1 Introduction

Today, most amino acids are produced by fermentation. Methionine, an important additive in animal nutrition, is however still synthesized by a chemical process yielding the racemic mixture D,L-methionine. Due to hazardous raw materials required for chemical synthesis and indications that the L-isomer has a better bioavailability for breeding poultry and other animals (Baker and Boebel, 1980; D'Mello and Lewis, 1978; Friedman, 1999), biotechnological production, allowing exclusive formation of L-methionine, has gained increasing interest in recent years. One of the most promising candidates for such a process is *Corynebacterium glutamicum*, a Gram-positive soil bacterium, which displays the dominating microorganism applied in industrial production of other amino acids including L-glutamate and L-lysine (Eggeling and Sahm, 1999). In *C. glutamicum*, biosynthesis of L-methionine originates from aspartate and is thus closely linked to central metabolism (Figure 1.1).

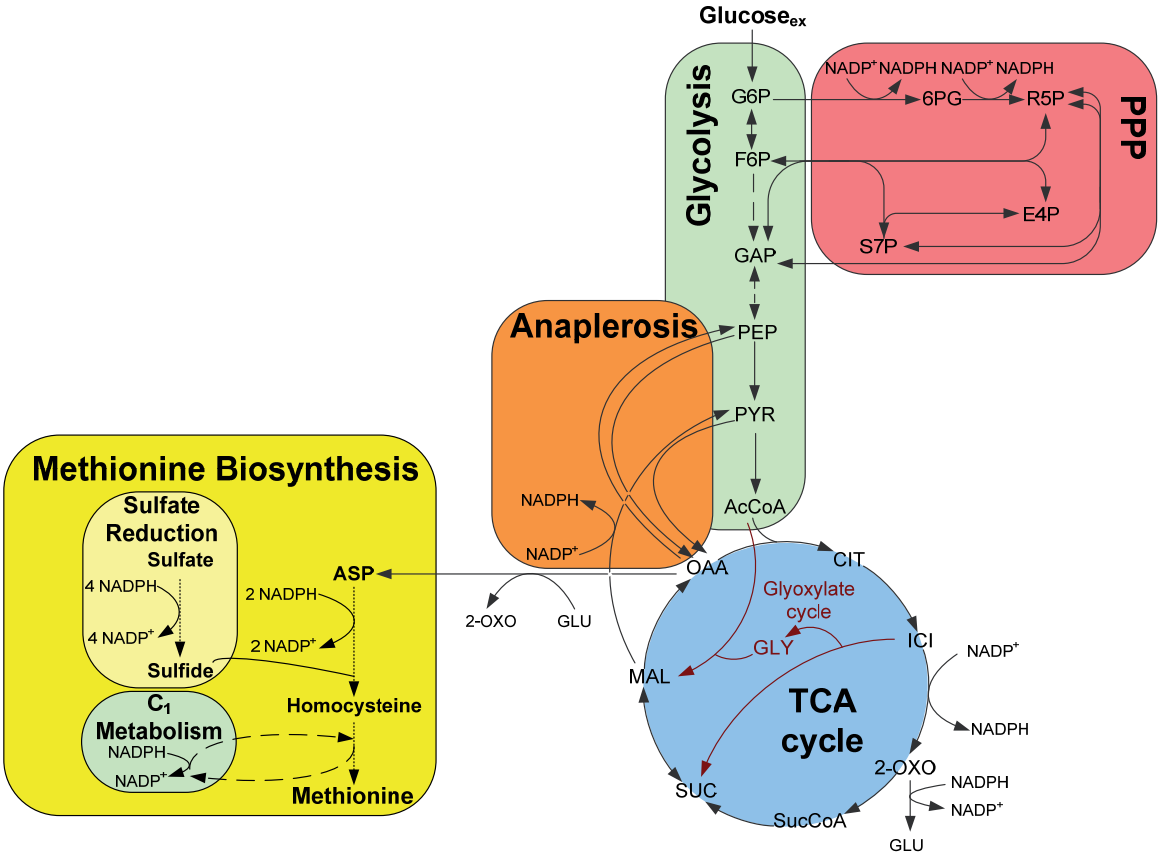

Figure 1.1: Central carbon metabolism and L-methionine biosynthesis in *C. glutamicum* from glucose and sulfate.

