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Study and engineering of methanol assimilation and L-lysine production in the thermotolerant bacterium *Bacillus methanolicus*

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PREFACE

The work presented in this thesis was carried out at Department of Biotechnology, SINTEF Materials and Chemistry, and at Department of Biotechnology, Norwegian University of Science and Technology (NTNU). It was financially supported by the Research Council of Norway.

The work has been supervised by Research Director and Adjunct Professor Trond E. Ellingsen at SINTEF Materials and Chemistry and Professor Svein Valla at NTNU. I would like to thank them both for offering me the Ph.D. position and for giving me the opportunity to learn from their knowledge and experience. Specifically, I would like to thank Trond for his care and commitment, for trusting me with a substantial share of responsibility throughout the project, and for introducing me to the challenges of the life behind the scenes of everyday research and development. Furthermore, I would like to thank Svein for valuable guiding of an amateur geneticist within the field of molecular biology, for his invincible optimism, his good humor and his ability to cheer anyone, anywhere, anytime.

I would like to acknowledge the ideas and work of Michael C. Flickinger, Arne R. Strøm, Kjell D. Josefsen and my supervisors for preparing the applications that lead to the initiation of the project "Microbial production of L-lysine from methanol for use as feed supplement to fish and animals", and thus laying the foundations for my Ph.D. work and what later was accomplished on this project.

Throughout my Ph.D. work, I have been in the very fortunate position of working in a project group with highly skilled people. In particular, I would like to express my gratitude to Trygve Brautaset who has more or less been involved in all my Ph.D. work. He has supervised and introduced me to new laboratory techniques, new theory and new ideas, and has been a good friend and colleague throughout my work. Furthermore, I would like to thank researchers, engineers and students that have worked on the lysine project throughout the years for their valuable contributions, in particular Kristin F. Degnes and Aline Benichou.

I would like to thank all friends at Department of Biotechnology at SINTEF Materials and Chemistry. You welcomed me warmly and have treated me as an equal

colleague ever since, included me in your social and professional lives, and always offered time to help. Especially I would like to thank Håvard Sletta for his friendship, ideas and help, Kathinka Q. Lystad and Randi Aune for teaching me the secrets of everyday laboratory work and Kjell D. Josefsen for his time and willingness to answer all thinkable and unthinkable scientific questions. Furthermore, I would like to thank Asgeir Winnberg, Geir Klinkenberg and Jan Erik Aastad for sharing their knowledge within the art of constructing and programming useful and useless gadgets: If a device works, it simply doesn't have enough features yet!

Likewise, I would like to thank the genetics group of the Department of Biotechnology at NTNU for guidance and help on the molecular biology laboratory, and for including me in their social network.

My parents and my brother deserve a heartfelt gratitude for being so supportive and for teaching me values I have come to appreciate very much. Their interest in my field is admirable, and I have yet to see a retired civil engineer with as good an insight into microbial production of essential amino acids as my father.

Halvor; thank you for new perspectives, new values and your enriching laughter.

Anita; thank you for your smile, and thank you for your support.

Trondheim, July 2008

Øyvind Mejdell Jakobsen

SUMMARY

Lysine is an essential amino acid and one of the most important biotechnological products with respect to production volume and value. Traditional feedstuffs normally contain less lysine than required by non-ruminants such as pigs and poultry. Feedstuffs may therefore be enriched with lysine to meet the nutritional requirements of the animals. Today's production of lysine exceeds 800,000 tons per year and is based on fermentation processes with the bacterium *Corynebacterium glutamicum* growing on sugar.

The work presented in this thesis was directed towards basic understanding of the biology of the candidate bacterium *Bacillus methanolicus*, in addition to the development of a new methanol-based fermentation process for lysine by this organism. Methanol is considered an alternative and attractive raw material for the biotechnological industry, and *B. methanolicus,* a Gram-positive, aerobic and thermotolerant methylotroph has previously been shown to be a potential candidate organism for high-level amino acid production.

The thesis presents fundamental results on the organization and regulation of genes involved in methanol assimilation by the ribulose monophosphate (RuMP) pathway in *B. methanolicus*. The findings are subsequently exploited from an applied point-of-view to improve the strain's methanol tolerance, an important requirement for a robust methanol-based microbial process. The latter part of the thesis demonstrates improved lysine productivity by overexpressing aspartokinase, a key enzyme of the lysine biosynthesis pathway.

