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Effects of mutations in metabolic genes of *Clostridium acetobutylicum*

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Content list

Abbreviations

°C	Centigrade
μ	micro
ad	Add to specific volume
Amp	Ampicillin
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Basepair
cDNA	Complementary deoxyribonucleic acid
CGM	Clostridial Growth Medium
cm	Centimeter
Cm	Chloramphenicol
cMM	Chloride minimal medium
CoA	Coenzyme A
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxy-ribonucleoside triphosphate
EDTA	Ethylene diamine tetraacetic acid
DSMZ	Deutsche Sammlung für Mikroorgasnismen und Zellkulturen
EDTA	Ethylene Diamine Tetraacetic Acid
Erm	Erythromycin
et al.	And the rest
ET buffer	Electroporation-transfer buffer
EtBr	Ethidium bromide
ETM buffer	Electroporation-transfer (magnesium) buffer
Fig.	Figure
g	Gram
GC	Gas chromatography
GS	Glutathione synthetase
GSH	Tripeptide glutathione
GSSH	Glutathione desulfide
h	Hour
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
k	Kilo
kb	Kilobase
KEGG	Kyoto Encyclopedia Genes and Genomes
kV	Kilo volt
L	Liter
LB medium	Luria Bertani Medium

Log	Logarithm
M	Molar/liter
MM	Minimal medium
mM	Milimolar/liter
min	Minute
mRNA	Messenger RNA
NaAc	Sodium acetate
NAD^+	Nicotinamide adenine dinuceotide, oxidized form
NADH	Nicotinamide adenine dinuceotide, reduced form
$NADP^+$	Nicotinamide adenine dinuceotide phosphate, oxidized form
NADPH	Nicotinamide adenine dinuceotide phosphate, reduced form
NCBI	National Center for Biotechnology Information
No.	Number
OD_{600}	Optical density at a wavelength of 600 nm
ORF	Open reading frame
pH	Negative decimal logarithm of the proton concentration
pmol	10^{-12} mol
PCR	Polymerase chain reaction
PTS	Phosphotransferase System
PLMM	Phosphate-limited minimal medium
RCM	Reinforced Clostridial Medium
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Rotation per minute
RT-PCR	Reverse transcription PCR
sec	Second
SDS	Sodium dodecyl sulfate
SLMM	Sulfur-limited minimal medium
SSC	Saline sodium citrate buffer
TAE buffer	Tris-acetate-EDTA buffer
TE	Tris-EDTA-buffer
Tm	Thiamphenicol
Tris	Tris-hydroxymethyl-aminomethane
Trx	Thioredoxin
V	Volt
v/v	Volume/volume
WT	Wild type
w/v	Weight/volume
γ-GCS	γ-glutamylcysteine synthetase
μM	Micromolar/liter

1. Introduction

1.1 Butanol producing bacteria

Butanol is an important industrial intermediate and extensively used in chemical industry, for instance synthesis of acrylate, methacrylate esters, butyl acetate and butyl glycol (Durre, 2008; Jones and Woods, 1986; Schiel-Bengelsdorf *et al.*, 2013). Butanol is also used in medical synthesis as an extracting agent and in a wide range of consumer products. Additionally, butanol is a primary four-carbon alcohol, which has two carbon atoms more than ethanol. Its characteristic, including less hygroscopic, lower vapour pressure and higher energy content, makes it more promising than ethanol as the next generation biofuel (Lee *et al.*, 2008). At present, butanol is mainly produced by a petrochemical route from propylene as chief substrate via hydroformylation and hydrogenation (Green, 2011). Correspondingly, the butanol price is mostly dependent on petroleum supply and price. It is well known that petroleum is an unsustainable fossil resource. As oil gets scarcer and more expensive year by year, it is increasingly urgent to find an alternative for butanol production.

Butanol fermentation has a long history and was developed in the UK in 1912 using a *Clostridium acetobutylicum* strain isolated by Dr. Chaim Weizmann. Due to the main products of *C. acetobutylicum* fermentation, i.e. acetone, butanol and ethanol (the ratio is 3:6:1), this fermentation was called solvent, ABE or AB fermentation (Jones and Woods, 1986). Commercial production spread around the globe during the first and second World Wars and declined in the early 1960s, because of the more favorable economics of the petroleum industry. However the study of solvent production was not discontinued in the past about 100 years. Many strains naturally producing butanol were isolated and among them, the classical butanol production strains for commercial industry all belong to genus *Clostridium*, including *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharobutylicum*,

and *Clostridium saccharoperbutylacetonicum* (Schiel-Bengelsdorf *et al.*, 2013). A lot of efforts have been made to clarify and modulate solventogenic pathways in clostridia. Until now, some potential strains for practical industrial production were obtained using traditional mutagenesis and gene manipulation. The hyperbutanol-producing mutant strain *Clostridium beijerinckii* BA101 (host strain was *Clostridium beijerinckii* NCIMB8052) was able to produce 18.6 g/L butanol in batch culture and the total ABE production achieved 32.6 g/L (Formanek *et al.*, 1997). Similarly, *C. acetobutylicum* EA2018, developed by chemical mutagenesis from host strain *C. acetobutylicum* ATCC824, produced 14.4 g/L butanol (accounting for 70% in total ABE) in a batch culture (Zhang Y, 1997). Additionally, an outstanding genetically engineered strain *C. acetobutylicum* BEKW (*pthl*AAD) produced 18.9 g/L butanol in a batch culture. In this stain, the two important genes (phosphotransacetylase, *pta*, and butyrate kinase, *buk*) related to butyrate formation were inactivated and a mutated aldehyde alcohol dehydrogenase (*adhE1D485G*) gene was overexpressed (Jang *et al.*, 2012). As a promising biofuel and an important chemical intermediate, isolation and construction of butanol producing stains is a research field of high practical significance.

1.2 The central metabolic pathways of *Clostridium acetobutylicum*

C. acetobutylicum is a Gram-positive, strictly anaerobic and endospore forming bacterium. In batch culture, the typical fermentation process of *C. acetobutylicum* is divided in two phases (Amador-Noguez *et al.*, 2011). The initial phase is called acidogenic growth (acidogenesis) with acetate and butyrate as main products and the second phase is called solventogenic growth (solventogenesis) with acetone and butanol as main products. Although, the fermentation process is divided two phases, there is no clear "boundary" between the two phases. It is difficult to study the acidogenesis and solventogenesis separate from each other. Usage of phosphate-limited continuous cultures allows to study the two phases independently and provides an effective method to investigate the transcriptomes and proteomes of

the different metabolic conditions of *C. acetobutylicum* (Bahl *et al.*, 1982; Grimmler *et al.*, 2010; Janssen *et al.*, 2010; Janssen *et al.*, 2012). Furthermore, *C. acetobutylicum* allows the characterization of more metabolic states in the continuous culture. In iron-limited continuous culture, lactate which is produced in trance amounts in batch culture becomes the main product during acidogenic growth (Bahl *et al.*, 1986). *C. acetobutylicum* grown on a mixture of glucose and glycerol as substrates produces mainly butanol and ethanol without acetone formation in continuous culture (Vasconcelos *et al.*, 1994). Therefore, the continuous cultures of *C. acetobutylicum* are more and more used in research.

C. acetobutylicum is able to ferment a variety of carbohydrates including hexoses, pentoses and starch. This is an advantageous characteristic and makes the fermentation of cellulosic biomass possible (Ezeji and Blaschek, 2008; Qureshi *et al.*, 2006). Moreover, *C. acetobutylicum* produces a variety of products including acids (acetate, butyrate and lactate), gas (carbon dioxide and hydrogen), and solvents (acetone, butanol and ethanol) (Jones and Woods, 1986). A lot of efforts are needed to clarify the regulation of the metabolism, especially the solvents production pathways and their regulation. The central metabolic pathways shown in Fig. 1.1 were worked out over the years by many research groups (Jones and Woods, 1986; Lutke-Eversloh and Bahl, 2011; Schiel-Bengelsdorf *et al.*, 2013). In the butanol production pathway, two operons play an important role and are investigated by many research groups. One is the well-known *sol* operon (CAP0162-0164) which is located on the *pSOL* megaplasmid. It consists of genes *adhE1*, *ctfA* and *ctfB* and is responsible for conversion of butyrate to butanol by the *bdhAB* genes (Durre *et al.*, 2002; Fischer *et al.*, 1993). The second operon is the *bcs* operon which consists of genes *hbd*, *crt*, *bcd* and *etfAB* (CAC2708-2712) (Boynton *et al.*, 1996; Schiel-Bengelsdorf *et al.*, 2013). This operon is responsible for butyryl-CoA formation, which is the precursor of butanol. Other important genes include acetoacetate decarboxylase gene (*adc*, CAP0165), the acetate and butyrate formation genes (*pta*, *ack*, *ptb*, and *buk*, in Fig. 1.1), which also are essential for solvents synthesis (Cooksley *et al.*, 2012; Green and

Bennett, 1998; Lehmann *et al.*, 2012).
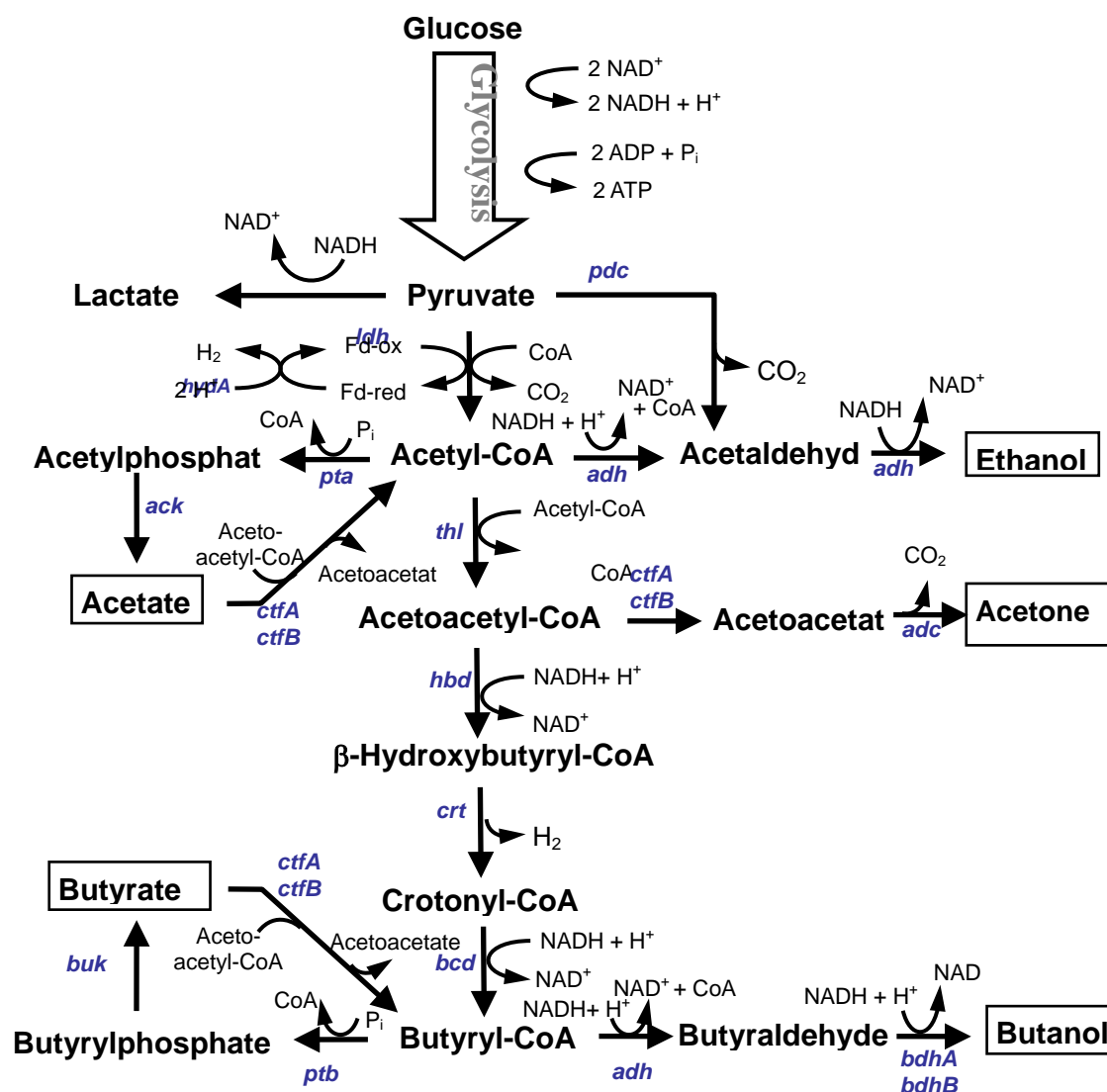
Fig.1.1 Schematic diagram of central metabolic pathways of *C. acetobutylicum*
Abbreviations: Ack, acetate kinase; HydA, hydrogenase; Pdc, pyruvate decarboxylase; Pta, phosphotransacetylase; Adh, aldehyde∕alcohol dehydrogenase; BdhA/B, alcohol dehydrogenase; Thl, thiolase; Hbd, 3-hydroxybutyryl-CoA dehydrogenase; Crt, crotonase; Bcd, butyryl-CoA dehydrogenase; Ptb, phosphotransbutyrylase; Buk, butyrate kinase; CtfA∕B, CoA transferase; Adc, acetoacetate decarboxylase; NADH, nicotinamide adenine dinucleotide (reduced form). Ldh, lactate dehydrogenas

Despite numerous research efforts, many metabolic pathways besides the central solvent metabolism in *C. acetobutylicum* remain incompletely understood. For instance, many aspects of the cysteine metabolism, the thiol redox system and the glucose transport system are unknown in this bacterium. These knowledge gaps prompted us to further study these metabolic pathways in *C. acetobutylicum*.

1.2.1 Cysteine synthesis pathways and global regulation of gene expression in response to cysteine availability during sloventogenic growth of *Clostridium acetobutylicum*

Sulfur is an essential element in the nutrition and is required by all living organisms for the synthesis of proteins and important cofactors. In the ecosystems, it is mainly present as sulfate. Sulfate assimilation means that inorganic sulfate is used as sulfur source in order to synthesize cysteine. This is the predominate pathway in most microorganisms (Albanesi *et al.*, 2005; Kredich, 1996; Sekowska *et al.*, 2000). Cysteine is an amino acid, which serves an important structural role in many proteins and it is the precursor for the synthesis of iron-sulfur cluster and the antioxidant glutathione (Ayala-Castro *et al.*, 2008; Zeller and Klug, 2006). Therefore, cysteine plays a central role in a variety of cellular functions. The sulfur assimilation and cysteine synthesis in *Bacillus subtilis* were studied extensively (Berndt *et al.*, 2004; Guillouard *et al.*, 2002; Mansilla and de Mendoza, 2000; van der Ploeg *et al.*, 2001). Two pathways for cysteine synthesis were described in *B. subtilis* (Hullo *et al.*, 2007; Soda, 1987). The first pathway was the thiolation pathway requiring sulfide which resulted from sulfate assimilation and *O*-acetylserine. The second pathway was the reverse transulfuration pathway. This pathway converts methionine or homocysteine to cysteine which is normally repressed and derepressed only under sulfur starvation conditions. Moreover, the exogenous cysteine could be directly imported by a cysteine transporter located in the membrane.

As an obligate anaerobic bacterium, little is known on sulfur assimilation and cysteine biosynthesis in *C. acetobutylicum*. However, it was reported that the mechanism of

cysteine synthesis was very similar to *B. subtilis*. Based on genome sequence analysis in *C. acetobutylicum*, a schematic diagram of the cysteine synthesis pathways is shown in Fig.1.2 (Andre *et al.*, 2008). As shown in Fig. 1.2, the function of a putative operon contained 9 tandem open reading frames (CAC0102-CAC0110) was speculated to convert sulfate to sulfite. The sulfite formation was first step of cysteine synthesis (Nolling *et al.*, 2001). However, there is no experimental evidence to verify its function in sulfur assimilation until now. Moreover, cysteine could be synthesized by the reverse transulfuration pathway as shown in Fig. 1.2. The *ubiGmccBA* operon, encoding AdoMet-dependent methyl-transgerase (*ubiG*, CAC0929), cystathionine γ-lyase (*mccB*, CAC0930) and cystathionine β-synthase (*mccA*, CAC0931) respectively, was pridicted to be involved in this process in *C. acetobutylicum*. The functions of genes, *mccB* and *mccA*, had been clarified through complementation experiments in the *mccA* and *mccB* mutants of *B. subtilis* (Andre *et al.*, 2008). Therefor, it is important to investigate their functions *in vivo*. Interestingly, like the well-known solventogenic *sol* operon, the transcription levels of these two putative operons, CAC0102-0110 and *ubiGmccBA*, were significantly increased during solventogenic growth as compared with acidogenic growth (Grimmler *et al.*, 2010). Therefore, the study of the functions of these two operons would provide insights in understanding cysteine biosynthesis and regulation in *C. acetobutylicum*. In the present work, *fer* (CAC0105) and *mccB* (CAC0930) genes were inactivated by ClosTron technology and the phenotypes of the mutants were studied during solventogenic growth in phosphate-limited continuous cultures.

Sulfate

$\quad\downarrow$ *CysDN* CAC0109 and CAC0110?

CAC0102-CAC0110?

APS

$\quad\downarrow$ *cysC* CAC0103?

PAPS

$\quad\downarrow$ **CAC0104?**

Sulfite $\qquad\qquad$ Serine＋ Acetyl-CoA

$\quad\downarrow$ *csrABC* $\qquad\qquad\qquad\downarrow$ *cysE*

Sulfide $\qquad\qquad\qquad$ *O*-acetylserine

$\qquad\qquad$ *cysK*

External Cysteine $\xrightarrow{\ tcyABC\ }$ **Cysteine**

$\quad$ *metI* $\updownarrow$ $\quad$ *mccB* CAC0930?

Cystathionine

$\quad$ *metC* $\updownarrow$ *mccA* CAC0931?

Homocysteine $\xleftarrow{\ luxS\ }$ SRH

$\qquad\qquad\downarrow$ $\qquad\qquad\uparrow$ *menN*

External Methionine $\xrightarrow{\ metNPQ\ }$ **Methionine**

$\qquad\downarrow$ *met K*

AdoMet $\xrightarrow{\qquad\qquad}$ SAH

$\qquad$ *ubi G* CAC0929?
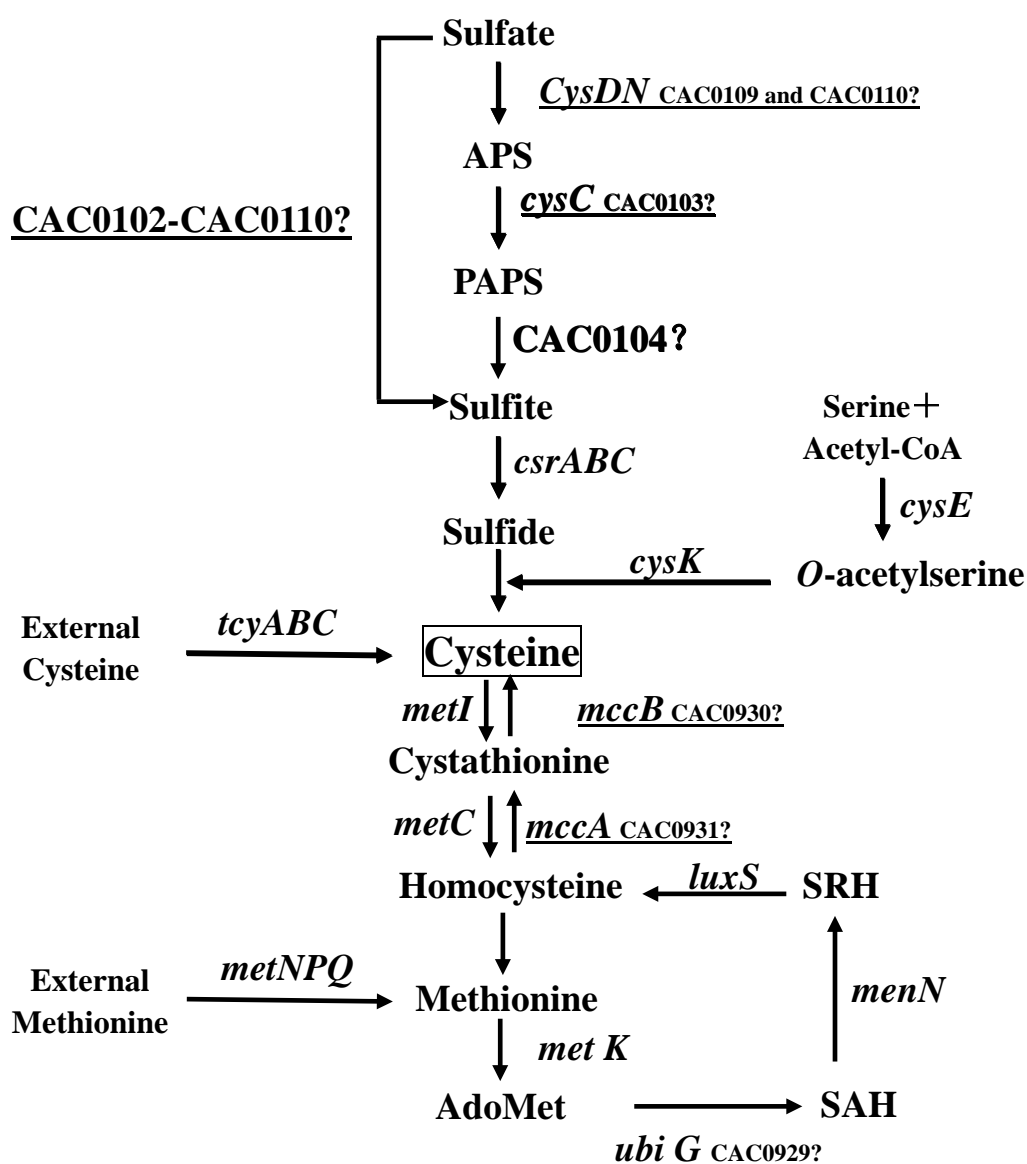
Fig.1.2 Schematic diagram of cysteine biosynthesis in *C. acetobutylicum.* The genes located in operons (CAC0102-CAC0110 and CAC0929-CAC0931) are underlined. A question mark indicates the genes probably involved in this pathway. AdoMet, *S*-adenosyl-methionine; SRH, *S*-ribosylhomocysteine; SAH, *S*-adenosylhomocysteine; Serine *O*-acetyltransferase, *cysE*; *O*-acetylserine-thiol-lyase, *cysK*; Methionine adenosyltransferase, *metK*; Adenosylhomocysteine nucleosidase, *mtnN*; S-ribosylhomocysteine lyase, *luxS*; Cystathionine β-synthase, *mccA*; Cystathionine γ-lyase, *mccB*; Cystathionineγ-synthase, *metI*; Cystathionine β-lyase, *metC*; ATP sulfurylase, *cysDN*; APS kinase, *cysC*; Anaerobic sulfite reductase, *asrABC*. The CAC numbers for *C. acetobutylicum* genes correspond to genome sequence (Nolling et al., 2001).

1.2.2 Cellular functions of the thioredoxin- and glutathione-dependent reduction pathways in *Clostridium acetobutylicum*

The tripeptide glutathione (GSH) and the protein thioredoxin (Trx) are involved in many crucial biological processes in prokaryotic and eukaryotic cells (Arner and Holmgren, 2000; Aslund and Beckwith, 1999; Penninckx and Elskens, 1993). GSH is synthesized by γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS) in a sequential two-step ATP-dependent pathway (Kino *et al.*, 2007; Lu, 2013). In contrast, thioredoxin is a small protein with a molecular mass of about 12 kDa (Arner and Holmgren, 2000). They belong to glutathione- and thioredoxin-dependent reduction systems respectively and both maintain the reduction environment of the cytosol. For example, they both catalyze the reduction of disulfide bonds by forming glutathione disulfide (GSSH) and oxidized thioredoxin, which in turn are reduced by glutathione reductase and thioredoxin reductase, respectively, at expense of NADPH, thus regenerating GSH and thioredoxin, respectively (Carmel-Harel and Storz, 2000; Rietsch and Beckwith, 1998).

To understand the cellular functions of GSH- and Trx-dependent reduction systems in *Escherichia coli* and yeast, many studies have been completed by constructing mutants of the genes belonging to the GSH and thioredoxin pathways. In *E. coli*, either of the two pathways is dispensable for normal growth. Only when the GSH- and Trx-dependent reduction pathways are simultaneously knocked out, *E. coli* is not able to grow aerobically (Fuchs and Warner, 1975; Greenberg and Demple, 1986; Prinz *et al.*, 1997; Ritz *et al.*, 2000). The results indicate that both pathways are functionally redundant and possibly partially substitute each other. In contrast, the *Saccharomyces cereivsiae* GSH- and Trx-dependant reduction pathways are clearly different from that of *E. coli* (Grant *et al.*, 1996). These two pathways are not functionally redundant and each has preferred or exclusive targets. The γ-glutamylcysteine synthetase mutant of *S. cereivsiae* is unable to grow in a minimal medium in the absence of exogenous GSH. Furthermore, there are two genes

encoding thioredoxin as part of the Trx-dependent reduction pathway, *Trx1* and *Trx2*. *Trx1* or *Trx2* single mutants are identical to the wild type with the regard to growth rate and cell morphology. In contrast, the double mutant becomes auxotrophic for methionine (Muller, 1991). In summary, although the GSH- and Trx-dependent reduction pathways are universal disulfide-reducing systems, the cellular functions fulfilled profoundly differ in different microorganisms.

According to genetic analysis, *C. acetobutylicum* possesses both GSH- and Trx-dependent reduction pathways. GSH is synthesized by two adjacent genes, γ-GCS (CAC1539, *gcs*) and GS (CAC1540, *gs*) in *C. acetobutylicum* (Kino *et al.*, 2007). Therefore, *gcs* was inactivated using ClosTron technology in present work to investigate the phenotype of the *gcs* mutant and the function of GSH-dependent reduction system in *C. acetobutylicum*. For the Trx-dependent reduction system, two sets of thioredoxin-thioredoxin reductase genes are found in the *C. acetobutylicum* genome. They are *trxA1-trxB1-gpx3* (CAC1547-CAC1549) and *trxA2-trxB2* (CAC3082-CAC3083). The former contains three genes, which encode a thioredoxin, a thioredoxin reductase, and a glutathione peroxidase homologue, respectively, and are localized tandemly as a polycistronic unit. The latter contains two tandem genes encoding a thioredoxin and a thioredoxin reductase, respectively. Furthermore, the former was strongly upregulated within 10 min after O_2 flushing, while the latter was transcribed and expressed constitutively (Kawasaki *et al.*, 2005). Therefore, *trxB* (CAC1548) was chosen and inactivated using ClosTron technology in the present work to investigate the phenotype of the corresponding *trxB* mutant and the function of Trx-dependent reduction system in *C. acetobutylicum*.

1.2.3 Confirmation of glucose transporters through targeted mutagenesis and transcriptional analysis in *Clostridium acetobutylicum*

Clostridium acetobutylicum is able to grow on a variety of carbohydrates and thus encodes a diverse set of metabolic enzymes and transporters for the uptake of hexoses and pentoses. Transcriptional analysis of differential carbohydrate utilization by

C. acetobutylicum showed that the hexoses were primarily taken up by the phosphoenolpyruvate phosphotransferase systems (PTS) (Mitchell and Tangney, 2005; Servinsky *et al.*, 2010). A typical PTS contains two soluble components, enzyme I and histidine-containing protein (HPr) and a membrane-bound enzyme II. The enzyme II complex incorporates three domains, termed IIA, IIB, and IIC, in which the IIC is integral membrane protein to translocate the substrate to cytoplasm (Saier and Reizer, 1992).

Bioinformatic analysis of the genome sequence of *C. acetobutylicum*, which has been completed in 2001, has identified 13 putative PTS EII enzymes (Mitchell and Tangney, 2005; Nolling *et al.*, 2001). Although the family of these 13 putative PTS EII domains were assigned by phylogenetic analysis and the probable substrates were annotated, for most of them experimental confirmation is still required. For glucose uptake, it was reported that the *glcG* gene (CAC0570) codes for a glucose transporter in *C. acetobutylicum*. However, when *glcG* was inactivated, the mutant fermented glucose as efficiently as the parent strain in a batch culture (Tangney and Mitchell, 2007; Xiao *et al.*, 2012). This result showed that a further glucose transporter existed in this organism. Despite its importance, it is currently unknown how many glucose transporters exist in *C. acetobutylicum*. In contrast, glucose uptake in *Escherichia coli* had been studied extensively. There is a main glucose transporter, which is very efficient for glucose uptake, and the maltose, mannose and galactose transport systems, which are also able to transport glucose into cytoplasm (Boos and Shuman, 1998; Hunter and Kornberg, 1979; Steinsiek and Bettenbrock, 2012). Therefore, it is very interesting to investigate whether a similar complex glucose transport mechanism exists in *C. acetobutylicum*. Moreover, more detailed physiological analysis will further increase our knowledge of the PTS in this organism.

In the present work, the ClosTron technology was used to inactivate the genes of a putative *C. acetobutylicum* glucose transporter. The mutants and wild type strain were cultured in batch and continuous culture. Then the growth phenotype and

transcriptome analysis of the mutants compared to the wild type were investigated.

1.3. Analytical and engineering tools for *Clostridium acetobutylicum*

With the completion of genome sequencing in *C. acetobutylicum* and rapid development of methods for constructing gene knockouts, nearly all the genes responsible for the central metabolic pathways (in Fig.1.2) were inactivated (Cooksley *et al.*, 2012; Green and Bennett, 1998; Lehmann *et al.*, 2012). Among the "knock-out" methods, the ClosTron technology was the most widely used. It was developed by the research group of Nigel Minton (Nottingham, UK). Nowadays, the website (www.clostron.com) which allows to plan gene knockouts using the ClosTron technology is open and free for academic research (Heap *et al.*, 2010; Heap *et al.*, 2007). The system has been refined and streamlined to minimize the labour-intensity and maximize the accessibility of the mutagenesis method.

The multiple fermentation products in *C. acetobutylicum* indicates complexity of the whole metabolic regulatory net. Transcriptome analyses provides useful insights to study metabolism on the full-scale (Ehrenreich, 2006). Especially, the DNA microarray technology was used widely to study sporulation, solventogenesis, and butanol stress at the transcriptome level in *C. acetobutylicum* (Alsaker and Papoutsakis, 2005; Hillmann *et al.*, 2008; Paredes *et al.*, 2007; Tomas *et al.*, 2003). These results provided a lot of important information. However, most of these experiments were performed using typical batch fermentations in which many physiological parameters of *C. acetobutylicum*, such as growth rate, substrate consumption, pH and solvent productivity were changing continuously during the different phases of growth of the culture. This made it impossible to understand many details of the special expression pattern. Furthermore, the batch fermentation of *C. acetobutylicum* is a biphasic fermentation process as described above (in Fig. 1.1). The metabolic feature during acidogenic and solventogenic growth are profoundly different (Jones and Woods, 1986; Lutke-Eversloh and Bahl, 2011). It is important to

study acidogenesis and solventogenesis of *C. acetobutylicum* independently. Since a phosphate-limited continuous culture is able to make the cells in the culture consistent and separate the fermentation into acidogenesis and solventogenesis independent of each other, it has been used in transcriptome research (Grimmler *et al.*, 2010; Janssen *et al.*, 2010; Janssen *et al.*, 2012; Schwarz *et al.*, 2012).

In this study, ClosTron technology, phosphate-limited continuous fermentations and DNA microarray technology were combined to study and evaluate the functions of key genes (operons) of cysteine biosynthesis, thiol redox systems and glucose transporter systems in *C. acetobutylicum*. The schematic technical route is described in Fig. 1.3 below.
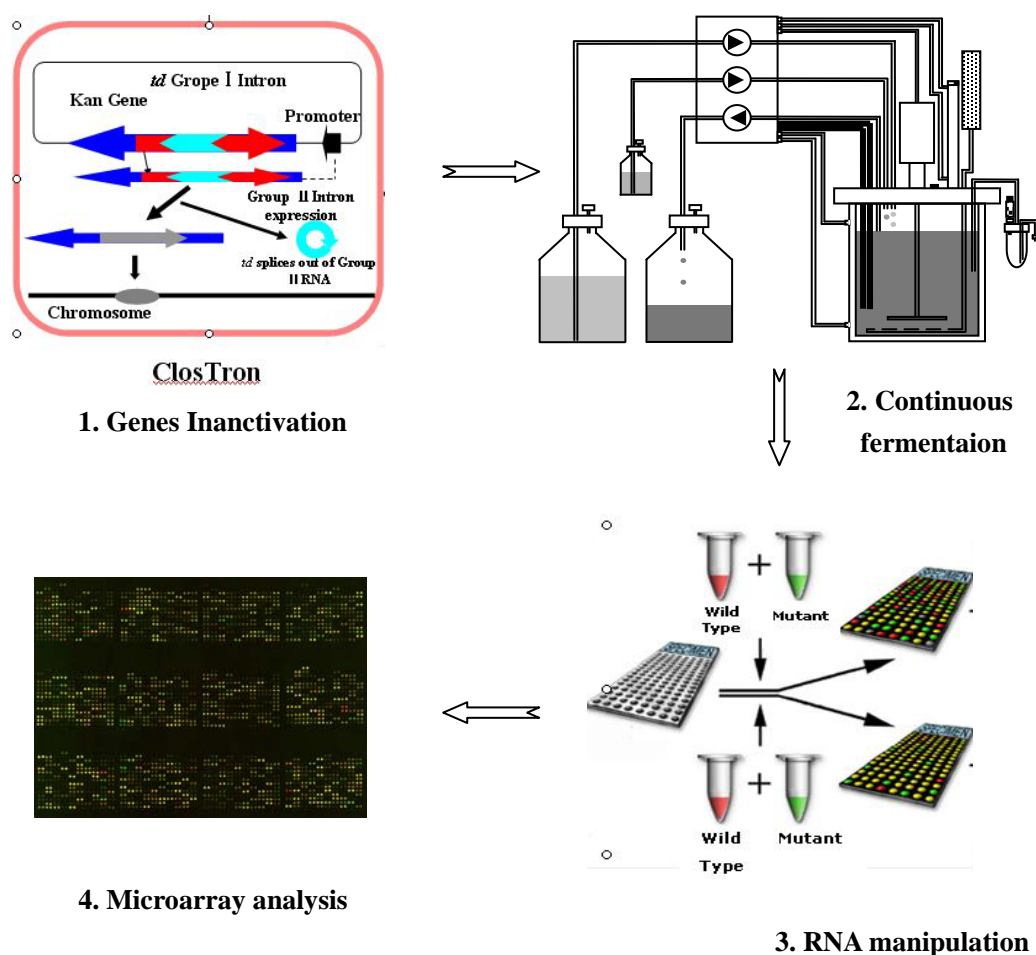
Fig.1.3. Technical route

2 Materials and methods

2.1 Gas, chemicals and equipment

The gas, pure nitrogen and mix gas (90% N_2+10% H_2), used in the anaerobic culture and chamber was supplied by Firma AirLiquide AG (Düsseldorf). Common chemicals were bought from Merck KGaA (Darmstadt), Sigma-Aldrich Chemie GmbH (Deisenhofen), Roth Chemie GmbH (Karlsruhe) or Applichem (Darmstadt). Enzymes and equipment used are listed in tables below.

Table 2.1 Enzymes and reagents used in this study

Enzyme and reagents	Company
*Bsr*GI (10 U/μL)	NEB, Frankfurt am Main, Germany
*Hin*dIII (10 U/μL)	NEB, Frankfurt am Main, Germany
Rnase-free DNAse I (10U/μL)	Roche, Mannheim, Germany
Random hexamers p(dN)6 (5 μg/μL)	Roche, Mannheim, Germany
dATP, dTTP, dCTP, dGTP (100 mM)	Roche, Mannheim, Germany
Random hexamers p(dN)6 (5 μg/μL)	Roche, Mannheim, Germany
dNTP Mix, (10 mM each)	Roche, Mannheim, Germany
Phire Hot Start II DNA-Polymerase	Finnzymes, Espoo, Finnland
Qiagen OneStep RT PCR Kit	Quiagen, Hilden
SuperScript III Reverse Transcriptase	Invitrogen GmbH, Karlsruhe
Cy^{TM}3-dCTP, Cy^{TM}5-dCTP	GE Healthcare Europe GmbH, München, Germany
T4 DNA ligase (10 U/μL)	Thermo Scientific, Schwerte, Germany
6× DNA loading dye	Thermo Scientific, Schwerte, Germany
1 kb DNA ladder	Thermo Scientific, Schwerte, Germany

Table 2.2. Biochemical kits used in this study

Kit	Company
AxyPrep^{TM} Plasmid Miniprep Kit	Serva Electrophoresis GmbH Heidelberg
Wizard$^{®}$ SV Gel and PCR Clean-Up System	Promega GmbH Mannheim
Master Pure DNA Purification Kit	Epicentre, Madison, USA
QIAquick PCR Purification Kit	Qiagen, Hilden, Germany
Biotin DecaLabel DNA Labeling Kit	Thermo Scientific Schwerte, Germany
Biotin Chromogenic Detection Kit	Thermo Scientific Schwerte, Germany
RNeasy Midi Kit	Qiagen Hilden, Germany
illustra^{TM} CyScribe^{TM}GFX^{TM} Purification Kit	GE Healthcare Europe GmbH, München

Table 2.2. (continued)

Kit	Company
D-glucose GOPOD kit	Megazyme GmbH Ireland
K-DLATE D-/L-Lactic Acid	Megazyme GmbH Ireland

Table 2.3. Equipment used in this study

Equipment	Company
2.5 L Anaerobic jar/sachet	Oxoid Ltd., Basingstoke, England
Electrophoresis device	Bio-Rad Laboratories, California, USA
Benchtop Stirrer Mobio 60	Variomag, Daytona Beach, USA
Alpha Imager Mini	Biozym, Hessisch Oldendorf, Germany
Micro-Dismembrator U	Sartorius, Göttingen, Germany
Bio-flow superclean bench	Heraeus Instruments GmbH, Hanau, Germany
Peristaltic Pump 101 U/R	Watson-Marlow Pumps Group
NanoDrop ND1000 Spectrophotometer	PeqLab, Erlangen, Germany
Cuvettes	Ratiolab, Dreieich, Germany
Positively charged nylon membrane	Roche, Mannheim, Germany
20 L Ilmabor Boro 3.3 glass jar	TGI GmbH, Ilmenau, Germany
MasterFlex peroxide-cured silicone tubing	Cole-Parmer, Vernon Hills, USA
Electroporation cuvettes	PeqLab Erlangen, Germany
Electroporation device	Bio-Rad Laboratories California, USA
SteritopTM filter units	EMD Millipore Billerica, USA
ScanMaker 1000Xl	Microtek, Willich,Germany
pH electrochemical sensor	Hamilton, Bonaduz, Switzrlan
Centrifuge	Haereus Holding GmbH, Hanau
UV/VIS-Spectrometer Ultrospec®3300pro	Amersham Pharmacia Biotech
Anaerobic chamber	COY company, USA
FlexCycler	Jena Analytik, Jena,Germany
Automated Slide Processor	ASP Lucidea Amersham Pharmacia Biotech
Tecan Hybridization Station HS400Pro	Tecan Austria GmbH Grödig/Salzburg
Scanner GenePix 4000B	Axon Instruments Union City, USA
Mikro-Dismenbrator U ball mill	BraunBiotech, Melsungen,Germany
Gas chromotography	GC-2010, Shimadzu, Duisburg, Germany
One-liter Biostat B*plus* fermenter system	Sarorius BBI Systems, Melsungen, Germany

2.2 Strains, plasmids, primers and media

2.2.1 Anaerobic manipulation of strains

All anaerobic manipulations were carried out in an anaerobic chamber (Coy

Laboratory Products Inc., Michigan, USA). Agar plates with *C. acetobutylicum* were placed in 2.5 L anaerobic jars (Oxoid Ltd., Basingstoke, England) in the anaerobic chamber prior to incubation at 37 °C.

