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Molecular characterization of PrpR, the transcriptional activator of propionate catabolism in *Corynebacterium glutamicum*

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ABSTRACT

The 2-methylcitrate cycle is used to metabolize propionate in *Corynebacterium glutamicum*. The regulator, PrpR (Cg0800), of the *prpDBC2* operon was identified and characterized. The regulator has no similarities to the up to now known PrpR regulators from other organisms. Growth of a Δ*prpR* mutant revealed severe growth deficits and a prolonged lag phase if propionate was present in the medium. Transcriptome analyses demonstrated the inability of the Δ*prpR* strain to induce the *prpDBC2* genes in the presence of propionate indicating that PrpR represents a transcriptional activator. They also provided evidence that PrpR controls only the *prpDBC2* operon while transcription of the *prpR* gene was found to be independent of the used carbon source. GC–MS based metabolic profiling of the wild type and the Δ*prpR* strain grown with propionate revealed smaller pool sizes of the metabolites of the 2-methylcitrate cycle in the mutant strain. The transcriptional start sites and their putative promoters of the *prpDBC2* operon and the *prpR* gene were identified by RACE-PCR. Analyses of promoter test vector constructs led to the identification of a 121 bp operator region upstream of *prpDBC2*, which is essential for a propionate-induced transcription by PrpR. Finally, EMSA studies revealed that 2-methylcitrate most probably acts as co-activator of PrpR.

1. Introduction

The Gram-positive bacterium *Corynebacterium glutamicum* is widely used in industrial biotechnology for the production of amino acids. The determination of the complete genome sequence of the *C. glutamicum* wild type strain ATCC 13032 (Ikeda and Nakagawa, 2003; Kalinowski et al., 2003) facilitated genome-wide analyses in general and also rational strain improvement (Ohnishi et al., 2002). Each year, more than 1.5 million tons of glutamate and 0.75 million tons of lysine are produced with this bacterium (Kelle et al., 2005; Kimura, 2005). In their natural habitat, microorganisms must adapt to frequent and rapid changes in their environment. These changes include changing pH, temperature, oxygen concentration, and also nutrient availability. To cope with the latter challenge, *C. glutamicum* is known to be able to utilize a broad range of carbon sources including sugars, amino acids, alcohols and also organic acids (Arndt et al., 2008; Claes et al., 2002; Kiefer et al., 2002; Kotrbova-Kozak et al., 2007; Netzer et al., 2004). The carboxylic acid propionate, which is abundant in the soil, can be metabolized by *C. glutamicum* (Claes et al., 2002), although a toxic effect of propionate has been described for many bacteria at higher concentrations (Bramer et al., 2002; Brock and Buckel, 2004; Claes et al., 2002; Horswill et al., 2001). The toxic effect also occurs in *C. glutamicum* and results in a prolonged lag phase (Claes et al., 2002). For uptake of propionate, a passive diffusion has been suggested, but recently the involvement of a proton-driven secondary transporter was shown (Jolkver et al., 2009). The activation of propionate inside the cell is realized by acetate kinase (AK) and phosphotransacetylase (PTA), which is essential if propionate is the sole carbon source (Veit et al., 2009). If it is co-metabolized with glucose, the activation reaction can also be fulfilled by a CoA transferase (Veit et al., 2009). The resulting propionyl-CoA can afterwards enter the 2-methylcitrate pathway, which is exclusively used to degrade propionate to pyruvate. The function of the 2-methylcitrate cycle in *C. glutamicum* was demonstrated by generating and analyzing mutants in the *prpDBC2* genes, which are essential for growth with propionate as carbon source and encode the enzymes 2-methylcitrate dehydratase (PrpD2), 2-methylisocitrate lyase (PrpB2) and 2-methylcitrate synthase (PrpC2). In addition, in the presence of propionate an increased expression of the *prpDBC2* genes on the transcript and on the protein level was observed, respectively (Claes et al., 2002; Hüser et al.,

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2003). In both studies, the presence of a second cluster (*prpDBC1*), which is separated by about 38 kbp from the *prpDBC2* cluster, remains obscure. Two of the intermediates (2-methylcitrate and 2-methylisocitrate) of the 2-methylcitrate cycle were identified by GC–MS analyses and the toxic effect of propionate on growth of *C. glutamicum* cells was determined to arise from 2-methylcitrate (Plassmeier et al., 2007).

In enterobacteria for which a regulation similar to that in *C. glutamicum* was observed, the regulator of the *prp* genes (PrpR) was described for *Salmonella enterica* and a homologue was also found in *Escherichia coli* (Horswill and Escalante-Semerena, 1997). PrpR in *S. enterica* belongs to the sigma-54 dependent activator family and needs 2-methylcitrate as co-activator (Palacios and Escalante-Semerena, 2004). Other proteins are also involved in the regulation of the *prp* Operon in enterobacteria, these are σ^{54}, IHF and the cyclic AMP receptor protein (CRP) (Palacios and Escalante-Semerena, 2000). In *E. coli* the regulation of PrpR is at least in part dependent on the repressor AscG (Ishida et al., 2009), which regulates the expression of β-D-galactoside transport and catabolism. For *C. glutamicum*, the transcriptional regulatory network was analyzed and no homologue to PrpR from *E. coli* or *S. enterica* was identified (Brinkrolf et al., 2007; Brune et al., 2005). For *C. glutamicum*, a regulator designated PrpR was shown to be possibly involved in the regulation of the propionate catabolism, as *prpR* mutants displayed increased citrate and 2-methylcitrate synthase activities (Radmacher and Eggeling, 2007), although no direct effect of PrpR on the *prp* genes or the enzymes was shown.

