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The hybrid histidine kinase DokA is part of the osmotic response system of *Dictyostelium*

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We have used PCR to identify a *Dictyostelium* homolog of the bacterial two-component system. The gene *dokA* codes for a member of the hybrid histidine kinase family which is defined by the presence of conserved amino acid sequence motifs corresponding to an N-terminal receptor domain, a central kinase and a C-terminal response regulator moiety. Potential function of the regulator domain was demonstrated by phosphorylation *in vitro*. *dokA* mutants are deficient in the osmoregulatory pathway, resulting in premature cell death under high osmotic stress. Under less stringent osmotic conditions, cells grow at a normal rate, but development at the multicellular stage is altered. *dokA* is a member of a family of histidine kinase-like genes that play regulatory roles in eukaryotic cell function.

Keywords: *Dictyostelium discoideum*/histidine kinase/ osmotic stress/signal transduction/two-component system

Introduction

Transient protein phosphorylation is a critical component of many signal transducing systems in eukaryotes. Attention in the past 10 years has focused primarily on modifications of tyrosine, serine and threonine (Herskowitz, 1995). However, in bacterial systems, there is an extensive literature suggesting that autophosphorylation on histidine residues and transphosphorylation of specific aspartyl residues on response regulator proteins play critical roles in intracellular information processing (Bourret *et al.*, 1991). The most intensely studied bacterial histidine kinase systems involve only two components, a sensor histidine kinase and a response regulator–effector complex (Hess *et al.*, 1987). In general, the catalytic kinase domain of the histidine kinase responds to regulation by the sensory domain of the molecule (Parkinson and Kofoid, 1992; Alex and Simon, 1994). The sensor controls the rate of autophosphorylation of the kinase and/or the rate of subsequent transfer of the phosphoryl moiety from the kinase to an aspartyl residue on the response regulator molecule. The phosphorylated response regulator molecule then interacts with an effector domain, modifying effector function. More than 50 different systems that include variations on this theme have been described in bacteria (Parkinson and Kofoid, 1992; Alex and Simon, 1994; Stock, 1996). They regulate responses to changes in concentrations of amino acids and other metabolites, as well as to changes in the physical parameters of the cell including osmolarity (Mizuno *et al.*, 1982a,b), redox potential (Iuchi *et al.*, 1990) and light (Rudolph *et al.*, 1995). These changes are transduced into changes in enzyme activity, gene expression and chemotaxis.

Complex circuits of sensor kinases and response regulator–effector molecules have been described, and a number of these have been shown to be involved in developmental control in bacterial systems (Alex and Simon, 1994; Hoch and Silhavy, 1995). For example, sporulation in *Bacillus subtilis* is regulated by a phosphorelay circuit of protein–protein interactions which includes multiple histidine kinases and response regulators as well as protein phosphatases that integrate a variety of metabolic and physiological inputs to control the initiation and progression of the early stages of sporulation (Burbulys *et al.*, 1991). A variety of forms of complex histidine kinases have been described. One class of these multidomain proteins, the hybrid kinase, is represented by a molecule which includes a sensor domain, a histidine kinase catalytic domain and a built-in response regulator domain. In these proteins there is presumably an intramolecular autophosphorylation on histidine and transfer to the aspartyl residue of the response regulator portion of the same molecule. The response regulator domain is thought to act as a pseudo-substrate to modulate kinase activity (Iuchi, 1993).

Recently, a number of eukaryotic signaling systems have been shown to include proteins that are highly homologous to the bacterial hybrid histidine kinases (Alex and Simon, 1994; Chang and Meyerowitz, 1994). Thus, *etr1*, a gene in *Arabidopsis thaliana*, shows a high degree of homology and is involved in regulating the ethylene response of the plant (Chang *et al.*, 1993). In addition, a number of other genes have been cloned that appear to be part of the ethylene response pathway and show amino acid sequence homology to the bacterial hybrid histidine kinases (Hua *et al.*, 1995). In yeast, the *sln1* gene product, another hybrid histidine kinase (Ota and Varshavsky, 1993), couples to a response regulator, Ssk1, which is homologous to the bacterial response regulators and controls responses to osmolarity (Maeda *et al.*, 1994). Both the Etr1 and the Sln1 pathways appear to be part of larger phosphotransfer circuits. Etr1 is thought to interact with a Raf kinase homolog while the Ssk1 response regulator has been shown to function through a variety of serine/threonine kinases to eventually activate a homolog of the eukaryotic MAP kinase (Maeda *et al.*, 1994, 1995). These findings establish the presence of histidine kinases in eukaryotes that are similar to those in bacteria. There is evidence in eukaryotes for at least two other kinds of

protein histidine phosphorylating systems. Huebner and Matthews (1985) found phosphorylation of histidine residues, both in histones and in cytoplasmic proteins, that does not correspond to the characteristics of the histidine kinases found in bacterial systems. Recently, Crovello *et al.* (1995) showed that P-selectin can be transiently phosphorylated on histidine residues, and suggested that this histidine phosphorylation may be part of a signal transducing system that regulates P-selectin function. This type of histidine phosphorylation possesses few of the characteristics of the two-component systems and also suggests the existence of another mode of signal transduction.

In order to explore the extent to which the bacterial type of histidine kinases are distributed in eukaryotic organisms, we have screened for homologous gene products by PCR in a number of organisms. In *Dictyostelium discoideum* there are multiple hybrid histidine kinases found that resemble those in yeast, plants and bacteria.

Here we describe DokA, a hybrid histidine kinase that is developmentally up-regulated in *D.discoideum*, required for modulation of the cell's response to changes in osmolarity and involved in spore formation.

Results

Cloning and sequencing of DokA

A number of conserved amino acid motifs have been identified as integral parts of the kinase domain of many bacterial histidine kinases. These include the N and the H box-associated sequences (Parkinson and Kofoid, 1992) (see the alignment in Figure 1B) which are separated by ~100 amino acid residues. Degenerate oligonucleotide primers were designed based on these conserved sequences, and a number of DNA fragments were obtained by amplification with the polymerase chain reaction (PCR) from genomic *D.discoideum* DNA. One of these, a 375 bp fragment, was subcloned and its sequence determined. It encoded an open reading frame (ORF) that showed 46% sequence identity with the amino acid sequence of the bacterial sensor histidine kinase BarA (Nagasawa *et al.*, 1992) (see Figure 1B). This DNA fragment was used subsequently to screen *D.discoideum* genomic DNA libraries that were constructed using size-selected DNA. The nucleotide sequence of a 7 kb region identified by hybridization with the probe was determined. The analysis of this sequence predicts a coding region that encompasses 1671 amino acids resulting in a putative protein with an M_r of 186 kDa (Figure 1A). We have designated the gene corresponding to this putative product *dokA* (*Dictyostelium* osmosensing kinase A) on the basis of its possible involvement in the regulation of the cellular response to changes in osmolarity (see below).

