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Complex expression control of the *Corynebacterium glutamicum* aconitase gene: Identification of RamA as a third transcriptional regulator besides AcnR and RipA

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ABSTRACT

Expression of the aconitase gene *acn* of *Corynebacterium glutamicum* was previously shown to be repressed by the TetR-type regulator AcnR in response to a yet unknown stimulus and by the AraC-type regulator RipA in response to iron limitation. Here we have identified a third transcriptional regulator of aconitase, RamA. The RamA protein was enriched by DNA affinity chromatography with the *acn* promoter region from protein extracts of acetate-grown cells but not or only weakly from extracts of glucose-grown cells. In the wild type, aconitase activity is about 3-fold higher in acetate-grown cells compared to glucose-grown cells. In extracts of a *ramA* deletion mutant, acetate-grown cells possess the same aconitase activity as glucose-grown cells. Inspection of the *acn* promoter region led to the identification of a RamA binding motif (TGGGGGTGAGTAAGGGGGT), which was shown by electrophoretic mobility shift assays to be essential for binding of purified RamA. Furthermore, the functional relevance of this motif, which is located −180 to −162 bp upstream of the transcriptional start site, for RamA-dependent activation of *acn* expression was confirmed by promoter fusion assays. Thus, RamA was shown to be responsible for activation of *acn* expression in the presence of acetate. Furthermore, evidence was obtained in this work that RamB negatively regulates *acn* expression, but in an indirect manner.

1. Introduction

Corynebacterium glutamicum is a predominantly aerobic, non-pathogenic, biotin-auxotrophic Gram-positive soil bacterium that was isolated in a screen for bacteria that excrete L-glutamate (Kinoshita et al., 1957). It has gained considerable interest as a model organism for the *Corynebacterineae*, a suborder of the actinomycetes, which also includes the genus *Mycobacterium*, and because of its use in large-scale biotechnological production of L-glutamate (1.5 million tons per year) and L-lysine (0.8 million tons per year). The current knowledge on *C. glutamicum* has been summarized in two recent monographs (Burkovski, 2008; Eggeling and Bott, 2005).

Both L-glutamate and L-lysine are derived from intermediates of the tricarboxylic acid cycle (TCA cycle) and therefore we have initiated studies on the control of this pathway. Meanwhile, transcriptional regulation has been shown for the aconitase gene *acn* and for the succinate dehydrogenase operon *sdhCAB* (Krug et al., 2005; Wennerhold et al., 2005; Wennerhold and Bott, 2006). Moreover, a new type of post-transcriptional regulation of the 2-oxoglutarate dehydrogenase complex was discovered. In its unphosphorylated state, the OdhI protein binds to the E1 subunit OdhA and inhibits its enzymatic activity. Inhibition can be relieved by phosphorylation of a threonine residue in OdhI by the serine/threonine protein kinase PknG (Niebisch et al., 2006). This inhibition is of key importance for L-glutamate production (Schultz et al., 2007). An overview on the current knowledge on TCA regulation in *C. glutamicum* can be found in a recent review (Bott, 2007).

The first regulator of a TCA cycle gene to be identified in *C. glutamicum* was AcnR, a TetR-type repressor that represses expression of the aconitase gene *acn* by binding to between the −10 and −35 regions of the promoter. Until know the physiological function of AcnR is unclear, as it is not known which effector is responsible for triggering the dissociation of the AcnR-operator complex. Besides AcnR, *acn* expression was shown to be controlled by iron availability. Under iron limitation, the AraC-type regulator RipA is induced which reduces expression of *acn* and several other genes coding for prominent iron proteins of the cell, such as the *sdhCAB* genes (Wennerhold et al., 2005). The function of this type of regulation is presumably to reduce the iron demand of the cell under iron starvation. Induction of the RipA protein under iron limitation is due to inactivation of DtxR, the global iron regulator of

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Table 1
Bacterial strains and plasmids used in this work.

Strains or plasmids	Relevant characteristics	Reference
Strains		
C. glutamicum ATCC 13032	Biotin-auxotrophic wild-type strain	Kinoshita et al. (1957)
C. glutamicum RG2	Wild-type derivative with a 364-bp deletion of the 3′-terminal portion of the *ramA* gene	Cramer et al. (2006)
C. glutamicum RG1	Wild-type derivative with a 775-bp deletion of the 5′-terminal portion of the *ramB* gene	Gerstmeir et al. (2004)
E. coli DH5α	*supE44* Δ*lacU169* (Φ80*lacZ* ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
E. coli BL21(DE3)	*ompT hsdS*$_B$($r_B^-m_B^-$) *gal dcm* (DE3)	Novagen
Plasmids		
pET2	Km^r; promoter probe vector carrying a promoterless *cat* gene	Vasicova et al. (1998)
pET2_acn1	Km^r; pET2 carrying 563-bp *acn* promoter fragment (see Fig. 2A)	This work
pET2_acn2	Km^r; pET2 carrying 256-bp *acn* promoter fragment (see Fig. 2A)	This work
pET2-acn3	Km^r; pET2 carrying 327-bp *acn* promoter fragment (see Fig. 2A)	This work
pET2-acn4	Km^r; pET2 carrying 189-bp *acn* promoter fragment (see Fig. 2A)	This work
pET2-acn5-M1	Km^r; pET2 carrying 279-bp mutagenized *acn* promoter fragment (see Fig. 2A)	This work
pET2-acn5-M2	Km^r; pET2 carrying 279-bp mutagenized *acn* promoter fragment (see Fig. 2A)	This work
pET2-acn5-M12	Km^r; pET2 carrying 279-bp mutagenized *acn* promoter fragment (see Fig. 2A)	This work
pET28-RamAx6His	Km^r; plasmid for overproduction of RamA containing an N-terminal hexahistidine tag	Cramer et al. (2006)
pET29-RamBx6His	Km^r; plasmid for overproduction of RamB containing an C-terminal hexahistidine tag	Gerstmeir et al. (2004)

C. glutamicum, which represses *ripA* and more than 50 other genes under iron excess (Wennerhold and Bott, 2006; Brune et al., 2006).

