Development of a single-cell GlxR-based cAMP biosensor for *Corynebacterium glutamicum*

Julia Schulte, Meike Baumgart, and Michael Bott*

IBG-1: Biotechnology, Institute of Bio- and Geosciences, Forschungszentrum Jülich, 52425 Jülich, Germany

*Corresponding author
Tel +49 2461 613294
Fax +49 2461 612710
E-mail m.bott@fz-juelich.de

Running title: Development of a cAMP biosensor for *C. glutamicum*

Abstract

Cyclic adenosine monophosphate (cAMP) plays a regulatory role as second messenger in many species. In the industrial model organism *Corynebacterium glutamicum*, cAMP acts as effector of the global transcriptional regulator GlxR, a homolog of enterobacterial Crp. The cAMP-GlxR complex activates or represses the expression of about 200 target genes. CyaB, a membrane-bound class III adenylate cyclase, synthesizes cAMP from ATP, but another yet unknown cAMP-forming enzyme is likely present in *C. glutamicum*. Recently, we identified the cAMP phosphodiesterase CpdA, which catalyzes the conversion of cAMP to AMP. As a tool to search for additional cAMP-forming and degrading enzymes, we constructed a plasmid-based cAMP biosensor by fusing the promoter of cg3195, a gene strongly repressed by GlxR, to the *eyfp* reporter gene. In control experiments, the biosensor showed the predicted responses to increased levels of cAMP or GlxR. The biosensor was able to distinguish between *C. glutamicum* wild type and mutants with defects in cAMP biosynthesis or degradation. Most importantly, the sensor allowed successful sorting of mixtures of wild type and mutant strains by fluorescence activated cell sorting (FACS), thus meeting the requirements for high-throughput screening of libraries for single mutant cells with an altered cAMP level.

Keywords: cAMP, biosensor, *Corynebacterium glutamicum*, GlxR, FACS

1. Introduction

The Gram-positive soil bacterium *Corynebacterium glutamicum* is used industrially as a microbial cell factory for the biotechnological production of amino acids, in particular L-glutamate, L-lysine, and proteins (Becker and Wittmann, 2012; Eggeling and Bott, 2015; Wendisch et al., 2016). Furthermore, *C. glutamicum* production strains for a large variety of other chemicals have been reported in recent years, such as organic acids (Wieschalka et al., 2013), polyamines (Schneider and Wendisch, 2011), biofuels (Blombach et al., 2011; Jojima et al., 2015), or secondary metabolites (Kallscheuer et al., 2016). Due to its industrial importance, *C. glutamicum* has become a model organism that is intensively studied with respect to metabolism and regulation (Burkovski, 2008, 2015; Eggeling and Bott, 2005; Yukawa and Inui, 2013).

The second messenger cAMP plays various regulatory roles in different species, with the activation of eukaryotic protein kinase A being a very prominent one (Taylor et al., 1990). In the bacterial realm cAMP often influences the adaptation to varying environmental conditions via cAMP-dependent gene regulation, with CRP of *Escherichia coli* representing the most intensively studied example (Kolb et al., 1993). Additional regulatory functions of cAMP in bacteria are e.g. cAMP-dependent protein lysine acetylation (Nambi et al., 2013) or the modulation of the activity of ion channels (Brams et al., 2014). In *C. glutamicum*, the only known function of cAMP so far is as effector of the transcriptional regulator GlxR, which is a homolog of *E. coli* CRP and thus a member of the CRP/FNR family of transcription factors. GlxR was initially discovered as regulator of the glyoxylate bypass gene *aceB* (Kim et al., 2004). Subsequent studies characterized GlxR as global regulator in *C. glutamicum* that activates or represses the expression of about 200 genes in a cAMP-dependent manner (Jungwirth et al., 2013; Kohl et al., 2008; Toyoda et al., 2011). Many GlxR target genes belong to the COG categories energy production and conversion (C), carbohydrate transport

and metabolism (G), amino acid transport and metabolism (E), and secondary metabolite biosynthesis (Jungwirth et al., 2013). Crystal structures of apo- and cAMP-bound GlxR have recently been solved and revealed the structural changes upon cAMP-binding (Townsend et al., 2014). The crucial role of GlxR in *C. glutamicum* is demonstrated by the fact that *glxR* deletion mutants, which could only be obtained in two labs, had severe growth defects (Park et al., 2010; Toyoda et al., 2009).

As the activity of GlxR is dependent on cAMP, the question how *C. glutamicum* regulates the cellular cAMP level is of central importance. In general, the cAMP level is determined by the activities of the cAMP-forming and -degrading enzymes. In addition, export or import of cAMP might influence the intracellular concentration, but no evidence for such transport processes is currently available. A single membrane-bound class III adenylate cyclase, CyaB, forming cAMP from ATP was identified in *C. glutamicum* (Cha et al., 2010). In contrast to *glxR*, the *cyaB* gene could readily be deleted and the resulting mutant showed a growth defect in acetate minimal medium, but not in glucose minimal medium (Cha et al., 2010). The cAMP concentration was strongly reduced in glucose-grown cells of the Δ*cyaB* mutant, but a significant residual level of about 15% was still detectable (Cha et al., 2010). These results suggest the existence of one or more additional cAMP-synthesizing enzymes in *C. glutamicum* awaiting their identification. In *Mycobacterium tuberculosis*, which like *C. glutamicum* belongs to the order *Corynebacteriales*, 17 adenylate cyclases were identified (Bai et al., 2011; McCue et al., 2000; Shenoy and Visweswariah, 2006). With respect to cAMP degradation, we recently identified the cAMP-phosphodiesterase CpdA (Schulte et al., 2017). A *cpdA* deletion mutant showed a two-fold increased intracellular cAMP concentration during growth on glucose and clear growth defects on all carbon sources tested, including glucose. In several cases, the growth defects could be reverted at least partially by plasmid-based expression of genes for uptake or catabolism of the respective carbon sources, which are known to be repressed by GlxR (Schulte et al., 2017). This suggests that the growth

phenotypes of the Δ*cpdA* mutant are primarily mediated by GlxR. In view of the fact that neither all enzymes responsible for cAMP synthesis and perhaps degradation nor the signals controlling their activity are known, our understanding of cAMP signaling in *C. glutamicum* is still rather limited.

After description of the first sensor for intracellular cAMP in 1991 (Adams et al., 1991), a variety of cAMP biosensors have been constructed for eukaryotes based on protein kinase A, Epac proteins, or cyclic nucleotide-gated ion channels (Paramonov et al., 2015; Patel and Gold, 2015). Several of these are genetically-encoded sensors based on Förster resonance energy transfer (FRET) allowing real-time detection of changes in cAMP levels. Alternatively, reporter gene-based systems provide a robust means to detect long-term differences in cAMP levels, but do not allow registration of dynamic changes of intracellular cAMP. In recent years, we developed a number of reporter gene-based biosensors for other metabolites, such as L-lysine, L-methionine and branched-chain amino acids, or NADPH and used them for bacterial strain and enzyme development as well as for studying population heterogeneity (Eggeling et al., 2015; Mahr and Frunzke, 2016). The aim of our current study was to establish a reporter gene-based cAMP biosensor for *C. glutamicum* that allows differentiation and isolation of single cells differing in their cAMP level by fluorescence-activated cell sorting (FACS). To this end, we made use of GlxR and a suitable target promoter controlling expression of a reporter gene encoding a fluorescent protein.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

All strains used in this study are listed in Table 1. *C. glutamicum* strains were cultivated in brain heart infusion (BHI) medium (Difco Laboratories, Detroit, USA), tryptone-yeast extract (2xTY) medium (Sambrook and Russell, 2001), or CGXII minimal medium

(Keilhauer et al., 1993) supplemented with 30 mg l^{-1} 3,4-dihydroxybenzoate as iron chelator. *E. coli* was cultivated in LB medium (Sambrook and Russell, 2001). Media were supplemented with kanamycin (50 µg ml^{-1} for *E. coli*, 25 µg ml^{-1} for *C. glutamicum*) or chloramphenicol (10 µg ml^{-1}) if appropriate. For growth experiments with *C. glutamicum*, single colonies were used to inoculate 5 ml 2xTY overnight precultures, which were cultivated for 16-18 h at 170 rpm and 30°C. The cells of the precultures were washed twice with 0.9% (wt/vol) NaCl solution before they were used for the inoculation of 800 µl CGXII main cultures supplemented with the carbon sources indicated in the Results section. Main cultures were inoculated to an optical density at 600 nm (OD_{600}) of 1 and were cultivated in a BioLector microcultivation system (m2p-labs, Baesweiler, Germany) with online measurement of backscatter at 620 nm and eYFP fluorescence (excitation at 510 nm, emission at 532 nm) every 15 minutes.

