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A novel device of bacterial signal transducers

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The osmoregulatory expression of *ompC* and *ompF* in *Escherichia coli* is mediated by a pair of bacterial signal transduction proteins, EnvZ (sensory kinase) and OmpR (response regulator). We isolated previously multicopy suppressors which can complement a defect in the phosphotransfer signal transduction caused by an *envZ* deletion mutation. Among such suppressors, *arcB* and *barA* are of particular interest because these gene products are unique in the sense that they contain both an autophosphorylated histidine site (or transmitter module) and a phospho-accepting aspartate site (or receiver module) in their primary amino acid sequences. Here we report that ArcB and BarA possess in the C-terminal region a phosphorylated histidine site which has never been noticed, in addition to the authentic one identified previously. This newly identified histidine in ArcB and BarA was demonstrated to play a crucial role in the observed multicopy suppression. Furthermore, it was demonstrated *in vivo* and *in vitro* for ArcB that the C-terminal domain containing the histidine can function as an alternative phosphodonor (or transmitter). This novel type of sensory kinase was therefore revealed to contain two independent phosphodonor sites, together with a phospho-accepting site. These findings suggest that this unique feature of ArcB and BarA, in terms of the signaling modules, make it possible for these sensory kinases to function as dual-signaling transducers.

Key words: bacterial signal transduction/*E.coli* ArcB/*E.coli* EnvZ/phosphorylation/response regulator/sensory kinase

Introduction

Bacteria have devised sophisticated signaling systems for eliciting a variety of adaptive responses to their environment (for a review see Parkinson, 1993). These adaptive response systems often involve two families (or two components) of signal transduction proteins, namely the 'sensory kinases' and 'response regulators' (for reviews see Stock *et al.*, 1990; Parkinson and Kofoid, 1992). The former monitor some environmental parameter and the latter mediate changes in gene expression or cell behavior in response to environmental stimuli. Such signal transduction occurs through the transfer of a phosphoryl group from a sensory kinase to a response regulator. In *Escherichia coli*, such members of the sensor/regulator families have been identified in >20 different adaptive response systems (Stock *et al.*, 1989; Parkinson and Kofoid, 1992; Nagasawa *et al.*, 1993). The widespread occurrence of this type of mechanism, so far found in >40 different bacterial species, implies that it is a powerful device for a wide variety of adaptive responses in prokaryotes. It was reported recently that this type of signal transduction mechanism also occurs in eukaryotes, including plants (for a review see Hughes, 1994).

Most signal transduction proteins contain two common and characteristic modules, the 'transmitter' and the 'receiver' (for reviews see Stock *et al.*, 1989; Parkinson and Kofoid, 1992; Alex and Simon, 1994). In a typical case, a sensory kinase contains a C-terminal transmitter module preceded by an N-terminal signal-input domain; a response regulator contains an N-terminal receiver module followed by a C-terminal signal-output domain. The transmitter module of ~240 amino acids contains several short stretches of amino acids common to members of the sensory kinase family, which contain an invariant autophosphorylated histidine (see EnvZ in Figure 1). Response regulators share an N-terminal receiver module of ~120 amino acids, in which an invariant aspartate residue is located around the center (see OmpR in Figure 1). This particular aspartate residue presumably acquires a phosphoryl group from the phospho-histidine of its cognate transmitter module.

We have been studying the molecular mechanism underlying the regulation of expression of the *E.coli* outer membrane proteins (OmpF and OmpC) in response to an environmental osmotic stimulus (for a review see Mizuno and Mizushima, 1990). This adaptive response is mediated by EnvZ (sensory kinase) and OmpR (response regulator) (for a review see Parkinson, 1993). EnvZ contains a C-terminal transmitter module and is located in the cytoplasmic membrane (Tokishita *et al.*, 1990). EnvZ can phosphorylate OmpR, which contains an N-terminal receiver module followed by a C-terminal DNA binding domain (Figure 1; Aiba *et al.*, 1989a; Forst *et al.*, 1989; Igo *et al.*, 1989). Phospho-OmpR functions efficiently as a transcriptional activator specific for *ompF* and *ompC* (Aiba *et al.*, 1989b). We recently isolated a set of multicopy suppressor genes which can complement phenotypically a defect in the phosphotransfer signal transduction caused by an *envZ* deletion mutation (Nagasawa *et al.*, 1992, 1993). From among these suppressor genes, we focused particularly on *arcB* and *barA* in this study, which also belong to the sensory kinase family (Iuchi *et al.*, 1990; Nagasawa *et al.*, 1992), because they are unique in the sense that they contain both transmitter and receiver modules in their primary amino acid sequences (Figure 1;

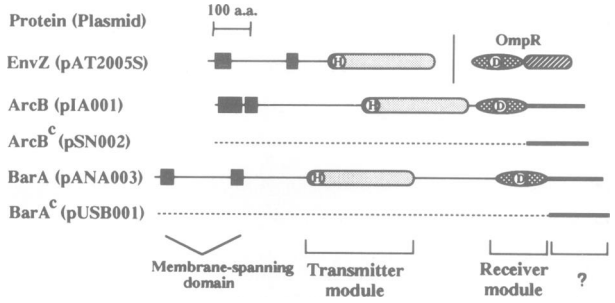

Fig. 1. Schematic representation of the structures of bacterial sensory kinases. A typical sensory kinase and response regulator, EnvZ and OmpR, are shown, which respectively contain a transmitter module and a receiver module. A presumed autophosphorylated histidine site is denoted by 'H' in the transmitter module, whereas a presumed phospho-accepting aspartate site is denoted by 'D' in the receiver module. ArcB and BarA have an unorthodox structural design (they contain both the transmitter and receiver modules). Note that ArcB and BarA contain an unknown C-terminal region, indicated by bold-faced lines. These regions are designated $ArcB^c$ and $BarA^c$, respectively. Filled rectangles indicate presumed membrane-spanning domains. Each plasmid, shown in parenthesis, is a high copy-number plasmid which can express the corresponding protein. Note that pSN002 and pUSB001 can each express a truncated form, $ArcB^c$ and $BarA^c$, respectively.

