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Single-gene knockout of a novel regulatory element confers ethionine resistance and elevates methionine production in *Corynebacterium glutamicum*

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Abstract Despite the availability of genome data and recent advances in methionine regulation in *Corynebacterium glutamicum*, sulfur metabolism and its underlying molecular mechanisms are still poorly characterized in this organism. Here, we describe the identification of an ORF coding for a putative regulatory protein that controls the expression of genes involved in sulfur reduction dependent on extracellular methionine levels. *C. glutamicum* was randomly mutagenized by transposon mutagenesis and 7,000 mutants were screened for rapid growth on agar plates containing the methionine antimetabolite D,L-ethionine. In all obtained mutants, the site of insertion was located in the ORF NCgl2640 of unknown function that has several homologues in other bacteria. All mutants exhibited similar ethionine resistance and this phenotype could be transferred to another strain by the defined deletion of the NCgl2640 gene. Moreover, inactivation of NCgl2640 resulted in significantly increased methionine production. Using promoter *lacZ*-fusions of genes involved in sulfur metabolism, we demonstrated the relief of L-methionine repression in the NCgl2640 mutant for cysteine synthase, *o*-acetylhomoserine sulfhydrolase (*metY*) and sulfite reductase. Complementation of the mutant strain with plasmid-borne NCgl2640 restored the wild-type phenotype for *metY* and sulfite reductase.

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Introduction

The two sulfur-containing amino acids methionine and cysteine are key elements in animal feed. While methionine is an essential amino acid, animals are able to convert supplemented methionine into cysteine. Both amino acids are not only essential for protein biosynthesis, but are also precursors of various metabolites such as glutathione, *S*-adenosylmethionine, polyamines and biotin, and are involved as the methyl group donor in numerous cellular processes. Since concentrations of methionine and cysteine are often low in edible plant sources (Nikiforova et al. 2002), methionine is an important feed additive, with an annual production in the range of 300,000 t, third to glutamate and lysine (Leuchtenberger 1996). Unlike other bulk amino acids, methionine is produced by chemical synthesis and thus is the last of the major commercial amino acids that is not produced by fermentation.

Corynebacterium glutamicum strains have proven to be effective producers of L-lysine and L-threonine (Sahm et al. 1996; Hermann 2003) which, together with L-methionine, belong to the aspartate family of amino acids. Although lysine titers of 120 g/l or more are routinely obtained in industrial fermentations with *C. glutamicum*, no such process exists for methionine. The key step that distinguishes the biosynthesis of lysine and methionine is the incorporation of sulfur into the carbon skeleton. The common source of sulfur is sulfate, that has to be taken up, activated and reduced by the consumption of 7 mol ATP and 8 mol NADPH per mole of methionine (Neidhardt et al. 1990). In terms of cellular energy demands, this makes methionine the most expensive amino acid.

Genes involved in methionine biosynthesis are scattered on the genome of *C. glutamicum* (Ruckert et al. 2003), while genes involved in assimilatory sulfate reduction are clustered and at least partially organized in the *cys*-operon (Fig. 1). Sulfite reductase (NCgl2718) generates sulfide and is one of the genes under the control of the putative *cys*-operon promoter. Sulfide is then incorporated into *o*-acetylhomoserine via direct sulfhydrylation (MetY), or into *o*-acetylserine to give cysteine via the transsulfhydrylation