A reliable gene delivery system was required as a basis for metabolic engineering of *B. methanolicus.* The work related to this thesis was therefore initiated by the development of such methods, resulting in an improved protocol for protoplast transformation and a new protocol for reliable transformation of *B. methanolicus* by electroporation.

B. methanolicus was shown to carry a plasmid harboring essential genes for methanol metabolism. The natural plasmid isolated from *B. methanolicus* wild type MGA3 was designated pBM19 and carries genes encoding methanol dehydrogenase

responsible for methanol oxidation and the five enzymes sedoheptulose-1,7-bisphosphatase, fructose-1,6-bisphosphate aldolase, transketolase, 6-phospho-fructokinase and ribulose-5-phosphate 3-epimerase, with assumed roles in methanol assimilation by the ribulose monophosphate pathway. This was the first demonstration of plasmid dependent methylotrophy in any microbial system. We further demonstrated that both chromosomal and plasmid-borne genes involved in methanol metabolism are transcriptionally upregulated in the presence of methanol, and that such upregulation is critical for the cell's methanol tolerance. The methanol sensitivity was shown to be caused by accumulation of toxic formaldehyde, and the cell's ability to detoxify this compound depends on the presence of a functional ribulose monophosphate pathway. Formaldehyde assimilation is dependent on the activity of 3-hexulose-6-phosphate synthase and 6-phospho-3-hexuloisomerase encoded by *hps* and *phi*, respectively. The copy-number of the plasmid-borne genes involved in methanol metabolism was estimated to 10 – 16 per chromosome, while *hps* and *phi* are chromosomally located. By increasing the copy-number of *hps* and *phi*, we were able to increase the cell's methanol tolerance and specific growth rate on methanol medium.

The key enzyme aspartokinase controls the flow of carbon into the aspartate pathway, of which the amino acids lysine, threonine and methionine are end products. To investigate the role of aspartokinase on lysine production by *B. methanolicus*, the genes encoding aspartokinase I and III were cloned and sequenced. In addition to the previously known gene for aspartokinase II, these genes represent three aspartokinase isozymes in this organism. By individual overexpression of each of these wild type genes in *B. methanolicus*, lysine production was demonstrated to increase up to 60-fold to 11 g/l (by overexpression of *yclM* encoding aspartokinase III), without similarly increasing the production of threonine and methionine. Such an increase in lysine production by overexpression of wild type aspartokinase, generally an enzyme efficiently regulated by allosteric feedback inhibition, has previously not been reported.

The findings related to the understanding and engineering of the methanol metabolism and the lysine biosynthetic pathway may contribute towards improved understanding of the biology of *B. methanolicus* and towards the exploitation of this organism for a future methanol-based lysine production process.

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Trygve Brautaset, Øyvind M. Jakobsen, Michael C. Flickinger, Svein Valla and Trond E. Ellingsen. 2004. *Plasmid-dependent methylotrophy in thermotolerant Bacillus methanolicus*. Journal of bacteriology, 186, 5: 1229-1238

Paper II

Øyvind M. Jakobsen, Aline Benichou, Michael C. Flickinger, Svein Valla, Trond E. Ellingsen and Trygve Brautaset. 2006. *Upregulated transcription of plasmid and chromosomal ribulose monophosphate pathway genes is critical for methanol assimilation rate and methanol tolerance in the methylotrophic bacterium Bacillus methanolicus*. Journal of bacteriology, 188, 8: 3063-3072

Paper III

Trygve Brautaset, Øyvind M. Jakobsen, Kjell D. Josefsen, Michael C. Flickinger and Trond E. Ellingsen. 2007. *Bacillus methanolicus*: a candidate for industrial production of amino acids from methanol at 50ºC. Applied microbiology and biotechnology, 74, 1: 22-34

Paper IV

Øyvind M. Jakobsen, Trygve Brautaset, Kristin F. Degnes, Michael C. Flickinger, Svein Valla and Trond E. Ellingsen. *Overexpression of wild-type aspartokinase increases L-lysine production in methylotrophic Bacillus methanolicus*. Submitted to Applied and environmental microbiology.

Contributions to related publications not included in this thesis:

Øyvind M. Jakobsen, Trygve Brautaset and Trond E. Ellingsen. 2008. *Patent application GB0809169: Method of L-lysine production*. Sinvent AS.

