CitA (citrate) and DcuS (C4-dicarboxylate) sensor kinases in thermophilic Geobacillus kaustophilus and Geobacillus thermodenitrificans

Sabrina Graf, Constanze Broll, Juliane Wissig, Alexander Strecker, Maria Parowatkin and Gottfried Unden

The thermophilic Geobacillus thermodenitrificans and Geobacillus kaustophilus are able to use citrate or C4-dicarboxylates like fumarate or succinate as the substrates for growth. The genomes of the sequenced Geobacillus strains (nine strains) each encoded a two-component system of the CitA family. The sensor kinase of G. thermodenitrificans (termed CitA_Gt) was able to replace CitA of Escherichia coli (CitA_Ec) in a heterologous complementation assay restoring expression of the CitA_Ec-dependent citC–lacZ reporter gene and anaerobic growth on citrate. Complementation was specific for citrate. The sensor kinase of G. kaustophilus (termed DcuS_Gk) was able to replace DcuS_Ec of E. coli. It responded in the heterologous expression system to C4-dicarboxylates and to citrate, suggesting that DcuS_Gk is, like DcuS_Ec, a C4-dicarboxylate sensor with a side-activity for citrate. DcuS_Gk, unlike the homologous DctS from Bacillus subtilis, required no binding protein for function in the complementation assay. Thus, the thermophilic G. thermodenitrificans and G. kaustophilus contain citrate and C4-dicarboxylate sensor kinases of the CitA and DcuS type, respectively, and retain function and substrate specificity under mesophilic growth conditions in E. coli.

INTRODUCTION

Bacteria of various taxa are able to grow at the expense of C4-dicarboxylates or citrate (Scheu et al., 2010; Unden & Kleefeld, 2004; Kröger, 1980; Kröger et al., 1992; Bott, 1997). The metabolic pathways for aerobic and anaerobic growth on C4-dicarboxylates and citrate are well characterized, whereas induction of the corresponding pathways has been studied only in a limited number of bacteria. Induction of C4-dicarboxylate metabolism is accomplished by three types of two-component sensor systems, DcuS–DcuR, DctS–DctR and DctB–DctR. Citrate catabolism is induced by the citrate-responsive two-component system CitA–CitB. DcuS–DcuR of enteric bacteria with sensor kinase DcuS represents the prototype of a C4-dicarboxylate-responsive two-component system (Janausch et al., 2002b; Scheu et al., 2010; Zientz et al., 1998; Golby et al., 1999). The DcuS sensor kinases constitute, together with the citrate sensor CitA, the CitA family of histidine kinases. The C4-dicarboxylate sensor kinases DctS_Bs from Rhodobacter capsulatus and DctS_Bs from Sinorhizobium meliloti, however, are members of the FixL and NtrB families of sensor kinases, respectively (Hamblin et al., 1993; Reid & Poole, 1998; Valentini et al., 2011; Janausch et al., 2002b; Scheu et al., 2010).

Sensors of the CitA family of γ-proteobacteria are membrane integral and have a common domain structure (Bott, 1997; Scheu et al., 2010). Sensing of the substrates is achieved by an extracytoplasmic PAS type sensor domain that is flanked by transmembrane helices TM1 and TM2 (Kaspar et al., 1999; Pappalardo et al., 2003; Kneuper et al., 2005). On the cytoplasmic side, TM2 is followed by a second PAS domain (PAS_C) that transmits the signal to the C-terminal kinase domain. DcuS of Escherichia coli (DcuS_Ec) responds to C4-dicarboxylates like fumarate, succinate or L-malate, and with lower sensitivity to citrate (Zientz et al., 1998; Golby et al., 1999; Kneuper et al., 2005). DcuS requires the transporters DctA under aerobic or DcuB under anaerobic conditions as co-regulators (Davies et al., 1999; Steinmetz et al., 2014; Witan et al., 2012; Kleefeld et al., 2009). Bacillus subtilis contains the sensor kinase DctS_Bs resembling DcuS_Ec in domain composition and function (Asai et al., 2000; Graf et al., 2014). DctS_Bs requires, like DcuS_Ec, the transporter DctA_Bs and an extra-cytoplasmic substrate-binding protein for function and response to the C4-dicarboxylates (Graf et al., 2014).

