Positive transcriptional control of the pyridoxal phosphate biosynthesis genes *pdxST* by the MocR-type regulator PdxR of *Corynebacterium glutamicum* ATCC 13032

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The *pdxR* (cg0897) gene of *Corynebacterium glutamicum* ATCC 13032 encodes a regulatory protein belonging to the MocR subfamily of GntR-type transcription regulators and consisting of an amino-terminal winged helix–turn–helix DNA-binding domain and a carboxy-terminal aminotransferase-like domain. A defined deletion in the *pdxR* gene resulted in the decreased expression of the divergently orientated *pdxST* genes coding for the subunits of pyridoxal 5′-phosphate synthase. The *pdxST* mutant *C. glutamicum* NJ0898 and the *pdxR* mutant *C. glutamicum* AMH17 showed vitamin B₆ auxotrophy that was restored by supplementing the growth medium with either pyridoxal, pyridoxal 5′-phosphate or pyridoxamine. The genetic organization of the 89 bp *pdxR*–*pdxST* intergenic region was elucidated by mapping the 5′ ends of the respective transcripts, followed by detection of typical promoter sequences. Bioinformatic pattern searches and comparative genomics revealed three DNA motifs with the consensus sequence AAAGTGGW(–/T)CTA, overlapping the deduced promoter sequences and serving as candidate DNA-binding sites for PdxR. DNA band shift assays with the purified PdxR protein demonstrated the specific binding of the transcription regulator to double-stranded 40-mer sequences containing the detected motifs, thereby confirming the direct regulatory role of PdxR in activating the expression of the *pdxST* genes.

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INTRODUCTION

The term vitamin B₆ collectively refers to the three vitamins pyridoxine, pyridoxamine and pyridoxal as well as to their respective 5′-phosphorylated forms (Eliot & Kirsch, 2004). The vitamin is an essential metabolite in all organisms and is required as a cofactor for numerous enzymes catalysing a wide variety of biochemical reactions, predominantly in amino acid metabolism. Pyridoxal 5′-phosphate (PLP) is the major biochemically active form of vitamin B₆ (Eliot & Kirsch, 2004). Two mutually exclusive pathways for the *de novo* biosynthesis of vitamin B₆ exist in bacteria, referred to as deoxyxylulose 5-phosphate (DXP)-dependent and DXP-independent (Fitzpatrick *et al.*, 2007). The DXP-dependent pathway is restricted, with a few exceptions, to the gammaproteobacteria, whereas the DXP-independent

pathway is much more predominant and is found in most eubacteria (Tanaka *et al.*, 2005). The key enzyme of the DXP-independent pathway is PLP synthase, consisting of the synthase subunit PdxS and the glutaminase subunit PdxT (Strohmeier *et al.*, 2006). PLP synthase catalyses the direct formation of PLP from pentose sugars (ribose 5-phosphate or ribulose 5-phosphate) and triose sugars (glyceraldehyde 3-phosphate or dihydroxyacetone phosphate) in addition to glutamine (Fitzpatrick *et al.*, 2007). Although the enzymic reaction of PLP synthase is of fundamental importance for bacterial metabolism, the transcriptional regulation of the *pdxS* and *pdxT* genes has not been investigated so far, but is addressed for the first time in the present study.

During our work on the systematic reconstruction of the transcriptional regulatory network in the Gram-positive model organism *Corynebacterium glutamicum* (Brinkrolf *et al.*, 2007; Kohl & Tauch, 2009), we recognized the Cg0897 (PdxR) protein as a candidate to control the expression of the *pdxST* genes in this species. According to genome annotation data, the Cg0897 protein was grouped

Abbreviations: Cy3, indocarbocyanine; DXP, deoxyxylulose 5-phosphate; E-b/O, effector-binding and/or oligomerization; IMPACT, intein mediated purification with an affinity chitin-binding tag; PLP, pyridoxal 5′-phosphate; RACE, rapid amplification of cDNA ends; SOEing, splicing by overlap extension.

into the GntR family of DNA-binding transcription regulators (Brune *et al.*, 2005). Inactivation of the *cg0897* (*pdxR*) gene by either random transposon mutagenesis with an IS6100-based mobile element (Mormann *et al.*, 2006) or directed gene replacement (McHardy *et al.*, 2003) resulted in vitamin B₆ auxotrophy of the respective mutant strains and indicated that the *pdxST* genes of *C. glutamicum* (*cg0898* and *cg0899*) are probably under positive transcriptional control by PdxR. Furthermore, the level of *pdxR* gene expression was enhanced during the response of *C. glutamicum* to severe heat shock conditions (Barreiro *et al.*, 2009), whereas the expression of the *pdxST* genes was slightly down-regulated in the *lexA* mutant *C. glutamicum* NJ2114 (Jochmann *et al.*, 2009). In the latter study, an SOS box was detected upstream of the *pdxST* gene region and the specific binding of the SOS response regulator LexA was demonstrated *in vitro* by DNA band shift assays, integrating the *pdxST* genes into the SOS regulon of *C. glutamicum*.

In the present study, we analyse the transcriptional regulation of the PLP synthase genes *pdxST*. We measure the expression of the *pdxST* genes in the wild-type strain and in a *pdxR* mutant in response to the presence of the B₆ vitamers and search for promoter sequences and candidate DNA-binding sites by a bioinformatic approach. The direct interaction of the purified PdxR protein with the candidate DNA-binding sites is shown *in vitro* by DNA band shift assays.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5 α MCR was grown at 37 °C in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) and used for standard cloning procedures. All *C. glutamicum* strains were routinely grown at 30 °C in LB medium or in minimal medium CGXII containing (per litre) 30 μ g protocatechuic acid and 420 μ g thiamin (Keilhauer *et al.*, 1993). To analyse the nutritional requirement of *C. glutamicum* NJ0898 and *C. glutamicum* AMH17, the CGXII medium was supplemented with 20 μ M of either

pyridoxal, PLP, pyridoxamine or pyridoxine. Growth of *C. glutamicum* strains was monitored at time intervals of 15 min by nephelometry by using a Nephelostar Galaxy nephelometer (BMG Laboratories). Antibiotics for plasmid selection were ampicillin (200 μ g ml⁻¹ for *E. coli*) and kanamycin (50 μ g ml⁻¹ for *E. coli* and 25 μ g ml⁻¹ for *C. glutamicum*). Growth of *E. coli* cultures was monitored by measuring OD₆₀₀ with an Eppendorf BioPhotometer.