The ORF of *dokA* is not interrupted by introns; however, there are long AT-rich sequences that encode polyasparagine stretches throughout the ORF. These are not flanked by consensus splice sequences (Kimmel and Firtel, 1980) and are presumably translated as part of the protein product. Extensive stretches of single amino acid repeats are found throughout the N-terminal region of the protein; up to 38 asparagine residues are consecutively repeated. Other stretches of repeats include glutamine, threonine, serine and cysteine. The function of these repeats, which have been found in a number of proteins in *D.discoideum*, is not known. These homopolymer stretches account for 65% of the first 1000 amino acids of the protein. When the repeats are subtracted, the overall size of the protein is similar to that of its prokaryotic and eukaryotic homologs (see Figure 1B).

Sequence analysis of *dokA* clearly indicates that it falls into the category of hybrid histidine kinases, which are distinguished by the presence of a response regulator domain. In bacteria, hybrid kinases such as ArcB (Iuchi *et al.*, 1990), BarA (Nagasawa *et al.*, 1992) and RcsB (Gottesman and Stout, 1991) have three discernible domains. All of the eukaryotic histidine kinases that have been cloned thus far show a similar pattern, with the exception of Ers (Hua *et al.*, 1995). Thus, the Etr homologs that regulate the response to ethylene in *A.thaliana* and the *sln1* gene product that is involved in the osmotic response in yeast are also found to be hybrid histidine kinases, i.e. they include a response regulator domain.

The N-terminal 'sensor' domain of DokA does not display any significant homology to other proteins found thus far in the protein database, while the kinase and the response regulator domains are clearly recognizable as members of the two-component histidine kinase family. The kinase domain of DokA shows 31% identity to the *Escherichia coli* protein BarA (Figure 1B), and the response regulator domain is most closely related to the regulatory domain of RcsB, showing 33% identity (Figure 1C, Table I).

Simultaneously with our discovery of DokA, another hybrid histidine kinase, DhkA, was discovered in *D.discoideum* (Wang *et al.*, 1996). Both kinases show a high degree of similarity in their domain structure and in their distribution of polar and non-polar amino acids. However, their overall degree of sequence identity is fairly low (27%). The fact that DokA and DhkA do not show extensive sequence overlap with each other suggests that they may function in different pathways. There is evidence for additional histidine kinase homologs beside *dokA* and *dhkA* in *D.discoideum* that comes from PCR and Southern blot analysis (C.K.Singleton, personal communication; S.C.Schuster, unpublished results). Thus, there may be an extensive family of homologous genes that control a variety of functions in *D.discoideum*.

Domains of DokA

Analysis of previously characterized bacterial and eukaryotic histidine kinases revealed extensive hydrophobic regions which are thought to correspond to transmembrane-spanning segments that define a sensory extracellular domain (Alex and Simon, 1994). Analysis of the *dokA* gene product using Kyte–Doolittle or Hopp–Woods algorithms suggests that it does not display extensive hydrophobic stretches of amino acids. It may, therefore, not be defined as a membrane-associated protein and may, therefore, function as a soluble intracellular signal transducer in contrast to the gene products that have been described previously.

Swanson *et al.* (1993), using the CheA histidine kinase, previously showed that the domains can be subcloned so that each of them can be expressed separately. The separated domains showed appropriate biochemical func-

A

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   1  MSSPHIELHS QRTLSPQPSS NNFELTGNKS CALSASLNGS IDDLNNNNNN
  51  NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNDGDKNDK KLLIITNAPT
 101  PPLTPPQPTK TQQTLLELNQ KFQEQQQQQQ QQQQLQKQNK QQQSQKQQED
 151  PPSSQQEEPP LSQQQQEQEQ EQEQEQEQEE QSKQKIEGKG GGGEEEECEG
 201  GGGGEGEGEE QFKEGDEEEH EGIEIDPNLP TTGYTGKRRS SLQINEASPK
 251  LIQTPSVDRT LCILDSLPCP IFQIGIIENT ENGNITLTGF ANERLTTLLS
 301  CSSNLLVDSL KSIPSPHYNT VPSLESCNSI PINNLAPGLL PNTDPTKILD
 351  PYISLYNKKQ LKERYSNNNN NTNTNNNNNN NNNNNNNNNN NNNNNNNDTT
 401  TTTTTTTTTT TTTTTTTTTT TTNESDNNNN NNNNNNNNNN NNNNNNNNNE
 451  EIKQNTKEGE EILHESTNST GTGNSISRPN SILQCILDSF HEQKTITEKV
 501  LLSNFLIRQQ YKSRATIKPF PDGCICIFEY IENINLNYQQ PPTSLNDRIN
 551  STNKLTNEME LLSLSPDSNH QHVQKLHNHN HNHHNHNHNN TTQRASSTDS
 601  PFIHSVSANS LSSMSSNSSV TSACASLSSN SSSNSNNNSN NASSSNCSSN
 651  ANSNNTNSSS SNCCCCCNNN NNNNTSSTSS TISSASSTKS KSYLHQQHKN
 701  SKVYSTLLNS FENLSFLLKS TPIIIWRADH NGEMVFFKKS DEIPIDEKKV
 751  VGNNFQDFLQ WVPQTYRTNF QEMFQNSLKC GKIFEFLFWF QTPQNYVQLF
 801  KIAGYPIFHI DGKDCIASKR YLIGWLGVTY NYLINDGAEI SIKGSANLDS
 851  MYEKFKIIYE MPNIERTKLT NNGISSGGDI TNANNNGGSS ESSNKILEKK
 901  KHLISEAEKE CFLKCKETYN ILFKLSLLGV MFSTFKGIIL DANDMLLQTI
 951  GYTRGDLENG KIDWMLLTPP EHFEISARAL QELKAKRWCQ PIEKAYIHKS
1001  GKRVPVLITS AMIDGSTEQC ITFVFDLSRY RQAEMAAIEA TRLKTQFITN
1051  ISHELRTPCH GIVGMSQLLL DSQLTNTQRD NIDSIKRSTD SLISLINDIL
1101  DFSKLEYGKV TLENESFELL PMIEEVLDSQ ATAANRKGID LIFVMGRDYP
1151  VPPVIFGDRN SLKKVLLNLV GNAVKFTETG FVLLEISTDY ESGDQISLRF
1201  TVKDSGIGIP ENKIEQIFVP FGQIDGSFSR KYGGSGLGLS FCKELVALMG
1251  GYIRVESGDQ EGGKGTTFWF AIKVSISSPS YLPNSVPAAN QFFYPEYRPS
1301  HYNNVIGNGD SPKNVLIIES NQMVIMSIQS ILLSMKCECI SASKAITALD
1351  LLESSKSTEN QIDIIVCSDK STFVQQILDY VTTEKVILYG VDPNSKYNEN
1401  SKVYSYLVTP ITHSKLISSI LLSKNLKSKN SFLTTTNNTN NTNNTNNIEN
1451  KSSIDSPLSI TSTSSSIITP ILTSNNLDLN NNNNNNNNSI LVSNNGGVDG
1501  GNNVPSTLTT IQQSQPKKYI LVAEDNDINI KVVVRQLEKL GYTAIVGING
1551  LKALEIIGSF PICLILLDCQ MPQMDGFTCS TILRQIEPTG QRIPIIAMTA
1601  NDSKDRCFEV GMDDYLSKPV RVDRLQKTLS DWIKTDENGN PTNSYNFYPL
1651  SYSLVYNNFI DTQLKKEKND D
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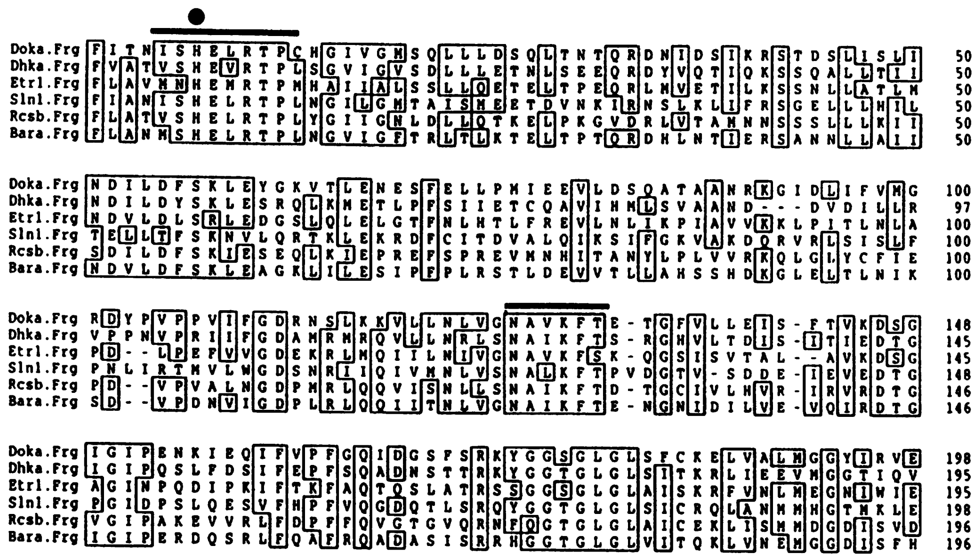
B



