ulm university universität ulm

Transcriptional organisation and regulation of genes encoding carbonic anhydrases in *Corynebacterium glutamicum*

Dissertation

For the fulfillment of the requirements for the doctoral degree **Dr. rer. nat.**
at the Faculty of Natural Sciences, University of Ulm

Submitted by

Adnan Shah
from Islamabad

University of Ulm, Germany

2015

The present work was performed at the Institute of Microbiology and Biotechnology, University of Ulm under the supervision of Prof. Dr. Bernhard J. Eikmanns.

Faculty Dean:	Prof. Dr. Peter Dürre
First Reviewer:	Prof. Dr. Bernhard J. Eikmanns
Second Reviewer:	PD Dr. Christian Riedel

Date of the doctoral examination: 12th November, 2015

Table of contents

Abstract……1

1 Introduction……4

2 Material and methods……10

2.1 Bacterial strains, plasmids/constructs and oligonucleotides……10
2.2 Chemicals, devices, kits and enzymes……14
2.3 Growth media and culture conditions……18
2.3.1 Growth media and additives……18
2.3.2 Cultivation conditions……21
2.4 Isolation and purification of DNA……21
2.4.1 Isolation of plasmid DNA from *E. coli*……21
2.4.2 Isolation and purification of genomic DNA from *C. glutamicum*……22
2.5 PCR techniques and gene cloning……23
2.5.1 PCR techniques……23
2.5.2 Gene cloning and sequence analysis……25
2.6 Transformation of *E. coli* and *C. glutamicum* cells……26
2.6.1 Transformation of *E. coli* cells……26
2.6.2 Transformation of *C. glutamicum* cells……26
2.7 Over-production and purification of proteins in *E. coli* BL21……27
2.8 SDS-PAGE analysis……28
2.9 Cloning of promoter regions in the promoter-probe vector pET2……30
2.10 Determination of TSPs of genes in *C. glutamicum*……30
2.10.1 RNA isolation……30
2.10.2 cDNA synthesis and 5'-RACE……31
2.11 Determination of specific CAT enzyme activity……33
2.12 DNA affinity chromatography……35
2.13 Electrophoretic mobility shift assays (EMSAs)……38
2.14 MALDI-TOF mass spectrometry……39
2.15 Chromosomal deletion of the *gca* gene in *C. glutamicum*……39

Table of contents

3 Results…….....…...41

3.1 Amplification of the *bca* and *gca* genes from *C. glutamicum* WT…….....…….....…..41

3.2 Over-production and purification of His-tagged Bca and Gca protein in *E. coli* BL21…….……..……42

3.3 The transcriptional organisation of the *bca* and *gca* genes in *C. glutamicum*…….....45

3.3.1 Cloning of the promoter regions of the *bca* and *gca* genes in promoter-probe vector pET2…….....……..45

3.3.2 Identification of TSPs for the *bca* and *gca* genes……..…….….47

3.4 Expression analysis of the *bca*, *gca* genes and the asRNA (within *bca* gene)….....49

3.4.1 Promoter activities of the *bca* and *gca* genes during growth of *C. glutamicum* with different carbon sources…..…….…….....….49

3.4.2 Effect of pH on carbon source-dependent regulation of *bca* gene in *C. glutamicum*……..……..…..……..…….....…....51

3.4.3 Characterization of the asRNA within the *bca* gene…….……..….....….52

3.5 Identification of proteins binding to the *bca* promoter region……...…….….....….54

3.6 Regulation of the *bca* gene by the transcriptional regulator RamA in *C. glutamicum*……...…….……...….....….55

3.6.1 Comparison of the *bca* promoter activity in *C. glutamicum*-Δ*ramA* and wild-type *C. glutamicum*…….…...……...……55

3.6.2 Over-production and purification of His-tagged RamA protein…….....…….….56

3.6.3 His-tagged RamA protein binds to the *bca* promoter region……...……..…..57

3.7 Creation of a *gca* deletion mutant of *C. glutamicum*……...…….……..…59

3.7.1 Comparison of the *bca* promoter activity in wild-type and *C. glutamicum*- Δ*gca*……...….....…61

4 Discussion…….…….…….63

5 References…….……...……...……..72

Abbreviations…….……..……..……82

Poster presentations.....……..……..……...……...…….85

Contribution of others to this work……..……....……..86

Acknowledgements.....87

Curriculum vitae…….…….….….89

Declaration……..……...…...….90

Abstract

The intracellular CO_2 and HCO_3^- balance is crucial in bacterial physiology, notably in carboxylation reactions which make use of HCO_3^- as substrate. As the tension of CO_2 is very low in the environment and it can freely move inside and outside of the cell, its conversion to HCO_3^- is important to trap CO_2 inside, to meet the cellular demands. The carbonic anhydrase (CA) (EC 4.2.1.1) enzyme catalyzes the reversible hydration of CO_2 to HCO_3^- and is likely to play an important role in carboxylation reactions of central metabolism, especially those of metabolic switch points of carbon flux distribution such as the PEP-pyruvate oxaloacetate node which is important for oxaloacetate anaplerosis, gluconeogenesis and amino acid production in *Corynebacterium glutamicum*.

In *C. glutamicum*, two putative genes namely the *bca* gene coding for beta-type carbonic anhydrase and the *gca* gene coding for gamma-type carbonic anhydrase have already been identified. The current work has focused on (1) over-production of the proteins encoded by the *bca* and *gca* genes, (2) transcriptional organisation of these genes, (3) regulation of expression of these genes in relation to carbon sources, pH and transcriptional regulators, (4) expression analysis of an antisense RNA (asRNA) within the *bca* gene and (5) creation of a *gca* deletion in *C. glutamicum* and its functional characterization.

Both the *bca* and *gca* genes were separately cloned into an expression vector pET-TEV1 and the corresponding His-tagged Bca and Gca proteins were over-produced in *Escherichia coli* BL21, purified and analyzed on an SDS-PAGE gel confirming the size of proteins. These purified Bca and Gca proteins can be used for further structural and functional characterization. The transcriptional start points (TSPs) were determined for both the *bca* and *gca* genes, using the pET2-*bca*-promoter and pET2-*gca*-promoter constructs respectively, by 5′-RACE method. It was observed that the *bca* gene codes for a leader-less transcript and its TSP was found to be “A”, which is the first nucleotide of the putative translational start site (ATG) of the *bca* gene. As the *gca* gene was already known to form an operon with an upstream gene cg0154, the TSP for the *gca* gene was determined as “A” nucleotide which was at -20 position relative to the putative translational start site of the cg0154 gene. Furthermore, a partially conserved -10 region was also found for both the *bca* and *gca* genes.

Abstract

To study the regulation of expression of both the genes in relation to carbon sources, the activity of the *bca* and *gca* promoters were determined using vectors pET2-*bca*-promoter and pET2-*gca*-promoter respectively on different carbon sources such as glucose and acetate at mid-exponential phase of growth in *C. glutamicum*. In general, the carbon source-dependent regulation was observed for both the genes, with higher activity on glucose than on acetate, as sole carbon source. It was also observed that the activity of the *bca* promoter was about 15 times higher than that of the *gca* promoter on glucose or acetate as carbon source. Furthermore, it was observed that the carbon source-dependent regulation of the *bca* gene was not affected by the initial pH of the medium as 6.3 or 6.8. As the RNA sequence analysis (RNAseq) had already reported an antisense RNA (asRNA) within the *bca* gene, the promoter region of this asRNA was shown to exhibit a very low activity on glucose or acetate as carbon source, using vector pET2-asRNA-promoter in *C. glutamicum.* This confirmed an upstream region acting as a promoter for this asRNA as well as expression of the asRNA during mid-logarithmic growth phase in *C. glutamicum*.

To study the regulation of the *bca* gene by transcriptional regulators, DNA affinity chromatography was performed for the *bca* promoter region with extracts of *C. glutamicum* cells grown with glucose as carbon source and harvested at mid-exponential growth phase. The enriched proteins were then analyzed by SDS-PAGE gel and identified by MALDI-TOF mass spectrometry. However, no transcriptional regulator was found to bind to the *bca* promoter region. As the comparative transcriptomic profile of a Δ*ramA* mutant had already shown the transcript level of the *bca* gene about 2.5 times higher than that of wild-type *C. glutamicum*, RamA was speculated to regulate the expression of the *bca* gene. To test this hypothesis, the pET2-*bca*-promoter was used to make a comparison of the *bca* promoter activity in Δ*ramA* and wild-type *C. glutamicum*. It was shown that the activity of the *bca* promoter was about four times higher in the Δ*ramA* mutant as compared to wild-type *C. glutamicum*. As the sequence analysis of the promoter region of the *bca* gene showed multiple RamA binding motifs, it was likely that the RamA protein binds to the *bca* promoter region. This was confirmed by binding of the over-produced and purified His-tagged RamA protein to the *bca* promoter region in EMSA experiments. Based on the higher transcript level of *bca* in the Δ*ramA* mutant as compared to wild-type *C. glutamicum*, higher activity of the *bca* promoter in the absence of a functional RamA regulator and binding of the His-tagged RamA to the *bca* promoter region, it is concluded

that RamA acts as a negative transcriptional regulator for the expression of the *bca* gene in *C. glutamicum*.

Finally, a deletion of the *gca* gene was created using the suicide vector pK19*mobsac*B in *C. glutamicum*. The growth pattern of *C. glutamicum*-Δ*gca* was shown to be nearly the same as that of wild-type *C. glutamicum*. Using pET2-*bca*-promoter, it was also observed that the activity of the *bca* promoter remains unaffected in the absence of a functional Gca protein in *C. glutamicum*.

1 Introduction

Corynebacterium glutamicum is an aerobic Gram-positive, biotin-auxotrophic soil bacterium that was isolated due to its ability to produce L- glutamate (Kinoshita et al., 1957). *C. glutamicum* is generally recognized as safe organism and suitable for biotechnological processes (Pátek et al., 2013). In addition to the industrial production of L-glutamate and L-lysine (Eggeling and Bott, 2005), the use in production of other amino acids such as L-valine (Blombach et al., 2007; Blombach et al., 2008; Hasegawa et al., 2013), L-arginine (Park et al., 2014), histidine (Cheng et al., 2013: Kulis-Horn et al., 2014), vitamins (Hüser et al., 2005), organic acids (Wieschalka et al., 2012), ethanol (Inui et al., 2004; Sakai et al., 2007), higher alcohols (Blombach and Eikmanns, 2011), diamines (Kind et al., 2010; Nguyen et al., 2015), 2-ketoacids such as 2-ketoisovalerate (Krause et al., 2010; Buchholz et al., 2013) and 2-ketoisocaprovate (Bückle-Vallant et al., 2014; Vogt et al., 2015), polymers (Song et al., 2012) and polyhydroxybutyrate (Jo et al., 2006) has widened the significance of *C. glutamicum*. Furthermore, *C. glutamicum* is also viewed as a model organism for the Corynebacterineae, which includes the genus *Mycobacterium* (Bott, 2007). The central carbon metabolism (CCM) is the integration of metabolic pathways related to transport and oxidation of carbon sources such as phosphotransferase system (PTS), glycolysis, gluconeogenesis, pentose phosphate pathway (PPP), the tricarboxylic acid (TCA) cycle and the glyoxylate bypass. The knowledge of regulation in CCM is important for metabolic engineering and re-directing the carbon fluxes towards production of industrially important metabolites (Papagianni, 2012).

In *C. glutamicum*, the PEP (phosphoenolpyruvate)-pyruvate-oxaloacetate node has been specifically focused due to its importance in carbon flux distribution as well as for supply of precursor, needed for the production of amino acids. *C. glutamicum* has two C-3 carboxylating anaplerotic enzymes namely the PEP carboxylase and pyruvate carboxylase converting phosphoenolpyruvate (PEP) and pyruvate to oxaloacetate, respectively. (Sauer and Eikmanns, 2005) Anaplerosis is the replenishment of intermediates which are removed from central metabolism during biosynthesis (Kornberg, 1965). Apart from these C3-carboxylating enzymes, *C. glutamicum* also has three C4-decarboxylating enzymes, namely the PEP carboxykinase converting oxaloacetate to PEP whereas oxaloacetate decarboxylase and malic enzyme converting oxaloacetate and malate respectively, to pyruvate (Sauer and Eikmanns, 2005) (Fig.

1). Bicarbonate is required as a substrate of the carboxylation reactions (Mitsuhashi et al., 2004) which highlights the importance of intracellular CO_2/bicarbonate balance for carboxylation reactions of central metabolism especially those of metabolic switch-points of carbon flux distribution. As bicarbonate is needed as substrate of metabolism, its significant source is the hydration of CO_2. Due to the low tension of CO_2 in the environment and its diffusion from the cell, the spontaneously formed bicarbonate is not sufficient to meet metabolic demands of the cell (Merlin et al., 2003).

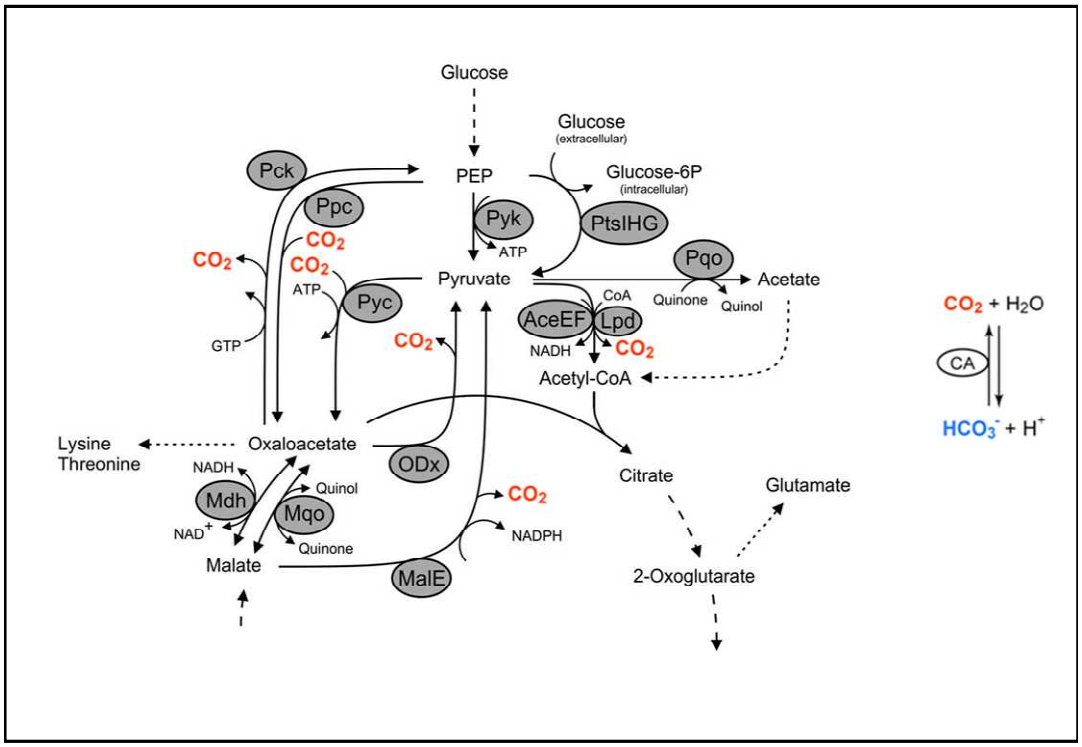


Figure 1: The PEP–pyruvate–oxaloacetate node in *C. glutamicum*. Abbreviations shows the products of genes that catalyze given reactions: AceEF, subunits E1 and E2 of the pyruvate dehydrogenase complex; Lpd, subunit E3 of the pyruvate dehydrogenase complex; Mdh, malate dehydrogenase; MalE, malic enzyme; Mqo, malate: quinone oxidoreductase; ODx, oxaloacetate decarboxylase, Pck, PEP carboxykinase; PtsIHG, phosphotransferase system; Pqo, pyruvate: quinone oxidoreductase; Pyc, pyruvate carboxylase; Pyk, pyruvate kinase and CA, carbonic anhydrase (after Sauer and Eikmanns, 2005).

The Carbonic anhydrase (CA) (EC 4.2.1.1) enzyme catalyzes the reversible hydration of CO_2 to bicarbonate and plays a key role in different biochemical and physiological processes (Kaur et

al., 2009). The catalytic mechanism of CA involves the first step with nucleophilic attack of zinc-bound hydroxide ion on the CO_2 (equation 1) followed by second step where active site is regenerated by ionization of the zinc-bound water molecule and removing proton from the active site (equation 2). The CAs with K_{cat} value higher than 10^4 S^{-1} need intermediate PSR (proton shuttle residue) for the transfer of proton from metal bound water to the external buffer "B" (equation 3 and 4) (Tripp et al., 2001).

$$Zn^{2+}\text{-}OH^- + CO_2 \rightarrow Zn^{2+} + HCO^{3-} \quad \text{(Eq. 1)}$$

$$Zn^{2+} + H_2O \rightarrow H^+ + Zn^{2+}\text{-}OH^- \quad \text{(Eq. 2)}$$

$$PSR + Zn^{2+}\text{-}H_2O \rightarrow Zn^{2+}\text{-}OH^- + PSR\text{-}H^+ \quad \text{(Eq. 3)}$$

$$PSR\text{-}H^+ + B \rightarrow PSR + B\text{-}H^+ \quad \text{(Eq. 4)}$$

CAs are ubiquitous in eukarya, bacteria and archaea domains of life (Smith and Ferry, 2000). There are five genetically distinct families of CA known so far, namely the α-, β-, γ-, δ-, and ζ-CAs (Supuran, 2011). The alpha class is predominantly found in mammals (Zimmermann and Ferry, 2008) while δ- and ζ-classes have been found in marine diatoms (Park et al., 2007) (Zimmermann and Ferry, 2008). The β and γ CAs are ancient and predominant classes in prokaryotes. The occurence of β and γ classes in species of archaea and bacteria domain shows their fundamental role in the biology of prokaryotes (Smith et al., 1999). The role of different classes of CA enzymes has been intensively studied in different organisms, especially in relation to CO_2/bicarbonate balance in the intracellular environment under physiological conditions. Bacterial β class of CAs are zinc metallo-enzymes, maintaining CO_2/HCO_3^- balance in the intracellular environment which is required for biosynthesis (Lotlikar et al., 2013). The β-CA has been shown to be required for capturing CO_2 from air and the requirement of CO_2 hydration appears to be higher for cells growing in logarithmic phase of growth in *Azospirillium brasilienses* (Kaur et al., 2009). Similarly, the role of β-CA enzyme (Cbp) in anaplerotic CO_2 fixation reactions by supplying bicarbonate to carboxlase enzymes, has also been suggested in *Clostridium perfringens* (Kumar et al., 2013). *Streptococcus pneumonia* having deletion of the CA gene, was unable to grow under poor CO_2 conditions but could be compensated by addition of HCO_3^- (Burghout et al., 2010). Similarly, *E. coli* is also unable to grow in air in the absence of

a functional CA and this inhibition of growth can be relieved by high CO_2 concentrations (Merlin et al., 2003). Also, in *Ralstonia eutropha* H16, the deletion of β-CA (*can* gene) affected the growth of organism but was compensated by addition of CO_2 to the culture (Gai et al., 2014). The CA along with urease has also been observed to play a major role in acid acclimation in *Helicobacter pylori* (Nishimori et al., 2008). The β-CA is the only enzyme, of the five distinct classes of CAs, which shows allosterism. The binding of bicarbonate (HCO_3^-) to an allosteric regulation site has been shown for HICA (*Haemophilus influenza* β-CA) and ECCA (*E. coli* β-CA) (Rowlett, 2010). The β-CA is also likely to be an important accessory enzyme for many other enzymes which utilize CO_2 or HCO_3^- (Rowlett, 2010), such as rubisco in chloroplasts, cyanase in *E. coli* (Guilloton et al., 1993), urease in *H. pylori* (Nishimori et al., 2008) and bicarbonate-dependent carboxylases in *C. glutamicum* (Mitsuhashi et al., 2004) (Rowlett, 2010). Furthermore, the β-CA (CpeCA) has also been observed to be inhibited by anions, such as (thio) cyanate, azide, bicarbonate, nitrate, nitrite, hydrogensulfite, hydrogensulfide, stannate, tellurate, pyrophosphate, divanadate, tetraborate, peroxydisulfate, sulfate, iminodisulfonate and fluorosulfonate with K_1S in the range of 1-10 mM, in *Clostridium perfringens* (Vullo et al., 2013). The γ-CA enzymes are widespread in procaryotes but their physiological role remains subtle. The γ-CA (Cam) of the methanogenic archeon *Methanosarcina thermophila* has been observed to exhibit CA activity (Kaur et al., 2010).

As genetic studies have shown CA to be dispensable under high CO_2 conditions in model organisms, it shows that microorganisms that are lacking CA can persist in nature by choosing high CO_2 niches (Ueda et al., 2012). Based on the crucial role of catalyzing the hydration of CO_2 to bicarbonate and proton, bacterial CAs probably play a role in steps of bacterial life cycle important for survival, invasion, and pathogenicity. Bacterial CAs also represent favorable targets for obtaining anti-bacterials without the problem of resistance (Supuran, 2011).

In *C. glutamicum*, two putative genes *bca* (locus-tag cg2954) and *gca* (locus-tag cg0155) coding for beta-type carbonic anhydrase and gamma-type carbonic anhydrase respectively, have been identified. The *bca* has been shown to be indispensable for growth of *C. glutamicum* and the growth phenotype in *C. glutamicum*-Δ*bca* could be restored with 5% of CO_2 supplementation.

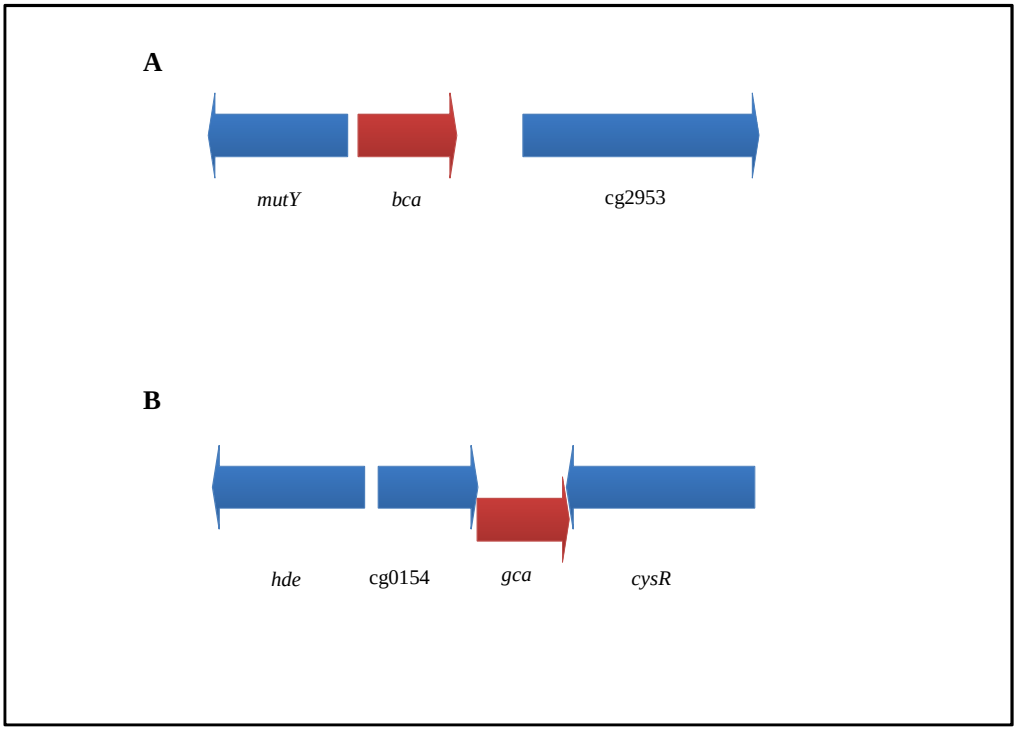


Figure 2: (A) The genetic organisation of the *bca* gene in the genome of *C. glutamicum*; *bca*: beta-type carbonic anhydrase, *mutY*: A/G specific adenine glycosylase and **(B)** genetic organisation of *gca* gene in the genome of *C. glutamicum*; *gca*: gamma-type carbonic anhydrase, *cysR*: bacterial regulatory protein, *hde*: probable esterase/lipase protein.

