chemotaxis. Acetoacetate or spermidine further promoted the responses of only AtoSC-expressing cells, while Ca^{2+} demonstrated its effects independently of AtoSC. Histamine regulated bacterial chemotaxis only in *atoSC*$^{+}$ cells in a concentration-dependent manner while reversed the AtoSC-mediated effects when added at high concentrations. The trAtoC-controlled motility effects were enhanced by acetoacetate or spermidine, but not by histamine. These data reveal that AtoSC system regulates the motility and chemotaxis of *E. coli*, participating in the transcriptional induction of the main promoters of the chemotactic regulon and modifying the motility and chemotactic phenotypes in an induction-dependent mechanism.

Keywords AtoSC · Two-component system · Chemotaxis · Histamine · Ca^{2+} · Motility

Abbreviations

AcAc	Acetoacetate
atoSC	Genetic locus encoding the AtoS and AtoC proteins
HI	Histamine
HK	Histidine kinase
MCPs	Methyl-accepting chemotaxis proteins
cPHB	Poly-(*R*)-3-hydroxybutyrate
SPD	Spermidine
RR	Response regulator
TCS	Two-component system
Asp-Ser	Aspartic acid-serine
Prop	Propionate
Glyc	Glycerol

Introduction

Regulatory networks involving two-component systems (TCS) dominate the adaptive signalling machinery of bacteria and allow them, amongst other multiple physiological processes, to direct their chemotactic movement to

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more favourable environments by changing their motility or altering gene expression and other cellular activities (Skerker et al. 2005). TCSs use transient phosphorylation of a sensory histidine kinase (HK) and a response regulator (RR) for signal transfer (Stock et al. 2000). Although *Escherichia coli* possess 32 of these phosphoryl transfer systems, many of their functions remain elusive (Mizuno 1997; Kyriakidis and Tiligada 2009).

The AtoSC TCS in *E. coli*, consisting of the AtoS HK and the AtoC RR (Lioliou et al. 2005; Grigoroudis et al. 2007; Filippou et al. 2008; Kyriakidis and Tiligada 2009), plays a pivotal role in many regulatory indispensable processes (Kyriakidis and Tiligada 2009). It constitutes together with *atoDAEB* operon the *atoSCDAEB* regulon and regulates growth on short-chain fatty acids through the transcriptional activation of the *atoDAEB* (Lioliou et al. 2005; Matta et al. 2007), as well as the biosynthesis and intracellular distribution of the inherent *E. coli* complexed poly-(*R*)-3-hydroxybutyrate (cPHB) (Theodorou et al. 2006). AtoC contains two putative phosphorylation sites, one at the conserved Asp55 (D55) residue and another at His73 (H73) located in an "H box" consensus sequence (Lioliou et al. 2005). Mutations at these putative phosphate-acceptor sites are capable of modifying the AtoSC effects (Lioliou et al. 2005; Theodorou et al. 2006, 2007). cPHB regulation by AtoSC TCS is achieved through its direct effects on *atoDAEB* operon (Theodorou et al. 2006) as well as through its involvement in fatty acid metabolism (Theodorou et al. 2011b). AtoSC-mediated signal transduction has been associated with acetoacetate (Theodorou et al. 2006), spermidine (Theodorou et al. 2007) or intermediate metabolic compounds of the short-chain fatty acid pathway (Theodorou et al. 2011c). It has been also correlated with the biogenic amine histamine and the mast cell degranulating calmodulin inhibitor compound 48/80 (C48/80) (Kyriakidis et al. 2008), possibly in an extracellular Ca^{2+}-mediated manner (Theodorou et al. 2009). The emergent complexity of possible AtoSC-related regulatory networks are underlined by a recent *E. coli* genome-wide promoter analysis revealing new AtoC binding sites in additional potential downstream targets of AtoSC TCS (Pilalis et al. 2011). The expression of AtoSC in some pathogenic *E. coli* strains as well as other pathogenic bacteria, led very recently to identification of three inhibitors of the AtoSC signalling, belonging to different chemical classes, i.e. Closantel, TNP-ATP and RWJ-49815 (Theodorou et al. 2011a).

AtoSC has been implicated in flagella synthesis and chemotaxis in *E. coli* (Oshima et al. 2002; Parkinson et al. 2005), which can consume amino acids and other carbon sources in their environment, thereby creating gradients and migrating towards high concentrations of attractants and away from repellents with high sensitivity (Ames and Parkinson 2007). Around 50 genes constitute the flagellar regulon, which is regulated via yet poorly defined mechanisms in response to environmental cues that involve a number of transcriptional regulators and cross-regulatory TCS networks (Chilcott and Hughes 2000; Rajagopala et al. 2007). During chemosensory adaptation, the extracellular signals in *E. coli* are perceived by methyl-accepting chemotaxis proteins (MCPs) principally involving components of the chemotaxis operon associated with the CheAY TCS. Without excluding the contribution of other pathways, the two most abundant MCPs in *E. coli*, Tsr and Tar, sense serine or aspartate and certain repellents, respectively (Zhulin 2001; Vaknin and Berg 2006; Ames and Parkinson 2007). In its natural environment, this symbiotic microorganism of the gastrointestinal flora (Guarner and Malagelada 2003) elicits a chemotactic response that plays a pivotal role in bacterial homeostasis, pathogenesis and survival against host defense mechanisms (Verstraeten et al. 2008; Jean St et al. 2008). Histamine and polyamines have been reported to enable pathogenic bacteria to overcome these mechanisms (Crosa and Walsh 2002), while histamine that is a key mediator of gastric acid secretion may modulate adaptation of pathogens to drug therapies often via TCS (Hori et al. 2002; Guarner and Malagelada 2003; Kakavas et al. 2006).

Despite the extensive experimental information on the molecular modelling of the chemotaxis regulon in *E. coli* (Chilcott and Hughes 2000; Ames and Parkinson 2007; Kyriakidis and Tiligada 2009), data on the involvement of additional cellular components as well as on the physiological role of this phenotype is limited. This study aimed to investigate the involvement of AtoSC in *E. coli* motility and chemotactic behavior and the possible regulatory contribution of agents that affect the physiological role of this TCS in *E. coli*. The data of this paper provide evidence for the involvement of AtoSC TCS in bacterial motility and chemotaxis.

Materials and methods

Materials

Histamine dichloride and all the other compounds and reagents were purchased from Sigma Chem. Co. (St. Louis, MO, USA).

