RESEARCH LETTER

Characterization of the Arc two-component signal transduction system of the capnophilic rumen bacterium *Mannheimia succiniciproducens*

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Received 7 March 2008; accepted 29 March 2008.
First published online 6 May 2008.

DOI:10.1111/j.1574-6968.2008.01187.x

Editor: Dieter Jahn

Keywords
two-component signal transduction system; ArcB sensor kinase; ArcA response regulator; *Mannheimia succiniciproducens*.

Abstract

The ArcB/A two-component signal transduction system of *Escherichia coli* modulates the expression of numerous operons in response to redox conditions of growth. We demonstrate that the putative *arcA* and *arcB* genes of *Mannheimia succiniciproducens* MBEL55E, a capnophilic (CO_2-loving) rumen bacterium, encode functional proteins that specify a two-component system. The Arc proteins of the two bacterial species sufficiently resemble each other that they can participate in heterologous transphosphorylation *in vitro*, and the *arcA* and *arcB* genes of *M. succiniciproducens* confer toluidine blue resistance to *E. coli arcA* and *arcB* mutants. However, neither the quinone analogs (ubiquinone 0 and menadione) nor the cytosolic effectors (D-lactate, acetate, and pyruvate) affect the net phosphorylation of *M. succiniciproducens* ArcB. Our results indicate that different types of signaling molecules and distinct modes of kinase regulation are used by the ArcB proteins of *E. coli* and *M. succiniciproducens*.

Introduction

Mannheimia succiniciproducens is a facultative anaerobic, capnophilic Gram-negative bacterium isolated from bovine rumen (Lee *et al.*, 2002). Under anaerobic growth conditions, *M. succiniciproducens* is able to produce a large amount of succinic acid, which has various industrial applications for manufacture of diverse substances such as biodegradable polymers, synthetic resins and chemical intermediates, and additives (McKinlay *et al.*, 2007). *Mannheimia succiniciproduce* was first isolated in 2002, and since then intensive studies have been performed to optimize its succinic acid production using genome sequence analysis, metabolic flux analysis, and metabolic engineering techniques (Hong *et al.*, 2004; Lee *et al.*, 2006; Kim *et al.*, 2007; Song *et al.*, 2007). However, very little is known about its genetic regulatory mechanisms that regulate the genome-wide expression depending on the growth conditions such as aerobic or anaerobic conditions.

According to the genome sequence of *M. succiniciproducens* (Hong *et al.*, 2004), the ArcB/A system is one of five putative two-component signal transduction systems present in this organism. This system was first reported in *Escherichia coli* (Iuchi *et al.*, 1990; Iuchi & Lin, 1992a; Malpica *et al.*, 2006). It comprises ArcB as the membrane-bound sensor kinase and ArcA as the cognate response regulator. ArcB is a tripartite protein having, in addition to a primary transmitter domain, a receiver domain and a secondary transmitter or a phospho-transfer domain (Iuchi *et al.*, 1990; Iuchi, 1993; Ishige *et al.*, 1994; Kwon *et al.*, 2003). ArcA is a typical response regulator having an N-terminal receiver domain and a C-terminal helix–turn–helix

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DNA-binding domain (Iuchi & Lin, 1988). Under anoxic growth conditions, the membrane-bound ArcB sensor kinase undergoes autophosphorylation, a process shown to be enhanced by certain fermentative metabolites, such as D-lactate, pyruvate, and acetate (Georgellis *et al.*, 1997, 1999; Kwon *et al.*, 2000a; Rodriguez *et al.*, 2004). The phosphoryl group is then transferred to the response regulator ArcA via a His → Asp → His → Asp phosphorelay, involving the His292, Asp576, and His717 of ArcB and Asp54 of ArcA (Georgellis *et al.*, 1997; Kwon *et al.*, 2000a, b). Phosphorylated ArcA (ArcA-P), in turn, modulates the expression of some 300 operons depending on the redox conditions of growth (Lynch & Lin, 1996; McGuire *et al.*, 1999; Oshima *et al.*, 2002; Liu & De Wulf, 2004; Salmon *et al.*, 2005). Under nonstimulating conditions, ArcB catalyzes the dephosphorylation of ArcA-P via an Asp → His → Asp → Pi reverse phosphorelay, involving the Asp54 of ArcA and the His717 and Asp576 of ArcB (Georgellis *et al.*, 1998; Pena-Sandoval *et al.*, 2005). During aerobiosis, the quinone electron carriers have been shown to act as the primary signals that inhibit the kinase activity of ArcB (Georgellis *et al.*, 2001a) through the oxidation of two redox-active cysteinyl residues that participate in intermolecular disulfide bond formation (Malpica *et al.*, 2004). Interestingly, these two cysteinyl residues (Cys180 and Cys241) are located in the Per-Amt-Sim (PAS) domain of the cytosolic linker region of ArcB (Taylor & Zhulin, 1999).

In this study, we report the characterization of the Arc proteins of *M. succiniciproducens* MBEL55E. Our results indicate that different types of signaling molecules and distinct molecular mechanisms are involved in regulating the signaling abilities of the *E. coli* and *M. succiniciproducens* ArcB proteins.

Materials and methods

Bacterial strains, plasmids, and growth conditions

Escherichia coli strains EOK101 ($\Delta arcA::Kan^R$) and EOK102 ($\Delta arcB::Tet^R$ $\Delta arcA::Kan^R$) were constructed by P1 transduction of a $\Delta arcA::Kan^R$ allele of strain PC35 (Cotter & Gunsalus, 1992) into strain ECL5002 (wild type) and ECL5012 ($\Delta arcB::Tet^R$) (Kwon *et al.*, 2000b), respectively. For routine growth of *E. coli* strains, L agar and L broth were used. The cultures were stored in glycerated-L broth at $-80\,^{\circ}C$. Plasmids pUC18 and pEXT20 (Dykxhoorn *et al.*, 1996) were used for cloning the *arc* genes of *M. succiniciproducens* for complementation tests. The vector pET28a+ (Novagen) was used to construct expression vectors for the Arc proteins of *E. coli* and *M. succiniciproducens*. Ampicillin, kanamycin, tetracycline, and chloramphenicol were used at concentrations of 100, 15, 40, and 34 $\mu g\,mL^{-1}$, respectively. *Mannheimia succiniciproducens* MBEL55E (Hong *et al.*, 2004) strain was grown at 39 °C in tryptic soy broth.