However, the *gca* gene has been shown to be dispensible for growth under ordinary atmospheric conditions. Furthermore, expression of the *bca* was also observed to be induced in high bicarbonate demand condition such as production of amino acid, L-lysine (Mitsuhashi et al., 2004).

The aim of the present study was to determine the transcriptional organisation of these genes in the genome of *C. glutamicum*. As *C. glutamicum* is capable to grows on different sugars and organic acids (Sauer and Eikmanns, 2005) and it has been shown that the expression of genes that are coding for enzymes of glycolysis, gluconeogenesis, pentose phosphate pathway as well as TCA and glyoxylate cycles can change with the source of carbon (Auchter et al., 2011a), it was therefore, also aimed to study the regulation of expression of genes encoding carbonic anhydrase enzymes in relation to different carbon sources in *C. glutamicum*.

The genes of central metabolism are under the control of several global regulators in transcriptional regulatory network, in *C. glutamicum* (Toyoda et al., 2013). The transcriptional

regulators such as FruR, SugR, GntR1, GntR2, AcnR, LldR, GlxR, RamB and RamA, have been identified, affecting the central metabolic pathways in *C. glutamicum* (Arndt and Eikmanns, 2008; Auchter et al., 2011a). A further aim of this work was to study the regulation of expression of the *bca* gene in relation to transcriptional regulators, in *C. glutamicum*. Furthermore, a deletion mutant was also created for the *gca* gene in *C. glutamicum* and functionally characterized including effect of the *gca* deletion on growth as well as on expression of the *bca* gene.

2 Material and methods

2.1 Bacterial strains, plasmids/constructs and oligonucleotides

All the bacterial strains, plasmids/constructs and oligonucleotides used in this work are listed in Table 1, 2 and 3 with their relevant characteristics:

Table 1: Strains used in the present work

Strain	Relevant characteristic(s)	Source or reference
E. coli DH5α	*supE44, hsdR17, recA1, endA1, gyrA96, thi-1, relA1*	Hanahan, 1983
E. coli BL21 (DE3)	ompT *hsd*S_B (r_B–m_B–) *gal dcm* (DE3)	Studier and Moffatt, 1986
E.coli DH5α (pET-TEV1)	*E. coli* DH5α carrying pET-TEV1 vector	S. Klaffl
E. coli DH5α (pET-TEV1-*bca*)	*E. coli* DH5α carrying pET-TEV1-*bca* construct	This work
E. coli DH5α (pET-TEV1-*gca*	*E. coli* DH5α carrying pET-TEV1-*gca* construct	This work
E. coli DH5α (pET2)	*E. coli* DH5α carrying pET2 vector	T. Laslo
E. coli DH5α (pET2-*bca*-promoter)	*E. coli* DH5α carrying pET2-*bca*-promoter construct	This work
E. coli DH5α (pET2-*gca*-promoter)	*E. coli* DH5α carrying pET2-*gca*-promoter construct	This work
E. coli DH5α (pET2-*as*RNA- promoter)	*E. coli* DH5α carrying pET2-asRNA-promoter construct	This work
E. coli DH5α (pK19-*mobsac*B)	*E. coli* DH5α carrying pK19-*mobsac*B vector	T. Laslo
E. coli DH5α (pK19*mobsac*B-Δ*gca*)	*E. coli* DH5α carrying pK19*mobsac*B- Δ*gca* construct	This work
E. coli DH5α (pET28a-*ramA*)	*E. coli* DH5α carrying pET28a-*ramA* construct	Cramer et al., 2006

C. glutamicum WT	Wild-type strain ATCC 13032	ATCC
C. glutamicum (pET2-*bca* -promoter)	*C. glutamicum* carrying pET2-*bca*-promoter construct	This work
C. glutamicum (pET2-*gca* -promoter)	*C. glutamicum* carrying pET2-*gca*-promoter construct	This work
C. glutamicum-Δ*atlR* (pET2-p4 fragment)	*C. glutamicum*-Δ*atlR* carrying pET2-p4	Gabris, 2011
C. glutamicum RES167-pET2	*C. glutamicum* RES167 carrying pET2 vector	Gabris, 2011
C. glutamicum (pK19*mobsac*B-Δ*gca*)	*C. glutamicum* carrying pK19*mobsac*B-Δ*gca* construct	This work
C. glutamicum-Δ*gca*	*C. glutamicum* with deleted *gca* gene in the genome	This work
C. glutamicum-Δ*ramA*	*C. glutamicum* with deleted *ramA* gene in the genome	Cramer et al., 2006

Table 2: Plasmids used in this study

Plasmids/constructs	**Relevant characteristic(s)**	**Reference/ source**
pET2 vector	Promoter probe vector, carrying the promoter-less *cat* reporter gene, Km^r	Vasicová et al., 1998
pET2-*bca*-promoter	pET2 carrying the *bca* promoter fragment cloned upstream of the *cat* gene	This work
pET2-*gca*-promoter	pET2 carrying the *gca* promoter fragment cloned upstream of the *cat* gene	This work
pET2-asRNA-promoter	pET2 carrying the asRNA promoter fragment cloned upstream of the *cat* gene	This work
pK19*mobsac*B vector	Km^r, Vector for integration of insert in the genome of the *C. glutamicum*	Schäfer et al., 1994
pK19*mobsac*B-Δ*gca*	pK19*mobsac*B vector carrying Δ*gca* insert	This work
pJET1.2/blunt	Linearized cloning vector, Amp^r	CloneJET PCR (Cloning kit)
pJET-*bca*-RACE	The *bca* 5'-RACE product cloned in pJET1.2/blunt vector	This work

pJET-*gca*-RACE	The *gca* 5'-RACE product cloned in pJET1.2/blunt vector	This work
pET-TEV1 vector	Over-expression vector, $P_{T7\ lac}$, *lac*I, His_{10}-tag, TEV site, Km^r	S. Klaffl
pET-TEV1-*bca*	pET-TEV1 over-expression vector carrying *bca* gene	This work
pET-TEV1-*gca*	pET-TEV1 over-expression vector carrying *gca* gene	This work
pET28a-*ramA*	pET-28a over-expression vector, carrying *ramA* gene	Cramer et al., 2006

Table 3: Oligonucleotides used in this study

Oligonucleotides	Sequence	Purpose
bca-fw	5'-GGGAATTCCATATGCCTTTGCGTAATGTTG-3'	Fw primer for amplification of *bca* gene
bca-rev	5'-ACGCGTCGACCTAACCCACGTTCTTGCTAA-3'	Rev primer for amplification of *bca* gene
gca-fw	5'-GGGAATTCCATATGACACCTCAACCACTGAT-3'	Fw primer for amplification of *gca* gene
gca-rev	5'-ACGCGTCGACTTACTCTCGTACCCTAACCTC-3'	Rev primer for amplification of *gca* gene
pET-TEV1-fw	5'-CGAAATTAATACGACTCACTATAGG-3'	Fw pET-TEV1 specific primer
pET-TEV1-rev	5'-ATGCTAGTTATTGCTCAGCGGTG-3'	Rev pET-TEV1 specific primer
bca-promoter-fw	5'-ACGCGTCGACTACACGCGACGCACGTTCGT-3'	Fw primer for *bca* promoter (SalI)
bca-promoter-rev	5'-CGGGGTACCTCAACATTACGCAAAGGCATAAGC-3'	Rev primer for *bca* promoter (KpnI)
gca-promoter-fw	5'-ACGCGTCGACCTCACCGGCACCCCAGCAAT-3'	Fw primer for *gca* promoter (SalI)
gca-promoter-rev	5' -CGGGGTACCGCCCCTGTAGTCAGCCGCGA-3'	Rev primer for *gca* promoter (KpnI)
asRNA-promoter-fw	5' -ACGCGTCGACCTAACCCACGTTCTTGCTAA-3'	Fw primer for promoter of asRNA (SalI)

asRNA-promoter-rev	5' -CGGGGTACCATCCTGTGGTGCAGTTGC-3'	Rev primer for promoter of asRNA (KpnI)
cm4	5' -GAAAATCTCGTCGAAGCTCG-3'	pET2-vector specific primer
cm 5	5' -AAGCTCGGCGGATTTGTC-3'	pET2 vector specific primer
cm6 (nested)	5' -ATTTGTCCTACTCAAGCTGG-3'	pET2 vector specific primer (designed primer)
pET2-rev	5' -CACACCATAGTGGCCATGAG-3'	pET2 vector specific primer
Oligo-(dT)	5' GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTT-3'	Primer for poly-(A) tail of cDNA
pJET-fw	5' -CGACTCACTATAGGGAGAGCGGC-3'	pJET vector specific fw primer
pJET-rev	5' -AAGAACATCGATTTTCCATGGCAG-3'	pJET vector specific rev primer
aceA-aceB intergenic-fw	5' -AGTCTGAGCAGACAACAGTTCCTG-3'	Fw primer for *aceA-aceB* inter-genic region
aceA-aceB intergenic-rev	5' -ACGTGGCTTTCCAACGTTTGACAT-3'	Rev primer for *aceA-aceB* inter-genic region
PF1-fw	5' -CTGTTTGAAAAGCTGTAAAT-3'	Fw primer for PF1 fragment of *bca* promoter
PF1-rev	5' -AAGCTTTATTATTCCACTCG-3'	Rev primer for PF1 fragment of *bca* promoter
Del-*gca*-upper-fw	5' -TGCACTGCAGTCAGGACATGGTTGATTATGT-3'	Fw primer for upper fragment of *gca* deletion (PstI)
Del-*gca*-upper-rev	5' -GTACCGTAATCGGCAATCCATGCGCTTTCATGGAT-3'	Rev primer for upper fragment of *gca* deletion
Del-*gca*-lower-fw	5' -CGATTACGGTACAGAAACATCAAAAGCCCAGGCTT-3'	Fw primer for lower fragment of *gca* deletion
Del-*gca*-lower-rev	5' -TGCACTGCAGAGCAGGGTTTCTGATCAGGGTATC-3'	Rev primer for lower fragment of *gca* deletion (PstI)
pK19*mobsac*B-fw	5' -TAATGCAGCTGGCACGAC-3'	pK19*mobsac*B vector-specific fw primer
pK19*mobsac*B-rev	5' -GTAGCTGACATTCATCCG-3'	pK19*mobsac*B vector-specific rev primer

2.2 Chemicals, devices, kits and enzymes

The chemicals, devices, kits and enzymes used in this study are listed in table 4, 5, 6 and 7:

Table 4: Chemicals used in this study

Chemical	Manufacturer
Acetyl Co-A	Genaxxon bioscience GmbH, Ulm, Germany
ROTIPHORESE® Gel 30 (37.5:1) [Acrylamide (30%) /Bisacrylamide (0.8%)]	Carl Roth GmbH, Karlsruhe, Germany
Agarose NEEO Ultra-Qualität	Carl Roth GmbH, Karlsruhe, Germany
Ammonium sulfate [$(NH_4)_2 SO_4$]	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Ammonium peroxydisulfate (APS)	Carl Roth GmbH, Karlsruhe, Germany
Ampicillin	Carl Roth GmbH, Karlsruhe, Germany
Bacto™ agar	Becto Dickinson GmbH, Heidelberg, Germany
Bacto™ trypton	Becto Dickinson and Company, Sparks, USA
Bacto™ yeast extract	Becto Dickinson and Company, Sparks, USA
Biotin	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Bovine Serum Albumin (BSA)	Thermo Scientific, Rockford, USA
Brain heart infusion (BHI)	Becto Dickenson and Company, Sparks, USA
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	Merck KGaA, Darmstadt, Germany
Chloramphenicol	Merck KGaA, Darmstadt, Germany
Chloroform/Isoamyl alcohol	Carl Roth GmbH, Karlsruhe, Germany
Coomassie® Blue G (CBB-G-250)	AppliChem GmbH, Darmstadt, Germany
D (+)- glucose monohydrate	Carl Roth GmbH, Karlsruhe, Germany
Di-potassium hydrogen phosphate (K_2HPO_4)	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4. 2H_2O$)	AppliChem GmbH, Darmstadt, Germany
Dimethyl Sulfoxide (DMSO)	Merck KGaA, Darmstadt, Germany

Dithiothreitol (DTT)	Carl Roth GmbH, Karlsruhe, Germany
Dithio-bis-(2-nitrobenzoic acid) DTNB	Boehringer Ingelheim Bioproducts, Heidelberg, Germany
Deoxynucleotide triphosphates (dNTPs)	Thermo Scientific, Darmstadt, Germany
Ethanol	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Ethidium bromide	Carl Roth GmbH, Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich Chemie GmbH, Steinheim, Germany
GeneRuler^{TM} 1 kb DNA Ladder	Thermo Scientific, Darmstadt, Germany
GeneRuler^{TM} 50 bp DNA Ladder	Thermo Scientific, Darmstadt, Germany
Glycerol	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Hydrochloric acid (HCl)	Merck KGaA, Darmstadt, Germany
Imidazole	Merck KGaA, Darmstadt, Germany
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Carl Roth GmbH, Karlsruhe, Germany
Isopropanol (2-propanol)	VWR prolabo® Chemicals, France
Kanamycin sulfate	Carl Roth GmbH, Karlsruhe, Germany
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	Merck KGaA, Darmstadt, Germany
Magnesium sulfate heptahydrate ($MgSO4.7H_2O$)	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Methanol	Sigma Aldrich Chemie GmbH, Steinheim, Germany
MOPS 3-(N-morpholino) propanesulfonic acid	Carl Roth GmbH, Karlsruhe, Germany
N, N, N, N-Tetramethylethylenediamine (TEMED)	Carl Roth GmbH, Karlsruhe, Germany
PageRuler^{TM} (unstained protein ladder)	Thermo Scientific, Darmstadt, Germany
Phenol	Carl Roth GmbH, Karlsruhe, Germany
Phenol/chloroform/isoamylalcohol	Carl Roth GmbH, Karlsruhe, Germany
Phosphoric acid (H_3PO_4)	Merck KGaA, Darmstadt, Germany

Poly [d (I-C)] (poly-deoxy-inosinic-deoxy-cytidylic acid)	Roche Diagnostics GmbH, Mannheim, Germany
Potassium acetate	Merck KGaA, Darmstadt, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Merck KGaA, Darmstadt, Germany
Potassium hydroxide (KOH)	Merck KGaA, Darmstadt, Germany
Restriction buffers (10x)	Thermo Scientific, Darmstadt, Germany
Reverse transcriptase reaction buffer (5x)	Thermo Scientific, Darmstadt, Germany
RiboLock RNase inhibitor	Thermo Scientific, Darmstadt, Germany
Sodium chloride (NaCl)	AppliChem GmbH, Darmstadt, Germany
Sodium di-hydrogen phosphate monohydrate (NaH_2PO_4. H_2O)	Merck KGaA, Darmstadt, Germany
Sodium dodecyl sulphate (SDS)	Carl Roth GmbH, Karlsruhe, Germany
Sorbitol	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Sucrose	Carl Roth GmbH, Karlsruhe, Germany
Tris (hydroxymethyl) aminomethane	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Triton X100	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Terminal deoxynucleotidyl transferase buffer (5x)	Thermo Scientific, Darmstadt, Germany
Urea	Carl Roth GmbH, Karlsruhe, Germany
β-Mercaptoethanol	Carl Roth GmbH, Karlsruhe, Germany

Table 5: Devices used in this study

Device	Manufacturer
ÄKTA™ purifier	Amersham Biosciences Europe GmbH, Freiburg
Amicon® Pro Ultra 15 purification system, 10 kDa	Merck KGaA, Darmstadt, Germany
Anthos hat III microplate-reader	Anthos Mikrosysteme GmbH, Krefeld, Germany
Biofuge pico Heraeus	Kindro Laboratory Products, Germany

Rotor F182464	Kindro Laboratory Products, Germany
Centrifuge 5804 R	Eppendorf AG, Hamburg, Germany
Rotor A-4-44	Eppendorf AG, Hamburg, Germany
Rotor F-45-30-11	Eppendorf AG, Hamburg, Germany
French® Pressure Cell Press	SLM instruments, USA
Gene pulser Xcell™	Bio-Rad Laboratories, USA
Glass beads (diameter 0.1 mm)	Sigma Aldrich Chemie GmbH, Steinheim, Germany
HisTrap™FF column (1 ml)	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
NanoDrop 2000 Spectrophotometer	Thermo Fisher Scientific, Waltham, USA
pH-Meter WTW pH521	Wissenschaftlich-Technische Werkstätten, Weilheim, Germany
Photo-documentation system	Decon Science Tec GmbH, Germany
PS-Microplate 96 well, flat bottom	Greiner Bio-One GmbH, Germany
RiboLyser FP 120	Thermo Hybaid GmbH, Heidelberg, Germany
Rock-N- Roller	Labinco BV, Netherlands
Thermo-cycler	Biometra GmbH, Göttingen, Germany
Ultracentrifuge L8-60M (Rotor TFT 65.13)	Beckman Coulter, USA
Ultrospec 2100 pro (Spectrophotometer)	Amersham Biosciences Biochrom Ltd, England
Water bath	Köttermann GmbH & Co KG, Germany

Table 6: Kits used in this study

Kits	Manufacturer
CloneJet™ PCR Cloning Kit	Thermo Fisher Scientific GmbH, Ulm, Germany
E.Z.N.A. Plasmid DNA Mini Kit	Omega Bio-tek, Inc., Norcross, USA
NucleoSpin® Gel and PCR Clean-up Kit	Macherey-Nagel GmbH & Co. KG, Düren, Germany

Pierce™ BCA protein Assay kit	Thermo Fisher Scientific GmbH, Ulm, Germany
Qiagen RNeasy Mini Kit	Qiagen GmbH, Hilden, Germany
HotStar HiFidelity Polymerase Kit	Qiagen GmbH, Hilden, Germany

Table 7: Enzymes used in this study

Enzymes	Manufacturer
DNase I	Thermo Scientific, Darmstadt, Germany
Fast AP™ thermosensitive alkaline phosphatase	Thermo Scientific, Darmstadt, Germany
Maxima reverse transcriptase	Thermo Scientific, Darmstadt, Germany
Phusion® polymerase	New England Biolabs, Ipswich, MA, USA
Taq DNA polymerase	Genaxxon bioscience GmbH. Ulm, Germany
Restrictions endonucleases	Thermo Scientific, Darmstadt, Germany
T4 DNA ligase	Thermo Scientific, Darmstadt, Germany
Terminal deoxynucleotidyl transferase (TdT)	Thermo Scientific, Darmstadt, Germany

2.3 Growth media and culture conditions

2.3.1 Growth media and additives

The nutrient media (described below) were prepared for the cultivation of *E. coli* and *C. glutamicum*. For preparation of agar plates, agar (18 g/l) was added before autoclaving of the medium. The additives such as micronutrients, biotin and antibiotics were added from stock solutions (see below) to the autoclaved medium. The antibiotics such as kanamycin (50 µg/ml) or ampicillin (100 µg/ml) was used for the cultures carrying plasmid/construct with respective antibiotic resistant gene as selectable marker. Furthermore, the sucrose (10%) containing complex medium plates were prepared by mixing the separately autoclaved sucrose (100 g/l) and 2xTY medium containing agarose (18 g/ l), before pouring the medium into the plates.

2X TY Complex media (Green and Sambrook, 2012)

Tryptone	16 g/l
Yeast extract	10 g/l
NaCl	5 g/l

BHIS complex medium (Liebl et al., 1989)

Brain heart infusion powder	37 g/l
Sorbitol	91g/l

The brain-heart-infusion medium was autoclaved and then combined with sorbitol solution after autoclaving.

TB complex medium (Tartof and Hobbs, 1987)

Trypton	12 g/l
Yeast extract	24 g/l
Glycerol	4 %

CgC minimal medium (modified after Eikmanns et al., 1991)

$(NH_4)_2SO_4$	5 g/l
Urea	5 g/l

MOPS	21 g/l
KH_2PO_4	1g/l
K_2HPO_4	1g/l
$MgSO_4$ x 7 H_2O	0.25g/l
$CaCl_2$ x $2H_2O$	0.01 g/l

Both the $MgSO_4$ x 7 H_2O and $CaCl_2$ x $2H_2O$ were prepared in stock solutions (1000x) each and calculated amounts (as shown above) were added to the medium. The pH of the medium was adjusted to 6.8 or 6.3 with the addition of KOH. After autoclaving, the following additives were added (1 ml/l) to the minimal medium.

Micronutrient solution	(1000 x)
Biotin	(200 mg /ml)

The glucose and/or acetate as sole or combined carbon source in concentrations of 1% (w/v) to 4% (w/v) was added to the autoclaved minimal medium.

Micronutrient solution (1000 x)

$FeSO_4$ x $7H_2O$	16.4 g/l
$MnSO_4$ x H_2O	10.0 g/l
$CuSO_4$ x $5H_2O$	0.2 g/l
$ZnSO_4$ x $7H_2O$	1.0 g/l
$NiCl_2$ x 6 H_2O	0.02 g/l

The salts were solved in water by the addition of the HCl (32%). The solution was sterile filtered and stored under N_2 atmosphere in screw cap bottle.

2.3.2 Cultivation conditions

The bacterial cultures stored as glycerol 30% (v/v) stock in screw cap tubes at -80 °C, were streaked out on complex medium (2xTY) agar plates. The plates for C. *glutamicum* were incubated at 30 °C for 48 h and plates for *E. coli* at 37 °C for 24 h. The single colonies appeared on the plates were then used for inoculation of the pre-culture.

Cultivation conditions for *Escherichia coli*

E. coli was cultured in 5 ml of 2xTY medium in tube (15 ml) or in 50 ml of 2xTY medium in 500 ml Erlenmeyer flask and then incubated at 37 °C on a rotary shaker at 120 rpm, until desired optical density was obtained.

Cultivation conditions for *Corynebacterium glutamicum*

For culturing of *C. glutamicum*, 5 ml of 2xTY medium in a tube (15 ml) was inoculated and incubated at 30 °C for 8 h on a rotary shaker at 120 rpm. Then 45 ml of the 2xTY medium was inoculated with 5 ml culture and incubated overnight under the same conditions. This pre-culture was then centrifuged at 4500 rpm for 10 minutes at 4°C, the pellet was washed twice with 5 ml of normal saline (0.9%) and then used to inoculate 50 ml of the CgC minimal medium containing glucose 1% (w/v) and/or acetate 1% (w/v) as source of carbon, to an OD_{600} of about 1 or 1.5 in 500 ml Erlenmeyer flask. The culture was then incubated at 30 °C on a rotary shaker at 120 rpm until desired OD was obtained.

2.4 Isolation and purification of DNA

2.4.1 Isolation of plasmid DNA from *E. coli*

E. coli was cultured in 5 ml of 2xTY medium (15 ml tube) and incubated at 37 °C on rotary shaker at 120 rpm, overnight. The plasmid DNA was isolated using the E.Z.N.A. Plasmid Mini Kit (Omega), according to the manufacturer's instructions. The plasmid (or also PCR amplified DNA) was then purified with NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel), according to the manufacturer's instructions.

