Polar accumulation of the metabolic sensory histidine kinases DcuS and CitA in *Escherichia coli*

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Signal transduction in prokaryotes is frequently accomplished by two-component regulatory systems in which a histidine protein kinase is the sensory component. Many of these sensory kinases control metabolic processes that do not show an obvious requirement for inhomogeneous distribution within bacterial cells. Here, the sensory kinases DcuS and CitA, two histidine kinases of *Escherichia coli*, were investigated. Both are membrane-integral and involved in the regulation of carboxylate metabolism. The two-component sensors were fused with yellow fluorescent protein (YFP) and live images of immobilized cells were obtained by confocal laser fluorescence microscopy. The fluorescence of the fusion proteins was concentrated at the poles of the cells, indicating polar accumulation of the sensory kinases. For quantitative evaluation, line profiles of the imaged fluorescence intensities were generated; these revealed that the fluorescence intensity of the polar bright spots was 2.3–8.5 times higher than that of the cytoplasm. With respect to the cylindrical part of the membrane, the values were lower by about 40 %. The polar accumulation was comparable to that of methyl-accepting chemotaxis proteins (MCPs) and MCP-related proteins. The degree of DcuS–YFP localization was independent of the presence of MCP and the expression level of *dcuS-yfp* (or DcuS concentration). The presence of effector (fumarate or citrate, respectively) increased the polar accumulation by more than 20 %. Cell fractionation demonstrated that polar accumulation was not related to inclusion body formation. Therefore, sensory kinases DcuS and CitA, which regulate metabolic processes without obvious polar function, exhibit polar accumulation.

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INTRODUCTION

Prokaryotes sense a large number of extra- and intracellular stimuli and adapt their metabolism, cell composition and development to the external environment. Environmental stimuli are frequently perceived by two-component systems consisting of a membrane-bound sensory histidine kinase and a corresponding response regulator (Mascher *et al.*, 2006). The perception of external stimuli induces autophosphorylation of the sensory histidine kinase, and then the phosphoryl group is transmitted to the response regulator, which triggers the cellular response mostly by gene regulation.

The C₄-dicarboxylate sensor DcuS of *Escherichia coli* represents a typical membrane-bound histidine protein

kinase. It contains a distinct periplasmic sensory domain for C₄-dicarboxylates that is situated between two transmembrane helices (Golby *et al.*, 1999; Zientz *et al.*, 1998). The structure of the periplasmic domain of DcuS in the liquid state has been solved by NMR (Pappalardo *et al.*, 2003), and the binding site for C₄-dicarboxylates has been characterized by mutagenesis (Kneuper *et al.*, 2005; Krämer *et al.*, 2007). The second (or C-terminal) transmembrane domain is followed by the cytoplasmic transmitter domain including the histidine kinase domain. DcuS controls the expression of genes required for fumarate respiration (*dcuB*, *fumB* and *frdABCD*) that encode the fumarate/succinate antiporter DcuB, fumarase FumB and fumarate reductase FrdABCD (Golby *et al.*, 1999; Jausch *et al.*, 2002a, b; Zientz *et al.*, 1998). Expression of *dcuB* occurs under anaerobic conditions, and strongly depends on induction by DcuS in the presence of C₄-dicarboxylates, such as fumarate or malate. Thus, expression of *dcuB-lacZ* reporter-gene fusions has been used to assay the functional state of DcuS *in vivo* (Kneuper *et al.*, 2005; Zientz *et al.*,

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Abbreviations: MCP, methyl-accepting chemotaxis protein; YFP, yellow fluorescent protein.

Supplementary data (including five movie files) are available with the online version of this paper.

1998). After signal perception and autophosphorylation of DcuS, the phosphorylated response regulator DcuR binds to target genes and activates their expression (Abo-Amer *et al.*, 2004; Janausch *et al.*, 2004). With all the attributes of a periplasmic sensory histidine kinase, the individual DcuS homodimers are assumed to function for signal perception and transmission to response regulators (Mascher *et al.*, 2006). CitA is a citrate-specific sensor kinase of the CitAB two-component system of *E. coli* and related bacteria (Kaspar *et al.*, 1999; Kaspar & Bott, 2002). Because of the similarities in sequence and overall topology, both CitA and DcuS are members of the CitA family of sensory kinases.

An uneven cellular distribution of histidine kinases controlling asymmetric processes in cell division and development has been observed in *Bacillus subtilis*, *Pseudomonas* and *Caulobacter* (Boyd, 2000; Shapiro *et al.*, 2002). The localization of these histidine kinases within the cell often relates to their site of function, and typically features a polar localization. The chemoreceptors of chemotaxis (Lybarger & Maddock, 2000) show also an asymmetric distribution within *E. coli* cells, although no asymmetric distribution is expected for chemotaxis regulation. A uniform distribution over the cell membrane is assumed for kinases controlling metabolic processes or perceiving stimuli that are evenly distributed over the cell.