The citrate sensor CitA is found in bacteria that are able to use citrate as the C- and energy source. CitA is highly specific for citrate and defined by the prototypic CitA sensor kinases of Klebsiella and E. coli (Bott et al., 1995; Bott, 1997; Kaspar et al., 1999). CitA of the proteobacteria, Corynebacterium and the homologous CitS of B. subtilis share the domain composition with the DcuS sensor
Bacteria and molecular genetic methods. The *Geobacillus* strains, derivatives of *E. coli* and plasmids used are listed in Table 1. All plasmids were isolated using the GeneJET plasmid miniprep kit and PCR products were purified using the GeneJET PCR purification kit (Fermentas). Oligonucleotides were synthesized by Eurofins MWG. For transformation of *E. coli*, electroporation (Dower et al., 1988) or heat shock were applied. Antibiotics were used at the following concentrations: 100 µg ampicillin ml⁻¹, 20 µg chloramphenicol ml⁻¹, 50 µg kanamycin ml⁻¹ and 15 µg tetracycline ml⁻¹. When two or more antibiotics were used the concentrations were halved.

Bioinformatics. BLASTP and DELTA BLAST were used for screening all non-redundant GenBank sequences (including cDNA sequence translations, PDB, Swiss-Prot, PIR and PRF databases, but excluding environmental samples from whole genome shotgun sequencing projects, with 69 159 658 sequences (14/07/2015 version).

Overproduction and isolation of His₆-DcuSGk and His₆-CitAGt, and autophosphorylation. DcuSGk and His₆-CitAGt were over-produced from pMW817 or pMW960, respectively, in *E. coli* C43BL21(DE3) as N-terminal His₆ fusion proteins after the cloning of dcuSGk and citAGt in pET28a (Table 1). The proteins were isolated from the membrane fraction of the bacteria by extraction with 2% Empigen and purified by Ni-NTA chromatography in 0.04% LDAO (N,N-diethyldecylamine-N-oxide)-containing buffer by the procedure described by Janausch et al. (2002a) for DcuSGk. For the proteins, 13.5 mg DcuSGk l⁻¹ and 4 mg CitAGt l⁻¹ were purified. For the autophosphorylation assay, the proteins were reconstituted in liposomes (protein : lipid ratio 1 : 20, w/w) produced from *E. coli* phospholipids (*E. coli* polar lipid extract; Avanti Polar Lipids) as described by Janausch et al. (2002a). Prior to reconstitution, the liposomes were frozen in liquid N₂ and thawed at room temperature three times. Then, the liposomes were destabilized by Triton X-100 (detergent : lipid ratio of 5:2, w/w) (Rigaud et al., 1988, 1995), mixed with the protein solution and the detergent was removed by BioBeads SM-2 (Bio-Rad) (Holloway, 1973; Janausch et al., 2002a). The proteoliposomes were sedimented by ultracentrifugation, dissolved in

### METHODS

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### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or characteristic(s)</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
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</tr>
<tr>
<td><em>G. kaustophilus</em> DSM7263</td>
<td>Wild-type</td>
<td>Nazina et al. (2001)</td>
</tr>
<tr>
<td><em>G. thermodenitrificans</em> DSM466</td>
<td>Wild-type</td>
<td>Nazina et al. (2001)</td>
</tr>
<tr>
<td><em>E. coli</em> K-12</td>
<td></td>
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</tr>
<tr>
<td>IMW260</td>
<td>MC4100, ZΦ(dcuB’-lacZ)hyb, bla^+ , ΔlacZ, dcuS’ : cam^R</td>
<td>Zientz et al. (1998)</td>
</tr>
<tr>
<td>IMW549</td>
<td>IMW279 (citA : : kan^R), ZΦ(citC’-lacZ) hyb, amp^R</td>
<td>Scheu et al. (2012)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pET28a</td>
<td>Overexpression plasmid, His₆-tag, kan^R</td>
<td>Novagen</td>
</tr>
<tr>
<td>pBAD33</td>
<td>Expression plasmid with pBR322 ori, arabinose induction, cam^R</td>
<td>Guzman et al. (1995)</td>
</tr>
<tr>
<td>pME6010</td>
<td>Low-copy plasmid, 8.3 kb, tet^R</td>
<td>Heeb et al. (2000)</td>
</tr>
<tr>
<td>pMW151</td>
<td>pET28a with dcuSGk-his₆ kan^R</td>
<td>This study</td>
</tr>
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<td>pMW817</td>
<td>pET28a with dcuSGk-his₆ kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>pMW960</td>
<td>pET28a with citAGt-his₆ kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>pMW1558</td>
<td>pHT304 with dctSp, with own promoter and ribosome-binding site, amp^R (E. coli) ery^R (B. subtilis)</td>
<td>This study</td>
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<td>pMW1599</td>
<td>pME6010, with citAcitB-his₆ behind citAGt promoter, tet^R</td>
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<tr>
<td>pMW1601</td>
<td>pME6010 with intergenic region upstream of citAGt</td>
<td>This study</td>
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<td>This study</td>
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<td>pMW1652</td>
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<td>This study</td>
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<tr>
<td>pMW1653</td>
<td>pBAD33, with citBC-his₆ and N-terminal ribosome-binding-site, cam^R</td>
<td>This study</td>
</tr>
</tbody>
</table>
buffer and stored in liquid N₂ (Janausch et al., 2002a). Autophosphorylation of the proteins was tested with different concentrations of [γ-32P]ATP (see Fig. 5) and varying times of incubation. The liposomes were then dissolved in SDS-containing buffer, subjected to SDS-PAGE. Radioactivity associated with the bands of DcuS or CitA was determined by exposure of the gels to a phosphoimager plate (FujiFilm) and evaluated with a FLA7000 fluorescent image reader (FujiFilm). For quantitative evaluation, slices of the SDS-PAGE gel with the labelled proteins were digested and measured for radioactivity by scintillation counting. From the radioactivity, the specific radioactivity and the amount of DcuS or CitA used in the experiment, the labelling was calculated as described by Janausch et al. (2002a).