Bacterial strains and plasmids

The genotypes of *E. coli* strains and the plasmids used are listed in Table 1. *E. coli* K-12 strains BW25113 (*atoSC*$^{+}$) (Haldimann and Wanner 2001) and BW28878 (Δ*atoSC*) (Oshima et al. 2002) were a gift from Dr Hirofumi Aiba (Nagoya University, Japan). *E. coli* K-12 strains RP437

Table 1 *Escherichia coli* strains and plasmids

Strain or plasmid	Genotype	References
Strains		
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)
RP437	*thr*(Am)-1 *leu*B6 *his*-4 *met*F(Am)159 *eda*-50 *rps*L136 [*thi*-1 *ara*-14 *lac*Y1 *mt*1-1 *xyl*-5 *ton*A31 *tsx*-78]/*jsp*/	Parkinson and Houts (1982)
RP1091	Δ(*che*A-*che*Z)2209 *thr*(Am)-1 *leu*B6 *his*-4 *met*F(Am)159 *eda*-50 *rps*L136 [*thi*-1 *ara*-14 *lac*Y1 *mt*1-1 *xyl*-5 *ton*A31 *tsx*-78]/*jsp*/	Parkinson and Houts (1982)
IMW353	*flgB*′-′*lacZ*	Lehnen et al. (2002)
YK4337	*fliA*′-′*lacZ*	Lehnen et al. (2002)
Plasmids		
pUC-Az	pUC19 containing the *atoS*, *atoC* genes and a part of the *atoDAEB* operon	Canellakis et al. (1993)
pUC-Az(AtoC$^-$)	pUC-Az with a frameshift mutation at codon 28 of *atoC*	Theodorou et al. (2006)
pCPC-Az	pIC20H with a *Sal*I/*Bam*HI fragment containing the *atoC* gene and the part of the *atoDAEB* operon	Canellakis et al. (1993)
pUC-Az(AtoDAEB$^-$)	pUC-Az derivative which lacks part of *atoDAEB* operon	Theodorou et al. (2006)
pUC-AtoC	pUC-Az derivative which lacks *atoS* and part of *atoDAEB* operon	Theodorou et al. (2011c)
pUC-AtoS	pUC-Az(AtoC$^-$) derivative which lacks part of *atoDAEB* operon	Theodorou et al. (2011b)
pAtoSC	pSC101 with a *Bam*HI/*Bst*XI fragment containing the *atoSC* locus	Theodorou et al. (2011b)
pAtoC	pSC101 with a *Sal*I/*Bst*XI fragment containing the *atoC* gene	Theodorou et al. (2011b)
ptratoC	pZErO 2.1 containing the *atoC* sequence encoding amino acids 140–461	Theodorou et al. (2011c)
pEM-Az	Reconstructed pUC-Az-like plasmid	Lioliou et al. (2005)
pEM-Az-D55	pUC-Az with a D55G mutation in *atoC*	Lioliou et al. (2005)
pEM-Az-H73	pUC-Az with a H73L mutation in *atoC*	Lioliou et al. (2005)
pEM-Az-D55/H73	pUC-Az with D55G and H73L mutations in *atoC*	Lioliou et al. (2005)
pFCZ6	Φ(flhDC′-′lacZ)6(hyb) in pMBL1034	Wei et al. (2001)

(wild type) and RP1091 (Δ*cheA-cheZ*) were a gift from John S. Parkinson (The University of Utah, USA) (Parkinson and Houts 1982). *E. coli* strains IMW353 (*flgB*′-′*lacZ*) and YK4337 (*fliA*′-′*lacZ*) were a kind gift from Dr Gottfried Unden (Johannes Gutenberg-Universität Mainz, Germany) (Lehnen et al. 2002). Plasmid pFCZ6 (*flhDC-lacZ*) was a kind gift from Dr Tony Romeo (University of Florida, USA) (Wei et al. 2001).

Plasmids pUC-Az, containing the *atoS*, *atoC* genes and a part of the *atoDAEB* operon (*atoD*, *atoA*, and two-thirds of *atoE*) or pCPC-Az, containing the *atoC* gene and the same part of the *atoDAEB* operon as pUC-Az, have been described previously (Canellakis et al. 1993). They express the whole AtoSC TCS or only AtoC protein, respectively. Plasmid pUC-Az(AtoC$^-$) is a pUC-Az derivative that does not express the AtoC protein but only the AtoS, due to the frame shift mutation introduced at codon 28 (Theodorou et al. 2006). Plasmid pUCAz(AtoDAEB$^-$) is a pUC-Az derivative, lacking the part of *atoDAEB* operon (Theodorou et al. 2006). Plasmid pUC-AtoC is a pUC-Az(AtoDAEB$^-$) derivative which does not express AtoS protein but only the AtoC protein (Theodorou et al. 2011c). Plasmid pUC-AtoS is a pUC-Az(AtoC$^-$) derivative which does not express AtoC protein but only the AtoS protein (Theodorou et al. 2011b). Plasmids pAtoSC and pAtoC, are pSC101 derivatives containing the *atoSC* locus or the *atoC* gene, respectively, have been described (Theodorou et al. 2011b). Plasmid ptratoC, which contains the *atoC* sequence encoding amino acids 140–461, expressing the N-terminal deleted AtoC form (trAtoC), lacking its 139 N-terminal amino acids, has been described (Theodorou et al. 2011c). Plasmids pEM-Az-D55, pEM-Az-H73 and pEM-Az-D55/H73 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 (Lioliou et al. 2005). Therefore, all three plasmids express AtoS together with a mutant form of AtoC.

Growth conditions and swarm agar plates assay

E. coli were grown at 37°C in LB medium supplemented with the requisite antibiotic Amp 100 μg/ml. Cell culture

samples of 5 µl were stabbed to swarm agar plates containing 1% (w/v) tryptone, 0.5% (w/v) NaCl and 0.3% (w/v) bacto-agar in the absence of antibiotic (Ames and Parkinson 2007). The medium also contained 5 mM Asp and Ser, or 2.5% (v/v) glycerol as chemoattractants and 1 mM propionate as chemorepellent. *E. coli* chemotaxis was investigated in the absence or presence of 10 mM acetoacetate, 2 mM spermidine, 0.005–20 mM histamine or 0.25–20 mM $CaCl_2$. Plates were incubated at 30°C in a humid environment for 10 h (Ames and Parkinson 2007).

β-Galactosidase assay

β-Galactosidase assay was performed using *E. coli* cells BW25113 and BW28878 carrying plasmid pFCZ6 (*flhDC-lacZ*) alone or together with plasmids pAtoSC or pAtoC, as well as strains IMW353 (*flgB′-′lacZ*) and YK4337 (*fliA′-′lacZ*) transformed with plasmids pUC-Az, pUCAz(AtoDAEB$^-$), pUC-AtoS, or pUC-AtoC. Strains were grown in modified M9 mineral medium (Miller 1992) in the absence or presence in the absence or presence of acetoacetate 10 mM.