DNA techniques. Preparation of plasmid DNA from *E. coli* cells by the alkaline lysis technique was performed by using a QIAprep Spin Miniprep kit (Qiagen). Chromosomal DNA of *C. glutamicum* was prepared as described previously (Tauch *et al.*, 1995). Restriction of DNA, analysis by agarose gel electrophoresis and DNA ligation were performed according to standard protocols (Sambrook *et al.*, 1989). DNA transfer was carried out by electroporation with competent *E. coli* and *C. glutamicum* cells (Tauch *et al.*, 1994, 2002). DNA amplification by PCR was performed with a PTC-100 thermocycler (MJ Research) and *Taq* DNA polymerase (Bioline) or Phusion DNA polymerase (Finnzymes). All PCR products were purified with a PCR Purification Spin kit (Qiagen).

Construction of the *pdxST* mutant *C. glutamicum* NJ0898. The gene SOEing (gene splicing by overlap extension) procedure (Horton *et al.*, 1989) was used to construct a *pdxST* mutant in *C. glutamicum* ATCC 13032. The PCR primers used were: *pdxSTdel1* (5'-GATC-TAGGATCCGCAACCACTCGTGTGAAG), *pdxSTdel2* (5'-TTCGG-ACTGCTCGATGAA), *pdxSTdel3* (5'-TTCATCGACGAGCCGAAC-GCTGGCCTGATCTATCT) and *pdxSTdel4* (5'-GATCTAGCATG-CCTTGGTGGATGCGGTAAT). The gene SOEing product was digested with *Bam*HI and *Sph*I and subsequently cloned in *E. coli* DH5 α MCR into the vector pK18*mobsacB* (Table 1). The resulting derivative pK18*mobsacB* Δ *pdxST* was suitable for performing an allelic exchange by homologous recombination in the chromosome of *C. glutamicum* ATCC 13032 (Schäfer *et al.*, 1994), resulting in the mutant strain *C. glutamicum* NJ0898.

Complementation of the *pdxR* mutant *C. glutamicum* AMH17. The complete *pdxR* (*cg0897*) gene was amplified from chromosomal DNA of *C. glutamicum* ATCC 13032 by PCR with the primer pair *compl_A* (5'-GATCTAGAGCTCGATGATCCAACGCCACAA) and *compl_B* (5'-GATCTACTGCAGCTCTCGGGCTGTTTTTC). The PCR product was digested with *Sac*I and *Pst*I and subsequently cloned in *E. coli* DH5 α MCR into the vector pEC-K18*mob2* (Table 1). The resulting plasmid pEC-K18*mob2* Δ *pdxR* was transferred to the mutant strain *C. glutamicum* AMH17 by electroporation.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>C. glutamicum</i> ATCC 13032	Wild-type strain	ATCC
<i>C. glutamicum</i> AMH17	Restriction-deficient strain with defined deletion in <i>pdxR</i>	McHardy <i>et al.</i> (2003)
<i>C. glutamicum</i> NJ0898	Wild-type strain with defined deletion in <i>pdxST</i>	This study
<i>E. coli</i> DH5 α MCR	<i>E. coli</i> strain used for standard cloning procedures	Grant <i>et al.</i> (1990)
<i>E. coli</i> ER2566	<i>E. coli</i> strain used for heterologous gene expression	New England Biolabs
<i>E. coli</i> TOP10	<i>E. coli</i> strain used for cloning of RACE-PCR products	Invitrogen
pK18 <i>mobsacB</i>	<i>sacB lacZ</i> Km ^r ; cloning vector for allelic exchange	Schäfer <i>et al.</i> (1994)
pK18 <i>mobsacB</i> Δ <i>pdxST</i>	pK18 <i>mobsacB</i> carrying a modified <i>pdxST</i> gene region	This study
pEC-K18 <i>mob2</i>	Km ^r ; <i>E. coli</i> / <i>C. glutamicum</i> cloning vector	Kirchner & Tauch (2003)
pEC-K18 <i>mob2</i> Δ <i>pdxR</i>	pEC-K18 <i>mob2</i> carrying the <i>C. glutamicum</i> <i>pdxR</i> gene	This study
pTXB1	Ap ^r ; <i>E. coli</i> vector for protein tagging with an intein/chitin-binding domain	New England Biolabs
pTXB1 Δ <i>pdxR</i>	pTXB1 carrying the <i>C. glutamicum</i> <i>pdxR</i> gene	This study
pCR2.1-TOPO	Ap ^r Km ^r ; cloning vector for PCR products	Invitrogen

RNA techniques. Preparation of total RNA from *C. glutamicum* cultures and hybridization of whole-genome DNA microarrays were carried out as described previously (Brune *et al.*, 2007; Jochmann *et al.*, 2009). Differential gene expression was measured by real-time RT-PCR with an OPTICON Continuous Fluorescence Detector (MJ Research) and SensiMix One-Step kit (Quantace). All measurements were performed with RNA probes from two biological replicates, each represented by two technical replicates. The amount of specific transcripts was normalized with respect to total RNA and the relative changes in the transcription rates were determined as $2^{-\Delta C_P}$, where ΔC_P is equal to the difference of the measured crossing points for the test and control condition. The 5' ends of transcripts were determined by using the second generation 5'/3' rapid amplification of cDNA ends (RACE) kit (Roche Diagnostics) according to the manufacturer's instructions. The resulting PCR products were cloned into the pCR2.1-TOPO vector and transferred to chemically competent *E. coli* TOP10 cells. Sequencing of cloned DNA fragments was performed by IIT Biotech.