1 INTRODUCTION

1.1 Biotechnological production of amino acids

1.1.1 Amino acids are essential building blocks for living organisms

Amino acids are carboxylic acids with an amino group. They are key compounds for living organisms and constitute the building blocks of proteins[1)]. Organisms require all the 20 amino acids commonly found in proteins. Higher animals need a sufficient supply of the so-called essential amino acids (lysine, methionine, threonine, tryptophan, leucine, isoleucine, valine and phenylalanine) which they cannot synthesize themselves. Non-ruminants of the food industry such as pig, poultry and fish represent a considerable demand for essential amino acids. Sufficient amounts of a natural diet will eventually meet this demand as proteins in the feedstuffs are broken down and the different amino acids are made available to the animal. However, as the content of the amino acids in the feedstuff does not necessarily correlate with the animal's requirements for optimal growth, fine-tuning of the amino-acid composition of the feedstuff may be desirable in order to increase the nutritional value of the feed. Extensive research has been carried out towards the isolation of crops with improved content of essential amino acids, both through conventional breeding-programs and targeted genetic engineering. Despite several advances within this field, commercial use of such crops has not yet occurred apart from the Quality Protein Maize lines with approximately double the amount of lysine and tryptophan (0.4% and 0.1% of total dry weight, respectively) compared to normal maize (49, 118, 144). The limited commercial success is believed to be due to recent legislation and a general concern about the use of genetically modified crops (12, 48).

In contrast to mammals, microorganisms such as bacteria can synthesize all the biologically required amino acids. Crude proteins may constitute 80% of the dry weight of bacteria (6), and so-called Single Cell Protein processes have evolved for the manufacture of protein-rich biological cell mass. Harvested biomass may be

[1] Amino acids can exist as D or L enantiomers, and with some exceptions, life has evolved to use L-amino acids rather than D (Madigan, M. T., M. Martinko, and J. Parker. 2002. Brock biology of microorganisms, 10th ed. Prentice Hall / Pearson Education, Upper Saddle River, NJ). In this thesis, the L enantiomer of amino acids is assumed if not specified.

subjected to downstream processing steps like washing, cell disruption, protein extraction and purification (47), and is used as protein supplementation of animal feed (6, 124).

Alternatively, bacteria may be engineered or mutagenized to overproduce specific amino acids so that the products of such processes may be used to balance the amino acid content of animal feed (section 1.1.3).

1.1.2 Essential amino acids are used as feed additives

Feed additives used by the animal feed industry constitute the majority of the global market for amino acids (91). The most important amino acids used as feed supplements are lysine and DL-methionine, but also threonine and tryptophan is used for this purpose.

Lysine is used almost exclusively as a feed additive. This essential amino acid is the first limiting amino acid in pig feed and the second limiting amino acid in poultry feed. The natural content of lysine in traditional feedstuffs such as corn, wheat and barley is less than or around 0.5% of total dry weight (122). For comparison, the demand of lysine in piglet feed is more than 1%. Soybean contains more than 2% lysine and in order to increase the feed efficiency, such lysine-rich crops can be added to traditional feedstuffs. However, as addition of soybean meal also supplies amino acids present in the feedstuff in sufficient amounts, direct addition of lysine may be a preferred method for rational optimization of the amino acid content. To illustrate, a mix of 0.5% pure lysine and 99.5% traditional feed-stuff will as an example offer a similar protein quality in regards of lysine content as a mix of about 20% soy meal and 80% traditional feed-stuff.

The content of methionine in corn, wheat and barley is below or around 0.2% of the feed dry weight. Methionine is the first limiting amino acid in poultry feed, and the second limiting amino acid in piglet feed (with a preferred DL-methionine content of about 0.4%) (122). The D-form of methionine, not commonly found in nature, can be converted into the nutritive L-form by the animal by means of an oxidase and a transaminase, which allows the direct use of a synthetic racemic mixture (91).

1.1.3 Towards 100 years of industrial amino acid production

A discovery by Dr. Kikunae Ikeda in 1908 (68 and references within) became the start of the amino acid industry. Pursuing a traditional Japanese flavoring component, he managed to isolate the amino acid glutamate from a brown kelp commonly used in Japanese cuisine and recognized that monosodium glutamate (MSG) represented a highly desirable flavor to foods. The Japanese company Ajinomoto began commercial production of this amino acid by extraction from acid-hydrolysate of wheat gluten and defatted soybean. This was the start of the first industrial scale amino acid production from natural raw material hydrolysates, and demonstrated a fundamental process possible also for other amino acids.