Here, the cellular distribution of DcuS and CitA, representing related sensory kinases of the CitA family, was studied in detail. The sensory kinases (DcuS and CitA) control metabolic processes (fumarate respiration and citrate fermentation, respectively) without obvious asymmetric distribution in the bacterial cell. Fusions of DcuS or CitA with a modified form of green fluorescent protein allowed careful analysis of the distribution of these kinases in the cell membrane of *E. coli* by confocal laser scanning microscopy.

METHODS

Bacteria and molecular genetic methods. *E. coli* K-12 strains and plasmids used in this study are listed in Table 1. Molecular genetic methods were performed according to standard procedures (Sambrook & Russell, 2001). Genomic DNA was isolated as previously described (Chen & Kuo, 1993); plasmids were isolated and purified using kits (Qiagen). PCR products were purified with the Qiaquick PCR purification kit (Qiagen). *E. coli* strains were transformed by electroporation (Dower *et al.*, 1988).

Construction of *dcuS-yfp* and *citA-yfp* fusions. The *yfp* gene was amplified from plasmid pEYFP (Clontech) by PCR with oligonucleotide primers gfpCfor (5'-CCATGGAGCTCAAGGGCGA-3') and gfpCrev (5'-AGCGACCGAAGCTTAGTTGG-3'). The PCR fragment lacking the sequence of the three N-terminal amino acids of EYFP was cloned via the *SacI* and *HindIII* sites of the oligonucleotides

Table 1. Strains of *E. coli* and plasmids used in this study

Strain or plasmid	Genotype	Reference or source
<i>E. coli</i> K-12		
MC4100	F ⁻ <i>araD139</i> Δ (<i>argF-lac</i>)U169 <i>rpsL150</i> Δ <i>lacZ</i> <i>relA1</i> <i>flbB530</i> <i>deoC1</i> <i>ptsF25</i> <i>rbsR</i>	Silhavy <i>et al.</i> (1984)
MG1655	CGSC 6300, <i>fmr</i> ⁻ , λ -F-P1-sensitive	Jensen (1993)
JM109	<i>recA1</i> <i>supE44</i> <i>endA1</i> <i>hsdR17</i> <i>gyrA96</i> <i>relA1</i> <i>thi</i> Δ (<i>lac-proAB</i>) F'[<i>traD36</i> <i>proAB</i> ⁺ , <i>lacI</i> ^q <i>lacZ</i> Δ M15]	Yanisch-Perron <i>et al.</i> (1985)
IMW205	MC4100 <i>dcuR</i> ::Kan ^r	Zientz <i>et al.</i> (1998)
IMW237	MC4100 λ [(<i>dcuB'</i> -' <i>lacZ</i>) <i>hyb</i> , <i>bla</i> ⁺]	Kneuper <i>et al.</i> (2005)
IMW260	MC4100 λ [(<i>dcuB'</i> -' <i>lacZ</i>) <i>hyb</i> , <i>bla</i> ⁺] <i>dcuS</i> ::Cam ^r	Zientz <i>et al.</i> (1998)
IMW262	MC4100, <i>dcuS</i> ::Cam ^r	Zientz <i>et al.</i> (1998)
IMW280	MC4100 λ [(<i>dcuB'</i> -' <i>lacZ</i>) <i>hyb</i> , <i>bla</i> ⁺] <i>citA</i> ::Kan ^r	Krämer <i>et al.</i> (2007)
IMW502	MC4100 Δ <i>dcuB</i>	This study
UU1250	Δ <i>tsr-7028</i> Δ (<i>tar-tap</i>)5201 Δ <i>trg-100</i> Δ <i>aer-1</i> (Kan ^r)	Ames <i>et al.</i> (2002)
VS100	Δ <i>cheY</i> , in-frame <i>cheY</i> deletion	Sourjik & Berg (2000)
Plasmids		
pBAD30	Expression vector; pACYC <i>ori</i> , P _{BAD} promoter (Ap ^r)	Guzman <i>et al.</i> (1995)
pDK108	Tar(1-331)-YFP expression plasmid; pTrc99a derivative (Ap ^r)	Kentner <i>et al.</i> (2006)
pET28a	Expression vector; pBR <i>ori</i> , T7 promoter, His-tag (Kan ^r)	Novagen
pEYFP	Vector containing enhanced GFP variant YFP (Ap ^r)	Clontech
pMW151	DcuS expression plasmid; pET28a derivative (Kan ^r)	Janausch <i>et al.</i> (2002a)
pMW384	DcuS-YFP expression plasmid; pMW391 derivative (Kan ^r)	This study
pMW391	<i>yfp</i> , protein fusion plasmid; pET28a derivative (Kan ^r)	This study
pMW407	DcuS-YFP expression plasmid; pBAD30 derivative (Ap ^r)	This study
pMW442	CitA-YFP expression plasmid; pBAD30 derivative (Ap ^r)	This study
pMW557	pMW442 with additional Cam ^r	This study
pVS1	CheY-YFP expression plasmid; pBAD18K derivative (Kan ^r)	Sourjik & Berg (2000)