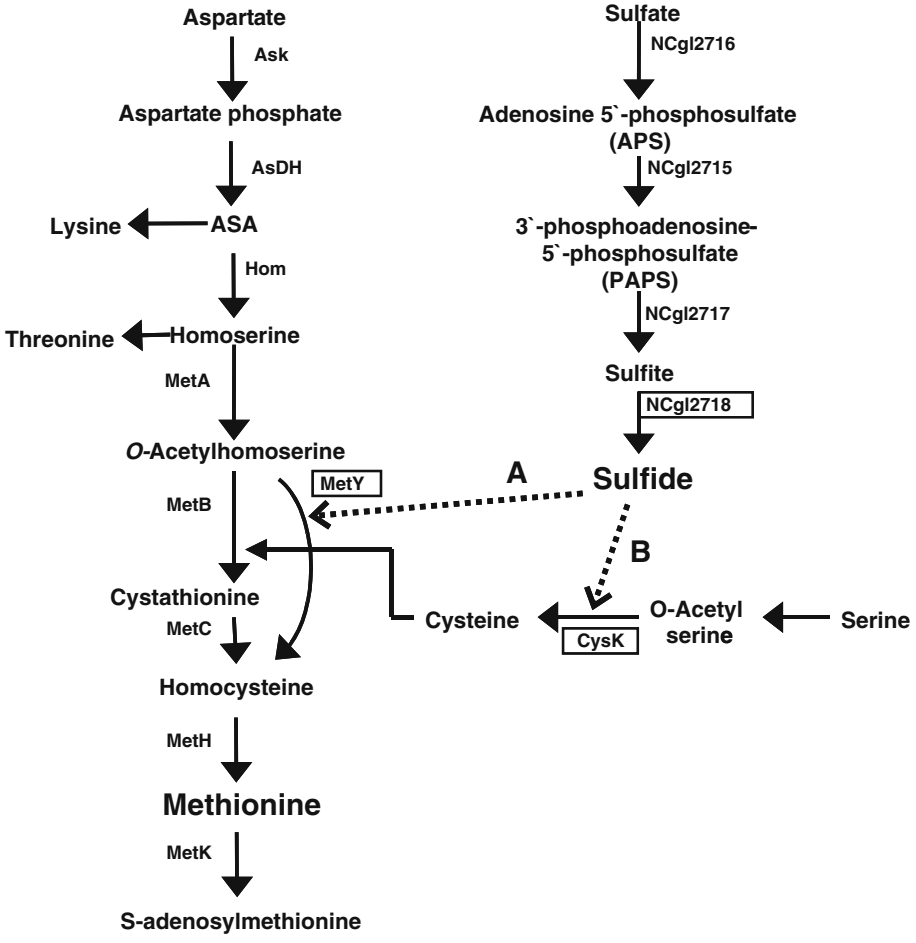

Fig. 1 The split pathway of sulfur incorporation in methionine biosynthesis. Sulfide is incorporated either via direct sulfhydrolation that is catalyzed by MetY (**a**), or by the transsulfhydrolation pathway (**b**). *lacZ* fusions were generated for promoters of the boxed gene products. The promoter of NCgl2718 governs the expression of genes involved in assimilatory sulfate reduction organized in a gene cluster (*cys*-operon, NCgl2715–NCgl2720). Cysteine synthase (NCgl2473) is not part of this cluster. *Ask* Aspartate kinase, *AsDH* aspartate semialdehyde dehydrogenase, *CysK* cysteine synthase, *Hom* homoserine dehydrogenase, *MetA* homoserine acetyltransferase, *MetB* cystathionine γ-synthase, *MetC* cystathionine β-lyase, *MetH* methionine synthase, *MetY O*-acetylhomoserine sulfhydrolase, *MetK S*-adenosylmethionine synthase, *NCgl2715* sulfate-adenosyltransferase subunit 1, *NCgl2716* sulfate-adenosyltransferase subunit 2, *NCgl2717* PAPS-reductase, *NCgl2718* sulfite reductase annotated as putative nitrite reductase

pathway (cysteine synthase CysK, NCgl2473; Fig. 1). *C. glutamicum* and related organisms use both pathways for methionine biosynthesis (Hwang et al. 2002; Lee and Hwang 2003). A diversity of regulatory mechanisms controlling the metabolic flux through these pathways has been described (Lee and Hwang 2003). Tight regulation avoids uneconomic depletion of cellular energy, but deregulation is indispensable for the overproduction of methionine. A detailed understanding of the regulatory mechanisms involved in sulfhydrylation is thus essential for the future rational design of methionine-producing strains.

There are only scattered reports on methionine-producing variants of *C. glutamicum*. Since 1975, when Kase and Nakayama (1975) reported on a multistep procedure using random mutagenesis and antimetabolite selection, no further improvement has been reported. Recently, a putative transcriptional repressor (McbR) involved in the regulation of the metabolic network directing the synthesis of sulfur containing amino acids was identified and a *mcbR* knockout strain was constructed (Rey et al. 2003). Still, methionine levels obtained in both examples fall short of commercial relevance.

Here, we report the identification of a novel regulator of methionine biosynthesis by screening a transposon library of *C. glutamicum* for ethionine-resistant strains. As a structural analogue of methionine, D,L-ethionine cannot be metabolized and thus mimics high concentrations of methionine. Consequently, methionine biosynthesis is downregulated and the organism finally starves from methionine depletion. One of several mechanisms to circumvent antimetabolite toxicity is to outcompete the toxic agent by overproduction of the natural metabolite (Mondal and Chatterjee 1994). Overproducing strains are typically based on mutations that relieve the feed-back inhibition of biosynthetic proteins (Kase and Nakayama 1975). Here, we screened for overproduction upon Tn*5531*-inactivation of genes involved in regulatory cascades.