**β-Galactosidase assays.** Expression of the *dcuB-lacZ* and *citC-lacZ* reporter gene fusions was measured as the β-galactosidase activity of exponential growing *E. coli* (ΔOD₆₀₀ 0.5 to 0.8). Cells were cultivated in 96-deep-well plates, anaerobically at 37 °C under an atmosphere of N₂ in enriched mineral (eM9) medium supplemented with acid-hydrolysed Casamino acids (0.1 %), L-tryptophan (0.005 %) and glycercol (50 mmol l⁻¹) plus DMSO (20 mmol l⁻¹) as the substrates. The effectors [citrate, glucose, fumarate, L-malate and succinate (20 mmol l⁻¹)] were included as indicated. β-Galactosidase activity (Miller, 1992) was quantified in 96-well microtitre plates (Menzel et al., 2013). Optical density (at 570 nm) and β-galactosidase activity (at 415 nm) were measured in a volume of 250 μl per well. For cell permeabilization, 200 μl cell culture were mixed with 800 μl buffer (0.1 mol potassium phosphate l⁻¹, 10 mmol potassium chloride l⁻¹, 1 mmol magnesium chloride l⁻¹, 0.005 % (w/v) cetyltrimethylammonium bromide, 0.0025 % (w/v) sodium deoxycholate and 0.027 % (v/v) 2-mercaptoethanol). For the assay, 150 μl of the permeabilized cells was incubated with 30 μl o-nitrophenyl-β-D-galactoside solution (0.4 %, w/v) at 30 °C. After 20 min, the reaction was stopped with 70 μl sodium carbonate (1 mol l⁻¹). The β-galactosidase activity was determined in triplicate in three induction experiments.

**Growth experiments.** Growth experiments with *G. kaustophilus* and *G. thermodenitrificans* were performed in supplemented White minimal medium (WM) (White, 1972). For *G. kaustophilus*, the WM was enriched with acid-hydrolysed Casamino acids (AHC, 0.1 %), L-tryptophan (0.01 %) and 0.1 % yeast extract (WM-Gk). *G. thermodenitrificans* was cultivated in WM enriched with acid-hydrolysed Casamino acids (0.1 %), L-tryptophan (0.01 %), 0.1 % meat extract and 0.5 % sodium chloride (WM-Gt). For aerobic growth, the media were supplemented with 20 mM of the substrates as indicated. The bacteria were grown at 60 °C (*G. thermodenitrificans*) or 55 °C (*G. kaustophilus*). For anaerobic growth, the media were supplemented with 50 mM glucose with or without the indicated electron acceptor (50 mM or as indicated). Growth experiments with *E. coli* were performed anaerobically at 37 °C in EMJ medium (Kim et al. 2007) containing glutonate (3 mM), glycercol (50 mM) and DMSO (50 mM) as growth substrates, plus effector (20 mM) as indicated.