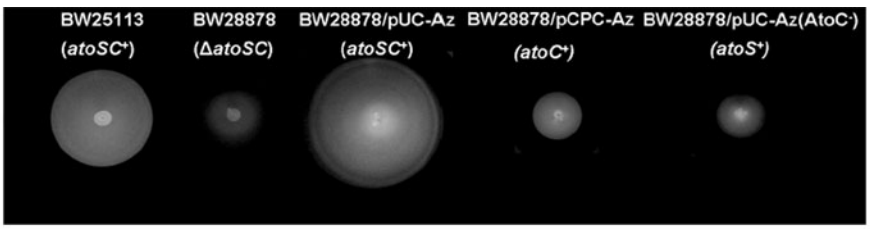
Results

AtoSC TCS participates in *E. coli* motility

The *atoSC*$^+$ (BW25113) wild-type strain produced the concentric ring patterns when grown in the motility agar in the absence of any chemoeffector (Fig. 1a). Conversely, the deletion of the *atoSC* locus in Δ*atoSC* (BW28878) cells diminished dramatically (approximately 49%) their motility property (Fig. 1a). The extrachromosomal introduction of the *atoS* and *atoC* genes through pUC-Az plasmid, in their BW28878/pUC-Az derivatives, complemented the Δ*atoSC* phenotype and further enhanced the bacterial motility (33% enhancement compared to the motility of the BW25113 wild-type cells). On the other hand, expression alone of either AtoS [pUC-Az(AtoC$^-$)-transformed derivatives] or AtoC (pCPC-Az-transformed derivatives) in Δ*atoSC* cells did not influence their motility response, resulting in a phenotype comparable to that obtained from the control Δ*atoSC* cells (Fig. 1a).

The presence of acetoacetate triggered a 13% motility enhancement of the AtoSC-expressing cells but not of the

A



B

Motility under AtoSC mediators (% modulation vs. control conditions)					
	BW25113	BW28878	BW28878/pUC-Az	BW28878/pCPC-Az	BW28878/pUC-Az(AtoC$^-$)
AcAc 10 mM	10.0 ± 0.8	0	13.0 ± 0.9	0	0
SPD 1 mM	-0.5 ± 0.1	0	-0.5 ± 0.1	0	0
SPD 2 mM	-12.2 ± 1.0	0	-16 ± 1.1	0	0
SPD 5 mM	-1.8 ± 0.1	0	-10.5 ± 0.1	0	0
$CaCl_2$ 1 mM	20.0 ± 1.6	9.0 ± 1.0	23.6 ± 1.7	9.0 ± 1.5	9.0 ± 1.5
$CaCl_2$ 2 mM	29.8 ± 2.4	18.0 ± 1.9	26.3 ± 2.6	18.0 ± 2.3	18.2 ± 2.1
$CaCl_2$ 5 mM	37.0 ± 2.5	27.2 ± 1.0	31.5 ± 2.3	27.2 ± 2.3	27.3 ± 2.2
HI 0.005 mM	14.3 ± 1.3	8.0 ± 1.2	15.1 ± 1.3	8.0 ± 1.1	8.0 ± 1.2
HI 0.05 mM	14.3 ± 1.3	8.0 ± 1.2	15.1 ± 1.3	8.0 ± 1.1	8.0 ± 1.2
HI 0.5 mM	14.3 ± 1.3	8.0 ± 1.2	15.1 ± 1.3	8.0 ± 1.1	8.0 ± 1.2
HI 2 mM	14.3 ± 1.3	8.0 ± 1.2	15.1 ± 1.3	8.0 ± 1.1	8.0 ± 1.2
HI 5 mM	35.7 ± 4.2	0.5 ± 0.9	45.5 ± 4.8	0.5 ± 0.1	0.5 ± 0.1
HI 10 mM	4.0 ± 0.8	0.5 ± 0.3	7.8 ± 0.9	0.5 ± 0.2	0.5 ± 0.2
HI 20 mM	13.2 ± 2.0	0.5 ± 0.1	15.0 ± 2.2	0.5 ± 0.1	0.5 ± 0.2

Fig. 1 The involvement of the AtoSC two-component system in *E. coli* motility. **a** Swarms produced by BW25113 (*atoSC*$^+$) or BW28878 (Δ*atoSC*) *E. coli* cells and their transformed derivatives with pUC-Az, pUC-Az(AtoC$^-$) or pCPC-Az plasmids, as determined by the soft agar plate assay in the absence of any compound or agent. **b** Effects of the mediators of AtoSC TCS in *E. coli* motility. Motility of the BW25113 (*atoSC*$^+$) or BW28878 (Δ*atoSC*) *E. coli* cells and their transformed derivatives with pUC-Az, pUC-Az(AtoC$^-$) or pCPC-Az plasmids' in the presence of the indicated concentrations of AcAc, SPD, $CaCl_2$ and HI expressed as the % modulation versus the motility under control conditions (absence of agents)

Δ*atoSC* derivatives (Fig. 1b). Spermidine addition in the motility agar regulated the motility phenotype only in AtoSC-expressing cells (Fig. 1b). Under spermidine 1 mM the cells moved in equal levels as in the control conditions. Higher polyamine concentrations (2 or 5 mM) elicited motility inhibition, yet at 2 mM caused the more pronounced 16% reduction of BW28878/pUC-Az cells compared with the control conditions.

Ca^{2+} produced an enhancement of *E. coli* motility in a concentration-analogous manner. The higher the Ca^{2+} concentration, the more motile *E. coli* cells of both the Δ*atoSC* or *atoSC*$^+$ genotypes either of *atoS*$^+$ or *atoC*$^+$ cells (Fig. 1b). However, the transformed strains expressing AtoS or AtoC demonstrated equal motility phenotypes under the respective Ca^{2+} concentrations to the control untransformed derivatives.

Histamine addition at the range of 0.0005–2 mM caused a weak enhancement of AtoSC-expressing *E. coli* motility, which was concentration-independent and remained standard under this histamine range. The maximal increases were obtained at 5 mM HI (Fig. 1b). However, the increase of histamine concentration to 10 or 20 mM limited the induction effect, producing swarms smaller than the maximal obtained at 5 mM. Yet Δ*atoSC* or the derivatives expressing only AtoS or AtoC were not sufficient to modulate their motility under histamine presence (Fig. 1b).

AtoSC TCS regulates *E. coli* chemotaxis: effects of AtoSC mediators

The *atoSC*$^+$ strain was responsive against the chemoattractants Asp and Ser, producing 11% larger chemotactic swarms compared with the control conditions (Fig. 2a, b). The Δ*atoSC* cells, however, showed that they had lost their chemotactic response, resulting in a phenotype comparable to that obtained in the absence of chemoeffectors (Fig. 2a). The response of those cells was restored by the extrachromosomal introduction of the *atoS* and *atoC* genes in their BW28878/pUC-Az derivatives (12.5% larger concentric patterns than in the absence of chemoeffectors), resulting to 167% chemotactic enhancement compared with their untransformed derivatives (Fig. 2a, b). On the other hand, expression of either AtoS or AtoC in Δ*atoSC* cells was insufficient to activate the response against Asp-Ser, restricting the swarms to the control diameters (data not shown).