Increased demand of MSG led to the development of improved bulk production technology. During the 1950s, Kinoshita and colleagues of Kyowa Hakko Kogyo Co. in Japan discovered that *Corynebacterium glutamicum* could produce significant amounts of glutamate directly from cheap sugar and ammonia (78). *C. glutamicum* is an aerobic, non-spore-forming, Gram-positive bacterium. The glutamate-producing *Brevibacterium lactofermentum* and *Brevibacterium flavum* were earlier regarded as closely related to *C. glutamicum* (165, 166), but were later taxonomically united in *C. glutamicum* (93). The 1950s discovery of glutamate production by *C. glutamicum* represented the beginning of industrial microbial production of amino acid, and caused the world market price of MSG to drop four-fold (46). In the late 1950s and 1960s, the use of random chemical mutagenesis and screening revealed *C. glutamicum*'s potential for the production of lysine (46). The discovery at Kyowa Hakko Kogyo Co. of a homoserine auxothroph mutant of *C. glutamicum* that produced large amounts of lysine (112) represented an important milestone in the history of amino acid production and enabled an industrial fermentation process also for this amino acid. Today, the major part of the amino acid production is based on microbial fermentation processes, although other types of production processes also exist (section 1.1.4).

1.1.4 Industrial amino acid production processes

Production processes of amino acids are broadly classified into four types: extraction, chemical synthesis, enzymatic synthesis and fermentation (68, 84). The method of choice depends on several aspects such as process economics, available

raw materials, market situation and environmental regulation. The preferred methods may therefore change both with time and locality.

The original **extraction** method for amino acids from natural raw materials is still an industrial process for a few amino acids such as isoleucine, leucine and tyrosine (e.g. 36, 133). However, limited availability of natural protein-rich resources such as meat, fish protein, hair, keratin, feather, blood meal or soybean has together with high purification costs impelled the development of alternative production methods. Consequently, the extraction method has been and is being substituted by enzymatic and fermentation processes for industrial production of several amino acids.

Amino acids produced by traditional **chemical synthesis** are obtained as D,L-amino acids and an additional optical resolution step is required to obtain the biologically active L-isomers. High production costs are associated with such a step, and therefore few amino acids are produced by chemical synthesis. However, methionine, of which both the L- and the D-form may be utilized by higher animals (section 1.1.2), is currently produced by this method (87). Chemical synthesis is also being applied to the production of glycine, the amino acid carrying no asymmetric carbon atoms.

Aspartic acid is produced in a continuous process from fumarate and ammonia with immobilized bacteria expressing aspartase (28). Aspartic acid can be further converted to alanine by aspartate β-decarboxylase (27). These examples illustrate the **enzymatic method** of amino acid production which yields optically pure amino acids. However, the competitiveness of enzymatic method processes relies on the cost of manufacturing the substrates and enzymes, and for most amino acid processes the fermentation method is preferred (68).

Since the pioneering discoveries of **fermentation processes** of glutamate and lysine, development of mutant strains of *Corynebacterium* and also Gram-negative bacteria such as *Escherichia coli* have made possible new processes for the production of amino acids such as phenylalanine, threonine, glutamine and arginine (46, 68, 133). Current fermentation processes for amino acids are usually based on large scale aerated agitated tank fermentors or airlift tank fermentors typically from 50 to 500 m^3. A representative amino acid batch fermentation process includes an inoculum and two seed tanks (1 – 2 m^3 and 10 – 20 m^3) which provide the inoculum for the main tank fermentor (68). The seed tanks ensure a high volumetric

fermentation yield in the shortest possible time in the main fermentor as well as better reproducibility. Today's industrial amino acid fermentation is mainly conducted using batch or fed-batch processes. Improvement of volumetric yield, substrate yield, purification yield and the productivity of the overall processes have over the last decades made possible efficient industrial fermentation processes to meet the demands from a rapidly growing amino acid market. Today, glutamate and lysine represent two of the world's most important biotechnological products, in respect to volume and value (section 1.1.5).

1.1.5 Amino acid production is a major biotechnological industry

After antibiotics, amino acids constitute the second most important category in the world market for fermentation products, in respect to market value (ethanol not included) (91).

The annual world production of amino acids in general is said to double every decade and was in 2001 estimated at more than 2 million metric tons per year (44, 46, 63). For a Norwegian comparison, this amount is more than four times the national salmon production for the same year (145). The annual market value for amino acids was in 2004 estimated to USD 4.5 billion (91).