Anaerobic Hungate-type tubes or Balch-type serum bottles (Müller+Krempel AG, Bülach, Switzerland) were prepared in a container filled with deionized water. First, they were placed below the water surface and filled with water to displace the air, and then inversed and simultaneously injected with nitrogen until all water was displaced. Afterwards, they were sealed by rubber stoppers plus plastic screw caps for Hungate-type tubes or by aluminum crimp seals for Balch-type serum bottles with a crimper. Prepared Hungate-type tubes and Balch-type serum bottles were autoclaved for 20 min at 121 °C.

2.2.2 Bacterial strains

C. acetobutylicum ATCC824 (wild type strain) and *E. coli* TOP10 were used as parental strains in this study. Seven *C. acetobutylicum* ClosTron mutants were constructed in this work. All strains are listed in Table 2.4.

Table 2.4 Strains used in this study

Strain	Relevant characteristics[a]	Source
Clostridium acetobutylicum ATCC 824	Wild type strain	American Type Culture Collection (ATCC)
Escherichia coli TOP10	F⁻, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), φ80*lac*ZΔM15, Δ*lac*X74, *rec*A1, *ara*D139, Δ(ara-leu)7697, *gal*U, *gal*K, *rps*L, (Str R), *end*A1, *nup*G	Invitrogen GmbH, Karlsruhe, Germany
C. acetobutylicum fer::int (276)	Group II intron inserted at 276./277. bp of *fer* (CAC0105), ErmR	This study
C. acetobutylicum mccB::int (414)	Group II intron inserted at 414./415. bp of *mccB* (CAC0930), ErmR	This study

Table 2.4 (continued)

Strain	Relevant characteristics[a]	Source
C. acetobutylicum gcs::int (465)	Group II intron inserted at 465./466. bp of *gcs* (CAC1539), Erm[R]	This study
C. acetobutylicum trxB::int (29)	Group II intron inserted at 29./30. bp of *mccB* (CAC1548), Erm[R]	This study
C. acetobutylicum glcG::int (1224)	Group II intron inserted at 1224./1225. bp of *fer* (CAC0570), Erm[R]	This study
C. acetobutylicum glcCE::int (193)	Group II intron inserted at 193./194. bp of *glcCE* (CAC0386), Erm[R]	This study
C. acetobutylicum glcG::int (1224)/ *glcCE*::int (193)	Group II intron inserted at 1224./1225. bp of *glcG* (CAC0570) and at 193./194. bp of *glcCE* (CAC0386), Erm[R]	This study

[a] Erm[R], erythromycin resistance gene.

2.2.3. Plamids used in ClosTron mutagenesis

Table 2.5 Plasmids used in this study

Plasmid	Relevant characteristics[a]	Source
pMTL007	Clostridial expression vector for expression of ClosTron containing Erm RAM, CmR, IPTG-inducible *fac* promoter	Heap *et al.* (2007)
pMTL007C-E2	Clostridial expression vector for expression of ClosTron containing Erm RAM, Cm[R].	Heap *et al.* (2010)
pAN2	Plasmid harboring φ3T I methyltransferase gene of *B. subtilis* phage φ3*t*I to methylate shuttle plasmids before their introduction into *C. acetobutylicum*, Tet[R]	Heap *et al.* (2007)
pMTL007C-E2-*fer*	ClosTron plasmid retargeted to *C. acetobutylicum fer* (CAC0105) gene, Cm[R].	This study
pMTL007C-E2-*mccB*	ClosTron plasmid retargeted to *C. acetobutylicum mccB* (CAC0930) gene, Cm[R].	This study
pMTL007C-E2-*gcs*	ClosTron plasmid retargeted to *C. acetobutylicum gcs* (CAC01539) gene, Cm[R].	This study

Table 2.5 (continued)

Plasmid	Relevant characteristics[a]	Source
pMTL007C-E2-*trxB*	ClosTron plasmid retargeted to *C. acetobutylicum trxB* (CAC1548) gene, Cm^R.	This study
pMTL007C-E2-*glcG*	ClosTron plasmid retargeted to *C. acetobutylicum glcG* (CAC0570) gene, Cm^R.	This study
pMTL007C-E2-*glcCE*	ClosTron plasmid retargeted to *C. acetobutylicum glcCE* (CAC0386) gene, Cm^R.	This study

[a] Cm^R, chloramphenicol/thiamphenicol resistance gene, Tet^R, tetracycline resistance gene.

2.2.4. Primers used in ClosTron mutagenesis, amplification of specific genes and Southern hybridization.

The basic principle of ClosTron technology is to make specific changes to a group II intron such that it preferentially inserts into the DNA region of interest. Four primers are needed, named as EBS, EBS1d, EBS2 and IBS, in which the EBS primer sequence is specific and universal in the ClosTron mutagenesis process. Afterwards, the retargeted plasmids are generated and the retargeted region must be sequenced. So, a primer, spofdx-seq-F, is needed in the sequencing process. When the putative mutants are obtained, the screening primers are needed to check that the intron has inserted into the target gene. These primers designed are cross the retargeted region in the chromosome and the products of PCR of the mutant are approximate 1.8 kb longer than that of the wild type. It is always necessary to verify that the isolated mutants contain a single intron insertion by Southern blot. An intron-specific probe is generated by PCR using Intron II primers. All the primers used in this study are listed in Table 2.6

Table 2.6 Primers used in this study

Primer	Sequence 5'→3'	Source
EBS universal	CGAAATTAGAAACTTGCGTTCAGTAAAC	Heap *et al.* (2010)

Table 2.6 (continued)

Primer	Sequence 5'→3'	Source
fer ClosTron mutant primers	*fer*-**IBS**:AAAAAAGCTTATAATTATCCTTAGGCAAC GAAAAGGTGCGCCCAGATAGGGTG *fer*-**EBS1d**:CAGATTGTACAAATGTGGTGATAACAG ATAAGTCGAAAAGATTAACTTACCTTTCTTTGT *fer*-**EBS2**:TGAACGCAAGTTTCTAATTTCGATTTTGC CTCGATAGAGGAAAGTGTC	This study
mccB ClosTron mutant primers	*mccB*-**IBS:**AAAAAAGCTTATAATTATCCTTAGTAAA CGCTATAGTGCGCCCAGATAGGGTG *mccB*-**EBS1d:**CAGATTGTACAAATGTGGTGATAAC AGATAAGTCGCTATATATAACTTACCTTTCTTTGT *mccB*-**EBS2:**TGAACGCAAGTTTCTAATTTCGATTTT TACTCGATAGAGGAAAGTGTCT	This study
gcs ClosTron mutant primers	*gcs*-**IBS:**AAAAAAGCTTATAATTATCCTTAGTCGCC AAAAGAGTGCGCCCAGATAGGGTG *gcs*-**EBS1d:**CAGATTGTACAAATGTGGTGATAACAG ATAAGTCAAAAGAAATAACTTACCTTTCTTTGT *gcs*-**EBS2:**TGAACGCAAGTTTCTAATTTCGATTGCG ACTCGATAGAGGAAAGTGTCT	This study
trxB ClosTron mutant primers	*trxB*-**IBS:**AAAAAAGCTTATAATTATCCTTAGCAGG CCCACTTGTGCGCCCAGATAGGGTG *trxB*-**EBS1d:**CAGATTGTACAAATGTGGTGATAAC AGATAAGTCCCACTTCCTAACTTACCTTTCTTTGT *trxB*-**EBS2:**TGAACGCAAGTTTCTAATTTCGATTCC TGCTCGATAGAGGAAAGTGTCT	This study
glcG ClosTron mutant primers	*glcG*-**IBS:**AAAAAAGCTTATAATTATCCTTAGAAGG CGTTAAGGTGCGCCCAGATAGGGTG *glcG*-**EBS1d:**CAGATTGTACAAATGTGGTGATAACA GATAAGTCGTTAAGAATAACTTACCTTTCTTTGT *glcG*-**EBS2:**TGAACGCAAGTTTCTAATTTCGATTCC TTCTCGATAGAGGAAAGTGTCT	This study

Table 2.6 (continued)

Primer	Sequence 5'→3'	Source
glcCE ClosTron mutant primers	*glcCE*-**IBS:**AAAAAAGCTTATAATTATCCTTACCACCCAATGAGGTGCGCCCAGATAGGGTG *glcCE*-**EBS1d:**CAGATTGTACAAATGTGGTGATAACAGATAAGTCAATGAGTCTAACTTACCTTTCTTTGT *glcCE*-**EBS2:**TGAACGCAAGTTTCTAATTTCGGTTGGTGGTCGATAGAGGAAAGTGTCT	This study
spofdx-seq-F	GATGTAGATAGGATAATAGAATCCATAGAAAATATAGG	This study
fer gene check primers	**CAC0105F:**ATGGGTGTTGCAACTATGGTAAC **CAC0105R:**GGGGATCTTGAAACATAGAGCAC	This study
mccB gene check primers	**CAC0930F:**ACGTGATATAAGAATCAAACTTCC **CAC0930R:**CTTGGAACTGCTGACATACTATG	This study
gcs gene check primers	**CAC1539F:**GATATACCAGTTGCACAATTTCC **CAC1539R:**ACACGAAGTGAAGTAGCATATC	This study
trxB gene check primers	**CAC1548F:**GTGAGGCTGTAGATAAGTATGG **CAC1548R:**TCCCGAAACATCGTATATTCC	This study
glcG gene check primers	**CAC0570F:**ACTTGCTGCATTCGCATCTG **CAC0570R:**CTTCAGTTCCAAAAAC	This study
glcCE gene check primers	**CAC0386F:**ACGTGATATAAGAATCAAACTTCC **CAC0386R:**CTTGGAACTGCTGACATACTATG	This study
Intron II probe primers	**IntronII-F:**CGCGACTCATAGAATTATTTCC **IntronII-R:**ATACTCAGGCCTCAATTAACC	This study
atpB gene primers	**atpB-F:** ATGGAGCTAGGTGCAAAGAC **atpb-R:** GAACCCATGAAGAGGTACTG	This study

2.2.5. Media used for bacterial cultivation

Clostridial growth medium (CGM) (Hartmanis and Gatenbeck, 1984) or CGM agar plate was utilized for general cultivation, mutant generation and recovery of

C. acetobutylicum strains. A minimal medium (MM) was usually used to culture the wild type and mutants. Due to its clear components, it was advantageous to study the effects of single components in the medium on the growth of *C. acetobutylicum*. In order to study sulfur assimilation study, all salts containing sulfate in the MM were substituted by the corresponding chlorides and the changed minimal medium was named chloride minimal medium (cMM) in this study. For the continuous cultures of *C. acetobutylicum* wild type and its mutant derivatives, the minimal medium (MM, for pre-culture) (Bahl *et al.*, 1982) and phosphate-limited minimal medium (PLMM) (Bahl *et al.*, 1982) media were utilized, respectively. In the sulfur assimilation study, the chloride minimal medium (cMM, for pre-culture) and sulfur-limited minimal medium (SLMM) were utilized in continuous culture. *E. coli* strains were routinely cultivated in Luria Bertani (LB) (Sambrook, 2001) liquid medium or LB agar plates. The ingredients of each medium are listed below.

Clostridial growth medium (CGM)	
(Hartmanis and Gatenbeck, 1984)	
Glucose $\times$ H_2O	50 g
K_2HPO_4	0.75 g
KH_2PO_4	0.75 g
$(NH_4)_2SO_4$	2 g
$MgSO_4 \times 7\ H_2O$	0.71 g
$MnSO_4 \times H_2O$	0.01 g
$FeSO_4 \times 7\ H_2O$	0.01 g
NaCl	1 g
Asparagine	2 g
Yeast extract	5 g
Agar (only solid medium was needed)	15 g
Deionized water	*ad* 1000 mL

The glucose, $FeSO_4$, and asparagine were dissolved independently and added into autoclaved medium with a 0.45 µm sterile filter, followed by sparging with pure nitrogen to ensure the anaerobic condition.

Minimal medium (MM)	
(Bahl *et al.*, 1982)	
Glucose $\times$ H_2O	50 g
$CaCO_3$	1 g
$K_2HPO_4 \times 3\ H_2O$	1 g
KH_2PO_4	1 g

$(NH_4)_2SO_4$	2 g
$MgSO_4 \times 7\ H_2O$	0.1 g
$MnSO_4 \times H_2O$	0.015 g
$FeSO_4 \times 7\ H_2O$	0.015 g
NaCl	0.01 g
$Na_2MoO_4 \times 2\ H_2O$	0.01 g
$CaCl_2 \times 2\ H_2O$	0.01 g
Biotin	0.1 mg
Thiamin-HCl	2 mg
ρ-Aminobenzoic acid	2 mg
Deionized water	*ad* 1000 ml

The glucose, $FeSO_4$, and three vitamins were dissolved independently and added into autoclaved medium with a 0.45 μm sterile filter, followed by sparging with pure nitrogen to ensure the anaerobic condition.

Chloride Minimal medium (cMM)	
Glucose $\times H_2O$	50 g
$CaCO_3$	1 g
$K_2HPO_4 \times 3\ H_2O$	1 g
KH_2PO_4	1 g
NH_4Cl	0.8 g
$MgCl_2 \times 6\ H_2O$	0.1 g
$MnCl_2 \times H_2O$	0.015 g
$FeCl_2 \times 4\ H_2O$	0.015 g
NaCl	0.01 g
$Na_2MoO_4 \times 2\ H_2O$	0.01 g
$CaCl_2 \times 2\ H_2O$	0.01 g
Biotin	0.1 mg
Thiamin-HCl	2 mg
ρ-Aminobenzoic acid	2 mg
Deionized water	*ad* 1000 ml

The glucose, $FeCl_2$, and three vitamins were dissolved independently and added into autoclaved medium with a 0.45 μm sterile filter, followed by sparging with pure nitrogen to ensure the anaerobic condition.

Phosphate-limited minimal medium (PLMM, the concentration of phosphate is 0.5 mM) (Bahl *et al.*, 1982)	
Glucose $\times H_2O$	600 g
$(NH_4)_2SO_4$	30 g
KH_2PO_4	1.05 g
$MgSO_4 \times 7\ H_2O$	1.5 g
NaCl	0.15 g
$Na_2MoO_4 \times 2\ H_2O$	0.15 g
$CaCl_2 \times 2\ H_2O$	0.15 g
$MnSO_4 \times H_2O$	0.225 g

FeSO$_4$ × 7 H$_2$O	0.225 g
Biotin	1.5 mg
Thiamin-HCl	30 mg
p-Aminobenzoic acid	30 mg
Deionized water	*ad* 15 L
	Adjust pH to 2.0 with H$_2$SO$_4$

The glucose, FeSO$_4$, and three vitamins were dissolved in 3 L deionized water and adjusted to pH 2.0. The solution was then sterized by a Steritop filter unit (EMD Millipore, Billerica, USA) and added into 12 L autoclaved saline solution, followed by sparging with pure nitrogen to ensure the anaerobic condition.

Sulfur-limited minimal medium (SLMM, the concentration of sulfate is 0.55 mM)	
Glucose × H$_2$O	600 g
NH$_4$Cl	12 g
KH$_2$PO$_4$	15 g
K$_2$HPO$_4$	15 g
MgSO$_4$ × 7 H$_2$O	1.5 g
NaCl	0.15 g
Na$_2$MoO$_4$ × 2 H$_2$O	0.15 g
CaCl$_2$ × 2 H$_2$O	0.15 g
MnSO$_4$ × H$_2$O	0.225 g
FeSO$_4$ × 7 H$_2$O	0.225 g
Biotin	1.5 mg
Thiamin-HCl	30 mg
p-Aminobenzoic acid	30 mg
Deionized water	*ad* 15 L
	Adjust pH to 2.0 with HCl

The glucose, FeSO$_4$, and three vitamins were dissolved in 3 L deionized water and adjusted to pH 2.0 with HCl. The solution was then sterized by a Steritop filter unit (EMD Millipore, Billerica, USA) and added into 12 L autoclaved saline solution, followed by sparging with pure nitrogen to ensure the anaerobic condition. In the SLMM, the sole sulfur source was sulfate. The total sulfate concentration of the three sulfate chemicals (MgSO$_4$ × 7 H$_2$O, MnSO$_4$ × H$_2$O and FeSO$_4$ × 7 H$_2$O) in the medium was 0.55 mM.

Sulfur-limited minimal medium (SLMM, the concentration of sulfate is 0.4 mM)	
Glucose × H$_2$O	600 g
NH$_4$Cl	12 g
KH$_2$PO$_4$	15 g
K$_2$HPO$_4$	15 g
MgCl$_2$ × 6 H$_2$O	0.465 g
MgSO$_4$ × 7 H$_2$O	0.945 g
NaCl	0.15 g

$Na_2MoO_4 \times 2\ H_2O$	0.15 g
$CaCl_2 \times 2\ H_2O$	0.15 g
$MnSO_4 \times H_2O$	0.225 g
$FeSO_4 \times 7\ H_2O$	0.225 g
Biotin	1.5 mg
Thiamin-HCl	30 mg
p-Aminobenzoic acid	30 mg
Deionized water	*ad* 15 L
	Adjust pH to 2.0 with HCl

The glucose, $FeSO_4$, and three vitamins were dissolved in 3 L deionized water and adjusted to pH 2.0 with HCl. The solution was then sterized by a Steritop filter unit (EMD Millipore, Billerica, USA) and added into 12 L autoclaved saline solution, followed by sparging with pure nitrogen to ensure the anaerobic condition. In the SLMM, the sole sulfur source was sulfate. The total sulfate concentration of the three sulfate chemicals ($MgSO_4 \times 7\ H_2O$, $MnSO_4 \times H_2O$ and $FeSO_4 \times 7\ H_2O$) in the medium was 0.4 mM.

Luria Bertani (LB) medium	
(Sambrook, 2001)	
Yeast extract	5 g
Tryptone	10 g
NaCl	10 g
Agar (only solid medium was needed)	15 g
Deionized water	*ad* 1000 mL

2.2.6. Antibiotics in the selected medium

Stock solutions of antibiotics used in this study were prepared in 70% ethanol or DMF (N,N-dimethylformamide). After complete dilution, each stock solution was sterilized by filtration and then stored at -20 °C. The appropriate volume of antibiotic stock solution was added to the liquid or agar medium whose temperature was below 50 °C.

Antibiotic	Stock solution (menstruum)	Final concentraiton
Tetracycline	10 mg/mL (70% Ethanol)	10 µg/mL
Chloramphenicol	10 mg/mL (70% Ethanol)	12.5 µg/mL in liquid, 25 µg/mL in solid
Thiamphenicol	15 mg/mL (DMF)	7.5 µg/mL in liquid, 12.5 µg/mL in solid
Erythromycin	5 mg/mL (70% Ethanol)	2.5 µg/mL in liquid, 5 µg/mL in solid

2.3 ClosTron-mediated gene deletion in *C. acetobutylicum*

2.3.1 Targeted gene and primers designation in the ClosTron technology

The genome sequence of *C. acetobutylicum* ATCC 824 has been completed in 2001 (Nolling *et al.*, 2001). The DNA sequence of the genes of interest could be obtained from NCBI. The plasmid pMTL007C-E2 was used for targeting genes of interest, allowing the plasmid-borne Ll.LtrB intron to insert into the specified region of the targeted gene. In order to let the intron recognize the target region of the gene, primers must be designed to modify the original intron located on pMTL007C-E2 using PCR. This step could be done in the ClosTron website (www.clostron.com), just following the directions in the website. In the end, four primers, EBS, EBS2, IBS and EBS1d, were obtained and synthesized by Eurofins Genomics (Ebersberg, Germany).

2.3.2 Splicing by overlap extension (SOE) PCR

SOE PCR was performed with the purpose of modification of the retargeting region of the intron which would insert into the gene of interest in *C. acetobutylicum*. Initially, two independent PCR reactions were carried out using a mixture of IBS/EBS primers and EBS2/EBS1d primers, respectively. The pMTL007 plasmid was utilized as the DNA template. Thereafter, a mixture of these two PCR products was employed as the template in the second round of PCR together with IBS/EBS1d primers. This PCR was to combine two fragments to form a PCR product (~350 bp) which contained three mutated sequences responsible for the recognition of the gene of interest. The sketch map of SOE PCR was shown in Fig.2.1 below. The composition and program of these two PCR reactions were listed below. Sequence information of primers (EBS1d, EBS2, IBS) for each gene of interest and of EBS Universal primer are listed in Tabel 2.6.

Primer Mixture	
(1) IBS+EBS (100 μM)	90 μL H_2O + 5 μL IBS + 5 μL EBS
(2) EBS2+EBS1d (100 μM)	90 μL H_2O + 5 μL EBS2 + 5 μL EBS1d
(3) IBS+EBS1d (100 μM)	90 μL H_2O + 5 μL IBS + 5 μL EBS1d
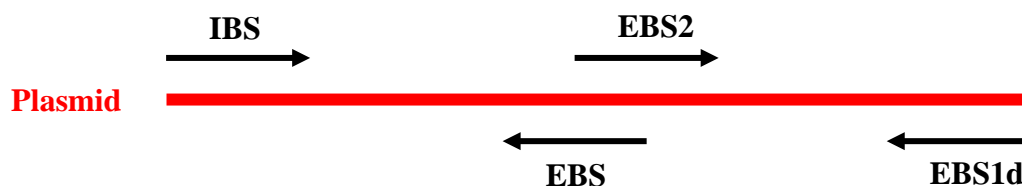
Fig.2.1 Sketch map of SOE PCR

Composition of the first round PCR	
pMTL007 (template)	1 μL (~ 50 ng)
Phire Buffer (5×)	10 μL
Primers mixture (1) or (2) (5 μM)	4 μL
Polymerase (Phire DNA Polymerase)	1 μL
dNTP (10 mM)	4 μL
Deionized H_2O	33 μL
Total volume	50 μL

Composition of the second round PCR	
Product (1)	1 μL
Product (2)	1 μL
Phire Buffer (5×)	10 μL
Primers mixture (1) or (2) (5 μM)	4 μL
Polymerase (Phire DNA Polymerase)	1 μL
dNTP (10 mM)	4 μL
Deionized H_2O	32 μL
Total volume	50 μL

In the first and second rounds of PCR reactions, use the following PCR cycle conditions: Denature 95°C for 2 min followed by 30 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 45 s with a final extension at 72°C for 2 min. The PCR product of the second round of reaction was then separated on a 1% agarose gel (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) and the ~350 bp DNA fragment was subsequently excised and purified. TAE buffer was used in electrophoresis and ingredients were listed below. Before running the gel, the nucleic acid samples were well mixed with 6 × DNA loading dye (Thermo Scientific, Schwerte, Germany) and 1 kb DNA ladder (Thermo Scientific, Schwerte, Germany) was used as the molecular

standard. The gel was then soaked in an ethidium bromide (EB) bath (10 μg/mL) and visualized under UV light at 302 nm in an Alpha Mini Imager (Biozym, Hessisch Oldendorf, Germany). The PCR product was purified using Wizard® SV Gel and PCR Clean-Up System (Promega GmbH, Mannheim, Germany) strictly based on the instruction of the manufacturer and directly used or stored at -20 °C.

50× TAE buffer (pH 8.0)	
Tris	242 g
EDTA (0.5 M, pH 8.0)	57 mL
Acetic acid	57 mL
Deionized water	*ad* 1000 mL

Prepared buffer was autoclaved for 20 min at 121°C, followed by dilution of 50-fold in work condition.

2.3.3 Double digestion and ligation of plasmids and inserts

The 350 bp DNA fragment harboured recognition sites for both *Bsr*GI and *Hin*dIII restriction enzymes (introduced by EBS1d and IBS primers respectively), so did the pMTL007C-E2 plasmid. Therefore, double digestion with *Bsr*GI and *Hin*dIII (NEB, Frankfurt am Main, Germany) was conducted for both plasmids and the purified DNA fragments (inserts) to form compatible cohesive ends. The digestion reaction lasted 4 h at 37 °C. The ingredients of the digestion reaction are listed below.

Composition of double digestion reaction	
Plasmid or inserts	20 μl (approximate 2 μg)
NEB Buffer 2 (10×)	5 μL
BSA 100×	5 μL
*Bsr*GI	2 μL
*Hin*dIII	2 μL
Deionized water	16 μL
Total volume	50 μL

Composition o ligation reaction	
pMTL007-CE2 (linearized)	5 μl (approximate 100 ng)
Retargeted region	15 μl (molar ratio 3:1 to plasmid)
Ligation buffer	3 μL
T4 DNA Ligase (10 U/μL)	1 μL
Deionized water	6 μL
Total volume	30 μL

After digestion with *Bsr*GI and *Hin*dIII restriction enzymes, the linear pMTL007C-E2 plasmid (~8.7 kb) and insert were visualized using 1% agarose gel electrophoresis, followed by excising and purifying. Then the purified insert was subsequently ligated into the linear pMTL007C-E2 plasmid with T4 ligase (Thermo Scientific, Schwerte, Germany) and the components of the reaction are listed above. The ligation reaction was performed overnight (12~16 h) at 14°C.

2.3.4 Transformation of constructed plasmid into *E. coli*

E. coli TOP10 competent cells were re-streaked on a LB agar plate without antibiotics. After an overnight cultivation, one clone was picked and inoculated into 5 mL LB medium (without antibiotics) and cultivated for ~15 h at 37 °C. In the next morning, 100 mL fresh LB medium (without antibiotics) was inoculated with 1 mL overnight culture and cultivated at 37 °C until its OD_{600} reached 0.5-0.6. Culture was then poured into a 50 mL Falcon tube and incubated on ice for 10 min. Collection of cells was then carried out by centrifugation (4,000 rpm, 10 min) at 4 °C, followed by addition of 10 mL cold $CaCl_2$ (100 mM) to re-suspend cells and incubation on ice for 30 min. After another centrifugation (4,000 rpm, 10 min) at 4 °C, supernatant was discarded and fresh 2 mL cold $CaCl_2$ was added and the pellet was re-suspended gently. Afterwards, 2 mL 30% glycerol was supplemented to a final glycerol concentration of 15%. The resultant culture was the competent cells which were dispensed into 1.5 mL centrifuge tubes (100 μL each) and immediately frozen in liquid nitrogen. *E. coli* competent cells prepared were stored at -80 °C.

After incubation for 16 h at 14 °C, the ligation reaction mixture (section 2.3.3) was transformed into *E. coli* TOP 10 competent cells (Invitrogen GmbH, Karlsruhe, Germany) by heat shock. 20 μL ligation mixture was added in to 100 μL *E. coli* TOP10 competent cells and gently blended, followed incubated on the ice for 30 min. Afterwards, it was heat shocked for 90 s at 42 °C and then immediately placed on ice for 2-3 min, followed by addition of 900 μL LB liquid medium without antibiotics and incubation at 37 °C (150 rpm) for 1 h for recovery. Recovered cells were then

collected by centrifugation (6,000 rpm, 5 min) at room temperature and re-suspended with fresh 100 µL LB medium. Thereafter, the cells were plated onto LB agar plates supplemented with chloramphenicol to select for transformants and incubated at 37°C overnight. Several transformants were picked and inoculated in 5 mL LB liquid medium containing 12.5 µg/mL chloramphenicol. After overnight cultivation, 4 mL culture was centrifugated (10,000 rpm, 1 min) and plasmid purification was carried out according to the instruction of the manufacturer of AxyPrepTM Plasmid Miniprep Kit. Plasmids were then digested with *Bsr*GI and *Hin*dIII restriction enzymes for verification. Correct plasmids resulted in two bands in 1% agarose gel. One was ~350 bp, and the other was ~8.7 kb. Correct plasmids were sent for sequencing (Eurofins Genomics, Ebersberg, Germany) using pMTL007C-E2 check primer (spofdx-seq-F). Sequence information obtained was subsequently aligned against predicted sequences of intron retargeting region (in the IBS, EBS2 and EBS1d primer sequences) which was given by the ClosTron site. The correct plasmids were stored at -20 °C or used immediately.

Due to the restriction system of *C. acetobutylicum* cells, retargeted plasmids, which were transformed into *C. acetobutylicum* had to be methylated in order to avoid degradation of the plasmid by the endonuclease. Thus, constructed plasmids were subsequently re-transformed into another *E. coli* recombinant strain containing pAN2 plasmids (Heap *et al.*, 2007), which possessed the gene of a DNA methylase from *Bacillus* phage Φ3T. The transformation process was the same as described above, so was the subsequent bacterial cultivation and plasmid purification. pMTL007C-E2 plasmids harbouring specific recognition region to genes of interest and pAN2 plasmid purified were the ultimate plasmids for transformation into *C. acetobutylicum*. The correct plasmids were stored at -20 °C or used immediately and the *E. coli* transformans with pMTL007C-E2 plasmid and pAN2 plasmid were stored at -80 °C with 30% glycerol.

2.3.5 Transformation of plasmids into *C. acetobutylicum* and generation of ClosTron mutants

C. acetobutylicum wild type strain was stored as the spore suspension in CGM liquid medium in a Hungate-type tube at room temperature. 500 μL spore suspension cells were inoculated into 5 mL fresh CGM liquid medium and were heated for 10 min at 80 °C to kill all vegetative cells and allow germination. Afterwards, the inoculum was cultivated for ~2 days in a shaker at 180 rpm at 37 °C as a pre-culture. 500 μL fresh wild type pre-culture was transferred to a new Hungate tube containing 5 mL CGM medium for the following overnight cultivation. 50 mL fresh CGM medium was inoculated in the next morning with 1 mL overnight culture and cultivated for ~5 h at 37 °C. As soon as the OD_{600} of the growing culture reached 0.6, electroporation could be started. All the manipulations were conducted in the anaerobic chamber. And a Falcon tube containing fresh culture or suspended cells was tightly closed when it was under aerobic conditions, i.e., during centrifugation and kept on the ice during the manipulation process. 40 mL of *C. acetobutylicum* fresh culture was poured into a 50 mL Falcon tube and centrifugated (5000 rpm, 10 min) at 4 °C. Afterwards, the pellet was re-suspended with 20 mL ETM buffer and centrifugated again under the same conditions as above. The resultant pellet was washed (re-suspended) using 10 mL ET buffer and centrifugated again (5000 rpm, 10 min) at 4 °C. The pellet after this process was finally re-suspended with 3 mL ET buffer. The competent cells of *C. acetobutylicum* were now ready for the subsequent electroporation. The compositions of ETM and ET buffers are listed below.

ETM Buffer	
Sucrose	9.2 g
$Na_2HPO4 \times H_2O$	0.001 g
$NaH_2PO4 \times H_2O$	0.06 g
$MgCl_2 \times 6\ H_2O$	4.3 g
Deionized water	*ad* 100 mL

This buffer was autoclaved for 20 min at 121 °C.

ET Buffer	
Sucrose	9.2 g
Na$_2$HPO4 $\times$ H$_2$O	0.001 g
NaH$_2$PO4 $\times$ H$_2$O	0.06 g
Deionized water	*ad* 100 mL

About 8 μg methylated plasmids (approximate 100μL in this study) were added to the Falcon tube containing 3 mL *C. acetobutylicum* competent cells. After gently mixing plasmids and cells, 600 μL was transferred into a cold electroporation cuvette (4 mm gap; PeqLab, Erlangen, Germany). Conditions of 50 μF, 600 Ω and 1.8 kV were set for the electroporation device (Bio-Rad Laboratories, California, USA). The cold electroporation cuvette was then fixed in the electroporation racket which was connected with the device. Subsequently, plasmids were electroporated into competent cells by starting the device at a duration time of electroporation of ~15 ms. 5 mL fresh CGM medium was inoculated with 600 μL transformed cells and cultivated at 37 °C for recovery. About 5 h later, cells were centrifugated (9,000 rpm, 10 min) at room temperature and re-suspended with 150 μL fresh CGM medium, followed by plating on a CGM agar plate containing thiamphenicol at a concentration of 15 μg/mL.

Two days after electroporation, colonies (putative transformants) were visible on the agar plates. To screen colonies containing the intron, which had inserted at the targeted site on the chromosome, transformants were directly transferred to new CGM agar plates containing erythromycin (5 μg/mL) using toothpicks. After an additional day, integrants carrying the mutation of the gene of interest appeared on the plate. These colonies were cultivated overnight in CGM liquid media and subsequently chromosomal DNA were isolated. Isolation of chromosomal DNA was done using Master Pure DNA Purification Kit (Epicentre, Madison, USA) as described below.

2 mL fresh overnight culture of *C. acetobutylicum* was centrifugated (12,000 rpm, 2 min) at room temperature and the pellet was re-suspended in 150 μL TE buffer (listed

below), followed by addition of 30 μL lysozyme (20 mg/mL). After well mixing, the blend was incubated for 30 min at 37 °C and then supplemented with 180 μL 2× T&C buffer and 1 μL proteinase K (20 mg/mL) for cell lysis and protein degradation. After incubation for 15 min at 65 °C, which was meanwhile vortexed every 5 min, 210 μL of MPC protein precipitation buffer was added to the sample and mixed for protein precipitation. Subsequently, the sample was centrifugated (12,000 rpm, 10 min) and the supernatant was transferred to a new centrifuge tube. Afterwards, 900 μL cold absolute ethanol was added for precipitation of nucleic acids, followed by additional centrifugation (12,000 rpm, 10 min). Supernatant was discarded and 70% ethanol was added for washing the pellet. Afterwards, the pellet was dried at room temperature for 10 min and then dissolved with 1 mL DNase-free water. 1 μL RNase A (10 mg/mL) was subsequently added to the sample, which was then incubated for 30 min at 37 °C for RNA degradation. Finally, the chromosomal DNA sample was stored at -20 °C.

TE Buffer	
Tris-HCl (1 M, pH 8.0)	1 mL
Na$_2$EDTA (0.5 M, pH 8.0)	200 μL
Deionized water	*ad* 100 mL

The putative ClosTron mutants were verified by PCR using gene-specific primers (Table 2.6) in order to detect the correct insertion of the intron into the chromosome. The PCR reaction system and program were listed below.

PCR reaction system (verified the ClosTron mutants)	
Chromosomal DNA	50 ng (approximate 1 μL)
5 × Phire reaction buffer	4 μL
Forward primer (10 μM)	1 μL
Reverse primer (10 μM)	1 μL
Phire DNA Polymerase	0.2 μL
Deionized water	*ad* 20 μL

Step	PCR program	Temperature	Time
1	Denaturation	95 °C	5 min
2	Denaturation	95 °C	45 s
3	Annealing	50~60 °C	45 s

4	Extention	72 °C	2 min 30 s
5	Back to 2 (× 31)		
6	Extension	72 °C	10 min

Annealing temperature in the step 3 depended on the Tm value of each corresponding primer pair.

Correct insertion of the intron into the desired site resulted in a PCR product approximate 1.8 kb (size of the intron) larger than that amplified using wild type chromosomal DNA as template, because specific primers were designed across the insertion sites in the chromosomal DNA. Confirmed clones were then streaked on a CGM agar plate without antibiotics and on the following day, several colonies were picked and inoculated into fresh CGM media. Afterwards, well-growing cultures were verified again by PCR using isolated chromosomal DNA, followed by storage at -80 °C after being supplemented with 30% glycerol in Hungate-type tubes.

2.3.6 DNA hybridization

2.3.6.1 Isolation of chromosomal DNA for Southern hybridization

When the ClosTron mutants were obtained, it was necessary to establish that they only contained a single intron insertion by Southern hybridization analysis. The phenol: chloroform method (Sambrook, 2001) was employed for extraction of chromosomal DNA from *C. acetobutylicum* when the resultant DNA was used for Southern hybridization.