2.2. Construction of plasmids and deletion mutants

All plasmids used in this study are listed in Table 1. Oligonucleotides and restriction enzymes used for the construction of plasmids and deletion mutants are listed in Table S1. The correct sequence of all recombinant plasmids was verified by DNA sequencing (MWG Operon, Ebersberg, Germany). For construction of plasmid pSenPcg3195, a 188-bp fragment of the cg3195 promoter was amplified from genomic DNA of *C. glutamicum* using the oligonucleotide pair Pcg3195-fw-BamHI/Promcg3195-rv-eYFP. The *eyfp* gene was amplified with the oligonucleotide pair eYFP-fw/eYFP-SpeI-rv from plasmid pEKEx2-*eyfp*. The two PCR fragments were fused by overlap extension PCR using the oligonucleotide pair Promcg3195-fw-BamHI/eYFP-SpeI-rv. The resulting fusion product and plasmid pJC1-*venus*-term were digested with BamHI and SpeI and ligated to create plasmid pSenPcg3195.

The chromosomal deletion of *cyaB* was performed by allelic exchange using the plasmid pK19*mobsacB* (Schäfer et al., 1994) as described before (Niebisch and Bott, 2001). Flanking

regions of the *cyaB* coding region of about 500 bp were amplified using the two oligonucleotide pairs cyaB_del1/cyaB_del2 and cyaB_del3/cyaB_del4. The flanking regions were fused in an overlap extension PCR with the oligonucleotide pair cyaB_del1/ cyaB_del4 and cloned into plasmid pK19*mobsacB* using the indicated restriction enzymes. The first recombination step into the genome was ensured via selection for kanamycin-resistant clones. For the second recombination step, sucrose-resistant clones were selected. The successful *cyaB* deletion was confirmed via colony PCR with the oligonucleotide pair cyaB_deltest_fw/cyaB_deltest_rv.

2.3. Fluorescence-activated cell sorting

Cells grown in 5 ml 2xTY medium were used to inoculate a 50 ml preculture in CGXII minimal medium with 100 mM glucose and cultivated for approx. 16 h at 130 rpm and 30°C. The cells of this preculture were washed once in 0.9% (wt/vol) NaCl solution and then used to inoculate the main cultures in 500 ml baffled shake flasks containing 50 ml CGXII medium with 100 mM glucose to an OD_{600} of 1. The main cultures were incubated at 130 rpm and 30°C until the exponential growth phase was reached (approx. 4-5 h). Culture samples were removed from the flasks and diluted in FACS flow buffer (Agilent) to an OD_{600} below 0.1 prior to FACS analysis using an FACS ARIA II high-speed cell sorter (BD Biosciences, Franklin Lakes, NJ, USA) as described previously (Kortmann et al., 2015). Mutant cells from 1:1 mixtures with wild-type cells were sorted on BHI agar plates using the gates P2 and P3 as indicated in Fig. 5 and Fig. 6. The BHI plates were incubated at 30°C for at least 24 h before the colonies were tested for deletion of *cyaB* and *cpdA* by colony PCR using the oligonucleotide pairs cyaB_deltest_fw/cyaB_deltest_rv and cpdA_deltest_fw/cpdA_deltest_rv.

3. Results

3.1. Choice of the GlxR target promoter and sensor construction

In order to find a promoter suitable for the construction of a GlxR-based cAMP biosensor for *C. glutamicum*, the literature was scanned for genes which are strongly transcriptionally activated or repressed by GlxR and which preferably are not regulated by other transcriptional regulators. Although GlxR directly regulates the expression of more than 200 genes in *C. glutamicum*, the identification of a suitable promoter for the biosensor construction proved to be very difficult. Many of the GlxR target genes are either also regulated by other transcription factors or regulation by GlxR is not strong enough to trigger detectable changes in the fluorescence output. Several native and modified promoters were tested for sensor construction, including those of *citH*, cg1735, and *tuf* of *C. glutamicum* and those of *galP* and *lacZ* of *E. coli*, but none of them was found to be suitable for one of the above mentioned reasons (data not shown).

Finally, the promoter of the cg3195 gene annotated as flavin-containing monooxygenase and predicted flavoprotein involved in K^+ transport (Kalinowski et al., 2003) was identified as promising candidate. The intergenic region between the divergently oriented cg3194 gene and cg3195 showed the highest enrichment factor of the entire genome in a ChIP-Seq analysis (Jungwirth et al., 2013) and one of the highest enrichment factors in a ChIP-Chip experiment with GlxR (Toyoda et al., 2011). Furthermore, cAMP-dependent binding of purified GlxR to a 40-bp double stranded oligonucleotide covering the GlxR-binding motif in the intergenic region of cg3194 and cg3195 was demonstrated, indicating that cg3195 is a direct GlxR target gene (Jungwirth et al., 2013). The proposed GlxR binding site AGTGAGTCACGACACT fits well to the consensus motif TGTGANNTANNTCACA (Kohl and Tauch, 2009) and overlaps with the transcriptional start site of cg3195 located 25 bp upstream of the proposed TTG start codon (Baumgart et al., 2013). This position clearly suggests that the cAMP-GlxR complex represses cg3195 (Fig. 1). Repression is supported by the findings that the mRNA level of cg3195 was lowered 14-fold in the Δ*cpdA* mutant having an elevated cAMP level (Schulte et

al., 2017) and increased 3-fold on glucose and even 24-fold on a glucose-acetate mixture in the Δ*cyaB* mutant having a decreased cAMP level (unpublished data). In addition to GlxR, expression of cg3195 is affected by IpsA, a LacI-type transcription factor which regulates genes involved in cell wall synthesis in *C. glutamicum* with *myo*-inositol as effector molecule (Baumgart et al., 2013). IpsA serves as activator for cg3195 expression and the IpsA binding site is located 151 bp upstream of the cg3195 transcription start site (Fig. 1).

For the biosensor construction, a 188 bp fragment of the cg3195 promoter region covering 163 bp upstream and 24 bp downstream of the transcription start site was fused to the reporter gene *eyfp*. In this way, the native ribosome binding site of cg3195 was positioned at the same distance to the start codon of *eyfp* as in the genome to the start codon of cg3195. The resulting fragment was cloned in a pJC1-derived vector resulting in plasmid pSenPcg3195 (Fig. 1). The first three nucleotides of the IpsA binding site, which were reported to be important parts of the palindromic binding motif (Baumgart et al., 2013), are missing in pSenPcg3195 and a control experiment showed that eYFP fluorescence is reduced only by about 20% in the Δ*ipsA* mutant carrying the sensor plasmid (data not shown).

3.2. Response of the cAMP biosensor to varying GlxR and cAMP levels

The anticipated behavior of the cAMP biosensor for *C. glutamicum* based on chromosomally encoded GlxR and plasmid pSenPcg3195 is depicted in Fig. 2: at high cAMP levels, expression of eYFP should be repressed by the GlxR-cAMP complex, whereas at low cAMP levels repression should be relieved. Thus, the cellular levels of both GlxR and cAMP should affect the fluorescence output. The influence of increased GlxR levels was tested using plasmid pXMJ19-*glxR* carrying *glxR* under control of the IPTG-inducible *tac* promoter (Schulte et al., 2017). As shown in Fig. 3A, increasing IPTG concentrations led to reduced growth rates of *C. glutamicum* wild type carrying pSenPcg3195 and pXMJ19-*glxR*, but did not influence the final cell density. In the presence of 100 µM IPTG, the specific eYFP

fluorescence was reduced by 85% compared to the culture without IPTG (Fig. 3B and C), whereas fluorescence output was not altered by IPTG in the control strain carrying pXMJ19 instead of pXMJ19-*glxR* (Fig. 3B and C).