The main applications of amino acids are as feed additives (section 1.1.2) and as flavor enhancers (63, 84). In respect to market shares, these applications contribute to about 56% and 32% of the total amino acid market value, respectively. In addition to flavor enhancers and additives for the food and feed industry, applications of amino acids include pharmaceuticals, cosmetics, polymer materials and agricultural chemicals (68).

In 2003, about 1.5 million tons glutamic acid was reported to be produced per year by fermentation processes with coryneform bacteria, mainly to support the use of MSG in prepared food, which typically consists of 0.1 to 0.8% MSG (63, 133). Major producers of MSG are Ajinomoto, Miwon, Kyowa-Hakko and Cheil Jedang.

As mentioned in section 1.1.2, lysine, DL-methionine, threonine and tryptophan are used as feed additives. Lysine is the most important biotechnologically produced feed amino acid, in respect to volume and value. In 2001, the world market for lysine was 550,000 tons with a growth rate of 7% per year (63), estimating the current lysine market to more than 800,000 tons per year.

A lysine world market of 850,000 tons per year was most recently reported by Sanchez and Demain (133). Main producers are Ajinomoto, ADM, Kyowa Hakko, Cheil Jedang, BASF and Degussa/Cargill (63). China and USA are the leading consuming countries, representing 20% and 17%, respectively of the global demand (100). The chemically produced DL-methionine represents a world market comparable to that of lysine (68). Threonine is primarily produced by *E. coli* strains and the 2002 world market was about 30,000 tons with an approximate annual growth of about 15% (63). The 2002 annual market for tryptophan was reported to be approximately 1,200 tons, produced by fermentation processes.

Other applications of amino acids are illustrated by glutamine which is used as a therapeutic agent (against gastroenterologic disorders, improvement of liver and brain functions, as an immunoenhancement agent, and against gastric ulcer and alcoholism) (63). Glutamine is also used as a food sweetener and in cosmetics, and is produced for an annual market of about 2,000 tons (87, 169). Phenylalanine, with a 2002 annual market of 14,000 tons, is used as a building block in the production process for the low-calorie sweetener aspartame (50, 63).

1.1.6 Methanol is an alternative substrate for biotechnological production

Low cost commodity carbohydrates such as cane molasses, beet molasses and starch hydrolysates (glucose) from corn and cassava are widely used as carbon sources for the industrial production of amino acids (122). The cost of the carbon source accounts for most of the total raw material price, and the choice may depend on the locality of the production plant. Starch hydrolysate from corn (corn syrup) is a common carbon source in the Unites States while cassava hydrolysates are abundantly used in South Asia. Due to cost and availability, the carbon sources of European and South American production plants are mainly cane and beet molasses, respectively (122).

In parallel, researchers have begun the exploration of alternative microorganisms utilizing more unconventional substrates for the fermentation industry. Methanol is considered as an attractive one-carbon compound both from a biotechnological and a bulk chemical point of view (94, 117). Compared to molasses, methanol is a pure substrate and can be completely utilized in the fermentation process. The price of methanol varies as other commodities according to supply and

demand, but has over the last years been comparable to that of raw sugar, as discussed in Brautaset et al. (17) (attached as Paper III in this thesis). Furthermore, methanol is easily stored and transported, highly water soluble, solutions are not explosive and residual methanol can easily be removed after completing the fermentation (136).

Methanol is a more reduced compound than sugar, and the oxygen demand for methanol-based microbial biosynthesis processes are considerably higher than for those using glucose as the raw material (section 3.1.1). As heat evolution increases proportionally with oxygen consumption, the cooling requirement for a methanol-based process may represent a significant increase in the production cost. Elevated operating temperatures may compensate for the increased cooling requirements as the cooling water may be heated to higher temperatures and the driving force for the heat exchange increases (83).

1.2 Methylotrophic bacteria

1.2.1 Methylotrophs

According to Colby and Zatman (31), microbes that utilize one-carbon compounds as sole carbon sources for growth can be divided into two groups. Methylotrophs are recognized by their ability to assimilate carbon as formaldehyde or a mixture of formaldehyde and carbon dioxide. Bacteria assimilating carbon dioxide for growth are on the other hand autotrophs in their terminology. Anthony (5) however, simply defines methylotrophs as microbes that are able to grow on one-carbon compounds irrespectively of the assimilation pathway. Finally, Large and Bamforth (88) defined methylotrophy as the ability of a living organism to use, as the sole carbon source of growth, compounds other than carbon dioxide containing one or more carbon atoms but no carbon-carbon bonds.