into pET28a (Novagen) behind the inducible T7 promoter. The resulting construct (pMW391) served as the plasmid for cloning EYFP to the C-terminal end of proteins. *dcuS* was amplified by PCR with oligonucleotide primers *dcuS*petCfor (5'-CGGCAGCCATA-TGAGACATTC-3') and *dcuS*petCrev (5'-TGTTCGAGAGCTCCCC-GTC-3') from pMW151 (Janausch *et al.*, 2002a). The PCR fragment was cloned via the *NdeI* and *SacI* sites into pMW391 at the 5' end of *eyfp*. The resulting construct (pMW384) encoded DcuS(1–539)-(Lys)-EYFP(4–240) (hereafter referred to as *dcuS*-*yfp* or DcuS-YFP fusion). The fusion protein also carried an N-terminal His₆-tag and a thrombin cleavage site. The *dcuS*-*yfp* fusion was inserted into pBAD30 using restriction endonuclease *XbaI* (pMW407). CitA-YFP was constructed as described for DcuS-YFP. The *citA* gene was amplified from genomic DNA of *E. coli* MG1655 by PCR with oligonucleotide primers *citA*petCfor (5'-CAACCCGCTAGCAAAA-CAATG-3') and *citA*petCrev (5'-CGTCTCAGAGCTCAATAGG-3') and by cloning the PCR fragment via *NheI* and *SacI* sites into pMW391. The *citA*-*yfp* fusion was subsequently inserted into pBAD30 via *XbaI* (pMW442). The sequences of the resulting constructs were verified by DNA sequencing.

Growth and expression. For microscopy, cell fractionation and Western blot analysis, bacteria were grown aerobically in LB medium at 30, 33, or 37 °C for 3–4.5 h, corresponding to the mid-exponential phase of growth (Sambrook & Russell, 2001). *E. coli* strains IMW262/pMW407, JM109/pMW407, UU1250/pMW407 and JM109/pMW442 were induced from the beginning of incubation with 10–333 µM L-arabinose. Ampicillin was added to a concentration of 100 µg ml⁻¹. The reference strains VS100/pVS1 and JM109/pDK108 were grown aerobically in LB broth for 4 h at 33 °C in the presence of 333 µM arabinose and 50 µg kanamycin ml⁻¹, or 1 mM IPTG and 100 µg ampicillin ml⁻¹, respectively.

Cell fractionation. Arabinose-induced bacteria were harvested by centrifugation at 6300 g for 10 min. All further steps were done at 4 °C. Washed cells from 400 ml medium were resuspended in 8 ml buffer (50 mM Tris/HCl, pH 7.7, 10 mM MgCl₂). The bacteria were broken using a French press (3 times at 8274 kPa) and then centrifuged at 8600 g for 10 min to sediment debris and potential inclusion bodies (low-speed pellet) and to separate them from the soluble fraction and cytoplasmic membranes contained in the supernatant (low-speed supernatant). For (semi-dry) Western blot analysis, proteins from the low-speed pellet and the low-speed supernatant were separated by SDS-PAGE and transferred to nitrocellulose membranes as described by Towbin *et al.* (1979). Membranes were treated with mouse polyclonal antibodies (Qiagen) against the His₆-tag of the fusion protein. Protein bands were detected with secondary IgG antibodies coupled to peroxidase (Sigma-Aldrich). Protein bands of three independent preparations were visualized and quantified by using the Kodak Image Station 440CF and the Kodak Molecular Imaging software.

To sediment the membrane fraction, the low-speed supernatant was centrifuged at 200 000 g for 65 min. The membrane pellet was washed twice (1 mM Tris/HCl, pH 7.7, 3 mM EDTA), homogenized in buffer (50 mM Tris/HCl, pH 7.7, 10 %, w/v, glycerol), and solubilized by the addition of Empigen BB (30 %, w/v, Calbiochem) to a final concentration of 2 % (w/v) as described by Janausch *et al.* (2002a).

The low-speed supernatant fraction was fractionated in a sucrose step-gradient (0 %, 10 % and 40 % sucrose) by ultracentrifugation at 200 000 g for 2 h. Fractions of 1 ml were collected and 20 µl of each fraction was subjected to SDS-PAGE to examine banding of DcuS-YFP in the protein/membrane fraction and in the protein-aggregate fraction. Isolated and purified DcuS-YFP was used as a standard.

Western blot analysis of DcuS-YFP expression levels. *E. coli* cells induced by different arabinose concentrations were sedimented

by centrifugation and dissolved in SDS-containing sample buffer (Laemmli, 1970). The dissolved whole-cell proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Towbin *et al.*, 1979). Membranes were treated with rabbit polyclonal antiserum (Eurogentec) raised against the periplasmic domain of DcuS and detected with secondary IgG antibodies coupled to peroxidase (Sigma-Aldrich).