Recombinant DNA techniques and PCR

Chromosomal and plasmid DNA were isolated, respectively, using the AccuPrep genomic DNA extraction kit and the AccuPrep Plasmid extraction kit (Bioneer, Daejeon, Korea). DNA fragments were recovered from agarose gels using the AccuPrep gel purification kit (Bioneer). The oligonucleotides used in this study were synthesized by Bioneer. Sequence verification of PCR-amplified DNA fragments was performed by the SolGent Co. Ltd (Daejeon, Korea).

Cloning of the *M. succiniciproducens arcA* and *arcB* genes

The *arcA* and *arcB* genes of *M. succiniciproducens* were PCR amplified using chromosomal DNA of *M. succiniciproducens* as a template and primer pairs MsArcA-N (5′-GTGCTGCAGAACGATCTGATCGTAGGC-3′) – MsArcA-C (5′-GTGCTGCAGGGAAGAAATGAATCCTCC-3′) and MsArcB-N (5′-GTGCTGCAGCTGTTATGCCGATGGTAG-3′) – MsArcB-C (5′-GTGCTGCAGTATTGTTGCGGCATCAGC-3′), respectively. The purified 1.3-Kb PCR product containing the *MsarcA* gene was digested with PstI and ligated with PstI-digested pUC18, yielding pMSarcA. The *MsarcA* gene of pMSarcA was isolated by HindIII and XbaI digestion and cloned into corresponding sites of pEXT20, yielding pMSTarcA. The 2.4-kb PCR product containing the *MsarcB* gene was first cloned into pGEMT-easy, yielding pGTarcB. The 2.4-kb fragment containing the *MsarcB* gene was subsequently isolated by EcoRI digestion of pGTarcB and cloned into EcoRI-digested pUC18 and pMSarcA, yielding pMSarcB and pMSarcAB, respectively. The *MsarcB* gene of pMSarcB was isolated by PstI and XbaI digestion and cloned into corresponding sites of pEXT20, yielding pMSTarcB. The *MsarcA* gene of pMSarcA was isolated by PstI digestion and ligated with PstI-digested pMSTarcB, yielding pMSTarcAB.

Construction of vectors expressing MsArcA and ′MsArcB proteins

To construct the plasmid expressing the His_6-tagged version of MsArcA, chromosomal DNA of *M. succiniciproducens* was used as a template in PCR reactions with primer pairs, MsH6AA-N (5′-CACCATATGCTTTCTCCACAAATT-3′) – MsH6AA-C (5′-CACAAGCTTCTGCAGGGAGAGAAATGAATCC-3′). The 0.8-kb PCR product was digested with NdeI and HindIII and ligated with NdeI–HindIII-digested pET28a+(Novagen), yielding pMSHarcA.

To construct the plasmid expressing the His_6-tagged versions of MsArcB (hereafter referred to as ′MsArcB), which has a deletion of the transmembrane segment

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encompassing amino acid residues 1–77, chromosomal DNA of *M. succiniciproducens* was used as a template in PCR reactions with primer pairs, MsH6ABcyt-N (5′-CAC CATATGGAGCGTTTGGAGCTGTCC-3′) – MsArcB-C (5′-GTGCTGCAGTATTGTTGCGGCATCAGC-3′). The 1.8-kb PCR product was digested with NdeI and PstI and ligated with NdeI–PstI-digested pMSHarcA, yielding pMSHarcB^{cyt}.

Purification of His$_6$-tagged proteins

Escherichia coli BL21 (DE3) cells transformed with pMSHarcA or pMSHarcB^{cyt} were grown at 37 °C in 1 L of Luria–Bertani (LB) broth supplemented with kanamycin (40 μg mL^{-1}). The expression of the His$_6$-tagged proteins was induced at the mid-exponential phase (OD$_{600\,nm}$ of *c.* 0.7) by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside and the cultures were harvested 4 h after induction. The cells synthesizing ′MsArcB were incubated for 16 h at 20 °C after induction to avoid inclusion body formation. Protein purification was performed as described previously (Georgellis *et al.*, 1997).

Phosphorylation and transphosphorylation assays

Phosphorylation assays were carried out at room temperature in the presence of 40 μM [γ-^{32}P]ATP (specific activity, 2 Ci mmol^{-1}; New England Nuclear), 33 mM HEPES (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol. Where indicated, purified ′MsArcB and MsArcA peptides were used at 50 and 100 pmol, respectively. The reactions were initiated by the addition of [γ-^{32}P]ATP and terminated by the addition of an equal volume of 2 × sodium dodecyl sulfate (SDS) sample buffer, and the reaction products were immediately subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 12% polyacrylamide gels. The gels were dried and exposed to an X-OMAR AR (Kodak) film, and the radioactivity of the proteins resolved in the gels was quantified by the SCION IMAGE Program (Scion Co.). To determine the effects of quinone electron carriers, an autophosphorylation reaction of purified ′MsArcB was performed with ubiquinone-0 (250 μM) or menadione (1 mM) in the absence or in the presence of dithionite (500 μM). To determine the effects of metabolites on the kinase activity of ′MsArcB, D-lactate, acetate, pyruvate, fumarate, or succinate were used at 1 mM.

Toluidine blue O sensitivity test

Escherichia coli cells transformed with plasmid harboring *M. succiniciproducens arc* genes were cultured in LB overnight at 37 °C. Toluidine blue sensitivity was tested by streaking of overnight culture broth on a section of TST agar plates (10 g tryptone L^{-1}, 8 g NaCl L^{-1}, 15 g Bacto Agar L^{-1}, 2 mg toluidine blue mL^{-1}) and on a section of TS plates without toluidine blue. Dye sensitivity was scored after overnight incubation at 37 °C.