**RESULTS**

**Genes encoding two-component systems of the CitA family in Geobacillus**

The genomes of the *Geobacillus* strains that are available in databases were BLAST searched for genes encoding members of the CitA family of sensor kinases with the sequences of DcuS and CitA of *E. coli* as the query sequences. All strains (nine) encoded homologues of the CitA family (similarity > 51 %; identity > 29 %) with the respective domain composition. The sensor kinases were members either of the DcuS (*G. kaustophilus, Geobacillus subterraneus* and *Geobacillus thermoglucosidasius*), or of the CitA (*G. thermodenitrificans, Geobacillus vulcani, Geobacillus stearothermophilus*, *Geobacillus caldoxylolysiticus* and *Geobacillus thermoleovorans*) sensor kinases according to sequence similarity. The corresponding genes of *G. thermodenitrificans* and *G. kaustophilus* encoding citA- and dcuS-like homologues, respectively, were selected for analysis. The *G. thermodenitrificans* genes GTNG_1840 and GTNG_1839 (Fig. 1a) are arranged in a predicted operon and code for proteins similar to a tripartite tricarboxylate transport system. In *G. kaustophilus*, a gene cluster of *dcuS dcuR*-like genes was located upstream of genes for a dicarboxylate-binding protein similar to the binding protein DctB of *B. subtilis* and a C₄-dicarboxylate transporter DctA (Fig. 1a). The effector binding regions of the PASₚ domains of DcuSEc and CitA𝑥ₚ are known (Gerharz et al., 2003; Reinelt et al., 2003; Kneuper et al., 2005; Cheung & Hendrickson, 2008). The corresponding domains in the proteins of *G. kaustophilus* and *G. thermodenitrificans* show 67 and 65 % similarity, respectively to the domains of DcuSEc and CitA𝑥ₚ. The domains reveal signature sequences specific for C₄-dicarboxylate (DcuSEc or DctS_Bs) or citrate (CitA) binding (Fig. 1b). The signature sequence is composed of residues that are common for CitA- and DcuS-type sensors, and additional residues (M₁₂₂, S₁₄₄, K₁₅₂ and S₁₆₅, numbering of Klebsiella pneumoniae CitA) that are specific for CitA-like proteins. The conserved residues are mostly ligands for citrate or C₄-dicarboxylate binding in the citrate or L-malate co-crystals of the CitA and DcuS PASₚ domains (Reinelt et al., 2003; Cheung & Hendrickson, 2008). The sensor kinase from *G. thermodenitrificans* contained nine of the CitA-specific residues, whereas that of *G. kaustophilus* carried the C₄-dicarboxylate motif and lacked the CitA-specific residues. The similarities suggest that *G. thermodenitrificans* encodes a citrate sensor of the CitA type (*CitA_Gk*) and *G. kaustophilus* a C₄-dicarboxylate sensor of the DcuS type (*DcuS_Gk*).

**Growth on C₄-dicarboxylates and citrate**

Citrate, various sugars and polyols have been shown, among others, to support growth of *G. thermodenitrificans* (strain DSM 466) and *G. kaustophilus* (Nazina et al., 2004; Manachini et al., 2000). In the experiment shown in Fig. 2, the bacteria were tested for growth on C₄-dicarboxylates that had not been used as substrates before. Under aerobic conditions, succinate or fumarate supported growth of *G. thermodenitrificans*, but the final cell densities were significantly lower than on glucose (Fig. 2a). Anaerobic conditions allowed only poor growth on glucose, which was stimulated by neither fumarate nor nitrate. *G. kaustophilus* grew under aerobic conditions with C₄-dicarboxylates including L-malate, and the cell densities reached 54 % or more of growth on glucose (Fig. 2b). Under anaerobic conditions, glucose enabled significant growth, provided that
nitrate was included as an electron acceptor. Overall, *G. kaustophilus* and *G. thermodenitrificans* are able to use C4-dicarboxylates for aerobic growth in addition to the citrate that was demonstrated earlier as a substrate. The lack of growth on l-malate by *G. thermodenitrificans* might be caused by the absence of specific l-malate transporters.
of the MaeN and YflS type. The transporters are essential in some bacilli for L-malate utilization, since L-malate is not transported by the general C_4-dicarboxylate transporter DctA in the bacteria (Tanaka et al. 2003).

**Citrate-specific complementation of E. coli citA mutants by the citA_Gt gene of Geobacillus**

The supposed citA_Gt and dcuS_Gt genes (or CitA_Gt and DcuS_Gt proteins) of *G. thermodenitrificans* and *G. kaustophilus*, respectively, were tested for their ability to complement citA- or dcuS-deficient strains of *E. coli*. The supposed CitA_Gt was tested in the *E. coli* citA mutant for its capacity to restore expression of the CitA–CitB-dependent citC–lacZ reporter gene fusion (Fig. 3a) and anaerobic growth on citrate (Fig. 3b). The coding regions of citA and citB of *E. coli* causes a lack of citB gene translation. For complementation by citA_Gt, the bacteria were supplied with citA_Gt and by full-length citB_Gt on a plasmid, and both genes were under the control of inducible promoters.