Parkinson and Kofoid, 1992). Here we explore the molecular mechanism underlying the multicopy suppression of the *envZ* mutation by *arcB* and *barA*, and report that ArcB and BarA have a novel phosphorylated histidine site in the C-terminal region which can function as an alternative phosphodonor *in vivo* and *in vitro*.

Results and discussion

Both the arcB and barA genes function as multicopy suppressors for the envZ deletion mutation

Strain DZ513 carries an *ompF′−lacZ*$^+$ transcriptional fusion gene on the chromosome, but exhibits the Lac^- phenotype due to an *envZ* deletion. To isolate multicopy suppressors for the *envZ* mutation, we searched previously for those that can complement the Lac^- phenotype. Indeed, a set of multicopy suppressors was isolated (Nagasawa *et al.*, 1992, 1993). From among them two genes (*arcB* and *barA*) were selected for this study. When DZ513 was transformed, with either pIA001 carrying the *arcB* gene or pANA003 carrying the *barA* gene (Figure 1), the β-galactosidase activities of the transformants increased substantially (Figure 2, bars 3 and 5). Their levels of activity were comparable with that observed for cells transformed with pAT2005S carrying the *envZ* gene (bar 2). However, when a Δ*ompR* derivative of DZ513 (named DB513) was used as the host strain the β-galactosidase activity did not increase (data not shown), indicating that the multicopy suppression is dependent upon OmpR. Based on the fact that both the gene products ArcB and BarA are members of the sensory kinase family containing a transmitter module, the multicopy suppression could be explained by assuming that these proteins, overproduced by a high copy-number plasmid, are capable of phosphorylating OmpR in the absence of EnvZ (i.e. 'cross-talk'; for this concept see Parkinson and Kofoid, 1992). This would result in an apparent complementation of the *envZ* mutation, as proposed previously (Nagasawa *et al.*, 1992). Surprisingly, the results presented in the next section seemingly contradicted this idea.

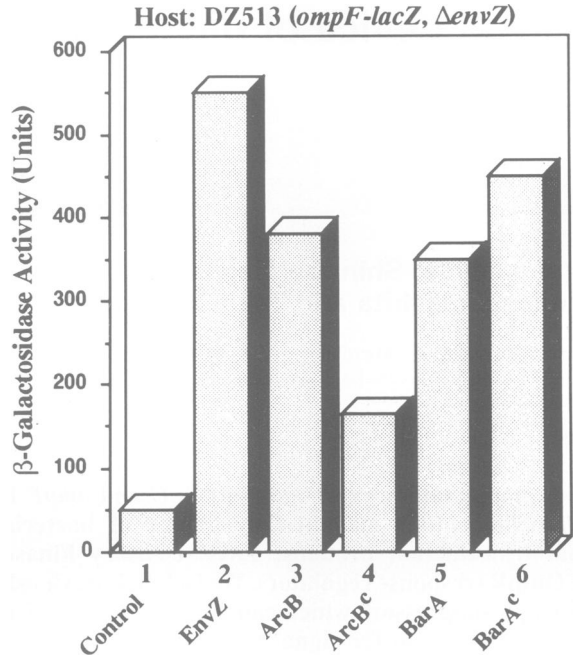

Fig. 2. β-Galactosidase activity expressed by DZ513 carrying the *ompF−lacZ* transcriptional fusion gene. DZ513 harboring an *envZ* deletion mutation was transformed with each plasmid shown in Figure 1 which can express the indicated protein (EnvZ, ArcB, $ArcB^c$, BarA and $BarA^c$, respectively). The control strain carried a vector, pBR322. The cells were grown in TY broth until the mid-logarithmic growth phase. The β-galactosidase activity expressed by these cells was determined.

A small C-terminal region is sufficient for ArcB and BarA to function as multicopy suppressors for EnvZ

ArcB and BarA consist of 778 and 918 amino acids, respectively. In contrast to EnvZ, both ArcB and BarA possess not only a transmitter module but also a receiver module (Figure 1). These two modules are followed by an unknown C-terminal region consisting of ~150 amino acids. When each C-terminal region of ArcB and BarA was expressed in DZ513 by a high copy-number plasmid (Figure 1, pSN002 and pUSB001, respectively), these truncated gene products were also able to function as multicopy suppressors, as judged from the β-galactosidase activities expressed by the transformants (Figure 2, bars 4 and 6). The C-terminal region of ArcB (named $ArcB^c$), presumably expressed in the cells, extends from Ile639 to the C-terminal Lys778, whereas that of BarA (named $BarA^c$) extends from Asn800 to the C-terminal Gly918. Therefore, both the authentic transmitter and receiver modules in ArcB and BarA turned out not to be crucial for multicopy suppression. Rather, the small C-terminal regions appear to be sufficient.

The C-terminal regions of ArcB and BarA may contain an alternative phosphodonor

How are the C-terminal regions of ArcB and BarA able to function as suppressors? Firstly, a computer-aided search for amino acid sequence homology between the C-terminal regions of ArcB and BarA was conducted. No