2 mL fresh culture in CGM medium was centrifuged (12,000 rpm, 2 min) at room temperature and the pellet was washed with 1 mL TE buffer twice. Afterwards, the pellet was re-suspended in 600 μL TE buffer, followed by addition of 200 μL lysozyme (20 mg/mL). After well mixing, the blend was incubated for 1 h at 37 °C and then supplemented with 70 μL 10% SDS for cell lysis, as well as 65 μL EDTA (0.5 M, pH 8.0), 3 μL Tris-HCl (1 M, pH 7.5) and 8 μL proteinase K (20 mg/mL). The sample was then gently mixed by reversion. After additional incubation for 1 h at 37 °C, 180 μL of 5 M $NaClO_4$ were added to the sample and mixed mildly. The

mixture was subsequently centrifuged (11,000 rpm, 10 min) and the supernatant (~ 800 μL) was transferred to a new 2 mL centrifuge tube followed by addition of 500 μL phenol and 500 μL chloroform in the ventilation hood. After reversion for several times, the sample was centrifuged (11,000 rpm, 10 min) and the resultant upper phase was transferred again to a new centrifuge tube and added with cold absolute ethanol for precipitation of nucleic acids. The sample was then centrifuged (11,000, 10 min) at 4 °C and supernatant was discarded. And then, 70% ethanol was added for washing the sample and this step was performed twice. The pellet was dried at room temperature and dissolved in 60 μL DNase-free water. RNase A (10 mg/mL, 2 μL) treatment was performed for 30 min at 37 °C as the last step. The concentration of chromosomal DNA isolated was determined with a NanoDrop ND-1000 spectrophotometer (PeqLab, Erlangen, Germany). 30 μg of chromosomal DNA was digested with *Hin*dIII (NEB, Frankfurt am Main, Germany) enzyme at 37 °C for 16 h, and 2 μL of the digested sample was checked by electrophoresis to ensure complete digestion which was able to be visualized as a smear pattern in a lane of the 0.8% agarose gel. The composition of the digestion reaction is given below.

Composition of *Hin*dIII digestion reaction	
Chromosomal DNA	30 μg
NEB Buffer 2 (10×)	5 μL
*Hin*dIII	3 μL
Deionized water	*ad* 50 μL

2.3.6.2 DNA probe labeling

The probe for DNA hybridization was amplified using intron specific primers (Intron II probe primers, Table 2.6) with the pMTL007 plasmid containing the PCR product fragment (probe) as template. The PCR products were purified with Wizard® SV Gel and PCR Clean-Up System (Promega GmbH, Mannheim, Germany). The concentration of the probes was determined using a NanoDrop ND-1000 Spectrophotometer (PeqLab, Erlangen, Germany). Afterwards, 1 μg of the probe was biotin-labeled using Biotin DecaLabel DNA Labeling Kit strictly according to the

official manual (Thermo Scientific, Schwerte, Germany).

2.3.6.3 Hybridization of probes with digested chromosomal DNA

Digested chromosomal DNA was separated on 0.8% agarose gel (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) for ~ 2.5 h at 80 V (voltage). Afterwards, the gel was placed on a vacuum blotter (Bio-Rad Laboratories, California, USA) with a piece of positively charged nylon membrane (~ 100 cm^2; Roche, Mannheim, Germany) under the gel. The rest bare area of the vacuum blotter surface was covered with a plastic foil to avoid escaping air when vacuuming. Depurination, denaturation and neutralization of DNA were carried out by starting the vacuum pump and pouring the according solution directly on the gel. Depurination and denaturation respectively lasted 30 min, and neutralization was for 20 min. After neutralization, 2 h of DNA transfer from the gel to the positively charged nylon membrane was conducted using a transfer solution (20× SSC solution) and during DNA transfer 20× SSC solution was supplemented on the gel every 30 min. The positively charged nylon membrane with DNA attached was then rinsed in deionized water shortly, followed by drying in sterile tissues. Afterwards, UV fixation was performed to the nylon membrane for 1 min at 302 nm. Thereafter, the DNA-attached nylon membrane was placed into a hybridization glass tube and pre-hybridization was subsequently performed for 2 h at 42 °C using 10 mL hybridization buffer without probes. The main hybridization step was finally carried out with fresh 20 mL hybridization buffer containing biotin-labeled probes (50 ng/mL) for ~16 h at 42 °C. Solutions used in these steps are listed below.

Denaturation solution	
NaCl	70.13 g
NaOH	16 g
Deionized water	*ad* 800 mL

Solution was then autoclaved for 20 min at 121°C

Neutralization solution (pH 7.5)	
Tris-HCl	48.46 g
NaCl	140.26 g
Deionized water	*ad* 800 mL

Tris was first dissolved in appropriate volume of deionized water and adjusted to pH 7.5 with HCl. Thereafter, NaCl was added and dissolved. Solution was then autoclaved for 20 min at 121°C

Transfer solution (20 × SSC, pH 7.0)	
NaCl	140.26 g
Trisodium citrate	70.58 g
Deionized water	*ad* 800 mL

Solution was then autoclaved for 20 min at 121°C

Maleic acid buffer (pH 7.5)	
Maleic acid	9.29 g
NaCl	7.01 g
NaOH	5.6 g
Deionized water	*ad* 800 mL

This buffer was prepared for dissolving blocking reagent used below.

10% Blocking stock solution	
Blocking reagent	10 g
Maleic acid buffer (pH 7.5)	*ad* 100 mL

Blocking reagent suspension was boiled shortly in a microwave oven and well mixed. Afterwards, it was stored at -20 °C.

Hybridization buffer	
20 × SSC (pH 7.0)	12.5 mL
10% Blocking stock solution	5 mL
10% N-lauryl sarcosine	0.5 mL
10% SDS	0.1 mL
Deinoized water	*ad* 50 mL

Hybridization buffer was stored at -20 °C when prepared. 10% N-lauryl sarcosine and 10% SDS were prepared in advance (w/v)

2.3.6.4 Detection of hybridization signals

The detection procedure of the positively charged nylon membrane (after hybridizing probes with genomic DNA attached) was conducted in the next morning according to the instructions of the manufacturer for the Biotin Chromogenic Detection Kit (Thermo Scientific, Schwerte, Germany). The hybridized nylon membrane was first

washed twice with washing buffer 1 for 10 min at 42 °C, followed by washing two times with washing buffer 2 for 10 min at 65 °C. Thereafter, the hybridized probe was detected with the reagents supplied in the Biotin Chromogenic Detection Kit. When detection procedure was completed, the hybridized nylon membrane was rinsed with deionized water to stop reactions and subsequently scanned in gray scale using a ScanMaker 1000Xl (Microtek, Willich, Germany). The resultant image was stored in a tagged image file format (TIFF). The ingredients of washing buffers are listed below.

Washing buffer 1	
20 × SSC (pH 7.0)	80 mL
10% SDS	8 mL
Deionized water	*ad* 800 mL

Washing buffer 2	
20 × SSC (pH 7.0)	4 mL
10% SDS	8 mL
Deionized water	*ad* 800 mL

2.4 Fermentation of *C. acetobutylicum*

2.4.1 Batch fermentation of *C. acetobutylicum*

C. acetobutylicum strains were cultivated in anaerobic conditions at 37 °C in Hungate-type tubes, 250-mL sealed serum bottles in a batch fermentation. For routine cultivation, *C. acetobutylicum* mutants were recovered from stock solutions on CGM agar plates. About 100 μL stock solution of mutant strains was streaked on a CGM agar plate in an anaerobic chamber (Coy Laboratory Products Inc., Michigan, USA) and then plates were cultivated upside down in a 2.5 L anaerobic jar (Oxoid Ltd., Basingstoke, England) for 1-2 days at 37 °C. Subsequently, colonies were picked and inoculated in 5 mL CGM liquid medium in Hungate tubes as pre-culture. For the wild

type strain, the spore suspension was inoculated in 5 mL fresh CGM liquid medium in the Hungate-type tube at a percentage of 10%. Then the Hungate-type tube was heated for 10 min at 80 °C, followed by cultivation for 1~2 days as pre-culture for batch culture. Therefore, CGM medium was routinely utilized for the construction process of ClosTron mutants and in reviving the mutants and wild type.

Yeast extract used for CGM medium is a complex chemical, which could provide various nutrition elements for bacterial growth. Therefore, it is not suitable for the characterization of physiologic requirements of *C. acetobutylicum*. Since minimal medium (MM, in section 2.2.5) consists of specific chemicals, it is better suited to compare the phenotype of wild type and mutants. In the sulfur assimilation study, the chloride minimal medium (cMM, in section 2.2.5) was utilized in the batch fermentation. Therefore, all the batch fermentations of *C. acetobutylicum* strains in this study were performed in 250-mL sealed serum bottles with 100 mL working volume. 5 mL well-growing strains in CGM medium in the Hungate-type tube was inoculated in 95 mL fresh MM or cMM and cultivated overnight at 37 °C and 180 rpm in a shaker as pre-culture. This well-growing pre-culture was then transferred to 95 mL fresh MM or cMM for fermentation study. Two independent cultivations were carried out for each strain (the wild type or mutants) and the average value of the OD_{600} was used for the growth curve.

2.4.2 Continuous fermentation of *C. acetobutylicum*

2.4.2.1 Fermentation device and inoculation of pre-cultures of strains into fermenter

A Biostat B plus fermenter system equipped with a 1 L fermenter (Sartorius, Göttingen, Germany), a 20 L Ilmabor Boro 3.3 glass jar (TGI GmbH, Ilmenau, Germany), MasterFlex peroxide-cured silicone tubing (Cole-Parmer, Vernon Hills, USA) and a peristaltic pump 101 U/R (Watson-Marlow Pumps Group, Wilmington, USA) were utilized for the continuous fermentation of *C. acetobutylicum* strains. The

fermenter was assembled according to the instructions of the manufacturer (Sartorius, Göttingen, Germany).

The pre-cultures of strains were prepared as described in above (section 2.4.1). In the phosphate-limited continuous culture, 650 mL of MM (saline components) was directly added into the fermenter vessel which was then autoclaved for 20 min at 121 °C. Afterwards, the vessel was connected to the control unit of the Biostat B plus fermenter system according to the instruction of the manufacturer. When the temperature of the medium dropped below 80 °C, 30 mL of vitamin solution, iron and glucose was sterilely supplemented via the inoculation well with a 0.45 μm filter. Then the fermenter was set to pH 5.7, 37 °C and 150 rpm. 100 mL pre-culture was then injected into the fermenter vessel using a rubber tubing equipped with manual switches at both ends. Pressure in the serum bottle containing the pre-culture drove the injection. The pH was controlled by automatic addition of 2 M KOH, because acids were produced by the culture. When the culture in the vessel was well-growing after inoculation (12~16 hours needed), acidic PLMM (or SLMM) was connected and pumped into the vessel. The drainage was also started to keep the volume of the culture constant. Once the continuous fermentation was initiated, stirring was adjusted to 200 rpm. The culture was growing acidogenically (pH 5.7). About 4~6 days after initiation of the continuous fermentation, cultures entered the steady-state acidogenic growth. This was determined by the OD_{600} of the culture, which does not further increase. 40 h to 48 h later, pH was set to 4.5 and accordingly addition of KOH was automatically stopped. The pH of the culture started to decrease due to formation of acids. When the pH dropped to 4.5, the KOH pump was switched on again to keep the pH and the culture grew under more acidic conditions (solventogenesis). Gradually, the OD_{600} of cells became stable at this pH in 1-2 days, and additional 2-3 days later, continuous fermentation was finished. The dilution rate of the continuous culture was 0.075 h^{-1} in present study, which was equal to the fact that the culture in the fermenter was completely renewed in 13.3 h.

For the sulfur-limited continuous culture, the operations were the same as those in the

phosphate-limited continuous culture. Only the MM was change to cMM in the pre-culture and MMLM was changed to SLMM in the continuous culture.

2.4.2.2 Taking samples in the continuous fermentation

Samples were taken from the continuous culture every 12 h. The OD_{600} was determined immediately. In addition, 4 mL sample was centrifuged at 4 °C and the supernatant was transferred to new tubes. The tubes were stored at -20 °C for the measurement of concentrations of glucose, sulfate, ethanol, acetone, butanol, acetic acid and butyric acid. Samples were taken in steady state during acidogenic and solventogenic growth for RNA preparation and subsequent microarray analysis. 15 mL Falcon tubes filled with 3 mL methanol were pre-cooled on ice. After well mixing 12 mL of the culture with methanol, centrifugation was immediately conducted (-20 °C, 9000 rpm) for 10 min. Supernatant was then discarded and the pellet was frozen in liquid nitrogen. Samples were then stored at -80 °C for RNA extraction.

2.5 RNA preparation from *C. acetobutylicum*

2.5.1 Reagent preparation for RNA extraction

TE buffer (pH 8.0, described as in section 2.3.5), deionized water, DNase buffer, sodium acetate (3.3 M, pH 5.0) were prepared in advance and autoclaved twice for 30 min at 121°C to completely inactivate RNase. The composition of solutions is listed below. In addition, centrifuge tubes (1.5 mL and 0.2 mL) and tips were prepared in advance and autoclaved twice for 30 min at 121°C.

NaAc (3.3 M, pH 5.0)	
NaAc $\times$ 3 H_2O	408.1 g
Deionized water	*ad* 1 L
	Adjust the pH to 5 with acetic acid

5 × **DNase buffer** (pH 5.0)	
MgSO4 × 7 H_2O	0.62 g
NaAc (3.3 M, Ph 5.0)	15.2 mL
Deionized water	*ad* 100 mL

2.5.2 RNA extraction

RNeasy Midi Kit (Qiagen, Hilden, Germany) was used for RNA preparation. Cell pellets (described in 2.4.2.2) of *C. acetobutylicum* were washed with 1 mL cold TE buffer (pH 8.0) and centrifuged (9000 rpm, 10 min) at 4 °C. After this washing step, the pellet was re-suspended with 500 μL cold TE buffer and subsequently disrupted in a liquid nitrogen-containing shaking flask assembled in a dismembrator (Micro-Dismembrator U, Sartorius, Göttingen, Germany) for 3 min at 1600 rpm. Afterwards, the frozen powder was re-suspended again with 4 mL supplied RLT lysis buffer which had been supplemented with 40 μL mercaptoethanol and transferred to a new 15 mL Falcon tube. After centrifugation (9,000 rpm, 10 min) of the lysate at room temperature, the supernatant was transferred again to a new Falcon tube, followed by addition of 2.8 mL cold absolute ethanol (molecular biology grade; Applichem, Darmstadt, Germany) and shaking the tube. The solution was then transferred to the supplied RNeasy Midi column using a pipette, followed by centrifugation (9,000 rpm, 10 min) at room temperature and three washing steps with supplied washing buffers. The nucleic acid sample bound in the RNeasy Midi column was subsequently eluted by 300 μL of RNase-free water (50 °C). In the next step, the nucleic acid sample was transferred to a new 1.5 mL centrifuge tube and DNase treatment was then performed for 3 h at 28 °C using 15 μL RNase-free DNase (10 U/μL; Roche, Mannheim, Germany) and 75μL RNase-free DNase Buffer (pH 5.0). Afterwards, phenol:chloroform extraction was carried out for inactivation of DNase and extraction of RNA (Sambrook and Russell, 2006). 500 μL phenol was added to the DNase-treated sample and mixed well, followed by centrifugation (12,000 rpm, 3 min) at 4 °C. The upper phase after centrifugation was transferred to a new centrifuge tube and 500 μL chloroform was then added and mixed well, followed by another

centrifugation (12,000 rpm, 3 min) at 4 °C. Upper phase after this step was transferred to a new centrifuge tube and 2.5 volume of cold absolute ethanol (molecular biology grade) were added for RNA precipitation, which lasted overnight at -20 °C. Precipitated RNA was centrifugated (13,000 rpm, 30 min, 4 °C) in the next morning and the RNA pellet was washed twice with 70% ethanol before it was dried in a towel-contained beaker for 1 h at 37 °C. Thereafter, the RNA sample was dissolved in 20 μL of cold RNase-free water and stored at -80 °C.

2.5.3 Verification of RNA integrity and determination of RNA concentration

To determine the integrity of the RNA sample, 1% agarose gel electrophoresis was employed. 1 μL RNA sample was transferred to a new centrifuge tube by a pipette and diluted with 9 μL of RNase-free water. And then, 5 μL diluted sample was used for electrophoresis and 1 μL diluted sample was used for measurement against RNase-free water. The residual diluted sample was used as template in the following PCR. After electrophoresis and staining with ethidium bromide (10 μg/mL), three bands for ribosomal RNA (23S, 16S, 5S) were supposed to be visible under UV light at 302 nm, which was regarded as the indicator of the RNA sample free of RNase contamination. The concentration of the RNA sample was measured using a NanoDrop ND-1000 Spectrophotometer (PeqLab, Erlangen, Germany). To confirm absolute elimination of genomic DNA by RNae-free DNase (10 U/μL; Roche, Mannheim, Germany), the diluted RNA sample was used as template for amplification of *atpB* gene in *C. acetobutylicum* using *atpB* Forward and Reverse primers (Table 2.6). The chromosomal DNA of *C. acetobutylicum* wild type was utilized as a positive control. Only the positive control was supposed to give a band, if the chromosomal DNA in RNA samples had been completely degraded. The composition and program of PCR is given below.

In the PCR reactions, the following PCR cycle conditions were used: Denature 95°C for 2 min followed by 30 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 45 s with a final extension at 72°C for 2 min.

Composition of the PCR	
Diluted RNA sample	1 μL
Phire Buffer (5×)	4 μL
atpB-F (10 μM)	1 μL
atpB-R (10 μM)	1 μL
dNTP (10 mM)	2 μL
Phire ployperase	0.2 μL
Rnase-free water	*ad* 20 μL

2.6 Transcription analysis

2.6.1 cDNA labeling

To investigate the metabolic effects brought by a mutated gene at the transcriptiona level, a series of transcriptome analyses were conducted using DNA microarrays (Janssen *et al.*, 2010, 2012; Grimmler *et al.*, 2010, 2011; Vasileva *et al.*, 2012; Schwarz *et al.*, 2012). For this, total RNA had to be reverse-transcribed into its complement DNA (cDNA) containing cyanine dye-labeled dCTP (GE Healthcare Europe GmbH, Munich, Germany) whose amount was used as an indicator for the quantification of gene expression, when slides with hybridized probes and labeled cDNA were subjected to a scanning process. Reverse transcription was employed using a SuperScript III Reverse Transcriptase Kit (Invitrogen GmbH, Karlsruhe, Germany), random hexamers p(dN)$_6$ (5 μg/μL; Roche, Mannheim, Germany) and dNTPs (Roche, Mannheim, Germany). Ingredients of the reaction are listed below.

Composition of labeling reaction system	
RNA	25 μg
Random hexamers p(dN)$_6$	4 μL
Rnase-free water	*ad* 10 μL

The above ingredients were mixed well in a 0.2 mL centrifuge tube and incubated for 10 min at 70 °C to allow denaturation of RNA and annealing of hexamers and RNA. Then the sample was immediately placed on ice for 3 min, followed by a short spin. Afterwards, the rest components for RNA labeling were added on ice.

Composition of labeling reaction system	
Hybridization mix obtained above	10 μL
5 × First-strand buffer	4 μL
0.1 M DTT	2 μL
dNTP mix (10 mM dATP/TTP/GTP; 4 Mm dCTP)	2 μL
dCTP (Cy3 or Cy5 dye-labeled nucleotide)	1 μL
Superscript III reverse transcriptase (200 U/μL)	1 μL
Total	20 μL

The Cy3 or Cy5 dye-labeled dCTP should be added in dark. Thereafter, the sample was incubated in a thermo cycler for at least 3 h at 42 °C to allow mRNA reverse transcription during which the cyanine dye-labeled dCTP was integrated into the resultant cDNA. For every RNA sample from a mutant or wild type, both Cy3 and Cy5 dye-labeled dCTP were employed. This meant that both Cy3 dye-labeled and Cy5 dye-labeled cDNA samples were prepared for every mutant as well as wild type, in order to eliminate effects based on the different size of Cy3 or Cy5. When reverse transcription of RNA was accomplished, samples were taken and immediately placed on ice for ~ 3 min. Thereafter, a short spin was performed and 2 μL of NaOH (2.5 M) was added to each reaction tube for hydrolyzing the mRNA. Well-mixed samples were placed in a thermo cycler and incubated for 15 min at 37 °C. Subsequently, samples were cooled on ice and 10 μL of 2 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) solution was added to each sample for neutralization of reaction. The ultimate reaction product (32 μL) was temporarily stored on ice for subsequent purification.

2.6.2 Purification of labed DNA for hybridization with microarray

Labeled cDNA was purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and manipulations were strictly based on the instructions of the manufacturer. Since Cy3 dye-labeled and Cy5 dye-labeled cDNA samples were sensitive to light, the whole operation process was done in dimmed light and the tubes were covered by aluminium foil against light. The purified cDNA samples in EB buffer (20 μL for each sample) were temporarily stored on ice and immediately used

in microarray experiments. The labeling efficiency was then determined using a NanoDrop ND-1000 Spectrophotometer where the concentrations of cyanine dye (Cy3 and Cy5) were measured. EB buffer supplied in the QIAquick PCR Purification Kit was used as the control. The ideal concentration of labeled cDNA was ~200 ng/μL and at least 10 pmol/μL was optimal for cyanine dyes.

Cy3 and Cy5 dyes displayed different fluorescence during the scanning process (Cy3 for green fluorescence and Cy5 for red). At least 80 pmol of each cyanine dye was used for one analysis (chip). Cy3- and Cy5-labeled cDNA samples of comparison group should be mixed before they were loaded in the microarry device. That meant Cy3-labeled mutant cDNA was combined with Cy5-labeled wild type cDNA to compare the amount of transcript through fluorescence intensity. Mixed cDNA samples (containing both labeled samples) were incubated for 5 min at 100 °C to eliminate any possible secondary structure. Tom-Freeman (TF) buffer was also incubated for 1 min under the same condition to dissolve the precipitated SDS. Samples and TF buffer were immediately placed on ice afterwards. Around 3 min later, both samples and TF buffer were centrifuged shortly and TF buffer was added to heat-treated samples to a final volume of 150 μL. This denatured sample in TF buffer was ready for hybridization using a Tecan Hybridization Station HS400Pro (Tecan Austria GmbH, Grödig/Salzburg, Austria). The whole procedure was done in dimmed light. The TF buffer was prepared as given below.

Tom-Freeman hybridization buffer (Fitzpatrick *et al.*, 2005)	
Deionized formamide	20 mL
50 × Denhart's solution	5 mL
20 × SSC	12.5 mL
Sodium pyrophosphate (100 μM)	0.5 mL
Tris-HCl (1 M, pH 7.4)	2.5 mL
10% SDS	0.5 mL
H_2O (HPLC grade)	*ad* 50 μL

Sterilized by a 0.22 μm filter and did aliquots to 1.5 mL centrifuge tubes. Prepared buffer was stored at -20 °C.

2.6.3 Hybridization of labeled cDNA with DNA microarray slides

The *C. acetobutylicum* array was constructed by spotting 5' amino-C6-modified

oligonucleotides with a length of 60-70 bases on CodeLink microarry slides (SurModics, Eden Prairie, Minn., USA) using a MicroGrid II microarray spotter (Zinsser Analytic, Frankfurt, Germany). Oligonucleotides were covalently coupled to the surface of the slides. The array contained 3,840 oligonucleotides representing 99.8% of all annotated open reading frames (ORFs) in *C. acetobutylicum* with one oligo per ORF, including all ORFs from pSOL1 megaplasmid.

The hybridization process started with a washing step with $0.1 \times$ SSC in the Tecan Hybridization Station HS400Pro. Thereafter, 150 μL denatured sample in TF buffer was injected into the hybridization chamber of the device using a pipette after the slides were placed in the instrument (positive side up) and the chamber was clamped. Up to four slides could be hybridized at the same time. The hybridization program as listed below was started subsequently (including washing and injection steps).

Step	Description	Condition and setting
1	Washing	45 °C, 30 s; $0.1 \times$ SSC
2	Injection	45 °C; Agitation
3	hybridization	45 °C, 15 h; Low agittation frequency
4	Washing	25 °C, 2.5 min; Soak time: 30 s; $1 \times$ SSC/0.2% SDS
5	Washing	25 °C, 1.17 min; Channel 3
6	Washing	25 °C, 1.17 min; 3 times; $0.1 \times$ SSC
7	Drying	30 °C, 3 min $0.1 \times$ SSC

The buffers used in the Channels

Channel	Composition
Channel 1	Deionized water
Channel 3	$1 \times$ SSC/0.2% SDS
Channel 4	$0.1 \times$ SSC

2.6.4 Quantification and normalization of microarray data

Around 16 h later, hybridization and cleaning steps were finished and slides were scanned for further analysis. Quantification of slides and normalization of data were performed using a GenePix 4000B Scanner and a GenePix 4.0 or 6.0 software (Axon Instruments, Union City, USA). First, pre-scanning was carried out for the

determination sensitively of the photomultiplier tube (PMT; PMT at 635 nm for Cy5, PMT at 532 nm for Cy3). The sensitivity was determined by measuring the fluorescence intensity of array spots of housekeeping genes whose expression levels were supposed to be constant and give an expression ratio of 1:1 when comparing mutant to wild type. Therefore, spots of these genes show bright yellow colour when scanning during which Cy5 displayed red colour and green fluorescence was for Cy3. When all housekeeping genes showed yellow colour during pre-scanning, according PMT (at 635 nm and 532 nm) irradiation strength was determined. After this pre-scanning process, main scanning was conducted using the previously-determined PMT irradiation strength.

The scanner produced two images for the fluorescence at 532 nm and 635 nm, respectively. They were stored in a tagged image file format (TIFF). Image analysis was subsequently performed with GenePix Pro 6.0 software (Axon Instruments, Union City, USA). Two TIFF images were imported in this software and automatically superimposed each other to become a single image. A GenePix array list (.gal) file, like a "mask", was used to identify features on the image and assign their annotations. This "mask" file contained 32 blocks which matched with the probes spotted on a slide. Each block contained rows of circles surrounding the spots (feature indicators) and the placement of feature indicators was automatically controlled by the algorithm of the software. Slight manually adjustment of placement of feature indicators had to be carried out for each spot, because to some extent spots were irregular and might contain artifacts. All pixels inside a feature indicator were regarded as the foreground and the local background was defined as entire adjoining pixels within a 3-fold radius of the feature diameter (Ehrenreich, 2006). After identification of all features, the image information was quantified by clicking the "analysis" button of the software. The fluorescence signal of each spot was automatically calculated into values which contained the feature foreground, local background and standard deviation for each spot and both dyes, as well as the ratio of medians, ratio of means and regression ratio of red and green channels for each spot.

Normalization was conducted by setting the arithmetic mean of the ratios equal to one. The ratio of medians, ratio of means and regression ratio were three different methods for calculation of expression ratios that were based on different mathematical methods. Each pixel in a spot had intensity values at two wavelengths (PMT 635 nm and PMT 532 nm), so did the background pixel surrounding the spot. The mean fluorescence intensity ratio of a spot (the ratio of median and the ratio of mean) was calculated from the individual pixel intensity where the background pixel intensity had been subtracted. The regression ratio was the linear regression between pixel intensities within a 2-fold radius of the feature diameter and it was independent from strictly defining the background or foreground pixels (Ehrenreich, 2006). The set of data of a microarray experiment was exported in a text (.txt) file format which was then opened with an Excel working sheet (Microsoft, Redmond, USA).

2.6.5 Analysis of microarray data

To obtain the actual data from a microarray experiment, fluorescence values of local background plus one standard deviation for two channels (PMT at 635 nm and PMT at 532 nm) had to be subtracted from the foreground intensity (background correction) to ensure the signal quality and to prevent artefacts. The resultant values were only considered when they were larger than zero, which was the first filtering criterion during data analysis (Ehrenreich, 2006). The other filtering criterion was the difference between the ratio of medians, the ratio of means and the regression ratio of each spot. Only those features could be taken into account where the deviation of these ratios was less than 30%. Afterwards, logarithmic ratios (to the basis of 2) of features were selected to represent values of expression ratios due to its convenience of discriminating up- and downregulated genes. Positive values in logarithmic ratios represent induced genes and repressed genes result in a negative ratio. In addition, fluorescence signals whose logarithmic ratios were greater than 1.60 were regarded as significantly induced, whereas values less than -1.60 indicate significant repression.

For a complete transcriptional analysis, duplicate microarray experiments had to be

performed with reverse-labeled samples (dye-flip). Only those expression ratios were taken into account where original and dye-flip results appeared in the last data analysis. From two microarray experiments, the average of two expression ratios of one gene was the final ratio of this gene. This filter criterion was applied in all microarray results analysis in present study. Microarray data were deposited at the ArrayExpress database (www.ebi.ac.uk/arrayexpress).

2.7 Analytical methods

2.7.1 Growth measurement

Growth of *C. acetobutylicum* strains was monitored by measuring the optical density at 600 nm (OD_{600}) of the culture using a UV/VIS-Spectrometer Ultrospec®3300pro (Amersham Pharmacia Biotech). Samples taken from Hungate tubes or serum bottles were routinely diluted 10-fold by deionized water (if the OD_{600} was larger than 1), followed by measurement against water in disposable plastic cuvettes. The reading was then multiplied by 10 and the resultant value was recorded.

2.7.2 Determination the concentration of glucose and L-lactate

The residual glucose and L-lactate were determined with D-Glucose Assay (GOPOD) Kit (Megazyme International, Wicklow, Ireland) and L-lactate Assay Kit (Megazyme International, Wicklow, Ireland) respectively. Samples taken from the fermentor or serum bottles were routinely centrifuged and supernatant samples were diluted appropriate fold by deionized water according to the range of measurement of the kits. A UV/VIS-Spectrometer Ultrospec®3300pro (Amersham Pharmacia Biotech) was used to determine the absorbance of the reaction system. The manipulations and subsequent calculation were done according to the instructions of the manufacturer.

2.7.3 Determination of sulfate ion (SO_4^{2-})

Sulfate ion was precipitated in a strongly acid medium as barium chloride ($BaCl_2$).

The resultant turbidity was measured photometrically at 420 nm. Based on this reaction, the concentration of sulfate in the continuous cultures of *C. acetobutylicum* was determined.

Precipitating solution and standard sulfate stock solution were needed and the compositions of these two solutions are given below.

Precipitating solution	
Barium chloride	10 g
Sodium chloride	10 g
Gelatin	0.25 g
Deionized water	500 mL
Stir the solution until dissolved absolutely (around 4 h)	
Hydrochloric acid (37%)	5 mL
Deionezed water	*ad* 1000 mL

This solution is stable for 1 month.

Standard sulfate stock (10 mM)	
Anhydrous sodium sulfate	1.421 g
Deionezed water	*ad* 1000 mL

This solution is stable for 6 months.

A standard curve was always measured alongside with the samples. For the standard curve, a series of dilutions were made from the standard sulfate stock (10 mM) to the following concentrations using deionized water: 0 mM, 2 mM, 4 mM, 6 mM, 8 mM and 10 mM. 750 μL deionized water was mixed with 150 μL sample or dilutions of standard sulfate stock. The optical density (OD) at 420 nm using a UV/VIS-Spectrometer Ultrospec[®]3300pro (Amersham Pharmacia Biotech) was determined as blank reading. Afterwards, 240 μL precipitating solution was added to cuvettes and mixed well. After 5 min, the optical density at 420 nm was determined again. If the OD values of samples were larger than 1.0, they were diluted until the OD values were below 1.0. Furthermore, measurments of samples and dilutions of standard sulfate stock were done triplicates.

2.7.4 Determination of the products in supernatant using gas chromatography (GC)

Acetone, ethanol, butanol, acetate and butyrate were main products in the cell-free supernatant. To measure fermentation products qualitatively and quantitatively, a Shimadzu GC-2010 gas chromatography (Shimadzu GmbH, Duisburg, Germany) was utilized. It was equipped with a Stabilwax-DA column (Restek Corporation, Bellefonte, USA) and nitrogen was used as the carrier gas. A set of standards (acetone, ethanol, butanol, acetate and butyrate) were prepared and the concentration of each product was 100 g/L. A standard solution mix (10 g/L) was prepared as below.

The standard solution	
Acetone (100 g/L)	100 μL
Ethanol (100 g/L)	100 μL
Butanol (100 g/L)	100 μL
Acetate (100 g/L)	100 μL
Butyrate (100 g/L)	100 μL
Deionezed water	*ad* 1 mL

For the standard curve, a series of dilutions were made from the standard solution (10 g/L) to the following concentrations using deionized water: 0 g/L, 2 g/L, 4 g/L, 6 g/L, 8 g/L and 10 g/L. 100 μL of dilution standard was blended with 350 μL deionized water (pH 2.5, acidified with HCl) in a glass vessel, followed by addition of 50 μL 1-propanol stock solution (5 g/L) to a final volume of 500 μL. The glass vessel was then tightly closed with a plastic cap. 1 μL dilution standard sample was injected into GC machine and the program of temperature of the oven is given below.

Time	Temperature
0 min	70 °C
5 min	98 °C
25 min	250 °C

The peaks were measured by a in the computer connected the GC machine. The acetone peak was the first to appear, followed by the ethanol peak, the 1-propanol peak, the butanol peak, the acetate peak and the butyrate peak in sequence. Therefore,

a standard curve was obtained by known concentrations of products and peak areas. It should be noted that 1-propanol was employed in each sample as an internal standard to ensure the accuracy of the metabolite quantification. Its peak area should vary less than 10% between each sample.

For the sample taken from fermentation culture, 100 μL of culture supernatant was blended with 350 μL deionized water (pH 2.5, acidified with HCl) in a glass vessel, followed by addition of 50 μL 1-propanol stock solution (0.5%, w/w) to a final volume of 500 μL. Then the products were determined by GC described above. The concentration of the products in the samples were determined by comparison with the standard curve.

3 Results

In the present study, seven ClosTron mutants of chromosomal genes in *C. acetobutylicum* were generated. They were mutants of *fer* (ferredoxin, CAC0105), *mccB* (cystathionine γ-lyase, CAC0930), *gcs* (γ-glutamylcysteine synthetase, CAC1539), *trxB* (thioredoxin reductase, CAC1539), *glcG* (PTS enzyme II, CAC0570), *glcGE* (cellobiose-specific component IIC, CAC0386) and a *glcG*/*glcCE* (CAC0570/0386) double mutant. According to their gene annotated functions, they were divided in three groups. Their phenotypes and transcriptome in batch or continuous cultures were described and discussed respectively. The first group was *fer* and *mccB* genes which were related to cysteine synthesis in sulfur source metabolic pathways. The second group was *gcs* and *trxB* genes which were possibly involved to maintain the reduction environment (thiol redox balance) in *C. acetobutylicum*. Finally, the third group contained *glcG* and *glcGC* genes, which belong to the phosphoenolpyruvate phosphotransferase system (PTS) in *C. acetobutylicum*.

3.1 Cysteine synthesis pathways and global regulation of gene expression in response to cysteine availability during sloventogenic growth in *Clostridium acetobutylicum*

3.1.1 Generation and verification of the *fer* and *mccB* mutants

In order to investigate the cysteine synthesis pathways in *C. acetobutylicum*, two genes (CAC0105, ferredoxin, *fer* and CAC0930, cystathionine γ-lyase, *mccB*) were targeted for inactivation using ClosTron mutagenesis as previously described (section 2.3). The primers for SOE PCR (section 2.3.2) are listed in Table 2.6. The length of *fer* is 315 bp and the target site was designed at 276/277 bp. The length of *mccB* is 1147 bp and the target site was designed at 414/415 bp. The correct retargeted plasmids, pMTL007C-E2-*fer* and pMTL007C-E2-*mccB* (in Table 2.5), were

constructed and were subsequently successfully transformed into *C. acetobutylicum*. In the end, putative mutants (the *fer* and *mccB* mutants) were obtained by screening CGM plates. Erythromycin-resistant clones (putative mutants) were verified (section 2.3.5) by check primers (CAC0105F, CAC0105R and CAC0930F, CAC0930R) listed in Table 2.6. The results showed that wild type genomic DNA gave a band at ~ 300 bp, while the correct mutants exhibited products of ~ 2.1 kb, which were 1.8 kb longer than that of wild type (Fig. 3.1.1).
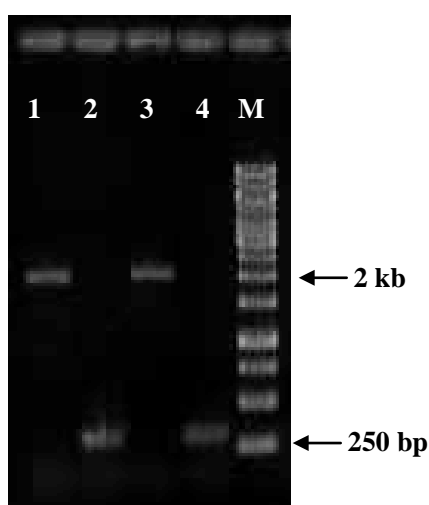
Fig. 3.1.1 PCR verification of *fer* and *mccB* mutans.
M, marker
lane 1, CAC0105F and CAC0105R primers, *fer* mutant genome DNA;
lane 2, CAC0105F and CAC0105R primers, wild type genome DNA;
lane 3, CAC0930F and CAC0930R primers, *mccB* mutant genome DNA;
lane 4, CAC0930F and CAC0930R primers, wild type genome DNA.

To double check that the intron had inserted into the target genes, the two 2.1 kb PCR products from *fer* and *mccB* genomic DNA as template were sequenced. Analysis of the sequences confirmed that the introns inserted in *fer* at 276/277 bp and the *mccB* at 414/415 bp respectively. These results showed *fer* and *mccB* were inactivated by insertion into desired positions of ClosTron-derived group II intron and *fer* and *mccB* ClosTron mutants were obtained. To further confirm that only one copy of the intron inserted in the chromosomal DNA of ClosTron mutants, Southern hybridization was carried out with the intro-specific probe (section 2.3.6.2). The results are shown in Fig.

