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

Institute for Microbiology and Wine Research, Johannes Gutenberg University Mainz, 55099 Mainz, Germany

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 (CitAEc) in a heterologous complementation assay restoring expression of the CitAEC-dependent cliclacZ reporter gene and anaerobic growth on citrate. Complementation was specific for citrate. The sensor kinase of G. kaustophilus (termed DcuSGk) 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. DcuSGw, 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 Sm–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 systems (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_{Sm} 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 PAS₂-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.
kinases (Bott et al., 1995; Brocker et al., 2009; Yamamoto et al., 2000). The CitA protein of E. coli functions unlike DcuSEc as a stand-alone sensor without the need for accessory proteins (Scheu et al., 2012).

Screening the genome sequences of thermophilic bacteria revealed that various Geobacillus and Deinococcus strains encode sensor kinases of the CitA family with features indicating that C4-dicarboxylate (DcuS-type) and citrate (CitA-type) sensor kinases are present in different strains. Thus, sensor kinases from Geobacillus thermodenitrificans and Geobacillus kaustophilus representing CitA- and DcuS-type sensor kinases from Geobacillus thermodenitrificans type) sensor kinases are present in different strains. Thus, sensor kinases from Geobacillus thermodenitrificans and Geobacillus kaustophilus representing CitA- and DcuS-type sensor kinases, respectively, were selected for characterization and comparison to the corresponding sensor kinases from proteobacteria. G. thermodenitrificans and G. kaustophilus are thermophilic with temperature optima at 60 and 55 °C, respectively (Nazina et al., 2001). The bacteria that are known to grow on citrate were tested for growth on C4-dicarboxylate. For identification as CitA- or DcuS-type systems, heterologous complementation was used in dcuS- or citA-deficient strains of E. coli. The E. coli strains were tested for gain of growth on citrate and C4-dicarboxylates, and for the expression of DcuS- and CitA-dependent reporter genes in response to C4-dicarboxylates or citrate.

METHODS

Bacteria and molecular genetic methods. The Geobacillus strains, derivatives of E. coli and plasmids used are listed in Table 1. The molecular methods were performed according to standard procedures (Sambrook & Russell, 2001). 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 HisS2-DcUSEc and HisS2-CitAEc and autophosphorylation. DcuSEc and HisS2-CitAEc were over-produced from pMW817 or pMW960, respectively, in E. coli C43BL21(DE3) as N-terminal HisS fusion proteins after the cloning of dcuSEc and citAEc 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-dimethyldodecylamine-N-oxide)-containing buffer by the procedure described by Janausch et al. (2002a) for DcuSEc. For the proteins, 13.5 mg DcuSEc l–1 and 4 mg CitAEc 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 Bio-Beads SM-2 (Bio-Rad) (Holloway, 1973; Janausch et al., 2002a). The proteoliposomes were sedimented by ultracentrifugation, dissolved in