2.4.2 Isolation and purification of genomic DNA from *C. glutamicum*

For isolation of chromosomal DNA, *C. glutamicum* was cultured in 5 ml of 2xTY medium for 8 hours, then inoculated into 50 ml of 2xTY medium in 500 ml Erlenmeyer flask and incubated at 30 °C on a rotary shaker at 120 rpm, overnight. The culture was centrifuged at 4500 rpm at 4 °C for 10 minutes and the pellet was dissolved in 2 ml of TE buffer. The re-suspended pellet (1 ml) was then filled in screw-cap tubes containing 250 µl of glass-beads (diameter 0.1 mm) (Sigma Aldrich) and cells were disrupted in the RiboLyser FP120 four times at speed of 6.5 for 45 seconds with cooling on ice for 5 minutes each time. The tubes were then centrifuged at 14000 rpm for 20 minutes at 4 °C and supernatant was collected and transferred to new 1.5 ml tubes.

TE buffer

Tris	10 mM
EDTA	1 mM

The pH of solution was adjusted to 7.6 with HCl

Purification of DNA (modified after Green and Sambrook, 2012)

The supernatant was mixed with equal volume of phenol/chloroform/isoamylalcohol [25:24:1 (v/v/v)] by inverting the tube and then centrifuged at 14000 rpm for 5 minutes at room temperature to collect the upper phase while the lower phase and interphase containing denatured proteins was discarded. The upper phase was taken and this process was repeated several times

until no protein interphase was visible. The upper phase was then taken and mixed with equal volume of chloroform/isoamylalcohol [24:1 (v/v)] for removal of phenol and then centrifuged at 14000 rpm for 5 minutes at room temperature. The supernatant was then transferred to a new 1.5 ml tube, mixed with equal volume of isopropanol and the precipitated chromosomal DNA was centrifuged at 14000 rpm for 10 minutes at room temperature. The pellet was washed with 70% ethanol and then dried and dissolved in 100 µl of water. The isolated genomic DNA was stored at -20 °C untill further use.

2.5 PCR techniques and gene cloning

2.5.1 PCR techniques

The DNA was amplified with PCR method described by Saiki et al., 1988, using the Thermo-cycler (Biometra). The primers were designed using the Clone Manager v.9 software and obtained from biomers.net (Ulm, Germany). The reagents of PCR including buffers, dNTPs and polymerase enzyme were obtained from Thermo Scientific. The composition of standard PCR reaction mix of 50 µl was as follow:

Standard PCR reaction

Phusion® GC reaction buffer	10 µl (5x)
dNTPs mix	5 µl (2 mM)
DMSO	1.5 µl (100%)
Primers forward and reverse (each)	2 µl (10 pmole/ µl)
Chromosomal or plasmid DNA as template	2 µl (10-100 ng/ µl)
Phusion® polymerase	1 U

Water was added to make the final volume up to 50 µl

Standard PCR program:

Initial denaturation	98 °C for 10 minutes
Denaturation	98 °C for 30 seconds
Annealing	55 °C for 45 seconds
Extension	72 °C for 1 minutes and 20 seconds
Final extension	72 °C for 10 minutes
Hold	4 °C

Colony PCR

Colony PCR (Woodman, 2008) was performed for verification of plasmid/construct in *E. coli* or *C. glutamicum* or for confirmation of gene deletions in *C. glutamicum*, using designed primers. The DNA was obtained by dissolving a single colony in 50 µl of water and heating at 95 °C for 10 minutes followed by centrifugation at 14000 rpm for 10 minutes at room temperature and supernatant collection. Due to insensitivity to impurities, the Taq DNA polymerase was used for amplification in the PCR. The composition for PCR reaction mixture for 25 µl was as follow:

Colony PCR reaction

Buffer S (with 15 mM $MgCl_2$)	2.5 µl (10x)
dNTPs mix	2.5 µl (2 mM)
DMSO	1 µl (100%)
Primers forward and reverse (each)	1 µl (10 picomole/ µl)
Template (supernatant)	16 µl
Taq DNA polymerase	1 U

Colony PCR program:

Initial denaturation	94 ℃ for 2 minutes
Denaturation	92 ℃ for 30 seconds
Annealing	50 ℃ for 30 seconds
Extension	72 ℃ for 2 minutes
Final extension	72 ℃ for 10 minutes
Hold	4 ℃

2.5.2 Gene cloning and sequence analysis

For cloning, both the DNA (insert) and vector were cut with appropriate restriction enzymes in buffers. The vector was additionally treated with FastAP^{TM} (thermosensitive alkaline phosphatase) enzyme to prevent re-ligation. Both the restricted DNA (insert) and vector were purified from the gel with NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). The vector and DNA (insert) were then ligated using T4 DNA ligase with buffer under recommended conditions. The enzymes and buffers used were obtained from Thermo Scientific and used according to the manufacturer's instructions. The ligated construct was then transformed into *E. coli* DH5α cells using a Gene pulser Xcell^{TM} (Bio-Rad). The positive clones of the *E. coli* DH5α carrying the construct with antibiotic resistant gene as selectable marker, were selected by growth on complex (2xTY) agar medium plate containing the respective antibiotic followed by colony PCR for verification of the construct/insert in the cells. The respective construct was then isolated from *E. coli* DH5α by the E.Z.N.A. Plasmid Mini Kit (Omega) and sequenced by GATC Biotech AG (Germany). The sequence was analyzed using NCBI database and Clone Manager v.9 software. The construct was then subsequently transformed into *E. coli* BL21 or *C. glutamicum* by electroporation.

2.6 Transformation of *E. coli* and *C. glutamicum* cells

2.6.1 Transformation of *E. coli* cells (modified after Dower et al., 1988)

For preparation of the electro-competent cells of *E.coli* DH5α, preculture was prepared by inoculating 5 ml of 2xTY medium in a tube (15 ml) and incubating at 37 °C on a rotary shaker at 120 rpm overnight. Then, 250 ml of the 2xTY medium was inoculated with 500 µl of the pre-culture, and incubated at 37 °C on a rotary shaker at 120 rpm, untill OD_{600} of 0.3 to 0.5 was obtained and cells were then harvested in 50 ml falcon tubes by centrifugation at 4500 rpm for 10 minutes at 4 °C. The pellet was then washed twice with 20 ml ice-cold water and then two times with 20 ml of ice-cold 10% (v/v) glycerol. The pellet was finally dissolved in 0.5 ml of 10% (v/v) glycerol and stored as 50 µl aliquots in 1.5 ml tubes at -80 °C, until further use. For electro-transformation, 2-6 µl of ligation reaction was mixed with 50 µl of the electro-competent cells and electroporation was performed in cuvettes (0.2 cm electrode distance) using Gene pulser Xcell™ with parameters (voltage of 2.5 kV, capacitance of 25 µF and resistance of 200 Ω). The cells were then mixed with 200 µl of 2xTY medium pre-heated at 37 °C in 1.5 ml tube and then incubated under shaking at 37 °C for one hour. The cells were then plated out on selective 2xTY agar plates containing 50 µg/ml of kanamycin or 100 µg/ml Ampicillin and incubated at 37 °C for 24 hours.

2.6.2 Transformation of *C. glutamicum* cells (modified after van der Rest et al., 1999)

Electro-competent *C. glutamicum* cells were prepared by inoculating 250 ml of BHIS medium in a 1L baffled Erlenmeyer flask with 5 ml of the overnight BHIS-culture, and incubated at temperature of 30 °C on a rotary shaker at 120 rpm. The culture was harvested at an OD_{600} of about 1.75, by centrifugation at 4500 rpm for 10 minutes at 4 °C. The cell pellet was washed twice with 20 ml of TG-buffer [(Tris 1 mM) (Glycerol 10% v/v) pH 7.5 adjusted with HCl] and then two times with 20 ml of 10% (v/v) glycerol, under the same conditions. Finally, the cell pellet was dissolved in 0.5 ml of 10% (v/v) glycerol and 150 µl aliquots in 1.5 ml tubes were stored at -80 °C. The electroporation was performed by mixing 2-6 µl of plasmid/construct with 150 µl of electro-competent cells in electroporation cuvette (electrode distance of 0.2 cm) in the Gene pulser Xcell™ at parameters set to voltage of 2.5 kV, capacitance of 25 µF and resistance

of 200 Ω. After electroporation, the cells were transferred to 4 ml of pre-heated BHIS medium in tube (20 ml) and incubated at 46 °C for 6 minutes, in water bath. The tubes were incubated at 30 °C for one hour on shaker at 120 rpm, then centrifuged and plated on selective 2xTY agar plates containing 15µg/ml or 50 µg/ml of kanamycin. The plates were then incubated at 30°C for the next 48 hours.

2.7 Over-production and purification of proteins in *E. coli* BL21

The His-tagged Bca, Gca and RamA proteins were over-produced in *E. coli* BL21 containing pET-TEV1-*bca*, pET-TEV1-*gca* and pET28a-*ramA* construct, respectively. For selection of the suitable colony for over-expression of respective protein, the freshly transformed colonies of *E. coli* BL21 carrying respective over-expression construct were streaked on plates containing kanamycin (50 µg/ml), plates containing kanamycin plus IPTG (1 mM) and plates containing IPTG (1 mM) only, simultaneously. The colonies showing normal growth on plates containing kanamycin but reduced growth on plates containing kanamycin plus IPTG and plates containing IPTG only were selected for further over-production. The selected colonies were then pre-cultured in 50 ml of TB medium in 500 ml Erlenmeyer flasks at 37 °C for 8 hours on a rotary shaker at 120 rpm. Then, 500 ml of TB medium in 2 L Erlenmeyer flask was inoculated with the pre-culture to an OD_{600} of 0.2 and incubated under the same conditions. The culture was grown to an OD_{600} of about 2 and then the over-production of respective protein was induced by addition of IPTG (1 mM) to the culture. The culture was grown further for up to 2 hours (OD_{600} of about 5) and then harvested by centrifugation , washed with respective loading buffer (see below) one time and stored at -20 °C, until further use

For purification of the His-tagged over-produced protein, the cell pellet was dissolved in respective loading buffer (as described below) and disrupted three times with a French press cell followed by centrifugation at 14000 rpm for 30 minutes at 4 °C and then ultracentrifugation of the supernatant at 45,000 rpm for 1 hour at 4 °C. The over-produced His-tagged Bca and Gca proteins were purified with a HisTrap^{TM} HP column using loading buffer (20 mM Na_2HPO4, 20 mM Imidazole, 0.5 M NaCl) and elution buffer (20 mM Na_2HPO_4, 0.5 M Imidazole, 0.5 M NaCl) while the His-tagged RamA protein was purified using loading buffer (NNIG-20: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, 5% glycerol (v/v), pH 8) and elution buffer (NNIG-

500: 50 mM NaH_2PO_4, 300 mM NaCl, 500 mM Imidazole, 5% glycerol (v/v), pH 7.8), in an ÄKTA purifier.

For subsequent EMSA experiments, desalting of the purified His-tagged RamA protein was performed where imidazole containing buffer was exchanged with TG buffer. The Amicon® Pro Ultra column was equilibrated with water, centrifuged at 4200 rpm at 4 °C for 10 minutes and the flow-through was discarded. Then 500 µl of the sample was added to the column, centrifuged at the same conditions for 30 minutes and about 500 µl was retained as supernatant on the column while the flow-through was discarded. Then, 15 ml of TG buffer was added to the supernatant, centrifuged at same conditions for 40 minutes and flow-through discarded. This step was repeated the second time and then the supernatant left in the column was collected. The concentration of protein was determined using Pierce BCA kit (Thermo Scientific) (see below).

2.8 SDS- PAGE analysis

The denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was used to separate purified His-tagged Bca, Gca, RamA protein or proteins enriched with DNA affinity chromatography. The SDS-PAGE was prepared with the following composition:

Resolving Gel (12%)

ROTIPHORESE® Gel 30	8 ml [Acrylamide (30%) /Bisacrylamide (0.8%)]
Tris-HCl (1.5 M, pH 8.8)	5 ml
SDS	200 µl (10%)
APS	200 µl (10%)
Water	6.6 ml
TEMED	8 µl (added at the end)

Stacking Gel

ROTIPHORESE® Gel 30	1 ml [Acrylamide (30%) /Bisacrylamide (0.8%)]
1.5 M Tris-HCl (pH 8.8)	750 µl
SDS	60 µl (10%)
APS	60 µl (10%)
Water	4.1 ml
TEMED	6 µl (added at the end)

SDS running buffer (10x) (pH 8.5)

Tris	250 mM
Glycine	2.5 M
SDS	1 %

The sample was prepared by mixing 15 µl of protein with 5 µl of the SDS loading buffer (2x) and heated at 95 °C for 5 minutes. The sample along with 5 µl of unstained pageRuler (Thermo Scientific) was loaded on the gel and run with 16 mA and 300 volts. The gel was then stained with colloidal coomassie overnight.

Colloidal coomassie staining solution

Coomassie Blue G (CBB-G250)	0.08 %
H_3PO_4	1.6 %
$(NH_4)_2 SO_4$	8 %
Ethanol	30 %

After staining, the gel was washed several times with water and the protein bands were visualized.

2.9 Cloning of promoter regions in the promoter-probe vector pET2

The promoter region of the *bca* and *gca* gene and that of the asRNA were amplified with primers *bca*-promoter-fw/*bca*-promoter-rev, *gca*-promoter-fw/*gca*-promoter-rev and asRNA-promoter-fw/asRNA-promoter-rev respectively. The respective amplified product was cloned into the multiple cloning site, upstream of promoter-less *cat* reporter gene in the pET2 promoter-probe vector and transformed creating pET2-*bca*-promoter, pET2-*gca*-promoter and pET-2-asRNA-promoter construct in *E. coli* DH5α cells separately. The transformed *E. coli* DH5α cells were capable to grow on plates containing kanamycin (50 µg/ml) due to the Km^r gene as selectable marker of the pET2 vector, and were also verified by colony PCR followed by isolation of construct and sequence verification using pET2 vector-specific primers namely cm4 and pET-rev. The pET2-*bca*-promoter, pET2-*gca*-promoter and pET2-asRNA-promoter construct were then transformed in *C. glutamicum* separately by electroporation.

2.10 Determination of TSPs of genes in *C. glutamicum*

2.10.1 RNA isolation

Total RNA was isolated from *C. glutamicum* carrying pET2-promoter plasmid (as performed by Auchter et al., 2011b) such as *C. glutamicum* carrying pET2-*bca* or pET2-*gca* promoter construct. The *C. glutamicum* carrying the respective construct was grown in minimal medium containing glucose 1% (w/v) as carbon source, untill mid-logarithmic phase of growth (OD_{600} of about 5), harvested by centrifugation at 4500 rpm for 10 minutes at 4 °C and stored at -80 °C until further use.

For isolation of total RNA, the phenol-chloroform extraction method was used (modified after Green and Sambrook, 2012). The cell pellet was re-suspended in 1 ml of the RLT buffer (Qiagen) (with β mercaptoethanol added) and distributed into two screw-cap tubes each containing 250 µl of glass-beads (diameter 0.1 mm) (Sigma Aldrich) and then 0.5 ml of acidic phenol was added to

each tube. The cells were disrupted three times in a RiboLyser FP 120 at speed of 6.5 for 45 seconds, with cooling for 5 minutes each time. The tubes were centrifuged at 14000 rpm for 10 minutes at 4 °C and supernatant was collected. The supernatant was then mixed with 1.5 ml of acidic phenol plus 1.5 ml of chloroform-isoamylalcohol (24:1) and 2.5 ml of acetate-EDTA (AE buffer, sodium acetate 40 mM, EDTA 1 mM) buffer. The tubes were shaken and put at 60 °C for 5 minutes. The tubes were then centrifuged at 4500 rpm for 5 minutes at 4 °C to separate phases, the supernatant was then taken into new tube and this process was repeated up to 4 times. The supernatant was then mixed with same volume of chloroform-isoamylalcohol (24:1) and centrifuged (4500 rpm, 4 °C and 5 minutes). Afterwards, 0.1 volume sodium acetate (3 M) and 2.5 volume ethanol (90 %) were added to the supernatant and precipitation took place at -20 °C, overnight. The precipitated RNA was then centrifuged at 4500 rpm at 4 °C for 30 minutes and washed with 70% ice-cold ethanol. After drying, the pellet was re-suspended in 400 µl of water.

To remove residual DNA from the RNA sample, the RNA preparation was then treated with DNase I (Thermo Scientific) enzyme, according to the manufacturer's instructions. The 40 µl reaction containing 10 µl of the RNA, 4 µl of DNase buffer, 2 µl of DNase I, 1 µl of RNase inhibitor and 23 µl of RNase free water, was incubated at 37 °C for 20 minutes and then inactivated at 70 °C for 10 minutes. The DNA free RNA was then purified using the RNeasy mini kit (Qiagen), according to the manufacturer's instructions.

2.10.2 cDNA synthesis and 5'-RACE

cDNA synthesis

The cDNA synthesis and 5'-RACE (described in Frohman et al., 1988) was performed for determination of TSPs of the *bca* and *gca* genes. The cDNAs for the *bca* and *gca* genes were created from total RNA isolated from *C. glutamicum* containing pET2-*bca*-promoter and pET2-*gca*-promoter respectively (discussed above) by reverse transcription with pET2 vector-specific primer cm5, using maxima reverse transcriptase (Thermo Scientific). The reaction was prepared in 1.5 ml tubes as follow:

Template RNA	1 pg – 5 ng
Primer (cm5)	15 – 20 pmole
dNTPs	0.5 mM
Water	up to 14.5 µl

The tubes were mixed gently then briefly centrifuged and incubated at 65 °C for 5 minutes and afterwards cooled on ice. Then the final volume was made up to 20 µl by addition of the following components:

Reaction buffer	4 µl (5x)
RiboLock RNase inhibitor	0.5 µl (40 U/ µl)
Maxima reverse transcriptase	1 µl (200 U/ µl)

The tubes were mixed gently then centrifuged briefly and incubated at 60 °C for 30 minutes. The reaction was terminated by heating at 85 °C for 5 minutes.

Poly-(A) tailing of the cDNA

The cDNA was subsequently tailed with poly-(A) at its 3'-end with dATP, using terminal deoxynucleotidyl transferase (Thermo Scientific). The reaction was prepared as follow:

Reaction buffer	4 µl (5x)
cDNA	5-10 µl
dATP	1 µl (100 mM/ml)
Terminal deoxynucleotidyl transferase	1.5 µl (20 U/µl)
Water	added up to total volume of 20 µl

The reaction mixture was incubated at 37 °C for 15 minutes. Afterwards, the reaction was terminated by heating at 70 °C for 10 minutes.

The 5'-RACE PCR

The poly-(A) tailed cDNA was amplified with primers Oligo-(dT) and cm5 using HotStar polymerase kit (Qiagen) in PCR. To get the amplified product, PCR was also repeated the second time, using 5 µl of the first PCR product as template. The PCR reaction composition was as follow:

Reaction buffer	10 µl (5x)
Q solution	10 µl (5x)
Primer oligo-(dT)	5 µl (10 pmole/ µl)
Primer cm5	5 µl (10 pmole/ µl)
cDNA (template)	5 µl
HotStar polymerase	1 µl (2.5 u/µl)
Water	added to final volume of 50 µl

The amplified PCR product was purified with NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) and cloned into pJET1.2/blunt vector of CloneJetTM PCR Cloning Kit with blunt end ligation according to the manufacturer's instructions and then sequenced. The sequence analysis was performed using the NCBI database and Clone Manager v.9 software.

2.11 Determination of specific CAT enzyme activity

The *C. glutamicum* containing pET2-*bca*-promoter or pET2-*gca*-promoter was grown in minimal medium containing glucose 1% (w/v) or acetate 1% (w/v) as carbon source, to OD_{600} of about 5 (mid-exponential growth phase) and then harvested by centrifugation at 4500 rpm for 10 minutes

at 4 °C. The cell pellet was washed one time with washing buffer [(200 mM) Tris/ HCl pH 7.8] and stored at -20 °C until further use.

For determination of the specific CAT enzyme activity, the cell pellet was dissolved in 1 ml of washing buffer [(200 mM) Tris/ HCl pH 7.8] and then 1 ml added to screw cap tube containing 250 µl of glass-beads (diameter 0.1 mm) (Sigma Aldrich). The cells were disrupted in a RiboLyser FP 120 at speed of 6.5 for 30 seconds three times with cooling on ice for 5 minutes each time. The samples were centrifuged at 14000 rpm for 30 minutes at 4 °C to sediment the cell debris and glass-beads and the supernatants were then transferred to new 1.5 ml tubes and used for enzyme assays. Appropriate dilutions (1:5, 1:10, 1:100 and 1:200) were prepared for the samples and enzyme activity was determined using spectrophotometer (Ultrospec 2100 Pro, Amersham) whereas the total protein in the samples was estimated using Pierce BCA Protein Assay kit (Thermo Scientific), according to manufacturer's instructions.

The method of Shaw (1975) with some modifications was used for determination of CAT enzyme activity in the samples. The reaction is shown below:

Chloramphenicol + 2 acetyl-CoA → Chloramphenicol-1, 3-diacetate + 2 CoA

CoA + DTNB → dithionitro benzyl-CoA + 5-thio-2-nitro benzoate

The increasing 5-thio-2-nitro benzoate was measured at 412 nm at temperature of 37 °C, using Ultrospec 2100 Pro spectrophotometer. The reaction mixture composition was as follow:

DTNB	500 µl (0.8 g/l; 200 mM Tris/ HCl pH 7.8)
Water	325 µl
Cell extract	100 µl
Acetyl CoA	25 µl (20 mM)
Chloramphenicol	50 µl (5 mM)

Protein quantification

The protein concentration was quantified for 1:10 and 1:20 dilutions of cell extract sample with duplicate measurement for each dilution using the Pierce BCA protein Assay kit (Thermo Scientific) in PS-Microplate 96 well, according to the manufacturer's instructions. The absorbance was measured at 562 nm using Anthos Htlll plate reader and protein concentration was calculated using the BSA standard curve.

Calculation of specific CAT enzyme activity

One unit (U) of CAT enzyme is the amount required to acetylate 1 µmol of chloramphenicol per minute. The specific CAT enzyme activity was calculated as "U/ mg of protein" with respect to estimated total protein in the samples, using the following formula:

$$\text{Specific activity (U/ mg)} = \frac{\Delta E / \text{min} * \text{total volume}}{2 * \varepsilon * d * \text{sample volume} * \text{protein concentration}}$$

ΔE / min = extinction change per minute

Ɛ = extinction co-efficient of 5-thio-2-nitro benzoate at 412 nm = 13.6 $mM^{-1} * cm^{-1}$

Light path (d) = 1 cm

Sample volume = 0.1 ml

2.12 DNA affinity chromatography

DNA affinity chromatography was performed to enrich proteins that bind to the promoter region of the *bca* gene. The buffers used for DNA affinity chromatography were as follow:

Protein Binding buffer (pH 7.5 – 7.8)

Tris	20 mM
EDTA	1 mM
Glycerol	10% (v/v)
Triton X100	0.01%
NaCl	100 mM
DTT	1 mM (freshly added)

2 x DNA binding buffer (pH 7.6)

Tris	10 mM
EDTA	1 mM
NaCl	2 M

Elution buffer (pH 7.8)

Tris	20 mM
EDTA	1 mM
Glycerol	10% (v/v)
Triton X100	0.01% (v/v)
NaCl	2 M
DTT	1 mM (freshly added)

The biotin-labelled probe (promoter region of the *bca* gene, as discussed above) was amplified, using primers *bca*-promoter-fw and biotin-labelled *bca*-promoter-rev and the amplified product was purified with NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel), according to the manufacturer's instructions. The amplified product was subsequently quantified using NanoDrop 2000 spectrophotometer and concentration was adjusted to 70 picomole in total of 200 µl volume.