Materials and methods

Bacterial strains, media and plasmids

C. glutamicum ATCC14752 or ATCC13032 were routinely cultivated in CGXII minimal medium (Keilhauer et al. 1993). *Escherichia coli* DH5α was used for standard cloning and *E. coli* ET12567 for plasmid amplification when plasmids were destined to be transformed in *C. glutamicum*. Strains and plasmids used in this study are listed in Table 1. Luria broth (LB) supplemented with appropriate antibiotics (50 μg/ml kanamycin, 50 μg/ml

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant phenotype	Source or reference
C. glutamicum strains		
ATCC13032	Wild type	ATCC
ATCC14752	Wild type	ATCC
13032::2640	Wild type with *NCgl2640*, Km^r	This work
14752::2640	Wild type with *NCgl2640*, Km^r	This work
14752-PcysK	ATCC14752 with reporter plasmid pClik-P*cysK*, Cm^r	This work
CG::2640-PcysK	14752::2640 with reporter plasmid pClik-P*cysK*, Km^r Cm^r	This work
CG::2640-PcysK-cpl	14752::2640 with reporter plasmid pClik-P*cysK*-cpl, Km^r Cm^r	This work
14752-PmetY	ATCC14752 with reporter plasmid pClik-P*metY*, Cm^r	This work
CG::2640-PmetY	14752::2640 with reporter plasmid pClik-P*metY*, Km^r Cm^r	This work
CG::2640-PmetY-cpl	14752::2640 with reporter plasmid pClik-P*metY*-cpl, Km^r Cm^r	This work
14752-Pcys	ATCC14752 with reporter plasmid pClik-Pcys, Cm^r	This work
CG::2640-Pcys	14752::2640 with reporter plasmid pClik-Pcys, Km^r Cm^r	This work
CG::2640-Pcys-cpl	14752::2640 with reporter plasmid pClik-Pcys-cpl, Km^r Cm^r	This work
E. coli strains		
DH5α	F^- *endA1*, *hsdR17*(rk^-mk^+) *supE44*, *thi-l* λ^- *recAI gyrA96 relA1* $\varphi 80\Delta lacAm15$	Hanahan (1983)
ET12567	*dam dcm hsd*, restriction deficient	MacNeil et al. (1992)
Plasmids		
pClik	*E. coli–C. glutamicum* shuttle vector; replicative in *C. glutamicum*, medium copy (10–20) number; Cm^r	EP 03/05423
pUC18	Ap^r, *lacZ*	Stratagene
pCGL0040	Donor of Tn*5531* (IS*1207* Km^r); Ap^r, $oriV_{E.c.}$	Ankri et al. (1996b)
pClik-SacB	Vector for allelic exchange by homologous recombination; nonreplicative in *C. glutamicum* Km^r, SacB	EP 03/05423
pSdel-NCgl2640	pClikSacB-based allelic exchange vector for chromosomal deletion of NCgl2640	This work
pClik-PcysK	*cysK*-promoter-probe vector *lacZ*-fusion, Cm^r	This work
pClik-PcysK-cpl	pClik-P*cysK* with NCgl2640, Cm^r	This work
pClik-PmetY	*metY*-promoter-probe vector *lacZ*-fusion, Cm^r	This work
pClik-PmetY-cpl	pClik-P*metY* with NCgl2640, Cm^r	This work
pClik-Pcys	*cys*-operon-promoter-probe vector *lacZ*-fusion, Cm^r	This work
pClik-Pcys-cpl	pClik-Pcys with NCgl2640, Cm^r	This work

chloramphenicol, 100 μg/ml ampicillin) was the standard medium for *E. coli* strains. LB medium supplemented with 4 mM $MgSO_4$ and 10 mM KCl (Psi-broth) was the recovery medium for chemically transformed *E. coli*. The recovery medium for electroporated *C. glutamicum* strains was LB with brain heart infusion and sorbitol (LBHIS; Liebl et al. 1989). Plasmid pCGL0040 (GenBank accession no. U53587) was used as the donor for Tn*5531* (IS*1207* Km^r) and was amplified in *E. coli* ET12567.

Recombinant DNA technologies

The transformation of *E. coli* cells with plasmid DNA was performed with chemically competent *E. coli* DH5α or ET12567. Cells were prepared according to the rubidium chloride method (http://micro.nwfsc.noaa.gov/protocols/) and transformed as described by Sambrook et al. (1989). Preparation of competent *C. glutamicum* cells and electrotransformation was done as described elsewhere (Liebl et al. 1989; Ankri et al. 1996a).

Genomic DNA was isolated using the Wizard genomic DNA purification kit (Promega). Plasmid preparation from *E. coli* cells was routinely done with the QIAprep miniprep kit (Qiagen). Restriction endonucleases were from Roche Diagnostics. Digested DNA fragments were recovered from agarose gels with the QIAEX II gel extraction kit (Qiagen). Standard DNA techniques were performed as described by Sambrook et al. (1989). DNA sequencing was performed using the Global edition IR2 system (LI-COR, Lincoln, Neb.).