In the citA citB negative strain, the expression of the citC–lacZ reporter drops to background levels (Fig. 3a). When citA_Gt was supplied on a plasmid (together with citB_Ec), citrate caused induction of citC–lacZ expression that exceeded the induction by complementation with *citA_Ec*. Complementation by both citA variants required the presence of citB_Ec (not shown) and for both variants the induction was specific for citrate, whereas fumarate or L-malate produced only a low response. Thus, the citA_Gt gene encodes a sensor kinase that is able to substitute CitA_Ec and CitA_Gt is citrate specific in the heterologous system. The *E. coli* citA mutant has lost anaerobic growth on citrate, whereas anaerobic growth by fumarate respiration is retained as expected (Fig. 3b). CitA_Gt restored the growth on citrate with an efficiency similar to CitA_Ec. This finding is in agreement with the citrate-dependent growth of the bacteria, but the missing response of CitA_Gt to fumarate suggests that the growth of *G. thermodenitrificans* on C_4-dicarboxylates is constitutive or independent from CitA_Gt.

The citA_Ec gene was also used to test complementation of a dcuS-deficient strain of *E. coli* (Fig. 3c). Plasmid-encoded citA_Gt allowed induction of the DcuS–DcuR-dependent dcuB–lacZ reporter gene at high levels that even exceeded those of plasmid-encoded dcuS of *E. coli*. However, restoration of dcuB–lacZ expression was maximal with citrate when CitA_Gt was present, whereas restoration was maximal with fumarate in the presence of DcuS_Ec. Therefore, CitA_Gt is apparently able to interact with and phosphorylate DcuR. Remarkably, in the CitA_Gt–DcuR_Ec-containing bacteria, the specificity for the stimulus (citrate) is that of the sensor CitA_Gt. DcuR retains the specificity for its target (dcuB promoter) demonstrating that the heterologous complementation involves a cross-talk between a CitA sensor kinase and a DcuR response regulator. There is, however, no cross-talk between the *E. coli* CitA_Ec and DcuR_Ec (Fig. 3c), since CitA_Ec, provided on a plasmid is not able to complement DcuS deficiency.
Complementation of \( dcuS \) of \( E. \) coli by \( dcuS \) of \( G. \) kaustophilus

In the same way, complementation of an \( E. \) coli \( dcuS \) mutant was tested by a plasmid encoding the supposed \( dcuS \) protein (Fig. 4a). The test strain (\( E. \) coli IMW260) is deficient of \( dcuS \) but proficient for chromosomally encoded \( dcuR \), and contains a (\( DcuS\)–\( DcuR \) dependent) \( dcuB\)–\( lacZ \) reporter gene fusion. The strain lacks \( dcuB\)–\( lacZ \) expression (Fig. 4a). Complementation with plasmid-encoded \( dcuS \) restored expression of \( dcuB\)–\( lacZ \) by \( \text{C}_4 \)-d carboxylates like fumarate and l-malate, and in agreement with earlier reports to lower extents by citrate (Zientz et al., 1998; Krämer et al., 2007). Plasmid-encoded \( dcuS \) was able to restore expression of \( dcuB\)–\( lacZ \), but the effector specificity was significantly different. Thus, l-malate and citrate stimulated the expression most efficiently, followed by fumarate. Therefore, \( DcuS \) is similar to \( DcuS \) (and different from the CitA proteins) in the broad specificity, but is has a high sensitivity to citrate as well, exceeding that for fumarate.

\( DcuS \) shows a high fumarate-independent background activity in the expression of \( dcuB\)–\( lacZ \) when the transporters \( \text{DctA} \) or \( \text{DcuB} \) are missing. The transporters function as co-regulators of \( DcuS \) and infer responsiveness for \( \text{C}_4 \)-dicarboxylates to \( DcuS \) (Davies et al., 1999; Kleefeld et al., 2009; Witan et al., 2012; Steinmetz et al., 2014).

Thus, in complementation by \( DcuS \), a high background activity of induction of \( dcuB\)–\( lacZ \) was observed in the absence of inducer (\( \text{C}_4 \)-dicarboxylates) (Fig. 4a), resembling the situation when in \( DcuS^+ \) strains transporters \( \text{DcuB} \) or \( \text{DctA} \) are deleted. This observation might be an indication that \( DcuS \) requires, like \( DcuS \), a co-regulator for adopting the ground-state and for full \( \text{C}_4 \)-dicarboxylate responsiveness, and that \( \text{DctA} \) cannot serve this function entirely.