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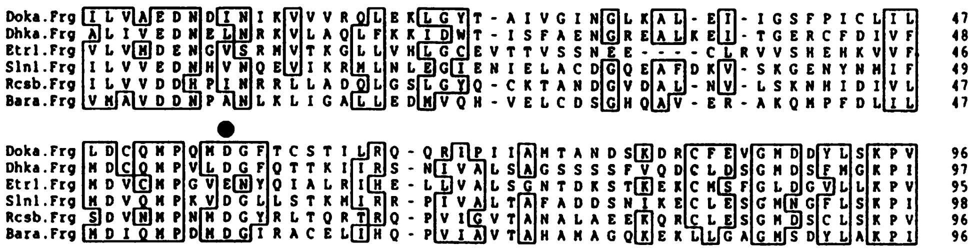

Fig. 1. (**A**) Protein sequence of DokA. The *dokA* gene encompassing 5010 bp is uninterrupted by introns. In *dokA*⁻ cells an internal sequence corresponding to 360 amino acids is omitted, including the conserved histidine [indicated by a closed circle in (B)]. (**B**) Alignment of the DokA kinase domain to the catalytic domains of DhkA (Wang *et al.*, 1996), Sln1 (Ota and Varshavsky, 1993), Etr1 (Chang *et al.*, 1993), BarA (Nagasawa *et al.*, 1992) and RcsB (Gottesman and Stout, 1991). The catalytic domains of histidine kinases are defined by the conserved phosphotransfer motif surrounding His1053 in DokA (H box), the ATP binding sites G1 and G2, two conserved phenylalanines and the N box motif of six amino acids. The initial PCR fragment includes the sequence inbetween the H and N box (H and N boxes are indicated by bars). For better alignment of the kinase domains 125 amino acids were omitted from the SLN1 sequence at position 138 corresponding to the sequence (DVRMKLLGEYDKEL-SEKKQYKEVYIKKGTEVTENLETTDKYDLPTLSNHRKSVDLESSATSLGSNRDTSTIQEEITKRNTVANESTYKKVNDREKASNDDVSSIVST-TTSSYDNAIFNSQFNKAPG) and 17 amino acids at position 143 corresponding to (EGGNLGRPIENPKTWVIS). (**C**) Response regulator domains of the above proteins were aligned on a conserved sequence of 100 amino acids. Two-component regulators are defined by an 'acidic pocket' which consists of three aspartate residues. In DokA they correspond to residues 1524, 1525 and 1575. Aspartate 1575 (indicated by a closed circle) is the presumed site of phosphorylation that receives the phosphoryl group from the His1053 of DokA. Lys1618, which is thought to interact with Asp1575, is also conserved throughout the regulator family. The six amino acids (DLGYTS) were omitted from the SLN1 sequence at position 70 in the alignment of the response regulator domains.