C. glutamicum exhibits a variety of central metabolic pathways, comprsing glycolysis, pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle, glyxoylate cycle and anaplerosis. These pathways provide the carbon skeleton and the required energy and reduction power. L-methionine biosynthesis requires eight molecules of NADPH using sulfate, the most common sulfur source. Thus, it is the most expensive amino acid in terms of reduction power (Mampel, et al., 2005). Hereby, the reduction of sulfate sulfur is one of the most costly steps (Krömer, et al., 2006a).

Stimulated by the high relevance towards biotechnological exploitation, the biosynthetic pathway for L-methionine has been studied intensively involving the unraveling of the underlying regulatory mechanisms (Haitani, et al., 2006; Hwang, et al., 1999; Hwang, et al., 2002; Krömer, et al., 2006a; Lee and Hwang, 2003; Lee, et al., 2009; Park, et al., 1998; Rey, et al., 2005; Rey, et al., 2003; Rückert, et al., 2003; Trötschel, et al., 2008). Hereby, the discovery of the transcriptional regulator McbR displays a remarkable finding (Rey, et al., 2005; Rey, et al., 2003). McbR represses the expression of almost all genes of the L-methionine biosynthesis (Rey, et al., 2005) and restricts the level of the corresponding proteins (Rey, et al., 2003) (Figure 1.2).

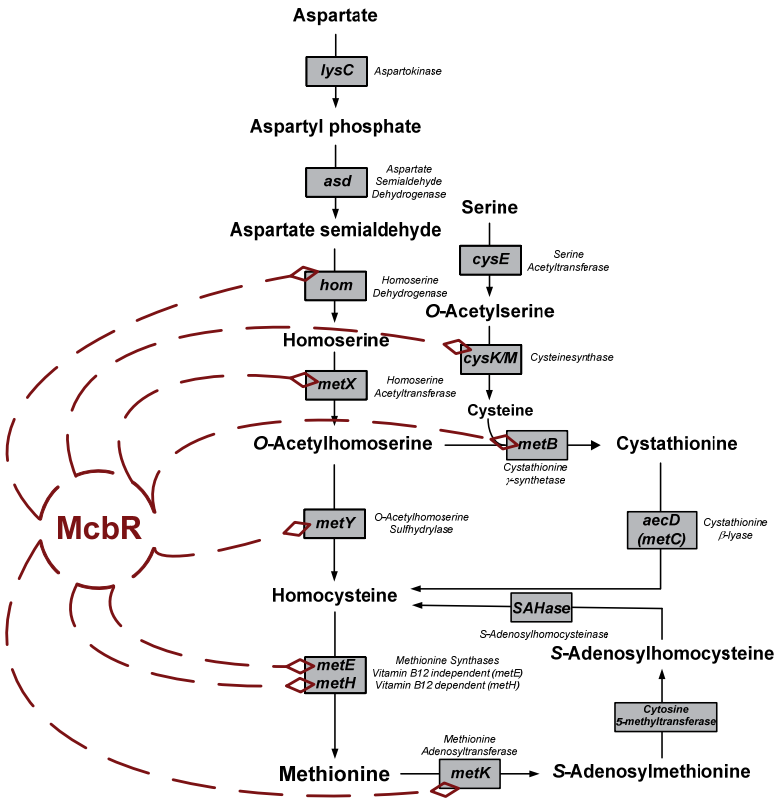

Figure 1.2: Repression of L-methionine biosynthesis in *C. glutamicum* by the transcriptional regulator McbR (Lee, 2005; Rey, et al., 2005).