Deletion of \( DcuS \) causes diminished aerobic growth of \( E. \) coli on fumarate or l-malate by decreased expression of \( \text{dctA} \) encoding the aerobic \( \text{C}_4 \)-dicarboxylate transporter \( \text{DctA} \) (Davies et al., 1999). Thus, the decreased growth of the \( dcuS \) mutant on fumarate or l-malate was restored by plasmid-encoded \( dcuS \) (Fig. 4b) and to nearly the same extent by \( dcuS \). Therefore, \( DcuS \) is able to restore the aerobic growth deficiency of a \( dcuS \) mutant by activating \( \text{dctA} \) expression in \( E. \) coli.

The data altogether indicate that \( DcuS \) is a typical \( DcuS \)-type sensor kinase. Some sensor kinases of this type, exemplified by \( \text{DctS} \) of \( B. \) subtilis, require in addition the function of an extracytoplasmic binding protein (Asai et al., 2000; Graf et al., 2014). Thus, in agreement with earlier data (Graf et al., 2014), \( \text{DctS} \) alone is not able to complement for \( \text{DcuS} \) deficiency and to restore \( dcuB\)–\( lacZ \) expression in the presence of fumarate or l-malate.
DcuSGk phosphorylation

For an initial characterization of one of the thermophilic sensor kinases, autophosphorylation of DcuSGk was studied after purification and reconstitution of the protein in liposomes. DcuSGk was overproduced heterologously in E. coli as a DcuSGk-His6 fusion protein. The protein was solubilized from the membrane fraction with detergent LDAO and purified to near homogeneity by Ni-NTA-chromatography (Fig. 5a). The purified DcuSGk showed only very weak autophosphorylation in the presence of [γ-33P]ATP. After incorporation into membranes produced from E. coli phospholipids, the protein was autophosphorylated efficiently (Fig. 5b). The degree of autophosphorylation exceeded that of DcuSEc, when treated under comparable conditions at 37 °C. For a more quantitative study, autophosphorylation of DcuSGk and of DcuSEc was performed in the presence of increasing concentrations of [γ-33P]ATP (Fig. 5c). The degree of phosphorylation was determined using the specific radioactivity of the ATP mixture, and after separating the protein by SDS-PAGE from non-bound [33P]. The radioactivity and phosphorylation in the bands corresponding to DcuSEc or DcuSGk was calculated from radioactivity incorporated and the specific radioactivity, as described earlier for DcuSEc (Janausch et al., 2002a). Phosphorylation reached saturation at high concentrations of [γ-33P]ATP. With 10 mM [γ-33P]ATP, about 18 % of the DcuSGk was phosphorylated, whereas only 2.2 % of the DcuSEc was phosphorylated after reaching maximal phosphorylation. The concentrations for half-maximal phosphorylation were approximately 43 and 420 μM ATP for DcuSGk and DcuSEc, respectively, indicating that DcuSGk when produced in E. coli and tested at 37 °C is active and exceeds DcuSEc in activity and affinity.

Relation of CitAGt and DcuSGk to sensor kinases of the CitA, DctSRc and DctBSm families

The three major classes of C4-dicarboxylate sensor kinases are represented by the CitA/DcuS, DctSRc and the DctBSm sensor kinases. The CitA family is characterized by sequence similarity to CitAKp and the domain composition with a PASp, two TM helices, PASc and the kinase domain (Bott et al., 1995; Zientz et al., 1998). DcuSGk and CitAGt are by domain composition and sequence similarity members of the CitA family. The DctSRc-type sensor kinases are defined by the R. capsulatus DctSRc that belongs to the FixL family of sensor kinases. DctSRc-type sensor kinases are found in R. capsulatus (β-proteobacteria) and ‘Aromatoleum aromaticum’ (β-proteobacteria) (Hamblin et al., 1993; Trautwein et al., 2012; Scheu et al., 2010). DctSRc has a predicted domain composition similar to CitA or DcuS with two transmembrane helices, a periplasmic and a cytoplasmic PAS domain, and the C-terminal kinase domain (UniProt; Magrane & UniProt Consortium, 2011). The periplasmic PAS domains of DcuSEc and DctSRc, however, are only distantly related (Golby et al., 1999; Krämer et al., 2007).