Test for DcuS and CitA function *in vivo*. *E. coli* IMW260 (*dcuS*::Cam^r, $\lambda[\Phi\text{dcuB}'\text{-}'\text{lacZ}]$) containing plasmids with various forms of *dcuS* as indicated was grown anaerobically at 37 °C in M9 mineral medium (Miller, 1992) with glycerol (50 mM), DMSO (20 mM) and fumarate (20 mM) as the inducer. Samples were withdrawn at OD₅₇₈ 0.5–0.7 for measurement of β -galactosidase activity. For testing CitA-YFP function, strain IMW280 (*citA*::Kan^r, $\lambda[\Phi\text{dcuB}'\text{-}'\text{lacZ}]$) was used, where expression of *dcuB*'-'*lacZ* responds to the presence of functional CitA (Krämer *et al.*, 2007). The bacteria with or without plasmid pMW557 (*citA*-*yfp* in pBAD30, Cam^r) were grown as described for strain IMW260, but with citrate (20 mM) as the inducer.

Spectroscopy. Absorption and fluorescence spectra were measured in 1 ml semi-microcuvettes with a Bruins Instruments Omega 20 spectrophotometer and a Jobin-Yvon Spex FluoroMax-2 spectrofluorimeter. Fluorescence spectra were corrected for the wavelength dependence of the fluorimeter and the inner filter effect.

Microscopy. For microscopy, *E. coli* cells were harvested, immobilized with poly-lysine films on backed coverslips, and washed with PBS buffer. All measurements were made in PBS buffer under ambient conditions using two different confocal fluorescence microscopes. On the one hand, a custom-built microscope (based on a Zeiss Axiovert 135 TV inverted microscope) was used, which has single-molecule sensitivity (Erker *et al.*, 2005; Kulzer *et al.*, 1999). Images with a typical size of 10 µm × 10 µm and 128 × 128 pixels were recorded with an integration time of 5 ms per pixel (objective: Zeiss Plan-Neofluar 100 × /1.30 oil). An argon-ion laser operating at 488 nm attenuated to a power of 0.5 µW was used for excitation. Emission was split into two beams (50:50), which simultaneously allowed the detection of total intensity (APD: Perkin-Elmer SPCM-AQR-14) and the recording of fluorescence spectra (spectrograph, Acton Spectra Pro-300i; Peltier-cooled CCD-camera, LaVision IM3 QE CAM). Images were recorded by moving the sample via a 3D piezo-scanner (Physik Instrumente P-731.20 and P-721.CLQ PIFOC). Alternatively, a commercial confocal microscope (Leica DM IRE2) with an argon-ion laser (488 nm, 0.4 µW) was used. The recorded images were typically 11.72 µm × 11.72 µm in size with a resolution of 512 × 512 pixels (objective: Leica HCX PL APO 40 × /1.25–0.75 oil).

The cellular brightness distributions of individual cells were analysed by line scans and 3D-reconstruction using self-written software based on Igor Pro (Wavemetrics). For 3D-reconstruction, a stack of images was recorded for each cell. The images differed with respect to focus position along the optical axis. Typical *z*-increments were in the range of 150–200 nm.

RESULTS

Functional state of DcuS-YFP and CitA-YFP fusion proteins

The *dcuS* gene of *E. coli* encoding the C₄-dicarboxylate sensory histidine kinase DcuS was fused to the *yfp* gene encoding the enhanced yellow fluorescent variant (YFP) of green fluorescent protein (GFP). For cloning, a low-copy

plasmid (pBAD30) was used to ensure low- or moderate-level expression of the cloned gene (Guzman *et al.*, 1995). The plasmid-borne *dcuS'*-*yfp* fusion encoding DcuS-YFP complemented anaerobic growth of a DcuS-deleted *E. coli* strain (IMW260) in the presence of glycerol and fumarate. In addition, the plasmid restored expression of the *dcuB'*-*'lacZ* reporter gene fusion in the presence of fumarate to a level approximately the same as that obtained with a plasmid carrying wild-type *dcuS* (Table 2). Both findings demonstrate that DcuS is functional upon fusion with YFP. The fluorescence properties of the bacteria containing DcuS-YFP were tested by fluorescence spectroscopy, which showed spectral characteristics for YFP fusion proteins (see supplementary data). Therefore, DcuS and YFP were both functional in the fusion protein. To ensure that the fluorescence was derived from membrane-integral DcuS-YFP, the presence of DcuS-YFP inclusion bodies and their contribution to the fluorescence of the cells was measured. Sucrose step-gradient centrifugation (not shown), cell fractionation and Western blot analysis showed that less than 32 % of DcuS-YFP protein and maximally 10 % of the total fluorescence of the cells resided in inclusion bodies (Table 3). Based on the small contribution of the inclusion bodies (<10 %), it can be concluded that the fluorescence in the bacteria corresponds to membrane-integral DcuS-YFP.

The functional state of CitA-YFP was confirmed in a similar way as for DcuS. The plasmid encoding CitA-YFP restored the function of CitA in a CitA-deleted *E. coli* strain (IMW280) to more than 90 % in the stimulation of *dcuB'*-*'lacZ* in the presence of citrate (not shown). Therefore CitA-YFP can be regarded as functional as well.

Fluorescence microscopy of single cells

The cellular distributions of the fusion proteins within *E. coli* were studied by live imaging of immobilized cells in buffer using confocal scanning fluorescence microscopy. Two different microscopes were used in order to reveal different aspects of the samples (see Methods). The custom-built microscope allowed synchronous recording of images and emission spectra (e.g. Supplementary Fig.