It was found that McbR also regulates the cysteine biosynthesis and the sulfur assimilation pathway and causes various secondary changes in the expression of genes without a McbR binding site, suggesting a global regulatory role in *C. glutamicum* (Rey, et al., 2005). Deletion of this repressor was regarded as a first major step towards an L-methionine producing strain. Metabolic studies showed that a *mcbR* deletion mutant of *C. glutamicum*, however, does not overproduce L-methionine (Krömer, et al., 2006a). Despite this could be partly attributed to the formation of a novel side product, homolanthionine, the overall response to the genetic modification was rather complex and is not fully understood. At this point it became clear that a more detailed analysis and understanding of the underlying pathways are required towards effective rational improvement for L-methionine overproduction.

For target finding, systems-oriented studies, employing omics technologies, display a valuable start of art approach (Lee, et al., 2005; Wendisch, et al., 2006; Wittmann and Becker, 2007). Hereby, the analysis of the metabolome, i.e. identification and quantification of intracellular metabolites, has been promised as key tool for profiling of biological systems towards superior production strains (Akinterinwa, et al., 2008; Buchinger, et al., 2009; Neuweger, et al., 2009; Wittmann, et al., 2002a). This technique is especially valuable for the investigation of selected metabolites of the complex L-methionine biosynthesis reaction network in *C. glutamicum* (Krömer, et al., 2005; Krömer, et al., 2006a) and seems a promising tool for a better understanding of this part of the metabolism.

Sampling is especially critical in metabolome analysis due to high exchange rates and small pool sizes of the metabolites of interest (Wittmann, et al., 2004c). Undoubtedly, metabolome analyses and also the conclusions drawn from the obtained data rise and fall with appropriate sampling, an aspect that has been not appropriately considered so far.

As central task of the present work, valid approaches for efficient metabolome analyses of *C. glutamicum* should be developed and subsequently applied for investigation of the methionine biosynthesis and supporting pathways. This should provide a detailed understanding of the underlying pathways and the regulation of the L-methionine biosynthesis towards superior strains.

2 Objectives

The main object of the present study was the characterization of the metabolic properties of *Corynebacterium glutamicum* via systems biology techniques in order to derive strategies for future biotechnological methionine production. The biosynthesis of L-methionine involves various supporting pathways. Due to the high relevance of the transcriptional repressor McbR, releasing almost all genes of the methionine, cysteine and sulfur metabolism from repression, the corresponding deletion strain *C. glutamicum* Δ*mcbR* should be studied in detail on the level of fluxome and metabolome. Additionally, engineering of the NADPH metabolism, providing the most important cofactor for L-methionine-biosynthesis, should be achieved by genetic engineering of *C. glutamicum*. Furthermore, alternative sulfur sources for *C. glutamicum* were supposed to be analyzed concerning their potential for L-methionine biosynthesis. These physiological studies should be combined with the data from different omics techniques, including metabolomics and fluxomics. Due to the lack of suitable sampling methods for metabolome analyses of bacteria, especially *C. glutamicum*, techniques first should be developed and validated prior to application for the detailed characterization of L-methionine biosynthesis in *C. glutamicum*.

3 Theoretical Background

3.1 *Corynebacterium glutamicum* as Biotechnological Production Strain

C. glutamicum is a fast-growing, Gram-positive, rod-like, non-motile, aerobic bacterium (Figure 3.1).

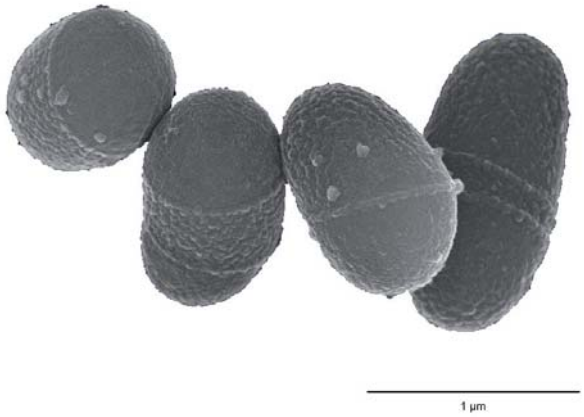

Figure 3.1: Raster electron micrograph of *Corynebacterium glutamicum* cultivated on minimal glucose medium.