![Fig. 4.](http://mic.microbiologyresearch.org) Complementation of dcuSEc by dcuSGk as tested by gene expression (a) and growth (b). (a) For testing complementation of dcuS-dependent gene expression, E. coli IMW260 (dcuS and FdcbB–lacZ) was grown anaerobically in eM9 medium with glycerol/glucanate/DMSO as the basic substrates plus one of the inducers (20 mM each) citrate (black bars), fumarate (light grey bars) or malate (dark grey bars), or without inducer (white bars). For complementation, strain E. coli IMW260 was transformed with plasmids encoding either DcuS Ec (pMW151), or DcuS Gk (pMW817) or DctBSr (pMW1558). Reporter gene activities (mean ± sd) are the mean of three biological replicates, and of at least four independent measurements. (b) For growth complementation, the same IMW260 strain was transformed with plasmids encoding either DcuS Ec (pMW151) or DcuS Gk (pMW817). Bacterial growth is given as the final OD578 after growth on fumarate or L-malate for the DcuS-negative strain and after complementation with DcuSEc or DcuSGk. Fum, Fumarate; L-Mal, L-malate. (Fig. 4a). This finding is in contrast to the efficient complementation by DcuSGk, indicating that the latter functions independent of a binding protein.
The γ-proteobacteria *Vibrio cholerae* and *Pseudomonas aeruginosa*, and the α-proteobacterium *S. meliloti*, contain C₄-dicarboxylate sensor kinases of the DctB Sm type (Reid & Poole, 1998; Valentini et al., 2011). DctB Sm belongs to the NtrB family of sensor kinases (Janausch et al., 2002b; Scheu et al., 2010; Valentini et al., 2011) and contains tandem extracytoplasmic PAS P domains with low similarity to DcuSEc and CitAKp. The cytoplasmic part is composed of a coiled coil CC domain and the kinase.

For a more detailed analysis, the sequences of the extracytoplasmic PAS P domains of the C₄-dicarboxylate or tricarboxylate sensor kinases were clustered by CLANS (Frickey & Lupas, 2004) using the sequences of the PAS P domains of DcuSEc, DctS Rc, DctS Bs and DctB Sm in the PSI-BLAST. Sequences with an e value cut-off of 10 (default) and 10 iterations were used resulting in the CitA/DcuS, DctS Rc and DctB Sm clusters of sensor kinases and two clusters of guanyltransferases (Fig. 6). CitA/DcuS represents the largest group and contains the prototypic DcuS and CitA sensor kinases from enteric bacteria. DcuSEc of *G. kaustophilus* and CitAKp of *G. thermodenitrificans* and the sensor kinases termed DctS (including DctS Rc) and CitS of the Gram-positive bacilli (Yamamoto et al., 2000), lactobacilli and clostridia are members of the CitA/DcuS cluster. The MalK malate sensor kinases from bacilli (Tanaka et al., 2003), Streptomyces and clostridia, and MaeK of *Lactobacillus casei* (Landete et al., 2010), are part of the CitA/DcuS cluster as well (Fig. 6). Interestingly, all of the sensor kinases of the CitA family that were characterized so far cluster in one subgroup within this family (Fig. 6). The bacteria of the second subgroup (left-hand side within the CitA/DcuS cluster in Fig. 6) without characterized CitA or DcuS proteins are mostly from the actinomycetales group of Gram-positive bacteria. The proteins of the DctS Rc cluster represent the smallest group and comprise the DctS proteins of the proteobacteria.

The sequence similarity of the PAS P domains of DcuSEc and other members within the CitA family is typically higher than 50 % (e.g. CitArc/DcuSEc 62 %, CitSrc/DcuSEc 59 % similarity), whereas that of DcuSEc with

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**Fig. 5.** Purification (a), reconstitution in liposomes and autophosphorylation of DcuSGk-His₆ and CitAGt (b, c). (a) DcuSGk and CitAGt solubilized and purified from *E. coli* BL21(DE3)(pMW817) and BL21(DE3)(pMW960), respectively, were separated by SDS-PAGE (12 % acrylamide) and stained with Coomassie blue (a). The SDS-PAGE gel shows protein marker, DcuSGk-His₆ (15 μg) and His₆-CitAGt (10 μg) as eluted from the Ni-NTA-agarose column. (b) Autophosphorylation of purified (Sol) and reconstituted (Rec) DcuSGk and DcuSGk-His₆-DcuSGk and His₆-DcuSGk were reconstituted in *E. coli* liposomes. Samples of Sol and Rec protein (5 μg each) were incubated for 30 min with 0.1 μM [γ-³²P]ATP in the presence of fumarate (20 mM). The samples were subject to SDS-PAGE (protein stain, lower part of b) and autoradiography (upper part of b). (c) Phosphorylation of DcuSGk and DcuSEc as a function of ATP concentration. DcuSGk or DcuSEc in proteoliposomes was incubated with [γ-³²P]ATP (0.1 μM to 10 mM) for 30 min. The proteoliposomes were dissolved in SDS sample buffer and subject to SDS-PAGE. After slicing the gel, the radioactivity in the protein bands was determined by scintillation counting, and the level of DcuS phosphorylation was calculated from radioactivity and protein content (Janausch et al., 2002a).
DctSRc is as low as 35%. In agreement with their separate clustering, the DctB Sm PASP domain (distal PASP of the PASP tandem structure) shares only 48 and 35% similarity with the domains of DcuS Ec and DctS Rc, respectively.