Table 2. Functional test of DcuS-YFP by induction of *dcuB'*-*'lacZ* expression

E. coli IMW260 [MC4100 *dcuS*::Cam^r, $\lambda(\Phi dcuB'$ -*'lacZ*)] was grown anaerobically in M9 medium containing glycerol and DMSO as growth substrates with and without fumarate (20 mM) as effector.

Strain	Expression of <i>dcuB'</i> - <i>'lacZ</i> (Miller units)	
	+ Fumarate	- Fumarate
IMW260 (<i>dcuS</i> ⁻)	5	8
IMW260 (pMW151; <i>dcuS</i> ⁺)	279	2
IMW260 (pMW384; <i>dcuS-yfp</i>)	332	9

Table 3. Distribution of DcuS-YFP fusion protein and fluorescence in cellular compartments of *E. coli*

The cell homogenate of *E. coli* IMW262/pMW407 (*dcuS*⁻) induced with 333 μ M arabinose for 4 h was separated into supernatant and pellet fraction by low-speed centrifugation. Quantitative Western blot analysis (mean of three independent preparations) was performed with both fractions. Fluorescence of low-speed supernatant (soluble proteins and membranes) and low-speed pellet fraction (debris and inclusion bodies) was detected after excitation at 488 nm.

Fraction	Western blot	Fluorescence
Low-speed supernatant	68 %	90 %
Low-speed pellet	32 %	10 %

S1) and was more sensitive (Supplementary Fig. S2). The commercial microscope was much faster and allowed the recording of more images per time interval, resulting in higher statistical significance.

In various *E. coli* wild-type strains expressing DcuS-YFP, the fluorescence intensity of most of the cells was not homogeneously distributed; instead, there were bright spots near the cell poles (Fig. 1, upper row). The individual cells showed spots at the poles that were much brighter than the fluorescence intensity at the membrane of the cylindrical part of the cell, and the fluorescence at the membrane was brighter than that in the cytoplasmic region. For quantitative evaluation of the fluorescence intensity in *E. coli* cells, line profiles were generated (lower row of Fig. 1) to reflect the fluorescence intensities of the cells along the lines indicated in the images of the upper row of Fig. 1. The line profile of the cell containing DcuS-YFP showed two (polar) peaks corresponding to the bright spots at the cell poles. The ratio of polar maximum to cytoplasm fluorescence intensity (ratio polar maximum/cytoplasm) was 6.9 for the first (polar) peak and 3.6 for the second one. For the same (polar) peaks, the ratios of polar maximum to the cylindrical part of the cell membrane (ratio polar maximum/membrane) were 4.3 and 2.2, respectively. The latter value might reflect the situation of membrane proteins more directly. For other proteins with polar localization mostly the former value is quoted (Lieberman *et al.*, 2004). Therefore, for reasons of comparability the former ratio (polar maximum/cytoplasm) will be used here as well.

The line profiles of most investigated cells revealed peaks at both cell poles. The fluorescence ratios (polar maximum/cytoplasm) of the brighter polar spots were typically in the range of 2.5–8.5 for the custom-built microscope (Supplementary Fig. S2) and in the range of 2.6–3.6 when measured with the commercial microscope (Table 4). For the data presented in Table 4, no preselection of cells was made, but all cells which gave a clear image were analysed. Heterogeneity of the analysed cells is represented by the histograms shown in Supplementary Fig. S3. Each value in Table 4 corresponds to the mean value of one of the histograms in Supplementary Fig. S3. 3D reconstruction of

Fig. 1. Localization of DcuS-YFP and CitA-YFP in *E. coli*. Upper row: single *E. coli* cells expressing DcuS-YFP (strain JM109/pMW407 and UU1250/pMW407) or CitA-YFP (strain JM109/pMW442) fusion protein recorded with a custom-built confocal microscope. Lower row: fluorescence-intensity profiles along the lines indicated in the images above.

individual cells confirmed the polar localization of DcuS-YFP (Supplementary data, movies 1–4).

Polar accumulation of the DcuS-YFP fusion protein was observed to the same extent not only in the DcuS-deficient strain (IMW262), but in the CheY-deleted strain (VS100) as well (data not shown). A similar result was observed in the chemoreceptor-deleted strain (UU1250) (Fig. 1), in which the five genes encoding the methyl-accepting chemotaxis protein (MCP) complexes had been deleted. DcuS-YFP also accumulated at the cell poles in strain UU1250, but the overall fluorescence intensity of the cells in this strain was, on average, 2.5-fold higher than that in the other strains (see line profiles).

The localization of CitA, a sensor kinase closely related to DcuS (Kaspar & Bott, 2002; Mascher *et al.*, 2006), was investigated in the same way. Again, a C-terminal fusion

with YFP was constructed (CitA-YFP) and its cellular localization was studied by fluorescence microscopy. The localization of CitA (Fig. 1) was the same as that of DcuS, i.e. the fusion protein was detected in the cell membrane and accumulated at the cell poles. The mean ratio (polar maximum/cytoplasm) obtained with the commercial microscope was in the range of 3.5–5.1 for CitA-YFP (Table 4).