In this study, we report the identification and characterization of the activator PrpR of the *prpDBC2* cluster (PrpR). In addition, the PrpR operator region was determined, as well as transcriptional start sites and the co-activator of PrpR. Metabolic analyses were used to identify the influence of the transcriptional regulator on the amount of metabolites formed by the 2-methylcitrate cycle.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

Minimal medium MM1 (Tauch et al., 2002), was used for growth of all *C. glutamicum* strains (Table 1) at a temperature of 30 °C, with 20 g/l glucose or 4 g/l of sodium acetate or 4 g/l of sodium acetate and 4 g/l sodium propionate as carbon sources. *C. glutamicum* strains carrying plasmids were selectively grown with kanamycin (25 μg ml^{-1}). Fermentations were performed in 1l BIOSTAT Q (Sartorius) culture vessels, with a constant pO_2 value of 30% and a constant pH value of 7 (using phosphorous acid and NaOH). Although *C. glutamicum* is able to use propionate as sole carbon source, a mixture of glucose and propionate is suitable for comparative studies due to the slow growth of *C. glutamicum* on propionate alone (Claes et al., 2002). Cultures for all kinds of analyses were harvested at an $o.D._{600}$ of 3, unless stated elsewhere in the text.

2.2. Chemicals

All chemicals and standard compounds were purchased from either Sigma–Aldrich–Fluka (Taufkirchen, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) or Macherey-Nagel (Düren, Germany).

2.3. DNA isolation, manipulation and transfer

Preparation of plasmid DNA from *E. coli* cells by an alkaline lysis technique was performed using the QIAprep Spin Miniprep Kit (Qiagen). Chromosomal *C. glutamicum* DNA was prepared as previously described (Tauch et al., 1995). Modification of DNA, analysis by agarose gel electrophoresis and ligation were performed by standard procedures (Sambrook et al., 1989). Transformation of *E. coli* and *C. glutamicum* cells was performed by electroporation, respectively (Tauch et al., 1994, 2002).

2.4. PCR methods and oligonucleotides

DNA amplification by PCR was carried out with KOD DNA polymerase (Merck) or Phusion DNA polymerase (Finnzymes) and chromosomal DNA as template in a PTC-100 thermocycler (MJ Research). PCR products were purified with the QIAquick PCR Purification Kit (Qiagen). All oligonucleotides used in this study were purchased from Metabion (Table S1).

2.5. Construction of defined mutant strains in C. glutamicum

A defined deletion was established within the *C. glutamicum cg0800* gene by means of the gene SOEing procedure, the primer sequence can be found in Table S1 (Lockhart et al., 1996). The resulting pK18*mobsacB* derivative was selected on PA plates (Antibiotic Medium No. 3) with kanamycin (25 μg ml^{-1}) and sequenced. After a transformation into *C. glutamicum*, a selection on CASO plates with kanamycin (25 μg ml^{-1}) and a subsequent selection on CASO plates (Tryptic Soy Broth) with 10% sucrose. The deletion of *cg0800* in the chromosome of *C. glutamicum* was verified by PCR (Schäfer et al., 1994).

2.6. Construction and transformation of plasmids

The plasmids pZ8-1::*prpR* and all pRIM2 derivatives were constructed and tested using the primers listed in Table S1. The primary PCR products were purified and digested with restriction enzymes corresponding to cleavage sites which were introduced via the primers and ligated into appropriately digested corresponding vector. The ligation mixture was used to transform *E. coli* JM109, the transformants were selected on PA plates containing 50 μg ml^{-1} kanamycin and 40 mg/l X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside). The correct inserts in the plasmids were verified by sequencing and afterwards the plasmids were transferred into *C. glutamicum* by electroporation. The transformants were selected on BHIS plates with 50 μg ml^{-1} kanamycin. After selection, the transformants were verified by PCR.

2.7. RNA preparation and real-time RT-PCR

Approximately 10^9 *C. glutamicum* cells were harvested from exponentially growing cultures at an $o.D._{600}$ of 3 by centrifugation for 15 s at 16,000 × *g*. The supernatant was decanted and the cell pellet was immediately frozen in liquid nitrogen. The frozen cells were resuspended in 800 μl RLT buffer provided with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and were disrupted using the Precellys instrument (Peqlab Biotechnologie, Erlangen, Germany) at a speed rate of 6.5 for 30 s. Subsequent purification of total RNA was performed using the RNeasy Mini Kit along with the RNase-Free DNase Set (Qiagen) and the DNase I Kit (Sigma–Aldrich, Taufkirchen, Germany) according to a previously published protocol (Hüser et al., 2003). Quantification of purified RNA samples was performed with the NanoDrop ND-1000 spectrophotometer (Peqlab Biotechnologie). Purified total RNA from *C. glutamicum* cultures was used in real time RT-PCR assays, performed with the LightCycler instrument (Roche Diagnostics) and the 2× SensiMix one step Kit (Quantace). The verification of the resulting RT-PCR products was performed by melting curve analysis. The differences in gene expression were determined by comparing the crossing points of two samples measured in duplicate. The crossing points