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>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. kaustophilus DSM7263</td>
<td>Wild-type</td>
<td>Nazina et al. (2001)</td>
</tr>
<tr>
<td>G. thermodenitrificans DSM466</td>
<td>Wild-type</td>
<td>Nazina et al. (2001)</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMW260</td>
<td>MC4100, αβ(ducB'–lacZ)hyb, bla', ΔlacZ, dcsA : cma'</td>
<td>Zientz et al. (1998)</td>
</tr>
<tr>
<td>IMW549</td>
<td>IMW279 (citA : kan'), αβ(citC'–lacZ) hyb, amp'</td>
<td>Scheu et al. (2012)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pET28a</td>
<td>Overexpression plasmid, HisS2-tag, kanR</td>
<td>Novagen</td>
</tr>
<tr>
<td>pBAD33</td>
<td>Expression plasmid with pBR322 ori, arabinose induction, camR</td>
<td>Guzman et al. (1995)</td>
</tr>
<tr>
<td>pME6010</td>
<td>Low-copy plasmid, 8.3 kb, tetR</td>
<td>Heeb et al. (2000)</td>
</tr>
<tr>
<td>pMWV151</td>
<td>pET28a with dcsA-kan, kanR</td>
<td>This study</td>
</tr>
<tr>
<td>pMWV817</td>
<td>pET28a with dcsA-his6, kanR</td>
<td>This study</td>
</tr>
<tr>
<td>pMWV960</td>
<td>pET28a with citA-his6, kanR</td>
<td>This study</td>
</tr>
<tr>
<td>pMW1558</td>
<td>pHT304 with dctSp with own promoter and ribosome-binding site, ampR (E. coli) eryR (B. subtilis)</td>
<td>This study</td>
</tr>
<tr>
<td>pMWV1599</td>
<td>pME6010, with citB-his6, behind citB, promoter, tetR</td>
<td>This study</td>
</tr>
<tr>
<td>pMWV1601</td>
<td>pME6010 with intergenic region upstream of citA</td>
<td>This study</td>
</tr>
<tr>
<td>pMWV1651</td>
<td>pMWV1601, with his6-citA, behind citA, promoter, tetR</td>
<td>This study</td>
</tr>
<tr>
<td>pMWV1652</td>
<td>pMWV1601, with his6-citA, behind citA, promoter, tetR</td>
<td>This study</td>
</tr>
<tr>
<td>pMWV1653</td>
<td>pBAD33, with citB-his6 and N-terminal ribosome-binding-site, camR</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 phosphomager 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 (ΔOD₅₇₈ 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 (Monzel 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 1⁻¹, 10 mmol potassium chloride 1⁻¹, 1 mmol magnesium chloride 1⁻¹, 0.005 % (w/v) cetethtrimethylammonium 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-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 EM9 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 caldoloxyslyticus and Geobacillus thermoeoverans) sensor kinases according to sequence similarity. The corresponding genes of G. thermodenitrificans and G. kaustophilus encoding citA-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 CitA and CitB from E. coli. The genes are located downstream of gene cluster tctCBA encoding 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 CitAKp 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 CitAKp. The domains reveal signature sequences specific for C₄-dicarboxylate (DcuSEc or DctSBg) 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 S₁₆₇, 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_Gt) 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.
0.01 % L-tryptophan, 0.5 % NaCl and 0.1 % meat extract enriched WM. The medium was enriched with 0.1 % AHC, 60\(^\circ\)C on glucose and carboxylates. The bacteria were grown at aerobic growth as indicated, or 50 mM (citrate 5 mM) for \(\text{citC}^\text{-lacZ}\) reporter gene fusion (Fig. 3a) and anaerobic growth on citrate (Fig. 3b). The coding regions of \(\text{citA}\) and \(\text{citB}\) of \(\text{E. coli}\) causes a lack of \(\text{citB}\) gene translation. For complementation by \(\text{citAGt}\), the bacteria were supplied with \(\text{citAGt}\), and by full-length \(\text{citB}\) on a plasmid, and both genes were under the control of inducible promoters.

In the \(\text{citA}\) \(\text{citB}\) negative strain, the expression of the \(\text{citC}^\text{-lacZ}\) reporter drops to background levels (Fig. 3a). When \(\text{citAGt}\) was supplied on a plasmid (together with \(\text{citB}\)), citrate caused induction of \(\text{citC}^\text{-lacZ}\) expression that exceeded the induction by complementation with \(\text{citAEc}\). Complementation by both \(\text{citA}\) variants required the presence of \(\text{citB}\) (not shown) and for both variants the induction was specific for citrate, whereas fumarate or L-malate produced only a low response. Thus, the \(\text{citAGt}\) gene encodes a sensor kinase that is able to substitute \(\text{CitA}\) and \(\text{CitG}\) is citrate specific in the heterologous system. The \(\text{E. coli}\) \(\text{citA}\) mutant has lost anaerobic growth on citrate, whereas anaerobic growth by fumarate respiration is retained as expected (Fig. 3b). \(\text{CitAGt}\) restored the growth on citrate with an efficiency similar to \(\text{CitA}\) of \(\text{E. coli}\) (Fig. 3b), indicating that citrate transport and citrate lyase production occur in the presence of \(\text{CitAGt}\). The heterologous complementation of \(\text{CitAEc}\) by \(\text{CitAGt}\) suggests that \(\text{CitAGt}\) functionally interacts with \(\text{CitB}\). Overall, the data show that \(\text{CitAGt}\) is a citrate-specific sensor that is able to replace \(\text{CitAEc}\) in the heterologous system and to interact with \(\text{CitB}\). This finding is in agreement with the citrate-dependent growth of the bacteria, but the missing response of \(\text{CitAGt}\) to fumarate suggests that the growth of \(\text{G. thermodenitrificans}\) on \(\text{C}_4\)-dicarboxylates is constitutive or independent from \(\text{CitAGt}\).