The genome database used to identify ORFs and promoters was ERGO (Integrated Genomics, Chicago, Ill.). The NCgl numbers refer to the *C. glutamicum* ATCC13032 genome sequence in the GenBank database at the National Center for Biotechnology Information (NCBI; GenBank accession no. NC_003450). The promoters deduced from the ERGO database for cysteine

synthase *cysK* (NCgl2473; promoter position 2721625–2721822), sulfite reductase (NCgl2718; promoter position 3005188–3005389) and *o*-acetylhomoserine-sulfhydrolase (*metY*; NCgl0625; promoter position 667771–668107) were PCR-amplified and fused by *Xho*I and *Bam*HI linkers to promoterless *lacZ* on plasmid pClik (Cm^r), opened by the same restriction enzymes. The resulting plasmids were termed pClik-PcysK, pClik-Pcys and pClik-PmetY, respectively (Table 1). A unique *Bgl*II site on these plasmids was used to introduce PCR-amplified NCgl2640, using primer pair 2640-fwd-BglII (5′-CCGCTGCTGCT GGTGGCGCTAGATCTGCTAACGGC-3′) and 2640-rev-BglII (5′-ATGTGTTGGGAGATCTCTTAAGTTATTTAG TCCAG-3′). The amplified DNA fragment comprised putative regulatory elements located up to 370 bp upstream of the NCgl2640 gene.

Transposon mutagenesis, screening and localization of transposon insertion sites

Plasmid pCGL0040 was isolated from *E. coli* ET12567 and *C. glutamicum* ATCC14752 was transformed with the plasmid by electroporation. Transposon insertion mutants were selected by plating on LBHIS containing 20 μg/ml kanamycin. All mutants were pooled, washed twice with sterile 0.9% NaCl and plated on CGXII containing kanamycin (20 μg/ml) and ethionine (7.5 g/l) in 100-μl aliquots of a 10^6 dilution of the pool. The most rapidly growing colonies were selected for further analysis.

For the localization of the transposon insertion sites, genomic DNA of the mutants was isolated and digested with *Eco*RI, as described by Simic et al. (2001). Insertion sites were determined by cloning transposon–chromosome junction sites into *Eco*RI-digested pUC18. Plasmids from kanamycin-resistant clones were isolated and subsequently sequenced with oligonucleotide Tn5531-Eco (5′-CGGG TCTACACCGCTAGCCCAGG-3′; Simic et al. 2001). The sequences thus obtained were analyzed using the BLASTn program applied to the NCBI GenBank sequence and the ERGO database. Different sequence analysis tools (http://www.expasy.org/, http://npsa-pbil.ibcp.fr or the protein family database, PFAM at http://pfam.wustl.edu/) were used for pattern and profile searches with the NCgl2640 sequence.

Chromosomal deletion of NCgl2640 in *C. glutamicum* ATCC13032

Using the two primer pairs 2640-SacB1 (5′-GAGAGGGC CCATCAGCAGAACCTGGAACC-3′) with 2640-SacB2 (5′-GATCCAGAGGTCCACAACC-3′) and 2640-SacB3 (5′-GATGGTTCAAGACGAACTCC-3′) with 2640-SacB4 (5′-GAGAGTCGACCAGAATCAATTCCAGCCTTC-3′), the upstream and downstream region of NCgl2640 was PCR-amplified from chromosomal DNA of *C. glutamicum* ATCC13032. The resulting fragments were digested with *Apa*I/*Xba*I and *Spe*I/*Sal*I, respectively, and cloned together into the nonreplicative vector pClik-SacB, that was digested with *Apa*I/*Sal*I, yielding plasmid pSdel-NCgl2640. *C. glutamicum* ATCC13032 was transformed by electroporation with the nonreplicative plasmid pSdel-NCgl2640. Kanamycin-resistant clones contained chromosomally integrated plasmids. Subsequently, we selected for loss of the plasmid by screening for sucrose-resistant mutants according to Schaefer et al. (1994). The deletion was verified by PCR analysis and Southern blotting.

LacZ activity measurements

Selected mutant strains of *C. glutamicum* were transformed with the plasmids pClik-PcysK, pClik-PmetY and pClik-Pcys or the NCgl2640-complemented derivatives thereof (Table 1). Transformants were grown in CGXII minimal medium containing chloramphenicol (15 μg/ml) in the presence or absence of 10 mM L-methionine. Cells were grown to an optical density at 600 nm (OD_{600}) of 1–3 and analyzed for β-Gal activity, as described by Sambrook et al. (1989). Assays were done in triplicates in four independent test series.