Expression and purification of the *C. glutamicum* PdxR protein. The PdxR protein was purified by using the intein mediated purification with an affinity chitin-binding tag (IMPACT) system (New England BioLabs). The *pdxR* coding region was amplified by PCR with the primer pair intein_A (5'-GGTGGTCATATGATGCT-TGCCGACCTTCCC) and intein_B (5'-GGTGGTTGCTCTCCG-CAGGCGCCTAGAGACACCGCATC), adding in this way an alanine residue (underlined) to the carboxy-terminal end of PdxR to ameliorate cleavage of the intein tag from the recombinant protein. The PCR product was digested with *NdeI* and *SapI* and subsequently cloned into the expression vector pTXB1, thereby fusing the recombinant C terminus of PdxR to a self-cleavable intein tag that contains a chitin-binding domain. The resulting plasmid pTXB1_ *pdxR* was transferred to competent *E. coli* ER2566 cells to overexpress the PdxR protein. *E. coli* ER2566 cultures were grown overnight at room temperature in 500 ml LB medium containing 200 µg ampicillin ml⁻¹. Induction of *pdxR* expression was carried out at an OD₆₀₀ of 0.5 by adding IPTG to a final concentration of 0.5 mM. Cells were harvested after 16 h incubation by centrifugation at 5000 g and 4 °C for 15 min. The cell pellet was resuspended in lysis buffer [20 mM Na₂HPO₄, 500 mM NaCl, 1 mM EDTA, 0.1 % (w/v) Triton X-100, 20 µM PMSF, 0.5 mM Tris(2-carboxyethyl)phosphine]. Cells were disrupted twice in a FRENCH Pressure Cell (Thermo Scientific) and the cell extract was cleared by centrifugation at 4 °C for 20 min. The PdxR protein was purified from the protein crude extract by means of the chitin-binding domain of the intein tag, applying a Protino Column (14 ml; Macherey-Nagel) that was packed with chitin beads (New England Biolabs). On-column cleavage was induced by loading the column with cleavage buffer containing 50 mM DTT, and incubating the assay at 4 °C for 16 h. The recombinant PdxR protein devoid of the intein tag was eluted with column buffer and stored at -20 °C. The protein concentration was determined with a Bio-Rad protein assay kit. The eluate was analysed by SDS-PAGE, and in-gel digestion with modified trypsin (Promega) was carried out to verify the purity of the PdxR protein. The peptide mass fingerprint of the purified PdxR protein was generated by MALDI-TOF MS, by using an Ultraflex mass spectrometer (Bruker Daltonics) and the MASCOT software. The LexA protein from *C. glutamicum* ATCC 13032 was purified as described previously (Jochmann *et al.*, 2009).

DNA band shift assays with purified PdxR protein. DNA band shift assays were performed with indocarbocyanine (Cy3)-labelled fragments generated by PCR with the primer pair shift_A (5'-Cy3-CGAGACGACGGATCTGTT) and shift_B (CGAGTGGTTGCTT-GGTAA) or with 40-mers that were annealed with complementary oligonucleotides to double-stranded DNA fragments by incubation at 94 °C for 5 min and subsequent cooling to 8 °C in steps of 1 °C per

10 s. The assays were performed in a final volume of 20 µl containing 0.05 pmol of the PCR product or double-stranded 40-mers, 40 pmol of the purified protein, 0.06 µg herring sperm DNA, 20 % (v/v) glycerol and binding buffer [20 mM HEPES, 30 mM KCl, 10 mM (NH₄)₂SO₄, 1 mM EDTA, 1 mM DTT, 0.2 % (w/v) Tween 20; pH 7.6]. The binding buffer was modified where appropriate by adding either 2 or 200 µM of the vitamers pyridoxal, PLP, pyridoxamine or pyridoxine. The assays were incubated at 30 °C for 30 min and separated in 2 % agarose gels prepared in gel buffer (40 mM Tris/HCl, 10 mM sodium acetate, 1 mM EDTA; pH 7.8); 70 V was applied for 1 h. The agarose gels were scanned with a Typhoon 8600 Variable Mode Imager (Amersham Biosciences Europe).

RESULTS

Molecular features of the PdxR protein from *C. glutamicum* ATCC 13032

The PdxR protein (Cg0897) of *C. glutamicum* ATCC 13032 has a predicted size of 453 amino acids and a theoretical molecular mass of 48.9 kDa (Kalinowski *et al.*, 2003). Protein domain predictions performed with the SUPERFAMILY tools (Gough *et al.*, 2001) and by Conserved Domain Database searches (Marchler-Bauer *et al.*, 2009) revealed that the PdxR protein consists of an amino-terminal winged helix-turn-helix DNA-binding domain of the GntR family of transcription regulators and a carboxy-terminal domain belonging to the aspartate aminotransferase superfamily. This fusion of domains in DNA-binding proteins is characteristic of the MocR subfamily of GntR-type transcription regulators (Hoskisson & Rigali, 2009; Rigali *et al.*, 2002). Aspartate aminotransferases are part of a large superfamily of PLP-binding enzymes (Schneider *et al.*, 2000) sharing a prominent mechanistic feature in such a way that the PLP group is bound covalently to a conserved lysine residue (Sigrist *et al.*, 2010). According to comparative genomics performed with the Integrated Microbial Genomes system (Markowitz *et al.*, 2006), proteins similar to PdxR from *C. glutamicum* are apparently encoded upstream of genes encoding subunits of PLP synthase in 12 other actinobacterial genomes. The PdxR protein from *C. glutamicum* ATCC 13032 showed 37–57 % amino acid sequence identity with the putative PdxR proteins from other actinobacteria (Fig. 1a). An amino acid sequence alignment of the PLP attachment sites present in the predicted aminotransferase domains of the PdxR-like proteins demonstrated that the lysine residue necessary for the covalent binding of PLP is only present in the respective protein from *Kocuria rhizophila* DC2201 (Fig. 1b). In the other PdxR-like proteins, the lysine residue is replaced by the amino acid serine or alternatively by a threonine or histidine residue. This result suggests that the aminotransferase domain of the actinobacterial PdxR-like proteins is probably unable to bind PLP covalently. At least in the case of *C. glutamicum* ATCC 13032, this indication fits the previous observation that no aminotransferase activity was detectable in enzyme assays with purified PdxR protein (Marienhagen *et al.*, 2005).