To analyze the influence of altered cAMP levels, *C. glutamicum* wild type carrying plasmid pSenPcg3195 was cultivated in CGXII glucose minimal medium supplemented with various cAMP concentrations between 0.01 and 10 mM. As shown in Fig. 3D, the presence of cAMP did not influence the growth behavior, but the specific eYFP fluorescence decreased gradually with increasing cAMP concentrations (Fig. 3E and F). The maximal specific fluorescence was reduced to approximately 50% in the presence of 10 mM cAMP, which is about 100-fold higher than the calculated intracellular concentrations (Schulte et al., 2017). The results described above prove that the cAMP biosensor shows the expected behavior to increased GlxR and cAMP levels.

During exponential growth, when the expression rate of eYFP was high, a kink in the curve of specific fluorescence was observed, which is assumed to be caused by oxygen limitation (Fig. 3B and E). The chromophore of the eYFP protein needs oxygen during maturation (Tsien, 1998) and although the Biolector cultivation system was designed for high oxygen transfer rates, the dissolved oxygen level was found to drop drastically when *C. glutamicum* grows exponentially in Flower plates and to rise again when the carbon source has been consumed (Unthan et al., 2015). As a consequence, maturation of apo-eYFP is probably hampered during exponential growth, leading to a decrease of the specific fluorescence, and is completed after carbon source consumption, causing an increase of the specific fluorescence in the stationary phase. To address this issue we performed a Biolector experiment in which we reduced the glucose concentration from 100 mM to 50 or 25 mM. By this change, the duration of the exponential growth phase as well as biomass formation should be reduced and oxygen supply should be improved. The results of this experiment are shown

in Fig. S1 and support the assmption that oxygen limitation is responsible for the kink in the curves of the specific fluorescence shown in Figs. 3B and 3E.

3.3 Response of the cAMP biosensor in adenylate cylase and cAMP phosphodiesterase mutants

To further characterize the functionality of the GlxR-based cAMP biosensor pSenPcg3195, it was tested in the *C. glutamicum* Δ*cyaB* and Δ*cpdA* mutants, which lack the adenylate cyclase CyaB or the cAMP phosphodiesterase CpdA, respectively (Fig. 4). When cultivated with 100 mM glucose as carbon source, the Δ*cyaB* mutant showed the same growth behavior as the wild type but had a higher specific eYFP fluorescence throughout the cultivation (Fig. 4A and B). The difference was most pronounced during the exponential growth phase (45% higher fluorescence) and diminished in the stationary phase (6% higher fluorescence). This result was in line with the expectation that the lowered cAMP level in the Δ*cyaB* mutant should result in a weakened repression of the cg3195 promoter by GlxR. As reported before (Schulte et al., 2017), the Δ*cpdA* mutant showed delayed growth and a reduced growth rate, but reached the same final cell density (Fig. 4A). The initial specific eYFP fluorescence of the Δ*cpdA* mutant decreased continuously throughout growth to reach a value of almost zero (Fig. 4B). The values in the exponential and stationary growth phase corresponded to only 41-66% and 6% of the wild-type levels. The Δ*cpdA* mutant has an increased intracellular cAMP concentration, which apparently causes a very strong repression of the cg3195 promoter by GlxR.

In a second series of growth experiments, the two mutants and the wild type carrying the biosensor pSenPcg3195 were cultivated with 100 mM acetate instead of 100 mM glucose. In several previous studies it has been reported that the intracellular cAMP concentration in the wild type is about 4-fold lower on acetate than on glucose (Cha et al., 2010; Kim et al., 2004; Toyoda et al., 2011). Both for the Δ*cyaB* mutant and the Δ*cpdA* mutant growth defects were

observed during cultivation on acetate (Fig. 4C). The Δ*cyaB* strain showed a reduced growth rate and lower maximal cell density, in agreement with a previous report (Cha et al., 2010). The Δ*cpdA* mutant showed an increased lag phase and a reduced growth rate, but reached the same cell density as the wild type, in line with our previous results (Schulte et al., 2017). The specific eYFP fluorescence of the wild type decreased continuously during exponential growth, but then rapidly increased to more reach about 230% of the initial level (Fig. 4D) which is probably caused by the temporary lack of oxygen for the eYFP maturation as described above. The specific fluorescence of the Δ*cyaB* strain increased slightly during the long growth phase and more strongly when the culture had reached the stationary phase (Fig. 4D). The maximal specific fluorescence measured after 40 h was 19% higher than the maximal specific fluorescence of the wild type, in agreement with the expected lowered cAMP level of the Δ*cyaB* mutant. The specific fluorescence of the Δ*cpdA* mutant showed similar kinetics as during growth on glucose. It continuously decreased to reach values close to zero (Fig. 4D). This suggests that the absence of the cAMP phosphodiesterase causes elevated cAMP levels also on acetate and thus strong repression of the cg3195 promoter by GlxR.

3.4. Flow cytometric analysis of wild type, ΔcyaB, and ΔcpdA strains carrying the cAMP biosensor and FACS of strain mixtures

One intended application of the cAMP biosensor is the identification of further enzymes beside CyaB and CpdA that are involved in cAMP synthesis or degradation by FACS-based high-throughput screening of genome-wide mutant libraries and isolation of cells having increased or lowered intracellular cAMP levels. To test the feasibility of such an application, we first analyzed cells of the wild type, the Δ*cyaB* mutant, and the Δ*cpdA* mutant, all carrying the sensor plasmid pSenPcg3195. The strains were cultivated in CGXII minimal medium with 100 mM glucose until mid-exponential phase and then used for FACS. 100 000 cells were

analyzed for each strain. The scatter plots (Fig. 5, upper panels) displaying eYFP fluorescence versus forward scatter (FSC) reveal comparable ranges of cell size (FSC) and differing ranges in eYFP fluorescence for the three strains, which become obvious via the number of cells in gates P2 and P3. The histograms shown below the scatter plots in Fig. 5, in which the number of cells is plotted against eYFP fluorescence intensity, reveal average fluorescence values of about 2000 for the Δ*cyaB* mutant, 750 for the wild type, and 80 for the Δ*cpdA* mutant. The flow cytometry data were qualitatively in agreement with the results shown in Fig. 4B.

In a next step, we tested the possibility to separate mixtures of mutant and wild-type cells via FACS. When analyzing a 1:1 mixture of wild-type and Δ*cyaB* cells (Fig. 6A), taken either in the exponential or the stationary growth phase, cells were sorted out using gate P2. Only 2 wild-type cells fell into gate P2 while it was 330 cells for the Δ*cyaB* mutant taken from the exponential phase. Individual cells from each growth phase were sorted on BHI agar plates, multiplied by overnight incubation and then 22 clones were tested by PCR for the *cyaB* deletion. 21 of the cells taken in the exponential phase (95%) and 20 of the cells taken in the stationary phase (91%) were found to be Δ*cyaB* mutants. When analyzing a 1:1 mixture of wild-type and Δ*cpdA* cells taken either in the exponential or the stationary growth phase, two distinct populations could be discriminated in the dot plot and the histogram (Fig. 6B). Cells were sorted using gate P3 which contained 79 wild-type and 22 792 Δ*cpdA* cells from the exponential phase. 23 individual cells each from the exponential and stationary growth phase were tested by PCR for the *cpdA* deletion. All 46 clones were identified as Δ*cpdA* mutants. We therefore demonstrated that the constructed cAMP biosensor is suitable for FACS sorting of mutants with altered intracellular cAMP levels.

4. Discussion

In this study we constructed and characterized a reporter gene-based single cell cAMP-biosensor for *C. glutamicum*. It is based on the chromosomally encoded cAMP-dependent

transcriptional regulator GlxR and plasmid pSenPcg3195, which carries the reporter gene *eyfp* under control of the promoter region of the cg3195 gene. This promoter contains a GlxR-binding site overlapping with the transcriptional start site and is repressed by the GlxR-cAMP complex. Initial experiments proved that the biosensor responds to both GlxR and cAMP levels, as both increased *glxR* expression and addition of cAMP to the medium resulted in lowered specific eYFP fluorescence. The observation that a relatively high concentration of 10 mM cAMP was required to reduce the sensor output by 50% suggests that *C. glutamicum* probably does not possess an active transport system for cAMP uptake, as in the presence of such a system much lower cAMP concentrations in the medium should have been sufficient to exert the effect.

