Cloning and Characterisation of Carbon Cycle Relevant Enzymes of *R. eutropha* H16

Diplomarbeit

durchgeführt von

Schwendenwein Daniel

Institut für Molekulare Biotechnologie

der Technischen Universität Graz

Leiter: Univ.-Prof. Dipl.-Ing. Dr. techn. Helmut Schwab

Univ.-Prof. Dipl.-Ing. Dr.techn. Helmut Schwab

Mag.rer.nat. Dr.rer.nat.Petra Köfinger

Acknowledgement

There are several people I want and have to thank for their support and encouragement.
First of all I want to thank Prof. Helmut Schwab for the opportunity to write this master thesis and the constant guidance and advice during my work.
Furthermore I want to thank my supervisor Dr. Petra Köfinger for her personal and scientific support through the whole time.
For his ongoing help and chemical advice I also want thank Dr. Jörg Schachner at this point.
My gratitude also belongs to the whole “Team Knallgas” and especially Steffen, for the constant assistance in personal and academic matters in the lab.
My special thanks goes to Liesa, she supported me throughout my whole studies and never lost the confidence in me.
Finally I want to thank my parents and my whole family for their never ending encouragement and trust.

Abstract

Ralstonia eutropha H16 is a Gram-negative, strictly respiratory facultative chemolithoautotrophic bacterium which is able to grow on H_2, O_2 and CO_2 as the sole sources of energy and carbon (Bowien und Kusian 2002). This bacterium is already widely used in different areas of biotechnology like wastewater-treatment or the production of a biopolymer called PHB (Louie et al. 2002; Budde et al. 2011). *R. eutropha* H16 is able to grow to very high cell densities with subsequently high product concentration (Srinivasan et al. 2002). This fact and several other abilities of *R. eutropha* H16 attracted the attention of biotechnological research.

In this diploma thesis the main objective was the cloning and characterisation of the four different carbonic anhydrases which allow the organism to convert CO_2 into carbonate. Besides RuBisCO, PEP-carboxylase (Ppc) and pyruvate-carboxylase (Pyc) are important enzymes for lithoautotrophic growth by CO_2 -fixation but they are not able to use CO_2 as a substrate directly. Carbonic anhydrases are responsible for the faster conversion of CO_2 to $HCO3^-$, which can be used by these carboxylases (Kusian et al. 2002). The ultimate goal of the study is the over expression of carbonic anhydrases to provide a basis for efficient carboxylation reactions and faster growth of *R. eutropha* H16 under lithoautotrophic conditions due to a more efficient CO_2 fixation next to RuBisCO. *R. eutropha* H16 has four carbonic anhydrases, which were identified by literature and bioinformatic approaches. All four carbonic anhydrases of *R. eutropha* H16 that are known up to now were cloned and expressed in *Escherichia coli*. The enzymes were examined in respect of their activity. Several enzyme assays were performed to measure the activity from CO_2 to carbonate as well as the reverse reaction from carbonate to CO_2. All enzymes show low but measurable activity in the direction to carbonate and no activity in the opposite direction. The best results were obtained with a slightly altered assay for quality control that measures the time that is needed for a pH drop due to the generation of H^+ in the reaction.

Zusammenfassung

Ralstonia eutropha H16 ist ein Gram-negatives, fakultativ chemolithoautotrophes Bakterium, welches in der Lage ist auf anorganischen Gasen (H_2, O_2und CO_2) als einzige Energie- und Kohlenstoffquelle zu wachsen (Bowien und Kusian 2002). Kohlenstoff wird dabei über verschiedene Wege in den Metabolismus eingebaut, unter anderem im Calvin-Zyklus über RuBisCO. *R. eutropha* wird bereits in vielen Bereichen der Biotechnologie, wie der Abwasserreinigung oder der Produktion von PHB eines Biopolymers, eingesetzt (Louie et al. 2002; Budde et al. 2011). Aufgrund dieser Fähigkeiten hat die biotechnologische Forschung ihre Aufmerksamkeit auf dieses Bakterium gerichtet.

Diese Forschungsarbeit beschäftigte sich mit der Klonierung, Expression und Charakterisierung von vier unterschiedlichen Carboanhydrasen. Die Aufgabe dieser Enzyme ist eine schnellere Reaktion von CO_2 zu HCO_3^- zu katalysieren, welche von der PEP-Carboxylase (Ppc) und der Pyc-Carboxylase (Pyc) verwendet werden kann, da diese CO_2 nicht direkt verwerten können (Kusian et al. 2002). Diese Carboxylasen stellen einen weiteren Weg dar, über den Kohlenstoff in den Metabolismus eingebracht werden kann.

Ziel dieser Studie ist die Überexpression dieser Carboanhydrasen um eine weitere Basis, neben RuBisCO, für eine effizientere Kohlenstofffixierung und schnelleres Wachstum von *R. eutropha* H16 unter lithotrophen Bedingungen zu bieten. Die vier bereits klassifizierten Carboanhydrasen von *R. eutropha* H16 wurden in dieser Studie mittels PCR und verschiedener Klonierungsmethoden in einen Expressionsvektor eingebracht und danach in *E. coli* Zellen überexpremiert. Im Anschluss wurden diese Enzyme mittels unterschiedlicher Methoden auf ihre Aktivität überprüft. Alle Enzyme zeigten geringe aber messbare Aktivität in Richtung Kohlensäure und keine Aktivität für die Rückreaktion. Die aussagekräftigsten Ergebnisse wurden mit einem Aktivitätstest, der für die Qualitätskontrolle von kommerziellen Carboanhydrasen entwickelt wurde, erhalten. Dieser Test basiert auf der pH-Änderung durch die bei der Reaktion entstehenden H^+-Ionen. Aus der Zeit, die für diese pH-Änderung benötigt wird, kann die Aktivität der Enzyme abgeleitet werden.

Table of contents

1 General Introduction

R. eutropha H16 was discovered in the late 1950's and clustered in the group *Hydrogenomonas* for its ability to utilize hydrogen, a source of energy for its metabolism. The classification of the organism changed over the time from *Hydrogenomonas eutropha* to its present name, C*upriavidus necator,* over several steps of reclassification due to new findings in morphology and genetics (Vandamme 2004). Its natural habitat is mud and soil in general (Wilde 1962). The organism is now divided as part of the *Burkholderiaceae* family in the class of betaproteobacteria. Its genome consists of two chromosomes and one megaplamid and is organized as a multi replicon genome (Pohlmann et al. 2006; Schwartz et al. 2003). In their genome several metabolic pathways are encoded, so the organism is able to use different sources of carbon and energy. The basic functions and housekeeping genes are mostly located on the biggest replication unit, chromosome 1 (4052032 bp), whereas the different genes for alternative carbohydrate pathways are located on the second chromosome (2912490 bp) (Fricke et al. 2009). The megaplasmid (452156 bp) pHG1 contains several features, which allow the organism to fix CO_2 over the Calvin – Benson – Bassham cycle (CBB-cycle). Also located on the megaplasmid is the Hox operon, which allows the organism to oxidize H_2 and therefore use hydrogen as a source of energy to perform the CBB-cycle (Schwartz et al. 2003; Schwartz et al. 1998). This ability allows *R. eutropha* H16 to grow under chemolithoautotrophic conditions. This competence makes this microorganism a model for the fixation of CO_2 and the use H_2 as a source of energy (Schwartz et al. 1998). The genus *Ralstonia* is even able to act as a predator of other microorganisms under nutrient limiting conditions (Makkar und Casida 1987). Since the discovery of its different abilities, it has served as a model organism and object of study in biotechnological research. It is able to synthesize a biopolymer called Polyhydroxybutyrate (PHB), a polyester which offers new opportunities for sustainable plastic as well (Doi und Steinbüchel 2002). PHB is naturally synthesized under physiological stress as a carbohydrate and thus as energy storage (Pötter et al. 2002). Though PHB is the most important bio plastic, several studies were performed to alter its composition or to use a variety of different substrates for production (Verlinden et al. 2011; Haas et al. 2008; Aneja et al. 2009).

1.1 Abilities

Several abilities of the genus *Ralstonia* and *R. eutropha* H16 are already mentioned. Probably the commercially most important ability used on industrial scale is the production of the bio plastic PHB (Potter 2004). This degradable bio plastic is one of the most important replacements for petroleum derived plastic (Doi und Steinbüchel 2002). Because this organism is able to generate huge amounts of this molecule as energy storage, it attracted great interest of the biotechnology companies. The production and purification on laboratory scale is simple and takes not more than a standard laboratory (Steinbüchel 2012). Different studies address the production of this material out of different resources. The most common approach is to use waste or cheap media such as waste starch or plant oil (Verlinden et al. 2011; Haas et al. 2008; Budde et al. 2011). Some studies also focus on alteration of the molecule and changing the properties of the whole material. This alteration is achieved through expression of heterologous enzyme in *R. eutropha* (Aneja et al. 2009). In this particular study, alternative polyhydroxyalkanoates synthase originating from *Allochromatium vinosum* ATCC 35206 were expressed in *R. eutropha*. This expression of heterologous genes in PHB negative mutant strains showed altered composition of PHB, which is useful for different applications of PHB. Since it is possible to manipulate the expression system, several attempts have been made to express different proteins to overcome some limitations of other expression hosts. One of these attempts is the combined expression and purification of protein through a fusion protein. To avoid costly downstream processing this approach uses a three-part fusion protein. The first part is the protein of interest, the second a sequence called Intein and the third part is a homologous protein of *R. eutropha* H16 called Phasin. The homologous part, the Phasin, binds to PHB granules and is purified easily with the PHB. After changing the buffer, the protein of interest is released, though the Intein is self-splicing under certain conditions (Barnard et al. 2005). One additional benefit is the ability of *R. eutropha* to grow to very high cell densities of over 300 (OD_{600}). The advantage of these high cell densities is the higher product concentration in the same fermentation volume. The organism does not tend to form inclusion bodies under overexpressing conditions, so this expression host is also suitable for the expression of proteins that tend to form inclusion bodies in *E. coli* (Srinivasan et al. 2002, 2003). On the other hand, some enzymes of *R. eutropha* H16 may gain importance in the future as an

application in hydrogen fuel cells. *Ralstonia* has oxygen-tolerant hydrogenases, which are rare in nature and potentially useful in a new enzyme-based type of hydrogen fuel cells (Vincent et al. 2005; Goldet et al. 2008; Ludwig et al. 2008). The advantage of a system that utilizes enzymes of *Ralstonia* is that the hydrogen concentration in the working media needed for efficient function is lower than in current fuel cell models and therefore less purified hydrogen is needed. Another approach using *R. eutropha* as a source of energy is the direct conversion of CO_2 into higher alcohols via the enzyme system of the organism. An electric current is applied directly to the membrane and this converts CO_2 to alcohol using the available enzyme system of *R. eutropha* (Li et al. 2012). One last field of application to mention is wastewater treatment. Since the organism has a great variety of different metabolic pathways, it also contains numerous metabolic routes for the degradation of several water pollutants. Combined with the high tolerance for heavy metals, this ability is widely used in treatment plants (Louie et al. 2002; Susanne Doris Juhnke 2004).

1.2 Lithotrophic growth

Lithotrophic growth is the capability of an organism to perform anabolic metabolism without the addition of organic compounds such as sugars or other energy conserving chemicals. In the case of the *Ralstonia* species only inorganic molecules such as carbon in CO_2, and an energy source such as H_2 and O_2 are needed for growth. Thus, the organism is capable of growing with these compounds in the gaseous phase. In nature this metabolic pathway follows two different paths:

1. The first path is the one used by *R. eutropha*, using inorganic substrates to gain the necessary electrons for the reduction of CO_2 to organic carbon in biological molecules. Inorganic molecules as electron donor are in this case H_2, H_2S or different metal ions (Glueck et al. 2009). *R. eutropha* is able to use nitrate as an alternative electron acceptor instead of O_2, so an aerobic environment is not always necessary (Armin K. Tiemeyer 2007).
2. The second path for reducing CO_2 is phototrophy. The energy is derived from sunlight and the electrons of water are used as the electron source. In this way O_2 is generated during the process (Glueck et al. 2009).

After reducing CO_2 through one of these processes, there are different pathways for the insertion of the reduced CO_2 in organic molecules. Up to now, four major pathways are known.

- Calvin-Benson-Bessham-Cycle
- Arnon-Buchanan-Cycle
- Wood-Ljungdahl-cycle
- Acyl-CoA carboxylase pathways

The first pathway, Calvin-Benson-Bessham-Cycle (CBB-cycle) is the most common route for fixing CO_2 in nature. *R. eutropha* uses this way for growth under absence of organic compounds (Glueck et al. 2009; Jeffke et al. 1999).

1.3 Hydrogen oxidation

The capability of *R. eutropha* H16 for growth under lithotrophic conditions is mediated by two NiFe-hydrogenases, which are capable of converting a H_2 molecule into energy and reduction equivalents (Bowien und Kusian 2002; Schwartz et al. 1998). The first hydrogenase is bound to the membrane and is called membrane bound hydrogenase (MBH). This enzyme converts the redox potential of H_2 and O_2 to energy in form of ATP. The second hydrogenase, the soluble hydrogenase (SH), provides $NADH/H^+$ for the fixation of CO_2. The hydrogen sensing system relies on a regulatory hydrogenase to activate a histidine kinase in the presence of H_2. The histidine kinase again interacts with HoxA, a transcription factor of the NtrC family, to trigger the expression of MBH and SH operons. SH is then deployed to reduce NAD^+ to NADH in the cytoplasm of *R. eutropha* H16 whereas MBH is mainly involved in electron transport coupled phosphorylation (Schwartz et al. 1998).

1.4 CBB-cycle

The main uptake of CO_2 under chemolithoautotrophic conditions is achieved through the enzymes of the Calvin-Benson-Bessham-cycle (Höfle 2005; Mueller-Cajar et al. 2011). The

key enzyme in this cycle is Ribulose-1,5-bisphosphat-carboxylase/-oxygenase (RuBisCO). This enzyme is probably the most common enzyme in the world and is used by all photosynthetic active plants and bacteria for this purpose, too (Kusian und Bowien 1997). This CBB-cycle in prokaryotes is encoded by a so called CBB-operon, which is quite common in various bacteria species.

These operons do not have the same genetic form across the different species of bacteria. The different enzymes that are encoded and therefore the mediated abilities are different in the investigated bacteria (Kusian und Bowien 1997). In the case of *R. eutropha* H16 some studies were performed to take a closer look at this operon and thereby gain more knowledge of the regulation mechanism and the general function of these different proteins (Jeffke et al. 1999; Thomas Jeffke 2000; van den Bergh et al. 1993). One of the major findings was that RuBisCO needs an activator to function properly, which is not always present under ambient air conditions (Mueller-Cajar et al. 2011). In *R. eutropha* H16, two copies of this CBB-operon are present. One copy is encoded chromosomally, whereas the other is encoded on the megaplasmid pHG1. As shown in figure 1, the two copies of the operon differ in the content of the genes (Bowien und Kusian 2002).

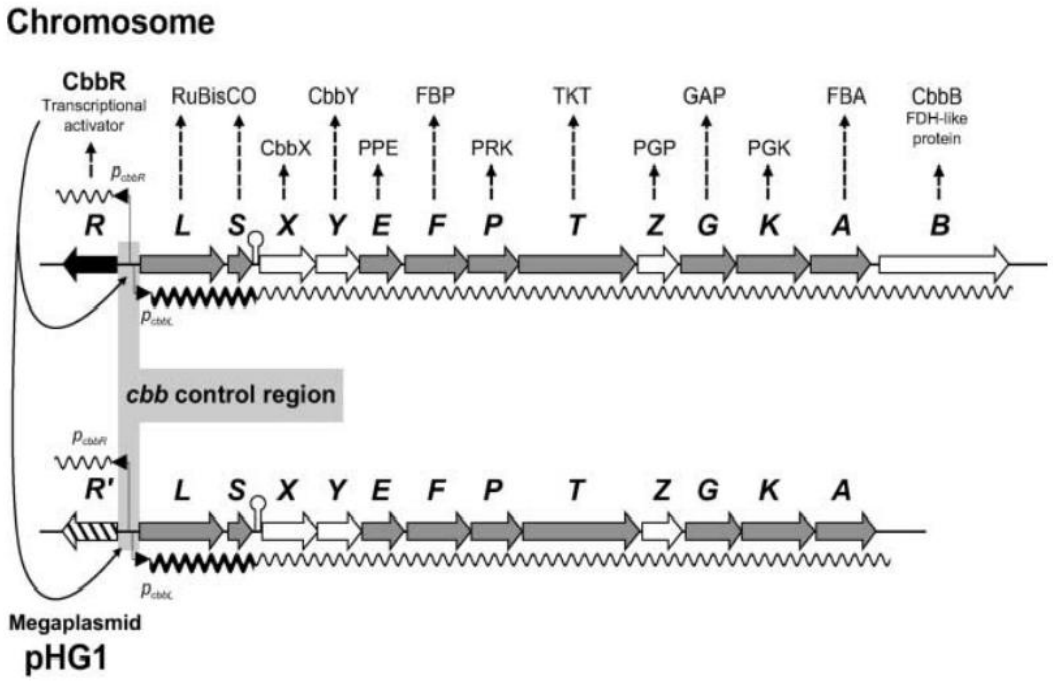


Figure 1: Location and organization of the cbb regulon of the chemo-autotroph *R. eutropha* H16 consisting of two highly homologous gene clusters. Gray-shaded genes encode CBB-cycle enzymes. Shortcuts of the gene products: CbbR LysR-type transcriptional activator binding to the operator of the cbb control region, RuBisCOribulose-1,5-bisphosphate carboxylase/oxygenase made up of large (CbbL) and small (CbbS) subunits, CbbX, CbbY products of unknown function, PPEpentose-5-phosphate 3-epimerase,FBPfructose-1,6-/sedoheptulose-1,7-bisphosphatase, PRK phosphoribulokinase, TKT transketolase, PGP2-phosphoglycolate phosphatase, GAPglyceraldehyde-3-phosphate dehydrogenase, PGK phosphoglycerate kinase, FBA fructose-1,6-/sedoheptulose-1,7-bisphosphate aldolase, CbbB formate dehydrogenase (FDH)-like protein. The hatched cbbR (R') is the defective cbbR gene located on megaplasmid pHG1 of the strain. pcbbL and pcbbR designate the strong, inducible cbb-operon and the weak, constitutive cbbR promoters, respectively. A hairpin structure (symbol between S and X) downstream of cbbS causes frequent premature transcription termination.

1.5 C3/C4 carbon metabolism in *R. eutropha* H16

Even though the CBB-cycle provides the major amount of carbon under lithotrophic conditions, there are several steps in the metabolism where CO_2 is either released or consumed. Two steps are particularly interesting because CO_2 is introduced in organic molecules in addition to the CBB-cycle. These steps mediate between the Entner-Doudoroff-pathway and the tricarboxylic-acid-cycle (TCC-cycle) (Bruland et al. 2010). These pathways are two of the most important metabolic routes for carbon metabolism so most carbohydrates pass this branching point.

CO_2 is incorporated through two carboxylases at this point.

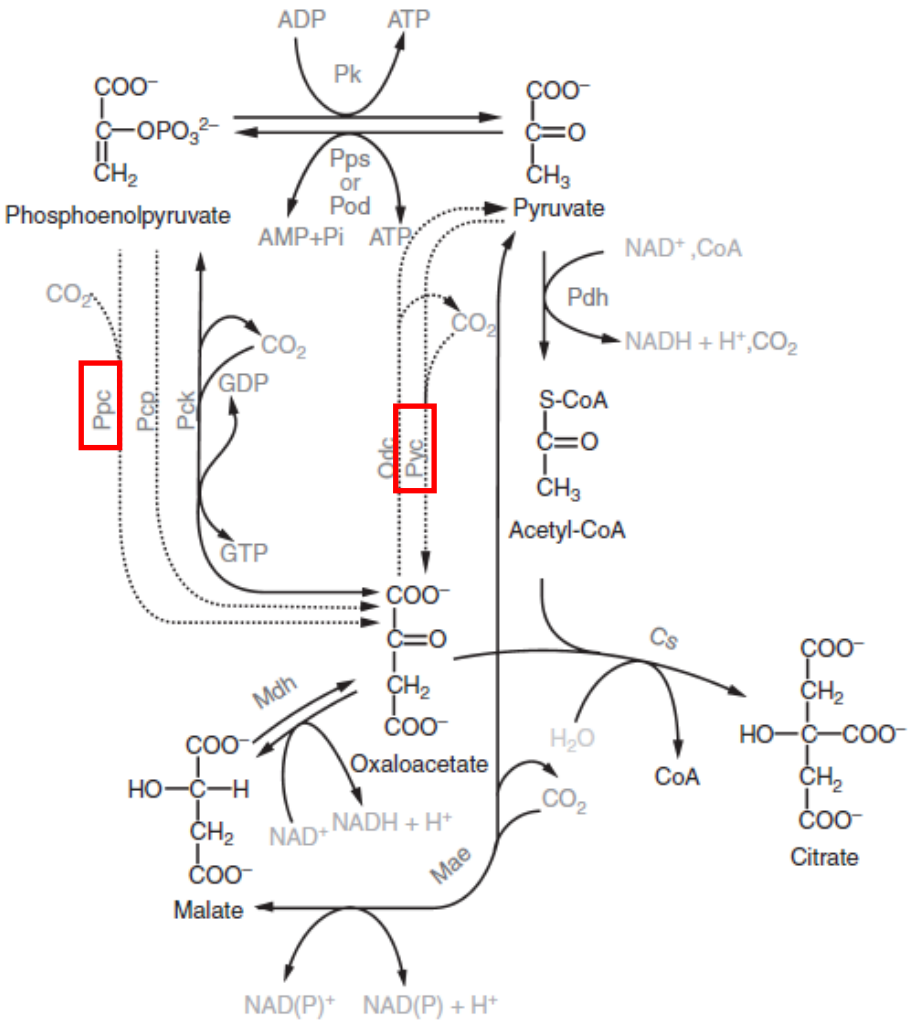


Figure 2: Pathways mediating between C_3 and C_4 metabolism in *R. eutropha* H16
Dotted lines are activities that are not proven, continuous lines are proven activities. Shortcuts of the gene products: Pk pyruvate kinase, Pps phosphoenolpyruvate synthase, Pod pyruvate orthophosphate dikinase, Pdh pyruvate dehydrogenase, Odc oxaloacetate decarboxylase, Pyc pyruvate carboxylase, Cs citrate synthase, Mdh malate dehydrogenase, Mae malic enzyme, Pck phosphoenol pyruvatecarboxykinase, Pcp phophoenol pyruvate carboxtransphorphorylase, Ppc phosphoenolpyruvate carboxylase (Bruland et al. 2010), marked in red are the two carboxylases, Ppc and Pyc

The first interesting enzyme is the phosphoenolpyruvate-carboxylase (Ppc), the second one the pyruvate-carboxylase (Pyc). As shown in figure 2 the pep-carboxylase as well as the Pyc-carboxylase are catalysing the reaction from phosphoenolpyruvate or pyruvate to oxaloacetate, which is a branching point at the beginning of the TCC-cycle. Both enzymes attach CO_2 to their substrates under avoiding the CBB-cycle (Britta Anderlei 2002). However, for these two carboxylases it is not possible to use CO_2 directly, it has to be converted to HCO^{3-} prior the carboxylation of the substrate. The conversion happens naturally at a low

rate. This low rate is often not sufficient for biological processes so all domains of life evolved special enzymes for this task. The family of enzymes that can accelerate this conversion to a very high level are the carbonic anhydrases.