DISCUSSION

In geobacilli, two-component systems of the CitA/DcuS family are widespread, and in domain composition and sequence similar to the DcuS and CitA proteins from proteobacteria. The cluster analysis shows that the DcuS/CitA group, which was originally defined by the proteins of proteobacteria, includes many two-component systems of Gram-positive bacteria including those from lactobacilli, bacilli, clostridia, corynebacteria, geobacilli, deinococcus and the actinomycetales. Indeed, a large number of the known C4-dicarboxylate/citrate sensor kinases in this group are from Gram-positive bacteria, whereas the C4-dicarboxylate sensor kinases from the proteobacteria are found in the DctS Rc and DctB Sm clusters as well.

Heterologous complementation of a dcuS-deficient mutant of *E. coli* by *dcuS* of *G. kaustophilus* confirmed the functional similarity of DcuS Gk with DcuS Ec. In the heterologous complementation, DcuS Gk showed functional interaction with DcuR Ec. Interestingly, the mesophilic growth conditions in *E. coli* and replacement of the *Geobacillus* lipid composition by the *E. coli* lipids obviously had no severe effects on the function of the *Geobacillus* DcuS Gk and CitA Gk in *E. coli*.

The substrate specificities of DcuS Gk were in agreement with those expected by the presence of the C4-dicarboxylate (‘DcuS’) signature in the binding site as defined in Fig. 1. Thus, DcuS Gk is a typical DcuS sensor with broad specificity to C4-dicarboxylates and to citrate. *G. kaustophilus* is able to grow aerobically on C4-dicarboxylates and on citrate. The lack of an additional CitA–CitB system suggests that DcuS Gk–DcuR Gk is responsible for induction of both metabolic systems, or that citrate metabolism is constitutively induced. Genes encoding a fumarase (FumC) and DctA next to the genes for the two-component system support the role of DcuS Gk–DcuR Gk in the control of C4-dicarboxylate metabolism.

DcuS-type sensor kinases require transporters like DctA or DcuB as co-regulators, and DcuSr and DctSr are in the permanent ON state when the transporters are missing (Davies et al., 1999; Kleefeld et al., 2009; Witan et al., 2012; Steinmetz et al., 2014). The high background activity of *dctB–lacZ* expression after complementation by DcuS Gk in the absence of effectors, indicates that DcuS Gk requires a transporter for adjusting the OFF or C4-dicarboxylate-responsive state as well. The *E. coli* transporters presumably cannot fully replace the *G. kaustophilus* transporters in this respect. Sensor kinase DctSr of *B. subtilis* requires an additional extracytoplasmic binding protein for function (Asai et al., 2000; Graf et al.,
2014). The high activity of DcuSGk in the absence of a binding protein indicates that DcuSGk functions independently of an extracytoplasmic binding protein. Overall, it appears that the DcuS-like protein in G. kaustophilus shares many properties with the corresponding sensor kinases of E. coli and that it is independent of an extracytoplasmic binding protein known from bacilli.

CitA_{GC} however, has the typical properties of a CitA-type sensor kinase and the signature of a citrate binding site. Remarkably, cross-talk between non-cognate systems (CitA_{EC} and DcuR_{EC}) was observed, whereas in the homologous system (CitA_{EC} with DcuR_{EC}) cross-talk was lacking, in agreement with earlier suggestions (Scheu et al., 2012). Therefore, in the heterologous system obviously some specificity in the sensor kinase/response regulator interaction is lost.

The data suggest that the CitA_{GC}–CitB_{GC} two-component system is suitable for inducing metabolism by citrate and growth on citrate, which is supported by the gene cluster tctABC that is located adjacent to the citAB genes and encodes a citrate transporter. Genome analysis shows that only the CitA-type sensor is present in G. thermodenitrificans; there is no DcuS-type sensor kinase. Therefore, the relatively weak growth on C_4-dicarboxylates depends on constitutive expression of the corresponding metabolism system, or the function of an additional unknown system.

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