Isolation of DNA-binding proteins

The principle of isolating DNA-binding proteins by DNA-affinity chromatography using magnetic beads is essentially described by Gabrielsen and Huet (1993) and a detailed protocol for *C. glutamicum* is available (Rey et al. 2003). We basically followed the latter protocol with a few exceptions. All buffers except the elution buffers were supplemented with 2.5 mM L-methionine. Crude extracts of cells grown in the presence or absence of 10 mM L-methionine were prepared separately and combined thereafter. Immediately after cell disruption, crude extract was protected against proteolysis by a protease inhibitor cocktail (phenylmethylsulfonyl fluoride, aprotinin, leupeptin; Rosenberg 1996). After ultracentrifugation of the crude extract (200,000 *g*, 40 min, 4°C), the protein solution was desalted by gel filtration (Sephadex G25). Biotinylated PCR-amplified promoter-DNA was immobilized on streptavidin-coated Dynabeads (M270; Dynal Biotech). As a negative control fragment, we amplified a 460-bp fragment from the upstream region of the *groES* gene of *C. glutamicum*. The washing buffer contained high amounts of unspecific competitor DNA (0.4 mg/ml salmon testes DNA; Sigma). 1D-SDS-PAGE was done with a 4% stacking gel and a 12% separative gel (Schägger and von Jagow 1987) and proteins were stained with colloidal Coomassie brilliant blue G-250 (Neuhoff et al. 1988). The protocol for tryptic digestion and matrix assisted laser desorption/ionization–time of flight (MALDI-TOF) analysis was essentially that described by Hermann et al. (2001).

Determination of extra- and intracellular methionine concentrations

Methionine was quantified as its *o*-phthaldialdehyde derivative by high-pressure liquid chromatography (HPLC; Molnar-Perl 2001). *C. glutamicum* was grown to the stationary phase in 500-ml shake-flasks with culture volumes of 50–100 ml at 30°C and 225 rpm. Cells were removed by centrifugation (10,000 *g*, 10 min, 4°C) and methionine concentrations were determined by HPLC analysis.

For the determination of intracellular methionine levels, cells were separated from the bulk liquid and inactivated by silica oil centrifugation, as described by Ebbighausen et al. (1989), and subsequently disrupted by sonification or by a blue-capped Ribolyser (FastPrep; Q-Biogene). Soluble protein in the supernatant was measured by a Bradford-type assay (Bradford 1976). The intracellular content of soluble protein in *C. glutamicum* was empirically determined as 250 mg/ml. Based on this value, we calculated the total internal cell volume of a sample in order to determine methionine concentrations upon HPLC analysis.

Results

Selection and identification of ethionine-tolerant transposon mutants

We hypothesized that ethionine tolerance can be acquired by overproduction of methionine, which in turn can be achieved by inactivation of a repressor involved in the regulation of methionine biosynthesis. Therefore, we transformed *C. glutamicum* ATCC14752 with pCGL0040 as the donor of Tn*5531* by electroporation. About 7,000 mutants were obtained on LBHIS plates containing kanamycin. All mutants were scraped off the plates, pooled, washed and plated onto CGXII-agar plates containing 7.5 g/l D,L-ethionine plus kanamycin. We plated about 100,000 colony-forming units (CFU) to ensure multiple recoveries of putatively ethionine-resistant mutants. Growth of the wild type was inhibited by 6 g/l ethionine for at least 4 days. After 2 days, 11 kanamycin- and ethionine-resistant mutants were isolated. All mutants contained the identical Tn*5531* insertion in ORF NCgl2640. This mutation was termed 14752::2640; and one clone was selected for further experimentation (Table 1).

Sequence analysis of transposon insertion loci

The site of transposon insertion in strain 14752::2640 was located in the C-terminal half of the putative protein at position 2,918,026/2,918,027 (GenBank accession no. NC_003450). NCgl2640 was separated from NCgl2639 by 7 bp, so both genes are presumably organized as an operon (Fig. 2). NCgl2639 is annotated as a putative hydrolase or acetyltransferase (GenBank). NCgl2640 encodes a protein of 42 kDa and homology searches identified more than 25 putative bacterial proteins of significant homology (*e*-value <2e−20), none of which were functionally assigned. A conserved domain search identified a consensus pattern for proteins of unknown function (COG2170) with high significance and for motif 04107 of the PFAM database that is characteristic for the glutamate-cysteine ligase family. No further consensus patterns were detected, in particular none for DNA-binding proteins.

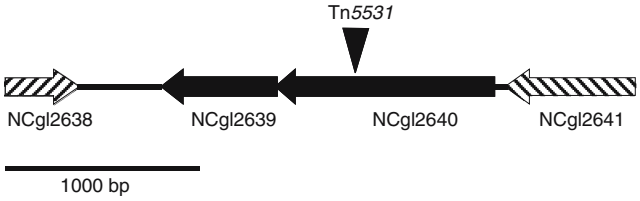

Fig. 2 Genomic context of NCgl2640. GenBank annotations available for the open reading frames: *NCgl2638* similarities to multisubunit Na^+/H^+ antiporter, *NCgl2639* similar to predicted hydrolases or acyltransferases, alpha/beta hydrolase superfamily, *NCgl2640* hypothetical protein/uncharacterized BCR, *NCgl2641* hypothetical protein, no annotation. *Tn5531* Transposon 5531 (*IS1207*). *Filled triangle* Site of insertion of Tn*5531* in NCgl2640

Verification of the ethionine-resistance phenotype

C. glutamicum ATCC14752 and the mutant strain were cultivated in shake-flasks in CGXII medium containing 3 g/l glucose in the presence or absence of 7.5 g/l D,L-ethionine. Growth of the mutant in the presence of ethionine was indistinguishable from growth without ethionine or growth of the wild type without ethionine (Fig. 3).