3.1.2. No hybridization signal was observed for wild type genome DNA, and genomic DNA of the *fer* and *mccB* mutants both exhibited a single band, demonstrating that the *fer* and *mccB* mutants possessed only one copy of intron on the chromosomal.
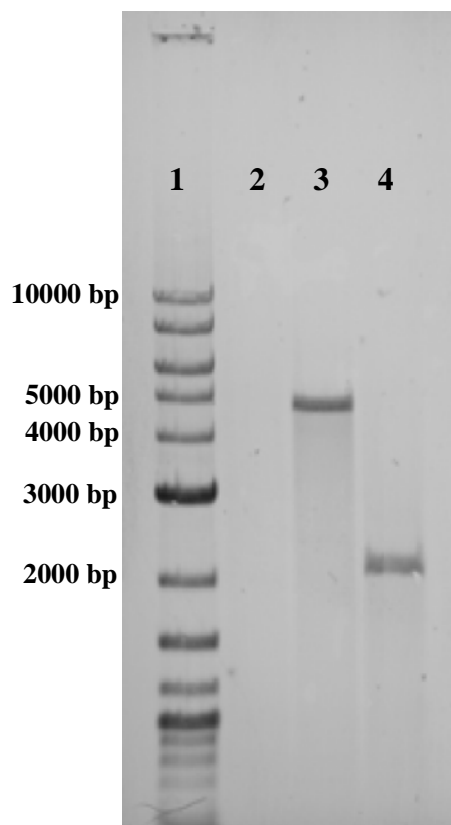
Fig.3.1.2. Southern hybridization analysis of the *fer* and *mccB* mutants using a specific intron probe.
Lane 1, Marker; Lane 2, Genomic DNA of wild type (negative control); Lane 3, Genomic DNA of the *fer* mutant; Lane, 4, Genomic DNA of the *mccB* mutant

3.1.2 Batch fermentation profiles of the *fer* and *mccB* mutants

The *fer* gene is located in a putative operon of 8,863-bp fragment (CAC0102-0110), together with other 8 open reading frames (Nolling *et al.*, 2001). The function of the nine gene products as deduced by analogy with *B. subtilis* was postulated to catalyze the reduction of sulfate to sulfite as shown in Fig. 1.3 (Andre *et al.*, 2008). In order to investigate the function of this operon, the *fer* mutant and wild type were cultured in minimal medium (MM, section 2.2.5) with sulfate as sole sulfur source. The results of the phenotypic characterization are shown in Fig. 3.1.3 A. The wild type grew normally, while the *fer* mutant was not able to grow in the minimal medium. These results suggested that the gene *fer*, which encoded a ferredoxin was involved in

sulfate reduction. A sequence of enzymatic steps reduce the inorganic sulfate to sulfite in *B. subtilis* and *E. coli* (Albanesi *et al.*, 2005; Kredich, 1996). Therefore, it was hypothesized that the function of putative operon of 8,863-bp is also the conversion of sulfate to sulfite in *C. acetobutylicum*. To test this hypothesis, sodium sulfite was added to minimal medium at 1 mM concentration when cultivating the *fer* mutant (Fig.3.1.3 A). This supplementation was able to complement the phenotype of the *fer* mutant in minimal medium. Sulfite is reduced to sulfide by sulfite reductase during sulfate assimilation. The O-acetyl-L-serine (thiol)-lyase incorporates the sulfide in serine, forming cysteine, which is in the central sulfur source in metabolism. Therefore, cysteine was also added to minimal medium at 1 mM concentration to complement the *fer* mutant (Fig.3.1.3A). The *mccB* mutant grew poorly in sulfur-free minimal medium in the presence of 1 mM methionine as sole sulfur source compared to the wild type under the same condition (Fig. 3B). This result provided evidence *in vivo* that the *ubiGmccBA* operon containing the gene *mccB* was involved in the conversion of methionine to cysteine.
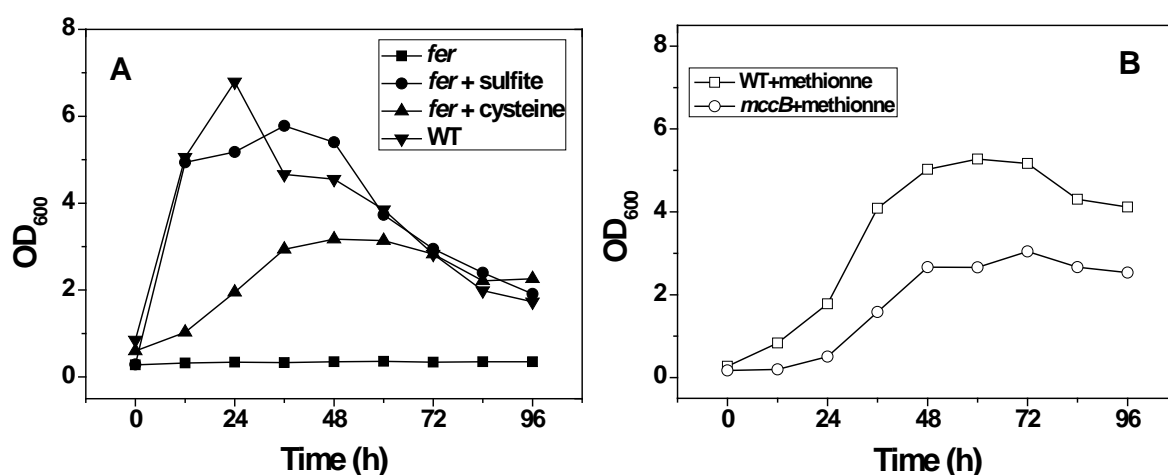
Fig.3.1.3. A, Wild type and the *fer* mutant cultured in minimal medium. B, Wild type and the *mccB* mutant cultured in chloride minimal medium in the presence of 1 mM methionine as sole sulfur source. (▼), Wild type; **(■),** The *fer* mutant; **(●),** *fer* mutant cultured in the presence of 1 mM sodium sulfite; **(▲),** *fer* mutant cultured in the presence of 1 mM cysteine; (□), Wild type; (○), The *mccB* mutant.

Compared to the growth phenotype of the wild type, the *fer* mutant grew poorly with cysteine as sole sulfur source. Altogether, the addition of sulfite and cysteine was able to complement growth of the *fer* mutant in batch fermentations in minimal medium. These results demonstrated that the putative operon of 8,863-bp length, containing *fer* was involved in conversion from sulfate to sulfite in the sulfur metabolism in *C. acetobutylicum. C. acetobutylicum* was able to grow in chloride minimal medium (cMM, section 2.2.5) with 1 mM methionine as sole sulfur source, which indicated that a biochemical pathway from methionine to cysteine exists in *C. acetobutylicum.*

3.1.3 Sulfur source consumption by the wild type and *fer* mutant during acidogenic and solventogenic growth

Chemostat cultivation is an excellent research tool to keep the cells at steady state and provide a suitable basis for studying sulfur metabolism of *C. acetobutylicum* during acidogenic and solventogenic growth respectively (Lutke-Eversloh and Bahl, 2011). Comparing acidogenic to solventogenic growth in phosphate-limited continuous culture, the two operons, CAC0102-0110 and *ubiGmccBA*, which were involved in cysteine synthesis were strongly induced during solventogenic growth (Grimmler *et al.*, 2010). Since sulfate is the sole sulfur source in continuous culture, these results implicated that more sulfate should be consumed during solventogenic growth. To investigate sulfate consumption in acidogenic and solventogenic growth, a phosphate-limited continuous culture of *C. acetobutylicum* was performed (section 2.2.5 and section 2.4.2) and the results are shown in Fig.3.1.4. The residual sulfate in the continuous culture was determined at 120h and 216h. All the cells in the fermenter showed nearly the same growth rate, nutrition and glucose uptake at these two time points representing steady-state metabolism during acidogenic (pH 5.7) and solventogenic growth (pH 4.5) respectively. The residual sulfate concentrations were 17.85 ± 0.14 mM at 120 h and 18.38 ± 0.09 mM at 216 h respectively. This result showed that less sulfate was needed to maintain steady-state growth during solventogenenic growth as compared to that during acidogenic growth. Considering a

sufficient supply of sulfur in the culture and strongly induced expression of the two operons (CAC0102-0110 and *ubiGmccBA*), it seemed to paradoxical to determine that less of the sulfur source was consumed during solventogenic growth in phosphate-limited continuous culture.

Fig.3.1.4 The fermentation profile of a phosphate-limited continuous culture of *C. acetobutylicum*. Dashed line indicated the dynamic pH shift from 5.7 to 4.5. (△), Glucose; (■), OD_{600}; (●), pH.

Due to the inactivation of the *fer* gene, the *fer* mutant was not able to grow in the minimal medium, but could be complemented by adding cysteine, which is the end-product of sulfur assimilation (Fig.1.2 and Fig.3.1.3). To further investigate sulfur consumption of *C. acetobutylicum* during solventogenic growth, the cysteine synthesis pathway was circumvented and the *fer* mutant was cultured using cysteine directly as the sole sulfur source in phosphate-limited continuous culture. Therefore, the *fer* mutant was cultured in a phosphate-limited continuous culture with 1 mM cysteine as sole sulfur source (Fig. 3.1.5).
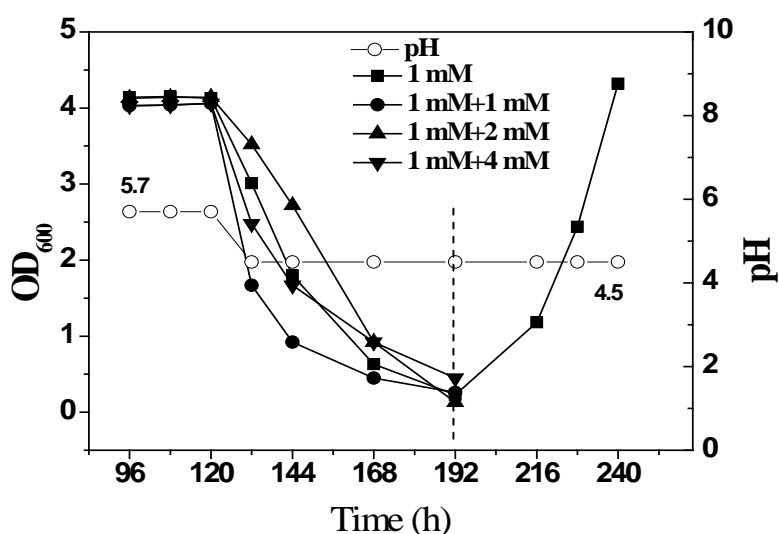
Fig. 3.1.5 The fermentation profile of the *fer* mutant in phosphate-limited continuous culture with cysteine as sulfur source
1 mM +1,2,4 mM, 1 mM, 2 mM or 4 mM cysteine was added after 120 h to the medium containing 1 mM cysteine to result in a a final cysteine concentration of 2, 3 or 5 mM. The dash line indicated that the continuous fermentation was stopped at 192h to result in a batch culture.

The result showed that the *fer* mutant could grow acidogenically, reaching steady state after 72 h. However, the mutant was not able to reach steady state solventogenic growth in continuous culture. The optical density decreased slowly and the cells in the fermentor were "washed out" in continuous culture. When the medium pump was stopped at 192 h and fermentation was continued as a batch culture at pH 4.5, the *fer* mutant grew again. In order to eliminate the possibility of insufficient cysteine in the medium during solventogenic growth, the experiment was repeated three times. Cysteine was added to PLMM at 120h when the pH began to decrease after switching off pH control. The final concentrations of cysteine in the PLMM were 2 mM, 3mM or 5 mM during solventogenic growth. The results showed that the phenotypes of the four fermentation runs were very similar during solventogenic growth. Although sufficient sulfur source (cysteine) was present in PLMM, the *fer* mutant still failed to grow during solventogenic growth in continuous fermentation. These results suggested that the efficiency of sulfur source consumption was lower during solventogenic growth than that during acidogenic growth. Thus, the *fer* mutant could

not consume cysteine efficiently enough to support growth during solventogenic growth in a continuous fermentation. Furthermore, the wild type could grow during solventogenic growth, while the *fer* mutant could not grow with cysteine as sulfur source in continuous fermentation. This indicated that exogenous cysteine was not able to substitute endogenous cysteine synthesis completely during solventogenic growth of *C. acetobutylicum.*

3.1.4 Comparison of gene expression profiles of the *mccB* mutant and wild type grown during solventogenic growth in phosphate-limited continuous culture

The expression of the *ubiGmccBA* operon (CAC0929-CAC0930) has been shown to be strongly induced during solventogenic growth in phosphate-limited continuous culture (Grimmler et al., 2010). This observation indicated that the operon, which is involved in the conversion of methionine to cysteine, probably plays a role during solventogenic growth in *C. acetobutylicum* (Andre *et al.*, 2008). The *mccB* gene (CAC0930) located in this operon was inactivated and the *mccB* mutant was cultivated in phosphate-limited continuous culture (Fig. 3.1.6).
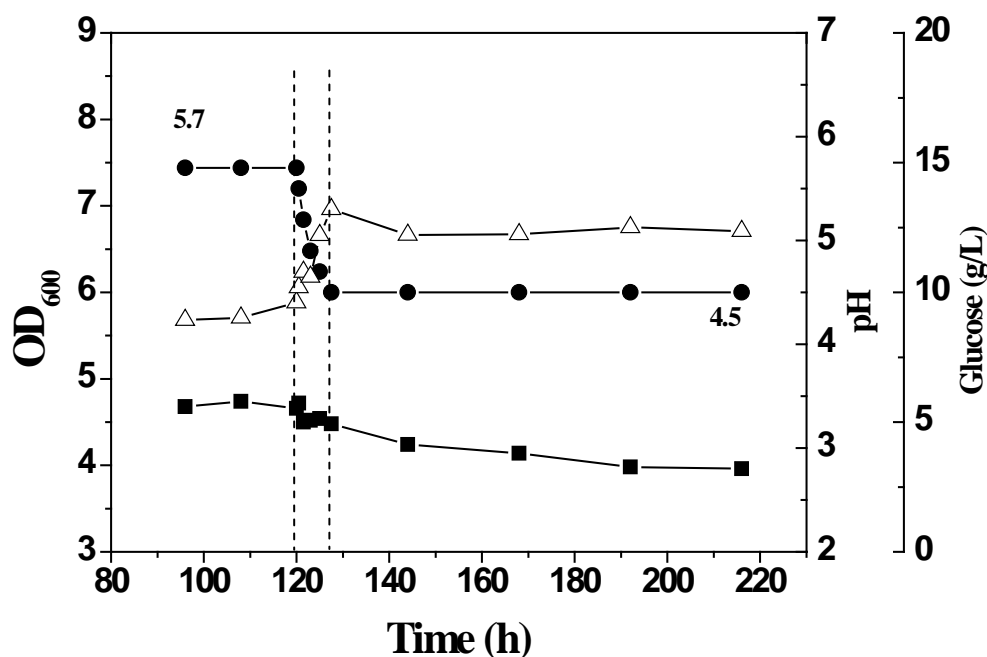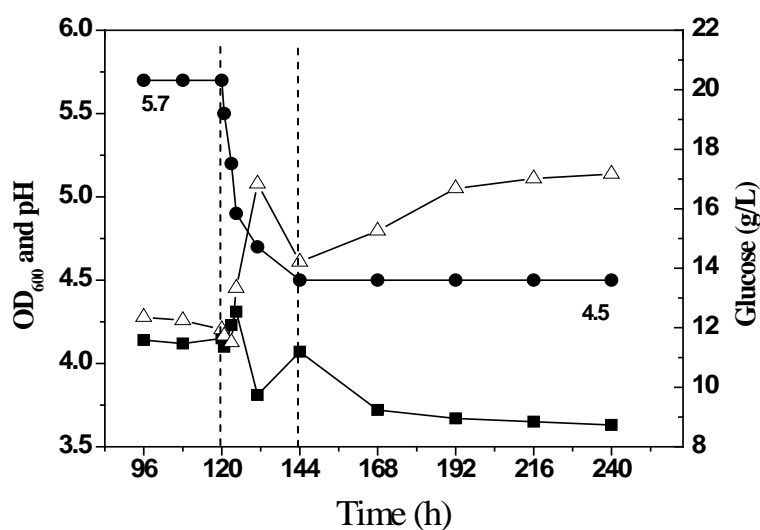

Fig.3.1.6 The fermentation profile of a phosphate-limited continuous culture of the *mccB* mutant. Dashed lines indicate the dynamic pH shift from 5.7 to 4.5. ($\triangle$), Residual glucose; ($\blacksquare$), OD$_{600}$; ($\bullet$), pH.

Product concentrations at 120 h and 240 h were determined andare listed in Table 3.1.1. Based on the measurement of optical density, glucose and product concentrations, the *mccB* mutant grew inferior, compared to the wild type in a phosphate-limited continuous culture (Fig. 3.1.4, Fig. 3.1.6 and Table 3.1.1).

To investigate the transcriptome changes caused by inactivation of the gene *mccB* (CAC0930), a DNA microarray experiment was done to compare the transcriptomes of the wild type and the *mccB* mutant grown in phosphate-limited continuous culture during solventogenic growth (216h for the wild type and 240h for the mutant). RNA isolation and manipulation for microarray hybridization are described in detail in section 2.5 and section 2.6. Microarray data were deposited in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under the accession number E-MTAB-2745. According to the filter criteria (described in section 2.6.4), 134 genes were found with significantly different transcription levels. These genes are listed in Table 3.1.2 and Table 3.1.3.

Table 3.1.1 Product concentrations in phosphate-limited continuous cultures of the wild type and the *mccB* mutant during steady-state acidogenic and solventogenic growth

Product	Wild type		The *mccB* mutant	
	120 h (mM)	216 h (mM)	120 h (mM)	240 h (mM)
Residual glucose[a]	48	69	66	95
Acetate	23	23	12.3	2.4
Butyrate	73	20.9	52	17
Acetone	0.2	20	0.4	12.8
Ethanol	5.3	7.3	1.7	2.1
Butanol	6	45	1.0	21

[a] The total glucose concentration in fresh PLMM was 40 g/L (222 mM). Residual glucose indicated that glucose was not consumped by strains and left in the culture.

Table 3.1.2 The expression profile of genes repressed in the *mccB* mutant during solventogenic growth, compared to the wild type in phosphate-limited continuous culture

ORF	Gene	Protein[a]	Ratio[b]
CA_P0130		Hypothetical protein	-2.05
CAC0102		O-acetylhomoserine sulfhydrylase	-4.40
CAC0103	*cysC*	Adenylylsulfate kinase	-4.84
CAC0104		Adenylylsulfate reductase	-5.16
CAC0105		Ferredoxin	-4.61
CAC0106		ABC-type probable sulfate transporter, periplasmic binding protein	-4.32
CAC0107		ABC-type sulfate transporter, ATPase component	-4.43
CAC0108		ABC-type probable sulfate transporter, permease protein	-4.44
CAC0109	*cysD*	Sulfate adenylyltransferase subunit 2	-4.05
CAC0110	*cysN*	GTPase, sulfate adenylate transferase subunit 1	-2.62
CAC0390	*metI*	Cystathionine gamma-synthase	-3.16
CAC0878		Amino acid ABC transporter permease component	-3.52
CAC0879		ABC-type polar amino acid transport system, ATPase component	-3.18
CAC0880		Periplasmic amino acid binding protein	-4.06
CAC0984		ABC transporter, ATP-binding protein	-2.91
CAC0985		ABC transporter, permease component	-2.30
CAC0986		Lipoprotein, attached to the cytoplasmic membrane, NLPA family	-2.23
CAC1356	*thiH*	Thiamine biosynthesis protein	-2.55
CAC1405	*bglA*	Beta-glucosidase	-1.70
CAC1825	*metB*	Homoserine O-succinyltransferase	-2.06
CAC1826		Hypothetical protein	-2.89
CAC1827		TldD-like protein fragment	-2.12
CAC2229		Pyruvate ferredoxin oxidoreductase	-1.68
CAC2235	*cysK*	Cysteine synthase/cystathionine beta-synthase, CysK	-2.63
CAC2236		Uncharacterized conserved protein of YjeB/RRF2 family	-1.62
CAC2783	*cysD*	O-acetylhomoserine sulfhydrylase	-2.43
CAC2841		Conserved membrane protein, probable transporter, YPAA	-1.61
CAC2928		Predicted membrane protein	-1.82
CAC2991	*metG*	methionine--tRNA ligase	-3.60
CAC3092		Germination specific (cwlC/cwlD B.subtilis ortholog)	-2.04
CAC3093		Phosphate permease	-1.87
CAC3094		Uncharacterized consrved protein, associated with phosphate permease	-1.77
CAC3309		Predicted membrane protein	-2.29
CAC3325		Periplasmic amino acid binding protein	-2.75
CAC3326		Amino acid ABC-type transporter, permease component	-2.59
CAC3327		Amino acid ABC-type transporter, ATPase component	-2.42
CAC3526		FMN-binding protein	-2.33
CAC3527		Ferredoxin	-2.31
CAC3664		Predicted flavodoxin	-2.57

Table 3.1.2 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC3665		Alpha/beta superfamily hydrolase	-2.71

Genes are listed in order of ORFs and considered as significantly downregulated when the logarithmic ratio was $\leq$ -1.6. The given values in the table are mean of results of two hybridizations with dys swaps.

[a] Protein name based on the whole genome sequence by Nölling *et al.* (2001)

[b] The expression ratio as the logarithm to the basis of 2

Compared to the transcriptional expression profile of the wild type, the repressed genes (40 genes) in the *mccB* mutant during solventogenic growth are listed in Table 3.1.2. The majority of genes repressed during solventogenic growth were related to the sulfur metabolism of *C. acetobutylicum*. The cluster of genes CAC0102-0110 is associated with conversion of sulfate to sulfite. According to KEGG pathway analysis, the *metI* (CAC0390), *metB* (CAC1825), *metG* (CAC2991), *cysK* (CAC2235) and *cysD* (CAC2783) genes all participate in cysteine and methionine metabolism (Andre *et al.*, 2008) (Fig.1.2). The results showed that compared to the wild type, the sulfur metabolic regulation changed in the *mccB* mutant during solventogenic growth. Another three clusters which were also repressed included two amino acid ABC-type transporters (CAC0878-0880 and CAC3325-3327) and a ferredoxin cluster (CAC3526-3527).

Table 3.1.3 The expression profile of genes induced in the *mccB* mutant during solventogenic growth, compared to the wild type in phosphate-limited continuous culture

ORF	Gene	Protein[a]	Ratio[b]
CA_P0044		Hypothetical protein	2.63
CA_P0045		Glycosyl transferase	2.40
CA_P0053	*xynb*	Xylanase, glycosyl hydrolase family 10	1.87
CA_P0054		Xylanase/chitin deacetylase family enzyme	2.03
CA_P0056		Pectate lyase, secreted, polysaccharide lyase family	1.70
CA_P0065		Predicted secreted metalloprotease	1.89
CA_P0116		Xylanase, glycosyl hydrolase family 10	2.28
CA_P0117		Possible beta-xylosidase diverged, family 5/39 of glycosyl hydrolases	2.86
CA_P0118		Possible xylan degradation enzyme	2.57
CA_P0119		Possible xylan degradation enzyme	3.35
CA_P0120		Possible xylan degradation enzyme	3.30
CA_P0168	*amyA*	Alpha-amylase	1.95
CAC0014		Aminotransferase	5.64

Table 3.1.3 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC0015	*serA*	D-3-phosphoglycerate dehydrogenase	4.78
CAC0016		Related to HTH domain of SpoOJ/ParA/ParB/repB family	4.96
CAC0017	*serS*	Seryl-tRNA synthetase	4.22
CAC0018		Putative NADPH-quinone reductase, YabF family	2.42
CAC0056		Hypothetical protein	1.98
CAC0057		Hypothetical protein	2.36
CAC0058		Hypothetical protein	2.13
CAC0059		Hypothetical protein	2.20
CAC0060		Predicted membrane protein	2.23
CAC0061		Phage-related protein, gp16	2.14
CAC0062		Hypothetical protein	1.94
CAC0063		Hypothetical protein	2.46
CAC0064		Hypothetical protein	2.59
CAC0065		hypothetical protein	2.49
CAC0316	*argF/I*	Ornithine carbomoyltransferase	4.12
CAC0328		Predicted metal-dependent hydrolase	2.03
CAC0380		Periplasmic amino acid-binding protein	2.71
CAC0528		ABC transporter, ATPase component (two ATPase domains)	1.78
CAC0552		Protein containing cell-adhesion domain	1.78
CAC0553		Hypothetical protein, CF-8 family	1.77
CAC0554	*lyc*	Autolytic lysozime (1,4-beta-N-acetylmuramidase)	2.21
CAC0561		Cellulase CelE ortholog, dockerin domain	2.58
CAC0562		Predicted membrane protein	2.16
CAC0563		Predicted membrane protein	1.91
CAC0574		Pectate lyase H (FS)	2.20
CAC0626	*trpS*	tryptophanyl-tRNA synthetase	2.36
CAC0717		Predicted membrane protein	1.95
CAC0826		Endoglucanase family 5	1.96
CAC0843		Ribonuclease precursor (barnase), secreted.	1.74
CAC0844		Barstar-like protein ribonuclease (barnase) inhibitor	1.79
CAC0910		Probably cellulosomal scaffolding protein precursor	4.89
CAC0911		Possible processive endoglucanase family 48,	4.98
CAC0912		Possible non-processive endoglucanase family 5	5.07
CAC0913		Possible non-processive endoglucanase family 9	4.73
CAC0914		Cellulosome integrating cohesin-containing protein, secreted	4.53
CAC0915		Endoglucanase A precursor (endo-1,4-beta-glucanase) (cellulase A)	4.46
CAC0916		Possible non-processive endoglucanase family 9	4.68
CAC0917		Cellulose-binding endoglucanase family 9	4.83
CAC0918		Possible non-processive endoglucanase family 5	4.82
CAC0919		Probably secreted sialidase, several ASP-boxes and dockerin domain	4.56
CAC0973	*argG*	argininosuccinate synthase	3.81
CAC0974	*argH*	Argininosuccinate lyase	3.85

Table 3.1.3 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC0980	*pflB*	Pyruvate-formate lyase	1.82
CAC0981	*pflA*	Pyruvate-formate-lyase-activating enzyme	1.62
CAC1390	*purE*	phosphoribosylaminoimidazole carboxylase catalytic subunit	2.22
CAC1391	*purC*	phosphoribosylaminoimidazole-succinocarboxamide synthase	1.88
CAC1392	*purF*	amidophosphoribosyltransferase	2.36
CAC1393	*purM*	phosphoribosylaminoimidazole synthetase	2.26
CAC1394	*PurN*	phosphoribosylglycinamide formyltransferase	2.16
CAC1395	*purH*	Phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP Cyclohydrolase	1.92
CAC1396	*purD*	Phosphoribosylamine--glycine ligase	1.91
CAC1655	*purQ/* *purL*	Bifunctional enzyme phosphoribosylformylglycinamidine (FGAM) synthase (synthetase domain/glutamine amidotransferase domain)	2.39
CAC1968		Pectate lyase related enzyme	1.95
CAC2388	*argD*	N-acetylornithine aminotransferase	3.96
CAC2389	*argB*	Acetylglutamate kinase	3.73
CAC2390	*argC*	N-acetyl-gamma-glutamyl-phosphate reductase	2.96
CAC2391	*argJ*	bifunctional ornithine acetyltransferase/N-acetylglutamate synthase	2.98
CAC2497		Hypothetical secreted protein	1.95
CAC2536		Glycosyltransferase	1.83
CAC2537		Predicted phosphatase	1.77
CAC2644	*carB*	Carbamoylphosphate synthase large subunit	2.93
CAC2645	*carA*	Carbamoyl-phosphate synthase small subunit	2.50
CAC2681		Hypothetical protein	2.05
CAC3019		Sensory transduction protein with GGDEF and EAL domains	2.19
CAC3020	*argJ*	Bifunctional ornithine acetyltransferase/N-acetylglutamate synthase	1.85
CAC3157	*trpA*	Tryptophan synthase alpha chain	2.75
CAC3158	*trpB*	tryptophan synthase subunit beta	1.94
CAC3159	*trpF*	Phosphoribosylanthranilate isomerase	3.22
CAC3160	*trpC*	Indole-3-glycerol phosphate synthase	2.99
CAC3161	*trpD*	Anthranilate phosphoribosyltransferase	3.39
CAC3162	*pabA*	Para-aminobenzoate synthase component II	3.07
CAC3163	*parB*	Anthranilate synthase component I	3.09
CAC3236		Possible transcriptional regulator from YAEG/LRPR family	1.76
CAC3421		Acyl carrier protein phosphodiesterase	2.98
CAC3469		Endoglucanase family 5, S-layer homology, cell-adhesion	3.29
CAC3470		Hypothetical protein	2.21
CAC3515		Alpha/beta superfamily hydrolase (possible peptidase)	3.07
CAC3617		Uncharacterized membrane protein, YHAG B.subtilis homolog	2.36
CAC3619		Amino acid ABC transporter, permease component	1.90
CAC3620		Amino acid (probably glutamine) ABC transporter	1.94
CAC3684		Polygalacturonase	1.61

Genes are listed in order of ORFs and considered as significantly upregulated when the logarithmic ratio was ≥ 1.6.

The given values in the table are mean of results of two hybridizations with dys swaps.

[a] Protein name based on the whole genome sequence by Nölling *et al.* (2001)

[b] The expression ratio as the logarithm to the basis of 2

A series of genes whose expression was strongly induced during solventogenic growth in the *mccB* mutant (Table 3.1.3) are mainly involved in the biosynthesis of three amino acids. The cluster of genes CAC0014-0018 is associated with serine biosynthesis. Serine is the precursor of cysteine. The genes with a function in the conversion of L-glutamate to L-arginine were strongly upregulated and consisted of three clusters CAC0973-0974, CAC2388-2391 and CAC3019-3021. The genes for tryptophan biosynthesis (CAC3157-3163) were also strongly induced during solventogeneic growth. An operon coding for a putative cellulosome (CAC0910-0912) was also identified to be induced during solventogenic growth despite the fact that *C. acetobutylicum* can not utilize cellulose (Lopez-Contreras *et al.*, 2003). According to the KEGG pathway analysis, a cluster (CAC1390-1396) which was induced during solventogenic growth was associated with conversion of phosphorbosyl pyrophosphate (PPRP) to inosinic monophosphate (IMP). PPRP and IMP are intermediates in the purine metabolism. Furthermore, the genes involved in xylan and starch metabolism were strongly induced during solventogenic growth. These genes located on the pSOL megaplasmid were *amyA* (CAP0168) and two clusters, CAP0053-CAP0055 and CAP0116-CAP0120.

3.1.5 Continuous fermentations of wild type in the presence of 0.55 mM and 0.4 mM sulfur source

In phosphate-limited minimal medium, the phosphate served as the limiting parameter. Under the condition chosen, it is not enough for sufficient growth of the wild type. To eliminate possible effects of phosphorus limitation on sulfur metabolism and further investigate sulfate (sulfur source) consumption and metabolism during solventogenic growth, a medium containing 0.55 mM and 0.4 mM sulfate (SLMM, section 2.2.5) was designed to cultivate the wild type. The results are shown in Fig.3.1.7 and Fig.3.1.8. The sulfate and product concentrations were determined at 120 h and 264 h

under the two fermentation conditions and listed in the Table 3.1.4.
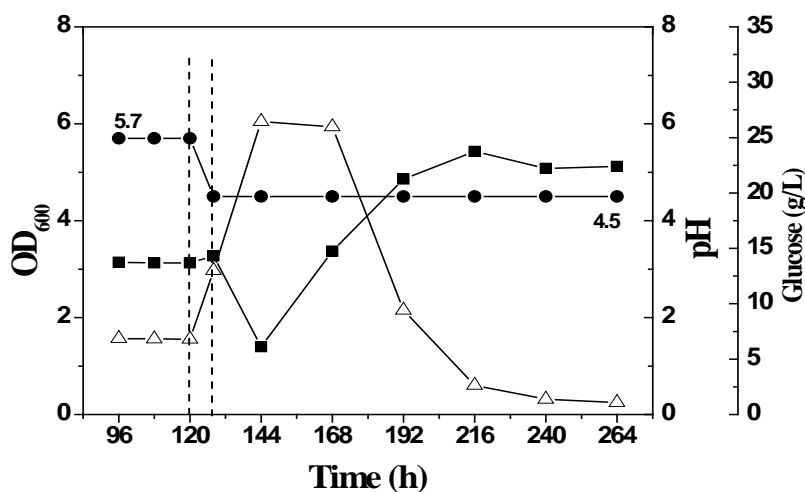
Fig.3.1.7 The fermentation profile of a continuous culture of *C. acetobutylicum* in the presence of 0.55 mM sulfate as sole sulfur source
Dashed line indicated the dynamic pH shift from 5.7 to 4.5. (△), Glucose; (■), OD 600 ; (●), pH
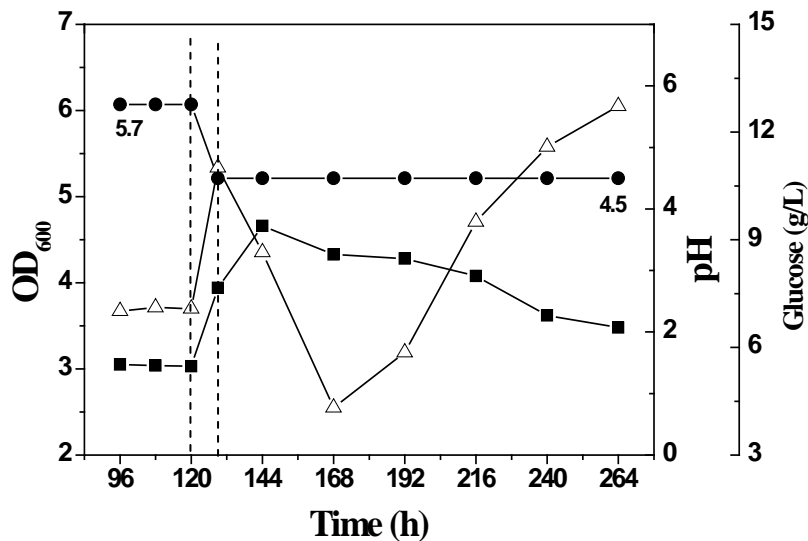
Fig.3.1.8 The fermentation profile of a continuous culture of *C. acetobutylicum* in the presence of 0.4 mM sulfate as sole sulfur source
Dashed line indicated the dynamic pH shift from 5.7 to 4.5. (△), Residue glucose; (■), OD600; (●), pH

During acidogenic growth under both fermentation conditions, sulfate was consumed completely and the products in the medium were comparable except for butyrate and

L-lactate, approximately 14 mM more butyrate and 1.4 mM more lactate were produced in the presence of 0.55 mM sulfate than in the presence of 0.4 mM sulfate (Table 3.1.4). The results showed that sulfate was the limiting factor and kept *C. acetobutylicum* in steady-state growth in the presence of 0.4 and 0.55 mM sulfate. Sulfur-limited continuous fermentation was achieved during acidogenic growth.

Table 3.1.4 Product concentrations in continuous cultures of wild type with 0.55 or 0.4 mM sulfate as sole sulfur source

Product	0.55 mM sulfate		0.4 mM sulfate	
	120 h (mM)	264 h (mM)	120 h (mM)	264 h (mM)
Sulfate	ND	0.04	ND	0.2
L-lactate	3.1	0.25	1.71	6.4
Acetate	23.3	29.7	22.7	37.8
Butyrate	57.0	20.9	43.3	40.1
Acetone	ND	34.0	ND	4.5
Ethanol	3.6	18.7	3.6	3.5
Butanol	5.6	83.1	6.3	23.0

ND: not detected

The wild type grew better during solventogenic growth than during acidogenic growth in the continuous culture in the presence of 0.55 mM sulfate. The optical density reached approximately 5.1 and kept steady state. 0.04 mM sulfate and 1.1 g/L glucose were determined and solvents (acteon and butanol) were the main products in the continuous culture at 264 h (Fig. 3.1.7 and Table 3.1.4). These results revealed that 0.55 mM sulfate in the continuous culture was enough for growth of wild type during solventogenic growth and the transition from acidogenic to solventogenic growth occurred normally through the pH control (from 5.7 to 4.5). However, 0.55 mM sulfate, served as a limiting factor to keep steady-state growth, was not enough for sufficient growth during acidogenic growth. This result showed that *C. acetobutylicum* consumed more sulfate during acidogenic growth than during solventogenic growth. It was consistent with the observation that more residual sulfate was determined during solventogenic growth in the phosphate-limited continuous

culture (Fig 3.1.4). *C. acetobutylicum* was not able to reach steady-state growth during solventogeic growth in the continuous culture in the presence of 0.4 mM sulfate. The residual glucose concentration in the continuous culture increased. The cells were washed out of the fermenter slowly and the optical density decreased. This growth phenotype was similar to the *fer* mutant cultured in a phosphate-limited continuous culture supplemented with 1 mM cysteine (Fig 3.1.5). Furthermore, acids (acetate, butyrate and L-lactate) became the main products at 264 h (Table 3.1.4). These results showed that 0.4 mM sulfate in continuous culture was not enough for growth of the wild type during solventogenic growth. Moreover, the sulfur source starvation affected the transition from acidogenic to solventogenic growth, although the pH was shifted from 5.7 to 4.5.

3.1.6 Comparison of gene expression profiles of wild type grown during solventogenic growth in the presence of 0.55 mM and 0.4 mM sulfur source

When *C. acetobutylicum* was cultivated in continuous culture in minimal medium at the dilution rate of 0.075 h^{-1} with 0.55 mM and 0.4 mM sulfate as sole sulfur source, the growth and metabolic phenotypes were different during solventogenic growth (Fig 3.1.7, Fig 3.1.8 and Table 3.1.4). 0.55 mM sulfate allowed *C. acetobutylicum* sufficient growth, while 0.4 mM sulfate was not enough for growth during solventogenic growth. To obtain new insights into the regulation in response to insufficient sulfur availability, we compared the transcriptome of *C. acetobutylicum* grown in the continuous culture with 0.55 mM or 0.4 mM sulfur source at 264 h. RNA isolation and manipulation for microarray analysis were described in detail in section 2.5 and section 2.6. Microarray data were deposited in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under the accession number E-MTAB-2745. According to the filter criteria (described in section 2.6.4), 261 genes were found with significantly different transcription levels. These genes were listed in Table 3.1.2 and Table 3.1.3.