1.6 Carbonic anhydrase

Carbonic anhydrases (CA) catalyses the reversible hydration of CO_2:

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$$

These enzymes were first identified and purified out of erythrocytes in 1933 (Tripp 2001). Since then, most effort was spend on the research on mammalian carbonic anhydrases. They play a great role in the transport and subsequent exhalation of CO_2 (Esbaugh und Tufts 2006). In humans they are found in all tissues involved in respiration. CA`s convert CO_2 to carbonate in muscles, which is then easily dissolved in blood and facilitates the conversion back to CO_2 in the lungs. From the lungs it is being excreted and therefore removed from the respiration system. On its way through the body it plays a great role in maintaining a constant pH (Geers und Gros 2000).

Up to now five subclasses of carbonic anhydrases are known (Sawaya 2006; So et al. 2004):

1. α-class: all mammal CA`s are belonging to the first class. The class is subdivided into four subclasses, depending on the localization. Also some prokaryotic enzymes are known.
2. β-class: most prokaryotic CA`s belong to this class. Several structures next to the active site suggest a distant relationship with the ε-class (So et al. 2004).
3. γ -class: first identified in methane-producing bacteria out of hot springs, by now several enzymes in different species are known.
4. δ-class: CA's of this type are found in diatoms
5. ε-class: are found in bacteria that contains carboxysomes like *Halothiobacillus neapolitanus*

The function of all CA's is depending on a zinc prosthetic group. This zinc is coordinated by three amino acid residues, which are slightly different between the subclasses. The two step reaction mechanism is the same in all classes. The first step is a nucleophile attack of a zinc-

bound hydroxide to CO_2. The second step is the regeneration of the active site by ionization of the likewise zinc-bound water molecule. At the second step, a proton is released from the active site (Smith und Ferry 2000).

In the last years several types of carbonic anhydrases were identified next to the mammalian ones.

The classes do not share a lot of sequence identity, although the coordinated zinc ion is present in all classes. One major difference is the quaternary structure. Most α-carbonic anhydrases are monomeric enzymes, whereas the other classes form at least dimers. The whole enzyme often contains some dimers, so it is suggested that dimers act as building blocks for the enzymes (Smith und Ferry 2000).

The four identified CA's of *R. eutropha* H16 are divided in the three main classes. The first two identified CA's are in the β-class. Additionally one α-class and one γ-class enzyme were discovered (Pohlmann et al. 2006). In nature it is not unusual that prokaryotes contain different subclasses of CA (Smith und Ferry 2000).

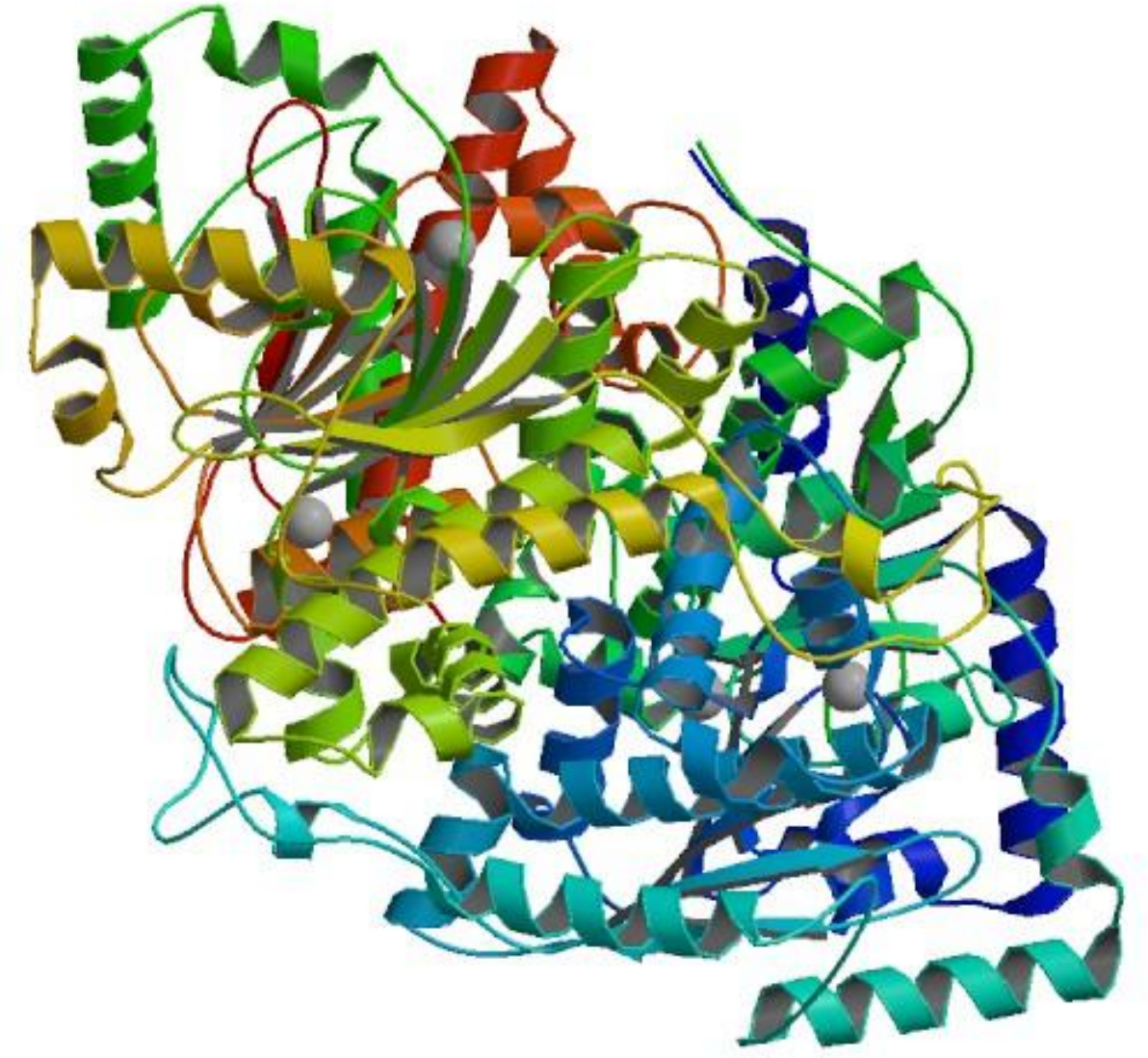


Figure 3: β-carbonic anhydrase from *Porphyridium purpureum*, the enzyme consist of two homodimers, the CA monomer is composed of two internally repeating structures, being folded as a pair of fundamentally equivalent motifs of an alpha/beta domain and three projecting alpha-helices (Mitsuhashi et al. 2000). The chains are coloured differently.

In *R. eutropha* H16 four carbonic anhydrases were identified by literature and bioinformatics searches (Kusian et al. 2002; Cramm 2009; Pohlmann et al. 2006). The activity of these enzymes is necessary for growth of *R. eutropha* H16 at ambient CO_2 concentrations, but the full physiological function still remains unclear (Kusian et al. 2002). Although some attempts have been made to identify the metabolic function of these enzymes, all functions of the different classes are not fully revealed yet (Merlin et al. 2003; Ueda et al. 2012). One important function may be the supply of sufficient CO_2 and carbonate levels for metabolic

processes which involve carboxylases such as the phosphoenolpyruvate-carboxylase and the pyruvate carboxylase (Ueda et al. 2012). Though CO_2 is easily lost for the cell through evaporation, it is possible that these enzymes shift the equilibrium of CO_2/carbonate to hold CO_2 for cellular metabolism in the cell. Whereas carbonate is only soluble in aqueous solutions, CO_2 can dissolve in lipids too and evaporate. The enzyme activity may provide this shift through speeding up the natural occurring conversion (Merlin et al. 2003). CA activity is only dispensable under elevated CO_2 concentrations or anaerobic growth, where CO_2 is generated in sufficient amounts in the cells (Ueda et al. 2012; Merlin et al. 2003; Smith und Ferry 2000).

The enzymes can be interchanged between species as long as the CA activity is still present. It is even possible to exchange enzymes of a distinct class with another enzyme of a different class without losing the ability to grow. In one study the β-carbonic anhydrase of *E. coli* was replaced by a γ-CA from *Methanosarcina thermophila* and restored the activity of the cell (Smith und Ferry 2000; Merlin et al. 2003; Kusian et al. 2002).

Another way how carbonic anhydrases serve in carbon fixation is the activity in the opposite reaction, the generation of CO_2 out of carbonate. The new found ε-class carbonic anhydrases are suggested to elevate the level of CO_2 in the so called carboxysomes (So et al. 2004). These structures are polyhedral protein micro compartments where the CO_2 fixation in prokaryotes takes place. RuBisCO is localized in this compartment; however this enzyme works best at higher CO_2 concentration. New findings suggest that membrane attached carbonic anhydrases are responsible for the elevation of the CO_2 concentration over several magnitudes and therefore the effectiveness of carbon fixation is increased (Cannon et al. 2010).

The activity of CA's is measured in Wilbur-Anderson (W-A) units. One W-A unit will cause the descend of the pH from 8,3 to 6,3 of a 0,012M TRIS buffer per min. This measurement is performed at 0°C. To obtain these units, several methods are described in literature; most of them were invented for the activity measurement of mammalian CA's (Shingles und Moroney 1997; McIntosh 1968).

1.7 Carbonic anhydrases in *R. eutropha* H16

In *R. eutropha* H16 four carbonic anhydrases are known as already mentioned above. The first identified CA was *can* of the β-class. This is the only CA out of *R. eutropha* H16 which has already been cloned and described as functional (Kusian et al. 2002). The three remaining CA's belong to different classes. *Can*2 is a β-class, *caa* a α-class and the remaining CA H16_A1192 a γ-class carbonic anhydrase. Though it is proven that *R. eutropha* H16 needs a carbonic anhydrase activity under ambient CO_2 concentrations, the specific function of all four carbonic anhydrases in the metabolism is still unknown. Since only *can* was mutated and the lack of this activity led to reduced growth, and the activity was restored by heterologous enzymes the function of the remaining three CA's in *R. eutropha* H16 should be clarified (Kusian et al. 2002; Cramm 2009). If the three CA genes encode functional enzymes with the same abilities, the lack of the activity would have been compensated by these three unless they have other undiscovered tasks in metabolism.

Table 1: Carbonic anhydrases in *R. eutropha* H16

Referred to	Name	Locus	Length [bp]	Replicon	Accession	Class
ca1	***can***	H16_A0169	672	Chromosome 1	Q0KFA0	β
ca2	***can2***	H16_B2270	708	Chromosome 2	Q0JYX2	β
ca3	***caa***	H16_B2403	750	Chromosome 2	Q0JYI9	α
ca4	**H16_A1192**	H16_A1192	525	Chromosome 1	Q0KCD8	γ

Recent studies suggest that the activity can be dispensed under elevated concentrations of CO_2 or anaerobic growth (Ueda et al. 2012). Though the activity is not compelling, some enzymes identified as carbonic anhydrase may have other tasks and functions next to the obvious ones. Maybe the remaining enzymes are only expressed under certain conditions which are not discovered yet. It is also conceivable that some carbonic anhydrases serve in

maintaining the internal pH constant as already reported in mammals (Esbaugh und Tufts 2006; Geers und Gros 2000).

1.8 Objective of this work

The main objective of the whole project is to establish a new expression system on basis of *R. eutropha* H16 with genetic engineering and the generation of a strain that is capable of incorporate more CO_2 than the wild type. With the obtained abilities for manipulation of the organism, a more efficient uptake of CO_2 via overexpressed carbon cycle relevant proteins is possible.

One other task, besides the more efficient CO_2 uptake over the RuBisCO-system, is the use of effective carboxylation mechanism present in *R. eutropha* H16, which can be applied for industrial application as well as for more efficient CO_2-uptake. Some industrial carboxylation reactions are hard to perform with conventional techniques, and some enzymes of *R. eutropha* H16 may overcome some limitations in this field. To reach the goal of an increased uptake rate of CO_2 besides RuBisCO over enzymes in different branching points of the carbon metabolism, an elevated level of carbonate as a precursor for carboxylation reactions is necessary. The understanding and knowledge about the equilibrium between CO_2 and carbonate under physiological conditions is essential to increase the uptake and utilization of CO_2 in effective carboxylation processes.

This diploma thesis focused on the four carbonic anhydrases of *R. eutropha* H16 that are known up to now. All four different carbonic anhydrases of *R. eutropha* H16 were cloned, expressed and characterized in more detail. An elevated carbonate concentration could favour different carboxylation processes which are commercially interesting, would guide more carbon to anaplerotic reactions and therefore lead it directly to the TCC. This detour could lead to a higher CO_2 uptake rate and subsequent to a higher cell mass.

2 Materials and methods

2.1 Organisms and Strains

Table 2: Overview of the strains used in this work

Strain	Genotype	Source
***E. coli* XL1**	Tc-r; recB, recI, sbcC201, uvrC, umuC::Tn5(Km-r), mcpA, mcrB, mrr, lac, Δ(hsdRMS), endA1, gyrA96, thi, relA1, supE44(F´), proAB, lacI9ZΔM15, Tn10(Tc-r)	Invitrogen
***E. coli* TOP10**	F´(proAB, lacIq, lacZΔM15, Tn10(tet-r)), mcrA, Δ(mrr-hsdRMS-mcrBC), Φ80ΔlacZΔM15, ΔlacX74, deoR, recA1, araD139(ara, leu), 7697, galU, galK, λ-, rpsL(streptomycin-r), endA1, nupG	Invitrogen
***E. coli* BL21**	*E. coli* B F- *dcm ompT hsdS*(r_B- m_B-) gal [$malB^+$]$_{K-12}$(λ^S)	Invitrogen
***R. eutropha* H16**	Wildtype	DSM 428

2.2 Plasmids forwarded to IMBT strain collection

Table 3: Overview over the used plasmids, forwarded to IMBT strain collection

Plasmid	Description	Source
pK470-Bg_EstC-1_Δ*Nco*I	Kan^r, P_{tac}, lacI cloning and expression vector for E. coli	IMBT strain collection # 4631
pK470_*ca*1	containing *ca1* (*can*) cloned with *Nde*I and *Hind*III	This work, forwarded to IMBT strain collection # 6902
pK470_*ca*2	containing ca2 (can2) cloned with *Nde*I and *Hind*III	This work, forwarded to IMBT strain collection # 6903
pK470_*ca*3	containing *ca3* (*caa*) cloned with *Nde*I and *Hind*III	This work, forwarded to IMBT strain collection # 6904
pK470_*ca*4	containing *ca4* (H16_A1192) with *Nde*I and *Hind*III	This work, forwarded to IMBT strain collection # 6905

2.3 Primers used in this work

Table 4: Primers for carbonic anhydrases and sequencing primers

Name	Sequence	Restriction enzyme	Complementary size [bp]	Annealing Temperature [°C]
CA1F	5' – attcatatgactgacgccatcgcc – 3'	*Nde*I	18	68
CA1R	5' – ataagctttcagcggatcgacgc – 3'	*Hind*III	15	64
CA2F	5' – cgcatatgcatcacatcgaacaactg – 3'	*Nde*I	21	64
CA2R	5' – ttaagctttcagggttcgcagggttc – 3'	*Hind*III	18	67
CA3F	5' – cttcatatgaacaccaggctgccg – 3'	*Nde*I	18	68
CA3R	5' – ttaaagcttctagtggctgacctgcac – 3'	*Hind*III	18	60
CA4F	5' – ccacatatggcgctttaccagctcg – 3'	*Nde*I	19	68
CA4R	5' – tcaaagctttcagccgatccgcttgag – 3'	*Hind*III	18	68
pJET1.2fwd	5'-cgactcactatagggagagcggc-3'			
pJET1.2rev	5'-aagaacatcgattttccatggcag-3'			
pMS_promfor	5'-gtgagcggataacaatttcacaca-3'			
TacpMS470 stop_new	5'-gtttttatcagaccgcttctgcg-3'			

The highlighted parts of the primers are the recognition sites for *Nde*I and *Hind*III restriction enzymes, these restriction sites were used for cloning. The binding nucleotides on the genes are underlined. The annealing temperature was calculated with the Tm-Calculator for Phusion® Polymerase of Thermo Scientific available online.

The last four primers were used for sequencing of the cloned PCR-products.

2.4 Cloning and expression vector pK470

All cloning and screening vectors were constructed on the basis of the pK470. This vector encodes the Tac promoter (P_{tac}), a kanamycin resistance gene and a ColE1 replication sequence originating from the pMS470 plasmid. Also encoded is *lac*I for induction of the inducible P_{tac} with Isopropyl-β-D-thiogalactopyranosid (IPTG). The original vector pMS470 contains an ampicillin resistance gene which was replaced by a kanamycin resistance gene. The Multiple Cloning Site (MCS) contains several restriction sites such as *Nde*I, *Hind*III and *Sph*I. The actual insert is a truncated version of an Esterase, *EstC*I, which was cloned in the vector by the same strategy. In this work all CA's were cloned in the vector via the restriction enzymes *Nde*I and *Hind*III. The advantage of *Nde*I cloning is that the first codon ATG is part of the recognition sequence. Therefor a frame shift in the coding gene can easily be avoided and the spacing between starting codon and Shine-Dalgarno-Sequence remains unchanged.

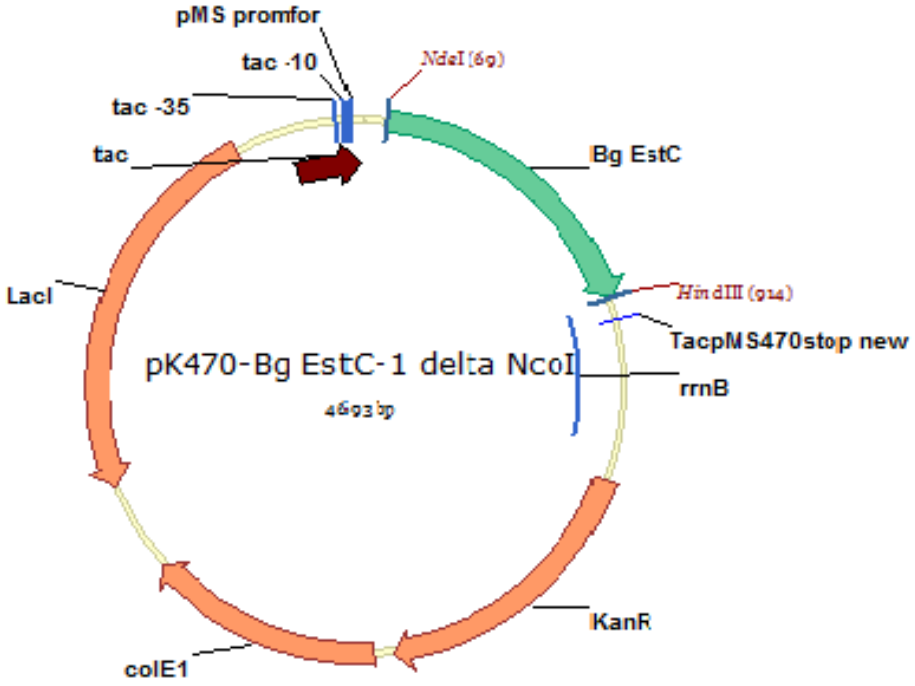


Figure 4: Cloning and Expression vector pK470-Bg_EstC-1_Δ*Nco*I
Shown in the figure are binding sites for the sequencing primers pMS_promfor and TacpMS470stop_new, the restriction sites for *Nde*I and *Hind*III, the original insert, EstC, ColE1 the replication origin, *Kan*R the Kanamycin resistance gene, rrnB the terminator sequence and the *lacI*-gene.

2.5 General Methods

2.5.1 Extraction of genomic DNA

The genomic DNA from *R. eutropha* H16 was extracted with Easy-DNA™ Kit (Invitrogen, Carlsbad, California, USA).

Preparation:

1 and 1,5 ml of overnight culture was centrifuged at 5000rpm for 5 min in a 1,5 ml Eppendorf tube to pellet the cells. The cell pellet was suspended in 200 µl of 1 x PBS

Isolation of DNA:

1. 350 µl of solution A were added to the cell suspension and vortexed in 1 second intervals until evenly dispersed. 2 µl of proteinase K were added after vortexing.
2. The cell suspension was incubated at 65°C for 30 min
3. 150 µl of solution B were added. The sample was vortexed vigorously until the precipitate moved freely in the tube and the sample was uniformly viscous
4. 500 µl of chloroform were added and the sample was vortexed until viscosity decreased and the mixture was homogenous.
5. The samples were centrifuged at maximum speed for 20 min at 4°C

DNA Precipitation:

1. 1 ml of 100 % ethanol (-20°C) was added to the sample and vortexed briefly
2. The samples were incubated on ice for 30 min.
3. Sample was centrifuged for 15 min at maximum speed and 4°C. Ethanol was removed from the pellet with a pipette.
4. 500 µl of 80 % ethanol (-20°C) were added and the sample was mixed by inverting the tube 3 – 5 times.
5. Sample was centrifuged at maximum speed for 5 min and 4°C. Ethanol is removed from the pellet with a pipette.

6. Sample was centrifuged for 3 min at 4°C. Residual ethanol was removed with a pipette. Additionally the sample was air dried for 5 min at room temperature.
7. The pellet was suspended in 100 µl TE buffer. 2 mg/ml RNAse A was added additionally to a final concentration of 40 µg/ml.
8. The sample was incubated at 37°C for 30 min to resolve the DNA and stored for further use at 4°C

2.5.2 Agarose Gel electrophoresis

To control and determine DNA amount and sizes, agarose gel electrophoreses were performed. Usually 1 % agarose gels were prepared. The appropriate amount of agarose (Biozym Scientific GmbH; Hessisch Oldendorf, Germany) was mixed with 1 x TAE buffer and ensuing heated by microwave for approximately three min to dissolve the agarose completely. The 1 x TAE buffer was diluted 1:50 from a 50 x TAE stock solution prior to use.

Table 5: Ingredients of 50 x TAE stock solution

50 x TAE stock solution (TRIS-acetate EDTA)
242 g TRIS
57,1 mL acetic acid
100 mL 0,5 M EDTA pH 8,0
H_2O bidest. up to 1000 ml
1000 ml final buffer volume

For size determination the 500 ng of the GeneRuler^{TM} DNA Ladder Mix (Thermo Science Inc, Waltham; USA) ranging from 100 bp to 10000 bp was used.

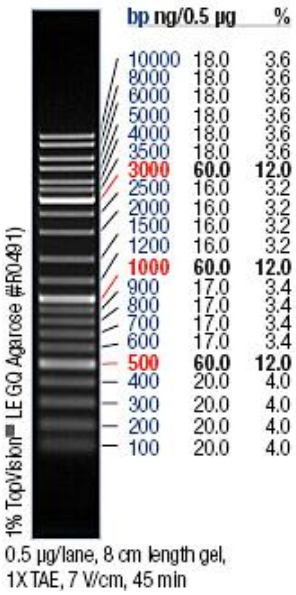


Figure 5: GeneRuler™ DNA Ladder Mix

Control gels were used at 120 V for approximately 45 min, whereas preparative gels were run at 90 V for about 90 min to enhance the separation process.

2.5.3 General PCR set-up

2.5.3.1 PCR set-up for gene amplification

PCR reactions were performed using Finnzymes Phusion® High-Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland), following the recommended protocol of the manufacturer.