To exclude second-site mutations that may have caused the ethionine-resistance phenotype, we tested the effect of NCgl2640 inactivation in *C. glutamicum* ATCC13032. NCgl2640 was excised by homologous recombination and selection of sucrose-tolerant mutant strains. This mu-

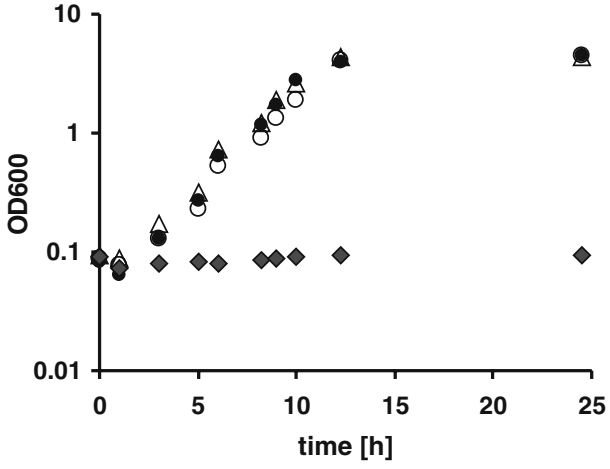

Fig. 3 Growth of *C. glutamicum* wild type and mutant grown in CGXII medium in the presence or absence of ethionine. The glucose concentration was 3 g/l, the D,L-ethionine concentrations was 7.5 g/l. *Filled symbols* Ethionine present during cultivation, *open symbols* cultivation without ethionine. *Filled triangles* Wild type, *filled circles* mutant strain

tant, designated 13032::2640, was readily resistant to D,L-ethionine at 7.5 g/l, while no growth of wild-type ATCC13032 was detected on these plates. Thus, we demonstrated that the ethionine-resistance phenotype depended on inactivation of NCgl2640.

Increased L-methionine levels in the NCgl2640 mutant

The resistance of *C. glutamicum* 14752::2640 to high ethionine concentrations may be due to upregulated biosynthesis of L-methionine. To test this hypothesis, we analyzed the production of L-methionine in the wild type and mutant in CGXII minimal medium batch cultures. Generally, the mutant accumulated about twice the amount of methionine compared with the wild type, both intra- and extracellularly (Fig. 4).

Altered expression levels of methionine biosynthesis genes in the NCgl2640 mutant

We assumed that tight regulation of sulfur incorporation would be a key element in methionine biosynthesis. Hence, inactivation of a repressor of sulfur gene expression would cause elevated methionine levels that confer ethionine resistance. Therefore, we decided to elucidate the impact of the NCgl2640 knockout on the expression levels of *metY*, *cysK* and *cys*-operon genes. For this purpose, strain 14752::2640 and the wild type were transformed with the *lacZ*-reporter plasmids pClik-PcysK, pClik-PmetY and pClik-Pcys (Table 1) and grown to an OD_{600} of 3.0 in CGXII medium with or without 10 mM L-methionine. Gene expression was then detected by monitoring LacZ activity.

In the wild type, the presence of methionine reduced the expression levels of all examined genes, but expression of the *cys*-operon was completely abolished. In the mutant strain, significant derepression was observed for the *cys*-operon (Fig. 5). A complementation assay was used to exclude the polar effects of the inactivation of NCgl2640 on the adjacent NCgl2639 (hydrolase or acetyltransferase; Fig. 2). Expression of NCgl2640 from medium-copy-number plasmids under the control of its endogenous promoter (370 bp of the upstream sequence were included) completely restored the wild-type phenotype in the mutant, i.e. methionine-induced repression of the *cys*-operon (Fig. 5).

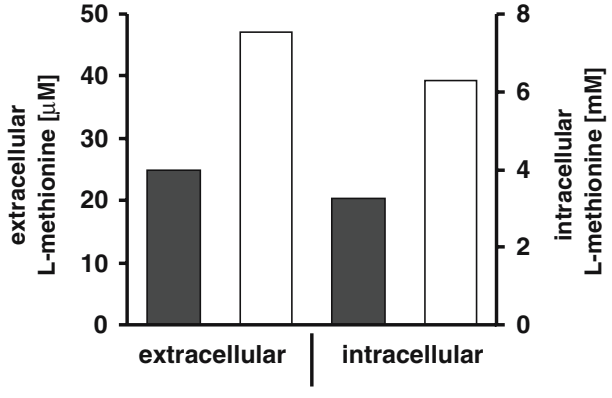

Fig. 4 Extra- and intracellular L-methionine levels in *C. glutamicum* wild type and the NCgl2640 knockout mutant in CGXII medium. *Dark bars C. glutamicum* wild type, *white bars* NCgl2640 knockout mutant