Evidence was obtained that at least one other transcriptional regulator is involved in *acn* expression besides AcnR and RipA, since even in a Δ*acnR* mutant the aconitase activity is 1.5- to 2.3-fold higher in acetate-, citrate-, and propionate-grown cells compared to glucose-grown cells and the *acn* mRNA levels as determined by primer extension are also higher during growth on these organic acids (Krug et al., 2005). In this work we have identified RamA as a transcriptional activator of *acn* expression, which is responsible for increased aconitase activity in acetate-grown cells.

2. Materials and methods

2.1. Bacterial strains, culture conditions, plasmids and oligonucleotides

All strains and plasmids used in this work are listed in Table 1, the oligonucleotides in Table 2. The *C. glutamicum* type strain ATCC 13032 was used as wild type. For growth in minimal medium, CGXII medium (Keilhauer et al., 1993) or CgC medium (Eikmanns et al., 1991) with glucose, glucose plus acetate or acetate at the indicated concentrations was used. The CGXII medium was supplemented with 3,4-dihydroxybenzoate (30 mg l^{-1}) as iron chelator. The trace element solution was always added after autoclaving. *C. glutamicum* cultures were incubated on a rotary shaker at 30 °C and usually 120 rpm. For all cloning purposes, *E. coli* DH5α (Invitrogen) was used as host, for overproduction of the RamA and RamB proteins *E. coli* BL21(DE3) carrying plasmid pET28-ramA6xHis or pET29-ramB6xHis. The *E. coli* strains were cultivated in LB medium or 2xTY medium (Sambrook et al., 2001) at 37 °C. When appropriate, the media contained kanamycin (25–50 μg ml^{-1} for *C. glutamicum*, 50 μg ml^{-1} for *E. coli*). Growth of the bacteria was followed by measuring the optical density at 600 nm (OD_{600}).

2.2. Recombinant DNA work

The enzymes for recombinant DNA work were obtained from Roche Diagnostics (Mannheim, Germany) or New England Biolabs (Frankfurt, Germany). Routine methods like PCR, restriction or ligation were carried out according to standard protocols (Sambrook et al., 2001). Chromosomal DNA from *C. glutamicum* was prepared as described (Eikmanns et al., 1994). Plasmids from *E. coli* were isolated with the QIAprepspin miniprep Kit (Qiagen, Hilden, Germany) or the GFX Microplasmid Prep Kit (GE Healthcare, Buckinghamshire, UK). *E. coli* was transformed by the RbCl method (Hanahan, 1985) or electroporation, *C. glutamicum* by electroporation (van der Rest et al., 1999). For the electrophoretic mobility shift assays (see Section 2.6), different portions of the *acn* upstream region were amplified by PCR (Fig. 2A) using the following oligonucleotide pairs: acn1 for/acn1 rev (fragment acn1), acn1 for/acn2 rev (fragment acn2), acn3 for/acn1 rev (fragment acn3), acn4 for/acn1 rev (fragment acn4), acn5-M1 for/acn1 rev (fragment acn5-M1), acn5-M2/acn1 rev (fragment acn5-M2) and acn5-M12/acn1 rev (fragment acn5-M12). For measuring promoter activity, fragments acn1, acn3, acn4 and acn5-M12 were cut with SalI and HindIII and cloned into the promoter probe vector pET2 resulting in plasmids pET2_acn1, pET2_acn3, pET2_acn4, and pET2-acn5-M12. The PCR-derived portions in these pET2 derivatives were subjected to DNA sequence analysis to exclude spurious mutations.

Table 2
Oligonucleotides used in this work.

Name	Sequence (5′ → 3′)	Features
Acn-for-3 (fish)	CAGGTTGGAAGTCATCACTGGAGT	
Acn-Biotin-rev-2	GAGGAGTCGTCGATGTGGAGACCGTCATAGGACTTGTCGCCAACTTC	
Biotin primer 1	GAGGAGTCGTCGATGTGGAGACC	5′-Biotin label
acn1 for	ACGCGTCGACTCAACAGCTCCTGCGCAATATC	SalI
acn1 rev	CGCGGATCCGTGAGATTTCGCGACGGCGTC	BamHI
acn2 rev	CGTGAGCCCGACACTAATGA	
acn3 for	ACGCGTCGACTCATTAGTGTCGGGCTCACG	SalI
acn4 for	ACGCGTCGACTTAGACACGCTAGCAGGCCA	SalI
acn5-M1 for	ACGCGTCGACAGCAATGG**TAT**TGAGTAAGGGGGT	SalI
acn5-M2 for	ACGCGTCGACAGCAATGGGGGTGAGTAAGG**TAT**T	SalI
acn5-M12 for	ACGCGTCGACAGCAATGG**TAT**TGAGTAAGG**TAT**T	SalI