Results and discussion

Identification of the putative *arc* genes of *M. succiniciproducens*

A close inspection of the complete genome sequence of the capnophilic rumen bacterium *M. succiniciproducens* MBEL55E revealed the putative *arc* genes, *MsarcA* (MS1730) and *MsarcB* (MS1504) (Hong *et al.*, 2004). The *MsarcA* and *MsarcB* genes are placed on distinct locations on the chromosome, as is the case in *E. coli*, and each gene is predicted to possess a monocistronic transcriptional unit. *MsarcA* encodes a protein that shows 73% amino acid sequence identity with the ArcA protein of *E. coli*, whereas the *MsarcB* encodes a protein that shows only 48% amino acid sequence identity with the *E. coli* ArcB. Moreover, the MsArcB lacks the regions corresponding to amino acids 96–116 and 134–280 of *E. coli* ArcB, which lie in the linker region connecting the transmembrane domain to the primary transmitter domain (Fig. 1). Curiously, this region of *E. coli* ArcB contains the two redox-active cysteine residues (Cys180 and Cys241) that have been shown to form intermolecular disulfide bonds, and thereby silencing the kinase activity of ArcB under oxic conditions of growth (Malpica *et al.*, 2004). Thus, the intriguing possibility that signal transduction by *E. coli* ArcB and *M. succiniciproducens* ArcB might be modulated by distinct molecular mechanisms is raised.

In vitro autophosphorylation of ′MsArcB and transphosphorylation of MsArcA

To test the *in vitro* activity of MsArcB and MsArcA, we cloned, overexpressed, and purified them as His$_6$-tagged proteins. The His$_6$-tagged version of ′MsArcB was expressed as an N-terminally truncated form by a deletion of the transmembrane segment encompassing amino acid residues 1–77, in order to facilitate its purification. Removal of the two transmembrane segments has been shown to result in constitutively active kinase proteins that are able to transphosphorylate their cognate response regulators efficiently (Forst *et al.*, 1989; Igo *et al.*, 1989; Iuchi & Lin, 1992b; Kwon *et al.*, 2000b).

The ability of purified ′MsArcB to autophosphorylate and transphosphorylate MsArcA was tested by incubation with [γ-^{32}P]ATP as described previously (Georgellis *et al.*, 1997, 1999). As can be seen in Fig. 2, ′MsArcB was able to autophosphorylate, and to transphosphorylate MsArcA. No phosphorylation of MsArcA was observed in the absence of ′MsArcB (data not shown). This result demonstrates that

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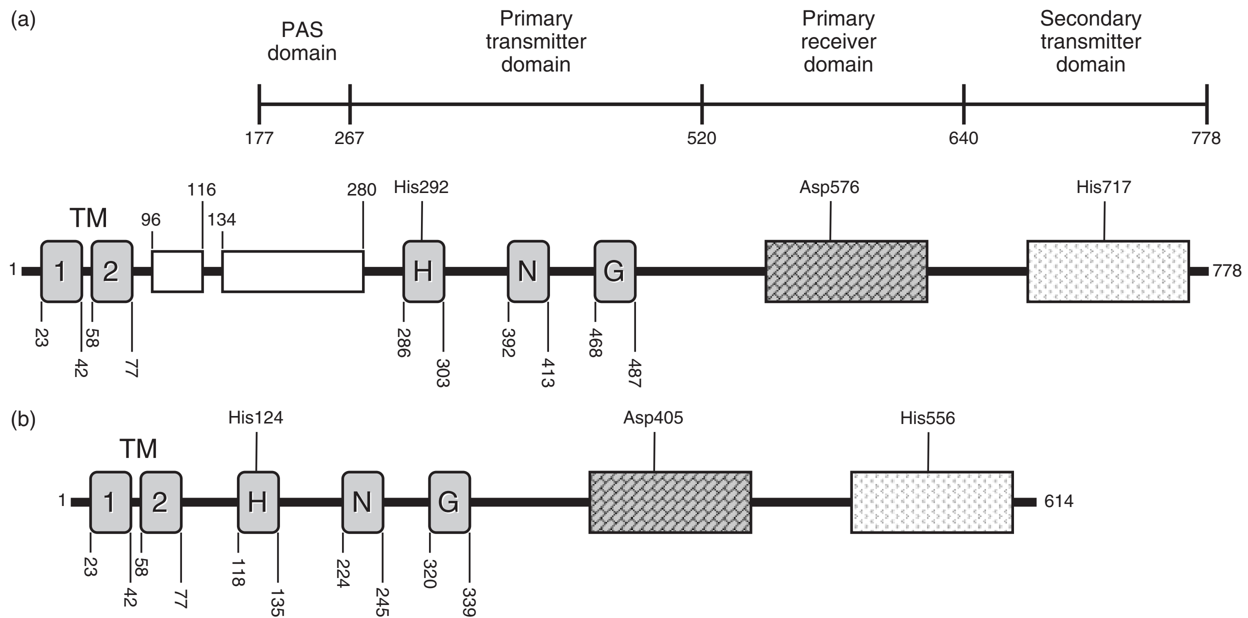

Fig. 1. Schematic representation of the ArcB sensor kinases of *Escherichia coli* and *Mannheimia succiniciproducens*. (a) The *E. coli* protein; (b) the *M. succiniciproducens* protein. The two N-terminal transmembrane segments (TM) were predicted on the basis of a hydrophobicity plot. The *M. succiniciproducens* ArcB lacks the regions corresponding to amino acids 96–116 and 134–280 of *E. coli* ArcB, which contains a PAS domain (residues 177–267) (Matsushika & Mizuno, 2000). The primary transmitter domain is shown with the catalytic determinants H, N, and G (Parkinson, 1995). The His124 of *M. succiniciproducens* ArcB corresponds to the conserved autophosphorylation site His292 of the *E. coli* ArcB. The Asp405 and His556 of *M. succiniciproducens* ArcB correspond to the conserved transphosphorylation sites in the receiver domain and the secondary transmitter domain of *E. coli* ArcB, respectively.

MsArcB and MsArcA are a functional sensor kinase and a response regulator, respectively.