C. glutamicum cells can be isolated from soil, soil contaminated with bird feces, sewage, manure, vegetables, and fruits (Liebl, 2005). The sequenced type strain of this species, *C. glutamicum* ATCC 13032, was originally isolated from a soil sample of the Ueno Zoo in Tokyo (Japan) due to its unique ability to secrete significant amounts of glutamate under suitable conditions (Udaka, 1960). Nowadays, *C. glutamicum* is applied in the fermentative production of amino acids, in particular L-glutamate and L-lysine, which play important roles in human and animal nutrition, respectively (Leuchtenberger, 1996). Biotechnological production of amino acids by fermentation with *C. glutamicum* represents a large market volume estimated annually at 1,500,000 metric tons L-glutamate and 900,000 metric tons L-lysine (Kohl and Tauch, 2009). Genetic engineering of this organism is established due to the available genome sequence. With regard to the importance of *C. glutamicum* for the biotechnological industry, the genome of the type strain ATCC 13032 was sequenced independently by different groups (Haberhauer, et al., 2001; Ikeda and Nakagawa, 2003;

Kalinowski, et al., 2003). Extensive biochemical studies in the last decades as reviewed in a recent handbook on *Corynebacterium glutamicum* (Eggeling and Bott, 2005) have contributed to a detailed knowledge of the reactions of lysine and glutamate biosynthesis and central metabolism in this microorganism (Wittmann and Becker, 2007). Beside glutamate and lysine, a variety of other amino acids as well as nucleotides and vitamins like pantothenate are produced by fermentation with *C. glutamicum* (Hüser, et al., 2005).

3.2 Industrial Methionine Production

The essential sulfur-containing amino acid methionine is the world's second most widely sold amino acid. It is notably used in feed for chickens and piglets. The global methionine animal feed market is estimated as 2.2 billion US$ in 2009 for a total output of 600 kilotonnes (FeedInfo, 2009). Although the sales market increases very fast, only a small number of companies such as Evonik (Essen, Germany) or Sumitomo (Sendai, Japan) are involved in global manufacturing of methionine. According to an OECD report, consumption of animal protein in China and Brazil, mainly chicken and pork, is growing by around 10 % and 6 %, respectively, per year. On the basis of these forecasts, the global market for methionine as a food additive could potentially grow by close to 6 % a year for the foreseeable future (OECD-FAO, 2009). Currently, feed grade methionine is exclusively produced as a racemic mixture by chemical synthesis. This process is based on the usage of hazardous chemicals and is accompanied by a costly purification of the product (Figure 3.2).

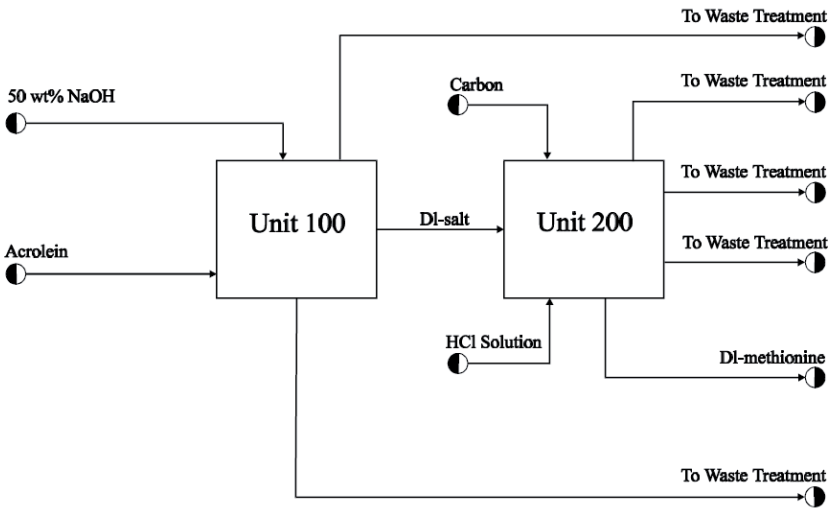

Figure 3.2: Schematic overview of the chemical production of D,L-methionine (Kawabe, et al., 2007).