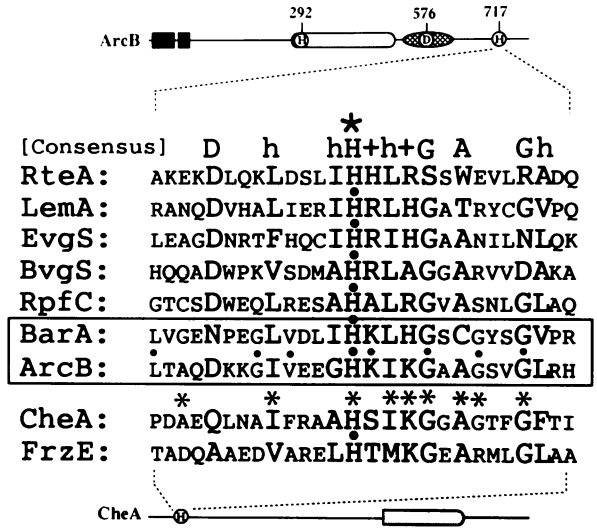

Fig. 3. Arrangement of sequences exhibiting a similarity for members of the unorthodox sensory kinase subfamily. References cited for sequences are: RteA (Stevens *et al.*, 1992); LemA (Hrabak and Willis, 1992); EvgS, (Utsumi *et al.*, 1994); BvgS (Arico *et al.*, 1989); RpfC (Tang *et al.*, 1991); BarA (Nagasawa *et al.*, 1992); ArcB (Iuchi *et al.*, 1990); CheA (Kofoid and Parkinson, 1991); FrzE (McCleary and Zusman, 1990). The positions of the compared sequences are shown in the schematic structures of the representatives ArcB and CheA. A weak consensus for these sequences was deduced. D, aspartate; H, histidine; G, glycine; A, alanine; h, hydrophobic residues; +, positively charged residues.

significant homology (or similarity) was revealed. When we looked for weak similarity more closely, ArcB and BarA were found to exhibit very weak similarity to each other in a limited region comprising a short stretch of amino acids located around the center of the respective C-terminal regions (Figure 3). These sequences consist of ~25 amino acids, and only eight residues are identical to each other at the corresponding positions. Although it was not certain whether or not this similarity was significant, we were interested because there was a histidine residue among the identical amino acids (His717 of ArcB and His861 of BarA). We extended this type of examination to other members of the sensory kinase family, particularly to those belonging to an unorthodox sensory kinase subfamily, including RteA (*Bacteroides thetaiotaomicron*; Stevens *et al.*, 1992), LemA (*Pseudomonas syringae pv. syringae*; Hrabak and Willis, 1992), EvgS (*E.coli*; Utsumi *et al.*, 1994), BvgS (*Bordetella pertussis*; Arico *et al.*, 1989) and RpfC (*Xanthomonas campestris*; Tang *et al.*, 1991). All these sensory kinases contain both transmitter and receiver modules, followed by a C-terminal region consisting of ~150 amino acids (for a review see Parkinson and Kofoid, 1992; however, it should be noted that the arrangements of signaling modules proposed in the review, particularly for ArcB/BarA/RpfC, appears not to be correct). In any case, we could easily align the corresponding short stretches for all of them (Figure 3). A weak consensus sequence could also be deduced. Although this putative consensus sequence is quite variable, it contains a single invariant histidine residue. It should be noted that this consensus does not resemble that proposed for the autophosphorylated histidine sites of authentic transmitter modules. Then, we compared these sequences with those encompassing the well-established autophosphorylated histidine sites of CheA (*E.coli*; Matsumura *et al.*, 1984; Mutoh and Simon, 1986; Kofoid and Parkinson, 1991) and FrzE (*Myxococcus xanthus*; McCleary and Zusman, 1990; Figure 3). The reason we examined these two was that they are known to have an unorthodox transmitter module. In other words, the short sequences surrounding their autophosphorylated histidine sites do not resemble those proposed for other orthodox transmitters. The basic structural design of CheA and FrzE is also significantly different from that of other transmitter-containing proteins (Figure 3). In fact, the consensus sequence in question is somewhat similar to the sequences surrounding the autophosphorylated histidine sites of CheA and FrzE. Based on these results, we assumed a priori that ArcB and BarA might each have a possible autophosphorylated histidine site in their C-terminal regions.

The newly identified histidine in ArcB and BarA is crucial for multicopy suppression

To examine the importance of the corresponding histidine residues in ArcB (His717) and BarA (His861), the following set of plasmids was constructed from the original ones shown in Figure 1. Plasmids pIA002 and pSN002H2 express the intact form and C-terminal region of ArcB, in which His717 was replaced by leucine, respectively. Similarly pANA003H2 and pUSB001H2 express the intact form and C-terminal region of BarA, in which His861 was replaced by arginine, respectively. These plasmids were transferred into the *envZ* deletion mutant, and then the β-galactosidase activities were measured (Figure 4). In all cases, the mutant plasmids failed to suppress the *envZ* deletion (bars 3, 5, 7 and 9). These results suggested that the histidine residues in the C-terminal regions of ArcB and BarA play a crucial role in the mechanism underlying multicopy suppression.

The results described above led us to envisage the following revised scenario. Each unorthodox sensory kinase, ArcB and BarA, has an alternative phosphorylated site (His717 of ArcB and His861 of BarA), in addition to the authentic one (His292 of ArcB and His302 of BarA). The region containing this C-terminal phosphorylated site may be capable of functioning as a phosphodonor; therefore it can efficiently phosphorylate the non-cognate response regulator, OmpR, provided that the alternative phosphodonor is overexpressed in the *envZ* deletion mutant. Consequently, the resultant phospho-OmpR can trigger *ompF*/*ompC* transcription even in the absence of the EnvZ function.

ArcB^c undergoes phosphorylation and functions as a phosphodonor for OmpR in vitro

To verify the hypothetical view described above, we focused our attention on ArcB since this sensory kinase has been characterized intensively in terms of its physiological and catalytic functions (Iuchi, 1993). ArcB^c was purified to near homogeneity (Figure 5A, lane 2). ArcB^c with the amino acid substitution of His717 to Leu was also purified (designated ArcB^c-H717L; Figure 5A, lane 3). Purified ArcB^c was incubated for 15 min at 37°C with 0.05 mM [γ-^{32}P]ATP, and then analyzed by SDS–PAGE, followed by autoradiography (Figure 5B). Under these conditions, ArcB^c alone was not radiolabeled (lane 4).