Table I. Sequence identity of DokA with various homologs

	Identity with DokA (%)	
	Histidine kinase domain (462 aa)	Response regulator domain (130 aa)
DhkA	33.2	33.8
BarA	31.0	31.8
RcsB	30.4	32.5
Sln1	30.2	25.4
Etr1	29.0	24.4

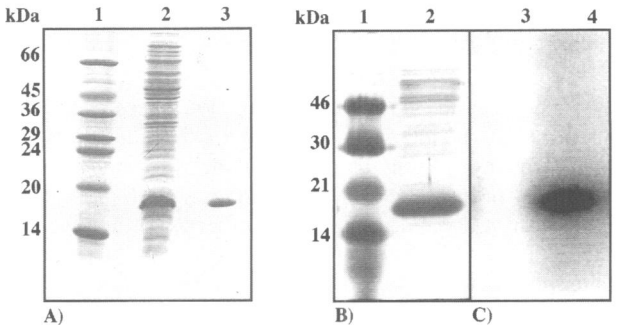

Fig. 2. (**A**) Expression of the DokA C-terminal domain. SDS–PAGE of heterologously expressed and purified C-terminus of DokA. Lane 1, molecular weight marker (sizes as indicated); lane 2, *E.coli* M15/pREP4/pQE12rr161 whole cell extract; lane 3, purified RR161. This fragment, termed RR161, holds the response regulator domain of the hybrid kinase. (**B**) and (**C**) Phosphorylation of the DokA regulator domain. SDS–PAGE and autoradiography of ^{32}P-labeled RR161. RR161 was incubated with [^{32}P]acetyl phosphate for 10 min. Excess label was removed by SDS-gel electrophoresis. Acetyl phosphate specifically transfers phosphoryl groups onto a conserved aspartyl residue in two-component response regulators.

tion. In order to demonstrate that the domains of the DokA protein indeed correspond to the functional regions of the molecule, the DNA sequence corresponding to the C-terminus of DokA was expressed and purified (see Figure 2A). The soluble protein was shown to act as a response regulator by incubation with radioactive acetyl phosphate (Figure 2B and C), a substrate that is known to phosphorylate specifically many response regulators (Lukat *et al.*, 1992). The phosphoryl transfer occurs to the conserved aspartate residue in the 'acid pocket' region of the response regulator protein, a hallmark of this class of proteins. The corresponding residue in the DokA sequence is Asp1575. The phosphorylated residue has the properties of a phosphoryl aspartate (data not shown) and the protein autodephosphorylates, again demonstrating an intrinsic activity of many response regulator proteins.

dokA messenger RNA expression is developmentally regulated

In order to study *dokA* gene expression during the development of *Dictyostelium*, RNA was isolated from the wild-type strain AX2 and was analyzed using a probe corresponding to nucleotides 2350–2700 of the *dokA* coding region. This is a region which shows little sequence identity with other histidine kinases, and appears to be a unique probe for the *dokA* mRNA. Similarly to *dhkA*, the *dokA* gene is expressed at lower levels in vegetative cells than in the late developmental stages of the organism. *dokA* expression increases markedly after 16 h of development

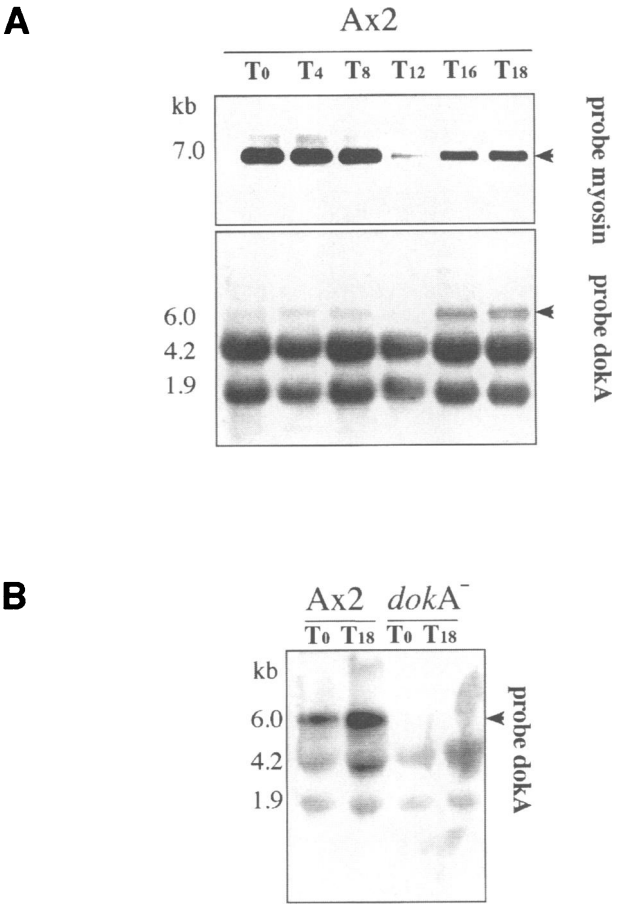

Fig. 3. (**A**) *dokA* mRNA expression in developing cells. Axenically grown cells were plated on phosphate agar and allowed to develop for 0, 4, 8, 12, 16 and 18 h. Total RNA was analyzed by Northern blot using a probe corresponding to a 350 nt fragment of *dokA*. The size of the *dokA* transcript and the quantity of RNA used was estimated by re-probing the blot with a probe derived from the myosin heavy chain gene (Noegel *et al.*, 1986). (**B**) Disruption of *dokA*. Northern blot analysis of total RNA from wild-type (AX2) (lanes 1 and 2) and *dokA*$^-$ cells (S2/25-1) (lanes 3 and 4) derived from T_0 (lanes 1 and 3) and T_{18} (lanes 2 and 4) developmental stages.

(Figure 3A), i.e. during the late culmination stage. The size of the transcript corresponds to the size predicted from the sequence of the gene. The mRNA appears to include 6 kb by comparison with myosin heavy chain RNA (Figure 3A).

Morphogenetic changes in dokA null mutants

In order to determine the role of the DokA histidine kinase, the gene was inactivated by a replacement vector. This vector was constructed by substituting 1079 nucleotides of the *dokA* gene (bp 3050–4129) with a G418 resistance cassette (Leiting and Noegel, 1988). The fragment of the *dokA* gene that was eliminated corresponds to the N-terminal portion of the kinase domain and includes a conserved histidine residue that has been shown in other histidine kinases to be required for function (His1053). Cells carrying the inactivated *dokA* gene were cloned, and the gene replacement was confirmed using Southern blot analysis. Figure 3B shows that the disruption of the *dokA* gene results in the complete absence of the transcript.