′MsArcB and ′EcArcB catalyze phosphorylation of heterologous ArcA proteins

As mentioned above, the ArcA and ArcB proteins of *M. succiniciproducens* show 73% and 48% of amino acid sequence identity with the corresponding proteins of *E. coli*. Therefore, we examined whether heterologous pairs of ArcB and ArcA from these two organisms could catalyze transphosphorylation reactions *in vitro*. To this end, we overexpressed and purified His_6-tagged EcArcA and a His_6-tagged version of EcArcB (hereafter referred to as ′EcArcB) deprived of the transmembrane segment corresponding to amino acid residues 1–77 (Georgellis *et al*., 1997).

Incubation of ′EcArcB with MsArcA and ′MsArcB with EcArcA in the presence of [γ-^{32}P]ATP revealed that both the ArcB kinases were able to efficiently transphosphorylate the ArcA proteins from the heterologous species (Fig. 2). These results clearly demonstrate that despite the limited amino acid sequence identity between the ArcB proteins of *M. succiniciproducens* and *E. coli*, the two kinases share the same substrate specificity in the transphosphorylation reaction. However, it has to be mentioned that the two ArcB proteins might have different specific activities because autophosphorylation and subsequent transphosphorylation of MsArcA by ′MsArcB were less effective than by ′EcArcB (Fig. 2). Moreover, ′EcArcB seems to be more efficient in autophosphorylation compared with ′MsArcB, and MsArcA appears to be a better substrate than EcArcA for transphosphorylation by both ′EcArcB and ′MsArcB. However, it is not clear whether these observations are specific for the *in vitro* reactions or whether they reflect the structural and functional differences between the Arc systems of *M. succiniciproducens* and *E. coli*.

Functional complementation of *E. coli arc* mutants by the corresponding *M. succiniciproducens arc* genes

A distinguishable phenotype of mutations in *arcA* or *arcB* genes in *E. coli* is the growth defect on medium containing the redox-cycling dye toluidine blue O (Iuchi & Lin, 1988). To address whether the *arc* genes of *M. succiniciproducens* are able to substitute for the *E. coli* genes under physiological conditions, we performed a functional complementation assay of the toluidine blue O sensitivity of *E. coli arc* mutants. For this purpose, the *arcA* and *arcB* genes of *M. succiniciproducens* were cloned with their own promoters

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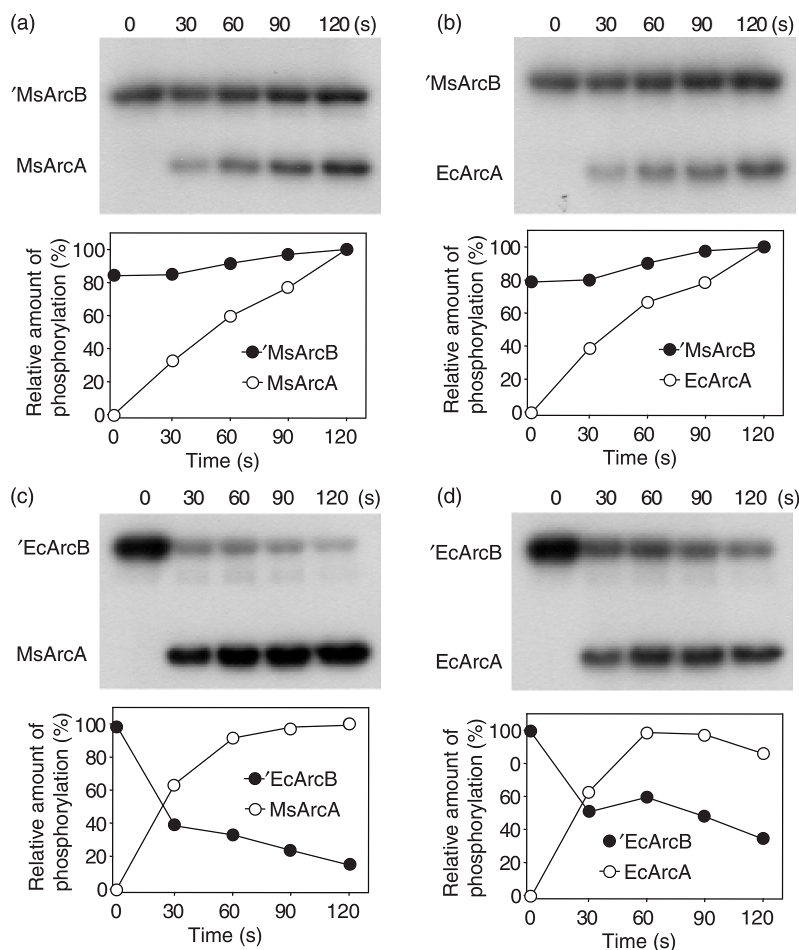

Fig. 2. Phosphorylation of ArcB and ArcA proteins of *Mannheimia succiniciproducens* and *Escherichia coli in vitro*. (a) Transphosphorylation of MsArcA by ′MsArcB. (b) Transphosphorylation of EcArcA by ′MsArcB. (c) Transphosphorylation of MsArcA by ′EcArcB. (d) Transphosphorylation of EcArcA by ′EcArcB. ArcB proteins were incubated in the presence of $[\gamma\text{-}^{32}P]$ATP for 1 min at 18 °C and ArcA proteins were added. At each time point, samples were withdrawn and analyzed by SDS-PAGE and autoradiography. The relative amounts of radioactivity incorporated into the Arc proteins were quantified by SCION IMAGE program.