Fig. 1. Comparison of actinobacterial gene regions encoding the transcription regulator PdxR and PLP synthase. (a) Schematic representation of the *pdxR*–*pdxST* gene region in the genomes of 13 actinobacteria. Genes are listed with their GenBank accession nos. The percentage identities [ID%] of the deduced proteins to the homologous counterparts from *C. glutamicum* ATCC 13032 are listed. The additional gene (*KRH_01140*) located between *pdxR* and *pdxST* in the *K. rhizophila* genome is indicated by a triangle. (b) Amino acid sequence alignment of PLP attachment sites detected in the aminotransferase-like domain of actinobacterial PdxR transcription regulators. The position of the conserved lysine residue generally used for the covalent binding of PLP is boxed. It is missing in the actinobacterial PdxR-like regulators, with the exception of the *K. rhizophila* protein *KRH_01150*. The amino acid sequence alignment was generated with the CLUSTAL W tool (Larkin *et al.*, 2007). (c) Schematic representation of the *pdxS* locus in corynebacterial genomes lacking a *pdxR*-like regulatory gene. Homologous genes share the same shading. The gene regions from *C. glutamicum* ATCC 13032 are shown for comparison.

The *pdxR* gene of *C. glutamicum* ATCC 13032 is located in a head-to-head arrangement with the *pdxST* genes (Fig. 1a). This divergent order of *pdxR* and *pdxST* genes is conserved in 13 actinobacterial genomes encoding PdxR-like proteins, with the exception of ‘*Corynebacterium genitalium*’ ATCC 33030 and *Corynebacterium matruchotii* ATCC 14266^T, both of which lack a *pdxT* gene downstream

of *pdxS* (Fig. 1a). Another exception was observed in the genome of *K. rhizophila* DC2201, which includes an additional gene between the *pdxR* and *pdxST* coding regions. It is interesting to note that the *pdxR*–*pdxST* gene clusters are located in different regions of the respective 13 actinobacterial genomes (Markowitz *et al.*, 2006). When comparing the mean G + C content of all coding regions of

the *C. glutamicum* ATCC 13032 genome (54.8 mol%) with that of the *pdxR* (64.5 mol%), *pdxS* (60.9 mol%) and *pdxT* (57.4 mol%) genes, a deviation in the DNA base composition was detected, i.e. *pdxR* and *pdxST* are characterized by a significantly higher DNA G+C content. A comparative genomics approach with the Integrated Microbial Genomes system (Markowitz *et al.*, 2006) revealed that the *pdxS(T)* genes from other corynebacteria that lack a *pdxR*-like regulatory gene, such as *Corynebacterium jeikeium*, *Corynebacterium kroppenstedtii*, *Corynebacterium urealyticum* and *Corynebacterium efficiens*, are located in a conserved region of the genome close to the *ruvCAB* operon (Fig. 1c). The corresponding gene locus is also present in the genome of *C. glutamicum* ATCC 13032, which, however, lacks the *pdxST* genes (Fig. 1c). The biosynthesis pathway for vitamin B₆ has been shown to be very dynamic with respect to evolutionary changes (Tanaka *et al.*, 2005), and a putative genetic replacement of *pdxST* by PdxR-regulated counterparts in *C. glutamicum* might be another example of gene gain and gene loss in this part of bacterial metabolism.

Deletion of the PLP biosynthesis genes *pdxST*

The PdxS and PdxT proteins represent the subunits of PLP synthase, which catalyses the *de novo* biosynthesis of PLP via the DXP-independent pathway (Fitzpatrick *et al.*, 2007). The PdxS protein from *C. glutamicum* ATCC 13032 showed 74–91 % amino acid sequence identity with predicted PdxS proteins from other actinobacteria, whereas the range of sequence identity of PdxT proteins was 44–64 % (Fig. 1a). To analyse the physiological role of the PLP synthase genes in *C. glutamicum* ATCC 13032, a *pdxST* mutant was constructed by introducing a marker-free deletion into the *pdxST* gene region (Table 1). The resulting mutant *C. glutamicum* NJ0898 was subsequently characterized by growth assays in CGXII minimal medium supplemented with the vitamins pyridoxal, PLP, pyridoxamine or pyridoxine (Fig. 2a). Deletion of the *pdxST* genes in *C. glutamicum* NJ0898 resulted in vitamin B₆ auxotrophy, which was restored by external pyridoxal, PLP or pyridoxamine (Fig. 2a). Growth of *C. glutamicum* NJ0898 in the presence of pyridoxal or PLP was indistinguishable from that of a wild-type control, whereas supplementing of CGXII medium with pyridoxamine led to a prolonged lag phase of the culture. No growth of *C. glutamicum* NJ0898 was observed when the medium was supplemented with the vitamin pyridoxine (Fig. 2a). This suggests that the *pdxST* genes are essential for the biosynthesis of PLP in *C. glutamicum* ATCC 13032. Moreover, the successful supplementation of the mutant strain with different vitamins indicates that a vitamin B₆ salvage pathway for the utilization of pyridoxal and pyridoxamine is functional in *C. glutamicum* ATCC 13032 (Fitzpatrick *et al.*, 2007). In this pathway, the vitamins can be phosphorylated through the action of the specific kinase PdxK. A corresponding gene encoding PdxK (*cg1041*) was predicted in the genome sequence of *C. glutamicum* ATCC 13032 (Ikeda & Nakagawa, 2003; Kalinowski *et al.*, 2003).