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

**Fig. 2.** Growth of \(\text{G. thermodenitrificans}\) (a) and \(\text{G. kaustophilus}\) (b) on glucose and carboxylates. The bacteria were grown at 60 \(^\circ\)C (\(\text{G. thermodenitrificans}\)) or 55 \(^\circ\)C (\(\text{G. kaustophilus}\)) in enriched WM. The medium was enriched with 0.1 % AHC, 0.01 % L-tryptophan, 0.5 % NaCl and 0.1 % meat extract (\(\text{G. thermodenitrificans}\)), or with 0.1 % yeast extract (\(\text{G. kaustophilus}\)). Growth substrates were added at 20 mM (citrate 5 mM) for aerobic growth as indicated, or 50 mM (citrate 5 mM) for anaerobic growth. Growth was measured as the \(\text{OD}_{578}\) after incubation for 7 h (\(\text{G. thermodenitrificans}\)) or 16 h (\(\text{G. kaustophilus}\)). Fum, Fumarate; Gluc, glucose; L-Mal, L-malate; Succ, succinate.

of the MaeN and YfS type. The transporters are essential in some bacilli for L-malate utilization, since L-malate is not transported by the general \(\text{C}_4\)-dicarboxylate transporter \(\text{DctA}\) in the bacteria (Tanaka et al. 2003).

**Citrate-specific complementation of \(\text{E. coli}\) \(\text{citA}\) mutants by the \(\text{citAGt}\) gene of \(\text{Geobacillus}\)**

The supposed \(\text{citAGt}\) and \(\text{dcuS}\) genes (or \(\text{CitAGt}\) and \(\text{DcuS}\) proteins) of \(\text{G. thermodenitrificans}\) and \(\text{G. kaustophilus}\), respectively, were tested for their ability to complement \(\text{citA}\)- or \(\text{dcuS}\)-deficient strains of \(\text{E. coli}\). The supposed \(\text{CitAGt}\) was tested in the \(\text{E. coli}\) \(\text{citA}\) mutant for its capacity to restore expression of the \(\text{CitA}\)-\(\text{CitB}\)-dependent \(\text{citC}^\text{-lacZ}\) reporter gene fusion (Fig. 3a) and anaerobic growth on citrate (Fig. 3b). The coding regions of \(\text{citA}\) and \(\text{citB}\) of \(\text{E. coli}\) overlap by 32 bp and, therefore, inactivation of \(\text{citA}\) causes a lack of \(\text{citB}\) gene translation. For complementation by \(\text{citAGt}\), the bacteria were supplied with \(\text{citAGt}\), and by full-length \(\text{citB}\) on a plasmid, and both genes were under the control of inducible promoters.
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$_{Gk}$ 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$_{Gk}$* restored expression of *dcuB–lacZ* by C$_4$-dicarboxylates 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$_{Gk}$* 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$_{Gk}$ is similar to DcuS$_{Ec}$ (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$_{Ec}$ shows a high fumarate-independent background activity in the expression of *dcuB–lacZ* when the transporters DctA or DcuB are missing. The transporters function as co-regulators of DcuS and infer responsiveness for 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$_{Gk}$, a high background activity of induction of *dcuB–lacZ* was observed in the absence of inducer (C$_4$-dicarboxylates) (Fig. 4a), resembling the situation when in DcuS$^{+}$ strains transporters DcuB or DctA are deleted. This observation might be an indication that DcuS$_{Gk}$ requires, like DcuS$_{Ec}$, a co-regulator for adopting the ground-state and for full C$_4$-dicarboxylate responsiveness, and that DctA$_{Ec}$ cannot serve this function entirely.