Table 6: General PCR mixture

Volume [µl]	Ingredient
5	5 x Phusion® HF Buffer
0,5	10 mM dNTPs
0,25	Phusion®High-Fidelity DNA Polymerase
1,25	forward primer(10 pmol/µl)
1,25	reverse primer(10 pmol/µl)
x	approximately 15 ng of template DNA
x	H2O bidest.
25	final PCR volume

The PCR cycles were chosen as followed:

Table 7: General PCR set-up

Step	Temperature [°C]	Time [s]
Initial Denaturation	98	x
Denaturation	98	x
Annealing	x	x
Extension	72	x
Final extension	72	x
Cycles	25	

PCR reactions were always performed with 25 cycles until specified otherwise. The annealing temperatures for the primers were determined by Tm-calculator of Thermo Scientific available online (Thermo Scientific) shown in table 4. The actual annealing temperatures as used for cloning are shown in the table below.

Table 8: Annealing temperatures and expected size for the genes *ca1-ca4*

Genes	Annealing temperature [°C]	Expected size [bp]
ca1	66	672
ca2	62	708
ca3	64	750
ca4	67	525

2.5.3.2 Colony PCR

Colony PCR reactions were performed after the recommended protocol applied with the FermentasCloneJET™ PCR Cloning Kit. These PCR reactions were performed using DreamTaq DNA polymerase (Fermentas, St. Leon-Rot, Germany).

Template DNA was added in form of cell material in small amounts. The breaking of the cells and the release of the template DNA was achieved through the initial denaturation. The primers were the same as used for the cloning PCR reaction.

Table 9: Colony PCR mixture

Volume [µl]	Ingredient
2	5 x Phusion HF Buffer
2	10 mM dNTPs
0,1	Phusion®High-Fidelity DNA Polymerase
0,4	forward primer(10 pmol/µl)
0,4	reverse primer(10 pmol/µl)
---	approximately 15 ng of template DNA
15,1	H2O bidest.
20	final PCR volume

The PCR cycles were chosen as followed:

Table 10: Colony PCR set-up

Step	Temperature [°C]	Time [s]
Initial Denaturation	95	300
Denaturation	94	30
Annealing	x	30
Extension	72	50
Final extension	72	180
Cycles	25	

2.5.4 General cloning strategies

General cloning were accomplished by using Fermentas restriction enzymes (Fermentas, St. Leon-Rot, Germany), Promega T4 DNA Ligase (Promega, Mannheim, Germany) and Phusion® High-Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland). Wizard® SV Gel and PCR Clean-Up System Kit (Promega, Mannheim, Germany) were used for gel and PCR product purification. PCR product cloning was either carried out with FermentasCloneJET™ PCR Cloning Kit (Fermentas, St. Leon-Rot, Germany) or directly to pK470-Bg_EstC-1_Δ*Nco*I restricted with *Nde*I and *Hind*III and therefore without insert. Sequencing of DNA molecules was performed by LGC Genomics (LGC Genomics, Berlin, Germany). Dephosphorylation of DNA molecules was performed by using Shrimp Alkaline Phosphatase (SAP) (Fermentas, St. Leon-Rot, Germany). Plasmid preparations were accomplished by using GeneJET™ Plasmid Miniprep Kit (Fermentas, St. Leon-Rot, Germany) following the recommended protocol.

2.5.4.1 Restriction mixtures

Control restriction mixtures contained 1,5 µl of suitable reaction buffer (10 x), determined according recommendation by the manufacture, 0,5 µl of each restriction enzyme and approximately 200 ng of DNA in solution. The reaction volume was filled up to 15 µl with H_20 bidest. .

The restriction reaction mixture was incubated at 37°C for at least four hours. Thermal inactivation was performed according to suggested protocols by Fermentas prior to further use.

Preparative restrictions were conducted in larger volumes up to 40 µl, depending on DNA concentration and were incubated at 37°C generally overnight.

2.5.4.2 Ligation mixtures

For ligation Promega T4 DNA Ligase (Promega, Mannheim, Germany) was used. A ligation mixture was set up to a total volume of 10 µl. It contained 1 µl of T4 DNA Ligase Buffer, 0,5 µl of T4 DNA Ligase, a variable volume of vector backbone DNA to a total amount of 100 ng

and an also variable amount of insert DNA to reach a vector to insert ratio of 1:3. The ligation mixture was filled up with H_2O bidest. to the total volume.

The ligation reaction mixture was incubated at 16°C overnight. Thermal inactivation was performed at 65°C for 20 min. Prior the electroporation, a desalting step was performed. The ligation was desalted on nitrocellulose filter plates (Millipore GSWP2500, pore size 0,22 µm) against H_2O bidest. for 30 min.

An amount of 2 µl was then used for transformation.

2.5.4.3 Protocol for gel elution and PCR product clean-up with Wizard® SV Gel and PCR Clean-Up System Kit (Promega, Mannheim, Germany)

Dissolving of the gel slice

10 µl of membrane binding solution were added per 10 mg of gel slice. The sample was vortexed and incubated at 65°C until gel slice was completely dissolved.

Processing PCR reactions

An equal volume of membrane binding solution was added to the PCR reaction

Binding of DNA

1. SV Minicolumn was inserted into the collection tube
2. The dissolved gel mixture or the prepared PCR product were transferred to the minicolumn assembly and incubated at room temperature for 1 min.

   The minicolumn was centrifuged for 1 min at 16,000 x g. The flow-through was discarded and the minicolumn was reinserted into the collection tube.

Washing

1. 700 µl of membrane was solution added. The sample was centrifuged for 1 min at 16,000 x g. The flow-through was discarded and the minicolumn was reinserted into the collection tube.
2. Step 1 was repeated with 500 µl of membrane wash solution. The sample was centrifuged for 5 min at 16,000 x g. The flow-through was discarded and the minicolumn was reinserted into the collection tube.
3. The column assembly was recentrifuged for 1 min at 16,000 x g.

Elution

1. The minicolumn was transferred to a clean 1,5 ml microcentrifue tube.

2. A volume of 30 µl of H_2O bidest. is added to the minicolumn and incubated at room temperature for 5 min. The sample was centrifuged for 1 min at 16,000 x g.

2.5.4.4 Protocol for cloning of PCR products with FermentasCloneJET™ PCR Cloning Kit (Fermentas, St. Leon-Rot, Germany)

The ligation reaction was set up on ice. One reaction contained 10 µl of 2 x Reaction buffer, a variable amount of purified blunt-end PCR product to reach 0,15 pmol ends, 1 µl of pJET1.2 vector backbone (50 ng/µl) with 0,05 pmol ends and was filled up to a total volume of 19 µl with H_2O bidest.. After mixing gently, 1 µl of T4 DNA Ligase was added to start the reaction.

The reaction mixture, in total 20 µl, was vortexed briefly and spinnned down. Afterwards the sample was incubated at 22°C for 10 min according protocol. The ligation mixture was inactivated at 65°C for 10 min and desalted over a nitrocellulose filter. The desalted DNA was then used for transformation.

2.5.4.5 DNA dephosphorylation with Shrimp Alkaline Phosphatase (SAP) (Fermentas, St. Leon-Rot, Germany)

Dephosphorylation reactions were done using Shrimp Alkaline Phosphatase. The reaction mixture contained about 1 µg (~1 pmol ends of a 3 kb plasmid) of linear DNA, 2 µl of 10 x SAP reaction buffer, 1 µl Shrimp Alkaline Phosphatase (1 U/µl) and was filled up with H_2O bidest. to a total reaction volume of 20 µl

The sample was mixed carefully, centrifuged for 5 sec and incubated at 37°C for 15 min. After these 15 min 1 µl of Shrimp Alkaline Phosphatase was added additionally, mixed briefly and centrifuged for 5 s again. Then the sample was incubated at 37°C for another 15 min. The reaction was stopped by heating the sample to 65°C for 15 min.

2.5.4.6 Protocol for plasmid preparation with GeneJET™ Plasmid Miniprep Kit (Fermentas, St. Leon-Rot, Germany)

Resuspend cells, lyse and neutralize

- Add 250 µl of resuspension solution and vortex
- Add 250 µl of lysis solution and inverted tube 4– 6 times
- Add 350 µl of neutralization solution and inverted tube 4 – 6 times
- Centrifuge with 16000 rpm for 10 min

Bind DNA

- Load supernatant to GeneJET™ spin column incubate for 5 min at room temperature and centrifuged for 1 min with 16000 rpm.

Wash column

- Add 500 µl of wash solution and centrifuged for 1min.Discarde the flow through. This washing step is performed twice. After second washing step centrifuge empty tube for 2 min.

Elute purified DNA

- Transfer the column to a new tube.
- Add 50 µl of H_2O bidest. to the column and incubated for 5 min.
- Centrifuged the column for 5 min and collect the flow through.

2.5.5 Transformation of *E. coli* strains

An aliquot of 45 µl electro competent *E. coli* cells was mixed with an amount of approximately 100 ng of DNA and incubated on ice for 15 min. The total volume of DNA never exceeded 4,5 µl, 10 % of the cell solution volume. All *E. coli* strains were transformed with Micro Pulser TM (Bio-Rad, Hercules, California, U.S.) program EC 2 (0.2 mm cuvettes, 2.5 kV). The cells were regenerated in 1 ml of LB or 2 x TY medium at 37°C and 750 rpm in a thermo mixer for 60 min before plating out on suitable plates. Further incubation was on 37°C over night, petri dishes with grown cultures were stored at 4°C.

2.5.6 Media, cultivation and harvesting

2.5.6.1 Cultivation of E. coli strains

E. coli TOP10, *E. coli* XL1 and *E. coli* BL21 cells were cultivated at 37°C in regular Lysogeny Broth medium (LB) or in 2 x TY media. The ingredients for 2 x TY media are 10 g/l yeast extract, 16 g/l peptone and 5 g/l NaCl. For petri dishes 20 g/l agar-agar was added to liquid media before autoclaving. Additionally 100 µg/ml ampicillin or 40 µg/ml of kanamycin, depending on the used plasmid, was added sterile after autoclaving.

2.5.6.2 Cultivation of R. eutropha H16

For the isolation of genomic DNA *R. eutropha* H16 for genomic DNA isolation was cultivated at 28°C in mineral salt medium (MSM) without antibiotic. The ingredients for one litre of MSM were made by mixing solution A and B with H_2O bidest. at a ratio of 1:1:28 before autoclaving. The pH was adjusted at 7 with diluted phosphorus acid. After autoclaving 0,01 volume solution C is added. Solution A (30 x) containing 270 g of Na_2HPO_4 x 12 H_2O and 45 g of KH_2PO_4 diluted in 1 litre of H_2O bidest. . One litre of solution B (30 x) contains 6 g of $MgSO_4$ x 7H_2O, 60 g of NH_4Cl, 30 ml of SL7, 125 mg of Fe(III)NH_4-citrate. Components of solution B were also diluted in one litre of H_2O bidest. . Solution C (100 x) contained 250 mg $CaCl_2$ x H_2O diluted in 250 ml of H_2O bidest. (Steinbüchel 2012). Solution C was filtered with a sterile filter and added sterile after autoclaving. Additionally 2 % w/w fructose was added after autoclaving.

2.5.6.3 Fermentation for activity assays

Fermentations for activity assays were done with *E. coli* BL21 [pK470 *ca*1-*ca*4].
The fermentation was performed in 2000 ml chicane piston with a volume of 300 ml 2 x TY media. Additionally 1 % of glucose and kanamycin to a final concentration of 40 µg/ml was added sterile after autoclaving. The main cultures were inoculated to an OD_{600} of 0,05 with an overnight culture in the same media.
After inoculation the cells were grown to an OD_{600} of one at 37°C with 120 rpm. When the cultures reached this OD_{600}, typically after 4-5 hours, the expression was induced by

Isopropyl-β-D-thiogalactopyranosid (IPTG) to a final concentration of 0,1 mM. For the expression phase the temperature was reduced to 28°C overnight.

2.5.6.4 Harvesting and disrupting of fermentations

The fermentations were harvested the next morning at an OD_{600} of approximately 10. The fermentation broth was centrifuged at 4000 rpm at 4°C for 15 min. The pellets were washed twice with 50 mM sodium phosphate buffer pH 7,5. For French press cell disruption the pellets were weighed and suspended in the same amount of buffer. For sonication the cells were solved in the tenfold volume of 50 mM sodium phosphate buffer pH 7,5.

- French Press (SLM-Aminco French Press Mini Cell; 19000 psi internal cell pressure). The instrument was chilled to 4°C in a fridge overnight. The cell suspension was homogenized twice to make sure the break down is complete. After two samples, (four runs) the press was chilled again to avoid degradation of protein through heat.
- The cell suspension was sonicated for 6 min using a Branson Sonifier 250 (Duty Cycle 80 %, Output Control 8).

In both cases the soluble fraction and the insoluble fraction were separated by centrifuging for 60 min at 20 000 rpm at 4 °C (Avanti ^{TM} J-20 XP centrifuge, JA-25.50 rotor, Beckman Coulter Inc.; Vienna, Austria). The soluble fraction was used for activity assays.

The supernatant was removed immediately and stored separately at 4°C. The protein concentration of the supernatant was determined by the Bradford method.

2.5.6.5 Measurement of protein concentration using Bradford

The protein concentration of the cell lysate and the purified proteins was determined by the standard Bio-Rad protein assay. Therefore the Protein Assay Dying Reagent (Bio-Rad Laboratories Inc; Hercules USA) was diluted 1:5 with bidest. before use. For measurement 10 µl of standard or sample were pipetted into a microtiterplate and mixed with 200 µl of dying reagent. After incubation for 5 min at room temperature the mixture was measured with a plate reader. The calibration was done using 0 mg/ml, 0,05 mg/ml, 0,1 mg/ml, 0,25 mg/ml,

0,4 mg/ml and 0,5 mg/ml of Bovine Serum Albumin (BSA) as standard solutions. Protein determination was assayed in triplicate.

2.5.7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The fermentations were analysed for proper expression by performing SDS-PAGE. The used gels were a combination of a resolving gel and a collection gel with different concentration of acrylamide. In the resolving gel up to 15 slots were embedded. The resolving gel has a total concentration of 12 % acrylamide, whereas the collection gel contains only 4 % of acrylamide.

Table 11 : Ingrediens SDS-PAGE

Ingredient	Collection gel [ml]	Resolving gel [ml]
H_2O	5,6	6
30 % acrylamide /bis solution (Bio-Rad Laboratories Inc)	6,4	1,5
0,5 M TRIS 0,4 % SDS pH 8,8	4	2,5
10 % ammonium persulfate (APS)	0,02	0,02
tetramethylethylenediamine (TEMED)	0,05	0,015
2 % bromphenol blue	0	0,015
Total Volume	16,07	10,05

For electrophoresis 10 x running buffer was prepared and diluted to 1 x concentration prior to use.

One litre of 10 x running buffer contains 30 g TRIS, 144 g glycine and 10 g SDS. All components are dissolved in 0,75 l water, the pH is adjusted to 8,6 and the filled up to a total buffer volume of one litre.

For sample preparation a Final Sample Buffer (5 x FSB) was prepared. This FSB contained 2,083 ml 1,5 M TRIS-HCl buffer pH 6,8, 1g SDS, 2,5 ml β-mercaptoethanol, 5 ml of glycerine and 2 mg of bromphenol blue. This mixture has a total volume of about 10 ml.

For electrophoresis three different samples were used.

1. **Washed pellet** resolved in an normalized amount of 50 mM Sodium phosphate buffer at pH 7,5 (whole cell extract). The amount of buffer was determined by the following equation.

   33,33*OD_{600}= µl of buffer added to pellet of 1 ml fermentation

   Two µl of this resolved pellet were mixed with 3,4 µl 5 x FSB, 1 µl of 20 % SDS, 1 µl of NaOH 0,1 M, 0,6 µl β-mercaptoethanol, 4 µl of potassium phosphate buffer 0,1 M pH 7,4 and 2 µl of sample solution to a final reaction volume of 12 µl.
2. **Supernatant (soluble fraction)**: the supernatant was normalized by protein concentration assayed by the Bradford method described before. For SDS-PAGE 10 µg of protein was used. The distinct amount of sample solution was mixed with 2,5 µl of FSB and filled up to 12 µl of total sample volume.
3. **Detached proteins (insoluble fraction)** during break down and centrifugation, a small piece of detached proteins was solved in 9,5 µl of 50 mM sodium phosphate buffer at pH 7,5 and 2,5 µl of 5 x FSB.

The sample solutions were cooked at 99°C for 10 min prior the electrophoresis. The incubated samples were loaded on the gel right away. The electrophoresis was performed in two stages with the SE 250 Mighty Small II electrophoresis unit (Hoefer Inc., Holliston, USA) and the PowerPac™ Basic Power Supply (Bio-Rad Laboratories Inc.; Hercules, USA). The first stage was run with 200 V for approximately ten min and then switched to 110 V for about 90 min in the second stage.

After electrophoresis the gels were stained for 30 min in coomassie Brilliant Blue staining solution.

200 ml of this staining solution were prepared by mixing 100 ml of methanol with 20 ml of glacial acetic acid, adding 100 mg of Brilliant Blue G250 and fill up to 200 ml with H_2O bidest.. After the 30 min staining step, the gels were decolorized in a 10 % w/w glacial acetic acid solution. To determine the size of the protein bands the PageRuler^{TM} Prestained Protein Ladder (Thermo Science Inc, Waltham; USA) was loaded onto the gel (Figure 6).

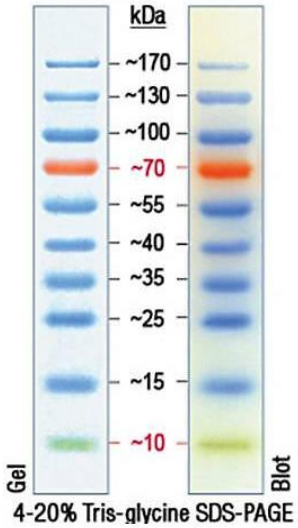


Figure 6: PageRulerTM Prestained Protein Ladder (Thermo Science Inc, Waltham; USA)

2.6 Activity Assays

2.6.1 Filter Assay

To obtain a quick indication for CA activity, some prior tests were performed. In order to get general information about *E. coli* [pK470 *ca1-ca4*] and *E. coli* [pK470-Bg_EstC-1_Δ*Nco*I] a new method was used. This method is similar to the widely used colony-based esterase assays. The assay is based on the release or consumption of acid to change the colour of a pH sensitive indicator substance. This principle was used in this work as the reaction of carbonic anhydrases is generating or consuming H^+ depending on the direction. Though it is a colony based assay it is easy to perform. In this case the rising pH should be measured.

The testing solution contained 40 mM Na_2CO_3 which forms hydrogen carbonate in aqueous solutions as a substrate and 0,005 % w/w phenol red as colour indicator. The pH was adjusted to 6,3 with acidic acid at room temperature. At this pH most of the carbonate should be in the form of hydrogen carbonate, which is the product/substrate of the enzyme reaction. The phenol red indicator is yellow from pH 1-7,3 and red-violet above it. The enzymes should work in the opposite direction, consuming H^+ and raise the pH.

The colonies were grown on LB-Agar plates with 40µg/ml kanamycin and 0,1 mM IPTG as inducing agent at 37°C overnight.

After incubation the colonies were transferred to a cellulose filter paper by pressing the paper on the surface of the plate by a Drigalski spatula and deducting it right away.

With this step, enough cell material should attach to the cellulose filter. The next step was drying the filter either by a hairdryer or at room temperature. The drying should cause disruption of the cells and releasing enough enzymes for a visible reaction.

Though it is not a method for quantity measurements it should give only an overview whether a clone is expressing protein in sufficient amounts for a visible reaction.

2.6.2 CO_2-Trapping

With this experiment the reaction from hydrogen carbonate to CO_2 should be measured. The experiment is based on the reaction of hydrogen carbonate to CO_2 with the subsequent removal of the generated gaseous CO_2 and trapping it in a $Ca(OH)_2$ solution.

The calcium hydroxide forms $CaCO_3$ with the CO_2 which is insoluble over a certain concentration. Therefor the CO_2 generated by the activity of the carbonic anhydrases should form a visible, insoluble precipitate which can be analysed.

For the experiment 80 ml of 40 mM $KHCO_3^-$ pH 8,3 solution was filled in a 200 ml glass bottle. At this pH all carbonate should be available as hydrogen carbonate for the reaction.The cover was penetrated by two small holes, one for air input and one with a tube leading into 40 ml of saturated $Ca(OH)_2$ solution in a 50 ml Greiner tube. A constant flow rate of air and thereby of generated CO_2 was created by a water jet pump, affiliated at the lid of the Greiner tube. The assay was performed at 22°C for 1 h.

2.6.3 Wilbur-Anderson-Assay

This assay is based on the decrease of pH over time. It is mainly used for activity determination of mammal enzymes. It is the standard procedure for determining carbonic anhydrase activity (Worthington Biochemical Corporation).

Blank Determination

Add 3,0 ml of chilled 0,02 M TRIS-HCl buffer, pH 8,0 to a 15-20 ml beaker. Maintain temperature at 0-4°C and record pH. Add 2 ml of chilled CO_2 saturated water to TRIS buffer. Immediately start a stop watch and record the time required for the pH to drop from 8,3 to 6,3. Record this time as T_0.

Activity Determination

Add 3 ml of chilled 0,02 M TRIS-HCl buffer, pH 8,0 to a 20 ml beaker. Maintain temperature at 0-4°C and record pH. Add 0,05 ml of freshly diluted enzyme or 0,05 ml of freshly prepared lysate. Quickly add 2 ml of CO_2 saturated water and record the time required for the pH to drop from 8,3 to 6,3. Record this time as T.

Equation 1: Formula for Wilbur-Anderson Units

$$\text{Units/mg} = \frac{2 \times (To - T)}{T \times \text{mg enzyme in reaction mixture}}$$

The buffer was chilled on ice for 20 min prior use. The CO_2 saturated water was prepared by bubbling CO_2 through 200 ml of H_2O bidest. for 30 min. The water was kept on ice during the saturation.

This experiment is designed for higher activities than it is expected in the crude lysate, to obtain more reliable results; a standard enzyme was used to create a standard curve for activities. The standard enzyme was obtained by Sigma-Aldrich (Product Number C3934-100MG) and has activity of 2986 Wilbur-Anderson units per mg and originates from bovine erythrocytes. The different standard solutions were measured according to the protocol (Worthington Biochemical Corporation).

Three standard solutions were prepared for 6 W-A units, 15 W-A units and 30 W-A units. These activities were diluted out of a 3000 W-A Units/ml solution in 1/10 and 1/5 dilution steps.

2.6.4 p-Nitrophenyl acetate assay

This procedure is based on a protocol published by Sigma-Aldrich for photometric measurement of carbonic anhydrase activity. This assay is based on a side reaction of, the conversion of p-Nitrophenyl acetate and H_2O to p-Nitrophenol and acetate. (Sigma-Aldrich 1994)

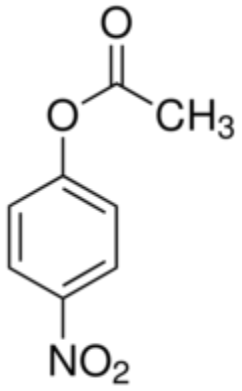


Figure 7: p-Nitrophenyl acetate

This conversion results in a changed OD_{348} by which the activity can be determined.