Fig. 2. Phenotypic characterization of *C. glutamicum* NJ0898 and *C. glutamicum* AMH17. Growth in CGXII minimal medium was monitored by nephelometry and is shown as relative turbidity. Values are the means of measurements of six biological replicates. (a) Growth of the *pdxST* mutant *C. glutamicum* NJ0898 and the wild-type strain *C. glutamicum* ATCC 13032. (b) Growth of the *pdxR* mutant *C. glutamicum* AMH17 and the wild-type control ATCC 13032. In (a) and (b): ◆, ATCC 13032; □, NJ0898/AMH17; ×, plus pyridoxal; ○, plus PLP; △, plus pyridoxamine; ■, plus pyridoxine. (c) Complementation of the *pdxR* mutant *C. glutamicum* AMH17. Plasmid pEC-K18mob2_ *pdxR* contains the *pdxR* gene for complementation (×). *C. glutamicum* ATCC 13032 carrying the empty cloning vector pEC-K18mob2 served as a control (△). ◆, ATCC 13032 (pEC-K18mob2).

Differential expression of *pdx* genes in response to B₆ vitamers

Expression of the *pdxST*, *pdxK* and *pdxR* genes was analysed in response to the presence of the four B₆ vitamers. The wild-type strain *C. glutamicum* ATCC 13032 was grown in CGXII minimal medium supplemented with pyridoxal, PLP, pyridoxamine or pyridoxine. Expression of the *pdx* genes was measured by real-time RT-PCR, by using total RNA probes from exponentially growing cultures. The wild-type strain grown in non-supplemented CGXII medium served as a reference. Expression of *pdxS* and *pdxT* was significantly reduced in the presence of pyridoxal or PLP, whereas supplementing with pyridoxamine or pyridoxine showed no effect on *pdxST* expression (Fig. 3a). Expression levels of *pdxK* and *pdxR* revealed no significant differences from the control assay under any experimental condition (data not shown). This RT-PCR assay suggests that the transcription of the PLP synthase genes *pdxST* is controlled in response to the presence of pyridoxal or PLP in *C. glutamicum*.

Activation of the PLP synthase genes *pdxST* by PdxR

The first evidence for a regulatory role of the PdxR protein in the expression of *pdxST* was obtained in *C. glutamicum* by random transposon mutagenesis (Mormann *et al.*, 2006) and by genome-based analysis of aminotransferase genes (McHardy *et al.*, 2003). In the present study, growth of the *pdxR* mutant *C. glutamicum* AMH17, containing a defined deletion in the *pdxR* gene (McHardy *et al.*, 2003), was monitored in CGXII minimal medium supplemented with different vitamers (Fig. 2b). *C. glutamicum* AMH17 revealed

the expected auxotrophy which was restored by supplementing the growth medium with either pyridoxal, PLP or pyridoxamine (Fig. 2b). As observed in the *pdxST* mutant *C. glutamicum* NJ0898, growth of *C. glutamicum* AMH17 was indistinguishable from that of a wild-type control when the CGXII medium was supplemented with pyridoxal or PLP, whereas the addition of pyridoxamine to the medium led to a prolonged lag phase of the culture. As expected, no growth of *C. glutamicum* AMH17 was observed in the presence of pyridoxine (Fig. 2b). Accordingly, the *pdxR* mutant *C. glutamicum* AMH17 showed the same growth phenotype as the *pdxST* mutant *C. glutamicum* NJ0898. Moreover, the vitamin B₆ auxotrophy of *C. glutamicum* AMH17 was complemented by plasmid pEC-K18mob2_*pdxR* (Fig. 2c), thereby confirming that the auxotrophic phenotype of this mutant strain was caused by the deletion of the *pdxR* gene.

To characterize further the phenotype of *C. glutamicum* AMH17 at the genetic level, expression of *pdxST* and *pdxK* was measured by real-time RT-PCR and compared with a wild-type control. Inactivation of the *pdxR* gene in *C. glutamicum* AMH17 resulted in decreased expression of *pdxS* and *pdxT* (Fig. 4), whereas no difference in *pdxK* expression was detectable (data not shown). This negative effect of the *pdxR* mutation on the expression of *pdxST* in *C. glutamicum* AMH17 was complemented by plasmid pEC-K18mob2_*pdxR*. Expression of the *pdxS* and *pdxT* genes remained slightly lower (two- to fourfold) in the complemented *C. glutamicum* strain when compared with the wild-type control carrying the empty cloning vector (Fig. 4). However, the differential expression pattern of the *pdxST* genes in *C. glutamicum* AMH17 suggested an activating role of the PdxR regulator in PLP biosynthesis.

Fig. 3. Relative gene expression of *pdxS* and *pdxT* in response to different B₆ vitamers. Expression levels of *pdxS* and *pdxT* were measured by real-time RT-PCR. Values are means ($\pm$ SD) of at least two measurements. The assays were performed with *C. glutamicum* cultures grown in CGXII medium in the presence of either pyridoxal (PL), pyridoxal 5'-phosphate (PLP), pyridoxamine (PM) or pyridoxine (PN) where appropriate. (a) Relative expression of *pdxST* in the wild-type strain *C. glutamicum* ATCC 13032. Values were normalized by using the measurements determined in *C. glutamicum* ATCC 13032 grown in non-supplemented CGXII medium. (b) Relative expression of *pdxST* in the *pdxR* mutant *C. glutamicum* AMH17. (c) Relative expression of *pdxST* in the complemented strain *C. glutamicum* AMH17 (AMH17 + *pdxR*). Values in (b) and (c) were normalized by using the measurements determined in *C. glutamicum* ATCC 13032 carrying the empty cloning vector pEC-K18mob2 and grown in non-supplemented CGXII medium.