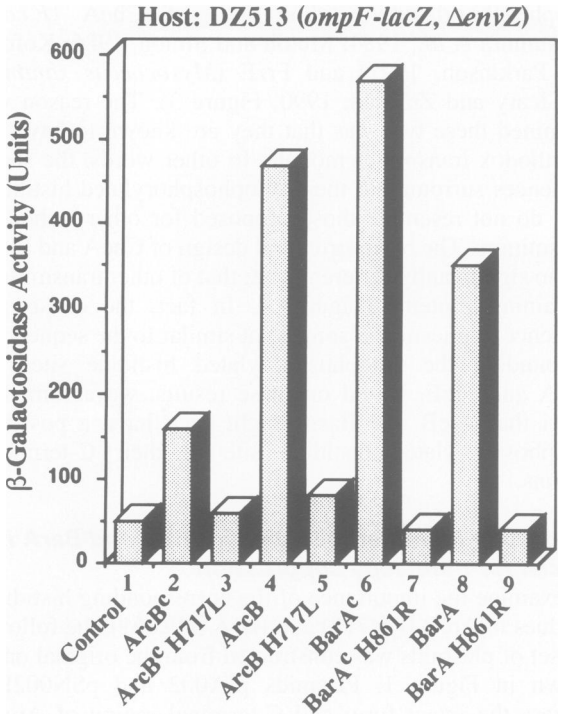

Fig. 4. β-Galactosidase activity expressed by DZ513 carrying the *ompF−lacZ* fusion gene. DZ513 was transformed with a set of plasmids (Figure 1), each being able to express the corresponding protein, as indicated, including, ArcB-H717L, ArcB^c-H717L, BarA-H861R and BarA^c-H861R (see Materials and methods). The control strain carried a vector, pBR322. The cells were grown in TY broth until the mid-logarithmic growth phase. The β-galactosidase activity expressed by these cells was determined.

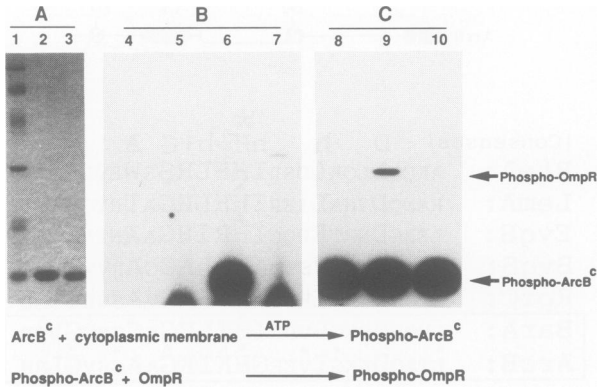

Fig. 5. *In vitro* analysis of phosphorylation with ArcB^c and OmpR. (**A**) SDS-PAGE of the purified ArcB^c protein: lane 1, molecular weight markers (from top to bottom, 94, 67, 43, 30, 20.1 and 14.4 kDa); lane 2, ArcB^c (3 μg); lane 3, ArcB^c-H717L (3 μg). (**B**) Autoradiogram showing ArcB^c phosphorylation by cytoplasmic membranes. The samples were analyzed by SDS−PAGE, followed by autoradiography. The samples analyzed were: lane 4, ArcB^c alone; lane 5, the cytoplasmic membrane alone; lane 6, ArcB^c and the cytoplasmic membrane; lane 7, ArcB^c-H717L and the cytoplasmic membrane. (**C**) Autoradiogram showing phosphotransfer between phospho-ArcB^c and OmpR. The samples were analyzed by SDS−PAGE, followed by autoradiography. The samples analyzed were: lane 8, phospho-ArcB^c alone; lane 9, phospho-ArcB^c and OmpR; lane 10, phospho-ArcB^c and OmpR-D55Q. Other details are given in Materials and methods.

However, when purified cytoplasmic membrane from DZ513 was added to the reaction mixture, ArcB^c was efficiently radiolabeled (lane 6). When ArcB^c-H717L was subjected to the same analysis, no radioactivity was incorporated into the form lacking His717 (lane 7). Phospho-ArcB^c was prepared *in vitro* and then purified (Figure 5C, lane 8). ArcB^c-phosphate was found to be stable in both neutral and alkaline media, but was labile under acidic conditions (data not shown), suggesting that it contains a histidinyl phosphate. When the purified phospho-ArcB^c was incubated with purified OmpR, this protein was phosphorylated (Figure 5C, lane 9). When a mutant OmpR lacking the presumed phospho-accepting site (named OmpR-D55Q; Kanamaru and Mizuno, 1992) was subjected to the same analysis, this particular mutant protein did not undergo phosphorylation in the presence of phospho-ArcB^c (Figure 5C, lane 10). These *in vitro* reactions are inferred to be as schematically shown in Figure 5 (see bottom).

From these results we conclude that ArcB has an additional phosphodonor site containing a crucial histidine (His717) in its C-terminal region, from which a phosphoryl group can be transferred to the aspartate residue (Asp55) in OmpR. Although the sequence surrounding this C-terminal region does not contain any other sequences common to the orthodox transmitter module including a presumed kinase domain, this novel phosphodonor site in ArcB will be referred to tentatively as an 'alternative transmitter module'.

Intact ArcB is capable of phosphorylating its own C-terminal site

It was demonstrated previously that His292 of ArcB constitutes a typical autophosphorylated site (Figure 1; Iuchi and Lin, 1992a,b). It was revealed here that ArcB possesses a second phosphorylated histidine in the C-terminal region. Our results indicate further that the purified ArcB^c alone lacks the ability to undergo autophosphorylation (Figure 5B). So, what is the determinant (or kinase) responsible for ArcB^c phosphorylation? It must be in the cytoplasmic membrane from DZ513. The most plausible candidate is the intact form of ArcB (DZ513 harbors the *arcB* gene on its chromosome). This was demonstrated to be the case, as shown in Figure 6. The cytoplasmic membrane was purified from a Δ*arcB* derivative of DZ513, and then subjected to *in vitro* ArcB^c phosphorylation. The time-course of ArcB^c phosphorylation was followed in comparison with the cytoplasmic membrane containing ArcB. ArcB^c phosphorylation was largely dependent on the presence of ArcB in the cytoplasmic membrane used. Furthermore, significantly enhanced ArcB^c phosphorylation was observed when the cytoplasmic membrane was purified from cells which had carried a plasmid overproducing ArcB. It is also worth mentioning that ArcB^c was phosphorylated upon incubation with the cytoplasmic membrane lacking intact ArcB, albeit with a very much lower efficiency. In any event, these results suggest that ArcB can phosphorylate its own C-terminal His717 site in an intermolecular fashion.