For preparation of cell extract, *C. glutamicum* was cultured in 250 ml of minimal medium (containing 2% glucose) in a 1L Erlenmeyer flask at 30 °C on a rotary shaker at 120 rpm until an OD_{600} of about 5 was obtained. The cells were then harvested and washed once with 20 ml of protein binding buffer and stored at -20 °C until further use. For cell disruption, 1g of pellet was dissolved in 1 to 2 ml of protein binding buffer and then disruption was carried out four times in screw-cap tubes each containing 250 µl of glass-beads (diameter 0.1 mm) (Sigma Aldrich) and 1 ml of dissolved cell pellet in the RiboLyser FP 120 at speed of 6.5 for 30 seconds with cooling on ice for 5 minutes each time. Then samples were centrifuged at 14000 rpm for 1 hour and the supernatant collected was then ultra-centrifuged at 45,000 rpm for 1 hour at 4 °C.

For affinity chromatography, 300 µl of dynabeads M-280 Streptavidin were taken into 2 ml tube, collected with magnet and washed twice with 500 µl of DNA binding buffer. The beads were then mixed with 200 µl of probe (biotin-labelled *bca* promoter) and 200 µl of DNA binding buffer. The tube was then incubated on a Rock-N- Roller for 1 hour at room temperature followed by collection of beads by magnet and washing three times with 300 µl of DNA binding buffer. The beads were then suspended with 300 µl of protein binding buffer and incubated for 5 minutes and then together with wild-type DNA in double concentration to that of probe, added to cell extract in 10 ml Falcon tube and incubated on a Rock-N-Roller for 1 hour at room temperature. The beads were then collected in fresh 2 ml tube in parts (500 µl each) using magnet, and the collected beads were then washed four times with 500 µl of protein binding buffer. The elution of bound protein was performed by mixing the beads two times with 350 µl of elution buffer, each time collecting the beads with magent and the elution collected in fresh tube.

The beads were then washed three times with 300 µl of elution buffer, then mixed with 300 µl of 1:2 dilution of DNA-binding buffer and stored at 4 °C. The 700 µl (2x 350 µl) elution was mixed with 700 µl of 30% TCA (Trichloroacetic acid) (end conc. 10-15%), incubated at 4 °C overnight and then centrifuged at 14000 rpm for 30 minutes at 4 °C. The pellet was washed one time with

500 µl of acetone followed by centrifugation at 14000 rpm for 30 minutes and then dried at 37 °C for 1-2 hours. The pellet was then suspended in 15 µl of 50 mM Tris (pH 7.5) and 1 µl of 1 M NaOH and then stored at 4 °C until further use.

2.13 Electrophoretic mobilitiy shift assays (EMSAs)

The binding of purified His-tagged RamA protein with the *bca* promoter region was tested using the electrophoretic mobility shift assay (EMSA) method. The *bca* promoter fragment, a sub-fragment of the *bca* promoter region (PF1) devoid of the RamA binding sites used as negative control for the RamA protein and the *aceA-aceB* inter-genic fragment with known binding affinity for RamA used as positive control for the RamA protein, were amplified with primers *bca*-promoter-fw/*bca*-promoter-rev, PF1-fw/PF1-rev and *aceA-aceB* intergenic-fw/*aceA-aceB* intergenic-rev respectively in the PCR and the amplified products were purified by NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). The *bca* promoter fragment (70 ng) was incubated with varying concentrations (0 to 2 µg) of His-tagged RamA and with 2 µg of BSA as negative protein control, in separate reactions while the *aceA-aceB* fragment (70 ng) with 0 and 1.2 µg of RamA and PF1 fragment (70 ng) with 0 and 2µg of His-tagged RamA protein. The composition of 20 µl of reaction mixture was as follow:

EMSA reaction composition

Tris	2 µl (100 mM)
DTE	2 µl (10 mM)
EDTA	2 µl (10 mM)
Glycerol	4 µl (50%)
Probe (DNA)	2 µl (about 70 ng)
Poly [d (I-C)]	1µg
Protein	0 – 1.2 µg
Water	added up to final volume of 20 µl

The reaction was incubated for 20 minutes at room temperature and then run on agarose gel (2%) at 70 volts, stained with ethidium bromide and the retardation was observed.

2.14 MALDI-TOF mass spectrometry

The identification of protein was performed by MALDI-TOF (Matrix Assisted Laser Desorption/ Ionization - Time Of Flight) mass spectrometry (described in Gerstmeir et al., 2004) and peptide mass fingerprinting. For identification of proteins that were enriched with DNA affinity chromatography as well as over-produced and purified His-tagged RamA, the samples were separated on an SDS-PAGE gel (as described above), the protein bands of interest were cut (approximately 5 by 1.5 by 1 mm in size) out of the gel with a scalpel and collected in 1.5 ml tubes and stored at -20 °C. The MALDI-TOF analysis was performed at Forschungszentrum Jülich (Germany) and the data (m/z values) obtained was then analyzed for identification of proteins using Mascot (PMF) Peptide mass fingerprint (http:/www.matrixscience.com).

2.15 Chromosomal deletion of the *gca* gene in *C. glutamicum*

Creation and cloning of Δ*gca* insert in pK19*mobsac*B vector in *E. coli* DH5α

The deletion of *gca* gene was carried out with allelic exchange by homologous recombination using suicide vector pK19*mobsac*B (Schäfer et al., 1994). To create a deletion version of the *gca* gene, the upper region (423 bp) of the *gca* gene using primer pair Del-*gca*-upper-fw (PstI restriction site) / Del-*gca*-upper-rev and lower region (423 bp) using primer pair Del-*gca*-lower-fw/ Del-*gca*-lower-rev (with PstI restriction site) were amplified in standard PCR. The amplified products were then purified using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). As a short complementary tail (about 13 nucleotides) was attached to the 5´-end of both the Del-*gca*-upper-rev and Del-*gca*-lower-fw primers (reverse primer of the fragment containing 5´ end and forward primer of fragment containing 3´end of the *gca* gene respectively), therefore the two amplified fragments (upper and lower region) were used as template and combined together as a cross-over product (about 834 bp) in a cross-over PCR (Ho et al., 1989) using primer pair Del-*gca*-upper-fw/ Del-*gca*-lower-rev. This resulted in creation of a truncated version of the *gca*,

deleting the internal region (about 422 bp) of the gene. Then both the amplified cross-over product and the pK19*mobsac*B vector were digested with PstI restriction enzyme, the insert (cross-over product) was cloned in the pK19*mobsac*B vector and transformed in *E. coli* DH5α cells. The success of transformation was confirmed by colony PCR as well as isolation of construct pK19*mobsac*B-Δ*gca* and verification of insert sequence using pK19*mobsac*B vector-specific primers pK19*mobsac*B-fw and pK19*mobsac*B-rev. The construct pK19*mobsac*B- Δ*gca* was then transformed in *C. glutamicum* by electroporation.

Replacement of the *gca* gene with Δ*gca* in the genome of *C. glutamicum*

To create a Δ*gca* mutant of *C. glutamicum*, the colonies of *C. glutamicum* containing pK19*mobsac*B-Δ*gca* construct were grown in 5 ml of 2xTY medium (15 ml tube) for 6 hours. Different dilutions (1:50, 1:100 and 1:1000) of the culture were prepared and 100 µl of each dilution was plated out on plates containing 10% sucrose. The plates were then incubated at 30 °C overnight. The colonies growing on sucrose-containing plates were selected because of integration of the insert and excision of the vector from the cells. Each of the selected colonies was further grown on 2xTY plates and 2xTY plates containing kanamycin (50 µg/ ml) (as master plates) simultaneously and incubated at 30 °C overnight. The colonies which showed growth on 2xTY plates but did not show growth on 2xTY plates containing kanamycin (50 µg/ ml) were selected and this further confirmed the loss of the vector (plasmid) that was carrying kanamycin resistant gene as selectable marker. The replacement of the native *gca* gene by Δ*gca* in the genome of *C. glutamicum* was confirmed by colony PCR of the selected colonies using primers *gca*-promoter-fw and Del-*gca*-lower-rev. The *C. glutamicum*-Δ*gca* was then preserved in glycerol 30% (v/v) as stock culture at -80 °C.

3 Results

The carbonic anhydrase (CA) enzyme catalyzes the reversible hydration of CO_2 to bicarbonate and plays an important role in maintaining the CO_2/HCO_3^- balance in the intracellular environment in *C. glutamicum*. The two putative genes, namely the *bca*, encoding beta-type carbonic anhydrase and the *gca*, coding for a gamma-type carbonic anhydrase enzyme, have been identified in *C. glutamicum*. Furthermore, the *bca* gene has also been shown to be indispensable for growth of *C. glutamicum* under normal environmental conditions (Mitsuhashi et al., 2004). Bicarbonate acting as a substrate for carboxylation reactions (Merlin et al., 2003; Mitsuhashi et al., 2004), underscores the importance of CO_2/HCO_3^- balance in physiological processes as well as production of amino acids in *C. glutamicum*. Therefore, the indirect role of CA in regulation of carboxylase/decarboxylase reactions especially those of PEP-pyruvate-oxaloacetate node which is important for oxaloacetate anaplerosis, gluconeogenesis as well as production of amino acids such as L-lysine, via maintaining CO_2/HCO_3^- balance can be presumed in *C. glutamicum*. The present work has focused on (1) over-production of proteins that are encoded by the *bca* and *gca* genes (2) transcriptional analysis of both the genes (*bca* and *gca*) in the genome of *C. glutamicum* by RNA/cDNA related studies (3) regulation of expression of these genes in relation to (i) carbon sources, (ii) pH and (iii) transcriptional regulators by determination of activities of their respective promoters, mutational analysis and DNA/protein interactions, (4) expression analysis of an asRNA within the *bca* gene as well as (5) creation of a deletion mutant of *gca* gene in *C. glutamicum* and its functional characterization.

3.1 Amplification of the *bca* and *gca* genes from *C. glutamicum* WT

The *bca* and *gca* genes were amplified using primers *bca*-fw/*bca*-rev and primers *gca*-fw/*gca*-rev respectively, from the genome of wild-type *C. glutamicum* and the amplified products (Fig. 3A) were then ligated separately into an expression vector pET-TEV1, creating pET-TEV1-*bca* and pET-TEV1-*gca* constructs, and transformed into *E. coli* DH5α cells (Fig. 3B and 3C). The success of transformation was confirmed by growth of *E. coli* DH5α carrying respective construct on plates containing kanamycin (50 µg/ml) (due to Km^r gene as selectable marker of the pET-TEV1 vector) followed by colony PCR using primers *bca*-fw/ pET-TEV1-rev for the pET-TEV1-*bca* construct and primers *gca*-fw/ pET-TEV1-rev for the pET-TEV1-*gca* construct as

well as by isolation of the respective construct from *E. coli* DH5α and verification of the insert by sequence analysis. The over-expression in pET-TEV1 vector provided His_{10}-tag (10 residues of histidine) (along with aTEV protease-cleavable site between the His_{10}-tag and respective protein) to the N-terminus of the respective over-produced protein (see below).

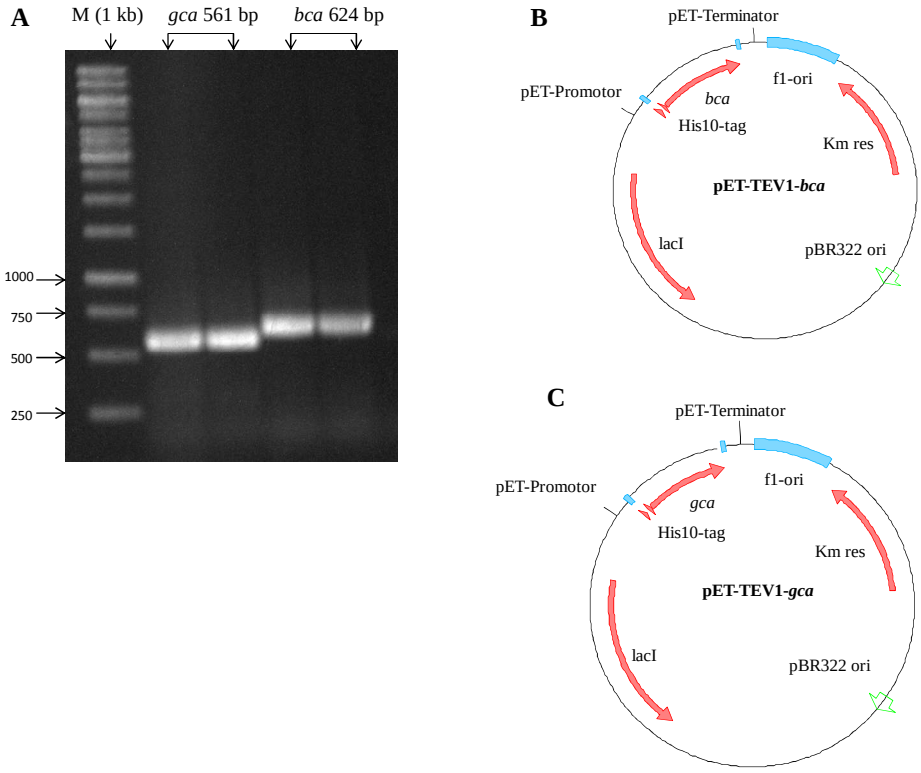


Figure 3: (A) The amplifed *gca* (561 bp) and the *bca* (624 bp) genes of *C. glutamicum* **(B)** *bca* gene over-expression construct pET-TEV1-*bca* and **(C)** *gca* gene over-expression construct pET-TEV1-*gca*.

3.2 Over-production and purification of His-tagged Bca and Gca protein in *E. coli* BL21

The two constructs pET-TEV1-*bca* and pET-TEV1-*gca* were isolated from *E. coli* DH5α and transformed into *E. coli* BL21 cells separately for over-production of the respective His-tagged Bca and His-tagged Gca proteins. The freshly transformed cells of *E. coli* BL21 containing the pET-TEV1-*bca* or pET-TEV1-*gca* construct were grown in complex medium (2xTY) plates

containing kanamycin (50 μg/ml) only, plates containing kanamycin (50 μg/ml) plus IPTG (1 mM) and plates containing IPTG (1 mM) only. The colonies showing normal growth on plates containing 'kanamycin' and slow growth on plates containing IPTG only and on plates with kanamycin plus IPTG (as shown in Fig. 4A and 4C), were chosen for further expression. This selection of suitable colonies for over-expression was based on the principle of reduced growth of *E. coli* BL21 containing pET-TEV1-*bca* or pET-TEV1-*gca* due to induction of the respective gene by IPTG. As shown in Fig. 4B and 4D, this reduced growth pattern was also observed in *E. coli* BL21 containing pET-TEV1-*bca* and pET-TEV1-*gca* for over-expression of His-tagged Bca or His-tagged Gca protein respectively, after induction by IPTG in the TB medium. The selected colonies of *E. coli* BL21 containing pET-TEV1-*bca* or pET-TEV1-*gca* construct were then grown in the TB medium and over-production of the respective His-tagged protein was induced by addition of IPTG (1 mM) to the culture medium. The cells were grown further for about 2 hours, then harvested by centrifugation and stored at -20 °C, untill further use.

To purify the over-produced His-tagged Bca and His-tagged Gca protein, the cells were disrupted by a French® Pressure Cell Press followed by centrifugation, ultra-centrifugation. The purification of the respective His-tagged protein was carried out with Ni^{2+}-NTA column affinity chromatography using a HisTrap™ HP column in an ÄKTA™ purifier. The purification was based on the principle of Ni^{2+}-NTA column affinity chromatography with binding of His-tagged protein to the Ni^{2+}-NTA column due to affinity of 'histidine' residues for 'nickel' and then using 'imidazole' for elution of the bound His-tagged-protein.

The purified over-produced proteins (His-tagged Bca and His-tagged Gca) were separated by SDS-PAGE gel. As shown in Fig. 5A and 5B, the respective protein bands were observed in the estimated size range for the corresponding His-tagged proteins. The sizes of the over-produced and purified His-tagged Bca and His-tagged Gca proteins were estimated to be 25.7 kDa and 22.7 kDa respectively.

A **B**

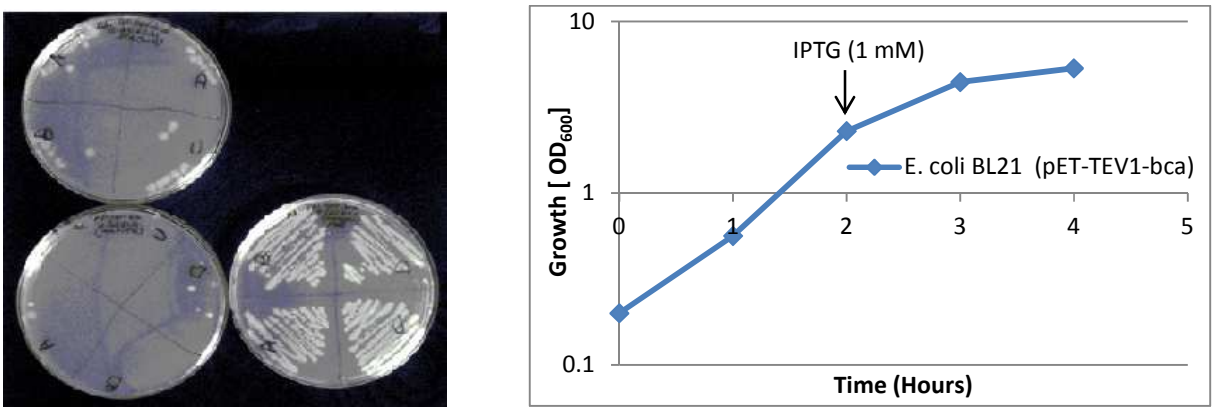


Figure 4: (A) The growth of *E. coli* BL21 containing pET-TEV1-*bca* construct on plates containing kanamycin (lower right), IPTG (upper) and kanamycin plus IPTG (1 mM) (lower left) **(B)** growth pattern of *E. coli* BL21 containing pET-TEV1-*gca* construct on TB medium for over-production of respective His-tagged-protein. The arrow indicates the time of induction for over-production of respective protein (His-tagged Bca) by addition of IPTG (1 mM) to the cultuure.

C **D**

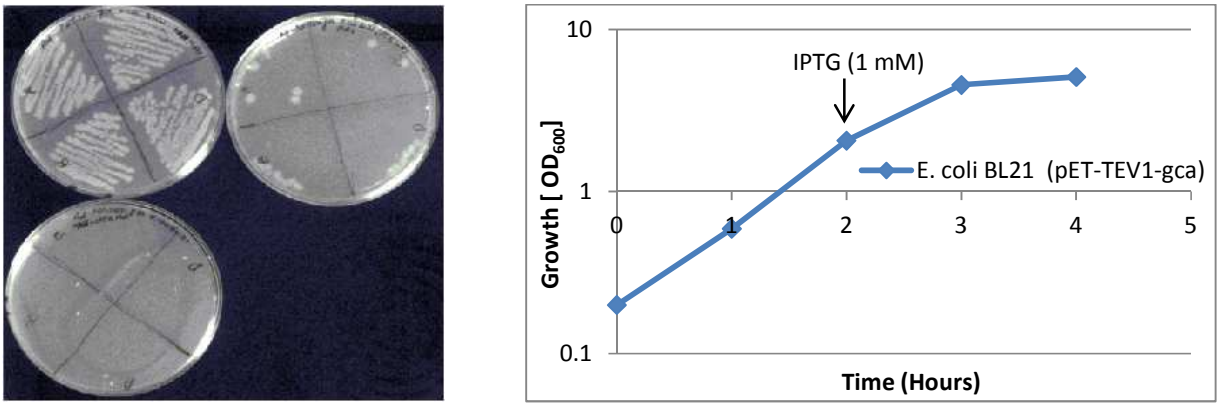


Figure 4: (C) The growth of *E. coli* BL21 containing pET-TEV1-*gca* construct on plates containing kanamycin (upper left), IPTG (1 mM) (upper right) and kanamycin plus IPTG (lower) **(D)** growth pattern of *E. coli* BL21 containing pET-TEV1-*gca* construct on TB medium for over-production of respective His-tagged-protein. The arrow indicates the time of induction for over-production of respective protein (His-tagged Gca) by addition of IPTG (1 mM) to the culture.

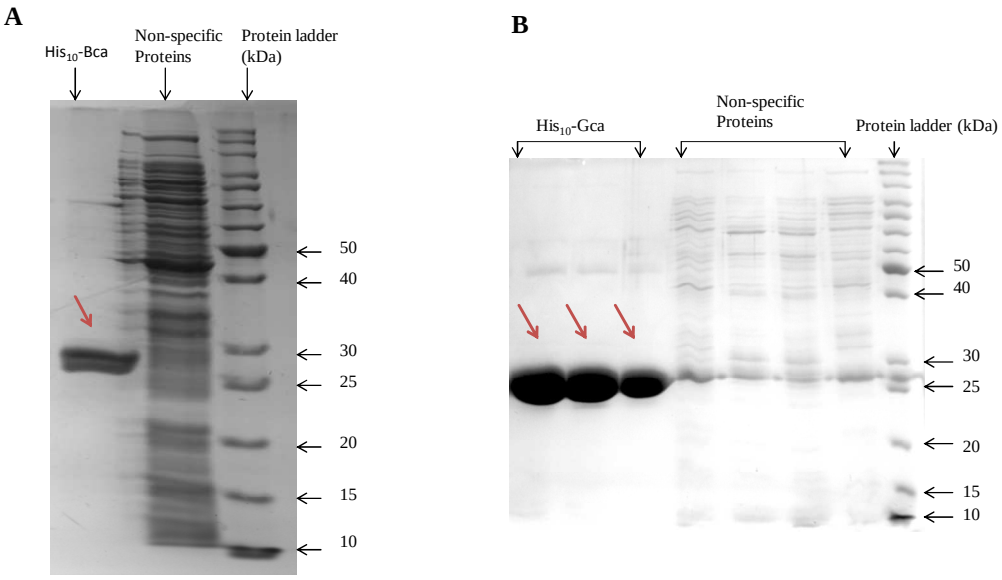


Figure 5: (A) The over-produced and purified His-tagged Bca protein in the size of 25.7 kDa on SDS-PAGE gel **(B)** over-produced and purified His-tagged Gca protein in the size of 22.7 kDa on SDS-PAGE gel.

3.3 The transcriptional organisation of the *bca* and *gca* genes in *C. glutamicum*

The transcriptional start points (TSPs) and transcriptional organisation was determined for both the *bca* and *gca* genes using RNA/cDNA and 5´-RACE analysis.

3.3.1 Cloning of the promoter regions of the *bca* and *gca* genes in promoter-probe vector pET2

The promoter region (about 520 bp) of the *bca* gene (-500 to +20 with respect to the annotated translational start site of the *bca* gene) using primers *bca*-promoter-fw and *bca*-promoter-rev and that of the *gca* gene (about 520 bp) (-262 to +258 with respect to the annotated translational start site of the cg0154 gene) using primers *gca*-promoter-fw and *gca*-promoter-rev, were amplified by PCR (Fig. 6A). The amplified promoter regions of the *bca* and *gca* genes were then separately

cloned upstream of the *cat* reporter gene coding for chloramphenicol acetyltransferase enzyme in the promoter-probe vector pET2, resulting in pET2-*bca*-promoter (Fig. 6B) and pET2-*gca*-promoter (Fig. 6C) respectively. The success of the cloning in *E. coli* DH5α was confirmed by growth of the transformed cells carrying the respective construct on complex medium (2xTY) plates containing kanamycin (50 µg/ml) (due to Km^r gene as selectable marker of pET2 vector) followed by colony PCR and verification of the insert sequence using cm4 and pET2-rev, pET2 vector-specific primers. The pET2-*bca*-promoter and pET2-*gca*-promoter constructs were isolated from *E. coli* DH5α cells and transformed separately into *C. glutamicum* by electroporation. The success of transformation was confirmed by isolation and restriction analysis of the respective construct from *C. glutamicum*. Afterwards, *C. glutamicum* cells containing the pET2-*bca*-promoter or the pET2-*gca*-promoter construct were used for determination of the TSPs of the respective genes as well as for functional studies such as determination of promoter activities of the respective genes in *C. glutamicum* (see below).