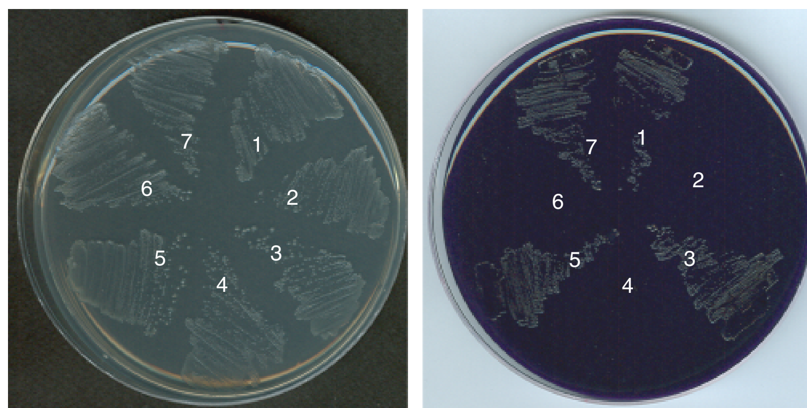

Fig. 3. Functional complementation of mutations in *arcA* and *arcB* genes in *Escherichia coli*. Left panel: tryptone plate (10 g tryptone L^{-1}, 8 g NaCl L^{-1}, 15 g Bacto Agar L^{-1}); right panel: tryptone plate containing toluidine blue O (2 mg mL^{-1}). To construct *E. coli* strains EOK101 (Δ*arcA*::*Kan*^{R}) and EOK102 (Δ*arcB*::*Tet*^{R} Δ*arcA*::*Kan*^{R}), the Δ*arcA*::*Kan*^{R} allele of strain PC35 (Cotter & Gunsalus, 1992) was P1-transduced into ECL5002 (wild type) and ECL5012 (Δ*arcB*::*Tet*^{R}) (Kwon *et al*., 2000a, b), respectively. 1, pEXT20/ECL5002; 2, pEXT20/EOK101; 3, pMSTarcA/EOK101; 4, pEXT20/ECL5012; 5, pMSTarcB/ECL5012; 6, pEXT20/EOK102; 7, pMSTarcAB/EOK102.

into plasmid pEXT20, resulting in pMSTarcA, pMSTarcB, and pMSTarcAB, respectively, and transformed into *E. coli* strains mutated in the corresponding *arc* genes.

As shown in Fig. 3, the wild-type *E. coli* strain was able to grow on the toluidine blue-containing agar plate, whereas the *arcA* and the *arcB* mutants were not. However, *E. coli arcA* or *arcB* mutant strains transformed with recombinant plasmids harboring the corresponding alleles of *M. succiniciproducens* regained the ability to grow on the toluidine blue-containing agar plate (Fig. 3).

Furthermore, the dye resistance of the *E. coli arcA arcB* double mutant was restored by the combined presence of the *arcA* and *arcB* genes of *M. succiniciproducens*. This is in agreement with the *in vitro* result demonstrating that the

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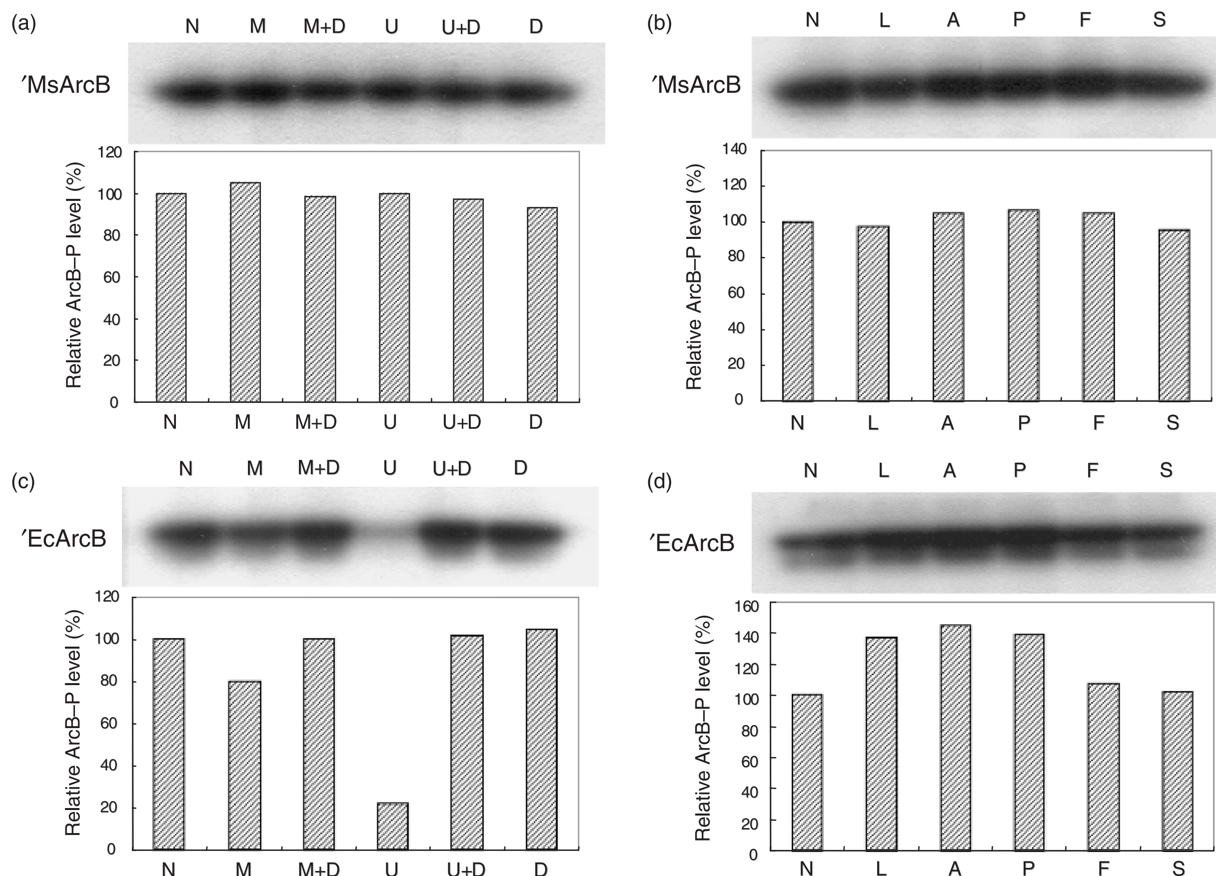

Fig. 4. Effects of quinone compounds and metabolites on the net phosphorylation of ArcB of *Mannheimia succiniciproducens* and *Escherichia coli*. (a) Autophosphorylation of ′MsArcB and ′EcArcB in the presence of quinone compounds. (b) Autophosphorylation of ′MsArcB and ′EcArcB in the presence of metabolites. Proteins were incubated in the presence of [γ-^{32}P]ATP for 3 min at 25 °C and analyzed by SDS-PAGE and autoradiography as described previously (Georgellis *et al.*, 1999, 2001a, b). N, none; M, MK_3; U, Q_0; D, dithionite; L, D-lactate; A, acetate; P, pyruvate; F, fumarate; S, succinate.

two ArcB kinases are able to transphosphorylate the ArcA proteins from the heterologous species. Also, it suggests that the *M. succiniciproducens* Arc system might have at least some functions equivalent to those of *E. coli* system, i.e. the ones required to confer toluidine blue O resistance to the cell.