The majority (181 genes) of the 261 genes were downregulated in the presence of 0.4

mM sulfate. These downregulated genes could be mainly assigned to 5 functional groups. The first group consisted of well-known solventogenic genes, including butanol dehydrogenase B (*bdhB*, CAC3298), acetoacetate decarboxylase (*adc*, CAP0165) and the genes *aad-ctfA-ctfB* (CAP0162-0164). This was consistent with the product concentrations of the fermentations where the solvent concentration was significantly lower in the presence of 0.4 mM sulfate as compared to the presence of 0.55 mM sulfate.

Table 3.1.5 The expression profile of genes repressed in continuous culture with 0.4 mM sulfur source during solventogenic growth, compared to gene expression in the presence of 0.55 mM sulfur source

ORF	Gene	Protein[a]	Ratio[b]
CA_P0004		Cysteine protease	-3.04
CA_P0053	*xynb*	Xylanase, glycosyl hydrolase family 10	-2.23
CA_P0065		Predicted secreted metalloprotease	-2.79
CA_P0098	*amyA*	Alpha-amylase	-2.47
CA_P0102		Membrane protein	-1.74
CA_P0128		Permease	-2.27
CA_P0162	*adhE1*	Aldehyde dehydrogenase (NAD$^+$)	-3.57
CA_P0163	*ctfA*	Butyrate-acetoacetate CoA transferase subunit A	-3.53
CA_P0164	*ctfB*	Butyrate-acetoacetate CoA transferase subunit B	-3.21
CA_P0173		Archaeal-type Fe-S oxidoreductase	-1.86
CAC0014		Aminotransferase	-2.91
CAC0015	*serA*	D-3-phosphoglycerate dehydrogenase	-2.51
CAC0016		Related to HTH domain of SpoOJ/ParA/ParB/repB family	-3.22
CAC0017	*serS*	Seryl-tRNA synthetase	-2.22
CAC0018		Putative NADPH-quinone reductase, YabF family	-3.11
CAC0078	*agrB*	Putative accessory gene regulator protein	-6.36
CAC0079		Hypothetical protein	-6.15
CAC0080	*agrC*	Histidine kinase-like ATPase	-3.40
CAC0081	*agrA*	Accessory gene regulator protein A	-2.20
CAC0082		Predicted membrane protein	-5.46
CAC0107		ABC-type sulfate transporter, ATPase component	-1.95
CAC0109	*cysD*	Sulfate adenylyltransferase subunit 2	-3.38
CAC0112	*glnQ*	Glutamine ABC transporter (ATP-binding protein)	-4.27
CAC0164		ABC transporter, ATP binding-protein	-2.46
CAC0165		Predicted ABC transporter, permease component	-2.28
CAC0252		Molybdate-binding protein	-3.70

Table 3.1.5 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC0253	*nifH*	Nitrogenase iron protein (nitrogenase component II)	-7.09
CAC0254	*nifHD*	Nitrogen regulatory protein PII (nitrogen fixation nifHD)	-8.22
CAC0255	*nifHD*	Nitrogen regulatory protein PII (nitrogen fixation nifHD)	-7.73
CAC0257	*nifK*	Nitrogenase molibdenum-iron protein, beta chain	-3.95
CAC0258	*nifE*	Nitrogenase iron-molibdenum cofactor biosinthesis protein	-6.07
CAC0259		Fusion nifN/K+nifB	-5.36
CAC0260	*nifV*	Homocitrate syntase, omega subunit nifV (nivO)	-4.67
CAC0261	*nifV*	Homocitrate synthase,alpha subunit nifV(nioA)	-3.70
CAC0282		Cytosine/guanine deaminase related protein	-3.08
CAC0353		2,3-cyclic-nucleotide 2'-phosphodiesterase	-2.40
CAC0365		Phosphoglycerate dehydrogenase	-3.86
CAC0366		Predicted permease	-3.68
CAC0367	*rocB*	Arginine degradation protein	-3.40
CAC0368		4-aminobutyrate aminotransferase	-2.88
CAC0375		Hypothetical protein	-3.61
CAC0376		N-dimethylarginine dimethylaminohydrolase	-3.25
CAC0384	*licB*	PTS system, cellobiose-specific component BII	-2.24
CAC0385		Beta-glucosidase	-2.60
CAC0386	*licC*	PTS cellobiose-specific component IIC	-3.08
CAC0387		Hypothetical protein	-2.23
CAC0537		Acetylxylan esterase, acyl-CoA esterase or GDSL lipase family	-2.21
CAC0542		Methyl-accepting chemotaxis protein	-2.17
CAC0561		Cellulase CelE ortholog, dockerin domain	-1.95
CAC0570		PTS enzyme II, ABC component	-4.80
CAC0574		Pectate lyase H	-3.05
CAC0625		Possible periplasmic aspartyl protease	-4.35
CAC0681	*nrgB*	Nitrogen regulatory protein PII	-2.56
CAC0682	*nrgA*	Ammonium transporter (membrane protein nrgA)	-5.79
CAC0702		Predicted lipoprotein, Med/BMP family	-2.09
CAC0709	*gapC*	Glyceraldehyde 3-phosphate dehydrogenase	-1.65
CAC0710	*pgk*	Phosphoglycerate kinase	-2.03
CAC0727	*yifK*	Amino acid permease	-1.69
CAC0746		Secreted protease metal-dependent protease	-4.87
CAC0815		Methyl-accepting chemotaxis protein	-3.44
CAC0816		Lipase-esterase related protein	-2.50
CAC0917		Cellulose-binding endoglucanase family 9	-2.13
CAC0918		Possible non-processive endoglucanase family 5	-2.24
CAC0935	*hisZ*	ATP phosphoribosyltransferase regulatory subunit	-2.28
CAC0936	*hisG*	ATP phosphoribosyltransferase	-1.99
CAC0968		Hypothetical protein	-2.70
CAC1000		Uncharacterized protein, homolog of yhfF B.subtilis	-2.02
CAC1078		Predicted phosphohydrolase, Icc family	-3.93

Table 3.1.5 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC1102		Predicted membrane protein	-2.23
CAC1405	*bglA*	Beta-glucosidase	-2.42
CAC1427	*gabT*	4-aminobutyrate aminotransferase (PLP-dependent)	-3.02
CAC1472		Amino acid permease	-3.15
CAC1532		Protein containing ChW-repeats	-2.43
CAC1634		Flagellin	-2.08
CAC1868		Uncharacterized secreted protein, homolog YXKC Bacillus subtilis	-2.16
CAC1876		N-acetylmuramidase, autolytic enzyme, family 25 of glycosyl hydrolase	-4.01
CAC1877		Hypothetical protein, CF-8 family	-3.78
CAC1878		Hypothetical protein	-3.13
CAC1879		Uncharacterized, phage-related protein	-3.54
CAC1880		Hypothetical protein, CF-35 family	-3.62
CAC1881		Hypothetical protein	-3.46
CAC1882		Hypothetical protein	-3.89
CAC1884		Hypothetical protein	-4.47
CAC1885		Hypothetical protein	-4.04
CAC1886		Uncharacterized phage related protein	-4.72
CAC1887		Hypothetical protein	-4.97
CAC1888		Uncharacterized phage related protein	-4.47
CAC1889		Uncharacterized phage related protein	-4.54
CAC1890		Phage related protein, possible DNA packing	-4.42
CAC1892		Hypothetical protein	-4.41
CAC1893	*ClpP*	Serine protease, possible phage related	-4.34
CAC1894		Phage-related, head portal protein	-4.19
CAC1895		Phage terminase-like protein, large subunit	-3.97
CAC1896		Phage terminase-like protein, small subunit	-2.90
CAC1897		Phage-related, Zn finger domain containing protein	-2.95
CAC1898		Hypothetical protein	-2.61
CAC1899		Hypothetical protein	-2.40
CAC1901		Hypothetical protein	-2.03
CAC1902		Hypothetical protein	-2.73
CAC1903		Hypothetical protein	-3.22
CAC1904		Hypothetical protein	-2.19
CAC1905		Hypothetical protein	-3.19
CAC1906		Hypothetical protein	-2.92
CAC1907		Hypothetical protein	-3.26
CAC1909		Ribonuclease D	-2.83
CAC1910		Predicted membrane protein	-2.48
CAC1913		Zn-finger containing protein	-2.50
CAC1915		Hypothetical protein	-2.20
CAC1918		Hypothetical protein	-1.94
CAC1920		Zn-finger containing protein	-2.16

Table 3.1.5 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC1935		hypothetical protein	-2.05
CAC1938		Predicted HD superfamily hydrolase	-1.82
CAC1942		Hypothetical protein	-1.76
CAC1944		Hypothetical protein	-2.42
CAC1949		Possible TPR-repeat contaning protein	-2.31
CAC2010		Predicted Fe-S oxidoreductase	-1.82
CAC2011	*fabH*	Possible 3-oxoacyl-[acyl-carrier-protein] synthase III	-1.81
CAC2012	*fadB*	Enoyl-CoA hydratase	-1.99
CAC2013		Hypothetical protein	-1.82
CAC2014		Predicted esterase	-1.93
CAC2015		Hypothetical protein	-2.32
CAC2017		Acyl carrier protein	-2.24
CAC2018		Aldehyde ferredoxin oxidoreductase	-2.14
CAC2019		Malonyl CoA-acyl carrier protein transacylase	-2.36
CAC2021	*moeA*	Molybdopterin biosynthesis enzyme, MoeA (short form)	-2.68
CAC2022	*moaB*	Molybdopterin biosynthesis enzyme, moaB	-2.09
CAC2023		Membrane protein	-2.61
CAC2025		Hypothetical protein	-2.50
CAC2026		Predicted flavodoxin	-2.26
CAC2293		Hypothetical secreted protein	-2.12
CAC2404		Glycosyltransferase	-1.97
CAC2407		CheY-like receiver domain of response regulator	-2.68
CAC2517	*nrpE*	Extracellular neutral metalloprotease	-5.03
CAC2518		Extracellular neutral metalloprotease	-2.78
CAC2584		Protein containing ChW-repeats	-2.24
CAC2658	*glnA*	Glutamine synthetase type III	-3.69
CAC2695		Diverged Metallo-dependent hydrolase(Zn) of DD-Peptidase family	-2.56
CAC2703	*groEL*	Chaperonin	-2.08
CAC2716		Predicted glycosyl transferase from UDP-glucuronosyltransferase family	-2.46
CAC2717		Ethanolamine ammonia-lyase small subunit	-3.45
CAC2718		Ethanolamine ammonia lyase large subunit	-3.83
CAC2719		Ethanolamin permease	-4.12
CAC2720		Sensory protein containing histidine kinase	-3.65
CAC2721		Response regulator (CheY-like reciever domain)	-3.34
CAC2806		Predicted phosphohydrolase	-4.19
CAC2807		Endo-1,3(4)-beta-glucanase family 16	-4.26
CAC2944		N-terminal domain intergin-like repeats	-2.29
CAC2959	*galK*	Galactokinase	-2.87
CAC3067		Predicted membrane protein	-2.22
CAC3068		Glycosyltransferase	-2.51
CAC3069		Predicted glycosyltransferase	-2.59
CAC3070		Glycosyltransferase	-3.22

Table 3.1.5 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC3071		Glycosyltransferase	-3.38
CAC3072		Mannose-1-phosphate guanylyltransferase	-3.48
CAC3073		Sugar transferase involved in lipopolysaccharide synthesis	-3.80
CAC3085		TPR-repeat-containing protein, Cell-adhesion domain	-3.22
CAC3086		Protein containing cell adhesion domain	-2.78
CAC3159	*trpF*	Phosphoribosylanthranilate isomerase	-2.11
CAC3160	*trpC*	Indole-3-glycerol phosphate synthase	-2.67
CAC3161	*trpD*	Anthranilate phosphoribosyltransferase	-2.60
CAC3162	*pabA*	Para-aminobenzoate synthase component II	-3.09
CAC3163	*parB*	anthranilate synthase component I	-3.88
CAC3279		Possible surface protein, responsible for cell interaction	-3.49
CAC3280		Possible surface protein, responsible for cell interaction	-3.30
CAC3285		Predicted amino acid transporter	-4.36
CAC3298	*bdhB*	NADH-dependent butanol dehydrogenase B (BDH II)	-1.87
CAC3319		Signal transduction histidine kinase	-3.31
CAC3408		NADH oxidase (two distinct flavin oxidoreductase domains)	-4.24
CAC3409	*alsR*	Transcriptional regulators, LysR family	-3.60
CAC3421	*acyl*	Carrier protein phosphodiesterase	-1.91
CAC3422		Sugar proton symporter (possible xylulose)	-6.56
CAC3423		Acetyltransferase (ribosomal protein N-acetylase subfamily)	-3.88
CAC3486		Multimeric flavodoxin WrbA family protein	-3.23
CAC3633		Hypothetical protein	-3.29
CAC3635		Oligopeptide ABC transporter, ATPase component	-2.68
CAC3636		Oligopeptide ABC transporter, ATPase component	-3.28
CAC3637		Oligopeptide ABC transporter, permease component	-3.68
CAC3638		Oligopeptide ABC transporter, permease component	-3.48
CAC3639		CRO repressor-like DNA-binding protein	-2.93
CAC3646		Predicted transcriptional regulator	-1.62
CAC3660		Uncharacterized protein, homolog of Desulfovibrio gigas	-5.74
CAC3683		Penicillin-binding protein 2	-1.68

Genes are listed in order of ORFs and considered as significantly downregulated when the logarithmic ratio was $\leq$ -1.6. The given values in the table are mean of results of two hybridizations with dys swaps.

[a] Protein name based on the whole genome sequence by Nölling *et al.* (2001)

[b] The expression ratio as the logarithm to the basis of 2

The second group was related to synthesis and transport of amino acids. One cluster of genes (CAC0014-0018) was putatively annotated as part of the serine biosynthesis pathway that converts 3-phospho-D-glycerate to serine which was the precursor of cysteine. CAC0107 and CAC0109 were in a cluster (CAC0102-0110) which was involved in the conversion from sulfate to sulfite. These two clusters of genes are both

related to cysteine biosynthesis and their expressions were depressed. This result indicated that the efficiency of sulfur uptake was lower in the presence of 0.4 mM sulfate than in the presence of 0.55 mM sulfate. Another cluster of genes (CAC3157-3163) was putatively annotated as the tryptophan biosynthesis pathway which converts chorismate into tryptophan. Besides, CAC0727, CAC1472 were putatively annotated amino acid permeases. Group 3 was a cluster which contained six genes (CAC0078-0083) that where reported to form an *agr* quorum sensing system which regulated granulose formation and sporulation in *C. acetobutylicum* (Steiner *et al.*, 2012). The group 4 contained genes involving sugar transport and metabolism. This group included two phosphoenolpyruvate phosphotransferase systems (PTS) for transporting sugars into the cytoplasm, including a predicted cellobiose transporter (CAC0384-0386) and a glucose transporter (CAC0570) (Mitchell and Tangney, 2005; Servinsky *et al.*, 2010). Moreover, CAC2959, CAP0098 and CAC3067-3073 are involved in sugar metabolism. The group 5 was related to nitrogen fixation and assimilation (Janssen *et al.*, 2012). The well-known *nif* cluster (nitrogen fixation) genes (CAC0253-0261) are required for the activity of the molybdenum-nitrogenase. Another cluster (CAC2016-2024) consists of nine genes which are predicted to be involved in molybdopterin biosynthesis. The molybdopterins are a class of cofactors found in most molybdenum enzymes. Two genes (CAC0681, CAC0682 coding for *nrgBA*) that were putatively related to ammonia transport had similar expression patterns as the *nif* cluster. The expression of the glutamate synthase gene (CAC2658, *glnA*) which plays an important role in the nitrogen assimilation significantly decreased in the presence of 0.4 mM sulfate. Furthermore, the expression of a large gene cluster CAC1876-2026 was strongly downregulated in the presence of 0.4 mM sulfate. These genes are involved in fatty acid synthesis and were reported to be strongly upregulated in the stationary phase in a batch culture and during solventogenic growth in a phosphate-limited continuous culture (Alsaker and Papoutsakis, 2005; Grimmler *et al.*, 2010). These results showed that this large gene cluster played a role in solvent synthesis. In our data, the expression of this cluster was significantly repressed in the presence of 0.4 mM

sulfate, compared to 0.55 mM sulfate. This suggested insufficient sulfur supply was not propitious for solvent synthesis and was consistent with our products measurement listed in Table 3.1.4.

79 genes were significantly upregulated in the presence of 0.4 mM sulfate, compared to the presence of 0.55 mM sulfate. These upregulated genes are listed in Table 3.1.6 in detail. Among these genes, the L-lactate dehydrogenase gene (*ldh*, CAC0267) was upregulated, its putative function is to catalyze the conversion of pyruvate to L-lactate in *C. acetobutylicum*. 6.4 mM L-lactate was determined in the presence of 0.4 mM sulfate at 264 h during solventogenic growth, which supported the observation of the upregulation of this gene. Genes with a function in the conversion of L-glutamate to L-arginine were strongly upregulated and consisted of three clusters CAC0973-0974, CAC2388-2391 and CAC3019-3021 (Janssen *et al.*, 2012). The pyruvate formate lyase (*pflB*, encoded by CAC0980) and its activating enzyme (*pflA*, encoded by CAC0981) were also strongly upregulated in the presence of 0.4 mM sulfate. The pyruvate formate lyase catalyzes the reversible conversion of pyruvate and coenzyme-A into formate and acetyl-CoA using radical chemistry and regulates anaerobic glucose metabolism in *E. coli* (Knappe and Sawers, 1990), but is part of purin biosynthesis in *C. acetobutylicum* (Hoeneke and Ehrenreich, unpublished). A cluster of genes (CAC1029-1031) was induced that contains genes annotated related to iron transport. Furthermore, another three clusters of genes (CAC2445-2446, CAC2571-2574 and CAC2585-2592) were also listed in the Table 3.1.6, and their function in the metabolism is unknown.

Table 3.1.6 The expression profile of genes induced in continuous culture with 0.4 mM sulfur source during solventogenic growth, compared to that in the presence of 0.55 mM sulfur source.

ORF	Gene	Protein[a]	Ratio[b]
CA_P0040		Xre family DNA-binding domain and TPR repeats containing protein	1.85
CA_P0078		Acetyl coenzyme A acetyltransferase	1.75
CA_P0090		ABC-type transporter, ATPase component	1.64

Table 3.1.6 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CA_P0091		Predicted regulator of stationary/sporulation gene expression	1.99
CA_P0107		HTH transcriptional regulator MerR family	2.09
CAC0228		Hypothetical protein	1.88
CAC0263	serB	Phosphoserine phosphatase related protein	2.03
CAC0267	ldh	L-lactate dehydrogenase	1.83
CAC0316	argF/I	Ornithine carbomoyltransferase	3.49
CAC0318		Membrane permease, predicted cation efflux pumps	2.21
CAC0319		ABC transporter ATP-binding protein	1.94
CAC0320		Predicted permease	1.97
CAC0371		Response regulator (CheY-like domain and HTH domain)	1.94
CAC0380		Periplasmic amino acid-binding protein	1.68
CAC0394	kdgA	Deoxyphosphogluconate aldolase (gene kdgA)	1.66
CAC0461		Mercuric resistance operon regulatory protein, MerR family	1.77
CAC0592	ribA	Riboflavin biosynthes protein	1.64
CAC0751		Permease	3.06
CAC0789	fhuB	permease	1.67
CAC0818		Diguanylate cyclase/phosphodiesterase domain containing protein	2.32
CAC0882		Predicted membrane protein, hemolysin III homolog	1.78
CAC0887	adeC	Adenine deaminase	1.75
CAC0973	argG	Argininosuccinate synthase	3.14
CAC0974	argH	Argininosuccinate lyase	2.34
CAC0980	pflB	Pyruvate-formate lyase	4.41
CAC0981	pflA	Pyruvate-formate-lyase-activating enzyme	4.47
CAC0983		Hypothetical protein	3.58
CAC0984		ABC transporter, ATP-binding protein	1.74
CAC0985		ABC transporter, permease component	2.03
CAC1031	feoB	FeoB-like GTPase, responsible for iron uptake	2.23
CAC1041	argS	Arginyl-tRNA synthetase	2.07
CAC1047		Ribonucleotide-diphosphate reductase alpha subunit	1.77
CAC1378	cbiT	Precorrin-6B methylase	1.69
CAC1465		Transcriptional regulator, MarR/EmrR family	2.00
CAC1584		Ribonuclease Z	2.10
CAC2388	argD	N-acetylornithine aminotransferase	3.12
CAC2389	argB	Acetylglutamate kinase	3.72
CAC2390	argC	N-acetyl-gamma-glutamyl-phosphate reductase	2.41
CAC2391	argJ	Ornithine acetyltransferase/N-acetylglutamate synthase protein	2.31
CAC2445		5-aminoimidazole-4-carboxamide ribonucleotide transformylase	3.82
CAC2446		Hypothetical protein	3.38
CAC2482		Carbonic anhydrase	1.87
CAC2495		Predicted transcriptional regulator	1.76
CAC2497		Hypothetical secreted protein	1.96
CAC2543	etfA	Electron-transferring flavoprotein large subunit	2.22

Table 3.1.6 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC2544	*etfB*	Electron-transferring flavoprotein small subunit	1.73
CAC2571		Predicted acetyltransferase	2.58
CAC2572		Possible aminoglycoside phosphotransferase (protein kinase related)	2.57
CAC2573		Predicted S-adenosylmethionine-dependent methyltransferase	2.18
CAC2585		6-pyruvoyl-tetrahydropterin synthase related domain	2.48
CAC2586		Predicted membrane protein	2.69
CAC2587		GGDEF domain containing protein	2.32
CAC2588		Glycosyltransferase	2.38
CAC2589		Glycosyltransferase	2.20
CAC2590		Hypothetical protein	1.96
CAC2591		Hypothetical protein, CF-41 family	2.56
CAC2592		6-pyruvoyl-tetrahydropterin synthase related domain	2.14
CAC2644	*carB*	Carbamoylphosphate synthase large subunit	2.44
CAC2645	*carA*	carbamoyl-phosphate synthase small subunit	2.50
CAC2841		Conserved membrane protein, probable transporter	2.36
CAC3019		Sensory transduction protein with GGDEF and EAL domains	2.13
CAC3021		Possible phosphoglycerate mutase	2.06
CAC3045		CPSB/CAPC ortholog, PHP family hydrolase	2.00
CAC3046		Transcriptional regulator, LytR family	1.99
CAC3048		Uncharacterized conserved membrane protein, possible transporter	2.00
CAC3049		Glycosyltransferase	2.02
CAC3050		AMSJ/WSAK related protein	1.96
CAC3051		Glycosyltransferase	1.70
CAC3056		Nucleoside-diphosphate-sugar pyrophosphorylase	1.82
CAC3281		ABC-type multidrug/protein/lipid transport system, ATPase component	1.71
CAC3282		ABC-type multidrug/protein/lipid transport system, ATPase component	1.70
CAC3387		Pectate lyase	1.77
CAC3551	*natA*	Na+ ABC transporter (ATP-binding protein), NATA	1.75
CAC3570	*accC*	Acetyl-CoA carboxylase	2.02
CAC3571	*fabZ*	(3R)-hydroxymyristoyl ACP dehydratase	1.69
CAC3618		ABC-type polar amino acid transport system, ATPase component	1.82
CAC3641		Oligopeptide ABC transporter, ATPase component	2.17
CAC3661		Glycosyltransferase	2.29
CAC3717	*rplI*	50S ribosomal protein L9	1.65

Genes are listed in order of ORFs and considered as significantly upregulated when the logarithmic ratio was ≥ 1.6.

The given values in the table are mean of results of two hybridizations with dys swaps.

[a] Protein name based on the whole genome sequence by Nölling *et al.* (2001)

[b] The expression ratio as the logarithm to the basis of 2

3.2 Cellular functions of the thioredoxin- and glutathione-dependent reduction pathways in *Clostridium acetobutylicum*

3.2.1 Generation and verification of the *gcs* and *trxB* mutants

To investigate the functions of glutathione- and thioredoxin-dependent reduction systems in *C. acetobutylicum*, two genes (CAC1539, γ-glutamylcysteine synthetase, *gcs* and CAC1548, thioredoxin reductase, *trxB*) that were assumed to play an important role in metabolism, were targeted for insertion inactivation using ClosTron mutagenesis as described above (section 2.3).
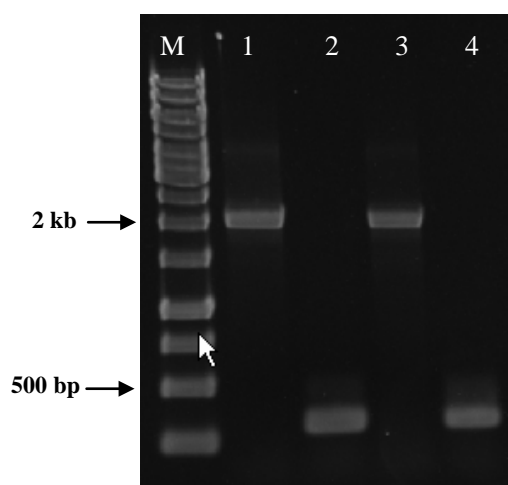
Fig. 3.2.1. PCR verification of *gcs* and *trxB* mutans
M, marker;
lane 1, CAC1539F and CAC1539R primers, wild type genome DNA;
lane 2, CAC1548F and CAC1548R primers, wild type genome DNA;
lane 3, CAC1539F and CAC1539R primers, *gcs* mutant genome DNA;
lane 4, CAC1548F and CAC1548R primers, *trxB* mutant genome DNA.

The primers for SOE PCR (section 2.3.2) were listed in Table 2.6. The length of *gcs* was 1446 bp and the target site was designed at 465/466 bp. The length of *trxB* was

876 bp and the target site was designed at 29/30 bp. The correct retargeted plasmids, pMTL007C-E2-*gcs* and pMTL007C-E2-*trxB* (in Table 2.5), were constructed and subsequently were transformed into *C. acetobutylicum*. In the end, putative mutants (the *gcs* and *trxB* mutants) were obtained by screening CGM plates. Erythromycin-resistant clones (putative mutants) were verified (section 2.3.5) by gene check primers (CAC1539F, CAC1539R and CAC1548F, CAC1548R) listed in Table 2.6. The results showed that wild type genomic DNA gave a band at ~ 300 bp, while the correct mutants showed products of ~ 2.1 kb, which were 1.8 kb longer than that of wild type (Fig. 3.2.1).
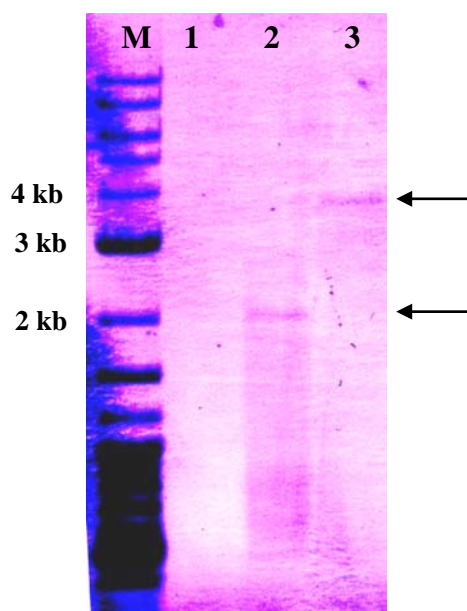
Fig. 3.2.2 Southern hybridization analysis of the *fer* and *mccB* mutants using and a specific intron probe
Lane 1, Marker; Lane 2, Genomic DNA of wild type (negative control); Lane 3, Genomic DNA of the *gcs* mutant; Lane, 4, Genomic DNA of the *trxB* mutant.
The arrows indicated the positions of hybridization bands.

To double check that the intron had inserted into the target genes, the two 2.1 kb PCR products from *gcs* and *trxB* genomic DNA as template were sequenced. Analysis of the sequences revealed that the intron inserted in *gcs* at 465/277 bp and *trxB* at 29/30 bp. These results showed that the *gcs* and *trxB* were inactivated by insertion into desired positions of ClosTron-derived group II intron and *gcs* and *trxB* ClosTron mutants were obtained. To further confirm that only one copy of the intron was present in the chromosomal DNA of the ClosTron mutants, Southern hybridization

was carried out with an intron-specific PCR-generated, biotin-labeled probe (section 2.3.6.2). The results are shown in Fig. 3.2.2. No hybridization signal was observed for wild type genomic DNA, and genomic DNA of the *gcs* and *trxB* mutants both exhibited a single band, demonstrating that the *gcs* and *trxB* mutants possessed only one copy of the intron on the chromosome.

3.2.2 The phenotypes of the wild type, the *gcs* and *trxB* mutants in batch and phosphate-limited continuous fermentations

The wild type, *gcs* and *trxB* mutants were cultured in minimal medium with 50 g/L glucose and showed similar growth phenotypes as shown in Fig. 3.2.3. The *gcs* and *trxB* mutants achieved the highest optical density at 36 h, which was later than the wild type. Moreover, the highest optical density achieved by the *gcs* mutant was slightly lower than that of the wild type in the batch fermentations.
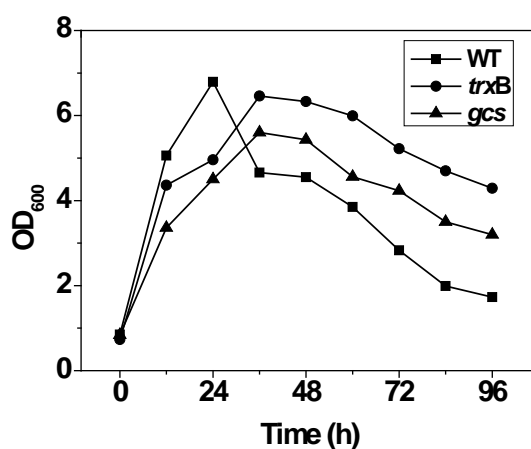
Fig. 3.2.3 **Wild type, the *fer* and *trxB* mutants cultured in minimal medium in the batch fermentation.** (■), Wild type; (▲), The *gcs* mutant; (●), The *trxB* mutant

To further evaluate the effects of *gcs* and *trxB* inactivation on growth and metabolism, three strains were cultivated in phosphate-limited continuous culture. Under the same fermentation conditions, all three strains achieved steady-state growth during acidogenic and solventogenic growth in the continuous fermentation. The optical

density and glucose concentration were followed in the continuous cultures and are shown in Fig. 3.2.4 (wild type), Fig. 3.2.5 (the *gcs* mutant) and Fig. 3.2.6 (the *trxB* mutant) respectively. The products of three strains were measured and listed in Table 3.2.1 during acidogenic and solventogenic growth.
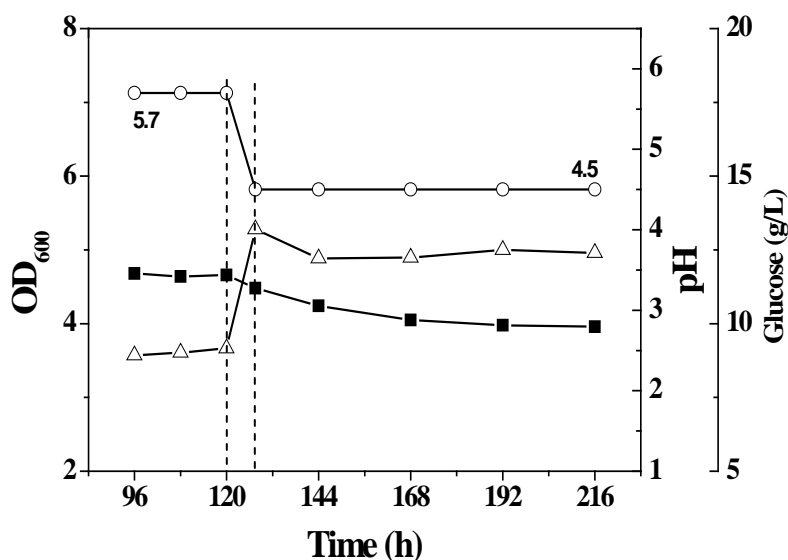
Fig. 3.2.4 The fermentation profile of a phosphate-limited continuous culture of *C. acetobutylicum* wild type

The dashed lines indicated the dynamic pH shift from 5.7 to 4.5. ($\triangle$), Residual glucose; ($\blacksquare$), OD_{600}; ($\circ$), pH.
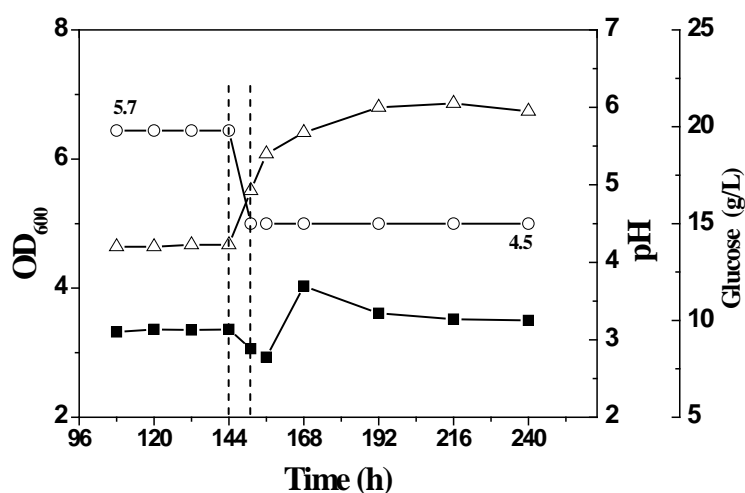
Fig. 3.2.5 The fermentation profile of a phosphate-limited continuous culture of the *C. acetobutylicum gcs* mutant

The dashed lines indicated the dynamic pH shift from 5.7 to 4.5. ($\triangle$), Residual glucose; ($\blacksquare$), OD_{600}; ($\circ$), pH.
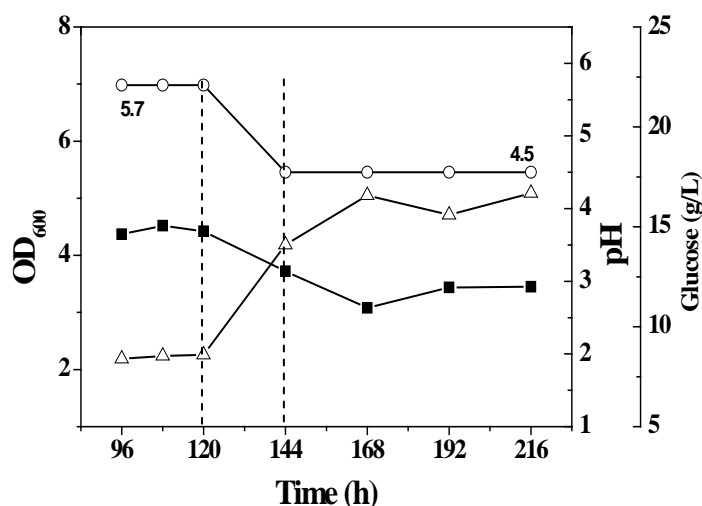
Fig. 3.2.6 The fermentation profile of a phosphate-limited continuous culture of the *C. acetobutylicum trxB* mutant

The dashed lines indicated the dynamic pH shift from 5.7 to 4.5. ($\triangle$), Residual glucose; ($\blacksquare$), OD_{600}; ($\bigcirc$), pH.

During acidogenic and solventogenic growth of the continuous culture of the wild type, the OD_{600} was approximately 4.6 and the concentration of residual glucose was approximately 9.6 g/L at 120 h (acidogenesis). 7.5 h after the pH shift, the wild type entered solventogenic growth, and the final steady-state OD_{600} was approximately 4 at 216 h (solventogenesis). Moreover, 12.4 g/L glucose was measured at 216 h in the continuous culture (Fig. 3.2.3). For the *gcs* mutant, the OD_{600} was approximately 3.4 and the concentration of residual glucose was 14 g/L at 144 h during acidogenic growth. 7 h after the pH shift, the steady-state OD_{600} was 3.5 and the concentration of residual glucose was 24 g/L at 240 h during solventogenic growth. Furthermore, the *gcs* mutant produced a lower concentration of acetate and butyrate during acidogenic growth and a lower concentration of acetone and butanol during solventogenic growth as compared to the wild type in the phosphate-limited continuous fermentation (Table 3.2.1). The results indicated that the glutathione-dependent reduction system plays an important role in the metabolism of *C. acetobutylicum* during acidogenic and solventogenic growth. Once this system was inactivated by mutation of the *gcs* gene, growth and glucose consumption were significantly affected.

Table 3.2.1 Product concentrations in the phosphate-limited continuous culture of *C. acetobutylicum* and mutants

Product	The wild type		The *gcs* mutant		The *trxB* mutant	
	120 h (mM)	216 h (mM)	144 h (mM)	240 h (mM)	120 h (mM)	216 h (mM)
Acetate	23	18	11.2	1.1	38.9	4.2
Butyrate	73	23	26.1	10.7	84.7	11.6
Acetone	0.2	20	0.1	5.6	1.9	25.5
Ethanol	5.3	7.3	1.6	2	4.2	5.1
Butanol	6	45	0.9	10.9	5.1	36.1

For the *trxB* mutant, the OD_{600} was 4.4 in the steady-state during acidogenic growth which was comparable to that of the wild type. The concentration of residual glucose in the culture at 120 h was 8.6 g/L which was slightly lower than that of the wild type. Moreover, the concentrations of acetate and butyrate were lower than with the wild type (Table 3.2.1). However, the OD_{600} was 3.5 in the steady-state during solventogenic growth, which was lower to that (OD_{600} ~ 4) of the wild type. Interestingly, the concentration of acetone was higher and the concentration of butanol was lower than that of the wild type during solventogenic growth (Table 3.2.1) at 216 h. The typical ratio of acetone to butanol of wild type was approximate 1:2 during solventogenic growth, as shown in Table 3.2.1. Thus these results indicated that the mutation of the *trxB* gene affected growth and solvents production of *C. acetobutylicum* during solventogenic growth. The ratio of acetone to butanol was higher than that of the wild type during solventogenic growth.