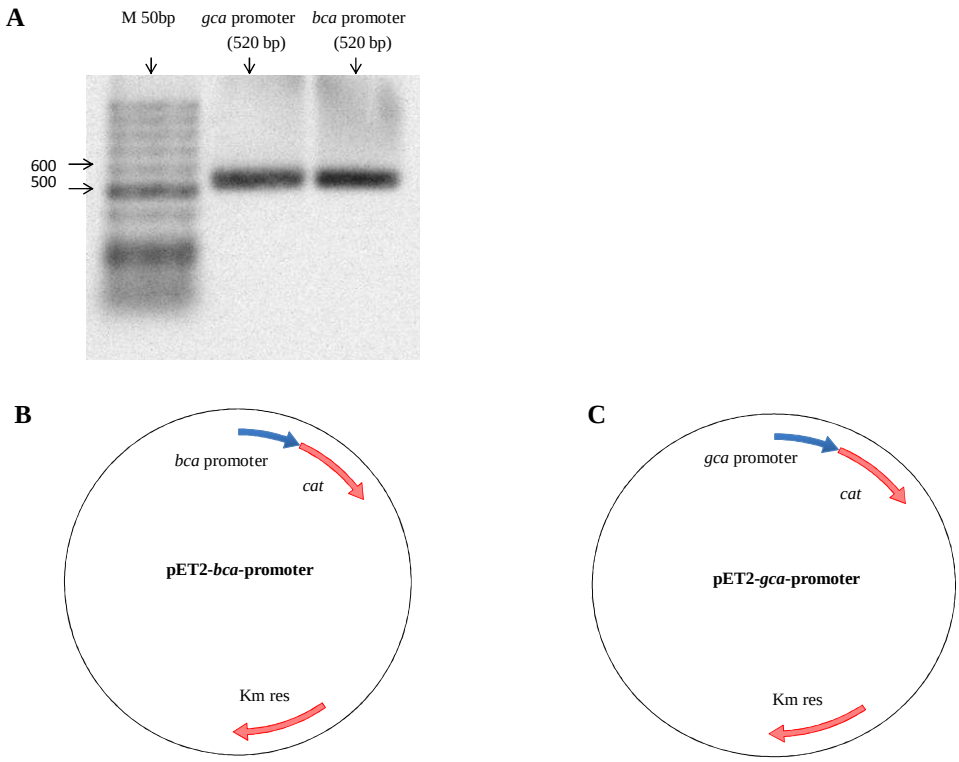


Figure 6: (A) The *bca* and *gca* promoter regions (each 520 bp) amplified from the genome of *C. glutamicum* WT. **(B)** cloning of the *bca* promoter region in pET2 vector (pET2-*bca*-promoter) in *E. coli* DH5α cells. **(C)** cloning of the *gca* promoter region in pET2 vector (pET2-*gca*-promoter) in *E. coli* DH5α cells.

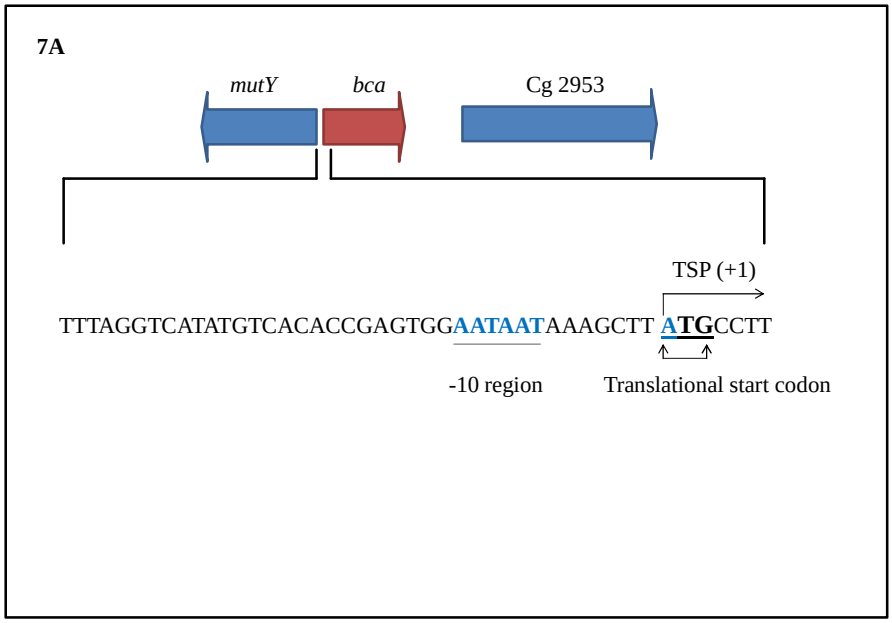
3.3.2 Identification of TSPs for the *bca* and *gca* genes

For identification of TSPs, the total RNA was isolated from pET2-*bca*-promoter and pET2-*gca*-promoter construct carrying *C. glutamicum* cells with aim to enhance the copy number of the respective transcripts for the *bca* and *gca* gene respectively for subsequent determination of their respective TSPs in *C. glutamicum*.

The cDNA synthesis was carried out with reverse transcriptase using the pET2 vector-specific primer cm5. The cDNA was then tailed at the 3′-end with poly-(A), using terminal deoxynucleotidyl transferase and the poly-(A)-tailed cDNA was purified using the RNeasy Mini Kit. The cDNAs for the *bca* and *gca* transcript were then amplified with PCR using anchor-primer oligo-(dT) and pET2 vector-specific primer cm5. The *gca* amplified product was then sequenced using the primers cm5/oligo-(dT) and cm6 (nested)/oligo-(dT). The *bca* amplified product was cloned into the pJET1.2/blunt cloning vector of the CloneJET PCR cloning kit via blunt-end ligation and subsequently sequenced using pJET1.2/blunt cloning vector-specific forward and reverse primers. The TSPs were identified for both the *bca* and *gca* genes by sequence analysis of the amplified products of the respective genes using the Clone Manager software and NCBI database. It was found that *bca* codes for a leader-less transcript, the TSP was observed to be the first nucleotide “A” of its annotated translational start codon (ATG) (Fig. 7A).

The TSP for *gca* was located to be residue “A” at - 20 position relative to the first nucleotide of the annotated translational start codon (ATG) of cg0154, located upstream of the *gca* gene (Fig. 7B). Identification of the TSP for the *gca* gene upstream of cg0154 gene was in agreement with previous results that both the genes (cg0154-*gca*) co-transcribe and form an operon, as observed by RNAseq experiments (Pfeifer-Sancar et al., 2013).

Furthermore, upstream of the TSPs, the motifs AATAAT and TAGGCT were observed for the *bca* and the *gca* genes, respectively (Fig. 7A and 7B), which are partially identical to the -10 consensus [TA (C/T) AAT] sequence for Corynebacteria (Pátek et al., 2003).



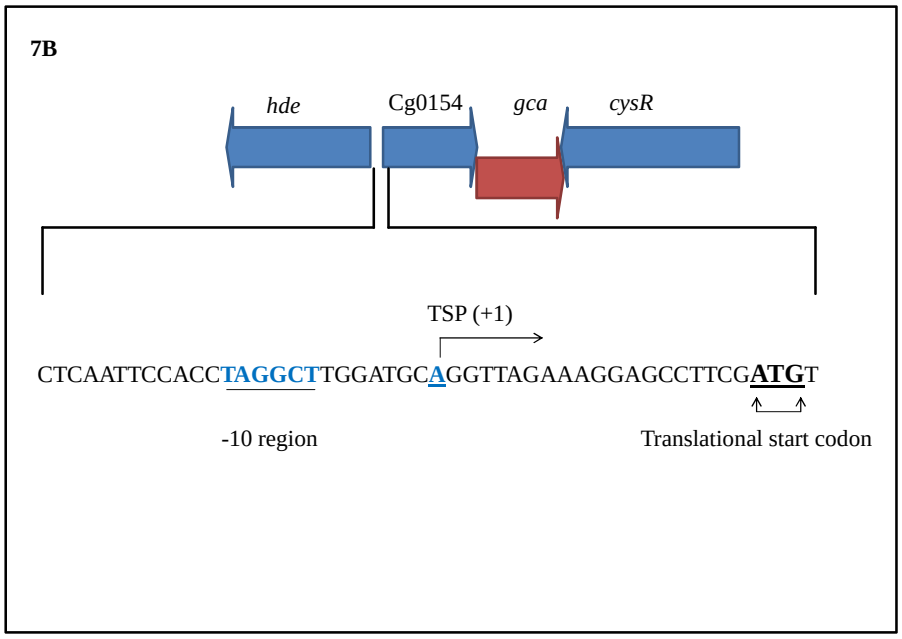


Figure 7: Transcriptional start points (TSPs) of the *bca* and *gca* gene, as identified by the 5'-RACE method. **(A)** TSP of the *bca* gene is the first nucleotide (indicated by arrow) of its putative translational start codon (ATG) (underlined) **(B)** TSP of the *gca* gene located at -20 position (indicated by arrow) relative to the first nucleotide of the putative translational start codon (ATG) (underlined) of cg0154, an upstream gene of *gca*. Furthermore, a partially conserved promoter consensus sequence was found around the -10 region (underlined) upstream of the TSP of the *bca* gene and *gca* gene, as shown in **(A)** and **(B)** respectively.

3.4 Expression analysis of the *bca, gca* genes and the asRNA (within *bca* gene)

Both the *bca* and *gca* genes and also the asRNA within the *bca* gene were characterized in terms of expression using the promoter-probe vector pET2, in *C. glutamicum*.

3.4.1 Promoter activities of the *bca* and *gca* genes during growth of *C. glutamicum* with different carbon sources

The expression profiles of both the *bca* and *gca* genes were determined by determination of activities of their corresponding promoters using pET2-*bca*-promoter or pET2-*gca*-promoter respectively, in *C. glutamicum*. The growth pattern of *C. glutamicum* containing pET2-*bca*-promoter or pET2-*gca*-promoter was observed to be the same as that of wild-type *C. glutamicum* (Fig. 8). For the determination of the promoter activites, the cultures were grown in minimal medium with different carbon sources such as glucose 1% (w/v) or acetate 1% (w/v) as sole source of carbon and energy with the pH of the medium adjusted to 6.8 and 6.3 for glucose and acetate respectively. The cells were then harvested at mid-logarithmic phase of growth (OD_{600} of about 5) and promoter activity of the respective genes were determined by determination of the specific CAT enzyme activity in crude cell extracts, using *C. glutamicum*-Δ*atlR* carrying pET2-P4 promoter (Gabris, 2011) as positive control and *C. glutamicum* RES167 carrying pET2 (Gabris, 2011) as negative control. The results of these experiments with *C. glutamicum* (pET2-*bca*-promoter) and *C. glutamicum* (pET2-*gca*-promoter), showed carbon source-dependent regulation of both the genes with glucose or acetate as sole source of carbon and energy **(**Table 8). The activity of the *bca* promoter was about 1.5 times higher on glucose as compared to acetate while the activity of the *gca* promoter was about twice as high in cells grown on glucose as in cells grown on acetate. Furthermore, the activities of the *bca* promoter were observed to be approximately fifteen-fold higher than the activities of the *gca* promoter on either of the carbon sources (glucose or acetate) in mid-logarithmic growth phase in *C. glutamicum* (Table 8). This carbon source-dependent regulation of expression of *bca* (and also of *gca*) might be explained by the higher demand of oxaloacetate anaplerosis during growth on glucose than on acetate, when used as sole source of carbon. This might indicate the possible indirect regulatory effect of the CA enzyme on carboxylating/decarboxylating enzymes of the PEP-pyruvate-oxaloacetate node, via CO_2/bicarbonate balance in the intracellular environment in *C. glutamicum*.

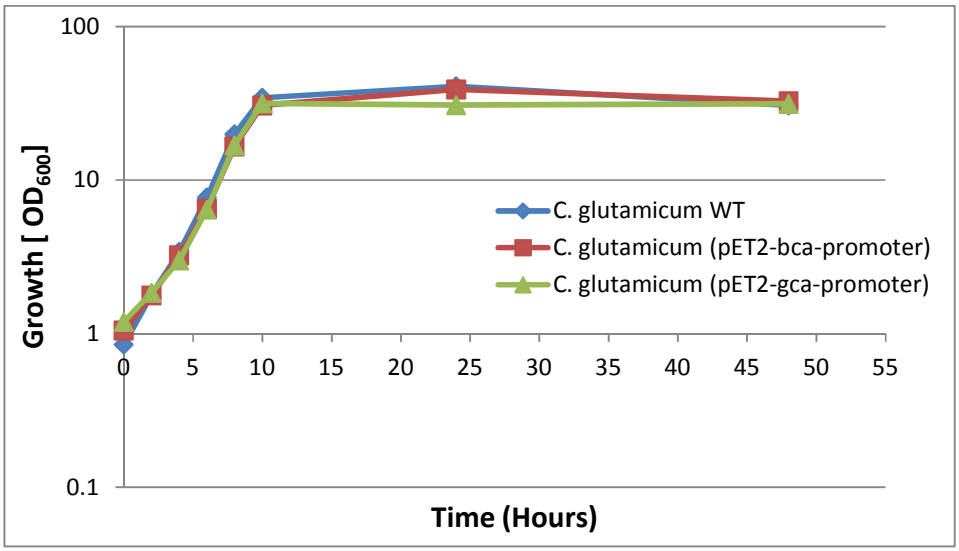


Figure 8: Comparison of the growth of *C. glutamicum* carrying pET2-*bca*-promoter, *C. glutamicum* carrying pET2-*gca*-promoter and wild-type *C. glutamicum* grown in minimal with glucose 4% (w/v) as source of carbon and energy.

Table 8: Comparison of activities of the *bca* and *gca* promoters in *C. glutamicum* grown with glucose 1% (w/v) or acetate 1% (w/v) as sole carbon source, determined as specific activity of the reporter chloramphenicol acetyltransferase (CAT) enzyme of the pET2 vector.

Strain	minimal medium	Specific CAT activity [U/mg of protein]
C. glutamicum (pET2-*bca*)	+ glucose	1.60 ± 0.14
C. glutamicum (pET2-*bca*)	+ acetate	1.15 ± 0.04
C. glutamicum (pET2-*gca*)	+ glucose	0.09 ± 0.005
C. glutamicum (pET2-*gca*)	+ acetate	0.05 ± 0.010

3.4.2 Effect of pH on carbon source-dependent regulation of *bca* gene in *C. glutamicum*

To determine possible effects of the pH on the carbon source-dependent regulation of the *bca* gene, a comparison of the *bca* promoter activities on glucose or acetate as sole carbon source was made, at two different initially adjusted pH (6.3 and 6.8) values of the minimal medium. *C. glutamicum* carrying the pET2-*bca*-promoter construct was cultured in minimal medium containing either glucose 1% (w/v) or acetate 1% (w/v) as sole source of carbon, with the pH of the medium adjusted at 6.3 or 6.8, and harvested at the mid-logarithmic growth phase (OD_{600} of about 5). The activities of *bca* promoter were determined by determining the specific CAT enzyme activities (as described above) in the cell extracts of *C. glutamicum* (pET2-*bca*-promoter), grown under different conditions. Again it was observed that activity of the *bca* promoter was about 1.5 times higher on glucose than on acetate, at both the pH values (6.3 or 6.8). Furthermore, the activity of the *bca* was also observed to be nearly the same on glucose as carbon source on both pH values (6.3 and 6.8) or acetate as carbon source at both pH values (6.3 and 6.8) (Table 9). This indicated that the observed carbon source-dependent regulation of the *bca* gene is independent of the initial adjusted pH (6.3 or 6.8) of the minimal medium, in shake flask fermentation experiments.

Table 9: Comparison of activities of the *bca* promoter in *C. glutamicum* grown under glucose 1% (w/v) or acetate 1% (w/v) as sole carbon source and with initial pH of 6.8 or 6.3 of the medium.

Strain	minimal medium	Specific CAT activity [U/mg of protein]
C. glutamicum (pET2-*bca*)	+ glucose (pH 6.8)	1.47 ± 0.003
C. glutamicum (pET2-*bca*)	+ acetate (pH 6.8)	0.71 ± 0.325
C. glutamicum (pET2-*bca*)	+ glucose (pH 6.3)	1.60 ± 0.184
C. glutamicum (pET2-*bca*)	+ acetate (pH 6.3)	0.97 ± 0.008

3.4.3 Characterization of the asRNA within the *bca* gene

The RNA sequence analysis revealed within the *bca* gene the presence of an antisense RNA (asRNA) (Pfeifer-Sancar et al., 2013) (Fig. 9A). To characterize this asRNA in terms of expression, the region from +91 bp to -206 bp (297 bp) with respect to the asRNA was amplified using primers asRNA-promoter-fw and asRNA-promoter-rev, as probable promoter for the asRNA within the *bca* gene. The amplified promoter region of the asRNA was then cloned upstream of the *cat* reporter gene coding for chloramphenicol acetyltransferase in the promoter-probe vector pET2 in *E. coli* DH5α cells (Fig 9B). The success of transformation was confirmed by colony PCR and sequence verification of the cloned insert in the pET2-asRNA-promoter construct with pET2 vector-specific primers pET2-rev and cm4. The pET2-asRNA-promoter construct was then isolated from *E. coli* DH5α and transformed into *C. glutamicum* by electroporation for functional analysis. Furthermore, the growth pattern of the *C. glutamicum* carrying the pET2-asRNA-promoter construct was observed to be nearly the same as that of wild-type *C. glutamicum* (Fig. 10)

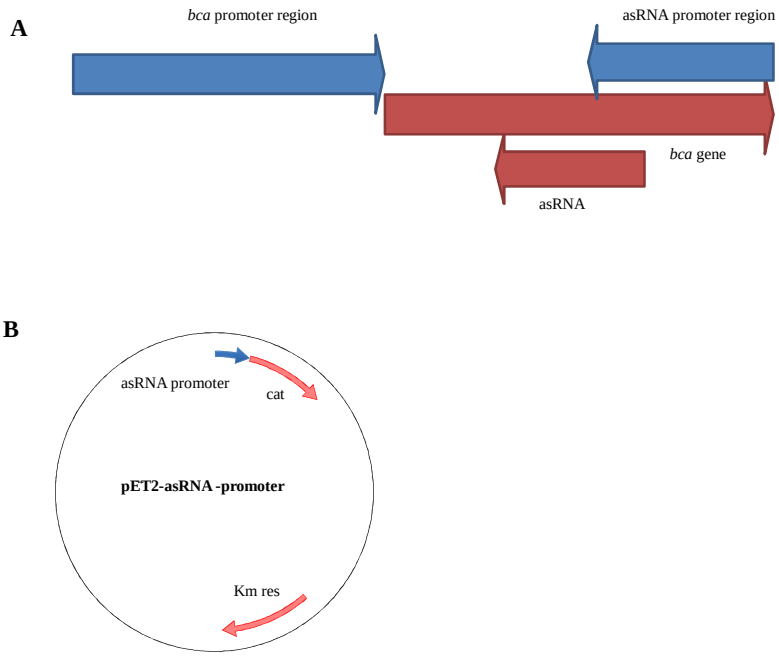


Figure 9: (**A)** The organisation of the *bca* gene and the asRNA (within the *bca* gene) in *C. glutamicum* as well as their promoter regions used for functional analysis **(B)** pET2-asRNA-promoter construct showing the asRNA promoter region cloned upstream of the *cat* gene in the promoter-probe vector, pET2.

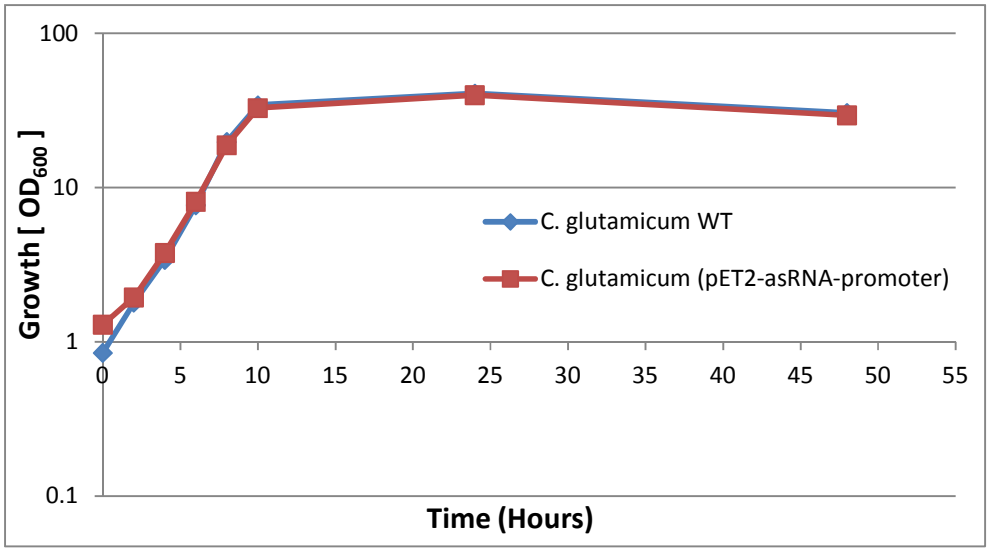


Figure 10: Comparison of the growth pattern of *C. glutamicum* carrying pET2-asRNA-promoter with wild-type *C. glutamicum* grown in minimal medium with glucose 4% (w/v) as source of carbon.

To determine the expression of the asRNA, the *C. glutamicum* carrying pET2-asRNA-promoter was grown in minimal medium containing either glucose 1% (w/v) or acetate 1% (w/v), as sole source of carbon and harvested at mid-logarithmic phase of growth (OD_{600} of about 5). The cells were disrupted and activity of the asRNA promoter was determined by determining the specific CAT enzyme activity, in crude cell extracts of *C. glutamicum*. It was observed that there is very low activity of the asRNA promoter region under conditions with glucose or acetate as sole carbon source (Table 10). These results confirmed that the cloned fragment (upstream region of asRNA) acts as promoter of the asRNA and was shown to be active with glucose or acetate as carbon source at mid-exponential phase of growth in *C. glutamicum*.

Table 10: Comparison of activity of the asRNA promoter in *C. glutamicum* grown with glucose 1% (w/v) or acetate 1% (w/v) as sole carbon source.

Strain	minimal medium	Specific CAT activity [U/mg of protein]
C. glutamicum (pET2-asRNA promoter)	+ glucose	0.005 ± 0.0013
C. glutamicum (pET2-asRNA promoter)	+ acetate	0.003 ± 0.0002

3.5 Identification of proteins binding to the *bca* promoter region

Proteins binding to the promoter region of the *bca* gene were enriched using DNA affinity chromatography and subsequently identified using MALDI-TOF mass spectrometry. The biotin-labelled *bca* promoter fragment was amplified using primers *bca*-promoter-fw and biotin-labelled *bca*-promoter-rev. The amplified biotin-labelled *bca* promoter fragment was bound to Dynabeads® M-280 Streptavidin and then incubated with cell extract of *C. glutamicum*, grown in minimal medium containing glucose 2% (w/v) and harvested at mid-logarithmic phase of growth (OD_{600} of about 5). The proteins with binding affinity for the *bca* promoter region were retained while other non-specific proteins were washed out. The promoter-bound proteins were then eluted and separated on a SDS-PAGE gel (Fig. 11). The protein bands were cut out of the gel and identified using MALDI-TOF mass spectrometry. The eluted proteins A1, A2, A3 and A4 (as shown in figure. 11), were identified as DNA polymerase I, Stress sensitive restriction system protein 2, DNA polymerase III epsilon subunit or related 3′-5′ exonuclease and Putative type II restriction endonuclease respectively. However, no putative regulator protein bound to the *bca* promoter was identified.