The *dokA* mutants grow well in axenic culture; however,

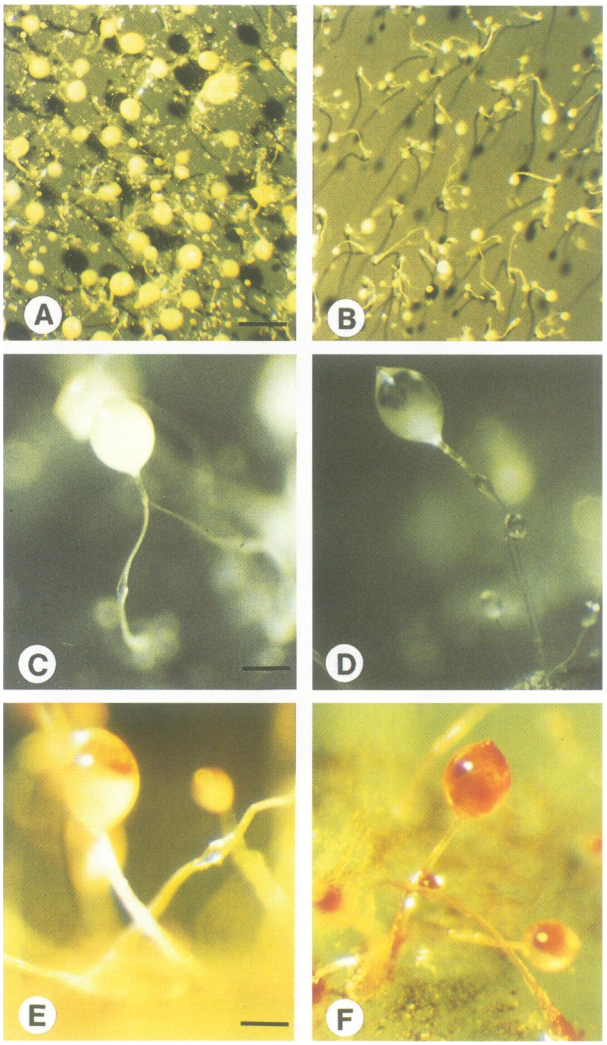

Fig. 4. The *dokA* null mutant phenotype. Wild-type AX2 (**A**) and *dokA*$^-$ cells (**B**) cultivated on bacterial plates in an overhead view (bar equals 555 μm). The spore heads of filter-grown *dokA*$^-$ strains are smaller than in the wild-type. They have a 'glassy' appearance (**D**) with only few spores in the sorus. Wild-type is shown in (**C**) (bar equals 140 μm). Neutral red staining of wild-type cells (**E**) leaves only few stains at the tip of the spore head after completion of development, while *dokA*$^-$ cells (**F**) show severe coloring of the liquid that fills the spore head (bar equals 140 μm).

they clearly show an altered morphology when compared with wild-type after development on filters (Figure 4A and B). The mutant spore heads have a glassy appearance with few spores accumulating in the lower part of the sorus (Figure 4C and D). Spores that were recovered from single fruiting bodies and plated with bacteria on agar resulted in ~30% of the colonies when compared with wild-type colonies (data not shown). Germination of the mutant spores was as efficient as that of wild-type spores.

When developing cells were stained with neutral red, the fluid surrounding the spores became stained. In *dokA*$^-$ fruiting bodies, almost the entire mutant spore head was stained (Figure 4F) whereas, in the wild-type, only little color was retained in the sorus at its tip (Figure 4E).

Histidine kinases are known to be involved in bacterial chemotaxis and phototaxis as well as in the detection of other environmental changes that lead to responses

Table II. Cellular responses investigated in *dokA*$^-$ cells

Investigated response	Observed phenotype
Phototaxis of slugs	normal
Motility of slugs	normal
Pre-stalk/pre-spore ratio	normal
Actin phosphorylation	normal
Spore germination	normal
Spore dormancy	normal
Growth on bacteria	normal
Growth on filters	altered fruiting body
Development in hyperosmotic conditions	impaired
Timing of developmental genes[a]	slightly delayed

[a]When probed with *car1*, *ecmA* and *ecmB* probes.

involving altered gene expression. We therefore have tested the *dokA* mutant for a variety of cellular responses (summarized in Table II). The mutant was found to be reasonably normal with respect to aggregation, phototaxis and slug motility. Furthermore, Northern blot analysis of mutant RNA taken from various time points during development showed little or no deviation from the wild-type pattern of expression when probed with *car1*, the gene coding for the cAMP receptor which is maximally expressed during the aggregation stage (Saxe *et al.*, 1991). Only slightly belated expression was observed in the case of mRNA corresponding to the developmental markers *ecmA* and *ecmB* (Jermyn *et al.*, 1987; Jermyn and Williams, 1991).

Deletion of dokA causes osmosensitivity

When wild-type cells were suspended in high osmolarity medium (400 mM sorbitol) they shrank by ~50% in volume. When the cells were rediluted in phosphate buffer, they re-established normal cell volume and cytoplasmic streaming within a few minutes. The *dokA*$^-$ mutant cells show shrinkage to about the same extent as the increase in osmolarity; however, upon redilution, these cells lysed. To study the osmotic response in detail, we have used a viability assay. Wild-type and mutant cells were exposed to high osmotic stress for increasing periods of time. Cells removed from the high osmolar medium were plated on agar with *Klebsiella aerogenes*. Recovery was assessed by counting colonies after 48 h. While AX2 wild-type cells survived the procedure with only a slight decrease in viability, the *dokA*$^-$ mutant cells were dramatically affected. Figure 5 shows that, upon a 2 h exposure to sorbitol, the mutant cells showed a mortality rate of up to 95%, while wild-type cells showed little or no decline in viability.

Figure 6A and B shows growth and development of wild-type and mutant on agar plates containing 200 mM sorbitol. The wild-type cells developed normal fruiting bodies (Figure 6C). In the *dokA*$^-$ mutant, only occasionally minute fruiting bodies were seen on the sorbitol agar (Figure 6D). Growth and migration of wild-type and mutant cells, however, remained unaffected (Figure 6A and B).

In response to the osmotic shock, synthesis of compatible osmolytes was investigated. Cells that have been exposed to sorbitol (400 mM) for up to 120 min were analyzed for an increase in intracellular concentration of naturally occurring amino acids, as well as glycerol.

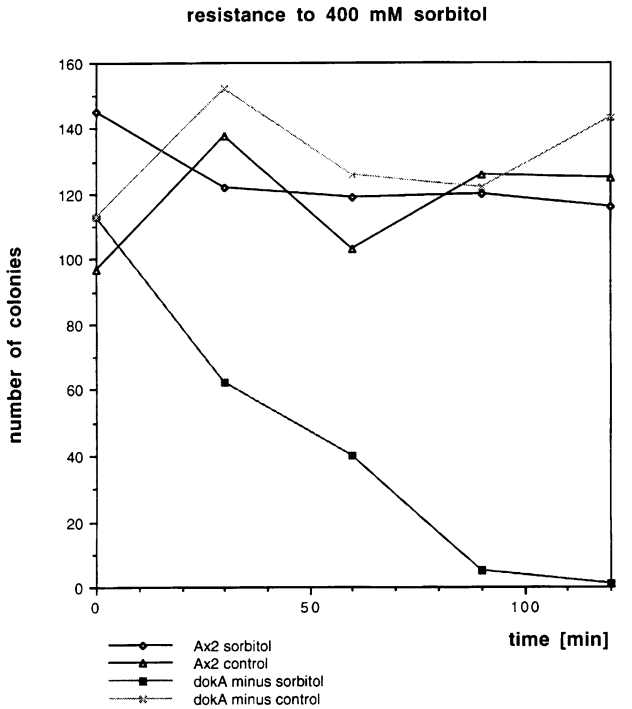

Fig. 5. Response to osmotic stress. Viability assay of osmotically shocked cells. Mutant cells that had been exposed to 400 mM sorbitol show decreasing viability over time (■). Wild-type viability was only marginally affected (◇). Wild type (△) and mutant cells exposed to buffer under identical conditions remained viable (×).