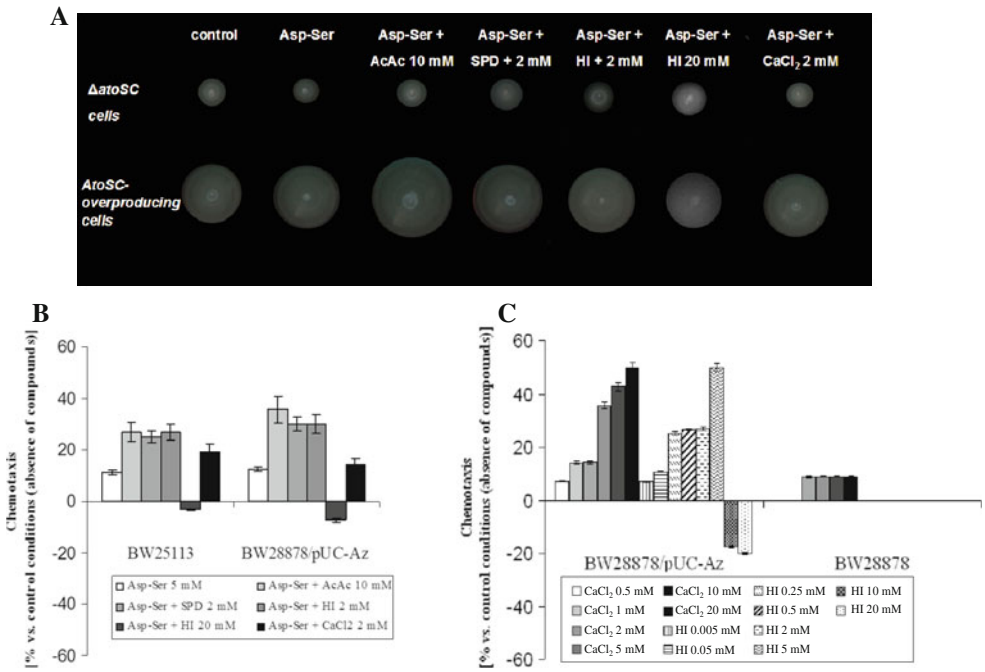


Fig. 2 AtoSC-mediated chemotaxis under Asp and Ser. **a** Swarms produced by BW28878 (Δ*atoSC*) and BW28878/pUC-Az derivatives (AtoSC-overproducing) against the chemoattractants 5 mM Asp and Ser in the absence or presence of the AtoSC mediators: AcAc 10 mM, SPD 2 mM, $CaCl_2$ 2 mM, HI 2 mM or 20 mM. **b** Chemotaxis of BW25113 and BW28878/pUC-Az cells against Asp and Ser, expressed as the % modulation versus the chemotaxis in the absence of any compound. **c** Ca^{2+} or HI effects on AtoSC-mediated chemotaxis towards Asp-Ser. Chemotaxis towards Asp and Ser in the presence of different concentrations of $CaCl_2$ or HI expressed as the % modulation versus the control conditions (absence of inducers)

Table 2 AtoSC-mediated chemotaxis towards glycerol

	BW25113	BW28878/ pUC-Az	Δ*atoS*C cells expressing either AtoS or AtoC
Glyc	6.1 ± 2.0	20.0 ± 2.1	0
Glyc + AcAc 10 mM	11.5 ± 2.2	27.5 ± 2.5	0
Glyc + SPD 2 mM	11.7 ± 2.1	22.5 ± 2.1	0
Glyc + HI 2 mM	28.1 ± 3.5	25.0 ± 3.1	0
Glyc + HI 20 mM	14.3 ± 3.2	25.0 ± 3.4	0
Glyc + $CaCl_2$ 2 mM	0	0	0

Chemotaxis towards 2.5% v/v glycerol in the absence or presence of the AtoSC mediators AcAc 10 mM, SPD 2 mM, $CaCl_2$ 2 mM, histamine (HI) 2 mM or HI 20 mM, expressed as the % modulation versus control conditions (absence of the chemoattractant or any compound)

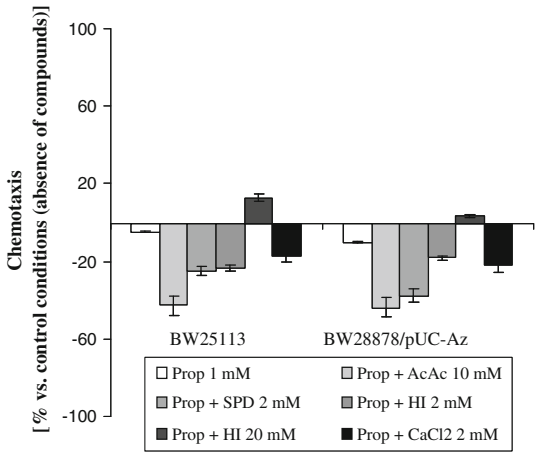


Fig. 3 AtoSC-mediated chemotaxis under propionate. Chemotaxis of BW25113 and BW28878/pUC-Az (AtoSC-overproducing) cells against the chemorepellent propionate (1 mM) in the absence or presence of the AtoSC mediators: AcAc 10 mM, SPD 2, $CaCl_2$ 2 mM, HI 2 mM or 20 mM, expressed as the % modulation versus the chemotaxis in the absence of any compound

When glycerol was used as chemoattractant at the best final concentration of 2.5% v/v, a substantial enhancement of the swimming phenotype was observed, 6% in *atoSC*$^+$ *E. coli* and 20% in the BW28878/pUC-Az derivatives expressing the AtoSC TCS, compared with the absence of any attractant (Table 2). Yet, the rings formed by the mutants containing neither or either AtoSC component were comparable to those under control conditions of motility in the absence of any attractant (Table 2).

In the case of the repellent, among the various concentrations tested (data not shown), 1 mM propionate optimally induced the chemorepellent phenotype, triggering approximately 5% inhibition of the swarm's extent in *atoSC*$^+$ *E. coli* (Fig. 3) and 10% in the extrachromosomally AtoSC-provided Δ*atoSC* strain (BW28878/pUC-Az derivatives) compared with its absence (Fig. 3; Supplement 1). Similar to the case of Asp and Ser, Δ*atoSC* bacteria (Supplement 1) and AtoS- or AtoC-expressing cells remained unresponsive in the presence of propionate (data not shown).