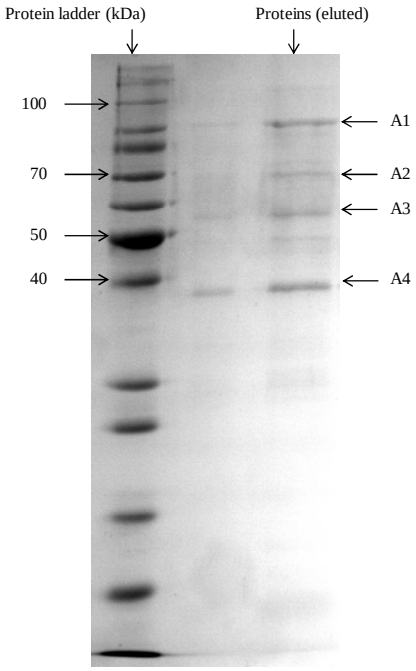


Figure 11: SDS-PAGE analysis of the proteins eluted from the *bca* promoter region in a DNA affinity chromatography.

3.6 Regulation of the *bca* gene by the transcriptional regulator RamA in *C. glutamicum*

The effect of the global transcriptional regulator RamA on expression of the *bca* gene was determined by mutational analysis and DNA/protein interactions such as electrophoretic mobility shift assays (EMSAs).

3.6.1 Comparison of the *bca* promoter activity in *C. glutamicum*-Δ*ramA* and wild-type *C. glutamicum*

The role of the global transcriptional regulator RamA on regulation of expression of the *bca* gene was determined by analyzing the activities of the *bca* promoter in a Δ*ramA* mutant and in wild-type *C. glutamicum.* The construct pET2-*bca*-promoter was transformed in both Δ*ramA* mutant and wild-type *C. glutamicum* and the promoter activities were determined by determining

the specific CAT enzyme activities in cell extracts of cultures grown in minimal medium containing glucose 1% (w/v) plus acetate 1% (w/v) and harvested at mid-logarithmic growth phase (OD_{600} of about 5). As shown in table 11, the activity of the promoter of the *bca* gene was about four-fold higher in the Δ*ramA* mutant as compared to wild-type *C. glutamicum*. This higher activity of the *bca* promoter in the absence of a functional RamA protein in the Δ*ramA* mutant indicated that the RamA acts as a negative transcriptional regulator of the *bca* gene in *C. glutamicum*. These results are also consistent with transcriptomic profile of the *ramA* mutant *C. glutamicum* which showed about 2.5 times higher transcript level of the *bca* gene in Δ*ramA* as compared to wild-type *C. glutamicum* (Auchter et al., 2011a).

Table 11: Comparison of the *bca* promoter activities in Δ*ramA* mutant and in wild-type *C. glutamicum* grown with glucose 1% (w/v) plus acetate 1% (w/v) as carbon sources.

Strain	minimal medium	Specific CAT activity [U/mg of protein]
C. glutamicum (pET-2-*bca*)	+ glucose + acetate	1.37 ± 0.13
C. glutamicum-Δ*ramA* (pET-2-*bca*)	+ glucose + acetate	5.19 ± 0.06

3.6.2 Over-production and purification of His-tagged RamA protein

The His-tagged RamA protein was over-produced using *E. coli* BL21 containing the pET28a-*ramA* construct (Cramer et al., 2006). *E. coli* BL21containing pET28a-*ramA* was cultured in TB medium for 2 hours and then the over-production of the His-tagged RamA protein was induced by addition of IPTG (1 mM) to the culture. The culture was then grown further for 2 hours and then harvested by centrifugation (Fig. 12A).

To purify the over-produced His-tagged RamA protein, the cells were disrupted by a French Press cell followed by centrifugation, ultra-centrifugation. The purification was carried out using HisTrap™ HP column on an ÄKTA™ purifier. The purified His-tagged RamA protein was then

separated on a SDS-PAGE gel and a band was observed in approximate range of size of the RamA, that is 30.8 kDa (Cramer et al., 2006) (Fig. 12B). The protein was then also identified as RamA by MALDI-TOF mass spectrometry.

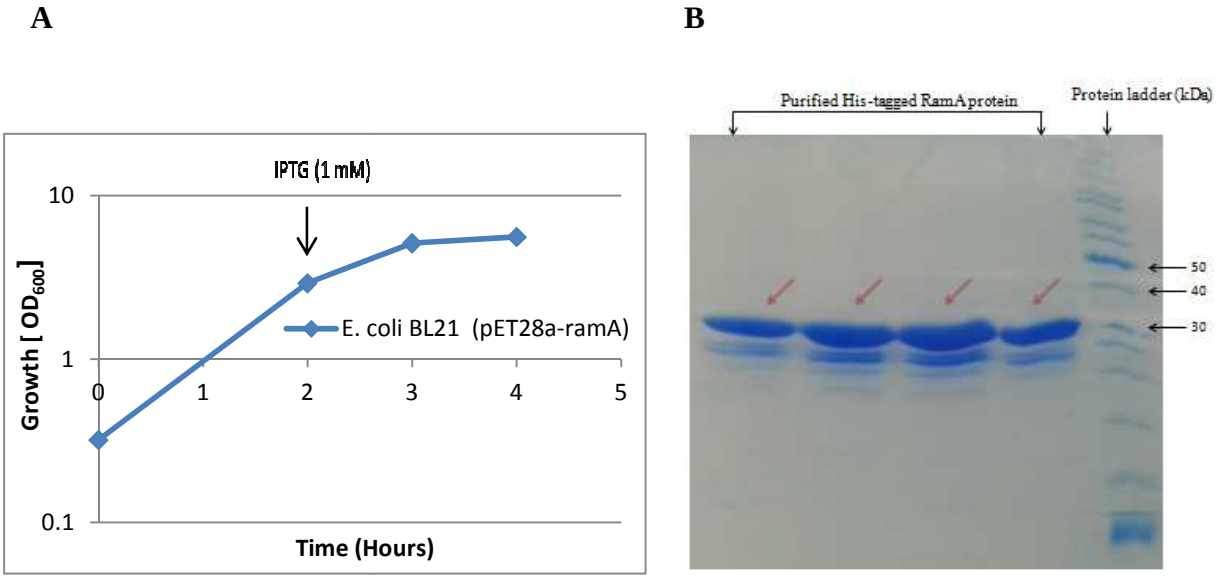


Figure 12: (A) Growth pattern of *E. coli* BL21 carrying pET28a-*ramA* during over-production of His-tagged RamA protein. The arrow indicates the time of addition of IPTG (1 mM) to the culture **(B)** SDS-PAGE analysis of the purified His-tagged RamA protein. The arrows indicate the purified His-tagged-RamA protein within approximate range of size of the RamA protein (30.8 kDa).

3.6.3 His-tagged RamA protein binds to the *bca* promoter region

The most common RamA binding motif has been identified as A/T/C-GGGG-N and with lower conservation A/T/C-CCCC-N (Auchter et al., 2011a). These binding sites were also observed in the sequence of the *bca* promoter region (Fig. 13) and the RamA protein was likely to bind to the *bca* promoter region. To analyze binding of the RamA protein to the *bca* promoter region, electrophoretic mobility shift assay (EMSA) experiment was performed.

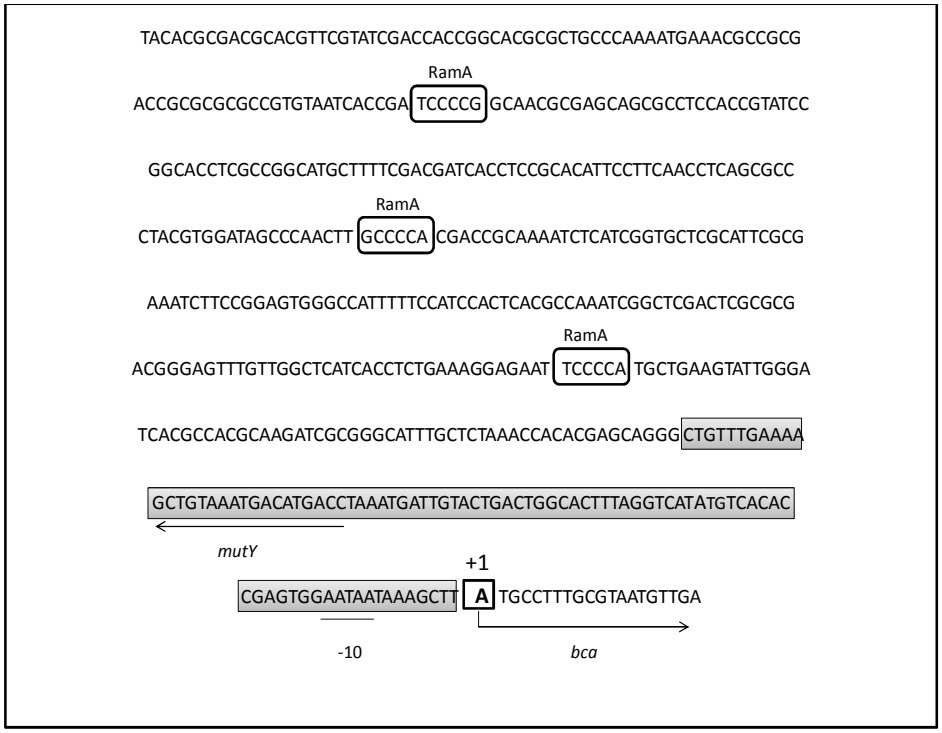


Figure 13: Sequence of the promoter region of the *bca* gene showing the RamA binding motifs. The region shown in grey color is a sub-fragment of the *bca* promoter region (91bp) named as PF1 that contains no RamA binding site.

In the EMSA experiments, the *bca* promoter fragment was incubated with varying amounts (0-2 µg) of purified His-tagged RamA protein and the assay mixture was separated on agarose gel to test for retardation. Bovine serum albumin (BSA) was used as negative protein control with *bca* promoter fragment in the EMSA experiment.The *aceA-aceB* inter-genic region was amplified with primers *aceA-aceB*-fw/*aceA-aceB*-rev and used as positive control for the His-tagged RamA protein as the RamA protein was already known to bind to this region (Cramer et al., 2006), while part of the *bca* promoter region devoid of the RamA binding sites, designated as PF1 (promoter fragment 1, as shown in grey shade in Fig. 13) was amplified using primers PF1-fw/PF1-rev and used as negative control for the RamA protein in EMSA experiments. As shown in Fig. 14, the *bca* promoter fragment was retarded by the RamA protein and retardation was observed to be proportional to increasing concentration of the His-tagged RamA protein, showing binding of the RamA protein to the *bca* promoter region. Taken together, the higher transcript level of the *bca* gene in the Δ*ramA* mutant as compared to wild-type *C. glutamicum* (Auchter et

al., 2011a), higher *bca* promoter activity in *C. glutamicum*-Δ*ramA* compared to wild-type and binding of the His-tagged RamA protein to the *bca* promoter fragment in the bandshift experiment showed that the global regulator RamA negatively regulates the expression of the *bca* gene in *C. glutamicum*.

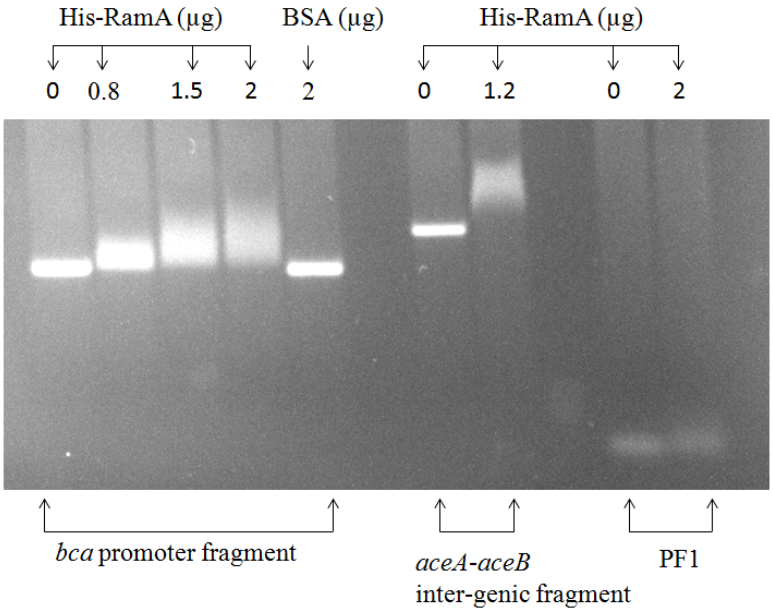


Figure 14: EMSAs for promoter region of the *bca* gene with purified regulator His-RamA protein showing retardation with increasing concentration of protein (0-2 µg). The *bca* promoter region showed no retardation with BSA (2 µg), used as negative protein control. The *aceA-aceB* intergenic region with known binding affinity for RamA showed retardation with His-RamA (1.2 µg). A sub-fragment of *bca* promoter PF1 (91 bp) having no binding sites for RamA showed no retardation with His-RamA protein (2 µg).

3.7 Creation of a *gca* deletion mutant of *C. glutamicum*

The *gca* gene (as shown in Fig. 15B) was replaced by a truncated *gca* gene in the genome of *C. glutamicum*. The truncated *gca* gene was created by amplifying the upper fragment (about 423 bp) using primers Del-*gca*-upper-fw and Del-*gca*-upper-rev and lower fragment (about 423 bp) using primers Del-*gca*-lower-fw and Del-*gca*-lower-rev, with deletion of the internal region (about 422 bp) of the *gca* gene. The fragments were then combined together in a cross-over PCR resulting in a truncated *gca* fragment. This truncated version of the *gca* gene was then cloned into pK19*mobsac*B vector creating pk19*mobsac*B-Δ*gca* and transformed into *E. coli* DH5α cells. The construct pk19mobsacB-*Δgca* was then isolated from *E. coli* DH5α and sequenced for

confirmation of the deleted portion of the *gca* gene, using vector specific primers pK19*mobsac*B-fw and pK19*mobsac*B-rev. The construct was then transformed into *C. glutamicum* and the *gca* (wild-type) gene was replaced by the *Δgca* version in the genome resulting in *C. glutamicum-Δgca* (Fig. 15C). The clones having successful replacement of the native *gca* gene with Δ*gca* and excision of vector, were selected by ability to grow on complex medium plates containing sucrose 10% (w/v) as *sac*B gene of the pK19*mobsac*B vector codes for levansucrase which produces "Levan" from sucrose, toxic for *C. glutamicum* as well as by loss of ability to grow on complex medium plates containing kanamycin (50 µg/ ml) (due to Km^r gene as selectable marker of the pK19*mobsac*B vector). The deletion in the *gca* gene was confirmed by colony PCR using primers *gca*-promoter-fw/ Del-*gca*-lower-rev and difference in size of the amplified fragments was observed for the *C. glutamicum-Δgca* (1368 bp) and wild-type *C. glutamicum* (1776 bp) (Figure. 15A).

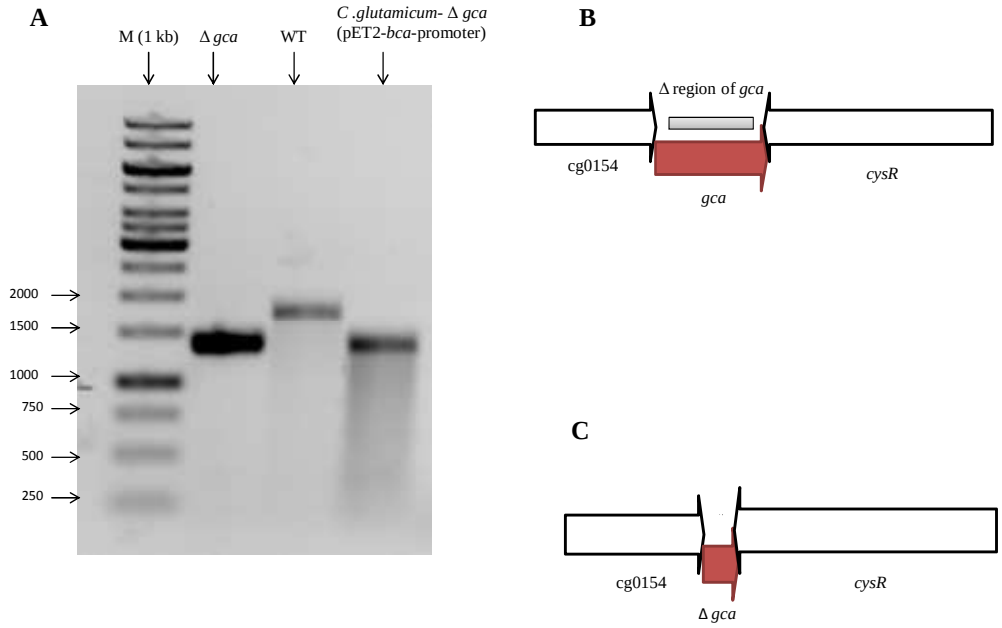


Figure 15: (A) Colony PCR for confirmation of deletion in the *gca* gene, showing fragment of about 1368 bp in *C. glutamicum*- Δ*gca* and *C. glutamicum*-Δ*gca* containing pET-2-*bca*-promoter construct in comparison to the fragment of 1776 bp size in wild-type *C. glutamicum*. **(B)** The genetic organisation of the *gca* gene with indicated deletion region (grey bar) in *C. glutamicum*-Δ*gca* and **(C)** shows the truncated version of the *gca* gene in *C. glutamicum*-Δ*gca*.

Furthermore, it was also observed that the growth pattern of *C. glutamicum*- Δ*gca* was nearly the same as that of wild-type *C. glutamicum* in minimal medium containing glucose 4% (w/v) as source of carbon (Fig. 16).

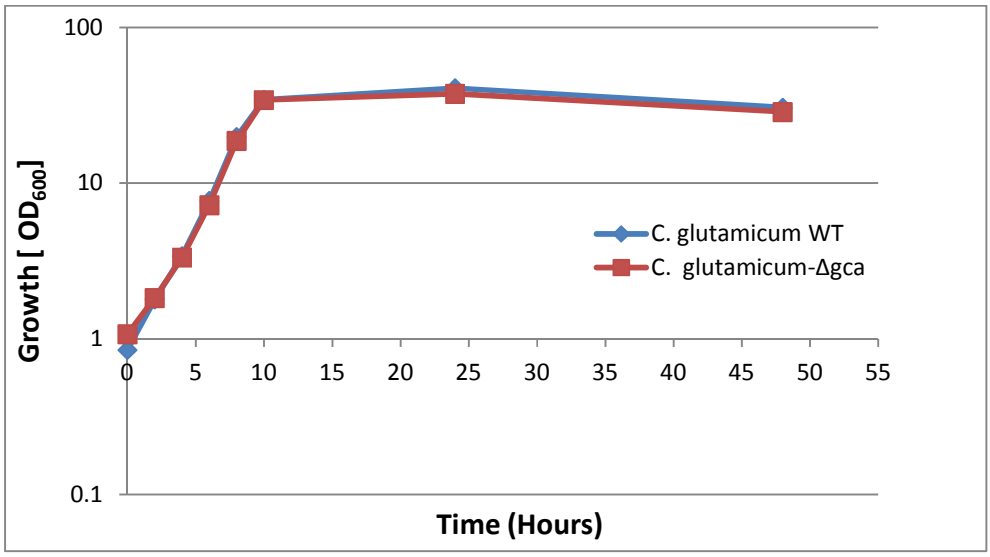


Figure 16: Comparison of growth pattern of *C. glutamicum*-Δ*gca* and wild-type *C. glutamicum* grown in minimal medium with glucose 4% (w/v) as source of carbon and energy.

3.7.1 Comparison of the *bca* promoter activity in wild-type and *C. glutamicum*-Δ*gca*

To investigate the possible complementation of function of *gca* by the *bca* gene, the pET2-*bca*-promoter construct was transformed into wild-type (as discussed above) and *C. glutamicum*-Δ*gca* by electroporation. A comparison of the *bca* promoter activity was made by determination of specific CAT enzyme activity in cell extracts of cultures of wild-type and *C. glutamicum*-Δ*gca* grown with glucose 1% (w/v) as carbon source and harvested at mid-exponential phase of growth (OD_{600} of 5). However, activity of the *bca* promoter was observed to be nearly the same in both wild-type and *C. glutamicum*- Δ*gca*, which indicated no significant effect of the *gca* deletion on expression of the *bca* gene in *C. glutamicum* (Table 12).

Table 12: Comparison of activities of the *bca* promoter in wild-type and *C. glutamicum*- Δ*gca* at mid-exponential growth phase grown with glucose 1% (w/v) as source of carbon.

Strain	minimal medium	Specific CAT activity [U/mg of protein]
C. glutamicum WT (pET2-*bca*)	+ glucose	1.77 ± 0.23
C. glutamicum-Δ*gca* (pET2-*bca*)	+ glucose	2.09 ± 0.07

4 Discussion

CO_2 is an important metabolite in living organisms and is in equilibrium with HCO_3^-, carbonic acid and carbonate and of these, the physiologically more important is HCO_3^- (Smith and Ferry, 2000), especially for carboxylation reactions. As CO_2 can diffuse in and out of the cell, conversion of HCO_3^- to CO_2 might facilitate its transport inside the cell while conversion of CO_2 to HCO_3^- might be important to retain CO_2 inside the cell. As chemical inter-conversion of CO_2 and HCO_3^- is slow at physiological pH, enzymatic conversion allows the cell to maintain the intracellular balance of CO_2/HCO_3^- for cellular processes (Smith and Ferry, 2000). CA is found in all three domains of life and catalyzes the reversible hydration of CO_2 [$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$] (Zimmerman and Ferry, 2008). It has been observed that in the absence of CA, the rate of bicarbonate generation is dependent upon the steady-state concentration of CO_2 as well as on the rate of uncatalyzed reaction, which is too low to meet the bicarbonate demand in *E. coli* under those conditions where CA is essential for growth (Merlin et al., 2003).

Bicarbonate acts as substrate for carboxylation reactions in the intracellular environment (Mitsuhashi et al., 2004; Merlin et al., 2003). The principal reactions in which CO_2/bicarbonate is consumed are those catalyzed by the enzymes, carbamoyl phosphate synthetase, 5-aminoimidazole ribotide carboxylase, biotin carboxylases (Merlin et al., 2003) such as acetyl Co-A carboxylase and pyruvate carboxylase and also phosphoenolpyruvate carboxylase (Merlin et al., 2003), the latter being key enzyme in PEP-Pyruvate-Oxaloacetate node in bacteria (Sauer and Eikmanns, 2005). The PEP-pyruvate-oxaloacetate node is metabolic switch-point of carbon flux distribution which is important for gluconeogenesis, oxaloacetate anaplerosis as well as amino acid biosynthesis such as L-lysine production in bacteria. Bicarbonate is also needed in fatty acid (FA) biosynthesis, where it is incorporated into malonyl-CoA by the action of biotin carboxylase (Merlin et al., 2003). Burghout et al. (2010) also observed that pneumococcal carbonic anhydrase (PCA) is linked to FA biosynthesis under CO_2 poor conditions in *S. pneumonia*. This shows that PCA provides HCO_3^- for carboxylation of acetyl-CoA by acetyl-CoA carboxylase to form malonyl-CoA, in fatty acid biosynthesis.