Localization of DcuS-YFP and CitA-YFP versus proteins with polar, membranous or cytoplasmic localization

As a reference for a protein with known polar accumulation, CheY-YFP was tested. CheY is a water-soluble intracellular protein that associates with the MCPs, which are localized at the cell poles. The polar localization of CheY is comparable with that of the MCPs (Sourjik & Berg, 2000). Images and

Table 4. Influence of effectors fumarate and citrate on the polar accumulation of DcuS-YFP and CitA-YFP

Bacteria containing pMW407 expressing DcuS-YFP or pMW442 expressing CitA-YFP were grown at 30 °C for 4 h with 133 µM arabinose in the presence or absence of effector (20 mM of fumarate or citrate, respectively). Each value in the table represents the mean $\pm$ SD of the ratio (polar maximum/cytoplasmic fluorescence intensity) obtained from different cells (cell number *n*) using the commercial microscope. The corresponding histograms are plotted in Supplementary Fig. S3. No preselection was made but all cells which gave a clear image were analysed.

Protein	Strain (relevant chromosomal genotype)	Mean ratio with effector	Mean ratio without effector	<i>n</i> with/without effector
DcuS-YFP	Wild-type	3.2 $\pm$ 0.2	2.6 $\pm$ 0.2	257/248
	IMW262 (<i>dcuS</i> [−])	3.4 $\pm$ 0.1	2.6 $\pm$ 0.1	106/114
	IMW502 (<i>dcuB</i> [−])	3.6 $\pm$ 0.1	2.7 $\pm$ 0.1	102/115
	IMW205 (<i>dcuR</i> [−])	2.9 $\pm$ 0.1	2.7 $\pm$ 0.1	101/67
CitA-YFP	JM109 (wild-type)	5.1 $\pm$ 0.2	3.5 $\pm$ 0.2	129/124

line profiles of CheY–YFP (Fig. 2) showed the polar accumulation of this protein. The fluorescence intensity at the poles was three times higher than the intracellular level. Thus CheY, DcuS and CitA show polar accumulation to similar extents. Slight differences in the polar accumulation could be due to some differences in genetic background of the strains used for this experiments. As a control for a protein with an even distribution over the cell membrane, a YFP fusion with truncated Tar(1–331) was used. Tar is a membrane-bound chemoreceptor which localizes at the cell poles, whereas the C-terminal truncated Tar(1–331) is membrane bound, but without polar accumulation (Kentner *et al.*, 2006). Images of cells expressing the truncated Tar(1–331) fused to YFP revealed that fluorescence was symmetrically distributed over the entire cell membrane (Fig. 2). As a cytoplasmic protein, wild-type YFP was used for comparison. Water-soluble YFP was homogeneously distributed in the cytoplasm, resulting in a line profile of roughly constant fluorescence level within the cell but with lower fluorescence at the membranes (Fig. 2). *E. coli* cells expressing no YFP variants produced only autofluorescence, with an intensity 10-fold lower than that of cells expressing YFP fusions (see line profiles). This autofluorescence resulted in relatively weak but not specifically localized fluorescence within the cells (Fig. 2).

Polar accumulation of DcuS–YFP is independent of DcuS expression rate and time

Expression of the DcuS–YFP fusion protein was induced by various concentrations of arabinose. The pBAD30 vector is

generally used for moderate-level expression due to its low copy number. After induction with 133 or 333 μ M arabinose, high expression of DcuS–YFP was detected by Western blotting with anti-DcuS antiserum (Fig. 3). In the presence of 10 μ M arabinose, the expression of DcuS–YFP sharply decreased by a factor of 10 but was still significantly higher than that of the endogenous DcuS. Compared to the endogenous DcuS expression level (maximally 0.004 % of the cell protein), the amounts of DcuS–YFP induced by 10, 133 or 333 μ M arabinose were estimated to be about 0.04 %, 0.4 % or 0.4 %, respectively, of the cell protein. The further localization studies were performed with cells induced by arabinose concentrations as low as 10 μ M, and up to 333 μ M arabinose for other experiments. With the induction by 10 μ M arabinose, measurements could be performed at a low DcuS level as close as possible to the *in vivo* conditions, but at the fluorescence detection limit. On the other hand, induction by 333 μ M arabinose resulted in DcuS levels which were higher than the endogenous levels, but the inducer concentration was still much lower than that commonly used for localization studies.

Concentration dependence of arabinose induction and time-course of polar accumulation of DcuS–YFP was studied. The influence of inducer concentration on DcuS–YFP localization was studied at the single-cell level. The fluorescence intensity ratios, (polar maximum/cytoplasm) and (polar maximum/membrane), were plotted as a function of the arabinose concentration used for fusion-protein expression (Fig. 4). Despite some fluctuations in the two ratios, polar accumulation of DcuS–YFP not only already appeared at low concentrations of both inducer and

Fig. 2. Controls for proteins with polar, membranous and cytoplasmic localization in *E. coli*. Upper row: images of single *E. coli* cells expressing CheY–YFP (strain VS100/pVS1) or Tar(1–331)–YFP (strain JM109/pDK108) fusion proteins, wild-type YFP (JM109/pEYFP), or no YFP (JM109). The image of Tar(1–331)–YFP was recorded with the commercial microscope, the other images with the custom-built microscope. Lower row: fluorescence-intensity profiles along the lines indicated in the images above.