Effect of quinone compounds on the autophosphorylation of ′MsArcB

It has been reported previously that the oxidized forms of ubiquinone or menaquinone inhibit the kinase activity of *E. coli* ArcB (Georgellis *et al.*, 2001a). Subsequently, it was shown that kinase silencing involves the oxidation of two redox-active cysteine residues located in the PAS domain of ArcB (Malpica *et al.*, 2004). Therefore, we asked whether the quinone electron carriers affect the *in vitro* kinase activity of ′MsArcB, which lacks the PAS domain and thus the two redox-active cysteine residues. To this end, purified ′MsArcB and ′EcArcB proteins were incubated with [γ-^{32}P]ATP in the absence or in the presence of the soluble quinone analogs ubiquinone-0 (Q_0) or menadione (MK_3). Autophosphorylation of ′EcArcB was inhibited by the oxidized forms of Q_0 and MK_3 (Fig. 4). The inhibition was stronger by Q_0 than MK_3, as reported previously (Georgellis *et al.*, 2001a). In contrast, neither Q_0 nor MK_3 were able to affect the autophosphorylation of ′MsArcB (Fig. 4), even though *M. succiniciproducens* is expected to produce menaquinone as the menaquinone biosynthetic genes are present in its genome sequence. Hence, it appears probable that redox regulation of the MsArcB activity uses a distinct molecular mechanism and signaling molecules other than the quinone electron carriers.

Effect of cytosolic metabolites on the autophosphorylation of ′MsArcB

It has been shown previously that autophosphorylation of the *E. coli* ArcB can be stimulated by the addition of certain anaerobic metabolites such as pyruvate, D-lactate, and

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acetate (Georgellis *et al.*, 1999). Moreover, it has been demonstrated that these metabolites act as physiologically significant effectors that amplify the kinase activity of ArcB but are unable to act as primary signals (Rodriguez *et al.*, 2004). Because the signaling molecules sensed by MsArcB do not appear to be the quinone electron carriers, we asked whether the above anaerobic metabolites affect the kinase activity of MsArcB. Purified ′MsArcB and ′EcArcB proteins