Several previous studies reported on *in vivo* changes of the cAMP concentration in *C. glutamicum*. It was shown that the cAMP level varies with the carbon source, being about 4-fold higher during growth on glucose compared to growth on acetate (Kim et al., 2004; Toyoda et al., 2011) and about 2.5-fold higher during growth on citrate compared to growth on glucose (Polen et al., 2007). The reports on changes of the cAMP level in different growth phases are contradictory. In a study with *C. glutamicum* strain ATCC 13032, the cAMP concentration was high in the exponential phase and decreased in the stationary phase during cultivation on glucose (Kim et al., 2004). In another study with *C. glutamicum* strain R, the cAMP level decreased in the exponential phase and increased in the stationary phase during cultivation on glucose (Toyoda et al., 2011). Online measurements of such dynamic cAMP level changes cannot be performed with our reporter gene-based biosensor, but require e.g. a FRET-based biosensor.

With respect to physiological changes of the cellular GlxR levels, current knowledge is meager. Four transcriptional regulators were reported to effect *glxR* expression. Negative autoregulation of *glxR* was indicated by binding of purified GlxR to a binding site overlapping the -10 region and the transcriptional start site of *glxR* (Jungwirth et al., 2008).

By DNA affinity chromatography and reporter gene studies RamB (Cg0444) was characterized as weak repressor of *glxR*, while GntR3 (Cg2544) and SucR (Cg0146) were identified as weak activators (Subhadra et al., 2015). The SucR protein was recently renamed AtlR, as its major function was shown to be the repression of genes involved in arabitol utilization (Laslo et al., 2012). The *glxR* mRNA level was found to be similar in the exponential and stationary growth phase in glucose medium (Hong et al., 2014). Besides transcriptional regulation, also regulation at the protein level has been reported for GlxR. The serine protease SprA was found to proteolyze apo-GlxR, but not the cAMP-GlxR complex (Hong et al., 2014). In summary, the data suggest that the GlxR level in the cell is subject to changes, which, however, are presumably moderate, as our results showed that overexpression of *glxR* has a negative impact on the growth rate. Growth-impeding concentrations of GlxR appear unlikely under physiological conditions. Further studies are required to obtain a clearer view of the impact of GlxR concentration changes compared to altered cAMP levels on the regulation of GlxR target genes. The cAMP biosensor provides a useful tool for such studies.

The responsiveness of the cAMP biosensor to altered cAMP levels was also confirmed with mutants that are defective in cAMP synthesis or degradation. The absence of adenylate cyclase in the Δ*cyaB* strain caused moderately increased specific eYFP fluorescence during growth, whereas the absence of the cAMP phosphodiesterase CpdA in the Δ*cpdA* strain blocked expression of *eyfp* from the cg3195 promoter so strongly that the specific eYFP fluorescence continuously decreased during cultivation. The observation that the absence of CpdA caused a much stronger repression of the cg3195 promoter than the addition of 10 mM cAMP to the medium supports the conclusion made above that cAMP uptake into *C. glutamicum* cells is inefficient. Nevertheless it shows that increased cAMP levels are sufficient to strongly effect GlxR target gene expression, in line with our previous transcriptome data of the Δ*cpdA* mutant (Schulte et al., 2017).

A key feature of the cAMP biosensor is the possibility to analyze single cells in high-throughput by flow cytometry. Cells with differing cAMP levels and therefore differing eYFP fluorescence could be clearly distinguished and isolated using FACS. Even in the case of a mixture of wild type and Δ*cyaB* mutant, where the two populations overlap, more than 90% of the cells sorted using a suitable gate proved to belong to the desired mutant strain (Fig. 6). In the case of a mixture of wild-type and Δ*cpdA* mutant cells, where the populations are clearly separated, even higher sorting efficiencies were reached. These results demonstrated the possibility to use the cAMP biosensor to analyze mutant libraries of *C. glutamicum* for cells having an increased or decreased cAMP level, caused e.g. by inactivation of adenylate cyclases other than CyaB or cAMP phosphodiesterases other than CpdA. In particular, the identification of the postulated, but yet unknown alternative adenylate cyclase(s) would be desirable for a better understanding of cAMP-dependent regulation in *C. glutamicum*.

Acknowledgements

This work was financially supported by the Fachagentur Nachwachsende Rohstoffe e.V. (FNR) within the ERA-IB project SCILS by grant no. 22029812A to M. Bott. We would like to thank Michael Bussmann for construction of strain *C. glutamicum* Δ*cyaB* and Eugen Pfeifer for help with the FACS analysis.

References

Adams, S.R., Harootunian, A.T., Buechler, Y.J., Taylor, S.S., Tsien, R.Y., (1991) Fluorescence ratio imaging of cyclic AMP in single cells. Nature 349, 694-697.

Bai, G., Knapp, G.S., McDonough, K.A., (2011) Cyclic AMP signalling in mycobacteria: redirecting the conversation with a common currency. Cell. Microbiol. 13, 349-358.

Baumgart, M., Luder, K., Grover, S., Gätgens, C., Besra, G.S., Frunzke, J., (2013) IpsA, a novel LacI-type regulator, is required for inositol-derived lipid formation in *Corynebacteria* and *Mycobacteria*. BMC Biol. 11, 122.

Becker, J., Wittmann, C., (2012) Bio-based production of chemicals, materials and fuels - *Corynebacterium glutamicum* as versatile cell factory. Curr. Opin. Biotechnol. 23, 631-640.

Blombach, B., Riester, T., Wieschalka, S., Ziert, C., Youn, J.W., Wendisch, V.F., Eikmanns, B.J., (2011) *Corynebacterium glutamicum* tailored for efficient isobutanol production. Appl. Environ. Microbiol. 77, 3300-3310.

Brams, M., Kusch, J., Spurny, R., Benndorf, K., Ulens, C., (2014) Family of prokaryote cyclic nucleotide-modulated ion channels. Proc. Natl. Acad. Sci. USA 111, 7855-7860.

Burkovski, A., (2008) Corynebacteria: genomics and molecular biology. Caister Academic Press, Norfolk, U.K.

Burkovski, A., (2015) *Corynebacterium glutamicum* - From Systems Biology to Biotechnological Applications. Caister Academic Press, Norfolk, U.K.

Cha, P.H., Park, S.Y., Moon, M.W., Subhadra, B., Oh, T.K., Kim, E., Kim, J.F., Lee, J.K., (2010) Characterization of an adenylate cyclase gene (*cyaB*) deletion mutant of *Corynebacterium glutamicum* ATCC 13032. Appl. Microbiol. Biotechnol. 85, 1061-1068.

Eggeling, L., Bott, M., (2005) Handbook of *Corynebacterium glutamicum*. CRC Press, Taylor & Francis Group, Boca Raton, Florida, USA.

Eggeling, L., Bott, M., (2015) A giant market and a powerful metabolism: L-lysine provided by *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. 99, 3387-3394.

Eggeling, L., Bott, M., Marienhagen, J., (2015) Novel screening methods-biosensors. Curr. Opin. Biotechnol. 35, 30-36.

Hanahan, D., (1983) Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166, 557-580.

Hentschel, E., Will, C., Mustafi, N., Burkovski, A., Rehm, N., Frunzke, J., (2013) Destabilized eYFP variants for dynamic gene expression studies in *Corynebacterium glutamicum*. Microb. Biotechnol. 6, 196-201.

Hong, E.J., Park, J.S., Kim, Y., Lee, H.S., (2014) Role of *Corynebacterium glutamicum sprA* encoding a serine protease in *glxR*-mediated global gene regulation. PLoS One 9, e93587.

Jakoby, M., Ngouoto-Nkili, C.E., Burkovski, A., (1999) Construction and application of new *Corynebacterium glutamicum* vectors. Biotechnol. Techniques 13, 437-441.

Jojima, T., Noburyu, R., Sasaki, M., Tajima, T., Suda, M., Yukawa, H., Inui, M., (2015) Metabolic engineering for improved production of ethanol by *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. 99, 1165-1172.

Jungwirth, B., Emer, D., Brune, I., Hansmeier, N., Pühler, A., Eikmanns, B.J., Tauch, A., (2008) Triple transcriptional control of the resuscitation promoting factor 2 (*rpf2*) gene of *Corynebacterium glutamicum* by the regulators of acetate metabolism RamA and RamB and the cAMP-dependent regulator GlxR. FEMS Microbiol. Lett. 281, 190-197.