Procedure:

1. Pipette 190 µl of 15 mM TRIS-Sulfate Buffer, pH 7,6 with a multichannel pipettes into a microtiterplate.
2. Immediately add 100 µl of freshly prepared 3 mM p-Nitrophenyl actetae solution. Mix by pipetting up and down and equilibrate at 20°C.
3. Add 10 µl of sample solution or water as standard.
4. Record OD_{348}

The final reaction volume was 300 µl. As a reaction vessel a microtiterplate with flat bottom with a volume of 300 µl was used. The absorbance was recorded for 20 min using a plate reader. All samples and standards were assayed at least in triplicates. The original assay was designed for cuvettes with a light path of 1 cm and a reaction volume of 3 ml. For measurement in microtiterplate the volume was changed to a tenth. Though the

microtiterplates has a light path of 1 cm if they are fully loaded, the light path remains unchanged.

Calculation:

The calculation was done according the following equation.

Equation 2: Wilbur-Anderson Units by absorption

$$\text{Units/mg enzyme} = \frac{(\Delta A_{348nm}/\text{min Test} - \Delta A_{348nm}/\text{min Blank})\ (1000)}{(5.0)\ (\text{mg enzyme/ml RM})}$$

1000 = conversion to µmol

5 = mM extinction coefficient of p-Nitrophenol at pH 7,6

RM = Reaction mixture

This value has to be multiplied with 1,5 to obtain Wilbur-Anderson Units according protocol.

2.6.5 Infrared spectroscopy

This method is based on the transmittance of carbonate and CO_2. With an infrared spectroscope it is possible to monitor concentration changes of different substances over time. In this case the generation or consumption of CO_2 and carbonate should be measured. The measurements were recorded with a Bruker Matrik MF FT-IR spectrometer.

Procedure:

1. Pipette 1,5 ml of 50 mM sodium phosphate buffer, pH 8 into a 5 ml glass well
2. Immediately add 1 ml of freshly prepared CO_2 saturated water
3. Blank the infrared spectroscope with this solution
4. Add 100 µl of sample solution or water as standard, maintain temperature at 25°C
5. Record data

The transmittance was recorded for 36 min. In total 6 measurements over this time, every 6 min, were performed.

3 Results

3.1 Genomic DNA Isolation

For genomic DNA isolation a volume of 30 ml of MSM-media was filled up in a 100 ml shaking flask. The shaking flask was inoculated with *R. eutropha* H16 by an inoculation loop and incubated at 120 rpm shaking and 28°C over night. The next morning different amounts of culture were centrifuged and the genomic DNA was isolated according the protocol. (See material and methods, page 24) For control a gel electrophoreses was performed.

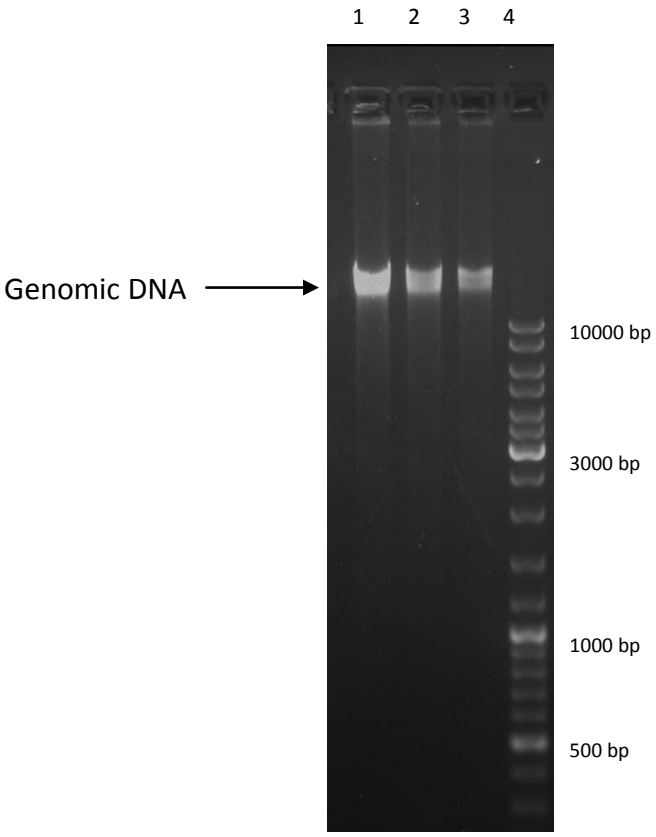


Figure 8: Isolated genomic DNA of *R. eutropha* H16
Slot 1:1 µl of genomic DNA isolation, 1 ml sample
Slot 2/3: 1 µl of genomic DNA isolation 1,5 ml sample
Slot 4: 500 ng GeneRuler^{TM} DNA Ladder Mix

For the first sample one ml of overnight culture (ONC) was centrifuged, for the following two samples 1,5 ml of the ONC were used. In this case it seems that the overall DNA concentration is the highest in sample number one. According to that, a 1:10 dilution of sample number one was used for all following PCR reactions.

3.2 PCR and expression vector construction

3.2.1 Construction of the Expression vector pK470 ca1 (can)

3.2.1.1 PCR conditions for ca1

For the PCR the primers CA1F with the attached *Nde*I restriction site and CA1R with the *Hind*III restriction site were used. (See material and methods, page 22) To obtain the gene *ca1* two successive PCR reactions with the same program were performed (Table 12). The first PCR reaction always led to unspecific products, even after alteration and improvement of the PCR program. To avoid the cloning of unspecific product, the PCR product was purified via a preparative agarose gel and the product at the right size, 672 bp, was cut out. The DNA was cleaned up according the protocol (Wizard® SV Gel and PCR Clean-Up System, see material and methods page 30) and used as new template for a subsequent PCR reaction with the same conditions. After this second PCR reaction a single, distinct product at the required size was obtained (Figure 9). The final PCR product was purified over a preparative agarose gel (1 % agarose) and subsequently eluted from the gel by Wizard®SV Gel and PCR Clean-Up System Kit (see material and methods page 30) for further cloning steps.

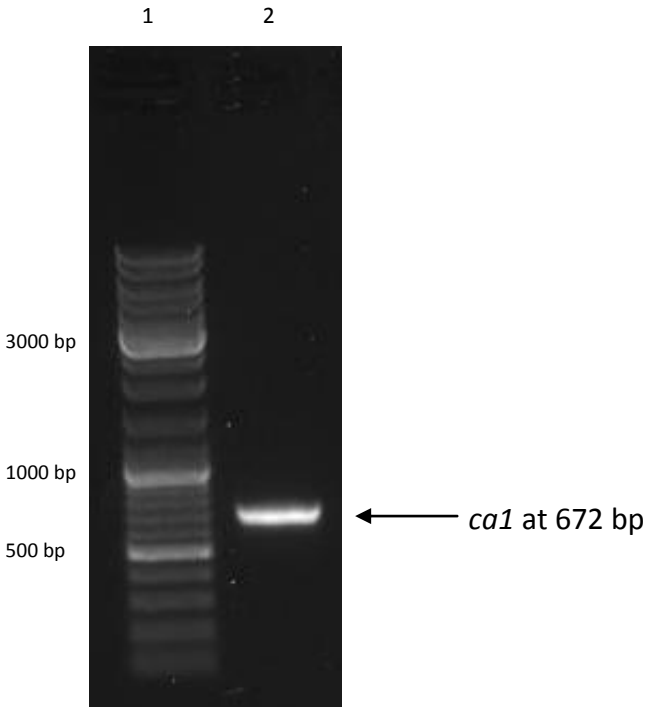


Figure 9: Control gel of the *ca1* PCR product after second PCR reaction
Slot 1: 500 ng GeneRuler™ DNA Ladder Mix
Slot 2: *ca*1 672 bp, this single product was obtained after the second PCR

As shown in figure 8, the two subsequent PCR reactions provide a single product at the required size of 672 bp.

Table 12: PCR set-up for the amplification of *ca*1

Step	Temperature [°C]	Time [s]
Initial Denaturation	98	360
Denaturation	98	15
Annealing	66	5
Extension	72	30
Final extension	72	300
Cycles	25	

The PCR program was designed after the recommended protocol of Finnzymes (Vantaa, Finland).

After finishing the program the PCR reaction was kept on 4°C until use.

The obtained PCR product was used for cloning into the pJET 1.2 vector. This step was performed according the protocol for FermentasCloneJET™ (see material and methods page 31). Afterwards 2 µl of the ligation mixture were transformed into *E. coli* XL1 Cells. For electrocompetent cells, the protocol recommended a additional purification step over the Wizard® SV Gel and PCR Clean-Up System Kit (see materials and methods page 30).

Plasmid DNA was isolated by GeneJET™ Plasmid Miniprep Kit (Fermentas, see material and methods page 32). Positive clones were identified by control restriction with *Nde*I/*Hind*III and sent for sequencing to LGC with the sequencing primers pJET1.2fwd and pJET1.2rev. After receiving the result, which showed no alteration of the sequence, the next cloning step was performed with the correct clone.

3.2.1.2 Transfer of the gene in expression vector pK470

For expression and activity studies *ca1* had to be separated from pJet1.2 and inserted into pK470. The insert (*ca1*) was isolated from pJET1.2 *ca1* by restriction with *Nde*I/*Hind*III. The gene of interest and pJET1.2 were separated by a preparative agarose gel and cleaned up by Wizard® SV Gel and PCR Clean-Up System (see material and methods page 30). In parallel, the vector, pK470-Bg_EstC-1_ΔNcoI, was digested with the same set of enzyme, purified via a preparative agarose gel and eluted out of the gel. The vector backbone was dephosphorylated with Shrimp Alkaline Phosphatase (SAP) (Fermentas) according protocol prior to the ligation to avoid religation (see material and methods page 31). Ligation was performed according protocol with T4 DNA Ligase (see material and methods page 29). After inactivation and desalting 2 µl of the ligation mixture were transformed into electro competent *E. coli* XL1 cells. The obtained clones were analyses by colony PCR and restriction according protocol with the primers CA1F and CA1R. As annealing temperature 66°C was chosen.

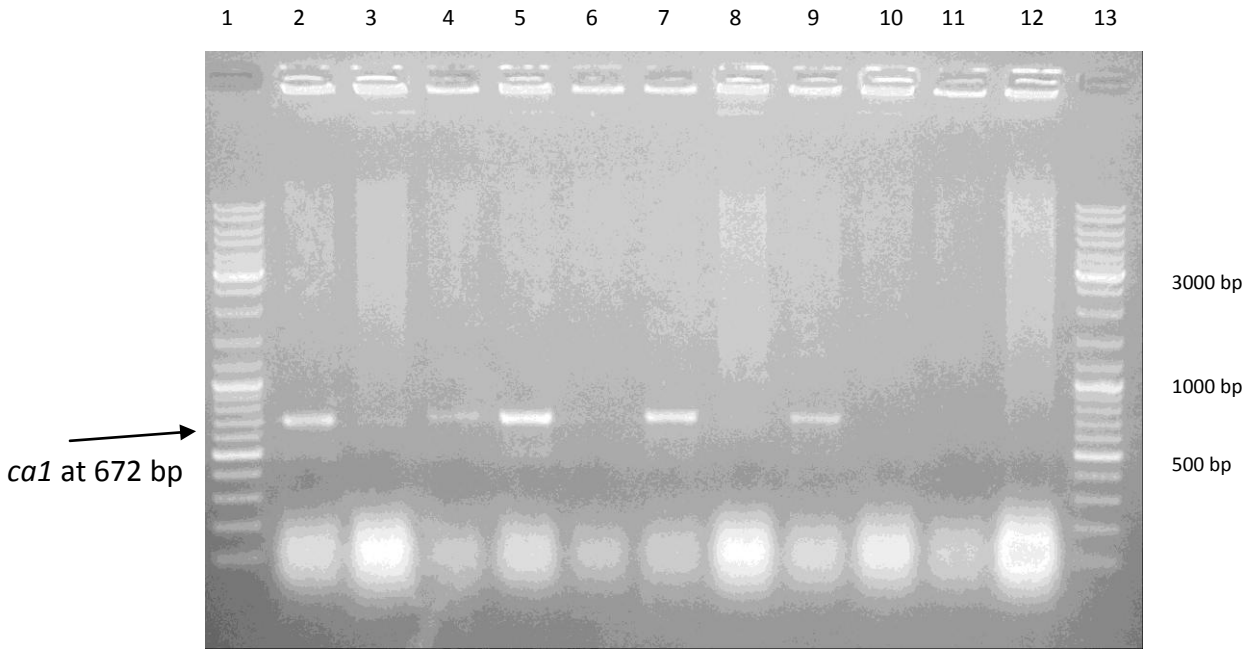


Figure 10: Colony PCR for *ca1*
Slot 1 and 13 500 ng GeneRuler™ DNA Ladder Mix, slot 2-12 show clones 1-11 of *E. coli* Top10 pK470 *ca*1. As shown, clone 1, 3, 4, 6 and 8 carry the gene at 672 bp. Visible below are the surplus primers.

Clone 1, visible in slot 2, was chosen for a subsequent restriction analyses. Plasmid DNA was obtained by plasmid isolation and restricted with *Nde*I and *Hind*III in a double digest according protocol for control restriction (see material and methods page 29, page 32). The double digest was the analysed by a control agarose gel.

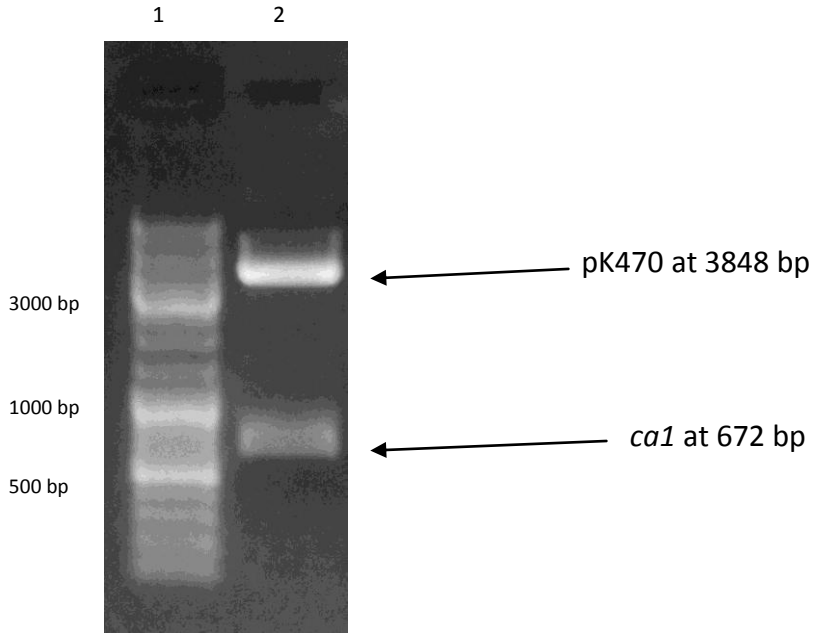


Figure 11: pK470 *ca*1 restriction analyses
Slot 1 500 ng GeneRuler DNA Ladder Mix
Slot 2 pK470 *ca*1 restricted with *Nde*I/*Hind*III 672 bp & 3848 bp

As shown in Figure 11 the control restriction with *Nde*I and *Hind*III worked as expected, both enzymes cut the vector and therefore the restriction pattern shows the vector backbone at 3848 bp and insert *ca1* at 672 bp. To be sure that no mutation occurred during cloning, this clone was sent to sequencing with primers binding on the vector backbone outside of the multiple cloning site, pMS_promfor and TacpMS470stop_new. The sequencing result revealed that no mutation had occurred.

3.2.2 Construction of the Expression vector pK470 ca*2* (*can2*)

3.2.2.1 *PCR conditions for ca2*

For the PCR the primers CA2F with the attached *Nde*I restriction site and CA2R with the *Hind*III restriction site were used (see materials and methods page 22). To get a specific product several PCR set-ups were tested.
The protocol in Table 13 gave the best results.

Table 13: PCR set-up for the amplification of *ca*2

Step	Temperature [°C]	Time [s]
Initial Denaturation	98	300
Denaturation	98	15
Annealing	62	15
Extension	72	30
Final extension	72	300
Cycles	25	

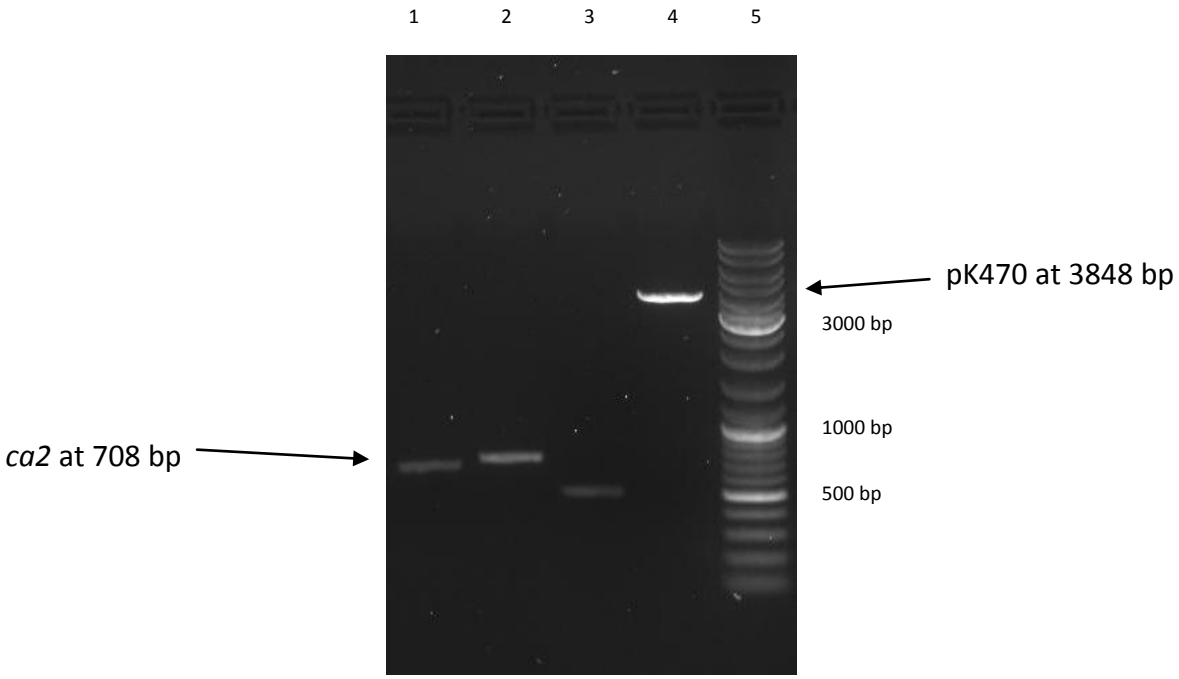


Figure 12: Control gel *ca2* PCR product
Slot 1: *ca2* 708 bp, Slot 2: *ca3* 750 bp, Slot 3: *ca4* 525 bp**,** Slot 4: pk470 restricted with *Nde*I/*Hind*III 3848 bp , Slot 5: 500 ng GeneRuler DNA Ladder Mix

The PCR program was designed after the recommended protocol of Finnzymes.

After finishing the program the PCR reaction was kept on 4°C until use.

The obtained PCR product was used for cloning into pJET1.2 vector (see material and methods page 31). The 2 µl of the ligation mixture were transformed into electro competent *E. coli* XL1 Cells.

Plasmid DNA was isolated by GeneJET™ Plasmid Miniprep Kit (Fermentas, see material and methods page 32). Positive clones were identified by a double digest with *Nde*I and *Hind*III.

3.2.2.2 Transfer of the gene in expression vector pK470

pJET1.2 *ca2* was restricted with *Nde*I/*Hind*III to divide the insert and pJet1.2. The gene of interest and pJET1.2 were separated by a preparative agarose gel. In parallel the vector, pK470-Bg_EstC-1_Δ*Nco*I, was cut with the same set of enzyme and also purified over a preparative agarose gel. Both fragments were eluted from the gel by the Wizard® SV Gel and PCR Clean-Up System (see material and methods page 30). The vector was dephosphorylated with Shrimp Alkaline Phosphatase (SAP) (Fermentas) prior the ligation to avoid relegation (see material and methods page 31). Ligation was performed according

protocol with Promega T4 DNA Ligase (see material and methods page 29). After inactivation and desalting the mixture was transformed into electro competent *E. coli* XL1 cells. The obtained clones were analyses by restriction only. A control restriction with *Nde*I and *Hind*III was performed according protocol (see material and methods page 29)

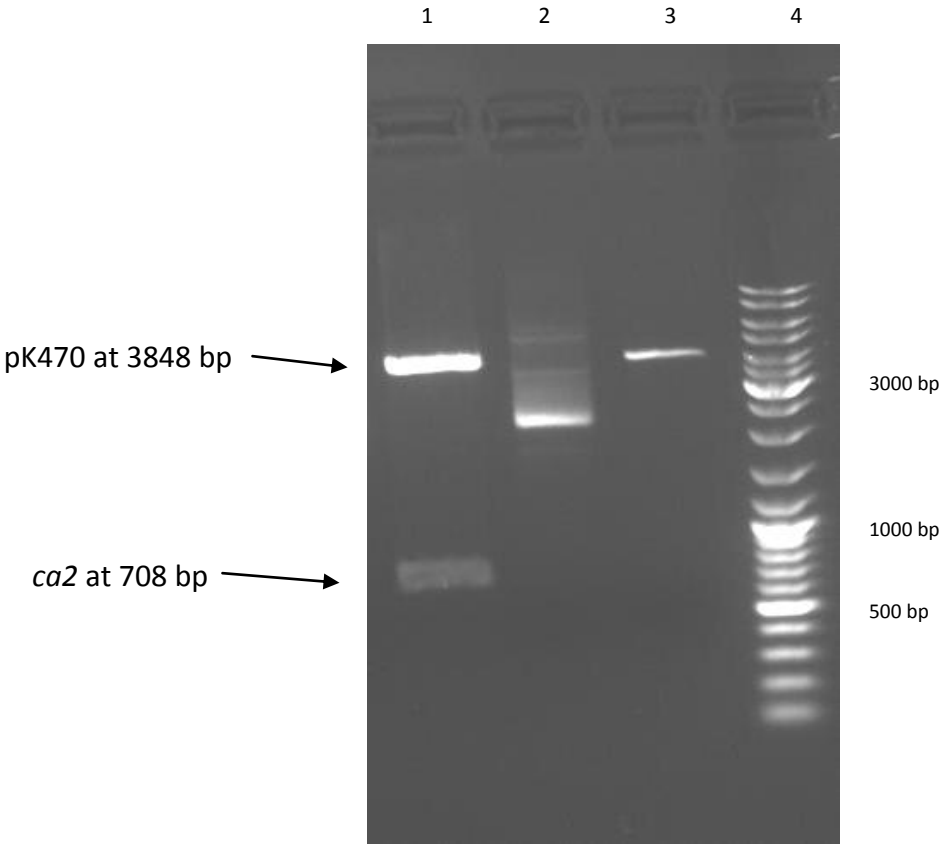


Figure 13: pK470 ca2 restriction analyses
Slot1: pK470 ca2 restricted with *Nde*I/*Hind*III, 708 & 3848 bp, Slot 2: pK470 ca2 uncut circular 4556 bp, Slot 3: pK470 restricted with *Nde*I/*Hind*III 3848 bp, Slot 4: 500 ng GeneRuler^{TM} DNA Ladder Mix.

After the correct restriction as shown in Figure 13, pK470 *ca*2 was sent for sequencing with the primers pMS_promfor and TacpMS470stop_new. The sequencing results showed no alteration to the published sequence.

3.2.3 Construction of the Expression vector pK470 ca3 (caa)

3.2.3.1 *PCR conditions for ca3*

For the PCR the primers CA3F with the attached *Nde*I restriction site and CA3R with the *Hind*III restriction site were used (see material and methods page 22). To get a specific product several PCR set-ups were tested.