From these results, one can then expect that when a Δ*arcB* derivative of DZ513 is used as a host strain, the multicopy suppression by ArcB^c should largely be abolished. This was indeed demonstrated, as shown in Figure 7. In the *arcB* deletion background, ArcB^c cannot function efficiently as a multicopy suppressor for the *envZ*

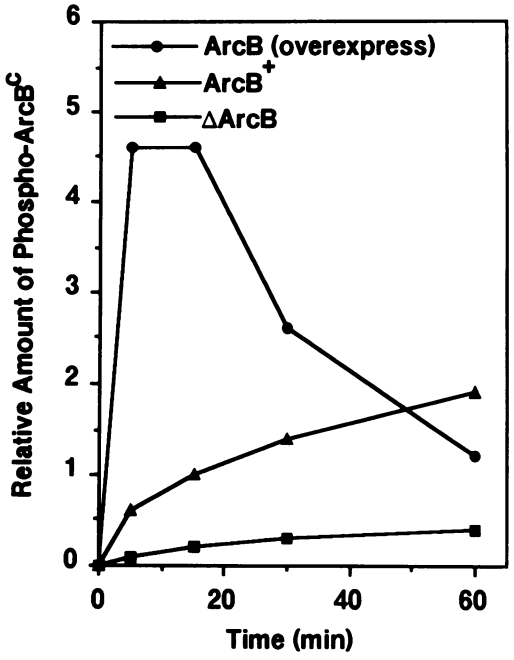

Fig. 6. Kinetic study of ArcB^c phosphorylation. Urea-treated cytoplasmic membranes were prepared from strains DZ513 (denoted ArcB$^+$), DZ513 carrying plasmid pIA001 [see Figure 1; denoted ArcB (overexpress)] and a derivative of DZ513 (named DAZ513) lacking the *arcB* gene (denoted ΔArcB). An ArcB^c phosphorylation experiment was carried out with the use of these purified cytoplasmic membranes under the conditions given in Materials and methods. The time-course of ArcB^c phosphorylation was examined by SDS−PAGE, followed by autoradiography. The amounts of phospho-ArcB^c at the indicated times were determined by densitometric quantification of the autoradiograms. The value (relative amount of phospho-ArcB^c) was expressed relative to the amount observed for ArcB$^+$ at 15 min, the value being taken as 1.

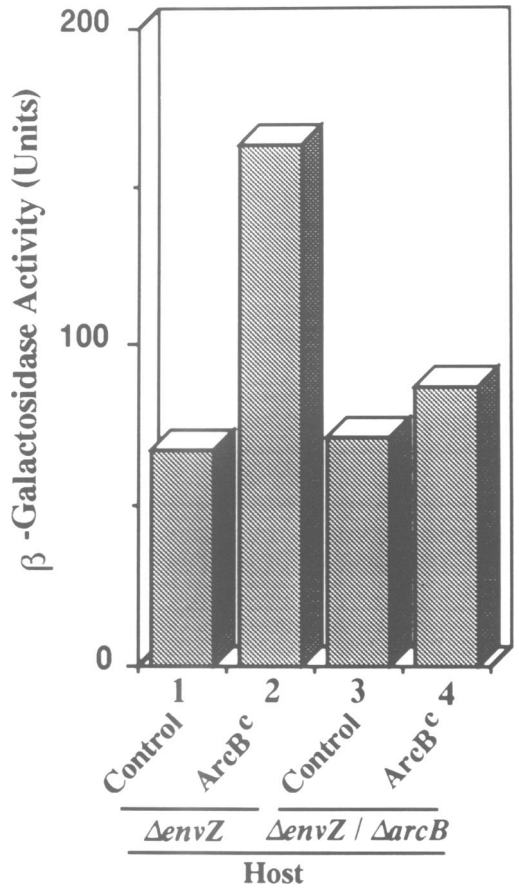

Fig. 7. β-Galactosidase activity expressed by strains carrying the *ompF−lacZ* fusion gene. Either DZ513 or DAZ513 lacking the *arcB* gene was transformed with pBR322 (control) and pSN002 expressing ArcB^c (see Figure 1). The cells were grown in TY broth until the mid-logarithmic growth phase. The β-galactosidase activity expressed by these cells was determined.

deletion (bar 4). Taking all these together, we propose a hypothetical model as to the mechanism underlying the multicopy suppression of the *envZ* mutation by ArcB^c (or ArcB), as shown in Figure 8. It can be easily imagined that the same is true in the case of BarA too.

Characterization of the ArcB function in relation to osmoregulation

ArcB has been characterized previously as a sensory kinase that controls the anaerobic repression of several operons in *E.coli* (Iuchi *et al.*, 1990; Iuchi and Lin, 1991, 1992a,b; Iuchi, 1993). Since we found that ArcB is capable of phosphorylating the non-cognate OmpR under certain conditions, we then wondered whether or not ArcB is somehow implicated in the regulation of *ompC* and *ompF* under physiological conditions. To address this issue, a strain producing ArcB-H717L, in which His717 is replaced by Leu, was constructed (named DA26 *arcB-H717L*). This strain is the wild-type with respect to both the *ompR* and *envZ* genes. We examined this *arcB* mutant in terms of the osmoregulatory profile of *ompC* and *ompF* under both aerobic and anaerobic growth conditions. Outer membrane proteins were analyzed directly by urea−SDS−PAGE, as described previously (Mizuno and Mizushima, 1987). We were unable to find any noticeable phenotypic alterations as to *ompC* and *ompF* expression on comparison with *arcB*$^+$ and *arcB-H717L* (data not shown). These results suggest that the multicopy suppression by ArcB (or ArcB^c)