Jungwirth, B., Sala, C., Kohl, T.A., Uplekar, S., Baumbach, J., Cole, S.T., Pühler, A., Tauch, A., (2013) High-resolution detection of DNA binding sites of the global transcriptional regulator GlxR in *Corynebacterium glutamicum*. Microbiology 159, 12-22.

Kalinowski, J., Bathe, B., Bartels, D., Bischoff, N., Bott, M., Burkovski, A., Dusch, N., Eggeling, L., Eikmanns, B.J., Gaigalat, L., Goesmann, A., Hartmann, M., Huthmacher, K., Krämer, R., Linke, B., McHardy, A.C., Meyer, F., Möckel, B., Pfefferle, W., Pühler, A., Rey, D.A., Rückert, C., Rupp, O., Sahm, H., Wendisch, V.F., Wiegrabe, I., Tauch, A., (2003) The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. J. Biotechnol. 104, 5-25.

Kallscheuer, N., Vogt, M., Stenzel, A., Gätgens, J., Bott, M., Marienhagen, J., (2016) Construction of a *Corynebacterium glutamicum* platform strain for the production of stilbenes and (2S)-flavanones. Metab. Eng. 38, 47-55.

Keilhauer, C., Eggeling, L., Sahm, H., (1993) Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. J. Bacteriol. 175, 5595-5603.

Kim, H.J., Kim, T.H., Kim, Y., Lee, H.S., (2004) Identification and characterization of *glxR*, a gene involved in regulation of glyoxylate bypass in *Corynebacterium glutamicum*. J. Bacteriol. 186, 3453-3460.

Kinoshita, S., Udaka, S., Shimono, M., (1957) Studies on amino acid fermentation. Part I. Production of L-glutamic acid by various microorganisms. J. Gen. Appl. Microbiol. 3, 193-205.

Kohl, T.A., Baumbach, J., Jungwirth, B., Pühler, A., Tauch, A., (2008) The GlxR regulon of the amino acid producer *Corynebacterium glutamicum*: *In silico* and *in vitro* detection of DNA binding sites of a global transcription regulator. J. Biotechnol. 135, 340-350.

Kohl, T.A., Tauch, A., (2009) The GlxR regulon of the amino acid producer *Corynebacterium glutamicum*: Detection of the corynebacterial core regulon and integration into the transcriptional regulatory network model. J. Biotechnol. 143, 239-246.

Kolb, A., Busby, S., Buc, H., Garges, S., Adhya, S., (1993) Transcriptional regulation by cAMP and its receptor protein. Annu. Rev. Biochem. 62, 749-795.

Kortmann, M., Kuhl, V., Klaffl, S., Bott, M., (2015) A chromosomally encoded T7 RNA polymerase-dependent gene expression system for *Corynebacterium glutamicum*: construction and comparative evaluation at the single-cell level. Microb. Biotechnol. 8, 253-265.

Laslo, T., von Zaluskowski, P., Gabris, C., Lodd, E., Ruckert, C., Dangel, P., Kalinowski, J., Auchter, M., Seibold, G., Eikmanns, B.J., (2012) Arabitol metabolism of *Corynebacterium glutamicum* and its regulation by AtlR. J. Bacteriol. 194, 941-955.

Mahr, R., Frunzke, J., (2016) Transcription factor-based biosensors in biotechnology: current state and future prospects. Appl. Microbiol. Biotechnol. 100, 79-90.

McCue, L.A., McDonough, K.A., Lawrence, C.E., (2000) Functional classification of cNMP-binding proteins and nucleotide cyclases with implications for novel regulatory pathways in *Mycobacterium tuberculosis*. Genome Res. 10, 204-219.

Nambi, S., Gupta, K., Bhattacharyya, M., Ramakrishnan, P., Ravikumar, V., Siddiqui, N., Thomas, A.T., Visweswariah, S.S., (2013) Cyclic AMP-dependent protein lysine acylation in mycobacteria regulates fatty acid and propionate metabolism. J. Biol. Chem. 288, 14114-11124.

Niebisch, A., Bott, M., (2001) Molecular analysis of the cytochrome bc_1-aa_3 branch of the *Corynebacterium glutamicum* respiratory chain containing an unusual diheme cytochrome c_1. Arch. Microbiol. 175, 282-294.

Paramonov, V.M., Mamaeva, V., Sahlgren, C., Rivero-Müller, A., (2015) Genetically-encoded tools for cAMP probing and modulation in living systems. Front. Pharmacol. 6, 196.

Park, S.Y., Moon, M.W., Subhadra, B., Lee, J.K., (2010) Functional characterization of the *glxR* deletion mutant of *Corynebacterium glutamicum* ATCC 13032: involvement of GlxR in acetate metabolism and carbon catabolite repression. FEMS Microbiol. Lett. 304, 107-115.

Patel, N., Gold, M.G., (2015) The genetically encoded tool set for investigating cAMP: more than the sum of its parts. Front. Pharmacol. 6, 164.

Polen, T., Schluesener, D., Poetsch, A., Bott, M., Wendisch, V.F., (2007) Characterization of citrate utilization in *Corynebacterium glutamicum* by transcriptome and proteome analysis. FEMS Microbiol. Lett. 273, 109-119.

Sambrook, J., Russell, D., (2001) Molecular Cloning. A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., Pühler, A., (1994) Small mobilizable multipurpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19 - Selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145, 69-73.

Schneider, J., Wendisch, V.F., (2011) Biotechnological production of polyamines by bacteria: recent achievements and future perspectives. Appl. Microbiol. Biotechnol. 91, 17-30.

Schulte, J., Baumgart, M., Bott, M., (2017) Identification of the cAMP phosphodiesterase CpdA as novel key player in cAMP-dependent regulation in *Corynebacterium glutamicum*. Mol. Microbiol. 103, 534-552.

Shenoy, A.R., Visweswariah, S.S., (2006) New messages from old messengers: cAMP and mycobacteria. Trends Microbiol. 14, 543-550.

Subhadra, B., Ray, D., Han, J.Y., Bae, K.H., Lee, J.K., (2015) Identification of the regulators binding to the upstream region of *glxR* in *Corynebacterium glutamicum*. J. Microbiol. Biotechnol. 25, 1216-1226.

Taylor, S.S., Buechler, J.A., Yonemoto, W., (1990) cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. Annu. Rev. Biochem. 59, 971-1005.

Townsend, P.D., Jungwirth, B., Pojer, F., Bussmann, M., Money, V.A., Cole, S.T., Pühler, A., Tauch, A., Bott, M., Cann, M.J., Pohl, E., (2014) The crystal structures of apo and cAMP-bound GlxR from *Corynebacterium glutamicum* reveal structural and dynamic changes upon cAMP binding in CRP/FNR family transcription factors. PLoS One 9, e113265.

Toyoda, K., Teramoto, H., Inui, M., Yukawa, H., (2009) Involvement of the LuxR-type transcriptional regulator RamA in regulation of expression of the *gapA* gene, encoding glyceraldehyde-3-phosphate dehydrogenase of *Corynebacterium glutamicum*. J. Bacteriol. 191, 968-977.

Toyoda, K., Teramoto, H., Inui, M., Yukawa, H., (2011) Genome-wide identification of in vivo binding sites of GlxR, a cyclic AMP receptor protein-type regulator in *Corynebacterium glutamicum*. J. Bacteriol. 193, 4123-4133.

Tsien, R.Y., (1998) The green fluorescent protein. Annu. Rev. Biochem. 67, 509-544.

Unthan, S., Radek, A., Wiechert, W., Oldiges, M., Noack, S., (2015) Bioprocess automation on a Mini Pilot Plant enables fast quantitative microbial phenotyping. Microbial cell factories 14, 32.

Wendisch, V.F., Jorge, J.M., Perez-Garcia, F., Sgobba, E., (2016) Updates on industrial production of amino acids using *Corynebacterium glutamicum*. World J. Microbiol. Biotechnol. 32, 105.

Wieschalka, S., Blombach, B., Bott, M., Eikmanns, B.J., (2013) Bio-based production of organic acids with *Corynebacterium glutamicum*. Microb. Biotechnol. 6, 87-102.

Yukawa, H., Inui, M., (2013) *Corynebacterium glutamicum*: Biology and Biotechnology. In: Steinbüchel, A. (Ed.), Microbiology Monographs. Springer, Heidelberg.