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

The data altogether indicate that DcuS$_{Gk}$ is a typical DcuS-type sensor kinase. Some sensor kinases of this type, exemplified by DctS$_{Bs}$ 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), DctS$_{Bs}$ alone is not able to complement for DcuS$_{Ec}$ deficiency and to restore *dcuB–lacZ* expression in the presence of fumarate or l-malate.
CitA sensor kinases of Geobacillus

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-32P]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-32P]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-32P]ATP. With 10 mM [γ-32P]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 CitAhp 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 CitAhp 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 (α-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.** 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 fdcuB–lacZ) was grown anaerobically in eM9 medium with glycerol/glucose/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 DcuSEc (pMW151), or DcuSGk (pMW817) or DctSBs (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 DcuSEc (pMW151) or DcuSGk (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.

---

http://mic.microbiologyresearch.org
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, DctSRc, DctSBs 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, DctSRc and DctSBs clusters of sensor kinases and two clusters of guanylate cyclases (Fig. 6). CitA/DcuS represents the largest group and contains the prototypic DcuS and CitA sensor kinases from enteric bacteria. DcuSGk of *G. kaustophilus* and CitAGt of *G. thermodenitrificans* and the sensor kinases termed DctS (including DctSRc) 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). Additionally, four strains of the *Deinococcus/Thermus* group and two *Meiothermus* strains contained CitA homologues. Remarkably, 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 DctSRc 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...
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 DctSRc and DctBSm 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 DcuSrGk and DctSbsGk 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 *dcuB–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 DctSbsGk of *B. subtilis* requires an additional extracytoplasmic binding protein for function (Asai *et al.*, 2000; Graf *et al.*,...
2014). The high activity of DcuS\textsubscript{Gk} in the absence of a binding protein indicates that DcuS\textsubscript{Gk} functions independently of an extracytoplasmic binding protein. Overall, it appears that the DcuS-like protein in \textit{G. kaustophilus} shares many properties with the corresponding sensor kinases of \textit{E. coli} and that it is independent of an extracytoplasmic binding protein known from bacilli.

CitA\textsubscript{GK} 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\textsubscript{EC} and DcuR\textsubscript{EC}) was observed, whereas in the homologous system (CitA\textsubscript{EC} with DcuR\textsubscript{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\textsubscript{GK}–CitB\textsubscript{GK} two-component system is suitable for inducing metabolism by citrate and growth on citrate, which is supported by the gene cluster \textit{tctABC} that is located adjacent to the \textit{citAB} genes and encodes a citrate transporter. Genome analysis shows that only the CitA-type sensor is present in \textit{G. thermodenitrificans}; there is no DcuS-type sensor kinase. Therefore, the relatively weak growth on C\textsubscript{4}-dicarboxylates depends on constitutive expression of the corresponding metabolism system, or the function of an additional unknown system.

**ACKNOWLEDGEMENTS**

Financial support by a grant from Deutsche Forschungsgemeinschaft to G. U. (UN49/17-1) is gratefully acknowledged. We are grateful to Drs J. Schultz, A. Lupas and J. Baßler (Tübingen) for introduction to and help with CLANS.

**REFERENCES**


White, P. J. (1972). The nutrition of *Bacillus megaterium* and *Bacillus cereus*. *J Gen Microbiol* 71, 505–514.


Edited by: S. Kengen