3.2.3 Comparison of gene expression profiles of the wild type and the *gcs* mutant during acidogenic and solventogenic growth

As a consequence of the inactivation of the GSH-dependent reduction system, the *gcs* mutant showed weaker growth as compared to the wild type and produced less

solvents and acids in a phosphate-limited continuous culture. In order to investigate which genes and metabolic pathways were affected in the *gcs* mutant, DNA microarray experiments were performed to compare the transcriptomes of *C. acetobutylicum* and the *gcs* mutant in a phosphate-limited continuous culture during acidogenic and solventogenic growth. Microarray data are available at the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2747.

During acidogenic growth, the samples were taken after 120 h of continous growth for the wild type and 144 h for the *gcs* mutant (Fig. 3.2.4 and Fig. 3.2.5). Transcriptional analysis identified 121 genes that were significantly induced and 36 genes that were significantly repressed during acidogenic growth according to the filter criteria as described above (section 2.6.4). All the induced and repressed genes are listed in Table 3.2.2 and in Table 3.2.3 respectively. The induced genes mainly included three groups based on the gene functions. Group 1 included sugar transporter genes (CAC0383-CAC0386, CAC0570) and a gene cluster putatively related to xylan degradation (CAP0116-CAP0120). The transporter (CAC0570) is a glucose transporter in *C. acetobutylicum* and the gene cluster (CAC0383-CAC0386) is reported to code for a cellobiose transporter (Mitchell and Tangney, 2005). Group 2 contained seven genes all of which are chemotaxis protein genes (CAC0118, CAC0304-CAC0305, CAC1600-CAC1601, CAC1233 and CAC2220). The genes in Group 3 are all related to flagellar biosynthesis (CAC1634, CAC2154-2162, CAC2203-CAC2214). Furthermore, genes involved in the cysteine and tryptophan biosynthesis (CAC0102-CAC0105 and CAC3158-CAC3163) were stongly expressed during acidogenic growth.

Table 3.2.2 Significantly upregulated genes in the *gcs* mutant compared to the wild type during acidogenic growth

ORF	Gene	Protein[a]	Ratio[b]
CA_P0116		Xylanase, glycosyl hydrolase family 10	1.72

Table 3.2.2 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CA_P0117		Possible beta-xylosidase diverged, family 5/39 of glycosyl hydrolases	2.80
CA_P0118		Possible xylan degradation enzyme	2.13
CA_P0120		Possible xylan degradation enzyme	3.44
CAC0102		O-acetylhomoserine sulfhydrylase	3.11
CAC0103	*cysC*	Adenylylsulfate kinase	3.90
CAC0104		Adenylylsulfate reductase	2.61
CAC0105		Ferredoxin	1.82
CAC0118	*cheA*	Chemotaxis protein cheA	2.67
CAC0149		Hypothetical protein	2.28
CAC0194		Glycosyltransferase involved in cell wall biogenesis	1.66
CAC0224		Response regulator (CheY-like receiver domain)	1.67
CAC0304	*motA*	Chemotaxis motility protein A, gene motA	2.40
CAC0305	*motB*	Chemotaxis motility protein B, gene motB	2.02
CAC0384	*licB*	PTS system, cellobiose-specific component BII	2.16
CAC0386	*licC*	PTS cellobiose-specific component IIC	2.06
CAC0409		Hypothetical protein	1.61
CAC0410		Uncharacterized small conserved protein, homolog of YUKE/YFJA	1.73
CAC0413		Uncharacterized small conserved protein, homolog of YUKE/YFJA	1.67
CAC0474		ACT domain containing transcriptional regulators	1.97
CAC0500		Membrane protein containing C-terminal PDZ domain	1.60
CAC0539	*manB*	Beta-mannanase ManB, contains ChW-repeats	3.09
CAC0540		Beta-mannanase ManB-like enzyme, contains ChW-repeats	1.78
CAC0562		Predicted membrane protein	1.99
CAC0563		Predicted membrane protein	2.35
CAC0570		PTS enzyme II, ABC component	1.64
CAC0626	*trpS*	Tryptophanyl-tRNA synthetase	1.73
CAC0706		Endo-1,4-beta glucanase (fused to two ricin-B-like domains)	2.96
CAC0946		ComE-like protein, Metallo beta-lactamase superfamily hydrolase	1.73
CAC0984		ABC transporter, ATP-binding protein	2.27
CAC0985		ABC transporter, permease component	1.63
CAC0998		Homoserine dehydrogenase	1.64
CAC1233	*cheV*	Chemotaxis protein CheV ortholog	2.32
CAC1243	*mreC*	Shape-determining protein	1.76
CAC1320	*glpP*	Glycerol-3-phosphate responsive antiterminator (mRNA-binding)	2.39
CAC1357		Uncharacterized predicted metal-binding protein	3.60
CAC1411		Similar to toxic anion resistance protein terA	2.01
CAC1412		Methyl methane sulfonate/mytomycin C/UV resistance protein	2.30
CAC1413		Similar to C-terminal fragment of toxic anion resistance protein	2.40
CAC1414	*TerE*	Ortholog of stress responce protein SCP2 (YCEC) *B.subtilis*	2.11
CAC1415	*TerC*	Ortholog of stress responce protein	1.97
CAC1429	*galE*	UDP-glucose 4-epimerase	2.29
CAC1540		Uncharacterized ATP-grasp enzyme	2.01

Table 3.2.2 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC1600		Methyl-accepting chemotaxis-like protein	2.53
CAC1601		Methyl-accepting chemotaxis-like protein	2.26
CAC1634		Flagellin	2.33
CAC1721	*rpiA*	Primosome assembly protein	1.68
CAC1776		HAD superfamily hydrolase	1.89
CAC1777		Nudix (MutT-like) hydrolase	1.63
CAC1825	*metB*	Homoserine O-succinyltransferase	1.84
CAC2043		Hypothetical protein	2.01
CAC2154	*flgE*	FlgE Flagellar hook protein	1.96
CAC2155		Hypothetical protein	2.53
CAC2156	*flgD*	Flagellar hook assembly protein	2.80
CAC2157	*fliK*	Flagellar hook-length control protein	3.39
CAC2162	*fliF*	Flagellar basal body M-ring protein	1.91
CAC2166		Nucleoside-diphosphate-sugar epimerase	1.60
CAC2174		Glycosyltransferase	1.79
CAC2203		Possible hook-associated protein, flagellin family	3.33
CAC2204		Hypothetical protein	1.71
CAC2205	*fliD*	Flagellar hook-associated protein	2.19
CAC2206	*fliS*	Flagellar protein	1.60
CAC2207		Hypothetical protein	2.56
CAC2208		Flagellin family protein, YVYC *B.subtilis* ortholog	1.94
CAC2209	*csrA*	Carbon storage regulator	1.76
CAC2210		Uncharacterized protein, YVIF *B.subtilis* homolog	2.57
CAC2211	*flgL*	Flagellin	2.95
CAC2212	*flgK*	Flagellar hook-associated protein	1.71
CAC2213		Hypothetical protein	1.88
CAC2214	*flgm*	Regulator of flagellin synthesis	1.60
CAC2220	*cheA*	Chemotaxis histidine kinase, CheA (contains CheW-like adaptor domain)	1.76
CAC2241		Cation transport P-type ATPase	2.59
CAC2242		Predicted transcriptional regulator, arsE family	1.87
CAC2252		Alpha-glucosidase fused to unknown alpha-amylase C-terminal. domain	2.56
CAC2337		Phosphomannomutase	1.71
CAC2382		Single-strand DNA-binding protein	2.31
CAC2433		HtrA-like serine protease (with PDZ domain)	1.77
CAC2435		Response regulator (CheY-like domain)	1.69
CAC2457		Hypothetical protein	4.57
CAC2533		Protein containing ChW-repeats	3.04
CAC2627	*trmB*	tRNA (guanine-N(7)-)-methyltransferase	1.96
CAC2628		Predicted metal-dependent peptidase	1.66
CAC2630		Uncharaterized conserved protein, YOME *B.subtilis* ortholog	2.06
CAC2631		Uncharacterized protein, ErfK family	1.61
CAC2743		Predicted permease, YXIO *B.subtilis* ortholog	1.91

Table 3.2.2 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC2758		Uncharacterized protein, YPUA *B.subtilis* ortholog	2.09
CAC2802		Predicted phosphoglycerol transferase	1.72
CAC2849		Proline/glycine betaine ABC-type transport system	2.24
CAC2850		Proline/glycine betaine ABC-type transport system, ATPase component	1.77
CAC2901		Predicted membrane protein	1.64
CAC3006		Zn-dependent peptidase, insulinase family	1.86
CAC3040		CPSC/CAPB subfamily ATPase	1.89
CAC3045		CPSB/CAPC ortholog, PHP family hydrolase	2.11
CAC3049		Glycosyltransferase	2.01
CAC3051		Glycosyltransferase	1.83
CAC3052		Glycosyltransferase	2.49
CAC3054		Phosphoheptose isomerase	3.01
CAC3059		Sugar transferase	4.00
CAC3154		RRNA methylase, YACO *B.subtilis* ortholog	2.08
CAC3155		Uncharacterized conserved protein, THY1 family	1.71
CAC3158	*trpB*	Tryptophan synthase subunit beta	1.63
CAC3159	*trpF*	Phosphoribosylanthranilate isomerase	1.64
CAC3161	*trpD*	Anthranilate phosphoribosyltransferase	2.20
CAC3162	*pabA*	Para-aminobenzoate synthase component II	1.83
CAC3163	*parB*	Anthranilate synthase component I	1.62
CAC3295		Probable cation efflux pump (multidrug resistance protein)	1.82
CAC3339		ATPase component of ABC transporter (two ATPase domains)	2.36
CAC3340		Uncharacterized conserved domain seen in the bacterial SpoT	2.26
CAC3341		Multimeric flavodoxin WrbA family protein	1.63
CAC3352		Membrane associated methyl-accepting chemotaxis protein	1.82
CAC3417		Flavodoxin	1.76
CAC3418		Transcriptional regulator, MarR/EmrR family	2.63
CAC3458		Uncharacterized protein, homolog of B. anthracis (gi 48942631)	2.80
CAC3461		Hypothetical protein	3.69
CAC3556		Probable S-layer protein	3.10
CAC3583		Predicted permease	2.56
CAC3584		Predicted permease	2.31
CAC3585		ABC-type transporter, ATPase component	3.13
CAC3617		Uncharacterized membrane protein, YhaG *B.subtilis* homolog	1.74
CAC3642		Oligopeptide ABC transporter, ATPase component	1.85
CAC3674		Two CBS domain containing protein	1.80

Genes are listed in order of ORFs and considered as significantly upregulated when the logarithmic ratio was ≥ 1.6.

The given values in the table are mean of results of two hybridizations with dys swaps.

[a] Protein name based on the whole genome sequence by Nölling *et al.* (2001)

[b] The expression ratio as the logarithm to the basis of 2

The repressed genes are mainly located in a large gene cluster (CAC1988-2022) (Table 3.2.3). The function of this cluster is reported to be involved in fatty acid synthesis and its expression was significantly induced in the solventogenic phase (Alsaker and Papoutsakis, 2005; Grimmler *et al.*, 2010). Except for the large cluster, a cysteine protease (CAP0004) gene and a carbon starvation protein (CAC1669, CstA) gene were also strongly repressed, although their functions are obscure until now.

Table 3.2.3 Significantly downregulated genes in the *gcs* mutant compared to the wild type during acidogenic growth

ORF	Gene	Protein[a]	Ratio[b]
CA_P0004		Cysteine protease	-2.37
CAC0204		Sortase (surface protein transpeptidase), YHCS *B.subtilis* ortholog	-1.70
CAC0542		Methyl-accepting chemotaxis protein	-1.81
CAC0666		Sugar permease	-1.89
CAC0790	*fhuD*	Ferrichrome-binding periplasmic proteinl	-1.70
CAC1078		Predicted phosphohydrolase, Icc family	-2.41
CAC1316		Predicted membrane protein	-1.83
CAC1527		Spore germination protein	-1.82
CAC1590		2-oxoglutarate/malate translocator	-1.87
CAC1669	*cstA*	Carbon starvation protein	-2.42
CAC1868		Uncharacterized secreted protein	-1.84
CAC1989		ABC-type iron (III) transport system, ATPase component	-1.77
CAC1990		ABC-type iron (III) transport system, permease component	-1.67
CAC1991		Uncharacterized protein, YIIM family	-1.92
CAC1994	*moaB*	Molybdopterin biosynthesis enzyme	-2.02
CAC2000	*iorB*	Indolepyruvate ferredoxin oxidoreductase	-1.74
CAC2004		Siderophore/Surfactin synthetase related protein	-1.69
CAC2006		Enzyme of siderophore/surfactin biosynthesis	-2.30
CAC2009	*mmgB*	3-Hydroxyacyl-CoA dehydrogenase	-1.69
CAC2011	*fabH*	Possible 3-oxoacyl-[acyl-carrier-protein] synthase III	-2.16
CAC2012	*fadB*	Enoyl-CoA hydratase	-1.62
CAC2013		Hypothetical protein	-1.73
CAC2016	*fadB*	Enoyl-CoA hydratase	-2.01
CAC2018		Aldehyde ferredoxin oxidoreductase	-1.87
CAC2019		Malonyl CoA-acyl carrier protein transacylase	-1.67
CAC2107		Contains cell adhesion domain	-2.22
CAC2366		Predicted membrane protein	-1.61
CAC2517		nrpE Extracellular neutral metalloprotease, NPRE	-1.76
CAC2695		Diverged Metallo-dependent hydrolase(Zn) of DD-Peptidase family	-2.87

Table 3.2.3 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC2772		Permease	-3.24
CAC2819	*murE*	UDP-N-acetylmuramyl tripeptide synthase, MURE	-1.66
CAC3280		Possible surface protein, responsible for cell interaction, contains cell adhesion domain and ChW-repeats	-2.12
CAC3320		Predicted secreted protein homolog of yjcM/yhbB *B.subtilis*	-1.74
CAC3422		Sugar proton symporter (possible xylulose)	-2.01
CAC3612		Hypothetical protein	-2.68
CAC3697		Uncharacterized, Zn-finger domain containing protein	-1.81

Genes are listed in order of ORFs and considered as significantly downregulated when the logarithmic ratio was $\leq$ -1.6. The given values in the table are mean of results of two hybridizations with dys swaps.

[a] Protein name based on the whole genome sequence by Nölling *et al.* (2001)

[b] The expression ratio as the logarithm to the basis of 2

During solventogenic growth, the samples were taken after 216 h of continuous growth for the wild type and 240 h for the *gcs* mutant (Fig. 3.2.4 and Fig. 3.2.5). Transcriptional analysis identified 28 genes as being significantly induced and 13 genes as being significantly repressed as compared to the wild type. All the induced and repressed genes are listed in Table 3.2.4 and in Table 3.2.5.

Table 3.2.4 Significantly upregulated genes in the *gcs* mutant compared to the wild type during solventogenic growth

ORF	Gene	Protein[a]	Ratio[b]
CAC0273		2-isopropylmalate synthase	1.76
CAC0274	*ansB*	Aspartate ammonia-lyase (aspartase) gene	1.70
CAC0384	*licB*	PTS system, cellobiose-specific component BII	2.63
CAC0385		Beta-glucosidase	2.19
CAC0386	*licC*	PTS cellobiose-specific component IIC	2.36
CAC0387		Hypothetical protein	2.42
CAC0570		PTS enzyme II, ABC component	1.74
CAC0910		Probably cellulosomal scaffolding protein precursor	2.53
CAC0911		Possible processive endoglucanase family 48	2.65
CAC0912		Possible non-processive endoglucanase family 5	2.07
CAC0913		Possible non-processive endoglucanase family 9	2.06
CAC0915		Endoglucanase A precursor (endo-1,4-beta-glucanase)	2.28
CAC0918		Possible non-processive endoglucanase family 5, ortholog of mannase A	2.40
CAC0919		Probably secreted sialidase, several ASP-boxes and dockerin domain	2.20

Table 3.2.4 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC1390	*purE*	Phosphoribosylaminoimidazole carboxylase catalytic subunit	2.14
CAC1391	*purC*	Phosphoribosylaminoimidazole-succinocarboxamide synthase	2.00
CAC1394	*PurN*	Phosphoribosylglycinamide formyltransferase	2.45
CAC1393	*purM*	Phosphoribosylaminoimidazole synthetase	2.13
CAC1394	*PurN*	Phosphoribosylglycinamide formyltransferase	2.13
CAC1395	*purH*	Bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	1.98
CAC1655	*purQ*	Bifunctional enzyme phosphoribosylformylglycinamidine	2.36
CAC2203		Possible hook-associated protein, flagellin family	1.70
CAC2207		Hypothetical protein	1.66
CAC2211	*flgL*	Flagellin	1.80
CAC1821	*purB*	Adenylosuccinate lyase	1.62
CAC2658	*glnA*	Glutamine synthetase type III	2.02
CAC2891		Fusion of alpha-glucosidase (family 31 glycosyl hydrolase)	1.67
CAC2959	*galK*	Galactokinase	1.67

Genes are listed in order of ORFs and considered as significantly upregulated when the logarithmic ratio was ≥ 1.6. The given values in the table are mean of results of two hybridizations with dys swaps.

[a] Protein name based on the whole genome sequence by Nölling *et al.* (2001)

[b] The expression ratio as the logarithm to the basis of 2

Like the gene expression profile during acidogenic growth, the genes related to sugar transporters and genes related to flagella biosynthesis were significantly induced during solventogenic growth in *gcs* mutant. These genes included CAC0570 (coding for a glucose transporter), CAC0383-CAC0386 (coding for a cellobiose transporter) and CAC2959 (coding for a galactokinase which is involved in galactose motabolism). Two genes, CAC2203 and CAC2211, are involved in flagella biosynthesis. Moreover, a gene cluster coding for a putative cellulosome (CAC0910-0919) was induced during solventogenic growth of the *gcs* mutant in the continuous culture. Despite that *C. acetobutylicum* was not able to grow with cellulose as the sole carbon source, a cluster coding for cellulosomal scaffolding protein was significantly expressed during solventogenic growth (Lopez-Contreras *et al.*, 2003). According to the KEGG pathway analysis, a cluster (CAC1390-1395) which was induced during solventogenic growth in the *gcs* mutant is associated with conversion of phosphorbosyl pyrophosphate (PPRP) to inosinic monophosphate (IMP). PPRP and IMP are

important intermediates in purine metabolism.

Table 3.2.5 Significantly downregulated genes in the *gcs* mutant compared to the wild type during solventogenic growth

ORF	Gene	Protein[a]	Ratio[b]
CA_P0173		Archaeal-type Fe-S oxidoreductase	-1.69
CAC0014		Aminotransferase	-3.06
CAC0015	*serA*	D-3-phosphoglycerate dehydrogenase	-3.19
CAC0016		Related to HTH domain of SpoOJ/ParA/ParB/repB family	-2.81
CAC0017	*serS*	Seryl-tRNA synthetase	-3.28
CAC0316	*argF/I*	Ornithine carbomoyltransferase	-2.39
CAC0380		Periplasmic amino acid-binding protein	-2.31
CAC0973	*argG*	Argininosuccinate synthase	-2.16
CAC0974	*argH*	Argininosuccinate lyase	-1.95
CAC2388	*argD*	N-acetylornithine aminotransferase	-1.94
CAC2389	*argB*	Acetylglutamate kinase	-1.79
CAC2390	*argC*	N-acetyl-gamma-glutamyl-phosphate reductase	-1.87
CAC2391	*argJ*	Bifunctional ornithine acetyltransferase/N-acetylglutamate synthase	-1.81

Genes are listed in order of ORFs and considered as significantly downregulated when the logarithmic ratio was $\leq$ -1.6. The given values in the table are mean of results of two hybridizations with dys swaps.

[a] Protein name based on the whole genome sequence by Nölling *et al.* (2001)

[b] The expression ratio as the logarithm to the basis of 2

Among the genes that were stongly repressed during solventogenic growth as compared to the wild type, a cluster of genes (CAC0014-0018) is putatively annotated as part of the serine biosynthesis pathway that converted 3-phospho-D-glycerate to serine. The expression of genes involved in the arginine biosynthesis (CAC0973-CAC0974 and CAC2388-CAC2391) was also strongly repressed during solventogenic growth. Moreover, a Fe-S oxidoreductase (CAP0173) showed the same the same expression pattern.

3.2.4 Comparison of gene expression profiles of *C. acetobutylicum* and the *trxB* mutant during acidogenic and solventogenic growth in continuous fermentation

To investigate which genes and metabolic pathways were affected in the *trxB* mutant, DNA microarray experiments were performed to compare the transcriptomes of the wild type and the *trxB* mutant in phosphate-limited continuous culture during acidogenic and solventogenic growth. Microarray data are available at the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2747.

During acidogenic growth, the samples were taken after 120 h of continuous growth for the wild type and the *trxB* mutant (Fig. 3.2.4 and Fig. 3.2.6). Transcriptional analysis identified 169 genes that were significantly induced and 107 genes that were significantly repressed during acidogenic growth according to the filter criteria as described above (section 2.6.4). All the induced and repressed genes are listed in Table 3.2.6 and in Table 3.2.7 respectively.

Table 3.2.6 Significantly upregulated genes in the *trxB* mutant compared to the wild type during acidogenic growth

ORF	Gene	Protein[a]	Ratio[b]
CA_P0004		Cysteine protease	2.40
CA_P0025	*pdc*	Pyruvate decarboxylase	2.40
CA_P0026		Hypothetical protein	2.11
CA_P0054		Xylanase/chitin deacetylase family enzyme	1.69
CA_P0057		Putative glycoportein or S-layer protein	5.55
CA_P0058		Rare lipoprotein A RLPA releated protein	7.45
CA_P0072		Hypothetical protein	1.96
CA_P0073		ABC ATPase containing transporter	2.83
CA_P0074		Hypothetical protein	2.70
CA_P0093	*tnpA*	Transposase (3' segment)	1.74
CA_P0096	*ipyR*	Inorganic pyrophosphatase	2.52
CA_P0097	*lipG*	Carboxyl esterase, a/b hydrolase	2.20
CA_P0112		Hypothetical protein	4.66
CA_P0129		Glycogen-binding regulatory subunit of S/T protein phosphatase I	5.41
CA_P0133		Antibiotic-resistance protein, alpha/beta superfamily hydrolase	2.86
CA_P0134		Hypothetical protein	3.25
CA_P0135		Oxidoreductase	3.15
CA_P0136		AstB/chuR/nirj-related protein	3.30
CA_P0137		Similar to C-ter. fragment of UDP-glucuronosyltransferases,	3.45

Table 3.2.6 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CA_P0138		hypothetical protein	3.02
CA_P0148	*phlC*	Phospholipase C	4.12
CA_P0162	*adhE1*	Aldehyde dehydrogenase (NAD+)	3.27
CA_P0163	*ctfA*	Butyrate-acetoacetate COA-transferase subunit A	3.70
CA_P0164	*ctfB*	Butyrate-acetoacetate COA-transferase subunit B	3.02
CA_P0165	*adc*	Acetoacetate decarboxylase	3.14
CA_P0168	*amyA*	Alpha-amylase	2.14
CA_P0173		Archaeal-type Fe-S oxidoreductase	1.79
CAC0011		Uncharacterized conserved of ErfK family	1.72
CAC0030		Hypothetical protein	1.82
CAC0175		Hypothetical protein	3.68
CAC0176	*appA*	Oligopeptide-binding protein, periplasmic component	3.25
CAC0177	*appB*	Oligopeptide transport permease protein	2.32
CAC0178	*appC*	Oligopeptide transport permease protein	1.80
CAC0180	*appF*	Oligopeptide ABC transporter, ATP-binding protein	1.71
CAC0183		Transcriptional regulators of NagC/XylR (ROK) family, sugar kinase	2.81
CAC0184		Predicted acetyltransferase	2.40
CAC0185		Uncharacterized membrane protein, YbbC *B.subtilis* ortholog	1.84
CAC0253	*nifH*	Nitrogenase iron protein (nitrogenase component II) gene nifH	2.53
CAC0255	*nifHD*	Nitrogen regulatory protein PII (nitrogen fixation nifHD)	2.84
CAC0256	*nifD*	Nitrogenase molybdenum-iron protein, alpha chain	2.93
CAC0258	*nifE*	Nitrogenase iron-molibdenum cofactor biosinthesis protein	1.92
CAC0259		Fusion nifN/K+nifB	2.38
CAC0488		Hypothetical protein	2.89
CAC0489	*acpS*	4'-phosphopantetheinyl transferase	2.19
CAC0490		Predicted sugar kinase	2.64
CAC0491		Uncharacterized probably secreted protein, homolog of YdcC *B.subtilis*	2.29
CAC0492	*alr*	Alanine racemase	2.02
CAC0546		Uncharacterized membrane protein, homolog of Methanobacterium	1.88
CAC0570		PTS enzyme II, ABC component	3.57
CAC0579	*prkA*	Serine protein kinase (prkA protein), P-loop containing	4.06
CAC0580		Hypothetical protein	5.95
CAC0581	*spoVR*	Stage V sporulation protein R	3.27
CAC0601		Unchracterized membrane protein, possible permease	1.83
CAC0660		Hypothetical protein, CF-26 family	6.44
CAC0666		Sugar permease	6.32
CAC0787		Uncharacterized conserved protein, repeats	2.37
CAC0814		3-oxoacyl-[acyl-carrier-protein] synthase III	3.20
CAC0815		Methyl-accepting chemotaxis protein	1.91
CAC0816		Lipase-esterase related protein	2.94
CAC0842		Hypothetical protein, CF-28 family	2.32
CAC1010		Predicted phosphohydrolase	3.14

Table 3.2.6 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC1073		Hypothetical protein	6.01
CAC1079		Uncharacterized protein, related to enterotoxins of other Clostridiales	1.69
CAC1246	*pbpA*	Penicillin-binding protein 2	2.31
CAC1694		Sigma factor E processing enzyme	2.82
CAC1695	*sigE*	Sporulation sigma factor	4.23
CAC1696	*sigG*	Sporulation sigma factor	4.34
CAC1775		Predicted membrane protein	5.75
CAC1841		Uncharacterized protein, related to Spore coat protein CotS	2.00
CAC1886		Uncharacterized phage related protein	2.56
CAC1889		Uncharacterized phage related protein	2.86
CAC1892		Hypothetical protein	2.92
CAC1893		ClpP family serine protease, possible phage related	2.89
CAC1894		Phage-related, head portal protein	2.68
CAC1909	*rnd*	Ribonuclease D	2.58
CAC1912		Uncharacterized phage related protein	2.03
CAC1942		Hypothetical protein	2.19
CAC1985		Hypothetical protein	2.01
CAC1986		Hypothetical protein	1.70
CAC1988		Ferrichrome-binding periplasmic protein	3.36
CAC1989		ABC-type iron (III) transport system, ATPase component	3.20
CAC1990		ABC-type iron (III) transport system, permease component	3.01
CAC1991		Uncharacterized protein, YIIM family	3.03
CAC1993	*moaA*	Molybdenum cofactor biosynthesis enzyme MoaA, Fe-S oxidoreductase	3.05
CAC1994	*moaB*	Molybdopterin biosynthesis enzyme, MoaB	2.51
CAC1995		Hypothetical protein	3.04
CAC1996		Hypothetical protein	2.99
CAC1997		Predicted glycosyltransferase	3.18
CAC1998		ABC-type transport system, ATPase component	2.99
CAC1999		Hypothetical protein	3.19
CAC2000	*iorB*	Indolepyruvate ferredoxin oxidoreductase	3.58
CAC2001	*iorA*	Indolepyruvate ferredoxin oxidoreductase, subunit alpha	3.20
CAC2003		Predicted permease	2.83
CAC2004		Siderophore/Surfactin synthetase related protein	2.44
CAC2005		Siderophore/Surfactin synthetase related protein	2.83
CAC2006		Enzyme of siderophore/surfactin biosynthesis	3.67
CAC2007		Predicted glycosyltransferase	3.41
CAC2008	*pksF*	3-oxoacyl-(acyl-carrier-protein) synthase	3.14
CAC2009	*mmgB*	3-Hydroxyacyl-CoA dehydrogenase	3.54
CAC2010		Predicted Fe-S oxidoreductase	3.30
CAC2011	*fabH*	fabH Possible 3-oxoacyl-[acyl-carrier-protein] synthase III	3.93
CAC2012	*fadB*	Enoyl-CoA hydratase	3.68
CAC2013		Hypothetical protein	3.62

Table 3.2.6 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC2014		Predicted esterase	3.50
CAC2015		hypothetical protein	3.53
CAC2016	*fadB*	Enoyl-CoA hydratase	3.64
CAC2017		Acyl carrier protein	3.53
CAC2018		Aldehyde ferredoxin oxidoreductase	3.24
CAC2019		Malonyl CoA-acyl carrier protein transacylase	3.19
CAC2040		ABC transported MDR-type, ATPase component	2.10
CAC2041		ABC transported MDR-type, permease component	2.11
CAC2086		Stage III sporulation protein AH, SpoIIIAH	5.34
CAC2089		Stage III sporulation protein AE, SpoIIIAE	3.74
CAC2091		Stage III sporulation protein AC, SpoIIIAC	3.97
CAC2137		Cation transport P-type ATPase	3.23
CAC2288		Acyl-protein synthetase, luxE	1.99
CAC2306	*sigF*	Sporulation sigma factor	2.74
CAC2307		Anti-sigma F factor	3.80
CAC2308		Anti-anti-sigma factor (antagonist of SpoIIAB)	3.80
CAC2342		Predicted membrane protein	4.12
CAC2353		hypothetical protein	2.48
CAC2354		Nifs family aminotransferase	2.34
CAC2365	*sspA*	Small acid-soluble spore protein	4.03
CAC2382	*ssb*	Single-strand DNA-binding protein	4.61
CAC2404		Glycosyltransferase	2.92
CAC2405		Predicted glycosyltransferase	2.70
CAC2406		Predicted permease, possible O-antigen transporter	2.52
CAC2407		CheY-like receiver domain of response regulator	2.58
CAC2408		Glycosyltransferase	2.58
CAC2460		Hypothetical protein	3.00
CAC2517	*nrpE*	Extracellular neutral metalloprotease, NPRE	1.91
CAC2518		Extracellular neutral metalloprotease	2.56
CAC2531		S-adenosylmethionine-dependent methyltransferase	2.26
CAC2543	*etfA*	Electron-transferring flavoprotein large subunit	1.94
CAC2556		Endoglucanase, family 26, S-layer homology domain	2.95
CAC2572		Possible aminoglycoside phosphotransferase	2.06
CAC2574		Predicted S-adenosylmethionine-dependent methyltransferase	2.88
CAC2575	*rubY*	Rubrerythrin	3.39
CAC2576		6-pyruvoyl-tetrahydropterin synthase related protein	2.54
CAC2577		GGDEF domain containing protein, (inactivated)	2.96
CAC2578		Glycosyltransferase	2.92
CAC2579		Hypothetical protein	2.70
CAC2580		Hypothetical protein, CF-41 family	2.73
CAC2581		6-pyruvoyl-tetrahydropterin synthase related domain	3.07
CAC2663		Protein containing cell-wall hydrolase domain	4.24

Table 3.2.6 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC2678		Hypothetical protein	3.02
CAC2682		Hypothetical protein	2.22
CAC2685		Trehalose/maltose hydrolase (phosphorylase)	1.76
CAC2695		Diverged Metallo-dependent hydrolase(Zn) of DD-Peptidase family	5.30
CAC2715		Hypothetical protein	1.91
CAC2716		Predicted glycosyl transferase from UDP-glucuronosyltransferase family	3.71
CAC2722		RCC1 repeats protein (beta propeller fold)	4.56
CAC2747		Protein containing LysM repeats (N-terminal domain)	2.34
CAC2791		MoaA/NirJ family Fe-S oxidoreductase	3.69
CAC2796		MoaA/NirJ family Fe-S oxidoreductase	1.70
CAC2798		NADH flavin oxidoreductase	1.64
CAC2808		Beta-lactamase class C domain (PbpX family) containing protein	3.15
CAC2903		LysM domain containing membrane protein	3.85
CAC2944		N-terminal domain intergin-like repeats	4.41
CAC2954		Galactose-6-phosphate isomerase	5.17
CAC2984		Hypothetical protein	2.10
CAC2985		Hypothetical protein	2.09
CAC3036		Superfamily I DNA helicase	2.50
CAC3355		Polyketide synthase	3.36
CAC3412		Predicted protein-S-isoprenylcysteine methyltransferase	3.88
CAC3419		S-adenosylmethionine-dependent methyltransferase	2.70
CAC3460		Hypothetical protein, CF-28 family	3.60
CAC3558		Probable S-layer protein	3.33
CAC3565		Uncharacterized secreted protein, containing cell adhesion domain	2.15

Genes are listed in order of ORFs and considered as significantly upregulated when the logarithmic ratio was ≥ 1.6.

The given values in the table are mean of results of two hybridizations with dys swaps.

[a] Protein name based on the whole genome sequence by Nölling *et al.* (2001)

[b] The expression ratio as the logarithm to the basis of 2

Genes related to sporulation, including *spoVR* (CAC0581), *spoIIGA* (CAC1694), *sigE* (CAC1695), *sigG* (CAC1696), *stageIII* sporulation protein (CAC2086-2093), *sigF* CAC2306 and anti-sigma F factor (CAC2307-CAC2308) were strongly induced as compared to the wild type. In addition, the genes of two sugar transporters, the glucose transporter (CAC0570) and the galactose transporter (CAC2952-2955), were also induced. The gene CAP0168, coding for an amylase located on the pSOL1 megaplasmid was also induced of the *trxB* mutant. Well-known solventogenic genes, such as the acetoacetate decarboxylase (*adc*, CAP0165), aldehyde dehydrogenase

(*adhE1*, CAP0162), butyrate-acetoacetate CoA-transferase (*ctfA-ctfB*, CAP0163-CAP0164), were strongly induced during acidogenic growth of the *trxB* mutant. The pyruvate decarboxylase gene (*pdc*, CAP0025) which is also located on the megaplasmid was also higyly induced during acidogenic growth of the *trxB* mutant. Furthermore, the large cluster of genes (CAC1988-2019) involved in fatty acid synthesis (Alsaker and Papoutsakis, 2005) and the cluster of genes (CAC0253-CAC0258) involved in nitrogen fixation were strongly induced during acidogenic growth of the *trxB* mutant as compared to the wild type.