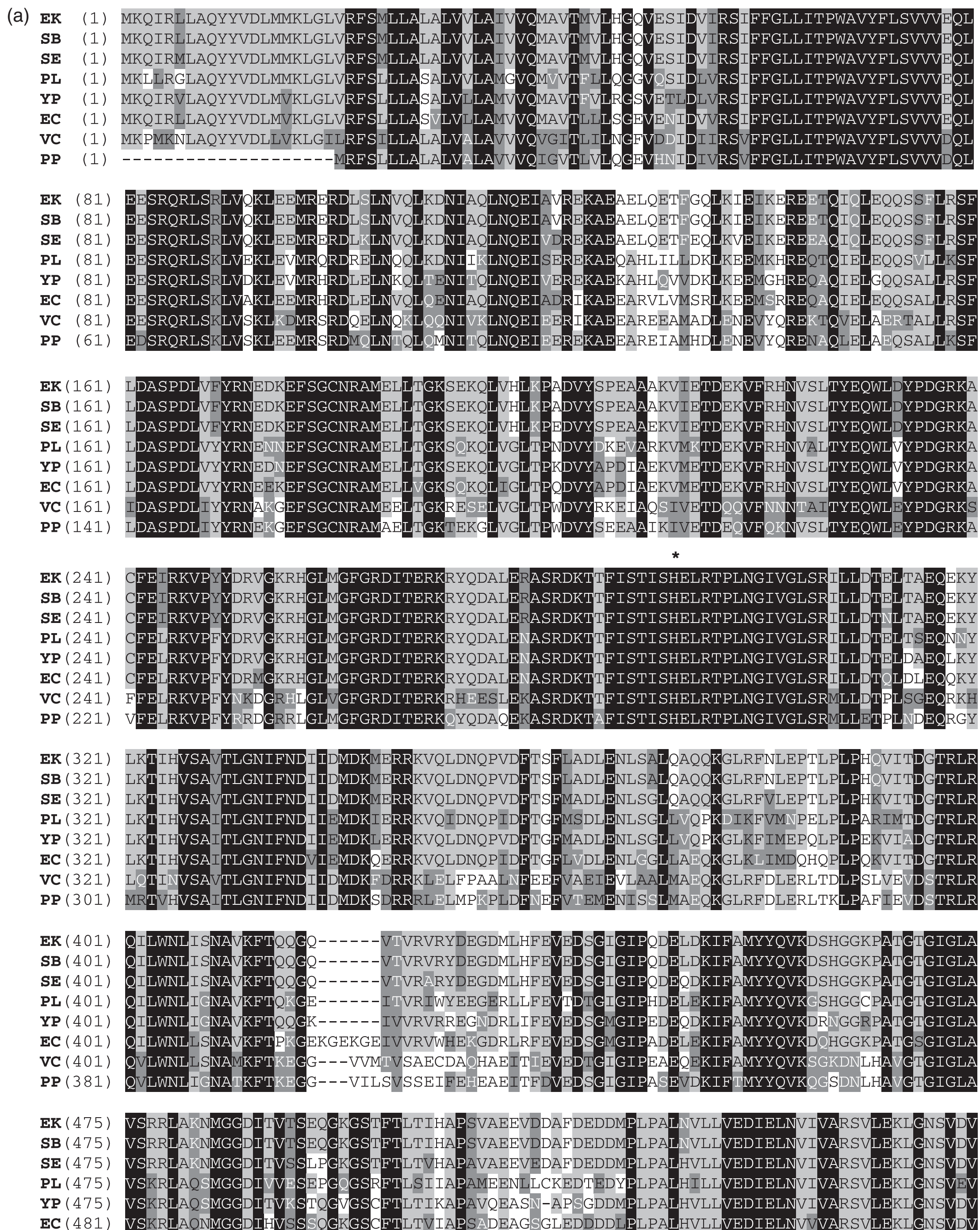

Fig. 5. Alignments of predicted ArcB proteins. (a) Type I ArcB proteins from *Escherichia coli* (EK), *Shigella boydii* (SB), *Salmonella enterica* (SE), *Photorhabdus luminescens* (PL), *Yersinia pestis* (YP), *Erwinia carotovora* (EC), *Vibrio cholerae* (VC), and *Photobacterium profundum* (PP). (b) Type II ArcB proteins from *Mannheimia succiniciproducens* (MS), *Actinobacillus succinogenes* (AS), *Haemophilus influenzae* (HI), and *Pasteurella multocida* (PM). Asterisks indicate essential amino acid residues involved in autophosphorylation and phosphorelay.

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VC(478) VSRQLIKLMGGDISVNSEEGFGSTFTVTIRVPLLAEAP--IEIEPQEPQTELNIFMVEDIELNITVARSLLESMGHKVTV
PP(458) VSRQLVQLMGGNILVTSDIGEGSTFTVTIRVPLVEAVVE-EEIMPVVPQASLSILMVEDIELNITVAKALLESLGHEVTV

                             *
EK(555) AMTGKAALEMFKPGEYDLVLLDIQLPDMTGLDISRELTKRYPREDLPPLVALTANVLKDKQEYLNAGMDDVLSKPLSVPA
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EK(635) LTAMIKKFWDTQDDEESTVVTTEENS------------------KSEALLDIPMLEQYLELVGPKLITDGLAVFEKMMPG
SB(635) LTAMIKKFWDTQDDEERTVVTTEENS------------------KSEALLDIPMLEQYLELVGPKLITDGLAVFEKMMPG
SE(635) LTAMIKKFWDATDKEESTVTPEESD-------------------KAQALLDIPMLEQYIELVGPKLITDGLAVFEKMMPG
PL(635) LTAIIEQYWGEQS-VHHMQPEEEESGL---------------MKKDEDLLDTEMLNQYIELVGPQLIRNSLSVFEKMMPG
YP(634) LTAMIKQFWDSK---PSSAVQKQEHKV---------------MQTHESLLDTTMLEQYIDLVGPQLIHQSLEMFEQMMPG
EC(641) LTAVIKQHWDTHTVWTEESVVEEGDEM---------------AKVKEDLLDIPMLEQYLDLVGPKLIHQSLEMFEQMMPG
VC(634) VREVIAKMTQHHAGESVAKVKSNKEKE-------------LPDDLYQQLLDLEMLQSYVEIVGSQPVIDSVHLFEQSMPA
PP(615) ITDVIQRLVLTCPLDDFDDDEEIETIEPISDTQPAVVVEHSNNEMFNQLLDLEMLESYVEIVGTKPVHESIAMFEKMMPD
                             *
EK(696) YVSVLESNLTAQDKKGIVEEGHKIKGAAGSVGLRHLQQLGQQIQSPDLPAWEDNVGEWIEEMKEEWRHDVEVLKAWVAKA
SB(696) YVSVLESNLTAQDKKGIVEEGHKIKGAAGSVGLRHLQQLGQQIQSPDLPAWEDNVGEWIEEMKEEWRHDVEVLKAWVAKA
SE(696) YLSVLESNLTARDKKGVVEEGHKIKGAAGSVGLRHLQQLGQQIQSPDLPAWEDNVAEWIEEMKQEWQHDVAVLKAWVANA
PL(699) YLSVLDSNMTAKDQKGITEEAHKIKGAAGSVGLRHLQQLAQQIQSPDLPAWWDNVQEWVDELKQEWRKDTEILGEWLTDA
YP(696) YLAVLDSNMTARDQKGITEEAHKIKGAAGSVGLRHIQQLAQQIQTPTLPAWWDNVQDWVDELKLEWRNDVQVLREWAAEV
EC(706) YLAILDSNMTARDQKGITEEGHKIKGAAGSVGLRHLQQIAQQIQTSSLPAWWDNVQEWIDELKHDWRHDVQVLRDWVAKA
VC(701) YLAVLDSNMVAKDQEGIVSEAHKIKGAAGSVGLKRIQKIAQKAQSPEAPAWWENISDWVEEIKNEYQSDIALLKRWLTQQ
PP(695) YIAILDSNMTAKDKDGIKFEAHKIKGAAGSIGLKHIQQVAQKAQSPELPAWWENINDWVDEIKNGYAHDLNVLREWVAEQ