Two annealing temperatures gave a similar result.

Table 14: PCR set-up for the amplification of *ca*3

Step	Temperature [°C]	Time [s]
Initial Denaturation	98	300
Denaturation	98	15
Annealing	64/66	5
Extension	72	30
Final extension	72	300
Cycles	25	

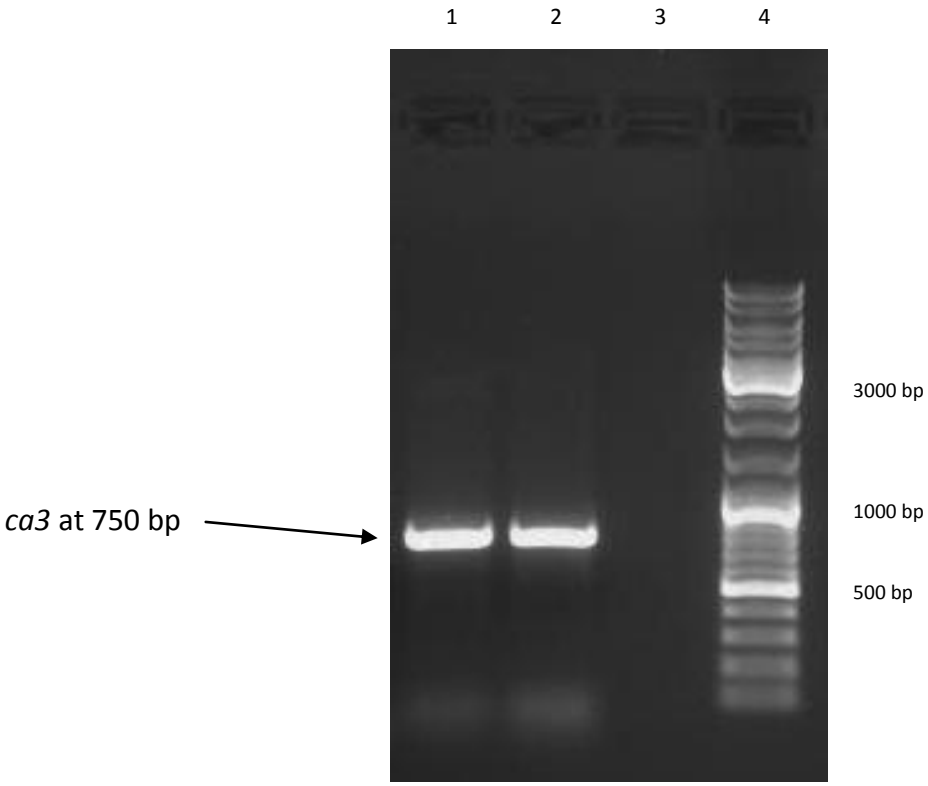


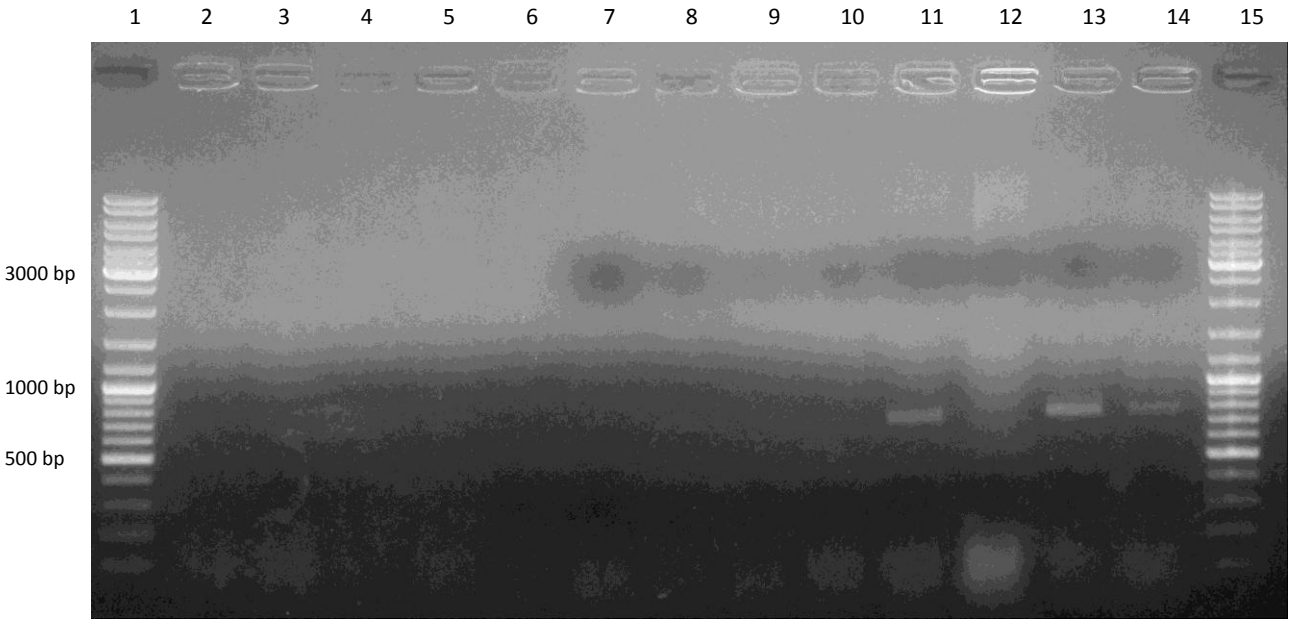
Figure 14: Control gel *ca*3 PCR product
Slot 1: *ca*3 with 64 °C annealing temperature 750 bp
Slot 2: *ca*3 with 66°C annealing temperature 750 bp
Slot 3: empty, Slot 4: 500 ng GeneRuler^{TM} DNA Ladder Mix,
Visible on the bottom is the primer surplus

Shown in Figure 14 are the obtained PCR products for *ca*3 out of genomic DNA. The two different products were received with different PCR programs. The expected size is 750 bp.

3.2.3.2 Transfer of the gene in expression vector pK470

For further cloning the approach with 64°C annealing temperature was used. The PCR product was cloned directly into the pK470 vector according the protocol (see material and methods page 29). For cloning into pK470-Bg_EstC-1_ΔNcoI the insert and the vector were digested with the same set of enzyme, *Nde*I and *Hind*III. The vector backbone was purified and separated from the original insert, EstC-1_ΔNcoI, over a preparative agarose gel. The insert fragment was digested and only purified by the Wizard® SV Gel and PCR Clean-Up System Kit (see material and methods page 30). The analysis of the obtained pK470 clones was done by colony PCR and restriction control. The colony PCR was done according the

protocol with the same primers as for cloning, CA3F and CA3R. The annealing temperature was 64°C.



ca3 at 750 bp

Figure 15: Results of the Colony PCR for *ca*3
Slot 1 and 15: 500 ng GeneRuler^{TM} DNA Ladder Mix, slot 2-14 clones 1-13 of *E. coli* pK470 *ca*3; positive clones are visible in slot 11, 13 and 14.

The insert should be at a size of 750 bp. As shown in figure 15 only three clones out of 13 carry the desired insert. For further cloning the clone 14, visible in slot 13, was used for further steps. To make sure that the restriction sites are functional and no mismatch had occurred during PCR, a double digest with *Nde*I and *Hind*III was performed (see material and methods page 29). The insert (*ca*3) is expected at a size of 750 bp and the vector at 3848 bp. As shown in, the expected restriction pattern after the double digest fits to the actual one. The vector backbone and the insert are the only bands that occurred, so the restriction sites are not altered.

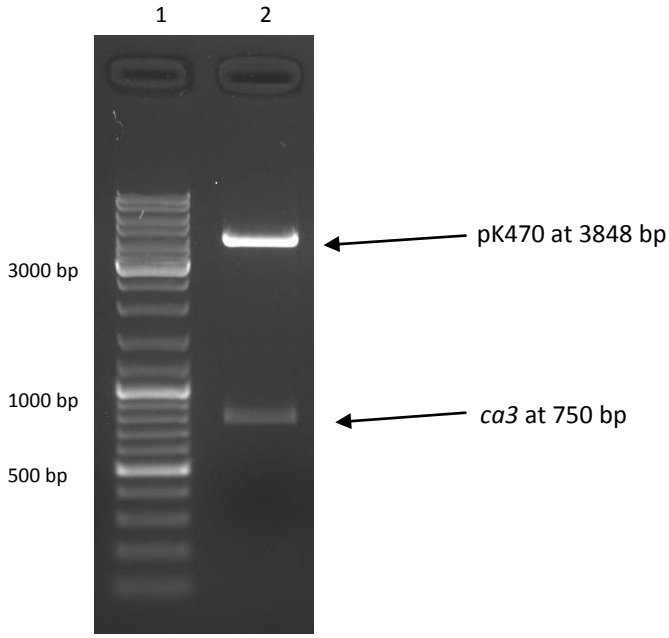


Figure 16: pK470 *ca*3 restriction analyses
Slot1: 500 ng GeneRuler DNA Ladder Mix, Slot 2: pK470 *ca*3 restricted with *Nde*I/*Hind*III 750&3848 bp

The correct clone was sent to sequencing with the same primer set as *ca*1 and *ca*2, pMS_promfor and TacpMS470stop_new. The sequence showed no differences to the sequence data, available online.

3.2.4 Construction of the Expression vector pK470 *ca*4 (H16_A1192)

3.2.4.1 PCR conditions for ca4

In order to amplify the gene H16_A1192 (*ca*4) the primers CA4F with attached *Nde*I restriction site and CA4R with the attached *Hind*III restriction sites were used (see materials and methods, page 22). The PCR program was designed after the recommended protocol of Finnzymes (Vantaa, Finland).

Table 15: PCR set-up for the amplification of *ca*4

Step	Temperature [°C]	Time [s]
Initial Denaturation	98	300
Denaturation	98	15
Annealing	67	15
Extension	72	30
Final extension	72	300
Cycles	25	

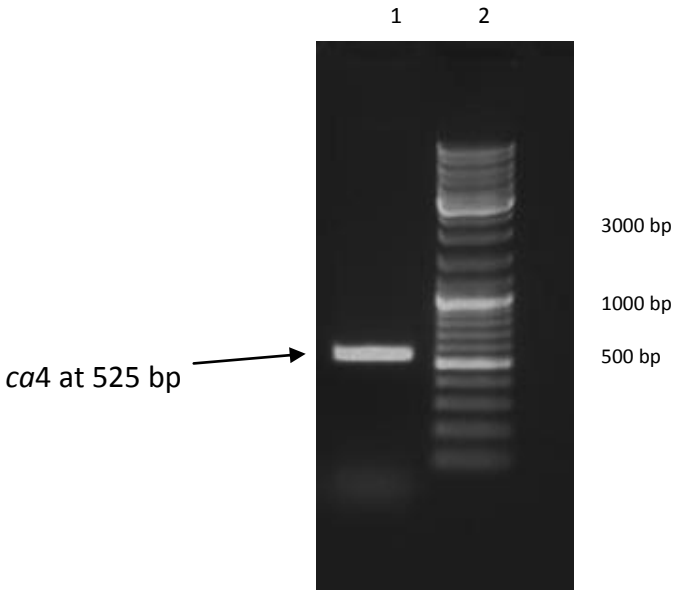


Figure 17: Control gel *ca4* PCR product
Slot 1: *ca*4 PCR product at a size of 525 bp
Slot 2: 500 ng GeneRuler DNA Ladder Mix

As shown in Figure 17 the PCR gave a distinct band at the right size. The PCR product was cut out and purified by Wizard® SV Gel and PCR Clean-Up System Kit (Promega). The purified product was used directly for cloning into pK470.

3.2.4.2 Transfer of the gene in expression vector pK470

For cloning the gene (*ca4*) into pK470-Bg_EstC-1_ΔNcoI the insert and the vector were digested with the same set of enzymes, *Nde*I and *Hind*III. The vector backbone was purified and separated from the original insert, EstC-1_ΔNcoI, over a preparative agarose gel. The insert fragment was digested and only purified by the Wizard® SV Gel and PCR Clean-Up System Kit (see material and methods page 30). To find out the concentration of both samples a control agarose gel was done. The two fragments were ligated according the protocol and 2 µl were transformed into electro competent *E. coli* XL1 cells (see material and methods page 29, 32). Plasmid preparation was done by GeneJET™ Plasmid Miniprep Kit (see material and methods page 32). The obtained clones were analysed by restriction in a double digest with *Nde*I and *Hind*III.

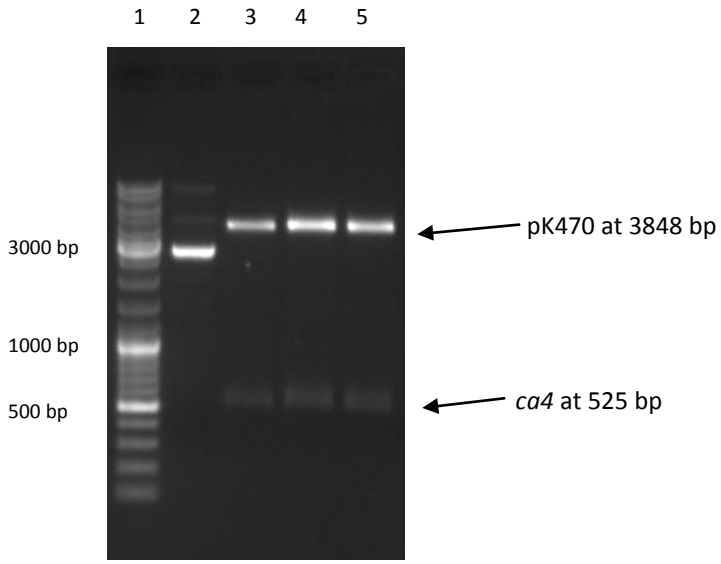


Figure 18: pK470 *ca*4 restricted with *Nde*I/*Hind*III
Slot 1: 500 ng GeneRuler DNA Ladder Mix, Slot 2: unrestricted pK470 *ca*4 clone 1, Slot 3: pK470 *ca*4 clone 1 restricted with *Nde*I/*Hind*III, vector backbone 3848 bp and *ca*4 525 bp, Slot 4: pK470 *ca*4 clone 2 restricted with *Nde*I/*Hind*III, vector backbone 3848 bp and *ca*4 525 bp, Slot 4: pK470 *ca*4 clone 3 restricted with *Nde*I/*Hind*III, vector backbone 3848 bp and *ca4* 525 bp

Clone 2 was sent for sequencing to LGC genomics. The results showed no mutation or base exchanges. For all following experiments this clone was used.

After cloning all four carbonic anhydrases into pK470, expression studies and activity assays were performed. The obtained vectors were isolated from either *E. coli* XL1 or *E. coli* TOP 10 and transformed to the expression strain *E. coli* BL21. Expression experiments were performed in this expression strain.

3.3 Expression and activity assays

The expression of the four carbonic anhydrases was done in 300 ml cultures with IPTG induction. The fermentations were kept on 28°C, shaking at 120 rpm overnight. The fermentations were analysed by SDS gels (12 % polyacrylamide) for expression.

3.3.1 Uninduced expression

In order to gain information about the promoter in this expression system, an uninduced expression was done. This experiment served as indication if carbonic anhydrases getting expressed without induction when nutrients are depleted. To carry out this experiment a sample of *E. coli* BL21 [pK470 *ca*4] and *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I] were grown on 28°C for 48 h at 120 rpm in a 100 ml shaking flask with a media volume of 30 ml LB.

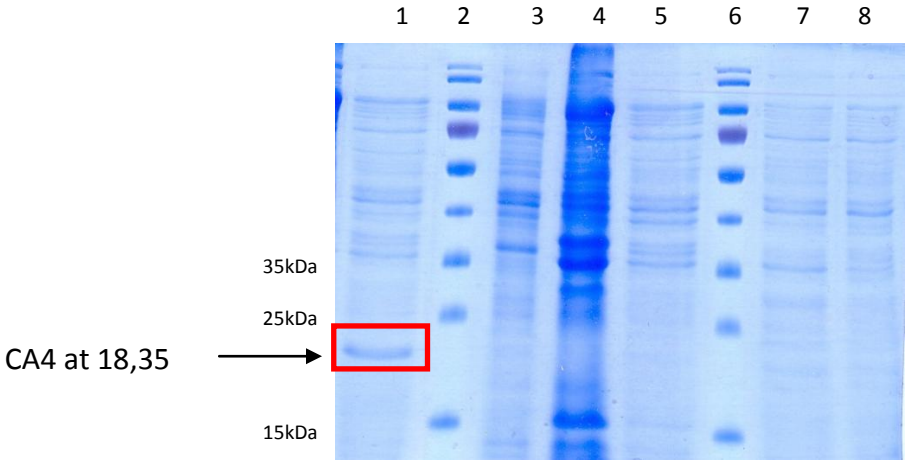


Figure 19: SDS-PAGE comparison uninduced vs. induced fermentation in *E. coli* BL21, Slot 1: *E. coli* BL21 [pK470 *ca*4] whole cell lysate induced, Slot 2/6: PageRuler™ Prestained Ladder, Slot 3-6: *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I] soluble fraction, Insoluble fraction, whole cell lysate induced, Slot 7: *E. coli* BL21 [pK470 *ca*4] whole cell lysate uninduced after 48h, Slot 8: *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I] whole cell lysate uninduced after 48h, marked in red CA4 at the expected size of **18,35 kDa** in the induced sample

This experiment showed no alteration of the protein profile in the uninduced sample (*E. coli* BL21 [pK470 *ca*4]), even after 48 h of growth at 28°C without addition of nutrients whereas the induced cells show a clear band at the size of 18,35 kDa, which is the expected size of CA4. The induced cells were harvested after 12 h of growth at 28°C. The uninduced sample was grown for 48, as already mentioned, and the OD_{600} was measured every three hours. In this sample, the OD_{600} remained unchanged after the first 14 h of growth. This experiment shows that the promoter P_{tac} is not leaky in this vector under these growth conditions. The expression of the carbonic anhydrases therefore has to be induced with IPTG for the desired product.

3.3.2 Filter Assay

The filter assay was performed according protocol (see material and method, page 37). The tested strains were streaked out on LB-Agra plates containing 40 µg/ml kanamycin and IPTG for induction to a total concentration of 0,1 mM.

E. coli XL1 [pK470-Bg_EstC-1_Δ*Nco*I] and *E. coli* TOP10 [pK470-Bg_EstC-1_Δ*Nco*I] as a negative control were streaked out on two plates. The two plates with the respective strains,

E. coli XL1 [pK470 *ca*1-4] and *E. coli* TOP10 [pK470 *ca*1-4] were grown at 28°C over night. On the next morning the assay was performed at the same time.

There was no difference visible between the negative control and the streaked out strains. The buffer did not turn red at *E. coli* [pK470-Bg_EstC-1_Δ*Nco*I] colonies or at the colonies of *E. coli* [pK470 *ca*1-4]. No colour change at all means that no sufficient consumption of H^+ takes place to raise the pH to change the pH indicator colour.

3.3.3 CO_2 Trapping

The assay was performed according protocol (see material and methods, page 38). As positive control one ml of a standard solution containing 30 W-A units was used.

The measurements showed no activity or change at all. There was no difference visible between the positive control, *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I] (soluble fraction), *E. coli* BL21 [pK470 *ca*1-4] (soluble fraction) and the negative control without any protein. From the soluble fractions a volume containing 10 mg of total protein was added to the reaction vessel. No activity or change of pH was measurable. The pH stayed the same as in the control solutions. No precipitate was visible or measurable. Also the pH remained unchanged through the experiment.

The pH of the 40 mM $KHCO_3^-$ stayed at 8,3 whereas the $Ca(OH)_2$ solution stayed at 11,1.

This indicates that no reaction takes place under these circumstances. Probably the activity was too low or the enzyme was inactivated during the experiment through the buffer.

3.3.4 p-Nitrophenyl acetate assay

For this assay two independent fermentations were performed. The cell disruption was done by sonication. Because of the sonication and the subsequent heat transfer in the sample, the pellet was solved in a high buffer volume to avoid protein precipitation and ensure cooling. Due to the high buffer volume the protein concentration was quite low.

For protein determination the Bradford assay according protocol was used (see material and methods, page 34). To control the soluble fraction for protein expression, SDS-PAGE was performed.

In the first fermentation, CA1 was not properly expressed, so in this case only the soluble fractions of *E. coli* BL21 [pK470 *ca*2-4] were measured. *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I] served as a control strain.

3.3.4.1 Protein Concentration

Table 16: Protein concentration for the p-Nitrophenyl acetate assay

Soluble fraction	1st Fermentation [mg/ml]	2nd Fermentation [mg/ml]
***E. coli* BL21 [pK470 *ca*1]**	---	7,7
***E. coli* BL21 [pK470 *ca*2]**	2,86	4,6
***E. coli* BL21 [pK470 *ca*3]**	3,86	7,1
***E. coli* BL21 [pK470 *ca*4]**	5,24	7,8
***E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I]**	3,47	8,3

3.3.4.2 Expression

As shown in Figure 20 no protein band is visible at the expected size of 24,77kDa for CA1 in the soluble fraction of *E. coli* BL21 [pK470 *ca*1]. Due to that fact, no measurements for the soluble fraction of *E. coli* BL21 [pK470 *ca*1] were performed.

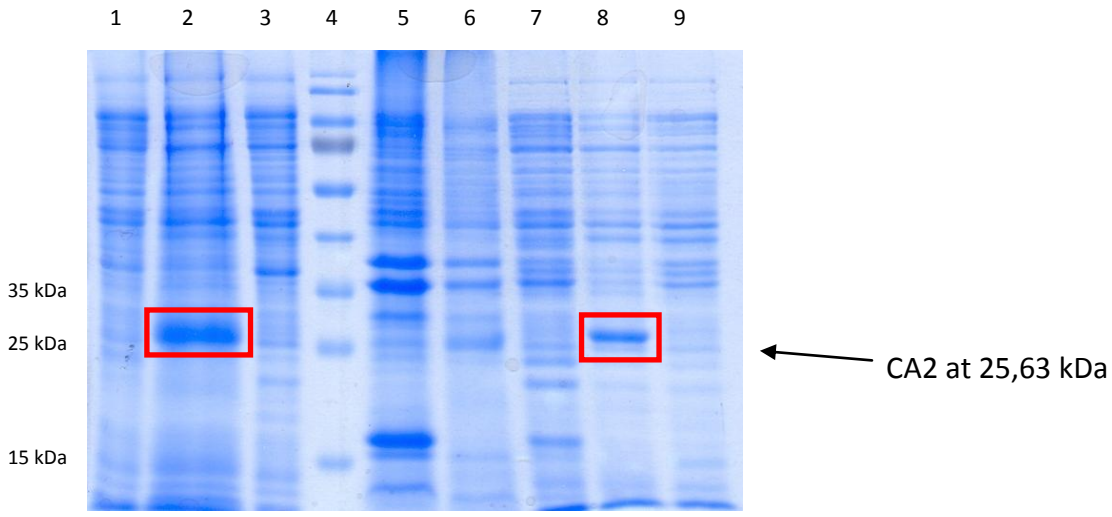


Figure 20: SDS-PAGE: fermentation *E. coli* BL21 [pK470-Bg_EstC-1_ΔNcoI] and *E. coli* BL21 [pK470 *ca*1/2] for p-Nitrophenyl acetate assay, Slot 1: *E. coli* BL21 [pK470 *ca*1] soluble fraction, Slot 2: *E. coli* BL21 [pK470 *ca*2] soluble fraction, Slot 3: *E. coli* BL21 [pK470-Bg_EstC-1_ΔNcoI] soluble fraction, Slot 4: PageRuler™ Prestained Protein Ladder, Slot 5: *E. coli* BL21 [pK470 *ca*1] insoluble fraction, Slot 6: *E. coli* BL21 [pK470 *ca*2] insoluble fraction, Slot 7: *E. coli* BL21 [pK470 *ca*1] whole cell lysate, Slot 8: *E. coli* BL21 [pK470 *ca*2] whole cell lysate, Slot 9: *E. coli* BL21 [pK470-Bg_EstC-1_ΔNcoI] whole cell lysate, Marked in red is CA2 at the expected size of **25,63kDa**, no band is visible at the expected size of CA1 at 24,77kDa

Figure 20 shows the expression pattern of *E. coli* BL21 [pK470 *ca*1/2]. For CA1, the expected band at the size of 24,77 kDa is not visible on this control SDS-PAGE. The soluble fraction of *E. coli* BL21 [pK470 *ca*2] shows the expressed protein at a size of 25,63 kDa.