C. glutamicum is an industrial workhorse organism which is widely used for the production of amino acids such as L-lysine, L- glutamate as well as other metabolites. The PEP-pyruvate-oxaloacetate node of this organism, being an important branch-point of carbon flux distribution

and having role in oxaloacetate anaplerosis, gluconeogenesis and amino acid biosynthesis such as L-lysine, involve several carboxylation/decarboxlation reactions and the effect of intracellular CO_2/bicarbonate balance on the physiology of this node can be presumed. In *C. glutamicum*, the two putative genes *bca* (cg2954) and *gca* (cg0155) code for beta-type carbonic anhydrase and gamma-type carbonic anhydrase enzymes, respectively (Mitsuhashi et al., 2004). The purpose of this work was to broaden our knowledge about the transcriptional organisation of these genes (*bca* and *gca*) as well as expression analysis in relation to media composition (carbon sources) and transcriptional regulators, so that to ultimately determine the role of these carbonic anhydrases in central carbon metabolism as well as their effect on the production of amino acids in *C. glutamicum*. In the present work, both the genes (*bca* and *gca*) were separately cloned in an over-expression vector pET-TEV1 in *E. coli* DH5α cells. The pET-TEV1 vector provides a His-tag (10 histidine residues) to the N-terminus of the respective over-produced protein. The affinity tags, such as the the intein chitin-binding tag (Chong et al., 1997; Chong et al., 1998), maltose-binding tag (di Guan et al., 1988; Maina et al., 1988), and the polyhistidine tags of the pET vector series (Rosenberg et al., 1987; Studier & Moffatt, 1986; Studier et al., 1990) has made possible the purification of different proteins more efficient, rapid and at higher yields than through native purification (Rocco et al., 2008). Furthermore, the pET-TEV1 vector also provides a "TEV protease" cleavable site for removal of the "His-tag" from the over-produced protein after purification. Though the addition of affinity tags makes the purification easy, however it sometimes brings the protein into a non-native state and the affinity tag can interfere during work with the protein. The TEV (tobacco etch virus) protease is used to cleave fusion proteins due to its specificity, insensitivity to protease inhibitors used in purification of protein, and the easy separation of both the affinity tag and protease from the target protein (Parks et al., 1994; Rocco et al., 2008).

The pET-TEV1-*bca* and pET-TEV1-*gca* constructs were separately transformed in *E. coli* BL21 and the respective His-tagged proteins (Bca and Gca) were over-produced by addition of IPTG to the culture. The *E. coli* BL21 (DE3) offer an inducible source of T7 RNA polymerase with basal expression level low enough to maintain most of the T7 genes under control of a T7 promoter in a multicopy plasmid. Plasmids that are maintainable in *E. coli* BL21 (DE3), the induction of the T7 RNA polymerase gene by IPTG results in the production of large amounts of target proteins (Studier and Moffatt, 1986). The over-produced His-tagged Bca and His-tagged Gca proteins

were purified using Ni^{2+}-NTA column HisTrap™ HP in an ÄKTA™ purifier. The immobilized metal-affinity chromatography (IMAC) method is used to purify recombinant proteins carrying affinity tags and is based on the interaction of transition metal ions such as Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}, immobilized on a matrix and specific amino acid residues. The amino acid "histidine" strongly ineracts with immobilized metal ion matrices and therefore, proteins which contain sequence of histidine residues are effectively retained on IMAC column matrices and eluted by pH adjustment of column buffer or using imidazole in the column buffer (Bornhorst and Falke, 2000). The purified proteins were separated by SDS-PAGE gel and the size of His-tagged Bca and Gca were observed to be about 25 kDa and 23 kDa respectively. These established over-expression systems for the products of both the genes can further be used for structural and functional characterization of these proteins.

In this work, a 520 bp promoter region for the *bca* (-500 to +20) relative to the first nucleotide of the putative translational start codon of *bca* gene and also for *gca* (-262 to +258) relative to the first nucleotide of the putative translational start codon of cg0154 gene (gene upstream of the *gca*) was amplified and each was separately cloned upstream of the promoter-less *cat* reporter gene in the promoter-probe vector pET2, creating pET2-*bca*-promoter and pET2-*gca*-promoter constructs. These constructs were used for determination of transcriptional start point (TSPs) of the respective genes as well as for functional characterization such as expression analysis of these genes in *C. glutamicum* (see below). The reporter systems are tools used for screening and measurement of activities of promoter (Pátek et al., 2013). The promoter-probe vector pET2 (Vasicova et al., 1998) has been frequently used for analysis related to promoter strength and transcriptional regulatory mechanisms (Pátek et al., 2013). The multicopy pET2 vector offers transcriptional fusion of a promoter fragment with promoterless chloramphenicol acetyltransferase (*cat*) gene which is carried by the vector and can be used for measurement of promoter activity as well as determination of transcriptional start sites and also to characterize the regulation of promoter (Vasicova et al., 1998).

It was further aimed to determine the transcriptional organisation such as the TSPs of both the genes (*bca* and *gca*) in *C. glutamicum* using the 5´-RACE method. To obtain higher copy number of the respective transcripts, total RNA was isolated from *C. glutamicum* carrying the pET2-*bca*-promoter and pET2-*gca*-promoter construct for the *bca* and *gca* gene respectively for subsequent

cDNA synthesis and 5′-RACE. Knowing the position of a 5′ transcriptional start point of an RNA molecule is important to identify the immediately flanking regulatory regions and for understanding the regulation of transcription (Kapranov, 2009). It was determined that TSP of the *bca* gene is the first nucleotide of its translational start codon (ATG) and the *bca* gene, therefore, codes for a leaderless transcript. These leaderless mRNAs that start with the AUG initiator codon are present in bacteria, archaea and eukaryotes (Moll et al., 2002). Leaderless transcripts are also frequently found in *C. glutamicum* genes (Pátek et al., 2003). The RNA sequence analysis (RNAseq) has revealed about 33% of mRNAs as leaderless in *C. glutamicum*. Furthermore, it was also observed that these leaderless mRNA have AUG (about 79%) or GUG (about 21%) start codon (Pfeifer-Sancar et al., 2013). For leaderless mRNAs that start with the AUG codon, there is no evidence of signals for recruitment of ribosomes downstream of the 5′-terminal AUG, which appears to be the only constant and necessary element (Moll et al., 2002). It has been shown in *E. coli* that the ratio of initiation factors IF2 and IF3 plays a final role in initiation of leaderless mRNA translation, which shows that the efficiency of translation of this mRNA class can be altered, dependent on availability of the translational machinery components (Grill et al., 2001; Moll et al., 2002). TSP for the *gca* gene was determined to be at -20 relative to the first nucleotide of the translational start codon (ATG) of cg0154 gene, which is located upstream of the *gca* gene. Location of the TSP for *gca* gene upsteam of cg0154 was based on previous results of the RNA sequence analysis (RNAseq) (Pfeifer-Sancar et al., 2013) which revealed that both the genes (cg0154-*gca*) co-transcribe and form an operon. The RNA-seq technology based on high-throughput sequencing of whole transcriptomes have offered new possibilities for genome-wide analysis of the transcripts (van Vliet, 2010; Pátek et al., 2013). Kaur et al. (2010) also observed that the gene coding for γ-CA co-transcribe with *argC* gene in *Azospirillium brasilense* Sp7, and suggested its role in biosynthesis of arginine instead of CO_2 hydration, as the protein expressed from this gene was observed to lack CO_2 hydration activity.

The determination of TSP as well as the respective promoter motif and its characterization in terms of activity make it possible to use promoters for biotechnological applications (Pátek et al., 2013). In addition to determination of TSPs for the *bca* and *gca* gene, it was also aimed to investigate the regulation of both of these genes in relation to carbon sources as well as transcriptional regulators, in *C. glutamicum*. The activities of promoters were determined for the *bca* and *gca* genes in cells grown with different carbon sources namely glucose and acetate. It

was observed that for both the genes, the activities of their promoters were higher on glucose as compared to acetate, when used as sole source of carbon and energy. Furthermore, activity of the *bca* promoter was observed to be about 15 times higher than activity of the *gca* promoter on glucose or acetate as carbon source in *C. glutamicum*. This lower level of expression of *gca* as compared to *bca* gene is also consistent with the results of Mitsuhashi et al. (2004) where Northern blot analysis showed that the transcript level of the *gca* was not detectable, possibly because the expression of *gca* is very low or tightly regulated due to which the transcript is not expressed with *bca* simultaneously under growth conditions. However, our results suggested that both the *bca* and *gca* genes are subject to carbon source-dependent regulation. Furthermore, for both the genes, the reduced promoter activity in acetate-grown cells than glucose-grown cells can be attributed to reduce CO_2/HCO_3^- demand and reduce need of oxaloacetate anaplerosis in *C. glutamicum*. In glucose-grown cells of *C. glutamicum*, the oxaloacetate anaplerosis is mediated by enzymes phosphoenolpyruvate carboxylase and pyruvate carboxylase. However, about 90% of the total oxaloacetate synthesis is contributed by pyruvate carboxylase in glucose-grown cells (Sauer and Eikmanns, 2005). Furthermore, it has been observed that pyruvate carboxylase has no significance for anaplerosis in acetate-grown cells of *C. glutamicum* (Peters-Wendisch et al., 1997). However the crucial role of *bca* seems not only confined to replenishment of oxaloacetate or other TCA cycle intermediates as Mitsuhashi et al. (2004) observed the *bca* gene to be indispensable for growth and complementation of the complex medium with end products of carboxylation reactions such as oxaloacetate, glutamate, aspartate, succinate, oleate, palmeate and adenine did not restore growth of *C. glutamicum*-Δ*bca*. It is also important to mention here that many bacteria are capable to utilize C4-carboxlates as sources of carbon, however, *C. glutamicum* is unable to use intermediates of TCA cycle such as fumarate, succinate and L-malate as sole source of carbon (Youn et al., 2008) but can use citrate as sole carbon source and can also co-utilize it with glucose (Polen et al., 2007).

Furthermore, it was also aimed to determine the effect of pH on the observed carbon source-dependent regulation of the *bca* gene. A comparison of the *bca* promoter activity was made in *C. glutamicum* grown in minimal medium with glucose or acetate as sole carbon source at two different initial adjusted pH of the minimal medium (6.3 and 6.8). However, it was again observed that expression of the *bca* gene was higher on glucose than on acetate in the same proportion as observed previously, under both the pH values of the medium. Furthermore, it was

also observed that the *bca* promoter activity was nearly the same on glucose as carbon source on both pH values (6.3 and 6.8) or on acetate as carbon source at both pH values (6.3 and 6.8). This proved that the observed carbon source-dependent regulation of the *bca* gene is independent of the initial adjusted pH of the medium, in the range of 6.3 to 6.8 in *C. glutamicum*. However, the pH of glucose-grown cultures was observed to be about 7.8 and that of acetate-grown cultures as 8.5 at the time of harvest (mid-exponential phase of growth; OD_{600} of about 5), irrespective of the initially adjusted pH of the medium as 6.3 or 6.8 for both carbon sources. Follmann et al. (2009) showed that in *C. glutamicum*, growth rate was optimal at pH of 7 to 8.5. The growth rate was, however, observed to decrease at an external pH below 6 and above 9 and showed effective homeostasis of internal pH at around 7.5 in the range of 6 to 9 of external pH. Furthermore, the growth was observed to be not significant at a pH of 4 as well as 10.5.

The RNA sequence analysis (RNAseq) has also revealed an anti-sense RNA (asRNA) within the *bca* gene in *C. glutamium* (Pfeifer-Sancar et al., 2013). The high-throughput transcriptome analyses has reported asRNAs in many bacteria, however the number of functional asRNAs is unknown as only few have been characterized (Lybecker et al., 2014). It was, therefore, attempted to characterize the expression of the asRNA within the *bca* gene in terms of expression via determining the activity of its promoter region. An upstream region (297 bp) as promoter of the asRNA, was cloned upstream of *cat* gene coding for CAT enzyme in the pET2 promoter probe vector creating pET2-asRNA-promoter construct, for expression analysis in *C. glutamicum*. It was observed that the promoter region of asRNA shows very low activity on either glucose or acetate as sole carbon source at mid-exponential growth phase in *C. glutamicum*, confirming the expression of the asRNA. In bacteria, regulatory RNAs are categorized into three main groups: (1) those present in the 5′ UTR region of the mRNA such as riboswtiches, pH sensors and thermosensors (2) the *trans*-encoded small RNAs (sRNAs) that are involved in regulation of one or several other target genes that are located somewhere else on the chromosome and (3) *cis*-encoded antisense RNAs (asRNAs), having complementarity with their target genes, located on the opposite strand of DNA of the same locus in the genome (Sesto et al., 2013). These asRNAs that are coded from the DNA strand opposite a gene, have the capability to interact and base pair with the respective sense RNA of the target gene (Thomason and Storz, 2010). The function of non-coding RNAs in regulation is related with the activity of RNases. The non-coding RNA interacts with its target transcript and can then recruit RNase and causes

degradation of the transcript (Sesto et al., 2013). Bacterial antisense RNAs interact with translation initiation regions (TIRs) and offer competition to the binding of ribosome, therefore repress translation and also cause the mRNA degradation. Furthermore, antisense RNA can also targets a sequence downstream of the TIR, which is within the coding region and causes the decay of mRNA (Wagner, 2009). On the other hand, the non-coding RNA can also increase stability of target RNA by preventing recognition of the transcript by RNases and protecting it from degradation (Sesto et al., 2013). In the present work, the asRNA within the *bca* gene might be involved in regulation of the *bca* gene and/or genes located elsewhere in the genome of *C. glutamicum*. Although the mechanism of this asRNA is not known so far, but as the asRNA transcribes within the coding region downstream of the TIR of the *bca* gene, the mechanism of this asRNA can be speculated based on a similar example where Hfq associated MicC sRNA in *Salmonella typhimurium* target the *ompD* mRNA within the coding sequence (CDS) and causes its repression (Pfeiffer et al., 2009).

In the present work, a deletion of the *gca* gene in *C. glutamicum* was also created. In the genome of *C. glutamicum*, the native *gca* (561 bp) gene was replaced with a truncated version of *gca* having deletion of the internal region of the gene (about 400 bp), by allelic exchange using suicide vector pK19*mobsac*B. Genome editing which includes gene knockout, knock-in and allelic exchange, is important technology for engineering of bacterial genome (Nakashima and Miyazaki, 2014). The *C. glutamicum*-Δ*gca* was also functionally characterized such as determination of its growth pattern in comparison of wild-type *C. glutamicum* as well as possible complementation of the function of *gca* by the *bca* gene in the absence of a functional Gca protein in *C. glutamicum*-Δ*gca*. It was observed that deletion of the *gca* gene is dispensable and the growth pattern of Δ*gca* mutant was observed to be nearly the same as that of wild-type *C. glutamicum*. Furthermore, the activity of the *bca* promoter was also observed to be the nearly the same in Δ*gca* and wild-type *C. glutamicum* which showed that absence of a functional Gca protein has no effect on expression of the *bca* gene in *C. glutamicum*. This is also consistent with previous results of Mitsuhashi et al. (2004) which showed the deletion of *gca* gene to be dispensable for growth of *C. glutamicum*.

To study regulation of the *bca* gene by transcriptional regulators, the proteins binding to the *bca* promoter region were enriched by DNA affinity chromatography and then subsequently identified using MALDI-TOF mass spectrometry. However, no transcriptional regulator protein was observed to bind to the *bca* promoter region in cell extract of *C. glutamicum* harvested at mid-exponential growth phase with glucose as source of carbon. Identification of proteins is performed by methods such as peptide mass fingerprinting by MALDI-MS and sequencing by tandem mass spectrometry after separation by two-dimensional gel electrophoresis, SDS-PAGE or liquid chromatography (Thiede et al., 2005). The transcriptional regulators bind to the DNA sequences in the promoter region of genes of *C. glutamicum* (Pátek et al., 2013). Based on the regulatory hierarchy level, transcriptional regulators have been categorized as "global" which are involved in regulation of expression of large number of genes directly or indirectly via cross-regulation, "master" which regulate expression of genes that are related in terms of function and belong to the respective functional module of regulatory network and "local" which control only few genes that are related in terms of function (Schröder and Tauch, 2010; Pátek et al., 2013). Upon binding of transcription factor to a promoter, it can either activate or repress initiation of transcription. Transcription factors function as sole activators or repressors, however, function of some can be either of these depending upon the target promoter (Browning and Busby, 2004).

The RamA, Regulator of Acetate Metabolism, is a LuxR type of global transcriptional regulator (Cramer et al., 2006). The RamA protein (281 amino acids) is encoded by the *ramA* (locus tag cg2831) gene with transcriptional start point (TSP) located 71bp upstream of the translational start codon (Cramer and Eikmanns, 2007). The C-terminus region of LuxR-family of DNA-binding protein contains a specific regulatory helix-turn-helix (HTH) domain, called the LuxR. The possible involvement of these LuxR regulators in virulence also makes them potential targets in disease causing microorganisms (Santos et al., 2012). RamA is indispensable for growth of acetate-grown C. *glutamicum* however, the *C. glutamicum*-Δ*ramA* mutant showed lower growth as compared to wild-type also with glucose as carbon source, indicating its role also in regulating expression of enzymes involved in glucose metabolism (Cramer et al., 2006). RamA has been shown to negatively auto-regulate its own expression (Cramer and Eikmanns, 2007). In addition to this negative auto-regulation, other regulators such as cAMP-dependent GlxR and the SugR act as activator and repressor for expression of the *ramA* gene respectively (Toyoda et al., 2013). The RamA regulator can act as activator or repressor for the target gene as shown by its

repressive effect on expression of the *pck* gene, coding for C-4 decarboxylating enzyme PEP carboxykianse, in glucose-grown cells and activating effect on the *pck* expression in cells grown in minimal medium with glucose plus acetate or in complex medium in *C. glutamicum* and this dual regulatory function might explained by interaction or competition of the RamA with other regulator or effector, under specific conditions. (Auchter et al., 2011a; Klaffl et al., 2013).

RamA is expected to have much broader role in regulation of further genes of central metabolism (Auchter et al., 2011a). As the comparative transcriptomic analysis showed the transcript level of the *bca* gene to be about 2.5 fold higher in Δ*ramA* mutant as compared to wild-type *C. glutamicum* (Auchter et al., 2011a), a comparison of the *bca* gene expression was performed and it was observed that activity of the *bca* promoter was about four times higher in Δ*ramA* mutant as compared to wild-type *C. glutamicum*. Furthermore, according to the RamA binding motif A/T/C-GGGG-N as identified by Auchter et al. (2011a), several potential RamA binding sites in the promoter region of *bca* gene were observed and it was likely that RamA protein binds to the *bca* promoter region. To test this, the His-tagged RamA protein was over-produced and purified and then purified His-tagged RamA protein was observed to bind to the promoter region of the *bca* gene in bandshift experiments. The electrophoretic mobility shift assay method is used for detection of protein complexes with nucleic acids, based on the slower mobility of protein-nucleic acids complex than the respective free nucleic acids in electrophoresis (Hellman and Fried, 2007). To conclude, the higher *bca* transcript level in Δ*ramA* than in wild-type *C. glutamicum* (Auchter et al., 2011a), higher activity of *bca* promoter in Δ*ramA* than in the wild-type *C. glutamicum* as well as binding of the purified His-tagged RamA to the *bca* promoter region showed that the RamA acts as a negative transcriptional regulator for the expression of the *bca* gene, in *C. glutamicum*.

5 References

Arndt, A and B. J. Eikmanns. 2008. Regulation of carbon metabolism in *Corynebacterium glutamicum*. In: Burkovski, A. (Ed.), Corynebacteria: Genomics and Molecular Biology. Caister Academic Press, Norfolk, UK, pp. 155–182.

Auchter, M., A. Cramer, A. Huser, C. Ruckert, D. Emer, P. Schwarz, A. Arndt, C. Lange, J. Kalinowski, V. F. Wendisch and B. J. Eikmanns. 2011a. RamA and RamB are global transcriptional regulators in *Corynebacterium glutamicum* and control genes for enzymes of the central metabolism. Journal of Biotechnology 154; 126‑139.

Auchter, M., T. Laslo, C. Fleischer, L. Schiller, A. Arndt, L. Gaigalat, J. Kalinowski and B. J. Eikmanns. 2011b. Control of *adhA* and *sucR* expression by the SucR regulator in *Corynebacterium glutamicum*. Journal of Biotechnology 152: 77–86.

Blombach, B and B. J. Eikmanns. 2011. Current knowledge on isobutanol production with *Escherichia coli*, *Bacillus subtilis* and *Corynebacterium glutamicum*. Bioengineered Bugs 2: 346-350.

Blombach, B., M. E. Schreiner, J. Holatko, T. Bartek, M. Oldiges and B. J. Eikmanns. 2007. L-Valine production with pyruvate dehydrogenase complex-deficient *Corynebacterium glutamicum*. Applied and Environmental Microbiology 73: 2079–2084.

Blombach, B., M. E. Schreiner, T. Bartek, M. Oldiges and B. J. Eikmanns. 2008. *Corynebacterium glutamicum* tailored for high-yield L-valine production. Applied Microbiology and Biotechnology 79:471–479.

Bornhorst, J. A and J. J. Falke. 2000. Purification of proteins using polyhistidine affinity tags. Methods in Enzymology 326: 245-254.

Bott, M. 2007. Offering surprises: TCA cycle regulation in *Corynebacterium glutamicum*. Trends in Microbiology 15: 417-425.

Browning, D. F. and S. J. W. Busby. 2004. The regulation of bacterial transcription initiation. Nature Reviews Microbiology 2: 57-65.

Buchholz, J., A. Schwentner, B. Brunnenkan, C. Gabris, S. Grimm, R. Gerstmeir, R. Takors, B. J. Eikmanns and B. Blombach. 2013. Platform engineering of *Corynebacterium glutamicum* with reduced pyruvate dehydrogenase complex activity for improved production of L-Lysine, L-Valine, and 2 Ketoisovalerate. Applied and Environmental Microbiology 79: 5566–5575.

Bückle-Vallant, V., F. S. Krause, S. Messerschmidt and B. J. Eikmanns. 2014. Metabolic engineering of *Corynebacterium glutamicum* for 2-ketoisocaproate production. Applied Microbiology and Biotechnology 98: 297–311.

Burghout, P., L. E. Cron, H. Gradstedt, B. Quintero, E. Simonetti, J. J. E. Bijlsma, H. J. Bootsma and P. W. M. Hermans. 2010. Carbonic Anhydrase is essential for *Streptococcus pneumoniae* growth in environmental ambient air. Journal of Bacteriology 192: 4054–4062.

Cheng, Y., Y. Zhou, L. Yang, C. Zhang, Q. Xu, X. Xie and N. Chen. 2013. Modification of histidine biosynthesis pathway genes and the impact on production of L-histidine in *Corynebacterium glutamicum*. Biotechnology Letters 35: 735–741.

Chong, S., F. B. Mersha, D. G. Comb, M. E. Scott, D. Landry, L. M. Vence, F. B. Perler, J. Benner, R. B. Kucera, C. A. Hirvonen, J. J. Pelletier, H. Paulus and M. Q. Xu. 1997. Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. Gene 192: 271–281.

Chong, S., G. E. Montello, A. Zhang, E. J. Cantor, W. Liao, , M. Q. Xu and J. Benner. 1998. Utilizing the C-terminal cleavage activity of a protein splicing element to purify recombinant proteins in a single chromatographic step. Nucleic Acids Research 26: 5109–5115.