Effect of acetoacetate on AtoSC TCS-mediated chemotaxis

Acetoacetate, at 10 mM, further enhanced the chemotactic response of AtoSC-expressing cells under Asp-Ser, producing 27% larger swarms of the wild-type *atoSC*$^+$ cells and 36% of cells overproducing the AtoSC TCS compared with the absence of the chemoattractants (Fig. 2a, b). However, a slighter acetoacetate-mediated chemotactic induction was observed under glycerol, where only 27.5% larger swarms of AtoSC-overproducing cells were observed (Table 2). Furthermore, it enhanced the repellent effect of propionate, resulting in 44% smaller ring patterns in AtoSC-overproducing cells (Fig. 3; Supplement 1). On the contrary, acetoacetate failed to induce the response of Δ*atoSC* (Fig. 2a, b; Table 2; Supplement 1), or of cells expressing either AtoS or AtoC (data not shown) providing further indication for the requirement of the complete AtoSC TCS to modulate *E. coli* chemotaxis.

Effect of spermidine on AtoSC TCS-mediated chemotaxis

Addition of 2 mM spermidine enhanced 30% the response of *E. coli* cells overproducing AtoSC, against Asp-Ser (Fig. 2), yet retaining smaller rings compared to the acetoacetate effect. Against glycerol the polyamine triggered chemotaxis at approximately 23% enlargement of the swarm diameters (Table 2). Spermidine significantly reduced (approximately 25% of the *atoSC*$^+$ parent strain and 38% of the AtoSC-overproducing derivatives) the diameter of the rings against propionate (Fig. 3; Supplement 1). For all substrates tested, spermidine failed to induce any alteration in the chemotactic behavior of Δ*atoSC* derivatives or of cells expressing either the AtoS or the AtoC component, compared with the absence of the polyamine (Fig. 2; Table 2; Supplement 1).

Effect of Ca^{2+} on AtoSC TCS-mediated chemotaxis

Addition of $CaCl_2$ at 0.5–20 mM resulted in a dose-related enhancement of the chemotactic phenotype (Fig. 2c). $CaCl_2$ at 2 mM enhanced 14% *E. coli* chemotaxis in cells overproducing the AtoSC TCS under Asp-Ser (Fig. 2) and 22% the repellent motility to propionate (Fig. 3; Supplement 1), whereas it had no effect against glycerol (Table 2). Moreover, in the presence of Asp and Ser, Ca^{2+}

slightly increased (approximately 9%) the motility of the Δ*atoSC* BW28878 bacteria (Fig. 2).

Effect of histamine on AtoSC TCS-mediated chemotaxis

When histamine was added at concentrations between 0.005 and 5 mM it further enhanced the effects of the chemoagents tested, i.e. enhanced the ring patterns under Asp-Ser (Fig. 2) and against glycerol (Table 2), while it reduced the diameters in the presence of propionate (Fig. 3; Supplement 1). Interestingly, when added at concentrations of 10 and 20 mM, histamine reversed the swimming phenotypes. It triggered a chemorepellent effect to Asp-Ser, resulting in approximately 20% reduction of the ring diameters at 20 mM compared with the absence of histamine (Fig. 2), while producing a chemoattractant phenotype under propionate (Fig. 3; Supplement 1) (14% larger swarms than in its absence). However, histamine could not reverse the attractant effect of glycerol (Table 2).

Effect of mutations in the AtoC phosphorylation sites on chemotaxis

The motility and chemotactic response to Asp-Ser of *E. coli* BW28878 derivatives bearing mutations in the AtoC phosphorylation sites: BW28878/pEM-Az-D55G (AtoC-D55G), BW28878/pEM-Az-H73L (AtoC-H73L) or BW28878/pEM-Az-D55G/H73L (AtoC-D55G-H73L) were next studied in the absence or in the presence of only Asp-Ser or together with the AtoSC inducers. Mutations in either one or both the AtoC D55 and H73 phosphorylation sites significantly reduced *E. coli* motility (Supplement 2). More potent reduction was exhibited by the single mutants compared with the double mutant form, yet the H73 mutation resulted in the most intense reduction (approximately 45%) of the motility compared with the cells overproducing the wild-type AtoSC (BW28878/pEMAz cells) (Supplement 2). In contrast, the D55 mutation reduced motility to approximately 26%. However, cells expressing the D55G/H73L double AtoC mutant form demonstrated enhanced motility compared with the single mutants, yet remaining 16% reduced compared with the wild-type AtoC. Under steady-state conditions, the mutations produced an almost non-chemotactic phenotype toward both the chemoattractants Asp-Ser (Supplement 2) and glycerol or the repellent propionate (data not shown).

Interestingly, the agents that physiologically affect the AtoSC TCS failed to promote the chemotactic phenotype in both single and double AtoC phosphorylation site mutants towards the chemoattractants or chemorepellent tested (Supplement 2, for Asp-Ser). An exception was observed in the case of Ca^{2+} that succeeded in enhancing approximately 22% of the mutants' chemotaxis under Asp-Ser, albeit significantly reduced compared with the wild-type *E. coli* strain (Supplement 2).

trAtoC regulates *E. coli* motility

BW25113 and BW28878 cells transformed with plasmid ptratoC, to express the constitutive active truncated form of AtoC, lacking its 139 N-terminal amino acids, were used to further study the mechanism of AtoSC-mediated regulation of *E. coli* motility.

trAtoC enhanced the motility of cells of both genotypes (Fig. 4a). However, the motility of BW28878/ptratoC cells was enhanced compared with their pUC-Az-transformed derivatives, which express the whole AtoSC TCS. In contrast, BW25113/ptratoC cells demonstrated weaker motility than their pUC-Az-transformed derivatives as well as the BW28878/ptratoC counterparts (Fig. 4a). trAtoC enhanced also the cellular response against the chemoagents used in the absence of the AtoSC TCS inducers (Fig. 4a). The presence of acetoacetate or spermidine up-regulated the trAtoC-mediated chemotaxis of cells of both genotypes, resulting in the observation of bigger patterns in the case of Asp-Ser or glycerol or smaller rings against propionate (Fig. 4b). However, the presence of histamine did not affect the chemotactic response of the trAtoC-expressing cells of either genotype (Fig. 4b) in contrast to the AtoSC-expressing derivatives, as observed earlier (Figs. 2, 3; Table 2).

AtoSC regulates positively the transcription of the *flhDC*, *fliAZY* and *flgBCDEFGHIJKL* operons

To study the possible mechanism of the AtoSC involvement in the regulation of the motility and chemotaxis processes, the transcriptional regulation of the early operon *flhDC* and the middle *fliAZY* and *flgBCDEFGHIJKL* operons by the AtoSC TCS was studied.