However, in no case was an increase observed for any of the investigated substances (data not shown).

Osmosensitive mutants have been found in *Dictyostelium* previously and some of these have been shown to be impaired in cyclic GMP production (Kuwayama *et al.*, 1996). A comparison of changes in cyclic GMP concentration in response to changes in osmolarity in wild-type and *dokA* mutant cells showed that the histidine kinase mutation does not affect this specific signaling process (data not shown).

Discussion

Two-component regulators in Dictyostelium

Autophosphorylation on a histidine residue in combination with subsequent phosphoryl transfer to a conserved aspartyl residue has been shown to constitute a predominant signaling pathway in prokaryotes. In contrast, the signaling network of the eukaryote *D.discoideum* is comprised of a complex circuitry of transient phosphorylations that fall into major eukaryotic signal transduction pathways involving serpentine receptors and tyrosine kinases, which eventually merge in a cascade of kinases that have been termed the MAP kinase pathway (Herskowitz, 1995; Segall *et al.*, 1995). The molecular motifs involved in these signal relays all display transient phosphorylation of tyrosine, threonine or serine residues. The finding of DokA, and also DhkA, is the first instance where histidine kinases have been demonstrated in *D.discoideum*. Comparison of their conserved kinase and regulatory domains (Figure 1B and C) reveals a high degree of homology to the bacterial-type kinases.

A rapidly growing number of histidine kinases has been found recently in eukaryotes such as yeast (Ota and Varshavsky, 1993), *Neurospora crassa* (Alex and Simon, 1994) and *A.thaliana* (Chang *et al.*, 1993). However, it has not been demonstrated that these histidine kinase domains function in a manner analogous to the bacterial systems. The phosphoryl group from acetyl phosphate is known to be transferred specifically to the conserved aspartyl residue of many response regulators (Stock, 1995), thereby substituting for the kinase as a donor. By phosphorylation *in vitro* through acetyl phosphate, we were able to demonstrate functionality of a response regulator domain of a eukaryotic hybrid kinase, using the C-terminus of DokA. The phosphoryl transfer from a 'small molecule phosphate donor' would allow direct activation of the hybrid histidine kinase via the regulator domain, thereby circumventing control of the kinase activity through the N-terminal signal detector domain. This reverse use of a hybrid kinase is in agreement with observation from ArcB where the autophosphatase activity of the regulator domain is modulated by the ligand lactate (Iuchi, 1993), which binds to the regulator domain but not to the N-terminal signaling domain.

A soluble hybrid kinase

The presence of two membrane-spanning regions in the N-terminal domain has been a hallmark for the subclass of hybrid kinases (Alex and Simon, 1994). DokA is very probably the first member of this subclass to deviate from this paradigm. Hydrophilicity plots performed with the Kyte–Doolittle (Kyte and Doolittle, 1982) and Hopp–Woods (Hopp and Woods, 1981) algorithms predict an entirely soluble signal detector domain. Calculations done by the 'positive inside rule' (Claros and von Heijne, 1994) suggest the rudimentary assets of two transmembrane domains that may have been lost in evolution. These domains are more evident in the case of DhkA, which shares a common ancestor with DokA. The only known cases where soluble histidine kinases have been described are CheA, NtrB and FrzE (Alex and Simon, 1994 and references therein). These proteins, however, do not share the domain organization of hybrid kinases. Two of them, FrzE and NtrB, detect an intracellular signal through a separate signal detector, which in the case of NtrB is the protein PII. The protein PII senses levels of α-ketoglutarate and glutamate in *E.coli* and subsequently controls the glutamine synthetase.

Location relative to the membrane can be indicative of a protein's role in a signal transduction pathway. In the case of DokA, a role in the response pathway to osmotic stress has been demonstrated. Its soluble state, however, suggests a different mechanism for sensing osmotic stress, when compared with the two membrane-bound sensor kinases Sln1 (Maeda *et al.*, 1995) and EnvZ (Mizuno *et al.*, 1982b), which are involved in the osmotic response in yeast or *E.coli*. Both these kinases have been suggested to possess a periplasmic receptor domain that senses and transmits a signal to the cytoplasmic kinase module.

The signal sensed by DokA is either the increase in the intracellular concentration of osmolytes that is caused by shrinkage of the cells, or a 'second messenger', that is generated by a primary osmosensing device. Second messengers could be polyamines, such as putrescine, or trehalose derivatives. Fluctuations in polyamine levels