Fig. 3. Detection of DcuS-YFP in lysed cells of *E. coli* by Western blotting with antiserum against the periplasmic domain of DcuS. *E. coli* JM109/pMW407 was grown under aerobic conditions in LB broth in the presence of 0 μ M (lane 3), 10 μ M (lane 4), 133 μ M (lane 5) and 333 μ M (lane 6) arabinose. For lane 2, *E. coli* wild-type (JM109) was grown anaerobically in M9 mineral medium with 50 mM glycerol and 20 mM fumarate. Cells were sedimented, the pellet was dissolved in SDS sample buffer, and 60 μ g (lanes 3–6) or 120 μ g (lane 2) of cell lysates were subjected to SDS-PAGE. After blotting onto a nitrocellulose membrane, DcuS and DcuS-YFP were detected by immunostaining with antiserum against the periplasmic domain of DcuS. Lanes 1 and 7 contain 1 μ g of purified DcuS or DcuS-YFP, respectively.

DcuS-YFP, but remained constant at higher concentrations of both inducer and DcuS-YFP. Therefore polar accumulation of DcuS-YFP is independent of the expression level.

The time-course of DcuS-YFP and CitA-YFP localization was also investigated. After induction of DcuS-YFP or CitA-YFP expression from the beginning of incubation, cell samples were collected every 30 min and analysed for polar localization of the fusion proteins at the single-cell level. The maximum fluorescence intensity per cell and the ratio (polar maximum/cytoplasm) are plotted in Fig. 5. DcuS-YFP and CitA-YFP behaved very similarly with respect to their fluorescence-intensity kinetics (Fig. 5a). After a lag phase of about 1.5–2 h, fluorescence intensity

Fig. 4. Dependence of DcuS localization on expression by various concentrations of inducer (arabinose). Expression of the DcuS-YFP fusion protein (governed by an *araBAD* promoter) in *E. coli* (strain IMW262/pMW407) was induced with increasing concentrations of arabinose. Samples were harvested after 4.5 h. Single cells were imaged with the commercial confocal microscope. The intensity ratios (polar maximum/cytoplasm, ●) and (polar maximum/membrane, ○) are plotted (means $\pm$ SD of 3–28 cells).

Fig. 5. Dependence of localization on the time after induction of fusion protein expression. Expression of the fusion protein (DcuS-YFP, strain IMW262/pMW407, ●; CitA-YFP, strain JM109/pMW442, ○) in *E. coli* was induced with 333 μ M arabinose. Samples were collected every 30 min and single cells were imaged with the commercial confocal microscope. The means $\pm$ SD of 5–39 cells are plotted. (a) Peak fluorescence intensity of the bright spots within the cells; (b) intensity ratio (polar maximum/cytoplasm).

increased continuously. By contrast, with respect to their ratio kinetics, the two proteins behaved differently (Fig. 5b). The ratio for DcuS-YFP was close to 2.0 after 30 min of induction and persisted for at least 2 h. The ratio for CitA-YFP was 2.5 after 30 min and increased with time. Despite the different behaviour of their ratio kinetics, the polar accumulation of both DcuS-YFP and CitA-YFP was established already within the first 30 min after their expression, and their localization persisted for several hours.

Polar accumulation responds to the presence of effector

Polar accumulation of DcuS-YFP and CitA-YFP was studied in the presence or absence of the corresponding effectors (fumarate or citrate, respectively). The ratios (polar maximum/cytoplasm) of individual cells from independent cultures were averaged (Table 4 and Supplementary Fig. S3) and analysed by Welch's *t*-test (test hypothesis: the average does not change upon adding effector, significance level of 5%). The DcuS-YFP fusion protein in the DcuS deleted strain (IMW262) revealed a significant increase in polar accumulation upon the presence of the effector, fumarate. The same increase in

polar accumulation of DcuS–YFP was observed for both the DcuB-deleted strain (IMW502) and wild-type strain (MC4100). No increase in polar accumulation of DcuS–YFP was observed in the presence of acetate (data not shown), suggesting that the effect of fumarate on polar accumulation of DcuS–YFP is specific. In the DcuR-deleted strain (IMW205), the polar accumulation of DcuS–YFP increased only to a very low extent in the presence of fumarate. Taken together, the data suggest that presence of fumarate increases the polar accumulation of DcuS–YFP, and the presence of DcuR is required for this effect.

The polar accumulation of CitA–YFP significantly increased upon addition of citrate to the wild-type strain JM109, but no increase in polar accumulation of CitA–YFP was observed in the presence of fumarate (data not shown), suggesting that the effect of citrate on polar accumulation of CitA–YFP is specific.