Fig. 4. Complementation of *C. glutamicum* AMH17 with the *pdxR* gene. Relative expression levels of *pdxS* and *pdxT* were measured by real-time RT-PCR and normalized by using the values determined in the plasmid-carrying wild-type *C. glutamicum* ATCC 13032 (pEC-K18*mob2*). The RNA probes were isolated from *C. glutamicum* strains grown in LB medium. Values are means ($\pm$ SD) of four measurements. AMH17, *C. glutamicum* AMH17 carrying the empty cloning vector pEC-K18*mob2*; AMH17+*pdxR*, *C. glutamicum* AMH17 carrying pEC-K18*mob2_pdxR* for complementation.

As global gene expression profiling by DNA microarray hybridization revealed no additional target gene with significant changes in gene expression levels (data not shown), PdxR most probably acts as a local activator of *pdxST* gene expression in *C. glutamicum*. According to the divergent orientation of the *pdxR* and *pdxST* genes, this genomic region of *C. glutamicum* ATCC 13032 represents a local gene regulatory unit commonly referred to as a divergon (Rodionov, 2007).

Furthermore, expression of the *pdxST* genes was measured by real-time RT-PCR in the *pdxR* mutant *C. glutamicum* AMH17 grown in CGXII minimal medium supplemented with pyridoxal, PLP or pyridoxamine (Fig. 3b). As expected, expression of the two genes was reduced under all growth conditions, most probably due to the absence of the *pdxR* gene product. Expression of *pdxST* was also monitored in the complemented mutant strain *C. glutamicum* AMH17 (Fig. 3c). In this case, expression levels of the *pdxST* genes were similar to those of the wild-type strain ATCC 13032 grown in the presence of the corresponding vitamers (Fig. 3a).

Transcriptional organization of the *pdxR*–*pdxST* intergenic region

For a more precise functional analysis of the 89 bp *pdxR*–*pdxST* intergenic region, *pdxR* and *pdxST* promoter

sequences and candidate DNA-binding sites of PdxR were predicted (Fig. 5). First, total RNA was isolated from exponentially growing *C. glutamicum* ATCC 13032 cells and used to amplify the 5' ends of the *pdxR* and *pdxST* transcripts by RACE-PCR. In both cases, the detected 5' ends were identical to the adenine residue of the annotated start codons (Fig. 5a), indicating the presence of so-called leaderless transcripts that are known to occur in *C. glutamicum* (Pátek *et al.*, 2003). The 5' ends were used to deduce potential –10 and –35 promoter sequences according to the characteristic features of corynebacterial promoters (Pátek *et al.*, 2003). The proposed promoter regions of *pdxR* (TTGCAT-N₁₇-GACGAT) and *pdxST* (CCGCTA-N₁₈-GATCAT) revealed weak similarity to the consensus sequence of *C. glutamicum* promoters, with three to four matches to the –10 and –35 hexamer consensus sequences (TTGCCA-N_{17/18}-TAYAAT).

The computer program REPuter (Kurtz *et al.*, 2001) was used to detect direct and inverted repeat sequences in the small *pdxR*–*pdxST* intergenic region (Fig. 5a). An 11 bp inverted repeat (motif 1 and motif 3) with one mismatch at position 8 and a spacing of 29 bp was found (AAAGTGG[A/T]CTA). Both DNA motifs are located between the mapped –10 and –35 hexamers of the *pdxR* and *pdxST* promoters (Fig. 5a). Interestingly, motif 1 overlaps the previously detected DNA-binding site (SOS box) of the SOS response regulator LexA from *C. glutamicum* (Jochmann *et al.*, 2009). An additional 12 bp sequence, termed motif 2 (AAAGTGGATCTA), showed similarity to motif 1 and motif 3 and was identified between the –35 promoter regions (Fig. 5a). A bioinformatic search for identical DNA motifs in the genome sequence of *C. glutamicum* ATCC 13032 with the PatScan program (Dsouza *et al.*, 1997) revealed an additional appearance of motif 1 within the coding region of *cg1490* (encoding a putative NTP pyrophosphohydrolase) and of motif 3 within the coding region of *cg0570* (encoding a putative dehydrogenase). Neither gene was detected as being differentially expressed by genome-wide transcriptional profiling of the *pdxR* mutant *C. glutamicum* AMH17 (data not shown), supporting the view that the transcription regulator PdxR acts as a local activator of the *pdxST* genes.

To provide more evidence for the biological significance of the three motifs, the nucleotide sequences of the *pdxR*–*pdxST* intergenic regions from six corynebacteria, *Propionibacterium acnes* and *Jonesia denitrificans* were aligned with the CLUSTAL W tool (Larkin *et al.*, 2007). This DNA alignment revealed that the direct and inverted repeat sequences represented by motif 1 to motif 3 are conserved in the *pdxR*–*pdxST* intergenic regions of the eight actinobacterial genomes (Fig. 5b). Motif 1 revealed the highest level of DNA sequence conservation, with eight of 11 matching bases. These DNA motifs are therefore likely candidates for DNA-binding sites of the transcription regulator PdxR. As knowledge of DNA-binding sites of MocR-type transcription regulators is very scarce (Rigali *et al.*, 2002), the functional relevance of the three motifs

Fig. 5. Genetic organization of the *pdxR*–*pdxST* intergenic region from *C. glutamicum* ATCC 13032. (a) Putative promoter sequences and candidate DNA-binding sites detected in the 89 bp *pdxR*–*pdxST* intergenic region. The mapped 5' ends are marked (TSP) and the deduced –10 and –35 hexamers of the *pdxR* and *pdxS* promoters are highlighted with grey boxes. The candidate DNA-binding sites (motifs 1–3) are shown and the location of an SOS box is indicated (Jochmann *et al.*, 2009). The ATG start codons are shown in bold letters. (b) Comparative DNA sequence analysis of the *pdxR*–*pdxST* intergenic regions from actinobacteria. The DNA regions corresponding to the candidate DNA-binding sites from *C. glutamicum* ATCC 13032 (motifs 1–3) are boxed. An additional motif (motif 4) was detected in the genome sequence of *P. acnes*. Start codons are shown in bold type. Pa, *P. acnes* KPA171202; Jd, *J. denitrificans* DSM 20603; Ca, *C. aurimucosum* ATCC 700975; Cd, *C. diphtheriae* NCTC 13129; Cge, '*C. genitalium*' ATCC 33030; Cm, *C. matruchotii* ATCC 14266; Cs, *C. striatum* ATCC 6940; Cgl, *C. glutamicum* ATCC 13032.

was investigated *in vitro* by DNA band shift assays with purified PdxR protein.