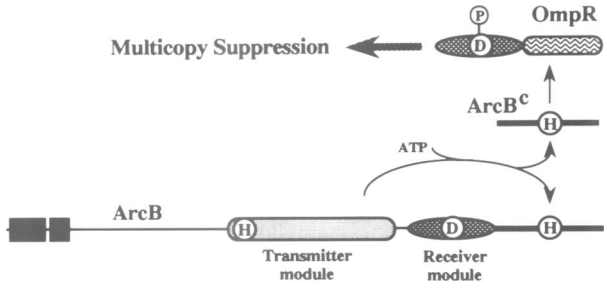

Fig. 8. A model explaining the multicopy suppression of the *envZ* deletion mutant by ArcB^c. For the structural features of the schematic illustrations of ArcB, ArcB^c and OmpR, see the legend to Figure 1. Arrows indicate possible flows of phosphoryl groups. Further details are given in the text.

observed for an *envZ* deletion mutation may be an artificial event, due to an overproduction of these proteins in cells. Although this issue must be addressed more extensively, there are a number of precedents for such a phenomenon (i.e. 'artificial cross-talk' between non-cognate pairs of sensory kinases and response regulators; Stock *et al.*, 1990). In any event, since the most intriguing finding in this study was that the unorthodox sensory kinases have a novel phosphodonor histidine site that has not been

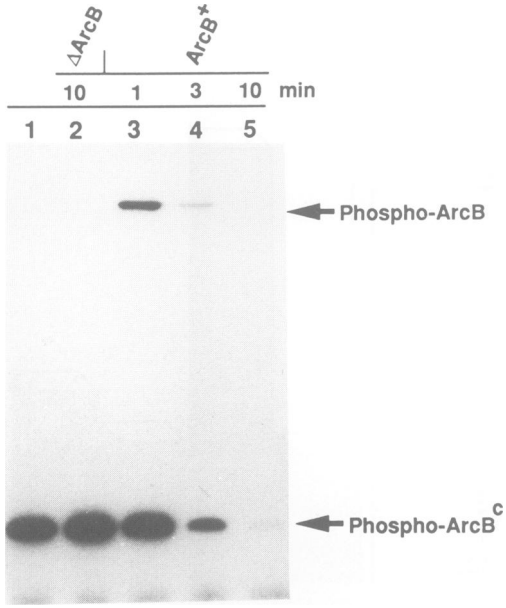

Fig. 9. *In vitro* phosphotransfer between phospho-ArcB^c and the intact form of ArcB. Radioactive phospho-ArcB^c was purified as described in Materials and methods (lane 1). The purified phospho-ArcB^c was incubated for the indicated times with cytoplasmic membranes either lacking (lane 2, denoted ΔArcB) or containing the intact form of ArcB (lanes 3–5, denoted ArcB$^+$), under the conditions given in Materials and methods. The samples were analyzed by SDS−PAGE, followed by autoradiography.

recognized previously, we wanted to address this general issue further.

The phosphoryl group in ArcB^c is capable of moving to another site in ArcB

Since the intact form of ArcB contains a receiver module in which a potential phospho-accepting aspartate residue (presumably Asp576) resides (Figure 1; Iuchi, 1993), we supposed that the phosphoryl group in phospho-ArcB^c may be transferred to ArcB itself. This was indeed the case, as shown in Figure 9. Radioactively phosphorylated ArcB^c was purified (lane 1) and incubated with cytoplasmic membranes either lacking (lane 2) or containing ArcB (lanes 3–5) in a Tris−HCl buffer without ATP. A band for the cytoplasmic membrane corresponding to an apparent molecular weight of 82 kDa was rapidly radiolabeled, but only in the case of the cytoplasmic membrane containing ArcB (lanes 2 and 3). During prolonged incubation, the radiolabel in both ArcB^c and the newly radiolabeled band disappeared rapidly (lanes 4 and 5). The apparent molecular weight of the newly labeled band was in good agreement with that of ArcB. These results suggested that the phosphoryl group in ArcB^c is rapidly and transiently transferred to the intact form of ArcB. Although there may be a possible phospho-accepting site in the receiver module of ArcB, the actual phospho-accepting amino acid remains to be determined. In any case, on the basis of the results of our *in vitro* studies (Figures 5, 6 and 9), together with those reported previously by Iuchi *et al.* (Iuchi and Lin, 1992a,b; Iuchi, 1993), we propose a probable schema as to the integrated complex phosphotransfer circuitry in ArcB based on the double transmitter and single receiver modules, as shown in Figure 10.

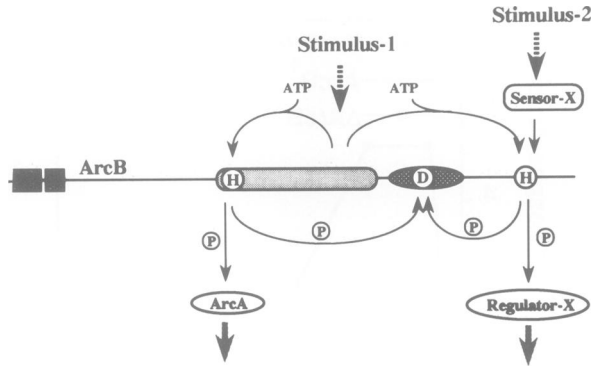

Fig. 10. A proposed schema for the integrated multi-signaling circuitry of the novel type of sensory kinase, ArcB. For each structural feature of ArcB, see the legend to Figure 1. Thin arrows indicate possible flows of phosphoryl groups, whereas bold arrows indicate those of signals. Further details are given in the text.