Table 1

Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference
Strains		
E. coli DH5α	F⁻ *endA1* Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 relA1 hsdR17*(r_K^- m_K^+) *deoR supE44 thi-1 gyrA96 phoA* λ^-; used for cloning purposes	(Hanahan, 1983)
C. glutamicum ATCC 13032	Biotin-auxotrophic wild-type strain	(Kinoshita et al., 1957)
C. glutamicum Δ*cyaB*	Wild type with chromosomal deletion of the adenylate cyclase gene *cyaB* (cg0375)	This work
C. glutamicum Δ*cpdA*	Wild type with chromosomal deletion of the cAMP phosphodiesterase gene *cpdA* (cg2761)	(Schulte et al., 2017)
Plasmids		
pK19*mobsacB*	Kan^R; plasmid for allelic exchange in *C. glutamicum*; (pK18 *ori*$V_{E.c.}$, *sacB*, *lacZ*α)	(Schäfer et al., 1994)
pK19*mobsacB*-Δ*cyaB*	Kan^R; pK19mobsacB derivative used for the chromosomal deletion of the adenylate cyclase gene *cyaB*; contains 500-bp flanking regions around *cyaB*	This work
pEKEx2-*eyfp*	KanR; $lacI^q$, P_{tac}; *C. glutamicum*/*E. coli* shuttle vector carrying the *eyfp* reporter gene	(Hentschel et al., 2013)
pJC1-*venus*-term	Kan^R; *C. glutamicum/E. coli* shuttle vector, carrying the *venus* coding sequence and additional terminators	(Baumgart et al., 2013)
pSenPcg3195	Kan^R; pJC1 derivative containing a 188-bp cg3195 promoter fragment cloned in front of the *eyfp* gene	This work
pXMJ19	Cm^R; P_{tac} $lacI^q$; *C. glutamicum*/*E. coli* shuttle vector (pBL1 ori_{Cg}, pK18 ori_{Ec})	(Jakoby et al., 1999)
pXMJ19-*glxR*	Cm^R; pXMJ19 derivative containing the *glxR* gene under control of the IPTG-inducible *tac* promoter	(Schulte et al., 2017)

Figure legends

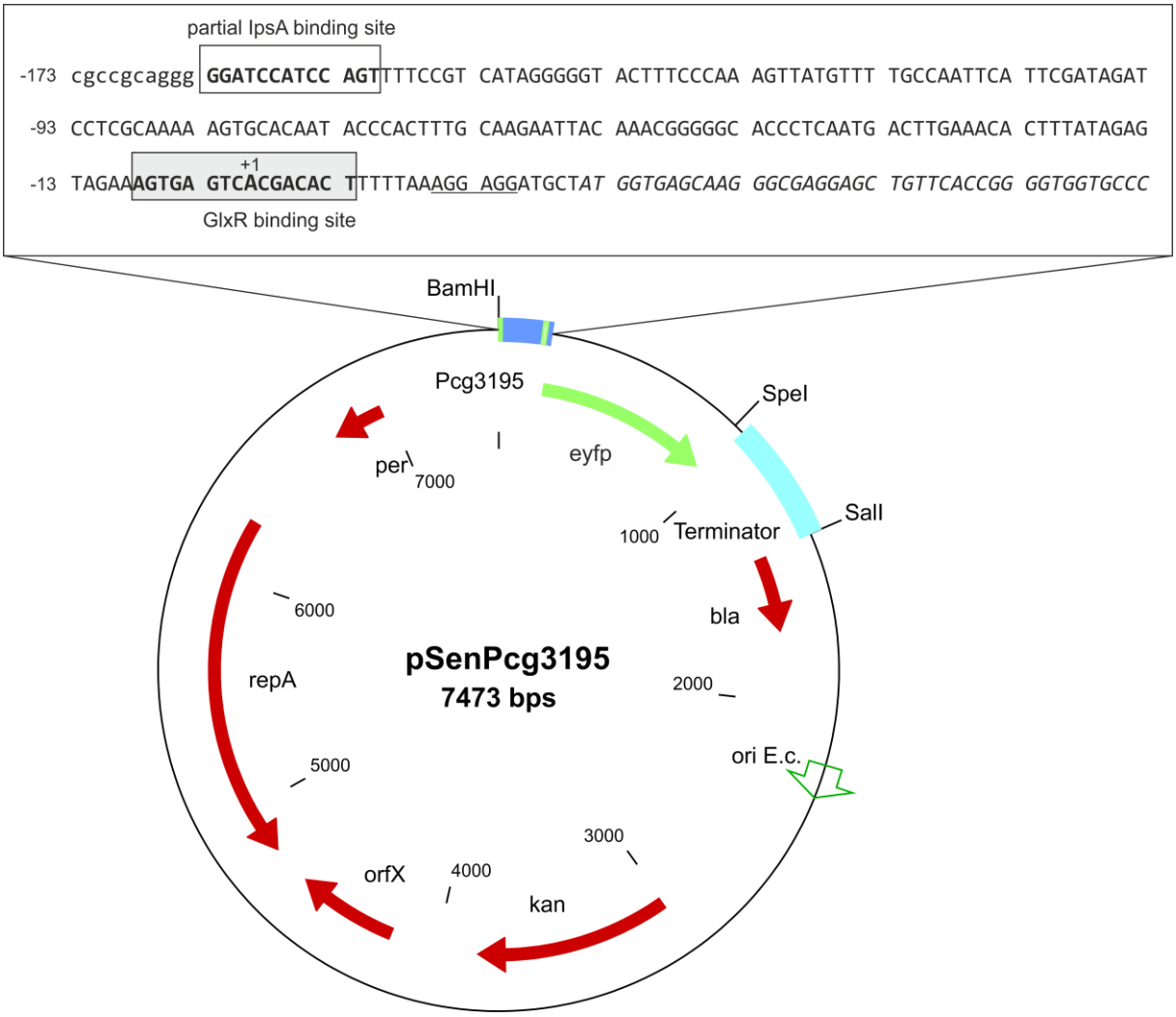


Fig. 1. Schematic overview of the sensor plasmid pSenPcg3195. The *eyfp* gene is under control of a 188 bp fragment covering 163 bp upstream and 24 bp downstream of the transcriptional start site of cg3195. In this way, the ribosome binding site of cg3195 (underlined) was positioned at the same distance to the start codon of *eyfp* as in the genome to the start codon of cg3195. The *eyfp* coding sequence is in italics. The binding sites for GlxR and IpsA are indicated with grey and white boxes, respectively. The IpsA binding site is not fully contained on the plasmid. The transcriptional start (+1) is annotated as described previously (Baumgart et al., 2013). An additional 10 bp upstream of the partial IpsA binding site that belong to the pJC1 backbone are indicated with non-capitalized letters.