DISCUSSION

DcuS–YFP and CitA–YFP accumulate at the cell poles of *E. coli*

The cellular localization of DcuS and CitA after fusion with YFP was studied. DcuS–YFP and CitA–YFP accumulated at one or both cell poles of *E. coli*, and the polar accumulation depended solely on the DcuS or CitA proteins, whereas YFP contributed neither to membranous nor to polar localization. The expression of recombinant proteins may result in the formation of inclusion bodies, which are frequently located close to the cell poles (Margolin, 2000), thus mimicking the polar localization of proteins. The potential artefact of inclusion bodies was excluded for DcuS–YFP by the following experiments. (i) After cells were fractioned, the fraction potentially containing inclusion bodies showed only a low fluorescence intensity (at most 10 % of the total). (ii) DcuS localization was found to be independent of the inducer (arabinose) concentration controlling *dcuS-yfp* expression, and therefore also independent of the protein level. (iii) Polar accumulation of DcuS–YFP or CitA–YFP became evident soon after induction at low levels of expression (Fig. 5b), implying that polar accumulation of DcuS–YFP or CitA–YFP was not due to overexpression of either fusion protein. Points (ii) and (iii) also argue against the formation of non-specific aggregates in the membrane. Altogether, the polar accumulation of DcuS and CitA appears to represent the native site of the kinases.

Polar accumulation can be quantified by a fluorescence intensity ratio (polar maximum/cytoplasm). The ratio for the polar accumulation of either DcuS–YFP or CitA–YFP was 2.5 or above, implying that these proteins accumulated but were not located exclusively at the cell poles.

Factors affecting polar accumulation of DcuS–YFP and CitA–YFP

The polar accumulation of DcuS–YFP and CitA–YFP was significantly increased in the presence of effector (fumarate

or citrate). In the case of DcuS, the increase depended on the presence of the related response regulator DcuR, suggesting that the polar accumulation is related to the functional state of the DcuSR two-component system. Both the polar accumulation at low concentrations of DcuS and its further increase in the presence of the effector fumarate could indicate that the polar accumulation is of physiological relevance, and that DcuR might play a role in DcuS localization. However, the mechanistic background for the supposed role of DcuR in polar accumulation of DcuS is not clear.

Comparison of DcuS accumulation with that of other localized proteins

MCPs represent the paradigm for polar-localized (or accumulated) sensory proteins (Lybarger & Maddock, 2000; Maddock & Shapiro, 1993; Sourjik & Berg, 2000). MCP-associated proteins, such as CheY, also accumulate at the cell poles to a similar extent. The fluorescence ratios (polar maximum/cytoplasm) for MCP-related proteins were reported to be in the range of 1.2–2.2, and for CheY about 1.6 (Sourjik & Berg, 2000). In the present study, fluorescence ratios (polar maximum/cytoplasm) in the range of 2.6–3.6 (Table 4) were determined for DcuS, and a similar range was observed for CitA. Thus, the amount of DcuS that accumulated at the cell poles was comparable to that of the chemotaxis proteins even if the methods to determine the ratios were slightly different in the two studies. However, the polar accumulation of DcuS did not depend on the presence of the MCP complex. In the absence of MCP (strain UU1250), DcuS still accumulated at the cell poles (Fig. 1); hence, there seems to be no direct interaction between DcuS and MCP.

So far, polar accumulation of sensory kinases has been shown for two-component systems that control reactions with an uneven or a polar distribution, such as the segregation of cell components in symmetric (Boyd, 2000) or asymmetric cell division and development (Jensen *et al.*, 2002; Shapiro *et al.*, 2002; Wheeler & Shapiro, 1999; Wingrove & Gober, 1996). In these examples, histidine kinases often localize at the sites of their regulated processes or their partners. As shown here for DcuS and CitA, polar or asymmetric localization can be found also for sensory histidine kinases that control metabolic processes without known or predicted asymmetric distribution in the cell. It is possible that such polar accumulation is not restricted to DcuS and CitA.

For DcuS and CitA, the degree of polar accumulation increased in the presence of the corresponding effector (fumarate or citrate, respectively). This could indicate that the polar accumulation is functionally relevant and that it supports proper function of the two sensor kinases in an unknown way. Clusters of chemotaxis sensors together with the sensor-related proteins CheA and CheW are well documented (Lybarger & Maddock, 2000). The clusters are believed to support stimulus integration and the sensitivity of

the sensors (Sourjik, 2004; Thiem *et al.*, 2007). However, chemotaxis requires no asymmetric distribution for the function of the sensory complexes, and the role of polar localization of the complexes is not understood. It could be related to the diffusion restriction of cell envelope proteins in the polar region (de Pedro *et al.*, 2004), which might extend to the cytoplasmic membranes. Furthermore, polar localization could be affected by phospholipids, e.g. in cardiolipin domains (Matsumoto *et al.*, 2006). Consequently, it has been suggested that membrane proteins can be trapped in lipid patches of specific composition due to increased solubility. Such a mechanism for polar accumulation is also feasible for DcuS and CitA.

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