The newly identified transmitter module and anaerobic regulation by ArcB

As mentioned above, ArcB controls the anaerobic repression of several operons by phosphorylating its cognate response regulator, ArcA (Drury and Buxton, 1985). This ArcA/ArcB system is also known to be responsible for the resistance of cell growth to certain dyes. These functions of ArcB have been characterized extensively (Iuchi *et al.*, 1990; Iuchi and Lin, 1991, 1992a,b; Iuchi, 1993). In these reports, however, the functional importance of the C-terminal region of ArcB was never noted or emphasized. To address this issue, the *arcB-H717* mutant was examined in terms of anaerobic regulation (e.g. repression of L-lactate dehydrogenase) and dye sensitivity (e.g. toluidine blue sensitivity), in comparison with those for the wild-type. In the mutant, L-lactate dehydrogenase was repressed under anaerobic conditions as in the wild-type (data not shown). The mutant strain as well as the wild-type exhibited resistance of growth against a certain concentration of toluidine blue (0.2 mg/ml; data not shown). Therefore, our results suggested that the C-terminal region of ArcB encompassing the newly identified alternative phosphodonor may not be important as far as anaerobic regulation and dye sensitivity are concerned. These observations were consistent with those reported by Iuchi *et al.* (1990). Although this particular issue must be addressed more carefully, our observations can be interpreted as meaning that the C-terminal transmitter module of ArcB could be involved in another as yet unknown processes of signal transduction and/or adaptive response. Examination of this interesting possibility is currently underway in our laboratory.

General implication

The multi-signaling modules demonstrated in this study for ArcB can make an integrated phosphotransfer circuitry (Figure 10). Although the general characteristics of signaling modules in bacterial signal transducers have been examined intensively, this fact has never been noted previously (Stock *et al.*, 1989, 1990; Parkinson and Kofoid, 1992; Parkinson, 1993; Alex and Simon, 1994). Among the putative members of the bacterial signal transducer family characterized so far, we propose that the following also belong to this novel type of signal transducer con-

taining double transmitters: EvgS (*E.coli*), BvgS (*B.pertussis*), LemA (*P.syringae*), RpfC (*X.campestris*) and RteA (*B.thetaiotaomicron*), as well as ArcB (*E.coli*) and BarA (*E.coli*) (for references see Figure 3). In this context, it is worth mentioning that VirA (*Agrobacterium tumefaciens*), RcsC (*E.coli*), SLN1 (yeast, *Saccharomyces cerevisiae*) and ETR1 (plant, *Arabidopsis thaliana*) do not contain the corresponding C-terminal region, although they possess both the authentic transmitter and receiver modules (Winans *et al.*, 1986; Stout and Gottesman, 1990; Chang *et al.*, 1993; Ota and Varshavsky, 1993; Maeda *et al.*, 1994). These assumptions were based on a brief inspection of current databases (the NCBI and EMBL databases). We proposed that the putative consensus for the novel phosphorylated histidine site is very short in amino acid length and is quite variable (Figure 3). Therefore, it should be noted that simple inspection with a computer may not always be helpful to find this site, as indeed happened in this study. We would thus expect that this novel type of signaling module may be in hiding even in the sequences of other signal transduction proteins that have been published already. In any event, it is most likely that the view with regards to ArcB, described above and below, is essentially applicable to other members. Thus, this finding should shed light on the general mechanism underlying signal transduction systems not only of bacteria, but also perhaps those of eukaryotes.

This novel type of composite signal transducer, typified by ArcB, is a unique example of integrated signaling circuitry. As shown in Figure 10, this signal transducer may be able to propagate external stimuli in a variety of ways, based on the integrated signaling circuitry. Firstly, in response to an external stimulus the authentic transmitter can be targeted to the receiver module of the cognate response regulator (e.g. ArcA; Iuchi, 1993). The same transmitter may also be targeted to its own attached receiver (Iuchi, 1993). These events can also be envisaged for the alternative transmitter (Figures 5 and 9). However, it is tempting to speculate that the alternative transmitter has its own specificity for another receiver (e.g. an as yet unknown response regulator, X). Although it appears that the phosphorylation event in the transmitters is an intrinsic property (i.e. 'autophosphorylation' that can happen in an intermolecular fashion; Iuchi, 1993; Figure 6), it is also tempting to speculate that the alternative transmitter may acquire a phosphoryl group from ATP through the function of another sensory kinase (e.g. an as yet unknown sensory kinase, X) which responds to another stimulus. A piece of evidence that supports this idea can be seen in Figure 6. ArcB^c is able to undergo phosphorylation *in vitro*, albeit with a very low efficiency, on incubation with the cytoplasmic membrane that lacks ArcB. So, this novel device may function as a dual-signal transducer by propagating dual input and output signals. More interestingly, these signaling processes could communicate (or interplay) with each other, either positively or negatively, through the phosphorylation of the attached receiver module. In fact, the receiver module of ArcB was suggested to function as a 'self-controlled switching device' by modulating (or inhibiting) the intrinsic kinase activity of the authentic transmitter module (for this concept see Parkinson and Kofoid, 1992; Iuchi, 1993). Verification of these views must await extensive experimentation. Finally, based on these considerations, we would like to propose that these unique features make it possible for this particular type of signal transducer to function as a powerful device for not only propagating multi-signals, but also making up signaling networks through more sophisticated ways than thought previously.

Materials and methods

Bacteria and media

E.coli K-12 strain DZ513 (F$^-$, Δ*envZ*, Δ*lacU169*, *araD139*, *rpsL*, *relA*, *thiA*, *flbB*), carrying an *ompF*–*lacZ* fusion gene, was constructed previously (Nagasawa *et al.*, 1992). A Δ*arcB* derivative of DZ513 was also constructed and used in this study. This Δ*arcB* strain, DAZ513, carries an *arcB*::Cm^r allele, which was constructed via *in vivo* homologous recombination according to essentially the same procedure as described previously (Mizuno and Mizushima, 1987). The *arcB*::Cm^r allele was transferred into a wild-type strain, CSH26, by P1 transduction to yield DA26. The *arcB-H717L* allele was cloned on a single copy-number plasmid, and then introduced into DA26 to established another strain, DA26 (*arcB-H717L*) (for the *arcB-H717L* allele, see below). These bacteria were grown in Luria broth unless otherwise indicated.