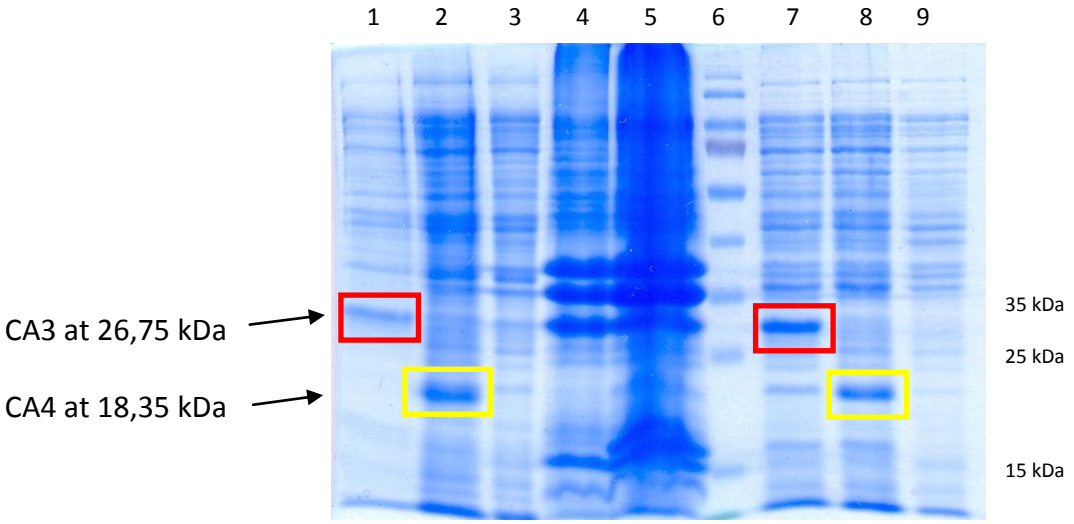


Figure 21: SDS-PAGE fermentation *E. coli* BL21 [pK470-Bg_EstC-1_ΔNcoI] and *E. coli* BL21 [pK470 *ca*3/4] for p-Nitrophenyl acetate assay
Slot 1: *E. coli* BL21 [pK470 *ca*3] soluble fraction, Slot 2: *E. coli* BL21 [pK470 *ca*4] soluble fraction Slot 3: *E. coli* BL21 [pK470-Bg_EstC-1_ΔNcoI] soluble fraction, Slot 4: *E. coli* BL21 [pK470 *ca*3] insoluble fraction, Slot 5: *E. coli* BL21 [pK470 *ca*4] insoluble fraction, Slot 6: PageRuler™ Prestained Protein Ladder, Slot 7: *E. coli* BL21 [pK470 *ca*3] whole cell lysate, Slot 8: *E. coli* BL21 [pK470 *ca*4] whole cell lysate, Slot 9: *E. coli* BL21 [pK470-Bg_EstC-1_ΔNcoI] whole cell lysate. Marked in red in figure 22 is CA3 at the expected size of **26,75 kDa**, marked in yellow is CA4 at a size of **18,35 kDa.**

As shown in Figure 21 the expression pattern shows strong protein bands at the correct size for these two carbonic anhydrases. The soluble fraction of *E. coli* BL21 [pK470 *ca*3] shows a band at the expected size of 26,75 kDa, as well as the soluble fraction of *E. coli* BL21 [pK470 *ca*4] at a size of 18,35 kDa. After it has been shown that the carbonic anhydrases are expressed, the soluble fraction was used directly for the activity assay and stored at 4°C.

In the second fermentation every carbonic anhydrase was expressed.

3.3.4.3 Measurements

The measurements were performed according protocol (see material and methods, page 40). The activity in the samples seems to be too low for fast measuring, so the test time was extended to 20 min in total.

Table 17: p-Nitrophenyl acetat assay: OD_{348} values 1st fermentation

	Run 1				Run 2			
Minutes	Soluble fraction of *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I]	Soluble fraction of *E. coli* BL21 [pK470 *ca*2]	Soluble fraction of *E. coli* BL21 [pK470 *ca*3]	Soluble fraction of *E. coli* BL21 [pK470 *ca*4]	Soluble fraction of *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I]	Soluble fraction of *E. coli* BL21 [pK470 *ca*2]	Soluble fraction of *E. coli* BL21 [pK470 *ca*3]	Soluble fraction of *E. coli* BL21 [pK470 *ca*4]
0	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
2	0,006	0,006	0,018	0,019	0,008	0,009	0,017	0,018
4	0,012	0,008	0,032	0,033	0,016	0,018	0,032	0,034
6	0,019	0,015	0,042	0,044	0,021	0,026	0,045	0,049
8	0,026	0,019	0,051	0,052	0,027	0,033	0,056	0,062
10	0,032	0,023	0,057	0,058	0,030	0,038	0,065	0,072
12	0,037	0,026	0,063	0,063	0,034	0,042	0,073	0,081
14	0,041	0,028	0,069	0,070	0,036	0,045	0,080	0,088
16	0,045	0,033	0,075	0,078	0,040	0,048	0,086	0,095
18	0,049	0,037	0,082	0,086	0,044	0,051	0,093	0,103
20	0,054	0,040	0,089	0,095	0,048	0,055	0,100	0,111

Table 18: p-Nitrophenyl acetat assay OD_{348} values 2nd fermentation

Minutes	**Soluble fraction of *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I]**	**Soluble fraction of *E. coli* BL21 [pK470 *ca*1]**	**Soluble fraction of *E. coli* BL21 [pK470 *ca*2]**	**Soluble fraction of *E. coli* BL21 [pK470 *ca*3]**	**Soluble fraction of *E. coli* BL21 [pK470 *ca*4]**
0	0	0	0	0	0
2	0,004	0,003	0,001	0,004	0,005
4	0,013	0,014	0,012	0,017	0,013
6	0,028	0,025	0,022	0,029	0,019
8	0,030	0,036	0,032	0,040	0,030
10	0,037	0,048	0,041	0,048	0,038
12	0,047	0,060	0,050	0,055	0,044
14	0,048	0,072	0,057	0,062	0,056
16	0,054	0,084	0,065	0,070	0,065
18	0,060	0,097	0,073	0,080	0,075
20	0,064	0,110	0,080	0,084	0,082

3.3.4.4 Calculation

The obtained extinctions were used to calculate the activity according the provided equation. Though the reaction volume was only 300 µl, the equation was adjusted to the smaller volume. The values of the control lysate (*E. coli* BL21 [pK470-Bg_EstC-1_ΔNcoI]) were used as a negative control and therefore deducted from the samples.

Table 19: Average enzyme activity measured with p-Nitrophenyl acetat assay

	Soluble fraction of *E. coli* BL21 [pK470 *ca*1]	**Soluble fraction of *E. coli* BL21 [pK470 *ca*2]**	**Soluble fraction of *E. coli* BL21 [pK470 *ca*3]**	**Soluble fraction of *E. coli* BL21 [pK470 *ca*4]**
Measurement 1 [W-A Units/mg]		1,08	3,61	2,78
Measurement 2 [W-A Units/mg]	2,87	3,76	1,70	1,18
Average [W-A Units/mg]	2,87	2,42	2,65	1,98
Standard deviation	0	1,34	0,95	0,80

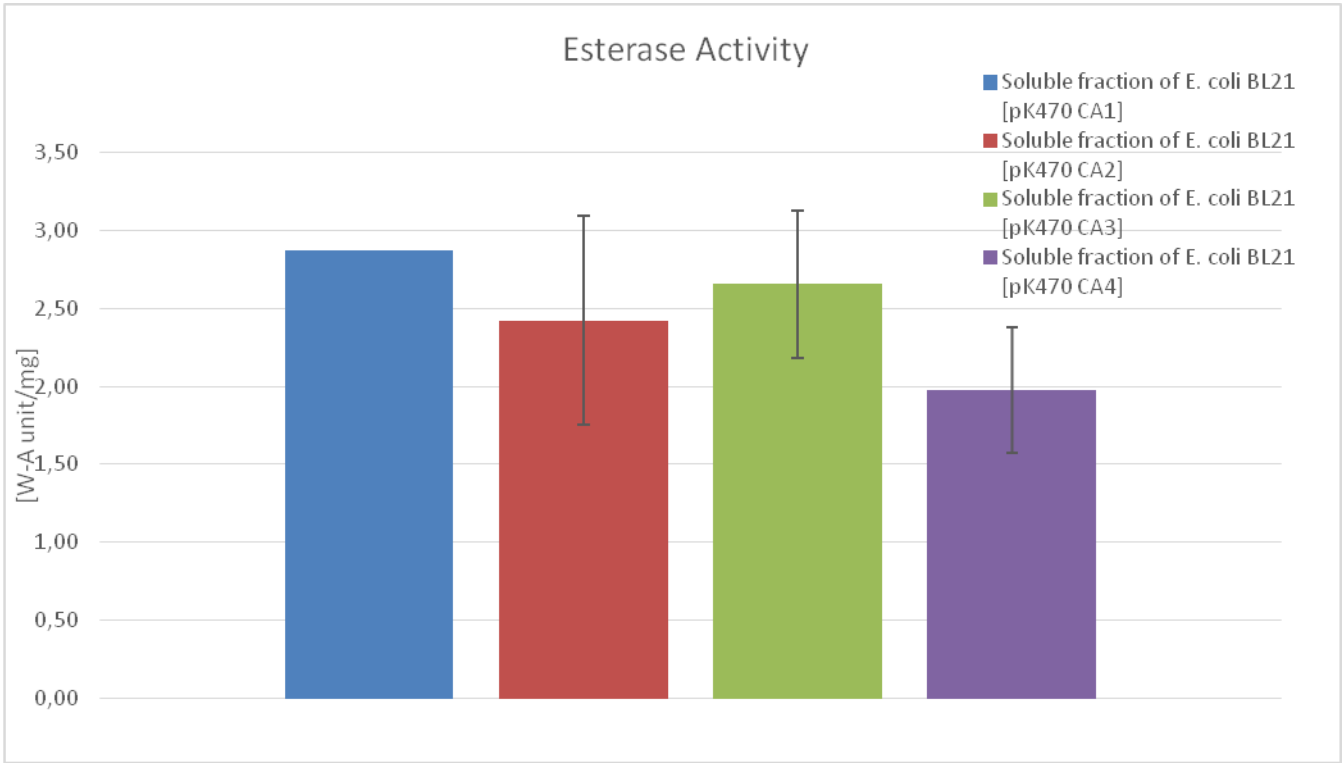


Figure 22: Activity according p-Nitrophenyl acetate assay in W-A-Units /mg protein

As shown in Figure 22, the specific activities of all samples were in a comparable range. For the soluble fraction of *E. coli* BL21 [pK470 *ca*1] only one value was measured, so no standard deviation can be calculated. The obtained results have a high deviation because of the different fermentations. No calculation about the actual proportion of carbonic anhydrase in the samples was done, so the varying activity is also due to the different content of carbonic anhydrase in the soluble fraction. The activity that was measured is not the main activity of the enzyme, so it is uncertain if the carbonic anhydrases of *R. eutropha* H16 are performing this side reaction at all or every different enzyme in the same extend.

3.3.5 Wilbur-Anderson Assay

For this assay two independent fermentations were performed. The fermentations were performed according protocol (see material and methods, page 33). The disruption of the cells was done by French Press. This method was chosen because of the high buffer volume after sonication due to methodical reasons. With the French Press the buffer volume can be reduced to get a higher protein concentration in the soluble fraction and therefore a higher activity. A prior experiment led to no result, the negative control without protein was in the same time range as the approaches with the soluble fractions of *E. coli* BL21 [pK470 *ca*1-4] and *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I].

The protein concentration of the already disrupted cells was lower for the 2nd fermentation because the added buffer volume was higher. However, to get comparable results the sample volume was doubled in the measurements of the second fermentation, so the protein content was similar. *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I] served as a negative control.

3.3.5.1 Protein concentration

The protein concentration was determined by the Bradford method (see material and methods page 34)

Table 20: Protein concentration of the soluble fractions used for the Wilbur-Anderson Assay

Strain	1st Fermentation [mg/ml]	2nd Fermentation [mg/ml]
***E. coli* BL21 [pK470 *ca*1]**	67,40	31,67
***E. coli* BL21 [pK470 *ca*2]**	44,62	29,30
***E. coli* BL21 [pK470 *ca*3]**	69,50	35,46
***E. coli* BL21 [pK470 *ca*4]**	62,23	36,44
***E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I]**	67,97	28,43

To ensure that the samples contain carbonic anhydrase control SDS-PAGE was performed and checked for proteins, respectively CA1-CA4.

3.3.5.2 Expression

As shown in Figure 23 and Figure 24, all carbonic anhydrases are expressed in a comparable amount. The bands for the different enzymes are visible constantly over the different samples soluble fraction, insoluble fraction and whole cell lysate. Though the bands at the height of the carbonic anhydrases are visible in every sample, it is presumable that a lot of protein does not solve in the buffer at all. Up to now it is unclear if the proteins get degraded during disruption of the cells or parts of the carbonic anhydrases does not fold right.
Marked in red is CA1 at the expected size of 24,77 kDa, marked in yellow is CA2 at a size of 25,63 kDa, marked in green is CA3 at a size of 26,75 kDa and marked in black is CA4 at a size of 18,35 kDa.

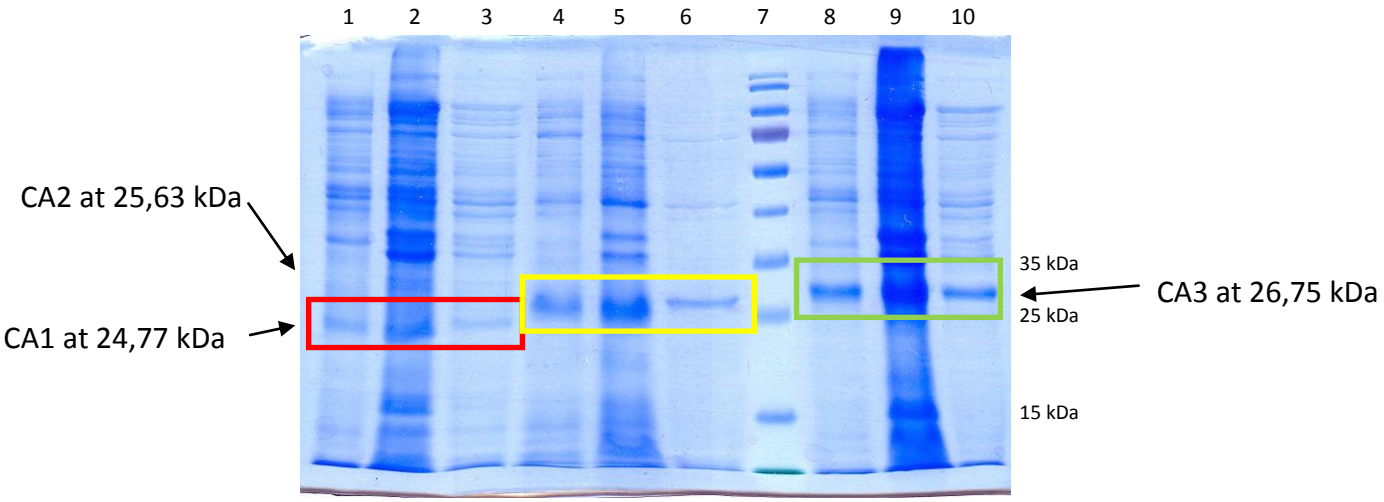


Figure 23: SDS-PAGE fermentation for Wilbur-Anderson Assay CA1/2/3
Slot 1: *E. coli* BL21 [pK470 *ca*1] soluble fraction, Slot 2: *E. coli* BL21 [pK470 *ca*1] insoluble fraction, Slot 3: *E. coli* BL21 [pK470 *ca*1] Whole cell lysate, Slot 4: *E. coli* BL21 [pK470 *ca*2] soluble fraction, Slot 5: *E. coli* BL21 [pK470 *ca*2] insoluble fraction, Slot 6: *E. coli* BL21 [pK470 *ca*2] whole cell lysate, Slot 7: PageRuler™ Prestained Protein Ladder, Slot 8: *E. coli* BL21 [pK470 *ca*3] soluble fraction, Slot 9 *E. coli* BL21 [pK470 *ca*3] insoluble fraction, Slot 10: *E. coli* BL21 [pK470 *ca*3] whole cell lysate, Marked in red is CA1 at the expected size of 24,77 kDa, marked in yellow is CA2 at a size of 25,63 kDa, marked in green is CA3 at a size of 26,75 kDa.

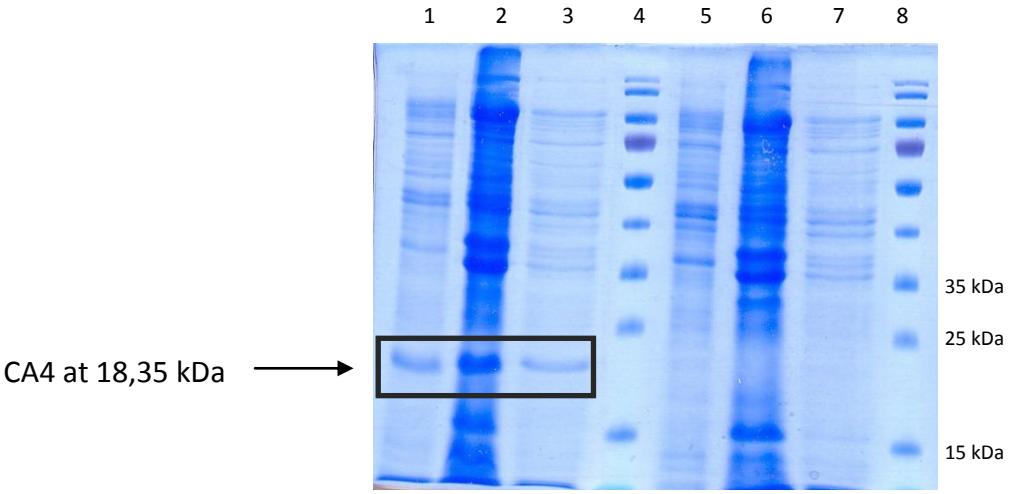


Figure 24: SDS-PAGE fermentation for Wilbur-Anderson Assay CA4 and control Slot 1: *E. coli* BL21 [pK470 *ca*4] soluble fraction, Slot 2: *E. coli* BL21 [pK470 *ca*4] insoluble fraction, Slot 3: *E. coli* BL21 [pK470 *ca*4] whole cell lysate, Slot 4: PageRuler™ Prestained Protein Ladder, Slot 5: *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I] soluble fraction, Slot 6: *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I] insoluble fraction, Slot 7: *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I] whole protein lysate, Slot 8: PageRuler™ Prestained Protein Ladder, marked in black is CA4 at a size of 18,35kDa

3.3.5.3 Calibration curve

This assay is not designed to measure whole cell lysates and low enzyme activities. Therefore a standard curve with an enzyme of defined activity was performed. The calibration curve was performed according to the same protocol as the samples (see material and methods, page 38). Every measurement was done at least in triplicate and the average was calculated. The negative control was the soluble fraction of *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I]. The standard enzyme (carbonic anhydrase from bovine erythrocytes with an activity of 2986 W-A units/mg) was diluted out of a 3000W-A Units/ml solution in 1/10 and 1/5 steps.

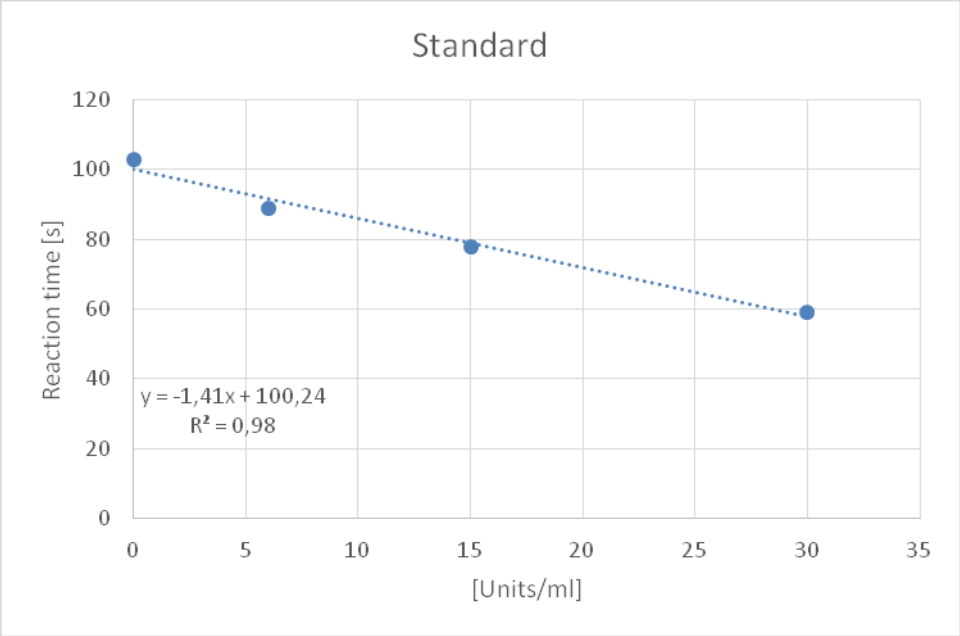


Figure 25: Calibration curve with a standard carbonic anhydrase Wilbur-Anderson Assay

Table 21: Standard enzyme measurement carbonic anhydrase

	Measurements [s]			
	Soluble fraction *E. coli* BL21 [pK470-Bg_EstC-1_ΔNcoI]	**6 U/ml**	**15 U/ml**	**30 U/ml**
	111	82	78	58
	101	91	78	63
	101	93	78	57
	101			
	103			
	101			
	103			
Average	103	88,7	78	59,3
Standard Deviation	3,4	4,8	0,0	2,6

The standard was measured three times at the beginning and between two different activities to detect, if unknown factors such as outgassing of the substrate solution lead to a shift in the measurements.

With the obtained calibration equation the amount of activity in the same volume of sample can be determined. After transformation of the equation the activity according the formula can be calculated.

Equation 3: Calibration curve equation for activity determination

$$x = -0,709y+71,092$$

3.3.5.4 Measurements

The samples were measured according protocol (see material and methods, page 38). Three standard measurements were performed before measuring the samples with defined activity. Between the different activities, also standard measurements were performed to detect a possible shift in the measurements due to changing CO_2 concentration in the substrate solution or other unknown factors.