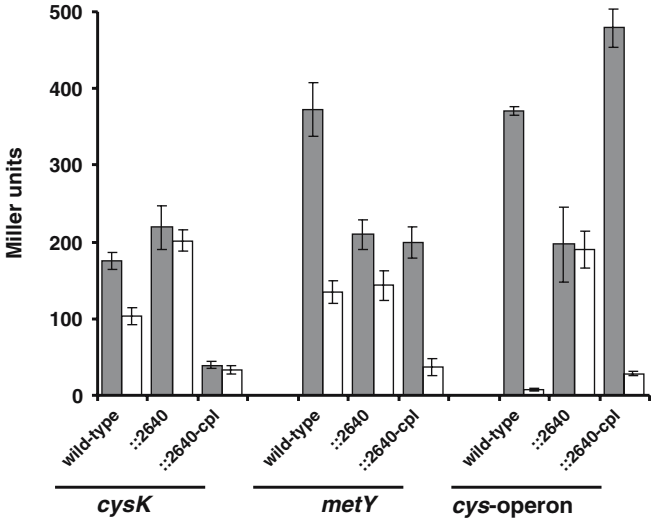

Fig. 5 Influence of the NCgl2640 knockout on methionine-dependent expression of cysteine synthase (*cysK*), *o*-acetylhomoserine sulfhydrolase (*metY*) and the *cys*-operon. *C. glutamicum* ATCC14752 (wild type), mutant strain 14752::2640 and mutant strains complemented with plasmid-borne NCgl2640 (::2640-cpl), harboring reporter plasmids pClik-P*cysK*, pClik-P*metY* or pClik-P*cys* were grown in CGXII medium (3 g/l glucose) in the presence (*white bars*) or absence (*dark bars*) of L-methionine (10 mM). Promoter activity was determined by LacZ activity reporter assays and quantified in Miller units

NCgl2640 does not bind to the *cysK*, *metY* and *cys*-operon promoters

We clearly demonstrated that expression of the *cys*-operon was modulated by NCgl2640. Since classic DNA-binding motifs could not be identified, we employed DNA-affinity purification in a pull-down assay to investigate whether NCgl2640 could bind to the respective promoter regions. PCR-amplified and bead-immobilized promoters were incubated in the presence of 2.5 mM L-methionine with combined crude extracts of *C. glutamicum* cells cultivated in the presence or absence of 10 mM L-methionine. Proteins that were eluted from the promoters at high salt concentrations (>200 mM) were separated by 1D-SDS-PAGE and analyzed by MALDI-TOF. Employing a similar approach, Rey et al. (2003) isolated the McbR-repressor along with four additional proteins that appeared to bind specifically to the *metY*-promoter. We largely confirmed these results in our study. In addition, we found that one of the proteins, exopolyphosphatase, reported by Rey et al. (2003) to bind specifically to the *metY*-promoter, also binds