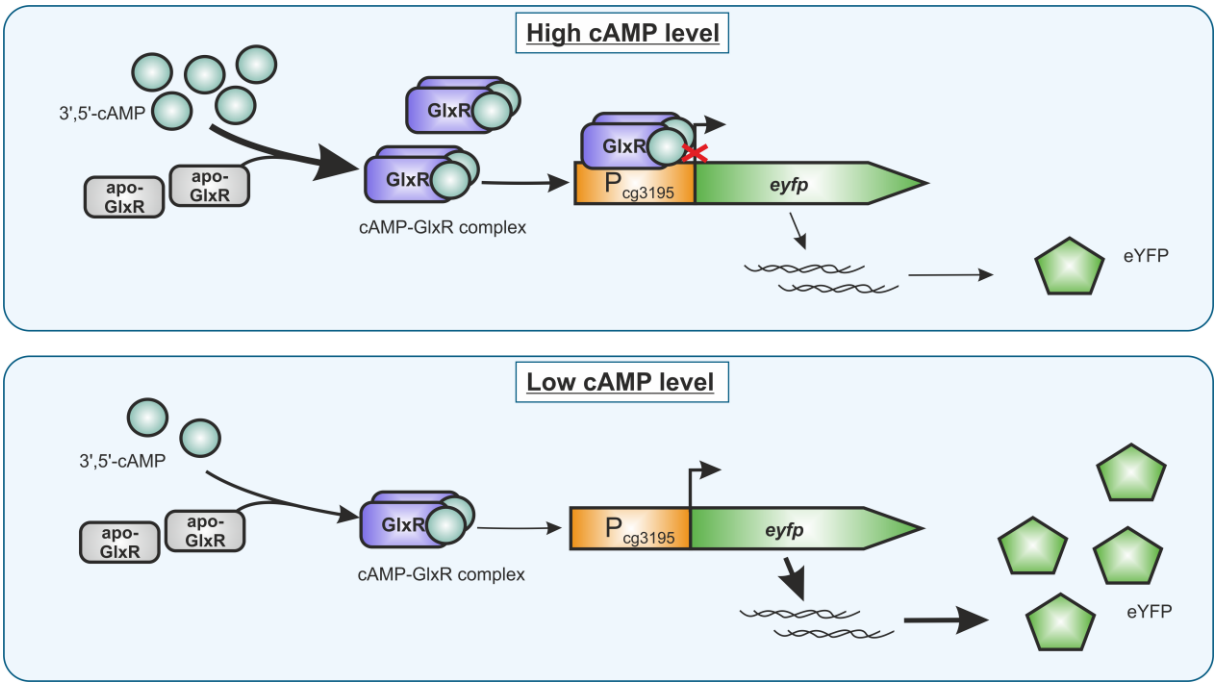


Fig. 2. Principle of the reporter gene-based cAMP biosensor for *C. glutamicum*. At high intracellular cAMP concentrations (upper scheme) formation of the cAMP-GlxR complex is favored which binds to the cg3195 promoter and represses expression of the *eyfp* reporter gene. Low cAMP levels (lower scheme) result in lower levels of the cAMP-GlxR complex and increased reporter gene expression.

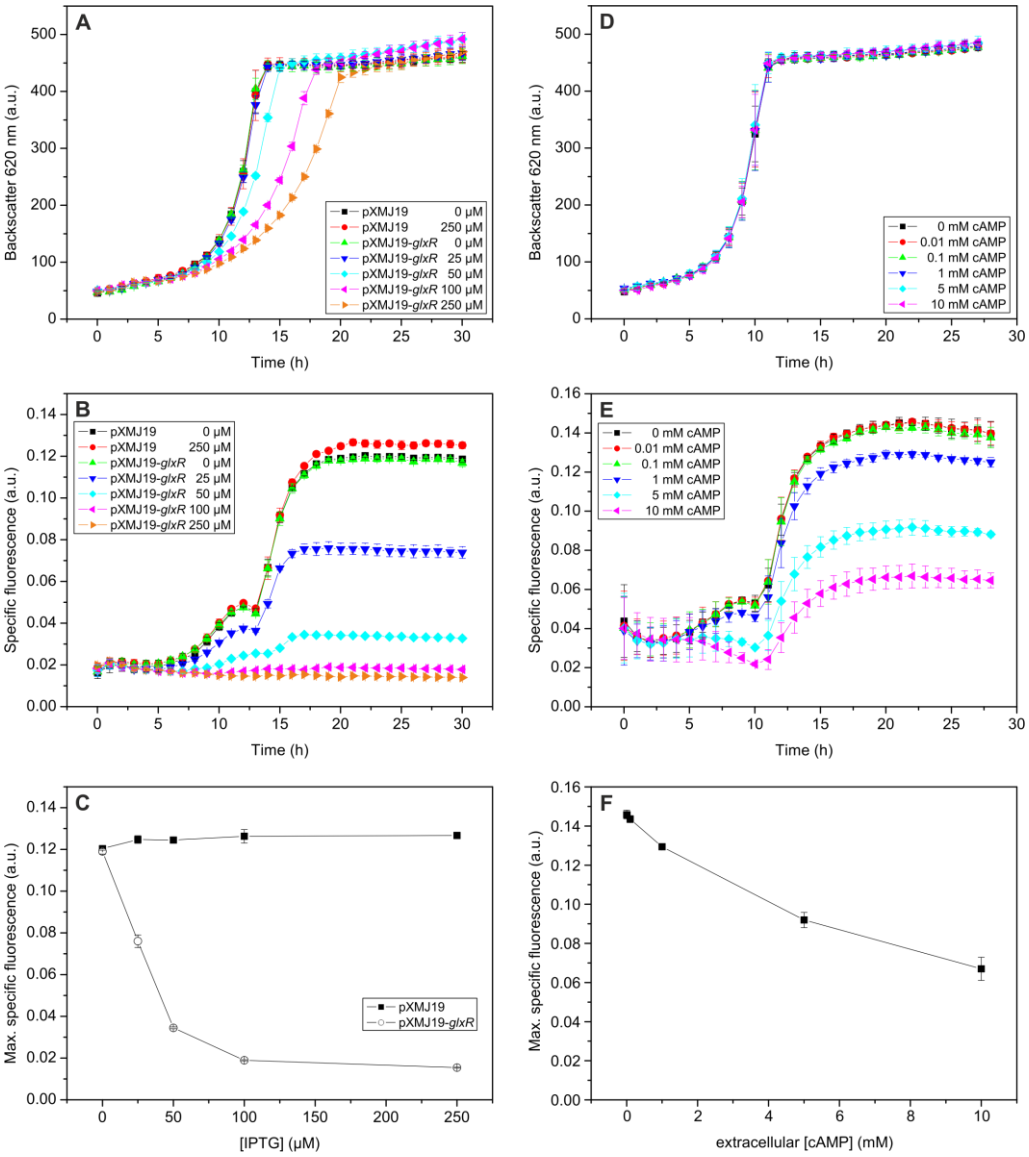
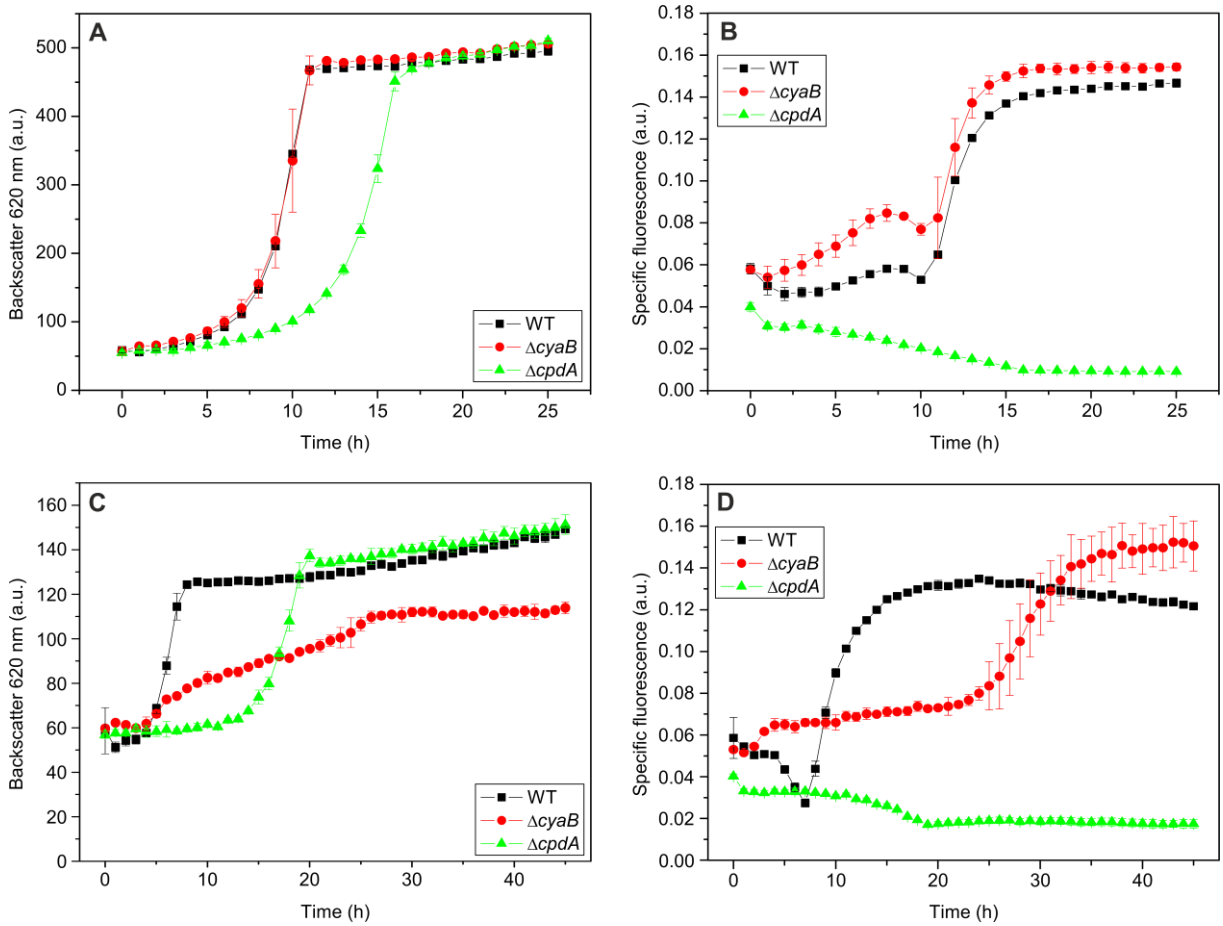