Table 22: Wilbur-Anderson time measurement for the 1st fermentation

	Soluble fraction *E. coli* BL21 [pK470-Bg_EstC-1_ΔNcoI]	Soluble fraction *E. coli* BL21 [pK470 *ca*1]	Soluble fraction *E. coli* BL21 [pK470 *ca*2]	Soluble fraction *E. coli* BL21 [pK470 *ca*3]	Soluble fraction *E. coli* BL21 [pK470 *ca*4]
	94	89	85	104	87
	100	70	70	90	40
	119	78	80	57	90
	111	59		66	
Average [s]	106	74	78,3	74,4	72,3
Standard Deviation [s]	8,6	9,8	5,4	16,7	19,8

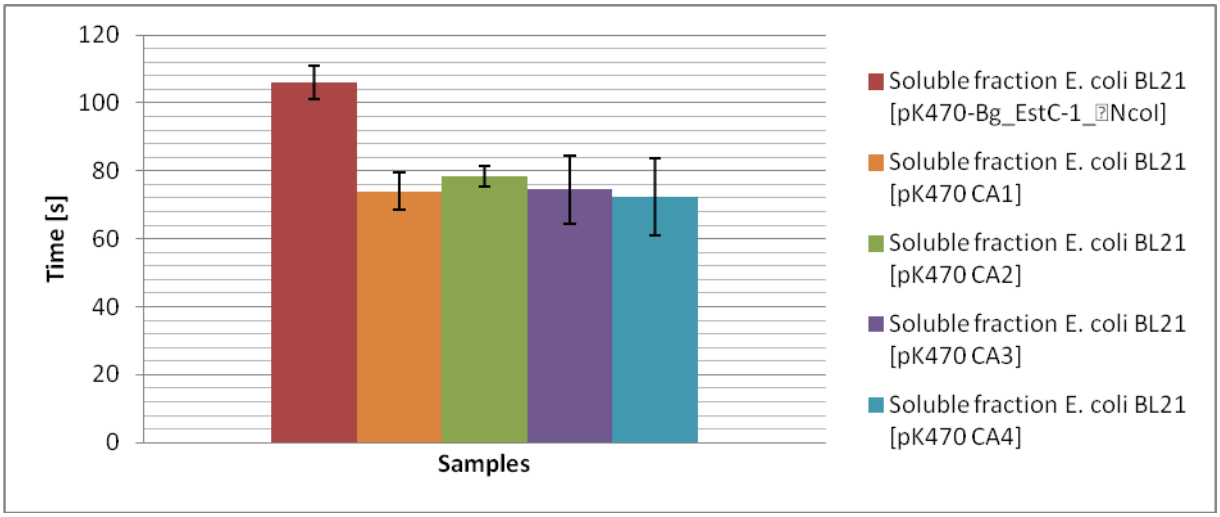


Figure 26: Comparison of the carbonic anhydrase activity in the 1st fermentation for Wilbur-Anderson units

Table 23: Wilbur-Anderson time measurement for the 2nd fermentation

	Soluble fraction *E. coli* BL21 [pK470-Bg_EstC-1_ΔNcoI]	Soluble fraction *E. coli* BL21 [pK470 *ca*1]	Soluble fraction *E. coli* BL21 [pK470 *ca*2]	Soluble fraction *E. coli* BL21 [pK470 *ca*3]	Soluble fraction *E. coli* BL21 [pK470 *ca*4]
	117	94	71	94	50
	110	73	89	68	83
	99	67	93	80	95
	103			86	80
Average	107,3	78	84,3	76,6	77
Standard Deviation	6,1	10,0	8,3	8,5	14,8

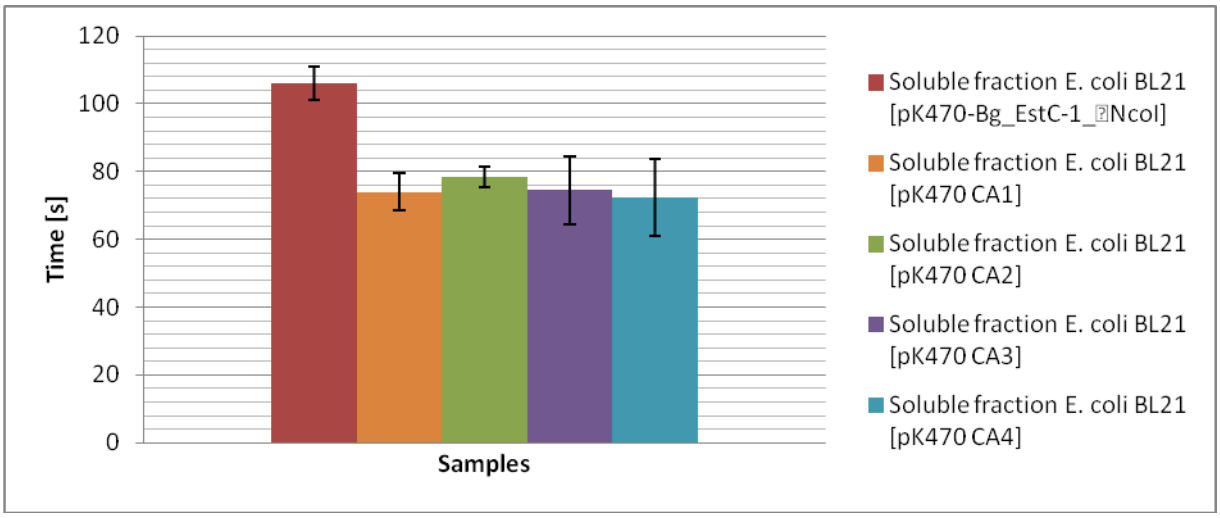


Figure 27: Comparison of the carbonic anhydrase activity in the 2nd fermentation for Wilbur-Anderson units

The enzyme activity of both fermentations was calculated according to two different methods. The activity can be calculated according the equation provided with the protocol. This equation is used for the standard application in quality control. Therefore this calculation method is designed to measure a higher enzyme activity than it is expected in the soluble fraction. The second method was the calculation with the obtained standard curve equation, which was measured according the altered protocol and in the range of the expected activity. Because of that, the results of this calculation method should be more accurate.

3.3.5.5 Calculation

The activity of both measurements was calculated according two methods mentioned above.

Table 24: Compared activities 1st Fermentation

	Soluble fraction *E. coli* BL21 [pK470-Bg_EstC-1_ΔNcoI]	**Soluble fraction *E. coli* BL21 [pK470 *ca*1]**	**Soluble fraction *E. coli* BL21 [pK470 *ca*2]**	**Soluble fraction *E. coli* BL21 [pK470 *ca*3]**	**Soluble fraction *E. coli* BL21 [pK470 *ca*4]**
Average Time [s]	106	74	78	74,4	72
Standard Deviation [S]	9,7	11	6,2	19,7	22,9
Activity according protocol [Units/ml]	0	8,6	7,1	8,5	9,3
Activity according protocol [Units/mg]	0	0,13	0,16	0,12	0,15
Calibration equation [Units/ml]	0	18,6	15,6	18,3	19,8
Calibration equation [Units/mg]	0	0,28	0,35	0,26	0,32

As shown in table 24 the calculated values with the calibration curve are approximately the double of the one obtained with the equation provided with the protocol. For further calculations and all charts only the values from the calibration curve were used.

Table 25: Compared activities 2nd fermentation

	Soluble fraction *E. coli* BL21 [pK470-Bg_EstC-1_ΔNcoI]	**Soluble fraction *E. coli* BL21 [pK470 *ca*1]**	**Soluble fraction *E. coli* BL21 [pK470 *ca*2]**	**Soluble fraction *E. coli* BL21 [pK470 *ca*3]**	**Soluble fraction *E. coli* BL21 [pK470 *ca*4]**
Average Time [s]	107	78	84	76,6	77
Standard Deviation [s]	6,9	12	9,6	10,6	19,0
Activity according protocol [Units/ml]	0	3,8	2,7	4,0	3,9
Activity according protocol [Units/mg]	0	0,12	0,09	0,11	0,11
Calibration equation [Units/ml]	0	7,9	5,6	8,4	8,2
Calibration equation [Units/mg]	0	0,25	0,19	0,24	0,23

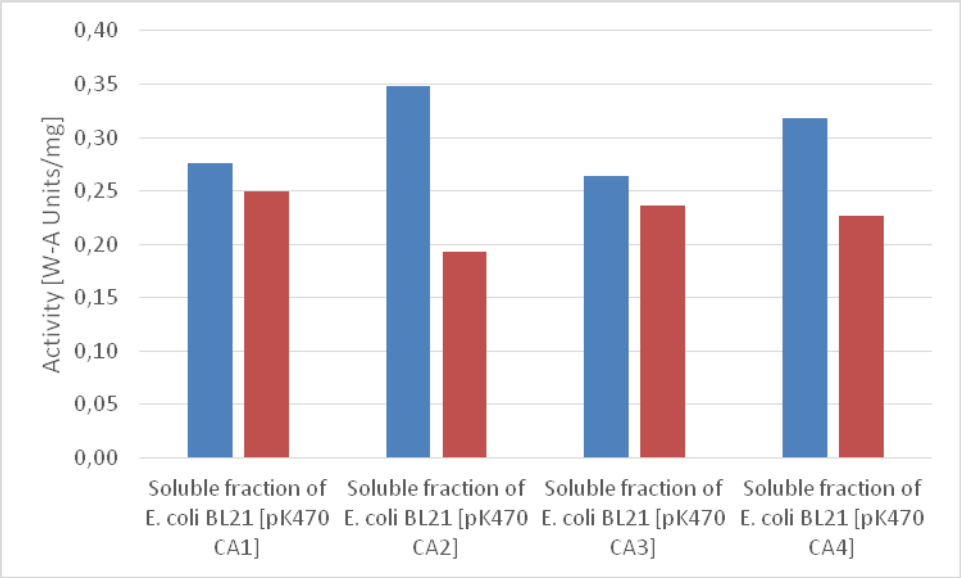


Figure 28: Compared activities according calibration curve equation, the specific activity is quite low because of the high protein background; activities were calculated according calibration curve equation.

As shown in Figure 28, the time in the second assay seems to be higher overall, which means that the activity is lower. The activities are in a comparable range, but still at a very low level. Though the specific activity is under 1 W-A Unit/mg further tests are required. The measurements are not exact enough to derive kinetic data. The calculation method according the standard curve seems to give more accurate results, because of the already included background reaction of the soluble fraction *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I] as a negative control and the defined activity in the range of the expected activity, which is much lower than usually measured. Still, one of the main problems is the different amount of activity present in the samples and the varying protein content due to the breaking down of the cells.

3.3.6 Infrared spectroscopy

Different measurements were done with the infrared spectroscope. It was tried to measure the reverse reaction from carbonate to CO_2 as well as the expected main reaction to carbonate. In the reaction from carbonate to CO_2, the generated CO_2 should be detected by the spectroscope. Changes in the transmittance were visible through all samples and in every testing condition, so the method is working in general.

Although changes were visible, this assay did not give exact results. There are several indications for activity but not all samples showed clear results. There are also different species of carbonate, which all has a different transmittance, which makes it hard to calculate the generated carbonate over the time.

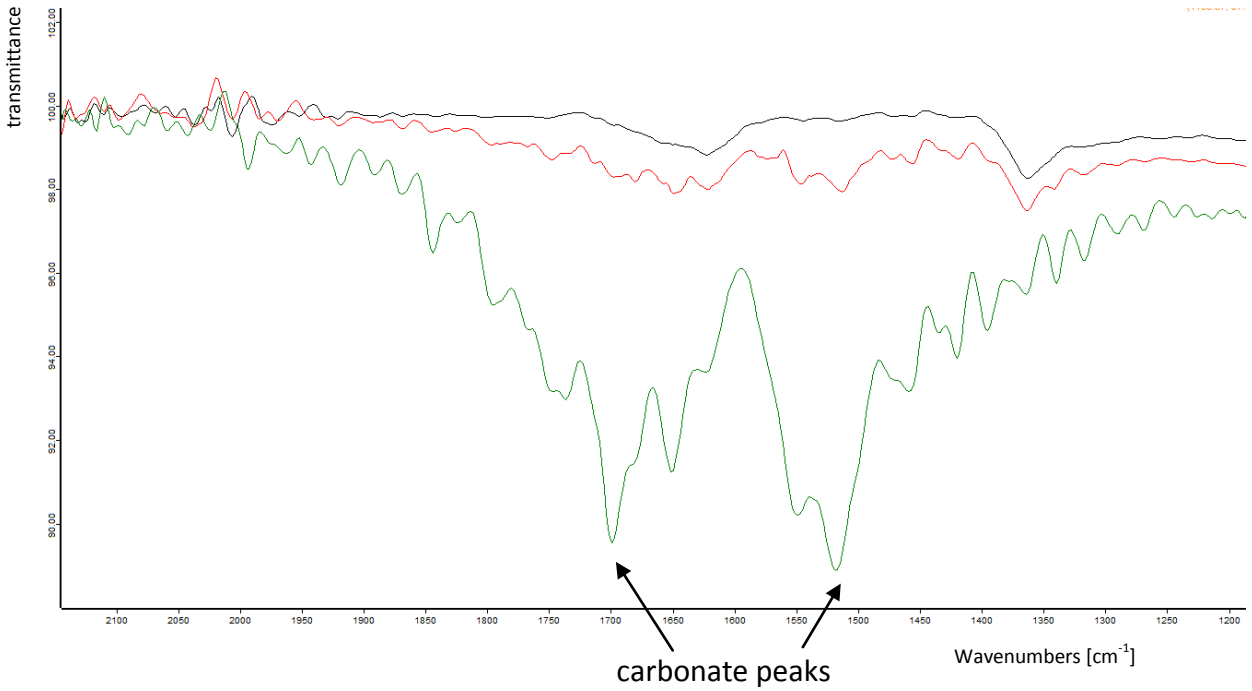


Figure 29: Background reaction from CO_2 to carbonate in the sample buffer without enzyme
In green is the standard, a 0,1% $NaHCO_3$ solution, in black is the sample solution without any enzyme at the time 0, in red is the sample solution without enzyme after 36min at the end of the experiment. Visible at 1700nm and 1515 are the carbonate peaks. The carbonate solution should give a short hint where the expected product should increase

As shown in Figure 29, the reaction takes place at a distinct level over time. That background reaction may be caused by the buffer. The CO_2 is converted to hydrogen carbonate whereas the buffer is converted to phosphoric acid. That background reaction is faster than the natural occurring conversion in water due to the buffer capacity and should be advanced

with the addition of carbonic anhydrase activity. Because of this natural occurring reaction it is even harder to calculate an activity due to this additional background reaction.

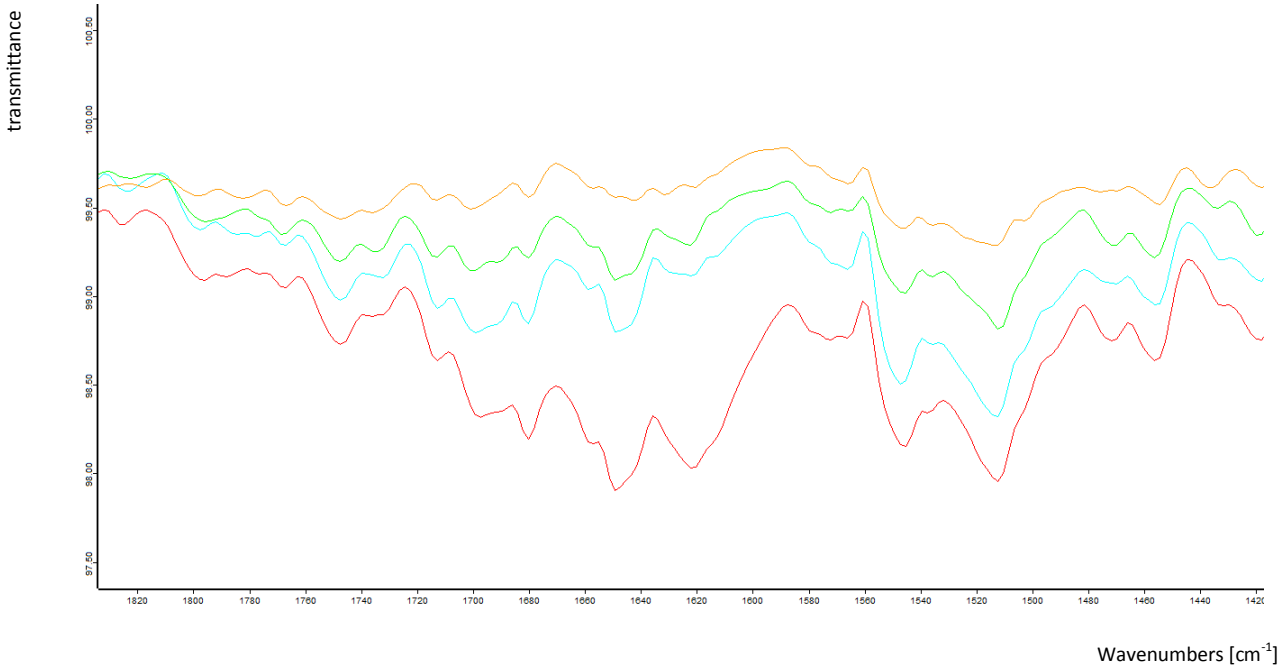


Figure 30: Different Activities Infrared Spectrometry In red is the negative control without enzyme, in blue is the sample solution with 1 Wilbur-Anderson Unit/ml, in green is the sample solution with 3 Wilbur-Anderson Units/ml, in orange is the highest activity of 10 Wilbur-Anderson Units/ml. All spectra are recorded at the end of the reaction after 36min.

As shown in Figure 30 the generation of hydrogen carbonate is surprisingly inhibited by increasing enzyme activity provided in the reaction volume. The generation of carbonate is decreasing the higher with a higher activity, which is contrary to the expected result. In theory, the addition of carbonic anhydrase should provide a much faster reaction than without.

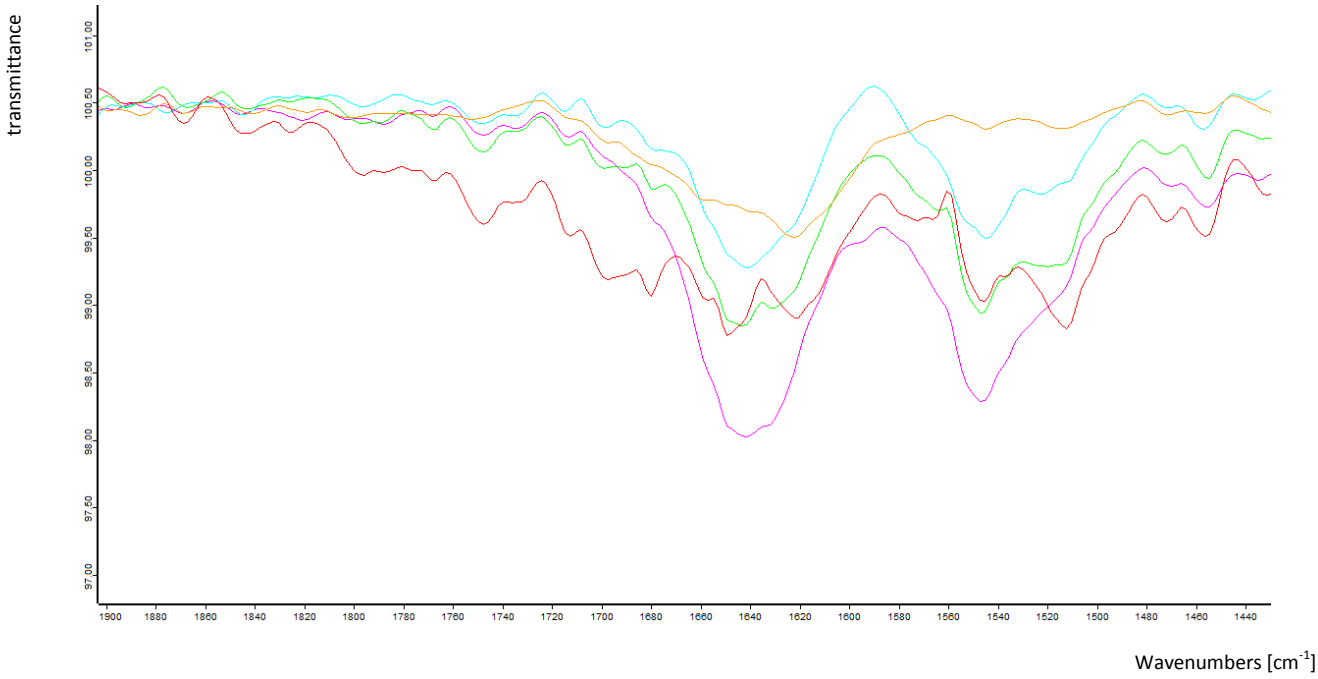


Figure 31: Infrared spectrometry with different soluble fractions of *E. coli* BL21, in red is the negative control without enzyme after 36min, in orange is the negative control at the time 0, in green is the sample solution with *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I](6 mg/reaction), in pink is the sample solution with *E. coli* BL21 [pK470 ca3](6 mg/reaction), in blue is the sample solution with *E. coli* BL21 [pK470 *ca*4](6 mg/reaction)All spectra are recorded at the end of the reaction after 36min except the orange at T=0.

In Figure 31, the soluble fractions of *E. coli* BL21 [pK470 *ca*3] and *E. coli* BL21 [pK470 *ca*4], vicarious for all four carbonic anhydrases, are shown in comparison to the negative control without enzyme and the control reaction with soluble fraction of *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I]. As visible, the generation of carbonate over time does not differ in a high amount between the negative control and the samples. Because of the different amounts of carbonic anhydrase in the sample solution it remains unclear if there is activity at all under this reaction conditions. It seems that CA3 is more and CA4 is less active than the control. The differences in the signal are modest and it is unlikely that activities can be determined without further testing.

4 Discussion

The abilities of *R. eutropha* H16 make it an interesting organism in which to study lithoautotrophic growth (Jeffke et al. 1999; Höfle 2005). The ability to grow in liquid media with the source of carbon and energy in the gaseous phase offers some advantages compared to established biological systems. However; there are several facts to consider. For example, little is known about its manipulation compared to *E. coli* strains. Under these circumstances, new knowledge, especially of the carbon metabolism under autotrophic conditions, is necessary to increase the understanding of the involved reactions and enzymes. Not all tasks of carbonic anhydrases are revealed yet, so a lot of research has to be done in this field (Ueda et al. 2012). Under this premise, this project focuses on new ways of manipulating this organism and increases its effectiveness in expression of proteins. A more efficient uptake of CO_2 and subsequent higher cell mass combined with the ability to grow to very high cell densities will offer some advantages compared to *E. coli.*

To accomplish a higher CO_2 uptake besides RuBisCO, this master thesis was focused on cloning and expression of carbonic anhydrases to generate more carbonate for as a precursor for following carboxylation reactions. The successful expression of all four carbonic anhydrases of *R. eutropha* H16 was followed by a characterization with different activity assays. This research suggests that these enzymes will provide an elevated level of carbonate and help to incorporate more CO_2 than in the wild type (Cramm 2009). The Pyc-carboxylase from *Corynebacterium glutamicum*, a subsequent reaction of the carbonic anhydrase activity, is an anaplerotic reaction in *E. coli*, but only when growing on lactate instead of glucose as sole carbon source (Britta Anderlei 2002). If the two carboxylases of *R. eutropha* H16 (phosphoenolpyruvate-carboxylase and pyruvate carboxylase) perform anaplerotic reactions too, more CO_2 should be incorporated via this pathway.