The transcription of the *flhDC* operon was enhanced in $atoSC^{+}$ cells (BW25113/pFCZ6) compared with their Δ*atoSC* derivatives (Fig. 5a). The acetoacetate-mediated induction of AtoSC up-regulated the *flhDC* transcriptional activation in $atoSC^{+}$ cells, while acetoacetate had no effect in Δ*atoSC* strains. The expression of *flhDC* operon was enhanced when Δ*atoSC* cells were also transformed with plasmid pAtoSC, resulting in the extrachromosomal expression of the AtoSC TCS (Fig. 5a). The in *trans* expression of only the AtoC response regulator in Δ*atoSC* bacteria (BW28878/pUCAtoC-pFCZ6) did not trigger any effect on the *flhDC* transcription.

The AtoSC TCS regulated positively also the transcriptional activation of the *fliAZY* (Fig. 5b) and

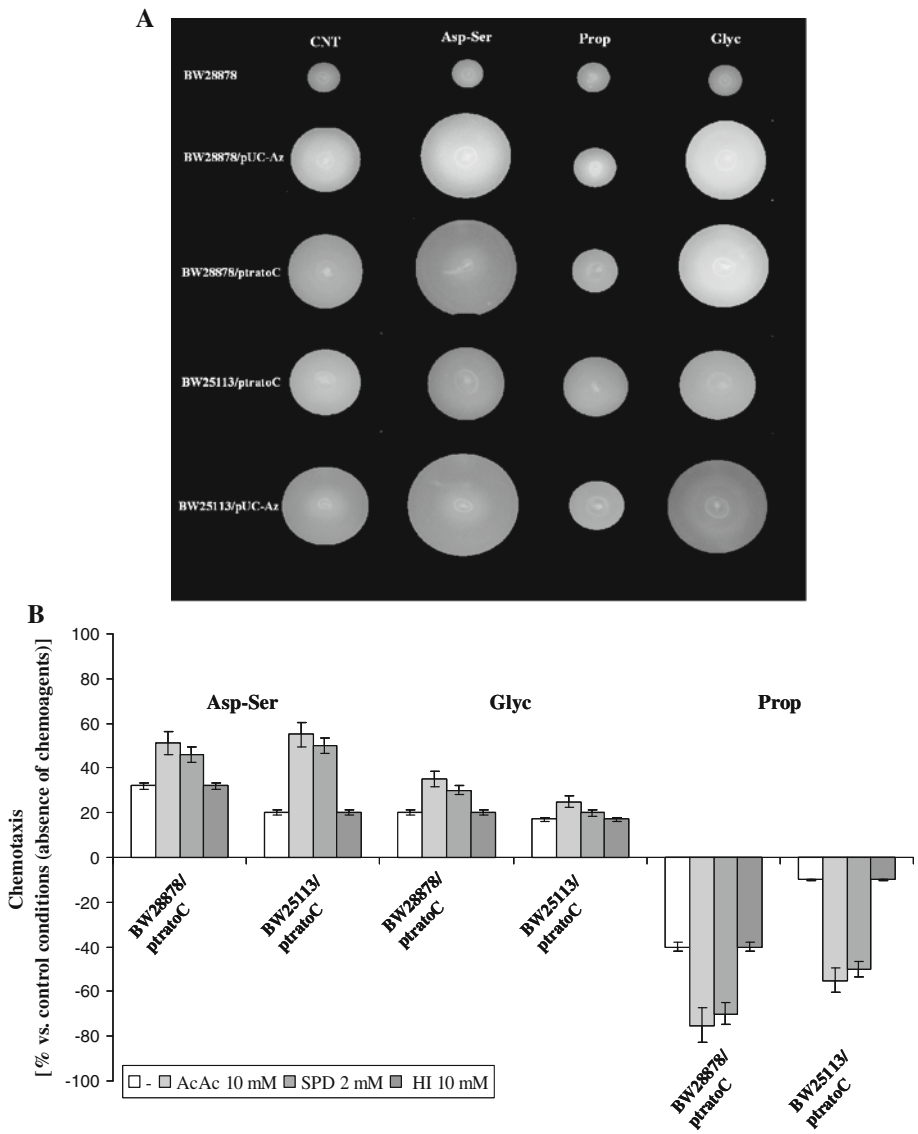


Fig. 4 Regulation of *E. coli* motility and chemotaxis by trAtoC. **a** Swarms produced by BW28878 (Δ*atoSC*) and BW25113 (*atoSC*$^+$) cells and their transformed derivatives with plasmids ptratoC or pUC-Az in the absence or presence of 5 mM Asp-Ser, 1 mM propionate or 2.5% v/v glycerol. **b** Effects of the AtoSC inducers on the trAtoC-mediated *E. coli* chemotaxis. Chemotaxis of BW28878 (Δ*atoSC*) and BW25113 (*atoSC*$^+$) cells transformed with plasmid ptratoC against 5 mM Asp-Ser, 1 mM Prop or 2.5% v/v Glyc in the absence or the presence of 10 mM AcAc, 2 mM SPD or 10 mM HI. The chemotaxis modulation is expressed as the % modulation versus the chemotaxis in the absence of any compound

flgBCDEFGHIJKL operons (Fig. 5c). However, AtoSC was more effective in the transcriptional activation of the *fliAZY* operon (Fig. 5b). The induction of AtoSC by acetoacetate enhanced the transcription of both the operons in cells expressing the AtoSC endo- or exo-chromosomally (pUCAz-transformed derivatives), while acetoacetate had no effect on the Δ*atoSC* strains. To study the effects of AtoS or AtoC separately in the transcriptional activation of the two operons, both strains YK4337 and IMW353 were transformed with pUCAtoS or pUCAtoC, to overproduce the two proteins, respectively. The overproduction of the AtoS or AtoC constituents separately triggered almost no effect on the operon expression, resulting in equal levels of expression of the two operons as in the control untransformed cells, in the absence or even in the presence of acetoacetate (Fig. 5b, c).