Table 3.2.7 Significantly downregulated genes in the *trxB* mutant compared to the wild type during acidogenic growth

ORF	Gene	Protein[a]	Ratio[b]
CA_P0003		Transglutaminase-like predicted protease domain fused to ChW-repeats	-3.87
CA_P0048		Related to methyl-accepting chemotaxis protein	-2.81
CA_P0098	*amyA*	Alpha-amylase	-4.44
CA_P0106		1-deoxyxylulose-5-phosphate synthase, dehydrogenase	-2.55
CAC0016		Related to HTH domain of SpoOJ/ParA/ParB/repB family	-1.78
CAC0022	*asd*	Aspartate-semialdehyde dehydrogenase	-2.74
CAC0107		ABC-type sulfate transporter, ATPase component	-2.94
CAC0108		ABC-type probable sulfate transporter, permease protein	-3.51
CAC0109	*cysD*	Sulfate adenylyltransferase subunit 2	-3.56
CAC0110	*cysN*	GTPase, sulfate adenylate transferase subunit 1	-3.26
CAC0117		Protein CheY homolog	-2.82
CAC0118	*cheA*	Chemotaxis protein	-2.73
CAC0120		Membrane-associated methyl-accepting chemotaxis protein	-3.41
CAC0149		Hypothetical protein	-3.61
CAC0304	*motA*	Chemotaxis motility protein A, gene motA	-3.14
CAC0384	*licB*	PTS system, cellobiose-specific component BII	-5.92
CAC0385		Beta-glucosidase	-5.77
CAC0386	*licC*	PTS cellobiose-specific component IIC	-6.53
CAC0387		Hypothetical protein	-5.28
CAC0432		Methyl-accepting chemotaxis protein	-2.12
CAC0474		ACT domain containing transcriptional regulators	-2.35
CAC0475		HD-GYP domain	-2.43
CAC0562		Predicted membrane protein	-2.19
CAC0563		Predicted membrane protein	-2.46
CAC0578	*metH*	Cobalamine-dependent methionine synthase I	-2.07
CAC0586		CheX protein (uncharacterized ORF in chemotaxis operon)	-1.65

Table 3.2.7 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC0631		Signal transduction protein	-2.03
CAC0848		Uncharacterized conserved protein, YitT family	-1.82
CAC0882		Predicted membrane protein, hemolysin III homolog	-3.40
CAC0986		Lipoprotein, attached to the cytoplasmic membrane, NLPA family	-1.91
CAC1092		Predicted metal-dependent phosphoesterase (PHP family), YciV ortholog	-1.78
CAC1232		Predicted lytic murein transglycosylase	-1.76
CAC1233	*cheV*	Chemotaxis protein CheV ortholog	-2.57
CAC1320	*glpP*	Glycerol-3-phosphate responsive antiterminator (mRNA-binding), GLPP	-3.17
CAC1321	*glpK*	Glycerol kinase	-4.52
CAC1322	*glpA*	Glycerol-3-phosphate dehydrogenase, GLPA	-3.27
CAC1357		Uncharacterized predicted metal-binding protein	-3.16
CAC1411		Similar to toxic anion resistance protein TerA	-2.48
CAC1412	*cdrC*	Methyl methane sulfonate/mytomycin C/UV resistance protein	-2.55
CAC1413		Similar to C-terminal fragment of toxic anion resistance protein terA	-2.54
CAC1472		Amino acid permease	-1.75
CAC1571		Glutathione peroxidase	-3.03
CAC1572		Fructose-1,6-bisphosphatase	-2.22
CAC1600		Methyl-accepting chemotaxis-like protein	-2.54
CAC1601		Methyl-accepting chemotaxis-like protein	-2.59
CAC1609		Zn-finger containing protein	-2.09
CAC1634		Flagellin	-3.28
CAC1778		Amidase from nicotinamidase family	-1.96
CAC1780		Nicotinate phosphoribosyltransferase	-2.71
CAC1783	*gltT*	Proton/sodium-glutamate symport protein	-1.87
CAC1966		Surface-layer related glycoprotein	-1.97
CAC2079		Hypothetical protein	-1.83
CAC2150	*fliP*	Flagellar biosynthesis protein	-1.97
CAC2151	*fliZ*	Flagellar biosynthesis protein	-2.13
CAC2152	*fliL*	Flagellar protein FliL	-2.15
CAC2155		Hypothetical protein	-3.06
CAC2156	*flgD*	Flagellar hook assembly protein	-2.98
CAC2157	*fliK*	Flagellar hook-length control protein	-2.97
CAC2158	*fliJ*	Flagellar protein FliJ	-1.88
CAC2166		Nucleoside-diphosphate-sugar epimerase	-2.32
CAC2167		Flagellin family protein	-2.29
CAC2171		Predicted glycosyltransferase	-2.01
CAC2172		Predicted glycosyltransferase	-2.20
CAC2173		Glycosyltransferase	-2.25
CAC2174		Glycosyltransferase	-2.09
CAC2175		Glycosyltransferase	-2.31
CAC2201		Hypothetical protein	-1.93
CAC2202		Hypothetical protein	-1.97

Table 3.2.7 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC2203		Possible hook-associated protein, flagellin family	-2.96
CAC2205	*fliD*	Flagellar hook-associated protein	-1.79
CAC2208		Flagellin family protein	-2.02
CAC2209	*csrA*	Carbon storage regulator	-2.00
CAC2210		Uncharacterized protein,	-2.27
CAC2211	*flgL*	Flagellin	-2.41
CAC2212	*flgK*	Flagellar hook-associated protein	-1.80
CAC2213		Hypothetical protein	-1.79
CAC2214	*flgM*	Regulator of flagellin synthesis	-1.78
CAC2219	*cheC*	Chemotaxis protein	-1.92
CAC2325		Possible cell wall hydrolase containing N-acetylglucosaminidase domain	-2.39
CAC2390	*argC*	N-acetyl-gamma-glutamyl-phosphate reductase	-1.64
CAC2490		Xre family DNA-binding domain and TPR repeats containing protein	-1.95
CAC2626	*fabG*	3-ketoacyl-(acyl-carrier-protein) reductase	-1.70
CAC2655		Uncharacterized membrane-associated protein, DedA family	-1.81
CAC2772		Permease	-3.07
CAC2841		Conserved membrane protein, probable transporter	-2.00
CAC2849		Proline/glycine betaine ABC-type transport system, permease component	-3.34
CAC2850		Proline/glycine betaine ABC-type transport system, ATPase component	-2.87
CAC3045		CPSB/CAPC ortholog, PHP family hydrolase	-2.08
CAC3047		Uncharacterized membrane protein, putative virulence factor MviN	-1.95
CAC3052		Glycosyltransferase	-3.20
CAC3054		Phosphoheptose isomerase	-2.94
CAC3055		Sugar kinase	-3.81
CAC3058		Mannose-1-phosphate guanylyltransferase	-4.39
CAC3059		Sugar transferase	-3.46
CAC3092		Germination specific	-1.90
CAC3093		Phosphate permease	-2.00
CAC3256		Predicted acetyltransferase	-1.89
CAC3263		Hypothetical protein	-3.03
CAC3285		Predicted amino acid transporter	-2.43
CAC3371		2-enoate reductase	-1.76
CAC3461		Hypothetical protein	-3.44
CAC3510		Membrane associated methyl-accepting chemotaxis protein	-2.34
CAC3556		Probable S-layer protein	-3.07
CAC3583		Predicted permease	-2.17
CAC3584		Predicted permease	-2.19
CAC3585		ABC-type transporter, ATPase component	-2.42
CAC3650		HD-GYP domain containing protein	-2.34

Genes are listed in order of ORFs and considered as significantly downregulated when the logarithmic ratio was $\leq$ -1.6. The given values in the table are mean of results of two hybridizations with dys swaps.

[a] Protein name based on the whole genome sequence by Nölling *et al.* (2001)

The genes, whose expression was strongly repressed during acidogenic growth as compared to the wild type are listed in Table 3.2.7. These genes can be classified in 4 groups. Group 1 contained the genes involved in sugar transport and energy metabolism. They were CAP0098 (coding for an amylase), CAC0383-CAC0386 (coding for a cellobiose transporter), CAC1320-CAC1322 (a gene cluster related to glycerol motablism) and a carbon storage regulator gene (*csrA*, CAC2209). Group 2 contained genes cording for flagellin family proteins. They were CAC1634, CAC2150-2158, CAC2203, CAC2205, CAC2208 and CAC2211-CAC2214 (Table3.2.7). The genes related to chemotaxis proteins were classified as group 3. They were CAC0117-CAC0120, CAC0304, CAC0586, CAC1233 and CAC1600-CAC1601. The rest of the genes in Table 3.2.7 were classified as group 4, mainly including cysteine biosynthesis genes (CAC0102-CAC0110) and some predicted protein genes.

During solventogenic growth, the samples were taken after 216 h of continous growth for the wild type and the *trxB* mutant (Fig. 3.2.4 and Fig. 3.2.6). Transcriptional analysis identified 103 genes that were significantly induced and 36 genes that were significantly repressed according to the filter criteria as described above (section 2.6.4). All the induced and repressed genes are listed in Table 3.2.8 and in Table 3.2.9 respectively.

Table 3.2.8 Significantly upregulated genes in the *trxB* mutant compared to the wild type during solventogenic growth

ORF	Gene	Protein[a]	Ratio[b]
CA_P0064		Fructose-bisphosphate aldolase class I	2.57
CA_P0130		Hypothetical protein	1.73
CA_P0141		Periplasmic hydrogenase small subunit, dehydrogenase	2.46
CA_P0142		Periplasmic hydrogenase large subunit, dehydrogenase	3.61
CA_P0143		Hydrogenase maturation protease delta subunit	3.56
CA_P0144		Possible steroid binding protein	3.61

Table 3.2.8 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CA_P0145		Hypothetical protein, CF-29 family	3.45
CAC0056		Hypothetical protein	2.75
CAC0057		Hypothetical protein	2.73
CAC0058		Hypothetical protein	2.76
CAC0060		Predicted membrane protein	2.19
CAC0061		Phage-related protein	2.53
CAC0062		Hypothetical protein	1.87
CAC0063		Hypothetical protein	2.49
CAC0064		Hypothetical protein	2.54
CAC0065		Hypothetical protein	2.51
CAC0469	*spmA*	Spore maturation protein A (gene spmA)	2.94
CAC0552		Protein containing cell-adhesion domain	2.61
CAC0553		Hypothetical protein, CF-8 family	2.70
CAC0554	*lyc*	Autolytic lysozime (1,4-beta-N-acetylmuramidase)	2.61
CAC0570		PTS enzyme II, ABC component	5.18
CAC0581	*spoVR*	Stage V sporulation protein R	2.75
CAC0613		Spore coat protein F	2.37
CAC0620		ABC transporter, periplasmic-binding	1.81
CAC0675		Hypothetical protein	1.86
CAC0750		Hypothetical protein	1.82
CAC0785		Hypothetical protein	2.61
CAC0787		Uncharacterized conserved protein, repeats	1.95
CAC0810	*hypF*	Hydrogenase maturation factor	2.43
CAC0846		Hypothetical protein, CF-29 family	2.21
CAC0989		General secretion family related protein	2.00
CAC1020		Hypothetical protein	2.59
CAC1043		Xre family DNA-binding domain and TPR-repeat containing protein	1.85
CAC1275		Germination protease precursor	2.28
CAC1336		Hypothetical protein	2.57
CAC1337		Spore coat protein COTJB	2.62
CAC1338		Spore coat protein COTJC	2.26
CAC1349		Aldose-1-epimerase	2.86
CAC1363	*sodC*	Superoxide dismutase, Cu-Zn family	1.84
CAC1455		Two-component system regulator (CheY domain)	1.89
CAC1575		Hypothetical protein	2.74
CAC1664	*glgP*	Glycogen phosphorylase	1.68
CAC1695	*sigE*	Sporulation sigma factor SigE	3.60
CAC1696	*sigG*	Sporulation sigma factor SigG	3.44
CAC1713	*spoIVA*	Coat morphogenesis sporulation protein	2.26
CAC2086	*spoIIIAH*	Stage III sporulation protein AH	3.74
CAC2087	*spoIIIAG*	Stage III sporulation protein AG	3.90
CAC2088	*SpoIIIAF*	Stage III sporulation protein AF	3.73

Table 3.2.8 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC2090	*SpoIIIAD*	Stage III sporulation protein AD	3.61
CAC2091	*SpoIIIAC*	Stage III sporulation protein AC	3.17
CAC2092	*spoAB*	Stage III sporulation protein	3.80
CAC2131		Hypothetical protein	1.69
CAC2135		ATP-dependent serine protease LA/LON family	2.47
CAC2137		Cation transport P-type ATPase	3.35
CAC2237	*glgC*	Glucose-1-phosphate adenylyltransferase	2.13
CAC2238	*glgC*	ADP-glucose pyrophosphorylase	2.48
CAC2241		Cation transport P-type ATPase	1.96
CAC2249		C-terminal domain of asparagine synthase	2.24
CAC2305	*spoVAC*	SpoVAC Stage V sporulation AC	2.47
CAC2342		Predicted membrane protein	2.07
CAC2343		LPS biosynthesis O-acetyl transferase	2.35
CAC2344		LPS biosynthesis protein , RfbU family	2.25
CAC2349		Hypothetical protein	2.55
CAC2350		Sugar transaminase	2.90
CAC2352		Hypothetical protein	2.71
CAC2353		Hypothetical protein	2.94
CAC2365	*sspA*	Small acid-soluble spore protein	2.18
CAC2382	*ssb*	Single-strand DNA-binding protein	1.91
CAC2383		Predicted xylanase/chitin deacetylase	3.76
CAC2428		Activator of 2-hydroxyglutaryl-CoA dehydratase	1.86
CAC2458		Ferrodoxin oxidoreductase beta subunit	1.84
CAC2460		Hypothetical protein	2.34
CAC2499		Pyruvate ferredoxin oxidoreductase	2.50
CAC2621		Cell wall hydrolase (autolysin), family 25 of glycosyl hydrolase	1.93
CAC2625		Predicted membrane protein	2.12
CAC2682		Hypothetical protein	2.86
CAC2685		Trehalose/maltose hydrolase (phosphorylase)	2.16
CAC2728		Hypothetical protein, CF-30 family	1.69
CAC2747		Protein containing LysM repeats (N-terminal domain)	2.22
CAC2791		MoaA/NirJ family Fe-S oxidoreductase	2.77
CAC2794		Transcriptional regulator, Lrp family	2.63
CAC2795		MoaA/NirJ family Fe-S oxidoreductase	3.14
CAC2827		S-adenosylmethionine-dependent methyltransferase	1.99
CAC2828		Nudix (MutT) family hydrolase/pyrophosphatase	2.04
CAC2859		spoIIID Stage III sporulation protein D, SpoIIID	2.99
CAC2862		UDP-N-acetylglucosamine 1-carboxyvinyltransferase	2.16
CAC2863		Predicted membrane protein	2.63
CAC2903		LysM domain containing membrane protein	3.18
CAC2905		Uncharacterized protein, YabG *B.subtilis* ortholog	2.81
CAC2906		Spore coat protein cotS related	3.49

Table 3.2.8 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC2909		Spore coat protein cotS related (diverged)	2.32
CAC2910		Spore coat protein cotS related	2.43
CAC2981		Mannose-1-phosphate guanyltransferase	2.19
CAC2984		Hypothetical protein	2.85
CAC2985		Hypothetical protein	3.11
CAC3009		Xylanase/chitin deacetylase family protein	1.88
CAC3244		Spore cortex-lytic enzyme, pre-pro-formin	2.63
CAC3278		Uncharacterized protein	2.55
CAC3307		TPR-repeats containing protein	2.25
CAC3308		Glycosyltransferase fused to TPR-repeat domain	2.45
CAC3318		Hypothetical protein	2.60
CAC3395		Predicted membrane protein	1.76
CAC3450		Hypothetical protein	2.12

Genes are listed in order of ORFs and considered as significantly upregulated when the logarithmic ratio was ≥ 1.6. The given values in the table are mean of results of two hybridizations with dys swaps.

[a] Protein name based on the whole genome sequence by Nölling *et al.* (2001)

[b] The expression ratio as the logarithm to the basis of 2

Among the genes, which were significantly upregulated as compared to the wild type, many genes encoded sporulation-associated proteins including CAC0469, CAC0581, CAC0613, CAC1337-CAC1338, CAC1695-CAC1696, CAC1713, CAC2086-CAC2092, CAC2305, CAC2365, CAC2859, CAC2906-CAC2910 and CAC3244 (Table 3.2.8). Activation of sporulation genes is often a signal indicating that the cells are stressed and "feel" bad. This result suggested that inactivation of *trxB* gene affected the metabolism of *C. acetobutylicum*. In addition, a gene (*lyc*, CAC0554) coding for an autolytic lysozyme was also upregulated. Moreover, some dehydrogenase genes (CAP0141-CAP013 and CAC0810) were upregulated, as well as some Fe-S-containing protein genes (CAC2458, CAC2499, CAC2791 and CAC2795). The expression of the glucose transporter gene (CAC0570) was strongly induced in the *trxB* mutant during solventogenic growth.

The genes whose expression was strongly repressed as compared to the wild type during solventogenic growth are listed in Table 3.2.9. A gene (CAC0386) in a cluster (CAC0383-CAC0386) coding for a putative cellobiose transporter was significantly

downregulated. Two genes (CAC0106 and CAC0109) located in a cluster (CAC0102-CAC0110)) were also significantly downregulated. The function of this cluster was to convert sulfate to sulfite which was an important step in cysteine synthesis. Genes with a function in the conversion of L-glutamate to L-arginine were downregulated strongly and consisted of two clusters CAC0973-0974 and CAC2388-2391.

Table 3.2.9 Significantly downregulated genes in the *trxB* mutant compared to the wild type during solventogenic growth

ORF	Gene	Protein[a]	Ratio[b]
CA_P0004		Cysteine protease	-1.84
CA_P0036		Uncharacterized, ortholog of YgaT gene of *B.subtillis*	-3.46
CA_P0037		Uncharacterized, ortholog of YgaS gene of *B.subtillis*	-3.88
CAC0106		ABC-type probable sulfate transporter, periplasmic binding protein	-1.93
CAC0109	*cysD*	Sulfate adenylyltransferase subunit 2	-3.29
CAC0316	*argF/I*	Ornithine carbomoyltransferase	-3.54
CAC0318		Membrane permease, predicted cation efflux pumps	-2.13
CAC0319		ABC transporter ATP-binding protein	-2.07
CAC0380		Periplasmic amino acid-binding protein	-3.00
CAC0386	*licC*	PTS cellobiose-specific component IIC	-3.84
CAC0387		Hypothetical protein	-3.29
CAC0973	*argG*	argininosuccinate synthase	-2.93
CAC0974	*argH*	Argininosuccinate lyase	-3.18
CAC0975		Predicted P-loop kinase	-1.80
CAC1078		Predicted phosphohydrolase, Icc family	-2.10
CAC1314		Hypothetical protein	-2.46
CAC1315		Peptodoglycan-binding domain containing protein	-1.99
CAC1316		Predicted membrane protein	-2.23
CAC1356	*thiH*	Thiamine biosynthesis protein	-1.73
CAC1634		Flagellin	-2.00
CAC1669	*cstA*	Carbon starvation protein	-2.31
CAC1745	*rpmF*	Ribosomal protein L32	-2.05
CAC2016	*fadB*	Enoyl-CoA hydratase	-1.77
CAC2107		Contains cell adhesion domain	-1.74
CAC2203		Possible hook-associated protein, flagellin family	-2.10
CAC2366		Predicted membrane protein	-2.05
CAC2388	*argD*	N-acetylornithine aminotransferase	-3.64
CAC2389	*argB*	Acetylglutamate kinase	-3.33
CAC2390	*argC*	N-acetyl-gamma-glutamyl-phosphate reductase	-3.70

Table 3.2.9 (continued)

ORF	Gene	Protein[a]	Ratio[b]
CAC2391	*argJ*	Bifunctional ornithine acetyltransferase/N-acetylglutamate synthase	-3.76
CAC2438		Predicted phosphatase	-1.80
CAC2517	*nrpE*	Extracellular neutral metalloprotease	-3.38
CAC2644	*carB*	Carbamoyl-phosphate synthase large subunit	-2.43
CAC2645	*carA*	Carbamoyl-phosphate synthase small subunit	-2.56
CAC3509		Transcriptional regulator, MerR family (duplicated domains)	-1.71
CAC3558		Probable S-layer protein	-2.40

Genes are listed in order of ORFs and considered as significantly downregulated when the logarithmic ratio was $\leq$ -1.6. The given values in the table are mean of results of two hybridizations with dys swaps.

[a] Protein name based on the whole genome sequence by Nölling *et al.* (2001)

[b] The expression ratio as the logarithm to the basis of 2

3.3 Confirmation of glucose transporters through targeted mutagenesis and transcriptional analysis in *Clostridium acetobutylicum*

3.3.1 Generation and verification of the *glcG*, *glcCE* and *glcG/glcCE* mutants

To investigate the glucose transporters in *C. acetobutylicum*, two genes (CAC0570, PTS enzyme II, ABC component, *glcG* and CAC0386, PTS cellobiose-specific component IIC, *glcCE*) were targeted for insertion inactivation using ClosTron mutagenesis as described above (section 2.3). The primers for SOE PCR (section 2.3.2) are listed in Table 2.6. The length of *glcG* was 1998 bp and the target site was designed at 1224/1225 bp. The length of *glcCE* was 1353 bp and the target site was designed at 193/194 bp. The correct retargeted plasmids, pMTL007C-E2-*glcG* and pMTL007C-E2-*glcCE* (in Table 2.5), were constructed and subsequently transformed into *C. acetobutylicum*. In the end, putative mutants (the *glcG* and *glcCE* mutants) were obtained by screening CGM plates. Erythromycin-resistant clones (putative mutants) were verified (section 2.3.5) by gene check primers (CAC0570F, CAC0570R and CAC0386F, CAC0386R) listed in Table 2.6. The results showed that wild type genomic DNA gave rise to a band at ~ 300 bp, while the correct mutants

exhibited products of ~ 2.1 kb, which were 1.8 kb longer than that of wild type (Fig. 3.2.1). For the double mutant (*glcG*/*glcCE*), the correct retargeted plasmid, pMTL007C-E2-*glcCE*, was transformed into the *glcG* mutant. Since the parent strain, the *glcG* mutant, contained an erythromycin marker, the concentration of erythromycin used for selection was increased from 5 μg/μL to 15μg/μL. The colonies obtained on the selection plates, which grew faster, were inoculated into fresh CGM medium and cultured overnight at 37°C. Then, these colonies were verified by PCR using the gene check primers (CAC0570F, CAC0570R and CAC0386F, CAC0386R) listed in Table 2.6. The correct mutants should exhibit products of ~ 2.1 kb, which were 1.8 kb longer than those of the wild type (Fig. 3.2.1).
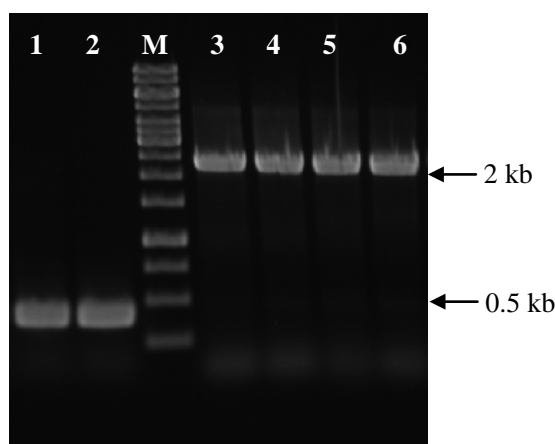
Fig. 3.3.1. PCR verification of *glcG*, *glcCE* and *glcG/glcCE* mutants
M, marker;
lane 1, CAC0570F and CAC0570R primers, wild type genome DNA;
lane 2, CAC0386F and CAC0386R primers, wild type genome DNA;
lane 3, CAC0570F and CAC0570R primers, *glcG* mutant genome DNA;
lane 4, CAC0386F and CAC0386R primers, *glcCE* mutant genome DNA.
lane 5, CAC0570F and CAC0570R primers, *glcG/glcCE* mutant genome

To double check that the intron had inserted into the target genes, the two 2.1 kb PCR products from the *glcG* and *glcCE* mutant genomic DNAs as template were sequenced. Analysis of the sequences revealed that the intron inserted the *glcG* at 1224/1225 bp and the *glcCE* at 193/194 bp respectively. These results showed the

glcG and *glcCE* were inactivated by insertion of ClosTron into the desired positions and the *glcG* and *glcCE* ClosTron mutants were obtained. To further confirm that only one copy of the intron was inserted in the chromosomal DNA of the *glcG* and *glcCE* mutants, Southern hybridization was carried out with the intron-specific probe (section 2.3.6.2). For the double mutant (*glcG/glcCE*), two copies of the intron should be inserted into the chromosomal DNA. Thus, two bands are expected in the Southern hybridization. The results are shown in Fig. 3.3.2. No hybridization signal was observed for wild type genomic DNA and genomic DNA of the *glcG* and *glcCE* mutants. Both exhibited a single band, demonstrating that the *glcG* and *glcCE* mutants possessed only one copy of the intron in the chromosome. The double mutant showed two bands in the Southern hybridization. One was same to that of the *glcG* mutant and the other was same as that of the *glcCE* mutant (Fig.3.3.2). This result indicated that two copies of intron were in the chromosomal DNA of the *glcG/glcCE* mutant and the introns were inserted correctly into *glcG* and *glcCE* genes.
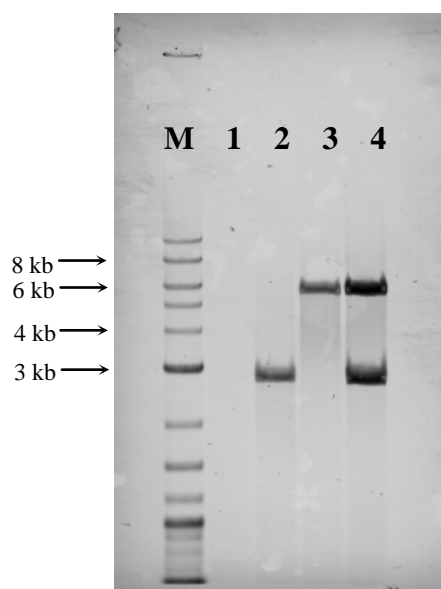
Fig.3.3.2 Southern hybridization to demonstrate the presence of intron in the constructed mutants. M, Marker; lane 1, The wild type (negative control); lane 2, The *glcG* mutant; lane 3, The *glcCE* mutant; lane 4, The *glcG/glcGE* mutant.

3.3.2 The phenotypes of the wild type and the *glcG* mutant in batch and phosphate-limited continuous fermentations

The wild type and *glcG* mutant were cultured in MM (minimal medium) with 50 g/L glucose and exhibited similar growth phenotypes as shown in Fig. 3.3.7 (in the section 3.3.4 below). This result was consistent with a previous report (Xiao *et al.*, 2012). To further evaluate the effects of *glcG* inactivation on glucose uptake, the two strains were cultivated in phosphate-limited continuous culture   (Fig. 3.3.3).
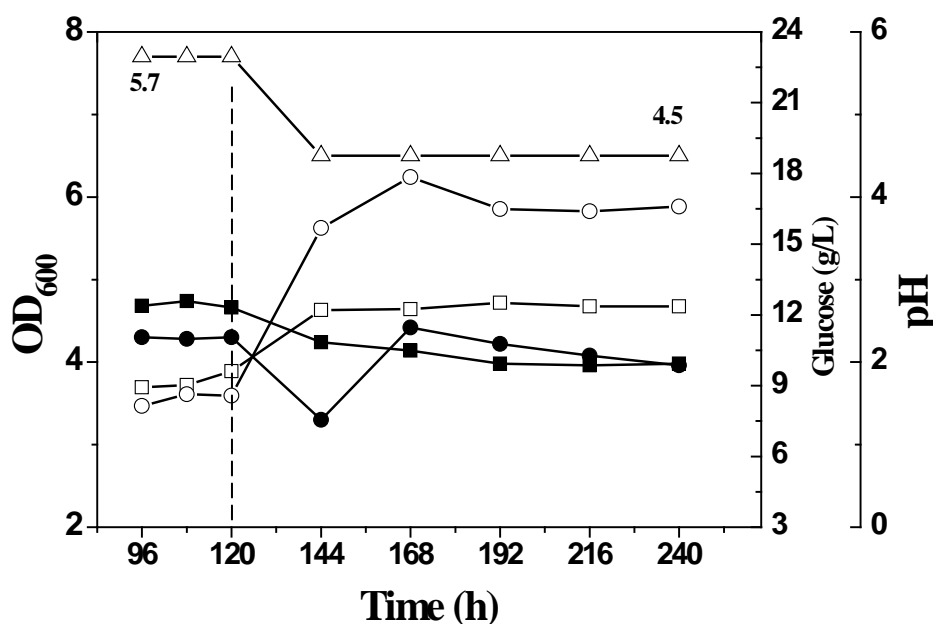
Fig. 3.3.3 Growth curves and residual glucose concentration in the phosphate-limited continuous culture of the wild type and *glcG* mutant. ($\triangle$), pH; ($\blacksquare$),OD_{600} of the wild type; ($\bullet$) OD_{600} of the *glcG* mutant; ($\square$), Residue glucose of wild type in the medium; ($\circ$), Residue glucose of the *glcG* mutant in the medium. The dash line indicated the initiation of dynamic pH shift from 5.7 to 4.5 in the continuous fermentation.

The optical density of the wild type was almost identical to that observed for the *glcG* mutant during acidogenic and solventogenic growth. There was 40 g/L glucose in PPLM (phosphate-limited continuous medium). Assays of the residual glucose concentrations in the continuous culture indicated that approximately 9 g/L glucose

was determined during acidogenic growth for the wild type and the *glcG* mutant, while approximately 12 g/L glucose was determined for the wild type and 17.5 g/L glucose for the *glcG* mutant in the continuous culture during solventogenic growth. Due to inactivation of the *glcG* gene, the rate of glucose uptatke by the *glcG* mutant was lower than that by the wild type during solventogenic growth. This result revealed that the GlcG protein (encoded by *glcG*, CAC0570) indeed is a PTS enzyme II protein for glucose transport. In addition, it can be assumed that an alternative glucose transporter exists in *C. acetobutylicum*, because still good growth on glucose can be observed in the *glcG* mutant.

3.3.3 Transcripttion analysis of all the phosphotransferase complement systems of the *glcG* mutant as compared to the wild type

C. acetobutylicum has a total 13 phosphotransferase systems for hexose transport. So, 13 PTS Enzyme II proteins, encoded by 26 genes, including GlcG, can be identified by sequence analysis (Mitchell and Tangney, 2005; Nolling *et al.*, 2001). The disruption of the *glcG* resulted in inactivation of glucose transport by GlcG. However, the *glcG* mutant was still able to ferment glucose efficiently during acidogenic and solventogenic growth (Fig.3.3.3). Therefore, other PTS were able to transport glucose in the cytoplasm in the *glcG* mutant. Thus its gene expression levels should be upregulated in the mutant as compared to the wild type. To test this hypothesis, DNA microarray experiments were carried out in order to detect changes in the transcription of all the 13 PTS Enzyme II protein genes caused by the *glcG* mutation as compared to the wild type. Cells of steady-state acidogenic ($t = 120$ h) and solventogenic growth ($t = 240$ h) of the chemostat culture of the *glcG* mutant and wild type were used for RNA isolation and subsequent microarray experiments. RNA extraction, cDNA production and labeling were described in section 2.5. Detailed experimental protocols for microarray experiments and methods for data analysis were described in section 2.6. The expression data of all the 13 PTS Enzyme II protein genes are given in Table 3.3.1.

Table 3.3.1 Transcription of all the PTS EnzymeⅡ protein genes of the *glcG* mutant and *glcCE* mutant compared to the wild type

ORF	Protein	The *glcG* mutant		The *glcCE* mutant	
		pH 5.7 $t = 120$ h	pH 4.5 $t = 240$ h	pH 5.7 $t = 120$ h	pH 4.5 $t = 240$ h
CA_P0066	PTS, mannose-specific IIAB component	—	—	—	—
CA_P0067	PTS, mannose/fructose-specific IIC component	—	—	—	—
CA_P0068	PTS, mannose-specific IID component	—	—	—	—
CAC0154	PTS, mannitol-specific IIBC component	1.34	1.10	1.01	1.05
CAC0156	PTS, mannitol-specific IIA domain	n.d.	n.d.	n.d.	n.d.
CAC0233	PTS, fructose-specific IIA component	4.99	3.74	-1.11	n.d.
CAC0234	PTS, fructose-specific IIBC component	2.06	1.88	-1.43	n.d.
CAC0383	PTS, cellobiose-specific IIA component	13.89	23.18	n.a.	n.a.
CAC0384	PTS, cellobiose-specific BII component	28.11	7.17	n.a.	n.a.
CAC0386	PTS, cellobiose-specific IIC component	40.23	2.83	n.a.	n.a.
CAC0423	Fusion PTS, beta-glucosides specific IIABC component	—	—	—	—
CAC0532	PTS, arbutin-like IIBC component	—	—	—	—
CAC0570	PTS enzyme II, ABC component	n.a.	n.a.	-1.01	1.02
CAC1353	PTS, N-acetylglucosamine-specific IIBC component,	1.06	n.d.	-1.49	n.d.
CAC1354	PTS, N-acetylglucosamine-specific IIA component	1.08	1.54	-1.58	-1.81
CAC1407	PTS, beta-glucosides-specific IIABC component	—	—	—	—
CAC1457	PTS, fructose(mannose)-specific IIA component	—	—	—	—
CAC1458	PTS, fructose(mannose)-specific IIB	—	—	—	—
CAC1459	PTS, fructose(mannose)-specific IIC	—	—	—	—
CAC2956	PTS, galactitol/fructose specific IIC component	—	—	—	—
CAC2957	PTS, galactitol/fructose specific IIB component	—	—	—	—
CAC2958	PTS, galactitol/fructose specific IIA component	—	—	—	—
CAC2964	PTS, lactose-specific enzyme IIBC component	—	—	—	—
CAC2965	PTS, lactose-specific enzyme IIA component	—	—	—	—
CAC3425	PTS, possibly glucose-specific IIBC component	n.d.	n.d.	n.d.	n.d.
CAC3427	PTS, possibly glucose-specific IIA component	1.15	n.d.	n.d.	-1.40

The numbers showed expression ratios in which the positive value was considered as upregulated and the negative value was considered as downregulated. Protein name based on the whole genome sequence by Nölling *et al.* (2001) Genes were listed in order of ORFs.

(—) indicated the genes were not transcribed both in the wild type and mutants; n.d.: No ratio was cacluated due to used filter criteria of the microarray (Not detected); n.a.: the ratio was not analyzed due to the inactivation of the genes (Not analyzed); ORF: Open reading frame.

Transcriptional analysis of all the PTS Enzyme Ⅱ protein genes of the *glcG* mutant as compared to the wild type indicated that the gene expression of 5 PTSs was upregulated (Table 3.3.1). Among these five PTSs, a cluster of genes (CAC0383-CAC0386) encoding a putative cellobiose-specific transporter exhibited the most significant increase in transcription in the *glcG* mutant during acidogenic and solventogenic growth in continuous culture. In addition, transcription of genes encoding a fructose-specific transporter (CAC0233-CAC0234) also increased as compared to the wild type. Furthermore, slight changes in the expression of the other three PTSs occurred, including a putative mannitol-specific transporter (CAC0154-CAC0156), a putative N-acetylglucosamine-specific transporter (CAC1345-CAC1346) and a putative glucose-specific transporter (CAC3425-CAC3427). The results suggested that the putative cellobiose transporter was possibly responsible for the transport of glucose into the cytoplasm when GlcG was inactivated.

3.3.4 The phenotypes of the wild type and the *glcCE* mutant in batch and phosphate-limited continuous fermentations

Sequence analysis provided compelling evidence that the operon (CAC0383-CAC0386) encoded a cellobiose tranporter in *C. acetobutylicum*. However, there was no experimental evidence to prove that *in vivo*. Therefore, the gene *glcCE* (CAC0386), which encodes a PTS Ⅱ C in this operon was inactivated by inserting an intron at position 1224/1225 bp. Then, the *glcCE* mutant and wild type were cultured in MM with 50 g/L cellobiose as sole carbon source. The growth phenotypes in batch culture are shown in Fig. 3.3.4.

Due to inactivation of the *glcCE* gene, the *glcCE* mutant grew very poorly in the MM with cellobiose as sole carbon source as compared to the wild type. The highest optical density of the *glcCE* mutant achieved was approximately 2, which was significantly lower than the wild type whose highest optical density was 5 in batch fermentations. The result confirmed that the transporter encoded by the operon

(CAC0383-CA C0386) was indeed a cellobiose-specific transporter. In addition, the weak growth of the *glcCE* mutant with cellobiose as sole carbon source suggested that a second transporter, able to transport cellobiose exists in *C. acetobutylicum*. This was not further investigated in this study.
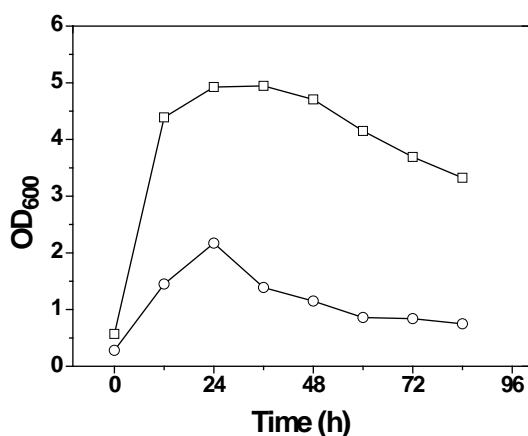

Fig. 3.3.4. The growth curves of the wild type and the *glcCE* mutant cultured in minimal medium with cellobiose as sole carbon source
(□), The wild type; (○), The *glcCE* mutant.

To investigate whether this cellobiose-specific transporter could transport glucose, the *glcCE* mutant was cultured in MM with 50 g/L glucose and in phosphate-limited continuous culture. The growth phenotypes are shown in Fig 3.3.7 (in the section 3.3.4 below) and Fig. 3.3.5. The *glcCE* mutant and wild type exhibited similar growth phenotypes in both batch and continuous fermentation. These results indicated that the *glcCE* mutation did not seriously affect glucose uptake and growth of *C. acetobutylicum*. The *glcCE* mutant even consumed slightly more glucose during acidogenic growth as compared to the wild type in the continuous fermentation (Fig. 3.3.5). To further investigate the effects of the *glcCE* mutation, DNA microarray experiments were carried out to detect transcription level changes of all the 13 PTS Enzyme II protein genes as compared to the wild type. Cells of steady-state acidogenic ($t = 120$ h) and solventogenic growth ($t = 240$ h) of the *glcG* mutant and wild type were used for RNA isolation and subsequent microarray analysis. The

microarray data of all the 13 PTS Enzyme II protein genes are listed in Table 3.3.1.
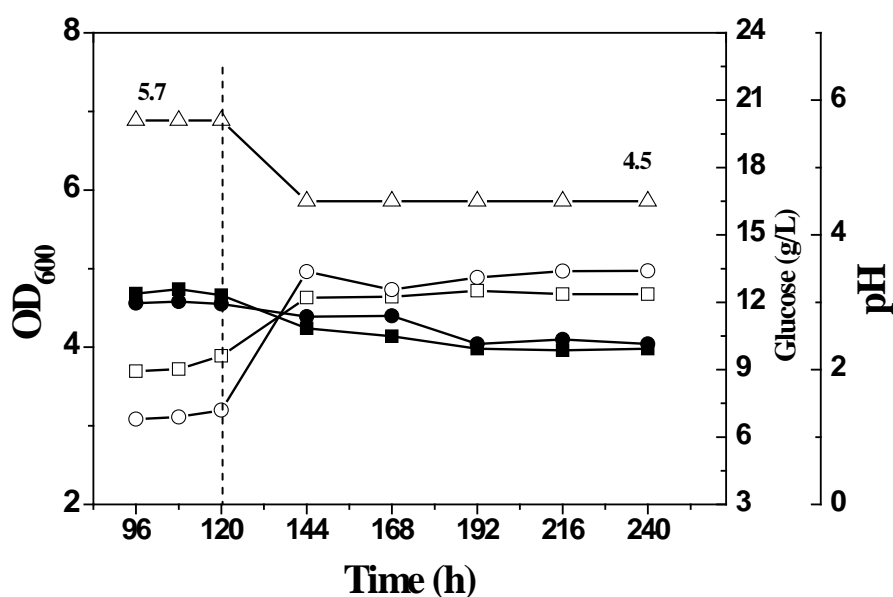

Fig. 3.3.5 Growth and residual glucose concentration in phosphate-limited continuous culture of the wild type and the *glcCE* mutant
($\triangle$), pH; ($\blacksquare$),OD_{600} of the wild type; ($\bullet$) OD_{600} of the *glcCE* mutant; ($\square$), Residue glucose of wild type in the medium; ($\circ$), Residue glucose of the *glcCE* mutant in the medium. The dash line indicated the initiation of dynamic pH shift from 5.7 to 4.5 in the continuous fermentation

The transcription assays of all the PTS Enzyme II protein genes in the *glcCE* mutant as compared to the wild type indicated that only 5 PTS genes were expressed (Table 3.3.1). However, the transcription levels of those 5 PTS genes changed slightly as compared to the wild type. During acidogenic and solventogenic stead-state growth of the *glcCE* mutant, the transcription levels of genes for glucose transporter (*glcG*, CAC0570) and a putative mannitol-specific transporter (CAC0154-CAC0156) were identical to that of wild type. The genes encoding for a putative fructose-specific transporter (CAC0233-CAC0234), a putative N-acetylglucosamine-specific transporter (CAC1345-CAC1346) and a putative glucose-specific transporter (CAC3425-CAC3427) were even slightly depressed compared to the wild type during acidogenic and solventogenic growth. As a consequence, the results revealed that GlcG (CAC0570) was the predominant glucose transporter in *C. acetobutylicum*.