Cramer, A and B. J. Eikmanns. 2007. RamA, the transcriptional regulator of acetate metabolism in *Corynebacterium glutamicum*, is subject to negative autoregulation. Journal of Molecular Microbiology and Biotechnology 12: 51–59.

Cramer, A., R. Gerstmeir, S. Schaffer, M. Bott and B. J. Eikmanns. 2006. Identification of RamA, a novel LuxR-type transcriptional regulator of genes involved in acetate metabolism of *Corynebacterium glutamicum*. Journal of Bacteriology 188: 2554–2567.

di Guan, C., P. Li, P. D. Riggs and H. Inouye. 1988. Vectors that facilitate the expression and purification of foreign peptides in *Escherichia coli* by fusion to maltose-binding protein. Gene 67: 21-30.

Dower, W. J., J. F. Miller and C. W. Ragsdale. 1988. High efficiency transformation of *E.coli* by high voltage electroporation. Nucleic Acids Research 13: 6127–6145.

Eikmanns, B. J., M. Metzger, D. Reinscheid, M. Kircher and H. Sahm. 1991. Amplification of three threonine biosynthesis genes in *Corynebacterium glutamicum* and its influence on carbon flux in different strains. Applied Microbiology and Biotechnology 34: 617-622.

Eggeling, L., and M. Bott. 2005. *Handbook of Corynebacterium glutamicum.* Boca Raton, FL: CRC Press.

Follmann, M., I. Ochrombel, R. Krämer, C. Trötschel, A. Poetsch, C. Rückert, A. Hüser, M. Persicke, D. Seiferling, J. Kalinowski and K. Marin. 2009. Functional genomics of pH homeostasis in *Corynebacterium glutamicum* revealed novel links between pH response, oxidative stress, iron homeostasis and methionine synthesis. BMC Genomics 10: 621.

Frohman, M. A., M. K. Dush and G. R. Martin. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. PNAS 85: 8998-9002.

Gabris, C. 2011. Involvement of xylulose kinase XylB in arabitol metabolism of *Corynebacterium glutamicum*. Diploma thesis. Institute for Microbiology and Biotechnology, University of Ulm.

Gai, C. S., J. Lu, C. J. Brigham, A. C. Bernardi and A. J. Sinskey. 2014. Insights into bacterial CO_2 metabolism revealed by the characterization of four carbonic anhydrases in *Ralstonia eutropha* H16. AMB Express 4: 2.

Gerstmeir, R., A. Cramer, P. Dangel, S. Schaffer and B. J. Eikmanns. 2004. RamB, a novel transcriptional regulator of genes involved in acetate metabolism of *Corynebacterium glutamicum*. Journal of Bacteriology 186: 2798–2809.

Green, M. R and J. Sambrook. 2012. Molecular Cloning, a laboratory maual, 4th edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor New York, USA.

Grill, S., I. Moll, D. Hasenöhrl, C. O. Gualerzi and U. Bläsi. 2001. Modulation of ribosomal recruitment to 5´- terminal start codons by translation initiation factors IF2 and IF3. FEBS Letters 495: 167-171.

Guilloton, M. B., A. F. Lamblin, E. I. Kozliak, M. Gerami-Nejad, C. Tu, D. S. Verman, P. M. Anderson and J. A. Fuchs. 1993. A Physiological role for cyanate-induced carbonic anhydrase in *Escherichia coli*. Journal of Bacteriology 175: 1443-1451.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. Journal of Molecular Biology 166: 557-580.

Hasegawa, S., M. Suda, K. Uematsu, Y. Natsuma, K. Hiraga, T. Jojima, M. Inui and H. Yukawa. 2013. Engineering of *Corynebacterium glutamicum* for high-yield L-Valine production under oxygen deprivation conditions. Applied and Environmental Microbiology 79: 1250–1257.

Hellman, L. M and M. G. Fried. 2007. Electrophoretic mobility shift assay (EMSA) for detecting protein–nucleic acid interactions. Nature Protocols 2: 1849-1861.

Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77: 51-59.

Hüser, A. T., C. Chassagnole, N. D. Lindley, M. Merkamm, A. Guyonvarch, V. Elišáková, M. Pa´tek, J. Kalinowski, I. Brune, A. Pühler and A. Tauch. 2005. Rational design of a *Corynebacterium glutamicum* pantothenate production strain and its characterization by metabolic flux analysis and genome-wide transcriptional profiling. Applied and Environmental Microbiology 71: 3255–3268.

Inui, M., H. Kawaguchi, S. Murakami, A. A. Vertès and H. Yukawa. 2004. Metabolic engineering of *Corynebacterium glutamicum* for fuel ethanol production under oxygen-deprivation conditions. Journal of Molecular Microbiology and Biotechnology 8: 243–254.

Jo, S-J., M. Maeda, T. Ooi and S. Taguchi. 2006. Production system for biodegradable polyester polyhydroxybutyrate by *Corynebacterium glutamicum.* Journal of Bioscience and Bioengineering 102: 233–236.

Kapranov, P. 2009. From transcription start site to cell biology. Genome Biology 10: 217.

Kaur, S., M. N. Mishra and A. K. Tripathi. 2009. Regulation of expression and biochemical characterization of a β-class carbonic anhydrase from the plant growth-promoting rhizobacterium, *Azospirillum brasilense* Sp7. FEMS Microbiology Letters 299: 149–158.

Kaur, S., M. N. Mishra and A. K. Tripathi. 2010. Gene encoding γ-carbonic anhydrase is cotranscribed with *arg*C and induced in response to stationary phase and high CO_2 in *Azospirillum brasilense* Sp7. BMC Microbiology 10: 184.

Kind, S., W. K. Jeong, H. Schröder and C. Wittmann. 2010. Systems-wide metabolic pathway engineering in *Corynebacterium glutamicum* for bio-based production of diaminopentane. Metabolic Engineering 12: 341–351.

Kinoshita, S., S. Udaka, and M. Shimono. 1957. Studies on the amino acid fermentation. I. Production of L-glutamic acid by various microorganisms. Journal of General and Applied Microbiology 3:193–205.

Klaffl, S., M. Brocker, J. Kalinowski, B. J. Eikmanns and M. Bott. 2013. Complex regulation of the phosphoenolpyruvate carboxykinase gene *pck* and characterization of its GntR-type regulator IolR as a repressor of *myo*-Inositol utilization genes in *Corynebacterium glutamicum*. Journal of Bacteriology 195: 4283– 4296.

Kornberg, H. L. 1965. Anaplerotic sequences in microbial metabolism. Angewandte Chemie International Edition 4: 558-565.

Krause, F. S., B. Blombach and B. J. Eikmanns. 2010. Metabolic Engineering of *Corynebacterium glutamicum* for 2-Ketoisovalerate Production. Applied and Environmental Microbiology 76: 8053–8061.

Kulis-Horn, R. K., M. Persicke and J. Kalinowski. 2014. Histidine biosynthesis, its regulation and biotechnological application in *Corynebacterium glutamicum*. Microbial Biotechnology 7: 5–25.

Kumar, R. S. S., W. Hendrick, J. B. Correll, A. D. Patterson, S. B. Melville and J. G. Ferry. 2013. Biochemistry and physiology of the β class carbonic anhydrase (Cpb) from *Clostridium perfringens* strain 13. Journal of Bacteriology 195: 2262–2269.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Liebl, W., A. Beyeri, B. Schein, U. Stillner and K. H. Schleifer. 1989. High efficiency electroporation of intact *Corynebacterium glutamicum* cells. FEMS Microbiology Letters 65: 299-304.

Lotlikar, S. R., S. Hnatusko, N. E. Dickenson, S. P. Choudhari, W. L. Picking and M. A. Patrauchan. 2013. Three functional β-carbonic anhydrases in *Pseudomonas aeruginosa* PAO1: role in survival in ambient air. Microbiology 159: 1748–1759.

Lybecker, M., B. Zimmermann, I. Bilusic, N. Tukhtubaeva and R. Schroeder. 2014. The double-stranded transcriptome of *Escherichia coli*. PNAS 111: 3134–3139.

Maina, C. V., P. D. Riggs, A. G. Grandea III, B. E. Slatko, L. S. Moran, J. A. Tagliamonte, L. A. McReynolds and C. di Guan. 1988. An *Escherichia coli* vector to express and purify foreign proteins by fusion to and separation from maltose-binding protein. Gene 74: 365-373.

Merlin, C., M. Masters, S. McAteer and A. Coulson. 2003. Why is carbonic anhydrase essential to *Escherichia coli*? Journal of Bacteriology 185: 6415–6424.

Mitsuhashi, S., J. Ohnishi, M. Hayashi and M. Ikeda. 2004. A gene homologous to β-type carbonic anhydrase is essential for the growth of *Corynebacterium glutamicum* under atmospheric conditions. Applied Microbiology and Biotechnology 63: 592–601.

Moll, I., S. Grill, C. O. Gualerzi and U. Bläsi. 2002. Leaderless mRNAs in bacteria: surprises in ribosomal recruitment and translational control. Molecular Microbiology 43: 239–246.

Nakashima, N and K. Miyazaki. 2014. Bacterial cellular engineering by genome editing and gene silencing. International Journal of Molecular Sciences 15: 2773-2793.

Nguyen, A. Q. D., J. Schneider, G. K. Reddy and V. F. Wendisch. 2015. Fermentative production of the diamine putrescine: System metabolic engineering of *Corynebacterium glutamicum*. Metabolites 5: 211-231.

Nishimori, I., S. Onishi , H. Takeuchi, and C. T. Supuran. 2008. The α and β classes carbonic anhydrases from *Helicobacter pylori* as novel drug targets. Current Pharmaceutical Design 14: 622-630.

Papagianni, M. 2012. Recent advances in engineering the central carbon metabolism of industrially important bacteria. Microbial Cell Factories 11: 50.

Park, H., B. Song and F. M. M. Morel. 2007. Diversity of the cadmium containing carbonic anhydrase in marine diatoms and natural waters. Environmental Microbiology 9: 403–413.

Park, S. H., H. U. Kim, T. Y. Kim, J. S. Park, S. S. Kim and S. Y. Lee. 2014. Metabolic engineering of *Corynebacterium glutamicum* for L-arginine production. Nature Communications 5: 4618.

Parks, T. D., K. K. Leuther, E. D. Howard, S. A. Johnston and W. G. Dougherty. 1994. Release of proteins and peptides from fusion proteins using a recombinant plant virus proteinase. Analytical Biochemistry 216: 413–417.

Patek, M., J. Nesvera, A. Guyonvarch, O. Reyes and G. Leblon. 2003. Promoters of *Corynebacterium glutamicum*. Journal of Biotechnology 104: 311-323.

Pátek, M., J. Holátko, T. Busche, J. Kalinowski and J. Nešvera. 2013. *Corynebacterium glutamicum* promoters: a practical approach. Microbial Biotechnology 6: 103–117.

Peters-Wendisch, P. G., V. F. Wendisch, S. Paul, B. J. Eikmanns and H. Sahm. 1997. Pyruvate carboxylase as an anaplerotic enzyme in *Corynebacterium glutamicum*. Microbiology 143: 1095-1103.

Pfeifer-Sancar, K., A. Mentz, C. Rückert and J. Kalinowski. 2013. Comprehensive analysis of the *Corynebacterium glutamicum* transcriptome using an improved RNAseq technique. BMC Genomics 14: 888.

Pfeiffer, V., K. Papenfort, S. Lucchini, J. C. D. Hinton and J. Vogel. 2009. Coding sequence targeting by MicC RNA reveals bacterial mRNA silencing downstream of translational initiation. Nature Structural & Molecular Biology 16: 840-846.

Polen, T., D. Schluesener, A. Poetsch, M. Bott and V. F. Wendisch. 2007. Characterization of citrate utilization in *Corynebacterium glutamicum* by transcriptome and proteome analysis. FEMS Microbiology Letters 273: 109–119.

Rocco, C. J., K. L. Dennison, V. A. Klenchin, I. Rayment and J. C. Escalante-Semerena. 2008. Construction and use of new cloning vectors for the rapid isolation of recombinant proteins from *Escherichia coli*. Plasmid 59: 231–237.

Rosenberg, A. H., B. N. Lade, D-S. Chui, S-W. Lin, J. J. Dunn and F. W. Studier. 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. Gene 56: 125–135.

Rowlett, R. S. 2010. Structure and catalytic mechanism of the β-carbonic anhydrases. Biochimica et Biophysica Acta 1804: 362–373.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487-491.

Santos, C. L., M. Correia-Neves, P. Moradas-Ferreira and M. V. Mendes. 2012. A Walk into the LuxR regulators of actinobacteria: Phylogenomic distribution and functional diversity. Plos One 7: e46758.

Sakai, S., Y. Tsuchida, S. Okino, O. Ichihashi, H. Kawaguchi, T. Watanabe, M. Inui and H. Yukawa. 2007. Effect of lignocellulose-derived inhibitors on growth of and ethanol production by growth-arrested *Corynebacterium glutamicum* R. Applied and Environmental Microbiology 73: 2349–2353.

Sauer, U. and B. J. Eikmanns. 2005. The PEP–pyruvate–oxaloacetate node as the switch point for carbon flux distribution in bacteria. FEMS Microbiology Reviews 29: 765–794.

Schröder, J and A. Tauch. 2010. Transcriptional regulation of gene expression in *Corynebacterium glutamicum*: the role of global, master and local regulators in the modular and hierarchical gene regulatory network. FEMS Microbiology Reviews 34: 685–737.

Schäfer, A., A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach and A. Pühler. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutumicum.* Gene 145: 69-73.

Sesto, N., O. Wurtzel, C. Archambaud, R. Sorek and P. Cossart. 2013. The excludon: a new concept in bacterial antisense RNA-mediated gene regulation. Nature Reviews Microbiology 11: 75-82.

Shaw, W. V. 1975. Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. Methods in Enzymol **43:**737–755.

Smith, K. S and J. G. Ferry. 2000. Prokaryotic carbonic anhydrases. FEMS Microbiology Reviews 24: 335-366.

Smith, K. S., C. Jakubzick, T. S. Whittam and J. G. Ferry. 1999. Carbonic anhydrase is an ancient enzyme widespread in prokaryotes. PNAS 96: 15184–15189.

Song, Y., K. Matsumoto, M. Yamada, A. Gohda, C. J. Brigham, A. J. Sinskey and S. Taguchi. 2012. Engineered *Corynebacterium glutamicum* as an endotoxin-free platform strain for lactate-based polyester production. Applied Microbiology and Biotechnology 93: 1917–1925.

Studier, F.W and B. A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. Journal of Molecular Biology 189: 113–130.

Studier, F. W., A. H. Rosenberg, J. J. Dunn and J. W. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods in Enzymology 185: 60–89.

Supuran, C. T. 2011. Bacterial carbonic anhydrases as drug targets: toward novel antibiotics? Frontiers in Pharmacology 2: 34.

Tartof, K. D and C. A. Hobbs. 1987. Improved media for growing plasmid and cosmid clones. BRL Focus 9: 12-14.

Thiede, B., W. Höhenwarter, A. Krah, J. Mattow, M. Schmid, F. Schmidt and P. R. Jungblut. 2005. Peptide mass fingerprinting. Methods 35: 237–247.

Thomason, M. K and G. Storz. 2010. Bacterial antisense RNAs: How many are there and what are they doing? Annual Review of Genetics. 44: 167–188.

Toyoda, K., H. Teramoto, W. Gunji, M. Inui and H. Yukawa. 2013. Involvement of regulatory interactions among global regulators GlxR, SugR, and RamA in expression of *ramA* in *Corynebacterium glutamicum*. Journal of Bacteriology 195: 1718–1726.

Tripp, B. C., K. Smith and J. G. Ferry. 2001. Carbonic Anhydrase: New insights for an ancient enzyme. The Journal of Biological Chemistry 276: 48615–48618.

Ueda, K., H. Nishida and T. Beppu. 2012. Dispensabilities of carbonic anhydrase in Proteobacteria. International Journal of Evolutionary Biology 2012: 324549.

van der Rest, M. E., C. Lange and D. Molenaar. 1999. A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. Applied Microbiology and Biotechnology 52: 541-545.

van Vliet, A. H. M. 2010. Next generation sequencing of microbial transcriptomes: challenges and opportunities. FEMS Microbiology Letters 302: 1-7.

Vasicová, P., Z. Abrhámová, J. Nesvera, M. Pátek, H. Sahm and B. Eikmanns. 1998. Integrative and autonomously replicating vectors for analysis of promoters in *Corynebacterium glutamicum*. Biotechnology Techniques 12: 743–746.

Vogt, M., S. Haas, T. Polen, J. V. Ooyen and M. Bott. 2015. Production of 2-ketoisocaproate with *Corynebacterium glutamicum* strains devoid of plasmids and heterologous genes. Microbial Biotechnology 8: 351–360.

Vullo, D., R. S. S. Kumar, A. Scozzafava, C. Capasso, J. G. Ferry and C. T. Supuran. 2013. Anion inhibition studies of a β-carbonic anhydrase from *Clostridium perfringens*. Bioorganic & Medicinal Chemistry Letters 23: 6706–6710.

Wagner, E. G. H. 2009. Kill the messenger: bacterial antisense RNA promotes mRNA decay. Nature Structural & Molecular Biology 16: 804 – 806.

Wieschalka, S., B. Blombach and B.J. Eikmanns. 2012. Engineering *Corynebacterium glutamicum* for the production of pyruvate. Applied Microbiology and Biotechnology 94: 449–459.

Woodman, M. E. 2008. Direct PCR of intact bacteria (Colony PCR). Current Protocols in Microbiology 9: A.3D.1-A.3D.6.

Youn, J. W., E. Jolkver, R. Krämer, K. Marin and V. F. Wendisch. 2008. Identification and characterization of the dicarboxylate uptake system DccT in *Corynebacterium glutamicum*. Journal of Bacteriology 190: 6458–6466.

Zimmerman, S. A and J. G. Ferry. 2008. The β and γ classes of carbonic anhydrase. Current Pharmaceutical Design 14: 716-721.

Abbreviations

%	percent
µg	microgram
µl	microliter
A	adenine
bp	base pair
BCA	bicinchoninic acid
BSA	bovine serum albumin
C	cytosine
Ca	calcium
CAT	chloramphenicol acetyltransferase (enzyme)
cat	chloramphenicol acetyltransferase (gene)
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
Co^{2+}	cobalt (II) ion
CoA	coenzyme A
Cu^{2+}	copper (II) ion
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
et al.	et alii (and others)
Fig.	figure
g	gram
G	guanine
h	hour
H^+	proton

H_2O	Water
HCO_3^-	bicarbonate
k	kilo
K	potassium
kDa	kilodalton
Km^r	kanamycin resistance
l	liter
Mg	magnesium
MALDI	Matrix Assisted Laser Desorption/Ionization
mg	miligram
min	minute
ml	milliliter
mM	milimolar
mol	mole
mRNA	messenger ribonucleic acid
N_2	nitrogen
Na	sodium
Ni^{2+}	nickel (II) ion
NTA	nitrilotriacetic acid
ng	nanogram
^oC	degree celsius
OD_{600}	optical density at wavelength of 600 nm
OH^-	hydroxide
Ori	origin of replication
PAGE	polyacrylamide gel electrophoresis

pH	negative logarithm of the hydrogen ion concentration
Poly [d (I-C)]	Poly-deoxy-inosinic-deoxy-cytidylic acid
Poly-(A)	polyadenylation
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
RT	reverse transcriptase
s	second
SDS	sodium dodecyl sulfate
T	thymine
TEV	Tobacco Etch Virus
TOF	Time Of Flight
Taq	*Thermus aquaticus*
TdT	terminal deoxynucleotidyl transferase
TSP	transcriptional start point
U	unit
v	volume
v/v	volume per volume
W	weight
w/v	weight per volume
Zn^{2+}	zinc (II) ion
µl	microliter
Ω	ohm (electric resistance)

Poster presentations

Adnan Shah and Bernhard J. Eikmanns. 2015. "Transcriptional analysis of genes putatively coding for carbonic anhydrase in *Corynebacterium glutamicum*."

Annuanl Conference of the Association for General and Applied Microbiology (VAAM) (1.3.2015 - 4.3.2015) held at Marburg, Germany.

Contribution of others to this work

The construction of pET2-asRNA-promoter and expression analysis of the asRNA on different carbon sources by determination of specific CAT enzyme activites using pET2-asRNA-promoter in *C. glutamicum* as well as comparison of the *bca* expression in Δ*gca* mutant and wild-type *C. gltuamicum* by determination of specific CAT enzyme activites of the *bca* promoter using construct pET2-*bca*-promoter, was performed in collaboration with David Sachpazidis.

The *C. glutamicum*-Δ*ramA* was provided by Annette Cramer. The pET28a-*ramA* construct and pET-TEV1 vector were provided by Simon Klaffl.

The *C. glutamicum*-Δ*atlR* carrying pET2-P4 and *C. glutamicum* RES167 carrying pET2 vector, used as positive and negative control respectively in determination of specific CAT enzyme activity in this study, were provided by Christina Gabris.

The MALDI-TOF mass spectrometry for identification of protein was performed in collaboration with Forschungszentrum Jülich.

Acknowledgements

I would like to express my sincere gratitude to my supervisor **Prof. Dr. Bernhard Eikmanns** for giving me the opportunity to join his research group under his supervision for my PhD. I, especially, express my sincere regards to him for his inspiring attitude, valuable advice and encouragement throughout my studies. Thanks a lot *Bernd*!

I express my deepest thanks and gratitude to the **HEC** (Higher Education Commission) of Pakistan and **DAAD** (German Academic Exchange Service) for offering financial support to me for my doctoral studies. I am especially thankful to DAAD for being very kind in helping me with administrative matters.

I would like to extend my thanks to **Prof. Dr. Peter Dürre**, Director, Institute of Microbiology and Biotechnology for his appreciable cooperation and kind help in academic and administrative matters.

I pay special thanks to **Dr. Christian Riedel** for his valuable suggestions, constructive discussions and extensive help during my research work. I am also thankful to him for being the second reviewer of this work.

With deep sense of gratitude, I offer thanks to Verena Bückle, Tanja Laslo and Katrin Breitinger for their cooperation, moral support and guidance during my doctoral studies. Being my lab fellows, they have always been very generous to offer their kind help and support to me.

I am also grateful to my other fellows Serin Weinmann, Tobias Hahn, Valeria Tschiginewa, Oliver Goldbeck, Stefan Wieschalka, Sonja Messerschmidt, David Sachpazidis, Karin Riegel and Christina Gabris for their admirable company and help.

I would also like to say thanks to Petra Dangel and Brigitte Zickner for their help and care during my research work. I am thankful to them for their friendly attitude and smiles.

Words are inadequate to express my gratitude to my parents, brother and sisters for their encouragement, moral support and care for me.

Last but not least, I am thankful to ALLAH for successful completion of my PhD and for His blessings on me in Life.

Curriculum vitae

Personal information

Name:	Adnan Shah
Date of birth:	23rd July, 1983
Place of birth:	Malakand

Academic Career:

1999	Secondary School Certificate (S.S.C) at Government High School Malakand
2001	Higher secondary school certificate (H.S.S.C) at OEA, Batkhela
2006	B.S (Hons) (Biotechnology) at University of Malakand
2008	M. Phil (Microbiology) at Quaid-i-Azam University, Islamabad
Nov, 2011- until now	Doctoral thesis at institute of Microbiology and Biotechnology, University of Ulm, Germany

Title: "Transcriptional organisation and regulation of genes encoding carbonic anhydrases in *Corynebacterium glutamicum*"

Declaration

I declare that the present thesis is my own work and all the sources of information and support used for its achievement have been mentioned in the text and in the references. With this, I apply for the doctoral degree (PhD) at the University of Ulm.

Ulm, September 2015

Adnan Shah