AtoSC cross-regulates *E. coli* chemotaxis with the Che system

To study the possibility that AtoSC TCS cooperates with CheAY, the established TCS for chemotaxis regulation in *E. coli*, we used isogenic pair of the wild-type RP437 (*che*$^+$) strain and the RP1091 (Δ*cheA-cheZ*) derivative. Both strains were also transformed with pUC-Az to overproduce the AtoSC TCS. The *che*$^+$ wild-type strain was responsive against the chemoattractants Asp-Ser or glycerol, as well as the chemorepellent propionate. AtoSC overproduction in the RP437/pUC-Az cells resulted in enhancement of the ring patterns of all the agents (Fig. 6). However, the Δ(*cheA-cheZ*) cells had lost their motility and chemotactic response against all agents. The extrachromosomal introduction of the *atoS* and *atoC* genes in their

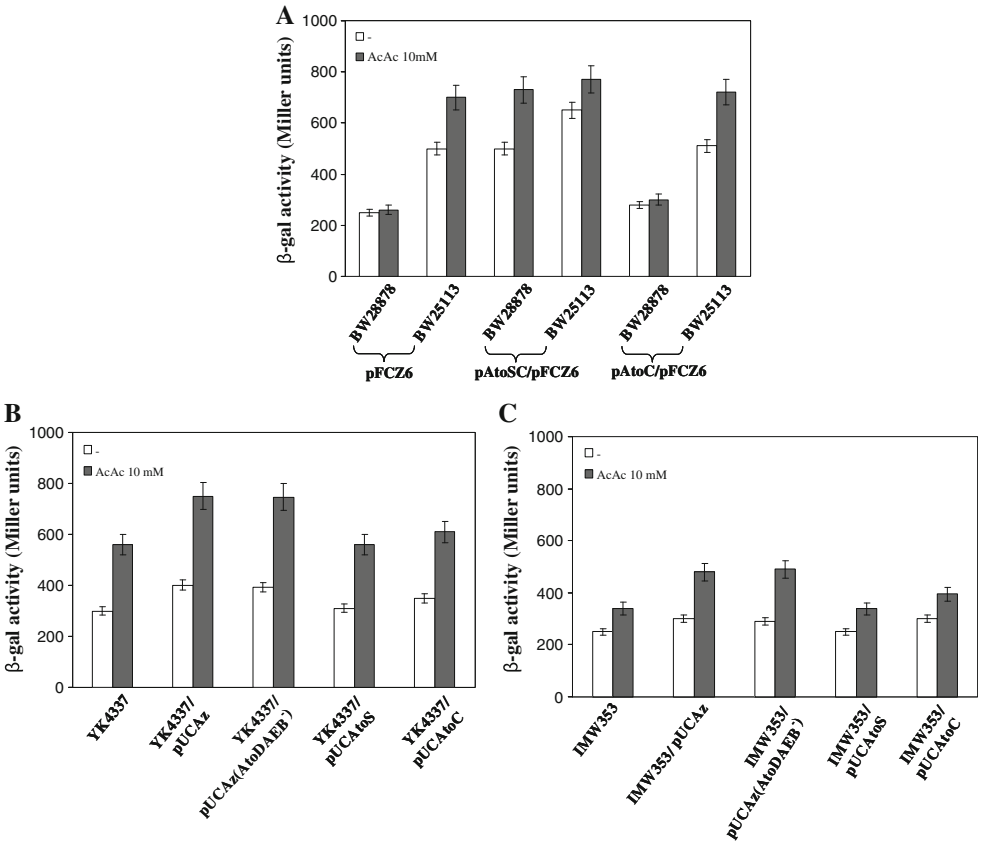


Fig. 5 Effects of the AtoSC TCS on *flhDC*, *fliAZY* and *flgBCDEFGHIJKL* operons' transcription. **a** BW25113 (*atoSC*$^{+}$) and BW28878 (Δ*atoSC*) bacteria transformed with plasmids pFCZ6 (*flhDC-lacZ*) alone or together with pAtoSC or pAtoC, as well as strains. **b** YK4337 (*fliA′-′lacZ*) and **c** IMW353 (*flgB′-′lacZ*) transformed with plasmids pUC-Az, pUCAz(AtoDAEB^{-}), pUC-AtoS, or pUC-AtoC were grown in the absence or presence of AcAc 10 mM. *β*-Galactosidase activity is expressed in Miller units. The values represent the average of two independent experiments. The error for each data point is <6%

RP1091/pUC-Az derivatives was insufficient to activate the motility or the response against the chemogents, in the absence of Che regulon (Fig. 6).

Discussion

The regulation of *E. coli* chemotactic response can be affected by multiple cross-regulatory mechanisms in response to environmental cues (Oshima et al. 2002; Chilcott and Hughes 2000). The participation of the AtoSC TCS and the flagellar regulon in many studies concerning the interplay between network structures and sensing mechanisms in *E. coli* as well as the implication that *E. coli* possesses an additional signal transduction system other than the flagellar regulon for the accomplishment of the chemotactic response (Eisenbach 2007) led this investigation on the involvement of AtoSC TCS in *E. coli* chemotaxis.

The deletion of the *atoSC* genetic locus from *E. coli* genome resulted in motionless as well as non-chemotactic bacteria independently to the attractant or repellent used. Analogous observations have been reported in previous studies (Oshima et al. 2002). The complementation of the Δ*atoSC* phenotype by the extrachromosomal introduction of the whole AtoSC pointed to the requirement of both TCS proteins for chemotaxis regulation. Additionally,

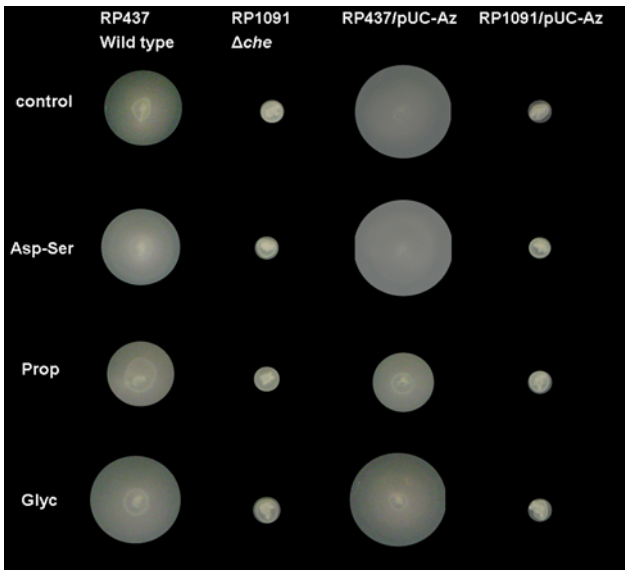


Fig. 6 AtoSC TCS cross-regulates chemotaxis with the Che regulon. Swarms produced by the RP437 (*che*$^{+}$) or RP1091 (Δ*cheA-cheZ*) strains and their pUC-Az-transformed derivatives (AtoSC-overproducing) in the absence (control) or presence of either the chemoattractants 5 mM Asp-Ser or glycerol 2.5%, or the chemorepellent propionate 1 mM

these data propose that only limited, if any, cross-talk takes place between the AtoSC components and other TCSs. Such cross-talk between the non-cognate UhpB HK and

AtoC has been implied in in vitro studies (Yamamoto et al. 2005). 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.