DNA band shift assays with purified PdxR protein

To demonstrate the specific binding of the regulatory protein PdxR to the detected sequence motifs in the *pdxR*–*pdxST* intergenic region of the *C. glutamicum* genome, DNA band shift assays were performed (Fig. 6). The PdxR protein was tagged and purified by means of the IMPACT system, and the identity of the obtained protein was verified by MALDI-TOF MS (data not shown). The purified PdxR protein was used in DNA band shift assays together with a Cy3-labelled PCR fragment of 195 bp, containing the 89 bp *pdxR*–*pdxST* intergenic region. This amplified DNA fragment showed different electrophoretic mobilities following incubation with either the PdxR protein or the SOS response regulator LexA (Fig. 6a). This result confirmed that both *C. glutamicum* proteins interact directly with the *pdxR*–*pdxST* intergenic region. A genomic DNA fragment containing the DNA-binding site of the transcription regulator LtbR from *C. glutamicum* served as a negative control (Fig. 6a). The binding of PdxR to the *pdxR*–*pdxST* intergenic region was not prevented *in vitro* by the addition of either pyridoxal, PLP, pyridoxamine or pyridoxine to the DNA band shift assay, although the more highly shifted band was reduced in the presence of pyridoxamine or pyridoxine (Fig. 6a). Moreover, Cy3-labelled, double-stranded 40-mers containing the sequence motifs 1–3 were applied in DNA band shift assays. Both PdxR and LexA were able to shift the specific 40-mer

oligonucleotide containing motif 1 and the SOS box (Fig. 6b). When using a mutated version of motif 1 by introducing transitions into the 11 bp sequence, the PdxR protein failed to shift the DNA fragment. On the other hand, transitions introduced into the genomic DNA regions flanking motif 1 did not affect the DNA binding of the PdxR protein (Fig. 6b). The PdxR protein also interacted with the 40-mers containing motif 2 or motif 3, but showed weaker interaction with these DNA fragments (Fig. 6c). As the three motifs are located very close to each other, the 40-mers representing motif 2 and motif 3 overlap with the adjacent motifs by a few bases. To clarify better the interaction of PdxR with motif 2 and motif 3, the native flanking regions of these DNA stretches were replaced by transitions. In both cases, the PdxR protein showed a weak interaction with the respective 40-mers, indicating that motif 2 and motif 3 can be recognized *in vitro* (Fig. 6c). These data demonstrated that the PdxR protein of *C. glutamicum* can interact *in vitro* with the three candidate DNA-binding sites that are located in the *pdxR*–*pdxST* intergenic region.

Additional DNA band shift assays were performed with 40-mer oligonucleotides containing motif 2 and motif 3. As observed with the single DNA motifs, a weak interaction of PdxR was detectable (Fig. 6d). The orientation of these motifs within the 40-mer was subsequently altered in such a way that they were arranged as direct repeats. In both cases, the PdxR protein interacted with the 40-mers much better than with the DNA fragment containing the native orientation of the two motifs (Fig. 6d). This probably

Fig. 6. DNA band shift assays performed with the purified PdxR protein from *C. glutamicum* ATCC 13032. (a) DNA band shift assays with a 195 bp PCR product covering the *pdxR*–*pdxST* intergenic region. The DNA band shifts were performed with PdxR and LexA proteins (+). The first lanes of each assay show controls that do not contain purified protein (–). In the DNA band shift assays with PdxR, modified binding buffers were used, containing either 2 or 200 μ M of the vitamins pyridoxal (PL), pyridoxal 5′-phosphate (PLP), pyridoxamine (PM) or pyridoxine (PN). The LtbR binding-site from *C. glutamicum* ATCC 13032 served as a negative control (Brune *et al.*, 2007). (b) DNA band shifts with oligonucleotides (40-mers) covering motif 1. The DNA sequences of the 40-mers are shown. The sequence of motif 1 is shown in bold type. Mutations were introduced by transitions into either motif 1 (motif 1*) or the native flanking regions (flank 1*). The replaced bases are shown in lower-case letters. The SOS box is underlined. (c) DNA band shifts with 40-mers covering motif 2 or motif 3. Mutations were introduced by transitions into the native flanking regions of motif 2 (flank 2*) and motif 3 (flank 3*). (d) DNA band shifts with 40-mers covering motif 2 and motif 3. The 40-mers were modified by inverting either motif 2 (motif 2inv) or motif 3 (motif 3inv). The orientation of the motifs is indicated by arrows. (e) DNA band shift assays with 40-mers covering the motif 1 region from *C. aurimucosum* (Ca), *C. diphtheriae* (Cd) and *P. acnes* (Pa). Motif 1 and the additional motif 4 in *P. acnes* are arranged as direct repeats, indicated by arrows.

indicates that a direct repeat favours the interaction of PdxR with the DNA *in vitro*. Moreover, DNA band shift assays were carried out with 40-mer oligonucleotides containing

motif 1 from *C. aurimucosum*, *Corynebacterium diphtheriae* and *P. acnes* to test whether the purified PdxR protein from *C. glutamicum* ATCC 13032 is able to interact also with

these motifs present in the *pdxR*–*pdxST* intergenic region of other actinobacteria (Fig. 6e). In all cases, a clear retardation of the 40-mer DNA fragment was observed, further supporting the idea that the conserved 11 bp region is relevant for the direct interaction of PdxR with the target DNA. The DNA band shift assay performed with a 40-mer containing motif 1 from *P. acnes* showed a different electrophoretic mobility when compared with the other assays (Fig. 6e). This difference may be due to the presence of an additional candidate DNA-binding site in this 40-mer sequence (motif 4) (Fig. 5b). By aligning the nucleotide sequences of the detected motifs, the DNA-binding site recognized by PdxR from *C. glutamicum* ATCC 13032 can be defined as AAAGTGGW(–/T)CTA.