Fig. 3. Response of *C. glutamicum* wild type carrying the cAMP biosensor plasmid pSenPcg3195 to varying levels of GlxR (A, B, C) and cAMP (D, E, F). Panel A shows the growth of *C. glutamicum*/pSenPcg3195/pXM19-*glxR* in CGXII minimal medium with 100 mM glucose and various concentrations of IPTG added at the start of the cultivation in the BioLector system. In the control strain, pXMJ19-*glxR* was replaced by the empty plasmid pXMJ19. Panel B shows the specific fluorescence (fluorescence/backscatter at 620 nm) of the cultures shown in panel A. In panel C, the maximal specific fluorescence shown in panel B is plotted as a function of the IPTG concentration used to induce *glxR* expression. In panel D, growth of *C. glutamicum*/pSenPcg3195 in CGXII minimal medium with 100 mM glucose and varying levels of cAMP added at the start of the cultivation is displayed. Panel E shows the specific fluorescence of the cultures presented in panel D. In panel F, the maximal specific fluorescence is plotted against the concentration of cAMP added to the medium. The results of three biological replicates with standard deviations are shown.



582
583 **Fig. 4.** Growth (A, C) and specific fluorescence (B, D) of *C. glutamicum* wild type, the Δ*cyaB*
584 mutant, and the Δ*cpdA* mutant, all carrying the sensor plasmid pSenPcg3195. Cells were
585 cultivated in CGXII minimal medium supplemented with 100 mM glucose (A, B) or 100 mM
586 sodium acetate (C, D) as carbon source in the BioLector. The results of three biological
587 replicates with standard deviations are shown.
588

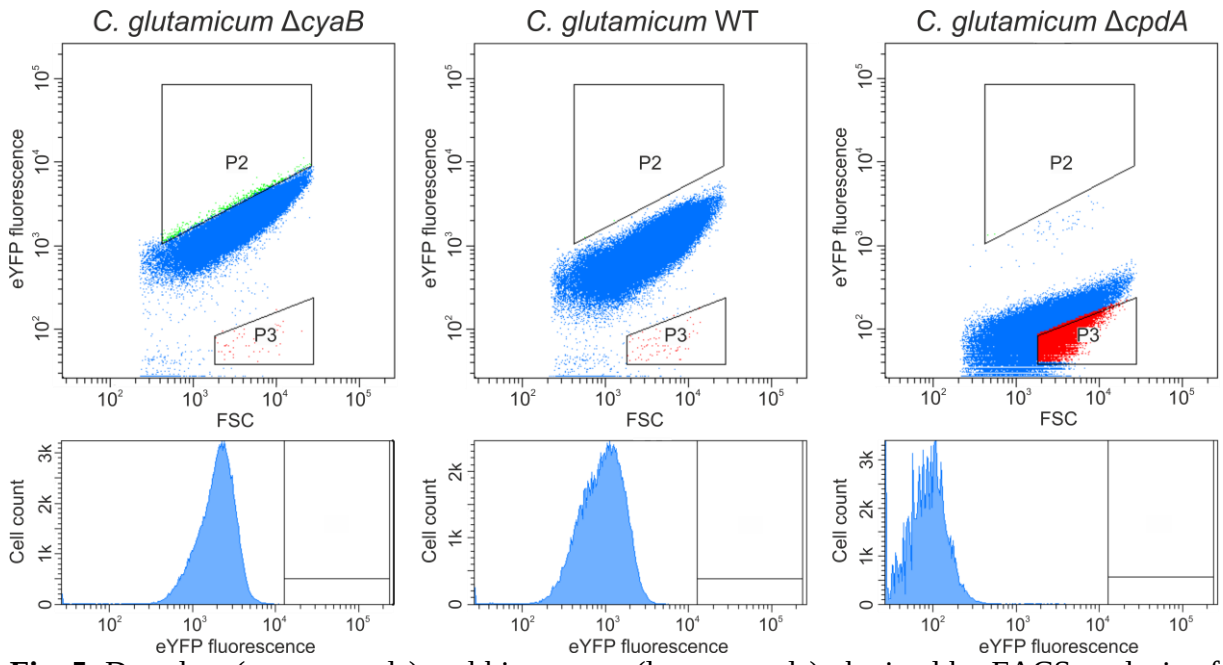


Fig. 5. Dot plots (upper panels) and histograms (lower panels) obtained by FACS analysis of the Δ*cyaB* mutant, the wild type, and the Δ*cpdA* mutant, all carrying the pSenPcg3195 plasmid. The strains were cultivated in shake flasks in CGXII minimal medium with 100 mM glucose as carbon source and cells of the mid-exponential growth phase (4-5 h) were used for FACS analysis. 100 000 cells were analyzed for each strain. The gates P2 and P3 were set for cell sorting.

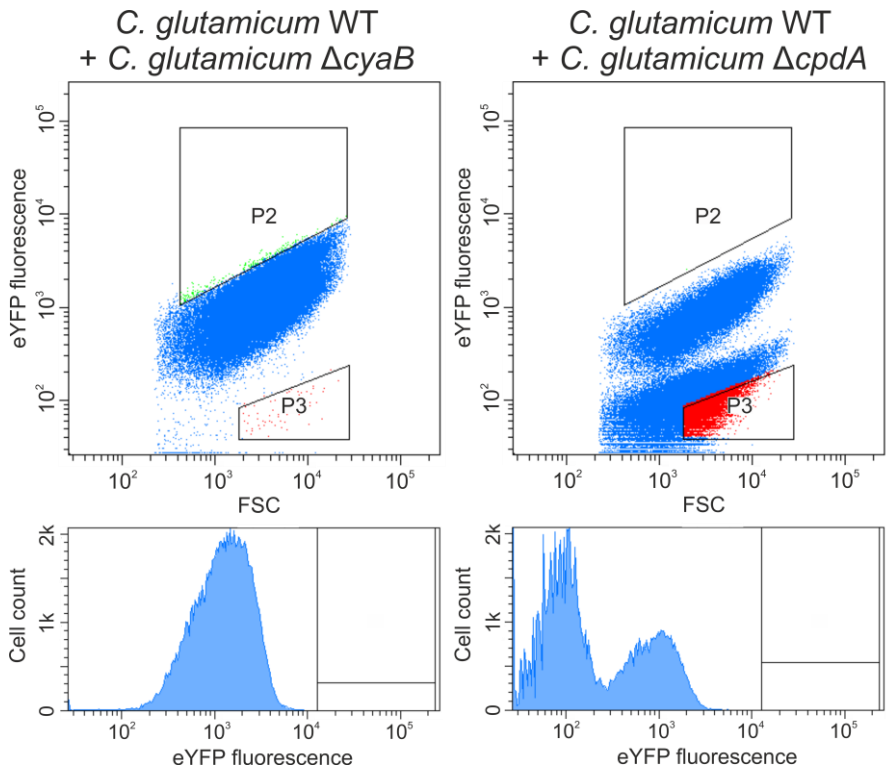


Fig. 6. Dot blots (upper panels) and histograms (lower panels) obtained by FACS analysis of 1:1 mixtures of wild-type and Δ*cyaB* cells (left panels) or wild-type and Δ*cpdA* cells (right panels). The strains were cultivated in shake flasks in CGXII minimal medium with glucose as carbon source and cells from the mid-exponential growth phase (4-5 h) were used for FACS analysis. 100 000 cells of each mixture were analyzed prior to the sorting. Gate P2 was used to select for Δ*cyaB* mutant cells, gate P3 was used to sort for Δ*cpdA* cells.