To provide enough precursors for efficient carboxylation, all four carbonic anhydrases of *R. eutropha* H16 were cloned into the expression vector pK470. This cloning step was accompanied with some difficulties.

The PCR conditions had to be optimized before obtaining a single, distinct product. This may be caused by the high GC-content of *R. eutropha* H16. This content varies between 62,3 % in the megaplasmid and 66,8 % in chromosome 2. This GC-content is very high compared to

50,8 % in *E. coli* K12 and can possibly cause problems in the PCR reactions as well as in the expression afterwards. After several attempts the optimal PCR conditions were obtained. However, the sequencing of the cloned genes showed no mutations at all. When the cloning step of all four enzymes was finished, the next important step was the expression of the obtained genes in *E. coli* BL21.

4.1 Expression

The expression of genes with this high GC-content in *E. coli* BL21 can lead to a non-functional gene product, although it seems to be expressed. The SDS-PAGE control gels showed expression for every single carbonic anhydrase in the soluble fraction but as well in the insoluble fraction.

Fermentation of heterologous genes so different from their own genes can lead to a high stress level for the cells, which can also interfere in the expression process. So the heterologous expression can cause problems in several different ways. In this study the expression worked quite well for all of the carbonic anhydrases. It is possible that the genes themself are interfering in the expression because of rare codons used and therefore the right folding is disturbed. It also may be that the gene product has an activity that inhibits growth or other basic functions of the cell. Since not all physiological tasks of the enzymes are revealed it is possible that the host, *E. coli,* is expressing some proteins that inhibit the function of the carbonic anhydrases. If the enzyme but not the activity can be dispensed there could be a possible interference between carbonic anhydrases originating in *E. coli* and the new introduced one (Merlin et al. 2003). One other fact to consider is the instability of heterologous protein. If the proteins are not correctly folded, the expressed protein may precipitate. The portion of the expressed carbonic anhydrase is found in the insoluble fraction may be one indication supporting this theory. The precipitation occurred at different times after centrifugation and should be further tested with different buffers to stabilize the enzymes in solutions. To overcome this problem the lysate was used directly after the centrifugation step for activity assays. At this point no precipitate was visible. The protein lysate was stored at 4°C and after several hours or days some precipitate occurred.

4.2 Activity

Most assays to determine the activity of CA`s are based on a pH change over time or at holding a distinct pH (Shingles und Moroney 1997; McIntosh 1968). To demonstrate the activity of the carbonic anhydrases from *R. eutropha* H16 several different experiments were performed. Not all of them were successful; especially the experiments to prove the reverse reaction from carbonate to CO_2. This may be due to reaction conditions that are not favourable for the enzymes. The filter assay that was performed for a first indication about activity with the *E. coli* colonies wiped from a petri dish also may cause problems because of the low activity under ambient conditions and the low concentration of expressed protein on the filter. If the main reaction is towards carbonate, the low concentration of enzyme and the low activity may vanish in the background reactions. One possible explanation why the CO_2-trapping experiment failed may be because of the high volume of carbonate solution. If the added activity is modest, the pH change and the generated CO_2 can be under the detection limit. The studies also suggest that the reaction in this direction is not the favoured one for these subclasses (So et al. 2004). Only one class, the ε-class, seems to have the distinct task to elevate the CO_2 concentration, the others work in the opposite direction (So et al. 2004; Sawaya 2006). Since *R. eutropha* H16 has no ε-class carbonic anhydrase, this activity may not be present at all.

However, the activity assays for the Wilbur-Anderson units directly as well as the p-Nitrophenyl acetate assay showed some activity for every enzyme measured, though the standard deviation for each enzyme activity is quite high.

Also this assay measures not the main activity of the enzyme, so in this context it is not certain that only the carbonic anhydrase is catalysing this reaction or if the CAs of *R. eutropha* H16 are performing this reaction at all. This experiment in particular is vulnerable to various interferences from the background proteins. Although all proteins are expressed quite well, the quantity of carbonic anhydrase in the lysate is not exactly known.

If the expression profile is altered through the expression of carbonic anhydrase, the background strain *E. coli* BL21 [pK470-Bg_EstC-1_Δ*Nco*I] may behave totally different to the other strains.

Therefore it is likely that the other proteins in the solution are interfering with the assays in different ways. The additional proteins in the lysate may function as an additional buffer or

some CA activity is present in the crude lysate. Especially the results of the Wilbur-Anderson-Assay indicate that the proteins of the background resulting from the strain itself have some activity in the opposite direction or inhibit the activity in some way. This is indicated by the negative control without any protein, which is about 25 s below the average of the negative control with protein of the background strain. It also may be that enzyme activity originating from *E. coli* BL21 is responsible for this delay. Under ambient conditions CA is expressed in *E. coli*, but this enzyme could be inhibited by a high concentration of CO_2, though it should keep only as much CO_2 in the cells as needed for metabolism (Merlin et al. 2003). One additional point is the drop of the pH. Different reactions are interfering with the change of pH, so the CA activity is not the only reaction which is influencing the measurement. This fact in particular is important for the measurements, because of the quality of the expressions. As already mentioned, it may be that the expression influences the cell in a certain way. Every carbonic anhydrase may have different tasks and therefore is influencing the cell in another way. If the expression profile of the background strain is altered by the expression, the background reactions are altered too.

If only one carbonic anhydrase is needed for growth of *R. eutropha* H16 under ambient conditions, as suggested by Kusian, et al, the others may have other tasks in metabolism that may be only needed under certain growth conditions (Kusian et al. 2002). The assay set-up does not meet these conditions, so the activity may not be measurable. Another concern is the inhibition that may come from the background or the buffer. If the enzymes are only active under certain conditions or may have a different task in metabolism, they may be inhibited under testing conditions (Smith und Ferry 2000).

The infrared spectroscopy showed the opposite of the expected result. The measurements with the standard enzyme suggest that the generation of carbonate out of CO_2 is inhibited by the higher activity.

One possible explanation for this phenomenon is that the reaction is already over when the first measurement is finished. There is a time delay between the start of the reaction and the measurement due to the methodology. In this time frame, the reaction may take place without the possibility of monitoring it. Though the infrared spectrometer is only measuring changes, it may be that the high activity is catalysing until equilibrium is reached and because of that no change over time can be observed. The background activity without any enzyme is explained by the reaction of CO_2 with the buffer. The phosphate in the buffer is

converted to phosphoric acid, whereas the CO_2 is converted to hydrogen carbonate. With all these variables, the possible reaction in both sides, the background reaction with the buffer, the possible interference of the background proteins as buffer or inhibiting activity, a general conclusion about the activity is not possible. Further testing and optimizing of this method is required to obtain reliable results. One option to get more reliable results would be the coupling of two activity assays. The time dependent assay and the infrared spectrometry can be performed at the same time. Though the generation of carbonate changes the pH, it would also be seen in the spectrometric data. With the combination of this two data, it should be possible to determine if the infrared spectrometer is recording the right reaction at the right time. With this method it should be further possible to exclude the measurement of non-target reactions.

In general the main problem for all activity assays is the unknown activity of the crude lysate affecting the measurements. For future work the assays should be performed with purified enzymes and different buffer conditions. Different buffers should be tested for all carbonic anhydrases to gain more information about activation and inhibition under certain conditions.

One possible experiment to eliminate even the reverse reaction is a coupled enzyme assay with a subsequent carboxylation step. This carboxylation would remove arising carbonate and therefore exclude the reverse reaction that may occur at a low level. This method is not yet described of the literature.

Only two methods used in this thesis are standardized and also used for quality control of CA. The sample solutions are always dissolved lyophilized powder and not the soluble fraction of cells (Sigma-Aldrich 1994; Worthington Biochemical Corporation). Though no enzyme assay for bacterial carbonic anhydrases is commercially available, further testing with different methods is required to obtain a reliable assay.

To sum up, all four carbonic anhydrases were successfully cloned and expressed in *E. coli*. Up to date only one carbonic anhydrase, *ca1,* is cloned and described as functional in literature (Kusian et al. 2002). In this master thesis the activity of all four enzymes discovered in *R. eutropha* H16 was demonstrated with slightly altered applied assays, originally designed to

measure higher activities with purified mammal enzymes (Sigma-Aldrich 1994; Worthington Biochemical Corporation). Up to now no kinetic data can be calculated with the data originated from the activity assays, because the specific activity varies between 0, 15 and 0, 35 W-A Units/mg, which is too low for a reliable statement. For a more detailed characterization, purified enzyme would be necessary. However, the activity between 2 and 3 W-A Units/mg in the p-Nitrophenyl acetate assay and 0,2 to 0,35 W-A Units/mg in the Wilbur- Anderson assay , all derived from the soluble fraction, is promising.

4.3 Outlook

The task of this diploma thesis was the cloning, expression and characterization of four different carbonic anhydrases out of *R. eutropha* H16. The aim is the faster conversion of CO_2 to carbonate. The subsequent activity of Ppc and Pyc should incorporate the generated carbonate in the metabolism and therefore increase the CO_2 uptake. The different assays show, that every carbonic anhydrase is active, but the background greatly affects the activity determination. Ideally all carbonic anhydrases should be expressed in *R. eutropha* H16 itself under lithoautotrophic conditions, to get the basic information if the carboxylases can be used for anaplerotic reactions and to purify the enzyme directly from its host. One advantage of expressing the enzymes in its own organism is that one can overcome the possibility of improper folding.

The activity assay should be repeated with purified enzymes to determine the specific activity in more detail and obtain kinetic data for characterization of the enzymes.

One possible new activity assay is a coupled enzyme assay with a subsequent carboxylation step as already mentioned above.

In conclusion, the enzymes should be expressed in their own organism and the activity determination should be performed with a purified enzyme to exclude other reactions that cause pH changes or interfere with the reaction in some way. The enzymes expressed in the originating host and purified enzyme will eliminate most of the varying conditions such as improper folding, different concentration of carbonic anhydrases in the soluble fraction and background reactions due to other enzymes.

5 References

Aneja, Kawalpreet K.; Ashby, Richard D.; Solaiman, Daniel K. Y. (2009): Altered composition of *Ralstonia eutropha* poly (hydroxyalkanoate) through expression of PHA synthase from *Allochromatium vinosum* ATCC 35206. In: *Biotechnol Lett* 31 (10), S. 1601–1612.

Armin K. Tiemeyer (2007): Reaktionstechnische Untersuchungen zur chemolithoautotrophen Nitratatmung von *Cupriavidus necator*. Disseration. Technische Hochschule München, München. Online verfügbar unter http://d-nb.info/98806829X/34.

Barnard, Gavin C.; McCool, Jesse D.; Wood, David W.; Gerngross, Tillman U. (2005): Integrated recombinant protein expression and purification platform based on *Ralstonia eutropha*. In: *Appl. Environ. Microbiol.* 71 (10), S. 5735–5742.

Bowien, Botho; Kusian, Bernhard (2002): Genetics and control of CO 2 assimilation in the chemoautotroph *Ralstonia eutropha*. In: *Archives of Microbiology* 178 (2), S. 85–93.

Britta Anderlei (2002): Untersuchungen zur Rolle der Pyruvat-carboxylase als neuer anaplerotischer Reaktion in *Escherichia coli*. Online verfügbar unter http://juwel.fz-juelich.de:8080/dspace/bitstream/2128/90/1/Juel_4016_Anderlei.pdf.

Bruland, N.; Voss, I.; Brämer, C.; Steinbüchel, A. (2010): Unravelling the C3/C4 carbon metabolism in *Ralstonia eutropha* H16. In: *J. Appl. Microbiol.* 109 (1), S. 79–90.

Budde, Charles F.; Riedel, Sebastian L.; Hübner, Florian; Risch, Stefan; Popović, Milan K.; Rha, ChoKyun; Sinskey, Anthony J. (2011): Growth and polyhydroxybutyrate production by *Ralstonia eutropha* in emulsified plant oil medium. In: *Appl Microbiol Biotechnol* 89 (5), S. 1611–1619.

Cannon, Gordon C.; Heinhorst, Sabine; Kerfeld, Cheryl A. (2010): Carboxysomal carbonic anhydrases: Structure and role in microbial CO2 fixation. In: *Biochim Biophys Acta* 1804 (2), S. 382–392.

Cramm, Rainer (2009): Genomic View of Energy Metabolism in *Ralstonia eutropha* H16. In: *J Mol Microbiol Biotechnol* 16 (1-2), S. 38–52.

Doi, Yoshiharu; Steinbüchel, Alexander (Hg.) (2002): Polyesters III. Applications and commercial products. Weinheim: Wiley-VCH.

Esbaugh, A.J; Tufts, B.L (2006): The structure and function of carbonic anhydrase isozymes in the respiratory system of vertebrates. In: *Respiratory Physiology & Neurobiology* 154 (1-2), S. 185–198.

Fricke, Wolfgang Florian; Kusian, Bernhard; Bowien, Botho (2009): The Genome Organization of *Ralstonia eutropha* Strain H16 and Related Species of the *Burkholderiaceae*. In: *J Mol Microbiol Biotechnol* 16 (1-2), S. 124–135.

Geers, C.; Gros, G. (2000): Carbon dioxide transport and carbonic anhydrase in blood and muscle. In: *Physiol. Rev.* 80 (2), S. 681–715.

Glueck, Silvia M.; Gümüs, Selcuc; Fabian, Walter M. F.; Faber, Kurt (2009): Biocatalytic carboxylation. In: *Chem. Soc. Rev.* 39 (1), S. 313.

Goldet, Gabrielle; Wait, Annemarie F.; Cracknell, James A.; Vincent, Kylie A.; Ludwig, Marcus; Lenz, Oliver et al. (2008): Hydrogen Production under Aerobic Conditions by Membrane-Bound Hydrogenases from *Ralstonia* Species. In: *J. Am. Chem. Soc.* 130 (33), S. 11106–11113.

Haas, Richard; JIN, Bo; ZEPF, Florian Tobias (2008): Production of Poly (3-hydroxybutyrate) from Waste Potato Starch. In: *Biosci. Biotechnol. Biochem.* 72 (1), S. 253–256.

Höfle, Caroline (2005): Untersuchungen zur genetischen Regulation der CO2-Assimilation in *Ralstonia* spp. Dissertation. Georg-August-Universität zu Göttingen, Göttingen. Online verfügbar unter http://d-nb.info/980154545/34.

Jeffke, T.; Gropp, N. H.; Kaiser, C.; Grzeszik, C.; Kusian, B.; Bowien, B. (1999): Mutational analysis of the cbb operon (CO2 assimilation) promoter of *Ralstonia eutropha*. In: *J. Bacteriol.* 181 (14), S. 4374–4380.

Kusian, B.; Bowien, B. (1997): Organization and regulation of cbb CO2 assimilation genes in autotrophic bacteria. In: *FEMS Microbiol. Rev.* 21 (2), S. 135–155.

Kusian, Bernhard; Sültemeyer, Dieter; Bowien, Botho (2002): Carbonic anhydrase is essential for growth of *Ralstonia eutropha* at ambient CO_2 concentrations. In: *J. Bacteriol.* 184 (18), S. 5018–5026.

Li, H.; Opgenorth, P. H.; Wernick, D. G.; Rogers, S.; Wu, T.-Y; Higashide, W. et al. (2012): Integrated Electromicrobial Conversion of CO_2 to Higher Alcohols. In: *Science* 335 (6076), S. 1596.

Louie, Tai Man; Webster, Christopher M.; Xun, Luying (2002): Genetic and biochemical characterization of a 2,4,6-trichlorophenol degradation pathway in *Ralstonia eutropha* JMP134. In: *J. Bacteriol.* 184 (13), S. 3492–3500.

Ludwig, M.; Cracknell, J. A.; Vincent, K. A.; Armstrong, F. A.; Lenz, O. (2008): Oxygen-tolerant H2 Oxidation by Membrane-bound [NiFe] Hydrogenases of *Ralstonia* Species: Coping with low level H_2 in air. In: *Journal of Biological Chemistry* 284 (1), S. 465–477.

Makkar, N. S.; Casida, L. E. (1987): *Cupriavidus necator* gen. nov., sp. nov.; a Nonobligate Bacterial Predator of Bacteria in Soil. In: *International Journal of Systematic Bacteriology* 37 (4), S. 323–326.

McIntosh, J. E. (1968): Assay of carbonic anhydrase by titration at constant pH. In: *Biochem. J.* 109 (2), S. 203–207.

Merlin, C.; Masters, M.; McAteer, S.; Coulson, A. (2003): Why Is Carbonic anhydrase Essential to *Escherichia coli*? In: *Journal of Bacteriology* 185 (21), S. 6415–6424.

Mitsuhashi, S.; Mizushima, T.; Yamashita, E.; Yamamoto, M.; Kumasaka, T.; Moriyama, H. et al. (2000): X-ray structure of beta-carbonic anhydrase from the red alga, *Porphyridium purpureum*, reveals a novel catalytic site for CO_2 hydration. In: *J. Biol. Chem.* 275 (8), S. 5521–5526.

Mueller-Cajar, Oliver; Stotz, Mathias; Wendler, Petra; Hartl, F. Ulrich; Bracher, Andreas; Hayer-Hartl, Manajit (2011): Structure and function of the AAA+ protein CbbX, a red-type RuBisCO activase. In: *Nature* 479 (7372), S. 194–199.

Pohlmann, Anne; Fricke, Wolfgang Florian; Reinecke, Frank; Kusian, Bernhard; Liesegang, Heiko; Cramm, Rainer et al. (2006): Genome sequence of the bioplastic-producing “Knallgas” bacterium *Ralstonia eutropha* H16. In: *Nat Biotechnol* 24 (10), S. 1257–1262.

Potter, M. (2004): The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous and paralogous phasins occur in *Ralstonia eutropha*. In: *Microbiology* 150 (7), S. 2301–2311.

Pötter, Markus; Madkour, Mohamed H.; Mayer, Frank; Steinbüchel, Alexander (2002): Regulation of phasin expression and polyhydroxyalkanoate (PHA) granule formation in *Ralstonia eutropha* H16. In: *Microbiology (Reading, Engl.)* 148 (Pt 8), S. 2413–2426.

Sawaya, M. R. (2006): The Structure of beta-Carbonic anhydrase from the Carboxysomal Shell Reveals a Distinct Subclass with One Active Site for the Price of Two. In: *Journal of Biological Chemistry* 281 (11), S. 7546–7555.

Schwartz, E.; Gerischer, U.; Friedrich, B. (1998): Transcriptional regulation of *Alcaligenes eutrophus* hydrogenase genes. In: *J. Bacteriol.* 180 (12), S. 3197–3204.

Schwartz, Edward; Henne, Anke; Cramm, Rainer; Eitinger, Thomas; Friedrich, Bärbel; Gottschalk, Gerhard (2003): Complete Nucleotide Sequence of pHG1: A *Ralstonia eutropha* H16 Megaplasmid Encoding Key Enzymes of H2-based Lithoautotrophy and Anaerobiosis. In: *Journal of Molecular Biology* 332 (2), S. 369–383.

Shingles, R.; Moroney, J. V. (1997): Measurement of carbonic anhydrase activity using a sensitive fluorometric assay. In: *Anal. Biochem.* 252 (1), S. 190–197.

Sigma-Aldrich (Hg.) (1994): Carbonic anhydrase Esterase Assay. Online verfügbar unter http://www.sigmaaldrich.com/img/assets/18220/Carbonic_anhydrase_-_CSRD.pdf, zuletzt aktualisiert am 21.01.1994, zuletzt geprüft am 12.06.2012.

Smith, Kerry S.; Ferry, James G. (2000): Prokaryotic carbonic anhydrases. In: *FEMS Microbiology Reviews* 24 (4), S. 335–366.

So, Anthony K. -C; Espie, George S.; Williams, Eric B.; Shively, Jessup M.; Heinhorst, Sabine; Cannon, Gordon C. (2004): A Novel Evolutionary Lineage of Carbonic anhydrase (ε Class) Is a Component of the Carboxysome Shell. Unter Mitarbeit von Anthony K. -C So, George S. Espie, Eric B. Williams, Jessup M. Shively, Sabine Heinhorst und Gordon C. Cannon. American Society for Microbiology (ASM). Online verfügbar unter http://www.ncbi.nlm.nih.gov/pmc/articles/PMC321498/, zuletzt aktualisiert am 01.01.2004, zuletzt geprüft am 23.03.2013.

Srinivasan, Sriram; Barnard, Gavin C.; Gerngross, Tillman U. (2002): A novel high-cell-density protein expression system based on *Ralstonia eutropha*. In: *Appl. Environ. Microbiol.* 68 (12), S. 5925–5932.

Srinivasan, Sriram; Barnard, Gavin C.; Gerngross, Tillman U. (2003): Production of recombinant proteins using multiple-copy gene integration in high-cell-density fermentations of *Ralstonia eutropha*. In: *Biotechnol. Bioeng.* 84 (1), S. 114–120.

Steinbüchel, Alexander (2012): Mikrobiologisches Praktikum. Versuche und Theorie. 2. Aufl. Berlin [u.a.]: Springer.

Susanne Doris Juhnke (2004): Untersuchungen zum Mechanismus der Chromatentgiftung in *Ralstonia metallidurans*. Dissertation. Martin-Luther-Universität Halle-Wittenberg, Halle-Wittenberg.

Thermo Scientific: Tm-calculator. Hg. v. Thermo Scientific. Online verfügbar unter http://www.thermoscientificbio.com/webtools/tmc/, zuletzt geprüft am 13.06.2013.

Thomas Jeffke (2000): Zur Struktur und Funktion regulatorischer Elemente des cbb-Regulons in *Ralstonia eutropha*. Dissertation. Georg-August-Universität zu Göttingen, Göttingen.

Tripp, B. C. (2001): Carbonic anhydrase: New Insights for an Ancient Enzyme. In: *Journal of Biological Chemistry* 276 (52), S. 48615–48618.

Ueda, Kenji; Nishida, Hiromi; Beppu, Teruhiko (2012): Dispensabilities of Carbonic anhydrase in Proteobacteria. In: *International Journal of Evolutionary Biology* 2012 (3), S. 1–5.

van den Bergh, E. R.; Dijkhuizen, L.; Meijer, W. G. (1993): CbbR, a LysR-type transcriptional activator, is required for expression of the autotrophic CO_2 fixation enzymes of *Xanthobacter flavus*. In: *J. Bacteriol.* 175 (19), S. 6097–6104.

Vandamme, P. (2004): Taxonomy of the genus *Cupriavidus*: a tale of lost and found. In: *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY* 54 (6), S. 2285–2289.

Verlinden, Rob A. J.; Hill, David J.; Kenward, Melvin A.; Williams, Craig D.; Piotrowska-Seget, Zofia; Radecka, Iza K. (2011): Production of polyhydroxyalkanoates from waste frying oil by *Cupriavidus necator*. In: *AMB Express* 1 (1), S. 11.

Vincent, Kylie A.; Cracknell, James A.; Parkin, Alison; Armstrong, Fraser A. (2005): Hydrogen cycling by enzymes: electrocatalysis and implications for future energy technology. In: *Dalton Trans.* (21), S. 3397.

Wilde, Erika (1962): Untersuchungen über Wachstum und Speicherstoffsynthese von *Hydrogenomonas*. In: *Archiv. Mikrobiol.* 43 (2), S. 109–137.

Worthington Biochemical Corporation: Carbonic anhydrase Assay. Hg. v. Worthington Biochemical Corporation. Online verfügbar unter http://www.worthington-biochem.com/ca/assay.html, zuletzt geprüft am 12.06.2013.

6 Appendix

6.1 Table directory

6.2 List of figures