DISCUSSION

Our results provide evidence that the transcription regulator PdxR controls the biosynthesis of PLP in *C. glutamicum* by activating the expression of the PLP synthase genes *pdxST*. Disruption of the *pdxR* gene resulted in decreased expression of the *pdxST* genes and consequently in vitamin B₆ auxotrophy. In an earlier study, growth assays performed with *Streptomyces venezuelae* ISP 5230 showed similar results, as disruption of the so-called ORF1 caused a growth requirement for pyridoxal (Magarvey *et al.*, 2001). The corresponding gene region was isolated by complementing the *pdx-4* mutation of *S. venezuelae*, indicating a relationship between the expression of ORF1 and the availability of pyridoxal. The protein encoded by ORF1 can be classified as a member of the MocR subfamily of GntR-type transcription regulators (Magarvey *et al.*, 2001; Rigali *et al.*, 2002). The PdxR protein from *C. glutamicum* was also grouped into this subfamily of regulatory proteins (Brune *et al.*, 2005; McHardy *et al.*, 2003). The GntR superfamily is one of the most abundant and widely distributed collections of metabolite-responsive transcription regulators in bacteria (Hoskisson & Rigali, 2009). In principle, these regulators contain a conserved N-terminal DNA-binding domain and a C-terminal effector-binding and/or oligomerization (E-b/O) domain that show extensive variation, apparently reflecting the large diversity of effector molecules that they bind. Based on comprehensive amino acid sequence alignments of the E-b/O domains and secondary structural predictions, GntR-like transcription regulators were classified into seven subfamilies (Hoskisson & Rigali, 2009). A characteristic feature of the MocR subfamily is the exceptional average length of the E-b/O domain that showed similarity to aspartate (class I) aminotransferases (Rigali *et al.*, 2002). These PLP-dependent enzymes catalyse the reversible transfer of an amino group from an amino acid substrate to an α -keto acid acceptor (Schneider *et al.*, 2000). This sequence similarity raises the possibility that the E-b/O domains of MocR-type transcription regulators have catalytic activity (Rigali *et al.*, 2002). However, the amino acid sequence alignment of PLP attachment sites

from the actinobacterial PdxR-like proteins revealed the lack of the highly conserved lysine residue that is generally used for the covalent binding of the PLP group (Sigrist *et al.*, 2010). Replacement of this essential lysine residue by serine, threonine or histidine may result in the loss of an enzymic function, which is consistent with a previous functional analysis in *C. glutamicum*, as aminotransferase activity was not detectable in enzyme assays with the purified PdxR protein (Marienhagen *et al.*, 2005). This result is in marked contrast with observations made with the regulatory protein GabR from *Bacillus subtilis*, a MocR-type transcription regulator that positively controls the utilization of γ -aminobutyric acid and represses its own expression (Belitsky & Sonenshein, 2002). The aminotransferase domain of GabR is probably essential for the proper functioning of this protein, as modification of the conserved lysine residue to alanine by site-directed mutagenesis led to a mutant form of the regulator that was unable to activate the expression of the divergently orientated *gabTD* operon, but retained the full ability to bind to the target DNA and to repress the *gabR* promoter (Belitsky & Sonenshein, 2002). The atypical amino acid sequence of the PLP attachment site in the actinobacterial PdxR-like proteins implies moreover that the covalent binding of PLP to the E-b/O domain is dispensable for the regulatory activity of PdxR, suggesting that the E-b/O domain has a different and hitherto unknown function in these proteins. Rigali *et al.* (2002) suggested that the E-b/O domain of MocR-type regulators may have been recruited to facilitate protein–protein interactions.

By using bioinformatic pattern searches, we detected three candidate DNA-binding sites for PdxR that are characterized by the consensus sequence AAAGTGGW(–/T)CTA. The 11 bp motifs 1 and 3 (AAAGTGGWCTA) and the 12 bp motif 2 (AAAGTGGATCTA) are apparently relevant for DNA binding of PdxR *in vitro*. Motif 1 and motif 2 represent a direct repeat that is separated by a spacer of 13 bp. Hence, these candidate DNA-binding sites are located on the same face of the DNA double helix. A similar genetic organization of candidate DNA-binding sites was detected in the nucleotide sequence linked to DNA binding of TauR, a MocR-type regulator that activates the expression of taurine utilization genes in *Rhodobacter capsulatus* (Wiethaus *et al.*, 2008). The respective DNA region contains a 10 bp direct repeat with the sequence CTGGAC(T/C)TAA and a 13 bp spacing (Wiethaus *et al.*, 2008). Interestingly, the candidate DNA-binding sites of TauR and the 12 bp motif 2 recognized by PdxR share the similar sequence TGGAYYTA, whereas the stretch of DNA sequence similarity to the 11 bp motif 1 is shorter (TGGWCY). DNA binding of the MocR-type regulator GabR was examined by DNase I footprinting and additional mutational studies of the recognized DNA region, revealing a 6 bp direct repeat with the sequence ATACCA located within the minimal GabR-binding region (Belitsky, 2004). Accordingly, short, directly repeated DNA sequences are present in the candidate operators of the

three MocR-type regulators. Another interesting aspect of MocR-type transcription regulators relates to their environmental sensing mechanism. The GabR protein from *B. subtilis* binds to its target DNA independently of PLP and γ -aminobutyric acid, but requires both metabolites for the activation of the *gabTD* operon (Belitsky & Sonenshein, 2002). Disruption of the *pdxR* gene in *C. glutamicum* resulted in decreased expression of the *pdxST* genes, which was also observed in the wild-type strain when supplementing the growth medium with either pyridoxal or PLP. How the PdxR regulator converts environmental information into the activation of *pdxST* gene expression is currently unknown and will be addressed in future studies.

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