When it worked normally in the culture with glucose as carbon source, all of the other PTSs did not become active, i.e., their encoding genes are not induced. To further test whether the cellobiose transporter could indeed transport glucose, it was necessary to construct a double mutant in which the *glcG* and *glcCE* genes were inactivated simultaneously.

3.3.5 The phenotypes of the *glcG*/*glcCE* mutant in the batch and phosphate-limited continuous fermentations

Growth of the *glcG*/*glcCE* mutant, lacking the glucose and cellobiose transporter systems, was analyzed in phosphate-limited continuous culture. This double mutant was inoculated into the fermentor and then the pH was set to 5.7. After cultivation overnight, the OD_{600} of the culture reached ~ 4.7 and then continuous fermentation of the double mutant started with PLMM (phosphate-limeted minimal medium) at a dilution rate 0.075 h^{-1}. The optical density of the culture was determined at 12 h intervals, yielding the growth curve shown in Fig. 3.3.6.
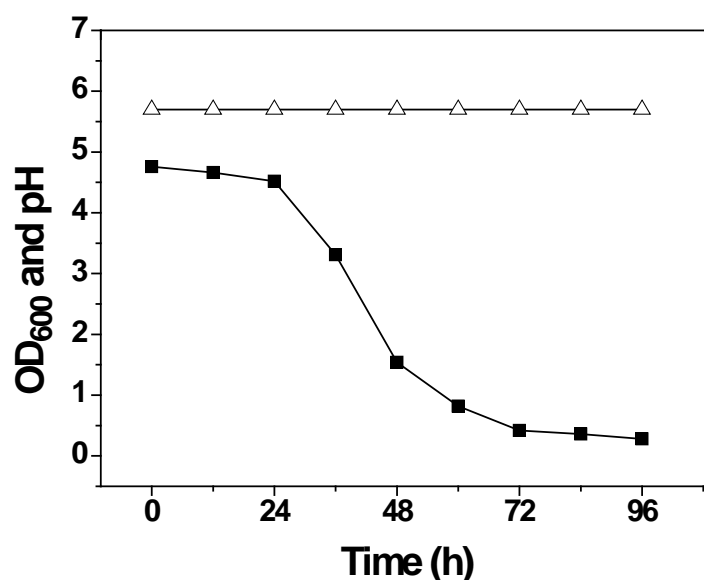
Fig. 3.3.6 Growth of the *C. acetobutylicum glcG/glcCE* mutant in the phosphate-limited continuous culture. (△), pH; (■),OD_{600} of the *glcG/glcCE* mutant

The growth result showed that the *glc*G/*glcCE* mutant was unable to achieve steady-state growth in the continuous fermentation. Under the same fementation conditions, the wild type, the *glcG* mutant and the *glcCE* mutants consumed approximately 32 g/L glucose (the total glucose concentration was 40 g/L) and exhibited comparable steady-state growth during acidogenic grow in continuous fermentation. It can be assumed that the double mutant could not consume enough glucose to support its steady-state growth in continuous fermentation, due to inactivation of the *glcG* gene and *glcCE* gene. Hence, the cells were washed out of the fermenter in continuous culture. This result provided experimental evidence that the cellobiose-specific transporter (encoded by CAC0383-CAC0386) can transport glucose into the cytoplasm.
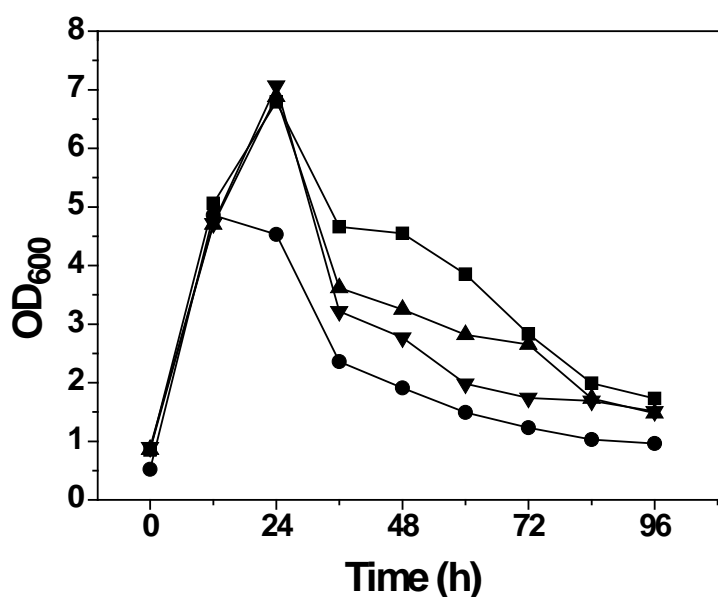
Fig. 3.3.7 Growth of the wild type *glcG, glcCE*, and *glcG/glcCE* mutants in the minimal medium with glucose as sole carbon source. (■) The wild type; (▲) The *glcG* mutant; (▼) The *glcCE* mutant; (●) The *glcG/glcCE* mutant.

Despite that the double mutant was not able to grow in the continuous fermentation, it was able to grow in batch culture in MM with 50 g/L glucose as sole carbon source. The highest optical density of the double mutant was approximately 5, which was lower than the wild type, as well as the *glcG* and the *glcCE* mutants, whose highest

optical density was approximate 7 in the batch fermentations (Fig. 3.3.7). This result showed that the simultaneous inactivation of the glucose tansporter and the cellobiose transporter impaired the growth of the double mutant.

3.3.6 Transcription analysis of all the phosphotransferase complement systems of the *glcG/glcCE* mutant as compared to the wild type

The inactivation of gene *glcG* and gene *glcCE* simultaneously resulted in weak growth of the double mutant, indicating that a third glucose transporter exists in *C. acetobutylicum*. To test, which PTS were able to transport glucose, except for the glucose transporter (CAC0570) and the cellobiose transporter, DNA microarray experiments were carried out to detect transcription level changes of all the 13 PTS Enzyme II protein genes as compared to the wild type in batch culture. Cells of the *glcG* mutant and the wild type at 12 h in batch culture were used for RNA isolation and subsequent microarray experiments (Fig. 3.3.7). RNA extraction, cDNA production and labeling were described in section 2.5. Detailed experimental processes of microarray experiments and methods for data analysis were described in section 2.6. The microarray data of all the 13 PTS enzyme protein genes were shown in Table 3.3.2.

Microarray analysis was used to identify any compensatory changes of gene expression of PTSs caused by mutation of the glucose transporter (CAC0570) and the cellobiose transporter (CAC0383-CAC0386) in the double mutant. The microarray result indicated that only 3 PTSs showed changes in the expression patterns in the double mutant as compared to the wild type. Among these 3 PTSs, the expression of genes encoding a mannitol-specific transporter (CAC0154-CAC0156) was upregulated 2.8-fold, while the expression of genes encoding a putative fructose-specific transporter (CAC0233-CAC0234) and a putative N-acetylglucosamine-specific transporter (CAC1345-CAC1346) were downregulated as compared to that of the wild type (Tabel 3.3.2). This result suggested that the mannitol-specific transporter possibly also has a function in glucose uptake, which

needs to be studied in more detail.

Table 3.3.2 Transcription levels of all the PTS enzyme Ⅱ protein genes of the *glcG* /*glcCE* mutant compared to the wild type in batch culture

ORF	Protein	The *glcG/glcCE* mutant
		$t = 12$ h
CA_P0066	PTS, mannose-specific IIAB component	—
CA_P0067	PTS, mannose/fructose-specific IIC component	—
CA_P0068	PTS, mannose-specific IID component	—
CAC0154	PTS, mannitol-specific IIBC component	2.80
CAC0156	PTS system, mannitol-specific IIA domain	n.d.
CAC0233	PTS, fructose-specific IIA component	-1.46
CAC0234	PTS, fructose-specific IIBC component	n.d.
CAC0383	PTS, cellobiose-specific IIA component	n.a.
CAC0384	PTS, cellobiose-specific BII component	n.a.
CAC0386	PTS, cellobiose-specific IIC component	n.a.
CAC0423	Fusion PTS, beta-glucosides specific IIABC component	—
CAC0532	PTS, arbutin-like IIBC component	—
CAC0570	PTS enzyme II, ABC component	n.a.
CAC1353	PTS, N-acetylglucosamine-specific IIBC component,	-1.34
CAC1354	PTS, N-acetylglucosamine-specific IIA component	-2.22
CAC1407	PTS, beta-glucosides-specific IIABC component	—
CAC1457	PTS, fructose(mannose)-specific IIA component	—
CAC1458	PTS, fructose(mannose)-specific IIB	—
CAC1459	PTS, fructose(mannose)-specific IIC	—
CAC2956	PTS, galactitol/fructose specific IIC component	—
CAC2957	PTS, galactitol/fructose specific IIB component	—
CAC2958	PTS, galactitol/fructose specific IIA component	—
CAC2964	PTS, lactose-specific enzyme IIBC component	—
CAC2965	PTS, lactose-specific enzyme IIA component	—
CAC3425	PTS, possibly glucose-specific IIBC component	—
CAC3427	PTS, possibly glucose-specific IIA component	—

The numbers showed expression ratios in which the positive value was considered as upregulated changed folds and the negative value was considered as downregulated changed folds. Protein name based on the whole genome sequence by Nölling *et al.* (2001) Genes were listed in order of ORFs.

(—) indicated the genes were not transcribed both in the wild type and mutant; n.d.: No ratio was cacluated due to used filter criteria of the microarray (Not detected); n.a.: the ratio was not analyzed due to the inactivation of the genes (Not analyzed); ORF: Open reading frame.

4 Discussion

4.1 Cysteine synthesis pathways and global regulation of gene expression in response to cysteine availability during sloventogenic growth of *Clostridium acetobutylicum*

As observed for several microorganisms (Hullo *et al.*, 2007; Kredich, 1996; Thomas and Surdin-Kerjan, 1997), *C. acetobutylicum* is able to grow in the presence of sulfate or methionine as sole sulfur source, indicating existence of a sulfate assimilation pathway to cysteine and a conversion pathway of methionine to cysteine. In *B. subtilis*, the 6.1-kb *cysH* operon and the *yrrT* operon are involved in cysteine synthesis from sulfate and methionine (Hullo *et al.*, 2007; Mansilla *et al.*, 2000). In *C. acetobutylicum*, the batch fermentation results of the *fer* and *mccB* mutants proved that the operon (CAC0102-CAC0110) and the *ubiGmccBA* operon (CAC0929-CAC0930) were involved in conversion of sulfate to sulfite and conversion of methionine to cysteine (Fig.3.1.3 A and B, in section 3.1.2) *in vivo*. In the cysteine synthesis pathway from methionine, MccB, which has homocysteine γ-lyase activity is the last enzymatic step that convertes cystathionine to cysteine (Fig. 1.2, in section 1.2.1). Interestingly, the mutation of *mccB* of *C. acetobutylicum* just resulted in a drastic growth defect instead of being lethal growth in the presence of methionine as sole sulfur source. This result indicated that there could be a second uncharacterized cystathionine γ-lyase in this organism. A similar phenotype occurred in *B. subtilis*. The mutation of the *mccB* gene (located in the *yrrT* operon) of *B. subtilis* was not lethal in the presense of methionine or homocysteine as sole sulfur source (Hullo *et al.*, 2007).

Growth of *C. acetobutylicum* is characterized by two distinct phases, acidogenic and solventogenic growth phase. Previous observations indicated that the expression of the operon (CAC0102-CAC0110) and the *ubiGmccBA* operon were strongly upregulated during solventogenic growth compared to acidogenic growth in a

phosphate-limited continuous culture (Grimmler *et al.*, 2010). This result suggested that more cysteine (sulfur source) was needed during solventogenic growth as compared to acidogenic growth in the phosphate-limited continuous culture. However, measurement of the residual sulfate showed that less sulfate was consumed during solventogenic growth (in section 3.1.3). To further test, whether cysteine was sufficient or not during solventogenic growth in a phosphate-limited continuous culture, the *fer* mutant, affecting sulfate reduction, was cultured by directly adding cysteine as sole sulfur source (Fig.3.1.4, , in section 3.1.3). Despite enough cysteine was supplied, the *fer* mutant could not be complemented during solventogenic growth. These results indicated that the cysteine metabolism was affected by unknown factors and the cysteine assimilation in the metabolism was reduced. *C. acetobutylicum* could not metabolize the sulfur source, cysteine, efficiently to support normal growth during solventogenic growth in a phosphate-limited continuous culture. Solventogenic growth could rely on key sulfur-containing components, such as several Fe-S-containing proteins. As a result, the expression of operons related to cysteine biosynthesis was strongly induced. In contrast, expression patterns of the operon (CAC0102-CAC0110) and the *ubiGmccBA* operon did not exhibit differences in acidogenesis and solventogenesis in batch fermentations of *C. acetobutylicum* (Alsaker and Papoutsakis, 2005). In order to keep the steady-state growth, there is only 0.5 mM phosphorus source in phosphate-limited continuous culture (Bahl *et al.*, 1982). Therefore, it could be speculated that phosphorus source limitation possibly affects cysteine metabolic efficiency during solventogenic growth in phosphate-limited continuous culture. However, it is not clear how phosphorus source limitation affects cysteine metabolism. Several questions remain to be answered in this respect. Unlike the *fer* mutant, the *mccB* mutant could grow in phosphate-limited continuous culture, but grew weaker and thus produced less products during solventogenic growth, compared to the wild type (Fig3.1.4, Fig.3.1.8 and Table 3.1.4, in section 3.1.3). The microarray results showed that the expression of almost all genes with a putative function in cysteine biosynthesis and metabolism was depressed during solventogeinic growth of the *mccB* mutant in phosphate-limited continuous

culture (Table 3.1.5 and Fig.1.2). In *B. subtilis*, a null mutation in the *cysK* gene encoding an *O*-acetylserine-lyase resulted in constitutive expression of almost all genes related to cysteine metabolism and was considered as a global negative regulator of genes involved in cysteine metabolism (Albanesi *et al.*, 2005). Hence, in analogy MccB could be seen s a possible global regulator of genes involved in cysteine metabolism during solventogeinic growth of *C. acetobutylicum.*

To eliminate the effect of phosphate limitation on sulfur metabolism, a sulfur-limited continuous culture was done to investigate how *C. acetobutylicum* responds to insufficient sulfur source during solventogenic growth. The phenotype and product concentrations were similar during acidogenic growth in presence of 0.4 and 0.55 mM sulfate in continuous culture, but changed significantly during solventogenic growth (Fig3.1.6, Fig.3.1.7 and Table 3.1.1, in section 3.1.3). These results indicated that *C. acetobutylicum* could not adapt to sulfur source deficiency during solventogenic growth in continuous fermentation at a dilution rate of 0.075 h^{-1}. Measurement of glucose uptake and optical density indicated that cells were gradually washed out (Fig.3.1.7). That was probably why 0.2 mM sulfate was determined at 264 h in this continuous culture (Table 3.1.1). From this perspective, it could be proposed that sufficient sulfur source is critical for the growth and metabolism of *C. acetobutylicum* in the solventogenic phase. Microarray results indicated that 261 genes had a significantly different transcription level in a continuous fermentation in the presence of 0.4 mM sulfate as compared to the presence of 0.55 mM sulfate (Table 3.1.2 and Table 3.1.3, in section 3.1.4). Expression of the majority of these 261 genes was downregulated. Since *C. acetobutylicum* grew poorly and failed to complete the transition from acidogenic to solventogenic growth, it was reasonable that the expression of genes related to solventogenesis, amimo acid biosynthesis and sugar transporters were downregulated. Surprisingly, the *ubiGmccBA* operon was not regulated and the CAC0102-CAC0110 operon was downregulated in response to sulfur insufficiency. This result further indicated that *C. acetobutylicum* can not respond to sulfur source deficiency during solventogenic growth in continuous

fermentations. Interestingly, among the genes whose expression was strongly induced (Table 3.1.3, in section 3.1.4), the *ldh* gene, the iron transporter genes and the riboflavin formation genes were included. These genes were also demonstrated to be induced significantly in the fermenations of *C. acetobutylicum* under conditions of iron limitation and the inactivation of the *fur* gene (*fur*, ferric uptake regulator, CAC1682) (Vasileva *et al.*, 2012). A larger number of Fe-S-containing proteins, such as pyruvate:ferredoxin oxidoreductase (PFOR) and ferredoxin, play important roles in metabolism of anaerobic bacteria (Ayala-Castro *et al.*, 2008). Hence a similar mechanism could occur under the conditions of iron or sulfur limitation. Moreover, the *ldh* gene encoding the lactate dehydrogenase was also induced during cysteine limitation in *Clostridium perfringens* (Andre *et al.*, 2010). Furthermore, the expression of the *pflBA* genes was strongly induced during solventogenic growth in presence of 0.4 mM sulfate. The pyruvate formate lyase (PFL, encoded by *pflB*) and its activating enzyme (PFL-AE, encoded by *pflA*) are involved in conversion of pyruvate and CoA to formate and acetyl CoA. This reaction is involved in the biosynthesis of nucleotides in *C. acetobutylicum* (Hönicke and Ehrenreich 2016, submitted). Importantly, the activity of PFL-AE is depended on Fe-S clusters (Buis and Broderick, 2005). In the present study, sulfur insufficiency probably affected the activity of PFL-AE, and then affected the conversion of pyruvate and CoA to acetyl CoA and formate. Pyruvate, as an important metabolic intermediate, is precursor both of acetyl CoA and lactate in *C. acetobutylicum*. In the present work, the expression of the *ldh* gene was strongly upregulated and 6.4 mM L-lactate was determined in the presence of 0.4 mM sulfate at 264 h (Table 3.1.1). These results revealed that the lactate formation pathway was probably a mechanism to dual with Fe and S limitations for clostridia, since the lactate producing pathway was activated when clostridia are cultured under sulfur or iron starvation.

In summary, despite less sulfur source was needed during solventogenic growth than that during acidogenic growth, solvents production and normal metabolism relied more on the sulfur source availability during solventogenic growth in

C. acetobutylicum. Considering that Fe-S-containing proteins play important roles in the metabolism during solventogenic growth, the biosynthesis and metabolism of cysteine are strictly regulated and the corresponding regulation mechanism is complicated in *C. acetobutylicum*. The observations presented here provided first insights in the sulfur regulation network in *C. acetobutylicum*.

4.2 Cellular functions of the thioredoxin- and glutathione-dependent reduction pathways in *Clostridium acetobutylicum*

Based on the whole genome sequence analysis, there are GSH- and Trx-dependent reduction systems in *C. acetobutylicum* (Nolling *et al.*, 2001). ClosTron mutants of *gcs* and *trxB* genes coding for major components of these two systems were constructed to investigate the effects of each system on the metabolism of *C. acetobutylicum*. In order to compare the phenotypes and trancriptomes between the wild type and the mutants during steady-state growth, the phosphate-limited continuous cultures were completed (Fig.3.2.4, Fig.3.2.5 and Fig.3.2.6, in section 3.2.2). These results provide valuable data to understand the GSH and Trx metabolism in *C. acetobutylicum*. Due to the biphasic fermentation of *C. acetobutylicum* and its many interdependencies, the phenotype of many mutants is hard to characterize by batch fermentation. The *gcs* and *trxB* mutants fall into this category. The growth phenotypes of the wild type and the *gcs* and *trxB* mutants were almost identical to batch fermentations (Fig.3.2.3). However, the continuous fermentation results allowed a more fine-grained characterization of phenotypic differences between the wild type and the mutants.

The *gcs* mutant exhibited a lower optical density during acidogenic and solventogenic growth as compared to the wild type (Fig.3.2.4, Fig.3.2.5, in section 3.2.2). Hence, the glucose consumption and concentrations of fermentation products were also less than with the wild type during acidogenic and solventogenic growth. The inactivation of *gcs* affected the growth, suggesting that the GSH-dependent reduction system plays an important role for the viability of *C. acetobutylicum*. This is in agreement to yeast

and *Candida albicans* where GSH was found to be essential for normal metabolism (Baek *et al.*, 2004; Grant *et al.*, 1996). On the other hand, this is different from *Escherichia coli* where GSH is totally dispensable for normal growth (Apontoweil and Berends, 1975; Fuchs and Warner, 1975; Greenberg and Demple, 1986). In order to further study, which genes and metabolic pathways were affected in the *gcs* mutant, DNA microarray technology was used to compare the transcriptomes of the wild type and the *gcs* mutant during acidogenic and solventogenic growth (Table 3.2.2, Table 3.2.3, Table 3.2.4 and Table 3.2.5, in section 3.2.3). The major differences were several upregulated genes. They were related to glucose transport, chemotaxis and flagella biosynthesis. *C. acetobutylicum* is a motile bacterium and the flagellum is responsible for locomotion (Belas, 2014; Gutierrez and Maddox, 1987). Chemotaxis is a normal character of motile bacteria and is in response to a chemical stimulus. According to chemotaxis, the bacteria direct their movement to find substrate molecules or flee from poisons (Maki *et al.*, 2000; Wadhams and Armitage, 2004). The genes related to chemotaxis and flagella biosynthesis were induced significantly in the *gcs* mutant. This result suggested that the inactivation of the GSH-dependent reduction system could trigger chemotaxis in *C. acetobutylicum*. It can be speculated that *C. acetobutylicum* could try to flee oxidative stress that could be signaled by the unfunctional GSH system. *C. acetobutylicum* could improve cellular resistance to oxidative stress, acid, and butanol when the GSH biosynthetic capability was introduced into the cell by cloning and over-expressing the *gshAB* genes frome *E. coli* (Zhu *et al.*, 2011). This observation confirms the finding that GSH plays an important role for increasing the stress tolerance of *C. acetobutylicum*.

The *trxB* mutant exhibited identical growth phenotype and glucose consumption as the wild type during acidogenic growth (Fig.3.2.4, Fig.3.2.6). The acid production was even higher than that of the wild type (Table 3.2.1, in section 3.2.2). However, during sloventogenic growth the *trxB* mutant reached a significantly lower optical density. The solvent production was less and the acetone-to-butanol ratio was higher than with the wild type. The results suggested that the Trx-dependent reduction

system plays an important role in the solventogenic growth phase. The acetone to butanol ratio is normally 1:2 in the typical batch and phosphate-limited continuous culture (Grimmler *et al.*, 2010; Jones and Woods, 1986). For *C. acetobutylicum*, several attempts related to changing the acetone/butanol ratio in the fermentation have been reported. *C. acetobutylicum* produces alcohol (butanol and ethanol) as main products in the presence of artificial electron carriers, such as neutral red and methyl viologen (Girbal *et al.*, 1995; Honicke *et al.*, 2012). The same phenotype occurs when the culture is sparged with carbon monoxide (CO) or when glycerol was added to glucose as carbon source in continuous culture (Datta and Zeikus, 1985; Vasconcelos *et al.*, 1994). In brief, all these mentioned exogenous manipulations affect the redox balance of *C. acetobutylicum* and increase the availability of reducing equivalents. As a result, the acetone/butanol ratio changes in the end-products compared with the typical batch fermentation. In the present study, the acetone to butanol ratio increased during solventogenic growth. Considering that the Trx-dependent reduction system is critical for keeping cellular protein disulfide/dithiol redox balance in many bacteria (Lu and Holmgren, 2014), this result revealed that the redox balance in the *trxB* mutant during solventogenic growth probably changed as compared to the wild type. The Trx-dependent reduction system plays an important role in maintaining the redox balance of *C. acetobutylicum*. Compared to the expression profile of the wild type, the most remarkable difference was the expression profile of genes related to sporulation in the transcriptome of the *trxB* mutant. Many sporulation genes were strongly induced during both acidogenic and solventogenic growth of the *trxB* mutant (Table 3.2.6, Table 3.2.7, Table 3.2.8 and Table 3.2.9, in section 3.2.4). In the batch culture, the solvent production is coupled with sporulation by the global regulator *spoOA* (CAC2071) in *C. acetobutylicum* (Scotcher and Bennett, 2008). The specific mRNAs of *spoOA*, *sigE* and *sigF* were found at higher amounts indicating the initation of sporulation in the *trxB* mutant (Jones *et al.*, 2011). In *Bacillus subtilis*, the expression of genes for the initiation of sporulation were also upregulated as in the *trxA* mutant (Mostertz *et al.*, 2008). This result was caused by the limition of cysteine and methionine following the depletion of TrxA in *B. subtilis*. Probably, a similar

mechanism exists in the *trxB* mutant of *C. acetobutylicum*.

Despite the GSH- and Trx-dependent reduction systems were assumed to play a similar role in maintaining the reductive environment in bacteria (Carmel-Harel and Storz, 2000; Rietsch and Beckwith, 1998), our observations presented here suggested different roles for the GSH- and Trx-dependent reduction systems in *C. acetobutylicum*. On the one hand, the phenotypes of both mutants were clearly different in continuous fermentations. On the other hand, the comparison of transcriptomes did not show identical expression profiles in both mutants. However, it should be noted that a second set of thioredoxin and thioredoxin reductase genes, *trxA2-trxB2* (CAC3082-CAC3083), can be identified in the *C. acetobutylicum* genome. Its role remains elusive until now. This is the first study to knock out the genes of GSH- and Trx-dependent reduction systems in *C. acetobutylicum*. More research is needed for deeper insights.

4.3 Confirmation of glucose transporters through targeted mutagenesis and transcriptional analysis in *Clostridium acetobutylicum*

The ClosTron technology is an excellent targeted mutagenesis tool and a widespread technology to construct single gene mutants in clostridia, especially in *C. acetobutylicum* (Cooksley *et al.*, 2012; Lehmann *et al.*, 2012). Under certain instances, mutagenesis of multiple genes is desirable in a sigle strain. Many multiple gene deletion mutants have been reported in *E. coli* and *B. subtilis* (Hullo *et al.*, 2007; Steinsiek and Bettenbrock, 2012). In ClosTron mutants of *C. acetobutylicum*, the *ermB* gene, as a selection maker, can be excised using FLP-recombinase and then the mutants lose erythromycin resistance (Heap *et al.*, 2010). Thus, multiple genes could be successively inactivated using ClosTron technology. Three double mutants, *agrA*/*spoOA*, *pta*/*adc* and *pta*/*ctfA*, were successfully contructed, using this strategy in previous studies (Heap *et al.*, 2010; Lehmann *et al.*, 2012). However, this strategy sometimes has problems and does not always work efficiently. In the present work, a double gene mutant (*glcG*/*glcCE*) was successfully constructed using ClosTron

technology without removing the antibiotic marker (erythtomycin resistance) (in section 3.3.1 and in section 3.3.2). The whole procedure was extremely efficient, rapid, and just requires increasing erythromycin concentration in selection plates and verifying putative tranformates promptly. The results of gene sequence and Southern blot showed that the double mutant was correct one, as designed. As a result, the work described here provided a novel solution how to construct a double mutant in *C. acetobutylicum* using ClosTron technology.

For *C. acetobutylicum*, it is well known that the fermentation can be divided in two distinct phases (Jones and Woods, 1986; Monot *et al.*, 1982). Most of the ClosTron mutants reported in the literature grow and consume carbon source similar to wild type in batch culture (Cooksley *et al.*, 2012; Lehmann *et al.*, 2012). Thus, it was necessary to construct a better fermentation model to investigate the differences between the wild type and the mutants. Then, the phenotype of the mutation could be more easily clarified. Phosphate-limited continuous fermentations makes it possible to compare the phenotypes of the wild type and the mutants during acidogenic and solventogenic growth (Grimmler *et al.*, 2010; Janssen *et al.*, 2010). In the present work, the results showed that it was a big advantage to study the glucose transporters in *C. acetobutylicum* using phosphate-limited continuous fermentation. The glucose consumption of the wild type and the *glcG* mutant were identical in the batch fermentations (Xiao *et al.*, 2012). In contrast, the glucose consumption of the wild type and the *glcG* mutant was clearly different during solventogenic growth in phosphate-limited continuous fermentation. Due to the same growth rate of all the cells in the continuous culture, it was possible to analyze the difference expression profiles caused by the gene mutation between the wild type and the mutant.

In *C. acetobutylicum*, hexoses are transported to the cytoplasm by PTS and there are in total 13 putative phosphotransferases (substrate-specific proteins, named as EⅡ in PTS) including one that is encoded on the pSOL plasmid (Mitchell and Tangney, 2005; Servinsky *et al.*, 2010). Among these phosphotransferases, the glucose-specific EⅡ

(GlcG, encoded by CAC0570) has been studied (Tangney and Mitchell, 2007; Xiao *et al.*, 2012). Despite inactivation of the *glcG* gene, the *glcG* mutant utilized glucose as efficiently as the wild type in batch fermentation. This result revealed that, like *E. coli*, *S.cerevisiae*, and other microbes, *C. acetobutylicum* has more than one glucose transporter and some of them are bifunctional (Hunter and Kornberg, 1979; Maier *et al.*, 2002; Steinsiek and Bettenbrock, 2012). The results described in this study are consistent with these previous observations. Disrupting the glucose-specific PTS Enzyme II led to a significant change in the expression of a cellobiose-specific PTS (CAC0383-CAC0386). Inactivation of both, the glucose-and the cellobiose-specific PTSs abolished growth in the phosphate-limited continuous culture, indicading that glucose transport in the *glcG* mutant takes placec mainly via the cellobiose-specific PTS (Fig.3.3.6 and Tabel 3.3.1, in section 3.3.3 and in section 3.3.5). Interestingly, this was observed only in the *glcG*/*glcCE* mutant; the *glcCE* mutant and the wild type had identical growth phenotypes in the phosphate-limited continuous culture. Inactivation of *glcCE* did not lead to changes in expression levels of the other 12 phosphotransferases in *C. acetobutylicum* (Tabel 3.3.1). Accordingly, the results showed that GlcG was the predominant glucose transporter in *C. acetobutylicum*. In the wild type growing on glucose, other glucose transporters are repressed. This phenomenon was extensively studied in *E. coli*. The glucose-specific PTS is the main glucose uptake system in *E. coli*. Moreover, the galactose transporter, the maltose transpoter and the mannose transporter can also transport glucose into the cytoplasm. Deletion of each of these three transporters do not lead to changes in the growth rate and gene expression profiles of remaining glucose transporter systems (Steinsiek and Bettenbrock, 2012). It is important to note that the *glcG*/*glcCE* mutant still grows in batch fermentation, indicating that another PTS except the glucose- and cellobiose-specific transporters could transport glucose into the cytoplasm. Analysis and comparison of transcriptomes of the *glcG/glcCE* mutant and the wild type in exponential phase of batch fermentations that the mannitol-specific tansporter could be a third glucose transporter in *C. acetobutylicum* (Tabel 3.3.2, in section 3.3.6). In summary, *C. acetobutylicum*, like many other bacteria, utilizes glucose as a preferred

sugar. The data presented here provide a foundation to study the glucose uptake mechanism in *C. acetobutylicum*.

5 Summary

Clostridium acetobutylicum is a rod-shaped, Gram-positive, spore-forming anaerobe that is of major biotechnological interest because it can produce organic solvents (acetone, butanol and ethanol) in industrial fermentations. Successful utilization of *C. acetobutylicum* in industry requires a detailed understanding of its physiology and regulatory mechanisms, especially concerning solvent formation. In this dissertation, cysteine biosynthesis pathways, GSH- and Trx-dependent reduction pathways and a glucose transporter were investigated in *C. acetobutylicum*. Six genes that are involved in these pathways were inactivated using ClosTron technology and the ClosTron mutants were studied in batch and continuous culture. The main conclusions are as follows:

1. The clustered genes (CAC0102-CAC0110) are involved in the conversion of sulfate to sulfite, which is the first reduction step in sulfur assimilation in *C. acetobutylicum*.

2. The *ubiGmccBA* operon is involved in the conversion of methionine to cysteine. The inactivation of the *mccB* gene is not a lethal mutation for *C. acetobutylicum*, indicating that another enzyme can substitute MccB to catalyze cystathionine to cysteine formation.

3. *C. acetobutylicum* consumes more sulfur source during acidogenic growth than during solventogenic growth. Furthermore, regulation of sulfur metabolism is significantly different during acidogenic and solventogenic growth.

4. Sulfur source starvation not only affects growth in solventogenesis of *C. acetobutylicum*, but also affects the normal transition from acidogenesis to solventogenesis.

5. *C. acetobutylicum* has a GSH-dependent reduction pathway, which plays an important role in metabolism. Inactivation of this pathway affects growth, the expression of genes of flegella synthesis and chemotaxis of *C. acetobutylicum*.

6. *C. acetobutylicum* has a Trx-dependent reduction pathway which plays an

important role in metabolism. Inactivation of *trxB* gene affects growth, acetone-to-butanol ratio and expression of genes for initiation of sporulaiton during solventogenic growth.

7. A simple approach was used to construct a double mutant in *C. acetobutylicum*. The *glcG/glcCE* mutant was constructed and verified using this approach.

8. GlcG, encoded by *glcG* (CAC0570), is the predominant glucose transporter in *C. acetobutylicum*. The cellobiose-specific transporter, encoded by CAC0383-CAC0386, can also transport glucose in the cytoplasm, which was shown after the GlcG gene was inactivated in *C. acetobutylicum*.

6 Zusammenfassung

Clostridium acetobutylicum ist ein stäbchenförmiger, Gram-positiver, sporenbildender Anaerobier, der von großem biotechnologischem Interesse ist, da er organische Lösungsmittel, wie Aceton, Butanol sowie Ethanol, in industriellen Fermentationen als Fermentationsprodukte bildet. Eine erfolgreiche Verbesserung der Anwendbarkeit in der Industrie erfordert ein detailliertes Verständnis über die Physiologie und Regulationsmechanismen von *C. acetobutylicum*, vor allem hinsichtlich der Bildung der Lösungsmittel. In dieser Dissertation wurden die Biosynthesewege der Cystein-Biosynthese, die GSH- und Trx-abhängigen Reduktionswege und ein Glukosetransporter von *C. acetobutylicum* untersucht. Sechs Gene, die an diesen Wegen beteiligt sind, wurden mit Hilfe der ClosTron-Technologie inaktiviert und die ClosTron Mutanten wurden in diskontinuierlichen und kontinuierlichen Kulturen charakterisiert. Die wichtigsten Schlussfolgerungen lauten wie folgt:

1. Der erste Reduktionsschritt der Schwefelassimilation in *C. acetobutylicum* ist die Umwandlung von Sulfat zu Sulfit. Daran sind die hintereinanderliegenden Gene (CAC0102- CAC0110) beteiligt.

2. Das *ubiGmccBA* Operon ist an der Umwandlung von Methionin zu Cystein beteiligt. Die Inaktivierung des Gens MccB ist nicht letal für *C. acetobutylicum*, was darauf hinweist, dass ein weiteres Isoenzym von *mccB* existiert, welches die Umsetzung von Cystathionin zu Cystein katalysieren kann.

3. C. acetobutylicum verbraucht mehr Schwefel während des acidogenen Wachstums als während des solventogenen Wachstums. Des Weiteren ist der Unterschied in der Regulierung des Schwefelmetabolismus zwischen acidogenem und solventogenem Wachtum signifikant.

4. Schwefelmangel hat nicht nur einen Effekt auf die Bildung von Lösungsmittel in *C. acetobutylicum,* sondern wirkt sich auch auf das Umschalten zwischen Säure- und Lösungsmittelbildung aus.

5. *C. acetobuylicum* besitzt einen GSH-abhängigen Reduktionsweg, welcher eine wichtige Rolle im Metabolismus spielt. Eine Inaktivierung beeinträchtigt das Wachstum und führt zur Expression von Genen der Flagellensynthese und der Chemotaxis.

6. *C. acetobutylicum* besitzt einen Trx-abhängigen Reduktionsweg, welcher eine wichtige Rolle im Metabolismus spielt. Wird das Gen *trxB* inaktiviert, wird das Wachstum, das Verhältnis von Aceton zu Butanol und die Expression der Gene für die Initiation der Sporulation während der Lösungsmittelbildung verändert.

7. Für die Konstruktion einer Doppelmutante in *C. acetobutylicum* wurde ein vereinfachter Ansatz verwendet. Damit wurde eine *glcG*/*glcCE* Doppelmutation konstruiert und anschließend verifiziert.

8. GlcG, kodiert durch *glcC* (CAC0570), ist der wichtigste Glukosetransporter in *C. acetobutylicum*. Nachdem das GlcG-Gen inaktiviert wurde zeigte sich, dass auch der Cellobiose spezifische Transporter, kodiert durch die ORFs CAC0383-CAC0386, Glukose in das Zytoplasma transportieren kann.

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Publications in the context of PhD thesis:

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In preparation

1. **<u>Ziyong Liu</u>**, Wolfgang Liebl, Armin Ehrenreich. Cysteine synthesis pathways and global regulation of gene expression in response to cysteine availability during solventogenic growth in *Clostridium acetobutylicum.*In preparation.

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