AtoSC-expressing *E. coli* were motile and chemotactic in the absence of any AtoSC inducer, signifying the requirement of AtoSC TCS for motile and chemotactic *E. coli* under all conditions and also indicating that multiple interactions may take place between the TCS constituents and the flagella regulon. Furthermore, AtoSC induction by acetoacetate or spermidine further enhanced the motility and chemotaxis of the cells. The diverse effects exerted by the polyamines on the expression of amongst other *E. coli* genes on genes involved in flagellar synthesis, sometimes described as the polyamine modulon (Yoshida et al. 2004), seem to be directed by the AtoSC TCS.

However, the incapability of AtoSC to regulate those phenotypes of Δ*che* strain reinforces the occurrence of cross-regulation mechanisms between the AtoSC TCS and the Che system towards the accomplishment of the chemotactic response, yet further studies are required for further elucidation of the exact mechanisms and interactions. The aforementioned data establish the participation of the signal transduction mechanism through AtoSC in this manifold adaptation. Previous transcriptome analysis has revealed the participation of other TCSs as well as in the positive or negative regulation of *E. coli* motility (Oshima et al. 2002).

Ca^{2+} has been documented to be involved in *E. coli* chemotaxis (Tisa and Adler 1992, 1995; Watkins et al. 1995; Tisa et al. 2000), but the possible mechanism still remains to be further determined (Tisa and Adler 1992). The Ca^{2+}-induced regulation of the chemotaxis of *atoSC*$^{+}$ cells was more pronounced than their Δ*atoSC* derivatives, suggesting that AtoSC signal transduction could be a means of Ca^{2+} participation in bacterial motility and adaptation. Concerning our studies about the involvement of the external Ca^{2+} on cPHB biosynthesis regulated by AtoSC (Theodorou et al. 2009), the cation-induced chemotactic response points to different or supplementary mechanisms underlying the interplay of Ca^{2+} with AtoSC signaling towards its downstream targets.

The observation that mutations altering the putative phosphate-acceptor sites in AtoC also diminish, or abrogate, the motility and chemotaxis of the cells even in the presence of the inducers or the other mediators of the AtoSC output imply that AtoC phosphorylation is required in this process. However, the relative effects of the single mutations on chemotaxis are inversed as to their effects on *atoDAEB* operon expression (Lioliou et al. 2005) and analogous to those on cPHB biosynthesis regulation by AtoSC under acetoacetate (Theodorou et al. 2006). The enhanced motility of the double AtoC mutant suggests that multiple regulatory interactions possibly take place between the constituents of the AtoSC TCS towards chemotaxis regulation.

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, the N-terminal deletion mutant AtoC (trAtoC), which preserves its in vitro activities without phosphorylation requirement, was used (Grigoroudis et al. 2007). The enhancement of the motility and response against chemoagents of trAtoC-expressing *E. coli* cells in the absence of inducers verified the in vivo effects of this constitutive active AtoC form, possibly as an outcome of its preserving ATPase and DNA-binding activities. Analogous activities of this constitutive AtoC form have been recently revealed for the other two established AtoSC targets, namely the *atoDAEB* operon and cPHB biosynthesis (Theodorou et al. 2011c). The presence of acetoacetate or spermidine corroborated the trAtoC-regulated motility and chemotactic response implying a possible additional role of these inducers on AtoC RR, a fact that has also been clarified in the case on *atoDAEB* and cPHB regulation (Theodorou et al. 2011c). However, a different mechanism of regulation seems to take place in the case of histamine, which triggers no effect in the trAtoC-mediated motility regulation. In contrast, histamine is able to up-regulate the other two established targets under trAtoC-mediated regulation (Theodorou et al. 2011c), suggesting that histamine acts strictly on the N-terminal AtoC receiver domain for the motility regulation while is able to act also through additional mechanisms on the central domain of AtoC for *atoDAEB* and cPHB regulation by AtoSC. The aforementioned data point to different modes of the AtoSC TCS induction by its signals towards the regulation of the downstream processes.

The enhancement of the transcriptional activation of the early *flhDC* operon in AtoSC-expressing cells, and most potently in the presence of acetoacetate, verify the involvement of the AtoSC TCS in the up-regulation of the flagellar regulon at the transcriptional level. Concerning the fact that FlhD and FlhC proteins form a heteromultimeric complex that directs the σ^{70}-dependent transcription of the middle operons (Chilcott and Hughes 2000), the AtoSC TCS acts a global regulator of the flagellar regulon of *E. coli*. Furthermore, AtoSC is involved in the transcriptional activation of some of the middle operons, the *flgBCDEFGHIJKL* operon but more effectively of the *fliAZY* operon, encoding the factor σ^{28} required for the transcription of the late genes including flagellar and chemotactic operons (Chilcott and Hughes 2000). AtoC RR belongs in the σ^{54}-RNA polymerase activators family (Kyriakidis and Tiligada 2009). A recent genome-wide analysis has revealed additional putative AtoC-binding

sites except its established *atoDAEB* promoter within *E. coli* genome, including amongst others the *fliA* promoter (Pilalis et al. 2011). The in vivo AtoC-binding was verified for 22 of those targets, located not only within intergenic promoters regions, but also within gene-encoding sequences, including the *fliA* promoter (Pilalis et al. 2011). Recent studies have revealed that the flhDC operon can be expressed either by the established σ^{70}-dependent transcription or by a newly identified σ^{54}-dependent transcription (Zhao et al. 2010). However, the aforementioned analysis has not predicted AtoC-binding sites in the *flhDC* or *flgB* promoter regions. Accordingly, a possible mechanism of their up-regulation by AtoSC could imply that AtoC regulates other required transcriptional factors or that a multicomponent complex including AtoC is required for transcriptional activation of those promoters.

Studies posit the involvement of the AtoSC TCS in the expression of a number of *E. coli* genes other than those of the *atoDAEB* operon. Indeed, the deletion of the *E. coli atoSC* locus results in the up-regulation of 11 genes and the down regulation of 32 genes (Oshima et al. 2002), suggesting both positive and negative roles for the AtoSC TCS. The intriguing issue that different yet unknown systems form regulatory networks (Chilcott and Hughes 2000; Oshima et al. 2002), as well as the implication that *E. coli* possesses an additional signal transduction system other than the Che regulon for the accomplishment of the chemotactic response (Eisenbach 2007), agrees with our findings that the AtoSC is another TCS that is involved in that response. It is clear that AtoSC is more than a regulator of the genes that participate in short-chain fatty acid catabolism but is a key mediator in the regulation of bacterial processes such as chemotaxis and motility, cPHB biosynthesis (Theodorou et al. 2006, 2007, 2009, 2011b, c; Kyriakidis et al. 2008) and processes that influence the sensitivity to aminoglycoside antibiotics (Oshima et al. 2002), establishing its homeostatic role in bacterial physiology and possibly pathophysiology or bacteria–host communication.

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Conflict of interest None to declare.

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