Plasmids

A set of plasmids, shown in Figure 1, was obtained during the course of the experiments described previously, namely pIA001 and pSN002 (Nagasawa *et al.*, 1993), pANA003 and pUSB001 (Nagasawa *et al.*, 1992) and pAT2005S (Tokishita *et al.*, 1992). Their vectors are either pIN-IIIA (Masui *et al.*, 1984), pUSI-2 (Shibui *et al.*, 1988) or conventional pUC-series plasmids. In this study, pIA001 and pSN002 was subjected to oligonucleotide-directed mutagenesis using an oligomer (dGAGGAA-GGCCTTAAAATTAAA). pANA003 and pUSB0011 were also subjected to the mutagenesis with an oligomer (dGATTTGATTCGAAAA-CTGCATG). The oligonucleotide-directed mutagenesis was carried out according to the method described by Morinaga *et al.* (1984). This yielded mutant genes *arcB-H717L*, *arcB^c-H717L*, *BarA-H861R* and *BarA^c-H861R*, respectively. Plasmids carrying these respective genes were named pIA002, pSN002H2, pANA003H2 and pUSB001H2. Another plasmid, pSU2, carries a gene encoding ArcB^c on the pUC119 vector, which was used for the purification of ArcB^c.

Recombinant DNA techniques

DNA-manipulating enzymes, such as restriction endonucleases, the Klenow fragment of *E.coli* DNA polymerase I and T4 DNA ligase, were used under the conditions recommended by the suppliers (Takara Shuzo Co. or Toyobo Co.). Other recombinant DNA techniques were carried out according to a standard laboratory manual (Maniatis *et al.*, 1982).

Enzyme assays

β-Galactosidase activity was determined by Miller's method (1972) with slight modifications. Cells were grown to the mid-logarithmic phase in a medium (named TY broth) containing Bacto-triptone (Difco) and Bacto-yeast extract (Difco; 10 and 5 g/l, respectively). The cells were collected and then suspended in 250 mM sodium phosphate (pH 7.1) for the accurate determination of cell density. A portion of the cell suspension was subjected to a β-galactosidase assay, after being permeabilized with toluene. Values were determined from triplicate cultures.

Preparation of urea-treated cytoplasmic membranes

Cells were grown in Luria broth; then urea-treated membranes were prepared as described previously (Tokishita *et al.*, 1990, 1992).

Purification of ArcB^c

ArcB^c was purified as follows. DZ513 transformed with plasmid pSU2 was grown until the late-logarithmic growth phase in the presence of ampicillin (50 μg/ml). To 10 g of cells was added 30 ml of sodium phosphate buffer (100 mM, pH 7.1) containing 5 mM $MgSO_4$, 2 mM 2-mercaptoethanol and 1 mg DNase I. The suspension was passed through an Aminco French pressure cell (FA#073) three times at 700 p.s.i. and centrifuged at 100 000 *g* for 3 h at 4°C to isolate the clear supernatant fraction. The volume of suspension was brought up to 100 ml by the addition of 100 mM sodium phosphate buffer (pH 7.1). Solid ammonium sulfate was added to 70% saturation at 4°C. Precipitates formed were

recovered and dissolved in 10 ml of a buffer containing 50 mM Tris−HCl (pH 7.8), 0.5 mM EDTA and 2 mM 2-mercaptoethanol. The sample was applied onto a DEAE column (1.5×10 cm, Whatman DE52) equilibrated previously with Tris−HCl buffer (pH 7.8). Proteins were eluted with a 200 ml linear NaCl gradient from 0 to 500 mM. $ArcB^c$-containing fractions were eluted at ~150 mM NaCl. This sample was further applied onto an FPLC column system with MonoQ (Pharmacia-LKB) under the same buffer conditions as described above. Proteins were eluted with a 30 ml linear NaCl gradient from 0 to 500 mM. Finally, $ArcB^c$-containing fractions were eluted at ~150 mM NaCl. This column chromatography was repeated. Note that the protein was monitored by means of SDS−PAGE.

Phosphorylation experiment

The urea-treated membrane (5 μg) was incubated at 37°C for various times in the presence of 0.05 mM [γ-^{32}P]ATP (10 000 c.p.m./pmol) and 200 mM KCl, 5 mM $MgCl_2$ in TEDG buffer (Tris−HCl, EDTA, dithiothreitol, glycerol) as described previously (Aiba *et al.*, 1989a). When necessary, the purified $ArcB^c$ and $ArcB^c$-H717L proteins (1 μg each) were added to essentially the same reaction mixture as described above. After incubation, the samples were immediately subjected to SDS−PAGE, followed by autoradiography. Other details were essentially the same as those described previously for the *in vitro* EnvZ/OmpR phosphorylation experiment (Aiba *et al.*, 1989a,b).

Phosphotransfer experiment

Purified $ArcB^c$ (20 μg) was incubated with the urea-treated cytoplasmic membrane (100 μg) under the conditions described above. The reaction mixture was immediately applied onto a Sephadex G-75 column equilibrated previously with TEDG buffer. The fraction containing radiolabeled $ArcB^c$, which was essentially free from the membrane and ATP, was collected. This purified phospho-$ArcB^c$ (2 μg) was incubated with either OmpR or OmpR[D55Q] in TEDG buffer containing 50 mM KCl and 5 mM $MgCl_2$ for 60 min at 37°C. Alternatively, the same phospho-$ArcB^c$ (2 μg) was incubated with the urea-treated cytoplasmic membrane (5 μg) in TEDG buffer containing 200 mM KCl and 5 mM $MgCl_2$ for various times at 25°C. The urea-treated cytoplasmic membranes were prepared from DZ513 carrying either pIA001 (ArcB overproduction) or only the vector (pIN-IIIA) ($ArcB^+$), as described above. The membrane was also prepared from DAZ513 (ΔArcB). These samples were subjected immediately to SDS−PAGE, followed by autoradiography.

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