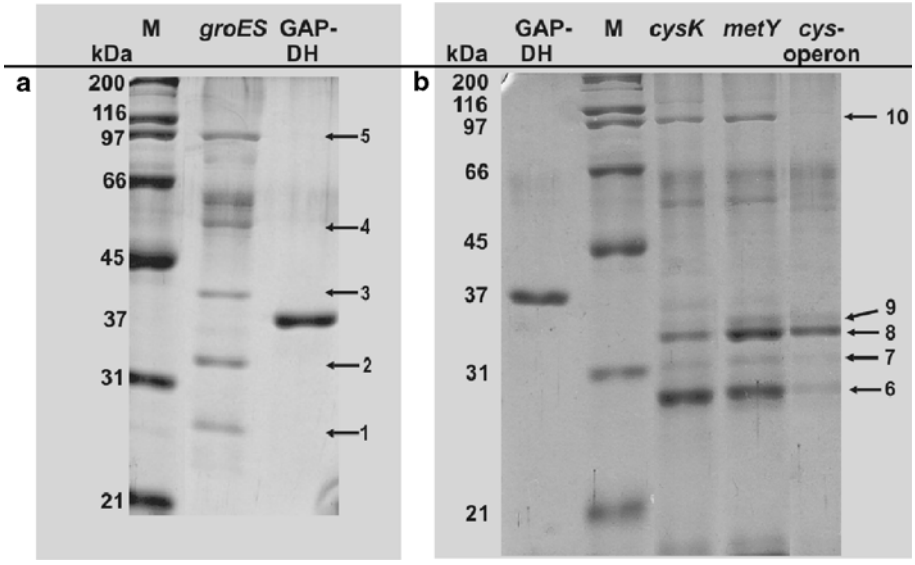

Fig. 6 SDS-PAGE of proteins binding to the putative control-promoter regions of *groES* (**a**) and to putative promoter regions of the *C. glutamicum* genes for cysteine synthase (*cysK*), *o*-acetylhomoserine sulfhydrolase (*metY*) and genes of the putative *cys*-operon (**b**). *1* Single-strand binding protein, *2* exopolyphosphatase, *3* diadenosine-tetraphosphate hydrolase, *4* DNA-polymerase III α-subunit, *5* DNA-polymerase I, *6* TetR-like regulator McbR that was shown to be involved in the regulation of methionine biosynthesis (Rey et al. 2003), *7* exopolyphosphatase (degradation product), *8* exopolyphosphatase, *9* ATP-phosphoribosyltransferase, *10* DNA-polymerase I. Except for exopolyphosphatase and DNA-polymerase I, none of the identified proteins bound to the *groES* control-promoter fragment (Fig. 6a). *GAP-DH* Glyceraldehyde-3-phosphate dehydrogenase used as additional marker-protein (37 kDa), *M* protein marker

to the control promoter of *groES*, indicating unspecific binding. Moreover, we could show that McbR also binds to promoters of *cysK* and the *cys*-operon; but binding to the *cys*-operon promoter was low compared to the *metY*-promoter (Fig. 6). Consistent in both studies, however, NCgl2640 was not detected, indicating that a direct DNA–protein interaction of NCgl2640 is not involved in the observed NCgl2640-mediated regulation.

Discussion

Using Tn*5531* mutagenesis and antimetabolite selection, we demonstrated that a single gene inactivation event renders *C. glutamicum* tolerant to high concentrations of ethionine and increases methionine biosynthesis. Classic strain development encompasses several rounds of random mutagenesis and antimetabolite selection (Sauer 2001); and the structural analogue of methionine, ethionine, is the major antimetabolite used for the selection of methionine overproducers (Lawrence et al. 1968; Kase and Nakayama 1975; Tani et al. 1988). Ethionine inhibition of methionine biosynthesis can be overcome by mutations that relieve feedback inhibition, e.g. by altering allosteric binding sites, but the resulting resistance is typically based on combinations of unidentified mutations. Here, we demonstrate that inactivation of NCgl2640 is sufficient to confer high-level tolerance to ethionine. To the best of our knowledge, this is the first report on a single-gene knockout that confers ethionine resistance.

Doubling of the methionine pool apparently suffices to confer resistance to a roughly 10-fold excess of the antimetabolite. The observed increase in extracellular methionine levels equals that reported recently for the *mcbR* mutant of *C. glutamicum* (Rey et al. 2002). Thus, inactivation of the putative master regulator of methionine biosynthesis has a similar effect on methionine production as the inactivation of NCgl2640, which underlines the importance of additional regulatory mechanisms in methionine and sulfur metabolism.

Based on *lacZ*-promoter assays, NCgl2640 was shown to exhibit a regulatory function in methionine biosynthesis that is most pronounced for genes involved in sulfur metabolism (*cys*-operon). In contrast to the putative transcriptional repressor McbR, whose activity does not appear to be directly induced by methionine, NCgl2640-based regulation is clearly dependent on extracellular methionine. Two arguments render a direct transcriptional regulation by NCgl2640 unlikely. First, no DNA-binding motif was detected, and second, we isolated McbR but not NCgl2640 with the DNA-affinity purification assay. Thus, NCgl2640 is probably an indirectly acting regulatory element involved in the repression of sulfur metabolism genes.

Two hypotheses on the actual regulation mechanism of NCgl2640 can be formulated from its annotation in the genome sequence of *C. glutamicum*. In the first, NCgl2640 might modulate gene expression indirectly via the putative master regulator McbR that was shown to bind directly to the *metY*-promoter (Rey et al. 2003). Based on one annotation of NCgl2640 as a putative glutamyl-cysteine-ligase, it might be speculated that interference with glutathione biosynthesis might affect McbR directly or indirectly, resulting in altered expression levels of McbR-

controlled genes. We observed a less pronounced effect of the inactivation of NCgl2640 on *cysK* and *metY* expression levels compared with *cys*-operon. By a proteomic analysis, Rey et al. (2003) showed that the levels of CysK, MetK and MetY were significantly altered in a *mcbR* knockout strain, although the levels of proteins encoded in the *cys*-operon were not affected. Thus, McbR seems to play a minor role in NCgl2640-mediated regulation.

The second hypothesis is based on the alternative annotation as a GTPase-activating protein published by Kyowa Hakko Kogyo Co. Ltd (GenBank accession no. BAC00130) and listed in ERGO, a commercial genome database that currently covers more than 600 genomes. GTPase-activating proteins are elements of GTPase-dependent regulation cascades that indirectly control gene expression (Voncken et al. 1995; Donovan et al. 2002; Litvak and Selinger 2003), mostly in eukaryotes (Caldon and March 2003) but also in bacteria (Lerner and Inouye 1991; Zhang and Inouye 2002). Thus, NCgl2640 may act as the methionine-sensing element that specifically enhances the GTP-hydrolyzing activity of its putative target GTPase that is involved in the regulation of methionine biosynthesis.

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