EK(776) TKK--
SB(776) TKK--
SE(776) EKK--
PL(779) EKK--
YP(776) EKK--
EC(786) EKES-
VC(781) ANAKQ
PP(775) EKAK-
```

Fig. 5. Continued.

were incubated with [γ-^{32}P]ATP in the absence or in the presence of various metabolites. In agreement with previous reports (Georgellis *et al.*, 1999; Rodriguez *et al.*, 2004), net phosphorylation of ′EcArcB was elevated in the presence of D-lactate, acetate, and pyruvate (Fig. 4). However, the effects of these metabolites on the net phosphorylation of ′MsArcB were negligible compared with those on the net phosphorylation of ′EcArcB. Finally, because *M. succiniciproducens* is a known succinate production strain, we tested the effect of fumarate and succinate on the kinase activity of the two ArcB kinases. No significant effect was detected (Fig. 4).

Conclusions

A computer search of available bacterial genomes (Fleischmann *et al.*, 1995; Blattner *et al.*, 1997; Heidelberg *et al.*, 2000; Manukhov *et al.*, 2000; May *et al.*, 2001; McClelland *et al.*, 2001; Deng *et al.*, 2002; Duchaud *et al.*, 2003; Bell *et al.*, 2004; Vezzi *et al.*, 2005; Yang *et al.*, 2005) revealed sequences with high levels of identity to the *E. coli* ArcB in *Vibrio*, *Salmonella*, *Shigella*, *Yersinia*, *Erwinia*, *Photobacterium*, and *Photorhabdus* (Type I), and also in *Mannheimia*, *Haemophilus*, *Pasteurella*, and *Actinobacillus* (Type II). Interestingly, we observed that these ArcB homologues cluster in two different groups: Type I that is represented by the *E. coli* protein, and Type II that is represented by the *M. succiniciproducens* protein (Fig. 5). The ArcB proteins of the second group lack almost the entire linker region, corresponding to amino acid residues 93–271 of *E. coli* ArcB, and thus the two regulatory cysteines. It is noteworthy to point out that all the bacteria having the Type I ArcB possess both ubiquinone and menaquinone in their electron transport system, whereas the bacteria having the Type II ArcB possess only menaquinone, except *Pasteurella,* which has both quinones. Despite this, the *Haemophilus influenzae arcB* gene has been reported to be able to complement *E. coli* strains containing *arcB* null mutations, and capable of mediating responses similar to those of the *E. coli* ArcB protein under a range of redox conditions (Georgellis *et al.*, 2001b). However, although the *arcB* gene of *M. succiniciproducens* was found to confer toluidine blue resistance to an *E. coli arcB* mutant, the purified ′MsArcB protein failed to

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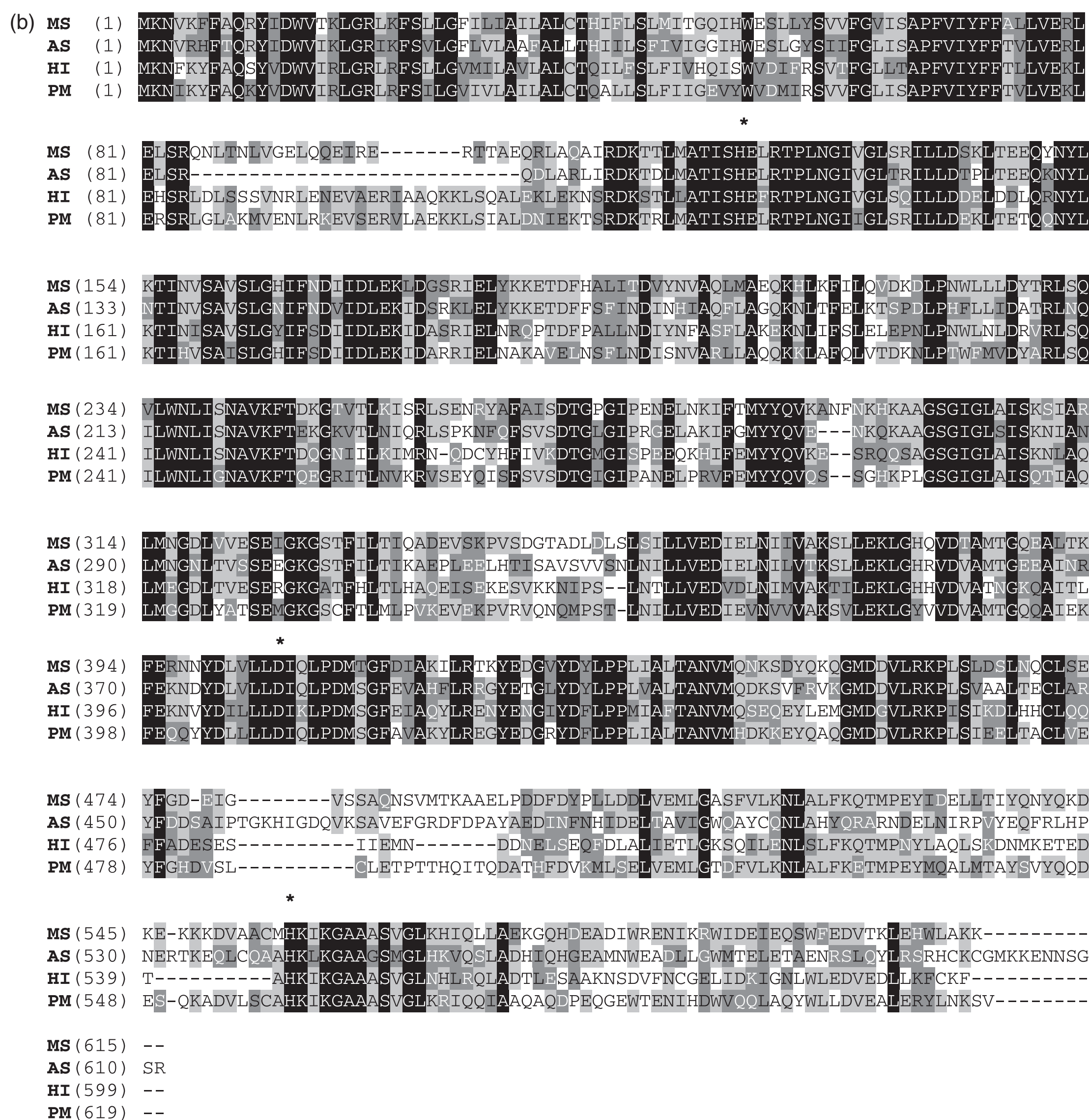

Fig. 5. Continued.

respond to both the quinone analogs (ubiquinone 0 and menadione) and the cytosolic effectors (D-lactate, acetate and pyruvate). It is possible that these observations are specific for the ArcB of *M. succiniciproducens* or it may be that a full-length ArcB protein is required for proper kinase regulation. Alternatively, it could be that the PAS-less ArcB proteins sense different types of signaling molecules and use different mechanisms for the regulation of their kinase activity. More detailed genetic and biochemical studies will be required to shed light on these questions.

Acknowledgements

This work was supported by the Genome-based Integrated Bioprocess Project of the Ministry of Science and Technology (MOST), the Korea Science and Engineering Foundation (KOSEF) grant (R01-2005-000-10288-0), the CONACyT/México Grant 47799, and DGAPA-PAPIIT-UNAM Grant IN221106.

Authors'contribution

W.S.J. and Y.R.J. contributed equally to this work.

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