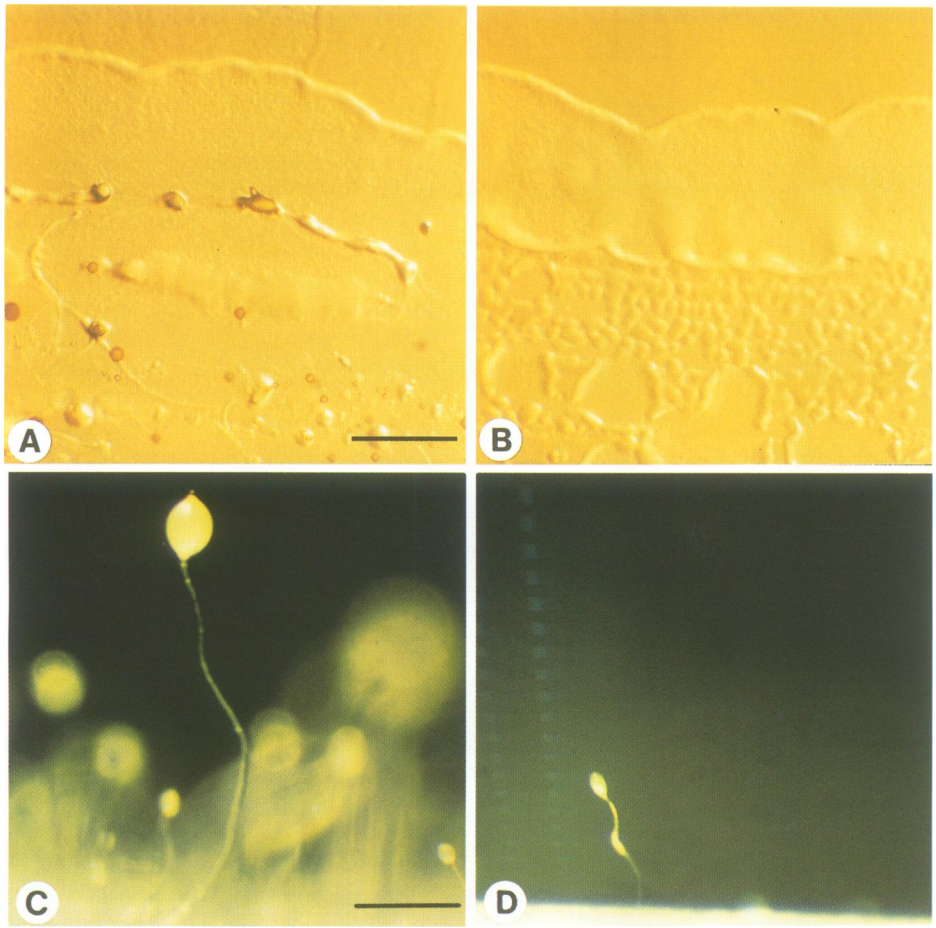

Fig. 6. Impaired development under osmotic stress. Growth of wild-type and *dokA* mutant cells on SM agar plates in the presence of 200 mM sorbitol. The bacterial lawn is consumed by the growing amoebae at an identical rate in (**A**) wild-type, (**B**) *dokA*-strain S2/25-1 (bar equals 1.11 mm). Whereas the wild-type AX2 (**C**) was capable of forming fruiting bodies of normal size, under these conditions *dokA*$^-$ cells only occasionally formed fruiting bodies that were of small size (**D**) (bar equals 280 μm).

have been observed in *D.discoideum* in response to changing osmotic strength.

Response to osmotic stress

Bacteria, amoebae and mammalian cells have three general means to respond to osmotic stress. As an immediate response, in order to re-equilibrate osmotically, the cells pump potassium ions into the cytoplasm. This response, however, has its limitations, as a 2-fold increase of the intracellular potassium concentration (from 140 to 280 mM) inhibits vital cell processes such as transcription and translation. In a situation of continuous stress, the cells synthesize or import 'compatible osmolytes' (small organic molecules) that are exchanged against potassium ions and allow them to maintain a minimal cell volume crucial for survival. Recovery through synthesis or transport takes up to several hours, making it an unfeasible mechanism for the immediate response. Transcriptional responses that improve the cell's osmotic stability can occur on the scale of one to several generation times (Kwon and Handler, 1995; and references therein).

The role of DokA in this response pathway remains unclear: *dokA* deletion causes a dramatic reduction in the viability of cells at high osmolarity (400 mM sorbitol) and inhibition of development at lower hypertonicity (200 mM sorbitol). The reduced stress at 200 mM sorbitol does not impair growth on bacterial lawns, suggesting that activation of genes that control development is most sensitive to the loss of *dokA*. In yeast, similar water stress leads to activation of glycerol-3-phosphate dehydrogenase (GPD1) via the MAP kinase pathway involving Ssk2/22, Pbs2 and Hog1 (Maeda *et al.*, 1995). This cascade of kinases is negatively regulated through the sensor kinase Sln1, a histidine kinase homolog, and through its cognate receiver Ssk1. Yeast has chosen glycerol as an osmoprotectant, taking advantage of its pronounced glycolysis (Mager and Varela, 1993). We have investigated whether glycerol plays a role in the response to osmostress in *D.discoideum*. However, no increase in the intracellular glycerol level was found. Other 'compatible osmolytes', such as α-ketoglutarate, glutamate and proline were also investigated, but the observed levels did not suggest any involvement in osmoprotection.

Osmosensitive mutants that have been found previously in *D.discoideum* are all directly or indirectly impaired in cytoskeletal functions. Adaptation to rapid changes in the cell's volume requires fast assembly and disassembly of actin and myosin filaments. Signaling pathways that coordinate these complex filamentous networks involve the activation of guanylyl cyclase. In a recent report, it was demonstrated that changes in the intracellular cGMP levels alter the cell's ability to regulate the assembly of

myosin II (Kuwayama *et al.*, 1996). We found that *dokA* mutant cells can regulate their cGMP level as can the wild-type, indicating that the histidine kinase acts either downstream of cGMP or in a parallel pathway.

Morphogenetic defects of dokA mutants

Inactivation of the *dokA* gene results in little or no effect on growth in axenic medium and on bacterial lawns. Starvation-induced development on filters, however, does clearly distinguish the *dokA* null mutant from the wild-type AX2. The most intriguing observation is the reduced number of spores which are found in the sorus. A reasonable hypothesis is that spore formation is inhibited through an alteration of the osmotic conditions in the spore head. Spores are formed by removing water from the cells and encapsulation of the condensed cytoplasm by a rigid cell wall. It is conceivable that this process is disturbed in mutant cells lacking an osmosensor. This notion coincides with the fact that PKA (cAMP-dependent protein kinase) mutants display an offset in their osmotic balance, resulting in a dysfunctionalized spore organization (P.Schaap, personal communication). Also, pre-spore-specific inhibition of PKA by preventing cAMP binding results in a glassy spore head (Hopper *et al.*, 1993), causing a phenotype similar to that of *dokA*$^-$ cells. In contrast, constitutive inhibition of PKA prevents cell aggregation and development in *D.discoideum* (Firtel and Chapman, 1990; Schulkes and Schaap, 1995). Whether DokA and PKA are involved in the same pathway is currently under investigation.

Materials and methods

Growth of Dictyostelium cells

Cells of the *D.discoideum* strain AX2-214 were cultivated axenically in shaken suspension at 23°C to a density of not more than 5×10^6/ml. For development, cells grown axenically to 5×10^6 cells/ml were washed in 17 mM Soerensen phosphate buffer, pH 6.0, and plated on filters (Type TA, Millipore) supported by phosphate-buffered agar, pH 6.0, or soaked Whatmann paper. Lids of the Petri dishes were supplied with Whatmann papers soaked in 1 M sodium/potassium phosphate buffer pH 6.0 (Newell *et al.*, 1969).

Transformations using the pn1b vector (Leiting and Noegel, 1988), G418 selection (Witke *et al.*, 1992), sporulation assays and vital staining by neutral red (Haugwitz *et al.*, 1994) were carried out as described.

Cloning and sequencing of the dokA gene

DNA sequences from four bacterial histidine kinases, BarA (Nagasawa *et al.*, 1992), ArcB (Iuchi *et al.*, 1990), RcsB (Gottesman and Stout, 1991) and LemA (Hrabak and Willis, 1992) were used to compute a consensus sequence of the kinase domain using the GCG package (University of Wisconsin). Degenerated oligonucleotides were designed against the H and the N box of histidine kinases (Parkinson and Kofoid, 1992) in accordance with the codon usage of *D.discoideum* (Sharp and Devine, 1989). The two primers used were H1 (5′-CAT/C GAI C/ATI AA/GI ACI CCI C/ATI-3′) and N1 (5′-CTA/G T/CTA/G CCI GGI CCI TAA/G/T-3′). PCR was used to amplify sequences of genomic *Dictyostelium* DNA. Amplified DNA was cloned into pSK II (+) pBluescript vector (Stratagene) and sequenced using automated fluorescent dye sequencing. The sequenced insert coded for a 375 bp fragment (125 amino acids) with a shared amino acid identity of 46% to the bacterial sensor kinase BarA. This fragment was used in the subsequent screening of genomic *D.discoideum* DNA libraries, which were constructed from size-selected DNA fragments of the corresponding size of the hybridization signals.

The complete ORF of the *dokA* gene was obtained by cloning a 6.5 kb *Eco*RI and a 4 kb *Eco*RV fragment of genomic AX2 DNA into the pSK II (+) vector (Stratagene, La Jolla). Sequencing was performed on an ABI 373 sequencer using dye-terminators. Single sequencer runs were analyzed and assembled using the software Seqed (Applied Biosystems) and Assemblyline (IBI Kodak). The obtained *Eco*RI and *Eco*RV fragments overlap by 925 bp.

dokA$^-$ cells

A gene replacement vector was constructed by cloning a 700 and a 684 bp PCR product, coding for base pairs 2350–3050 (plus engineered *Eco*RI–*Sac*I sites) and 4129–4813 (plus engineered *Xba*I–*Sph*I sites), respectively, of *dokA* into sites that flank the Geneticin (G418) resistance cassette of the pn1b vector (Leiting and Noegel, 1988). Thereby, 1079 bp of the gene were omitted from the ORF. The resulting vector pDicDelV was digested with *Sph*I–*Eco*RI, and the purified insert was electroporated into AX2 wild-type cells. Transformants were selected with 7.5 μg/ml of G418 in axenic media. Transformants were plated on bacterial agar plates, and single colonies were isolated and recloned. Twenty five clones from five independent transformations were analyzed by Southern blot analysis. Two to 15 clones of each individual transformation displayed an insertion within the *dokA* gene, as seen by a 1 kb shift in an *Eco*RI fragment. Isolated clones were tested subsequently on bacterial agar plates and on filters for morphological alterations. Cell biological experiments were conducted with the two strains S2/25-1 and M 2-1 obtained in independent transformation experiments.

Southern blotting and colony screening

Genomic DNA from wild-type (AX2) and the *dokA* mutant (S2/25-1) was prepared according to Noegel *et al.* (1985a). Twenty μg were digested with the appropriate enzyme and size separated on a 1% agarose gel. The DNA was transferred to a Hybond-N membrane by vacuum blotting for 45 min and fixed through UV cross-linking. Pre-hybridization and hybridization were carried out in Rapid-Hyb Mix buffer (Amersham) at 60°C for 20 and 120 min, respectively. ^{32}P-Labeled probes were prepared by the random prime method using prime-it II (Stratagene). PCR fragments (350 bp) were used as templates for the priming reactions. Washing of the Southern blots was performed in 2× SSC, 0.1% SDS at room temperature for 15 min and twice in 0.1× SSC, 0.1% SDS at 60°C. The membrane was then exposed to an X-ray film (Kodak) at −70°C with intensifying screens or a PhosphoImager screen (Fuji).

Northern blot analysis

Total cellular RNA was prepared as described (Noegel *et al.*, 1985b) or by use of the RNeasy kit (Qiagen). Ten μg of RNA per lane were separated by gel electrophoresis on a 1% formamide gel and transferred and immobilized as described (Sambrook *et al.*, 1989). Hybridization, probe preparation and washing were performed as for the Southern blot analysis.

Osmotic shock experiments

Axenically grown cells were harvested at a density of 5×10^6 cells/ml and washed in 17 mM Soerensen phosphate buffer, pH 6.0. Then 3×10^7 cells/ml were shaken in 25 ml of the buffer at 150 r.p.m. at 23°C for 1 h. Sorbitol (Sigma) was added to a final concentration of 400 mM. After various times of incubation (0, 30, 60, 90 and 120 min) in the hypertonic medium, cells were diluted into the phosphate buffer and plated at ~100 cells/plate on SM-agar with *K.aerogenes*. After incubation for 48 h at 23°C, colonies were counted. Wild-type and mutant control cells that had not been exposed to sorbitol were shaken for the times indicated above.

Intracellular osmolyte was determined in a lysate of osmotically shocked cells. Cells were lysed by incubation at 95°C for 2 min. Glycerol content was measured according to Bergmeyer (1974). The amino acid content of the lysate was determined by the use of an automated amino acid analyzer (Biotronik Amino Acid Analyzer LC 6001). The lysate was prepared by the following protocol: 3×10^7 cells were resuspended in 150 μl of 0.04 M lithium citrate, pH 2.2. Subsequently, the samples were heated for 90 s in a boiling water bath and centrifuged for 20 min at 20 800 *g* in an Eppendorf centrifuge; 5% w/v 5-sulfosalicylic acid was added to the supernatant and the mixture was shaken for 30 min. After removal of the precipitate by centrifugation (15 min, 20 800 *g*), LiOH (4 M) was added to adjust the pH of the supernatant to 2.2.

Heterologous expression of the regulator domain of dokA

A fragment corresponding to amino acids 1511–1671 of the *dokA* sequence was expressed in *E.coli* strain M15 (Qiagen). Using PCR, two restriction sites were engineered flanking the sequence of the fragment. Subsequent cloning into the *Eco*RI–*Bam*HI-cut vector pQE12 (Qiagen) resulted in a 161 amino acid fragment (RR161) with no residues added to the DokA sequence. The RR161 protein was purified from inclusion

bodies using standard protocols. Acetyl phosphate experiments were carried out according to Lukat *et al.* (1992). ^{32}P-Labeled acetyl phosphate was synthesized by the protocol of Stadtman (1957). ^{32}P-Labeled protein was run on a 18% SDS–PAGE with subsequent exposure to X-ray film (Kodak).

Accession number

The GenBank accession number for the *dokA* sequence is X96869.

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