1.2.2 One-carbon compounds occur abundantly in nature and support methylotrophic growth

A large group of microorganisms have evolved in order to utilize one-carbon compounds from the most reduced form as methane to the most oxidized form as carbon dioxide as carbon and energy sources (88, 94, 117).

Methane exists as abundant fossil deposits and is continuously formed by methanogenic bacteria living in habitats such as anaerobic lakes, marshes, and the digestive tract of ruminant mammals (88). The ability to grow on methane is referred to as methanotrophy, and methanotrophs play major roles in global carbon cycles, substantially reducing emissions of biologically generated methane to the atmosphere (159). Methane has been reported to be the most important natural reduced one-carbon compound for methylotrophic growth (88). In this thesis, further focus on utilization of one-carbon compounds for growth will be limited to methanol only.

In nature, ***methanol*** is formed by hydrolysis of methyl esters and ethers such as pectin and lignin from plants (41). Methanol is also formed by oxidation of methane by hydroxyl radicals in the trophosphere, and descends in low concentrations in rain (88). Microbial oxidation of methanol to formaldehyde (FA) is catalyzed by methanol dehydrogenase (MDH). Formaldehyde itself is too reactive to occur at a significant concentration in nature, but serves as a key intermediate in microbial one-carbon assimilation processes. Three types of MDH have been reported to exist in methylotrophs (80): An alcohol dehydrogenase using FAD as a cofactor operates in yeast (58), while Gram-negative bacteria harbor an NAD(P)-independent methanol dehydrogenase (MDH), using pyrroloquinoline quinone as a cofactor (5). Finally, Gram positive bacteria employ cytoplasmic MDH with NAD(P) as a cofactor (62, 126). Assimilation of FA into cell-carbon is discussed in section 1.2.3.

Additionally, several other one-carbon compounds are known to serve as growth substrates for various microorganisms. These compounds include, but are not limited to, carbon monoxide (formed by oxidation of methane by OH radicals in the trophosphere and man-made processes such as incomplete combustion of fossil fuel), cyanide (produced by electro-plating, steel and carbonization industry and by plants and fungi) and chloromethane (from industrial pollution and microbial activity) (88).

1.2.3 Methylotrophs employ different strategies for carbon assimilation

The biological diversity among one-carbon utilizing microorganisms is considerable and is reflected by the different assimilation pathways employed within this group. Carbon compounds are oxidized by the microorganism itself or by the environment to either formaldehyde or carbon dioxide and assimilated into cell carbon via certain assimilation pathways. Four different cyclic pathways have been

discovered and biochemically described (4, 41). In all these pathways, one-carbon compounds are assimilated by condensation to molecules that are regenerated by the completion of one cycle (Figure 1). The final outcome of all pathways is the synthesis of a three-carbon compound from three one-carbon compounds. The ***ribulose bisphosphate (RuBP) pathway*** is the main pathway in chemolitotrophic (autotrophic) bacteria, and carbon is assimilated from carbon dioxide. Sharing many of the RuBP pathway enzymes, the ***ribulose monophosphate (RuMP) pathway*** assimilate cell carbon from formaldehyde. The third option, the ***xylulose monophosphate (XuMP) pathway***, also called the *dihydroxyacetone (DHA) pathway,* is responsible for formaldehyde assimilation in yeast growing on methanol and shares many similarities with the RuBP and RuMP pathways. Finally, most distinct is the ***serine pathway****,* through which cell carbon is assimilated from carbon dioxide and formaldehyde. The three former pathways use carbohydrates as intermediates while the latter uses carboxylic acids and amino acids. In this thesis, further focus on assimilation of formaldehyde will be limited to the RuMP pathway.

1.2.4 The RuMP pathway assimilate formaldehyde into cell-carbon

FA is produced by oxidation of methanol by MDH (5, 41, 58, 62, 80, 88, 126) (Figure 1 and section 1.2.2). The RuMP pathway assimilates cell carbon through synthesis of a three-carbon compound from three molecules of FA (4, 41). The pathway can be divided into three parts (Figure 2). In the fixation part, FA is condensed with ribulose 5-phosphate (Ru5P) to form hexulose 6-phosphate (H6P). H6P is further converted to fructose 6-phosphate (F6P) which then enters the cleavage part**.** Here, F6P is converted to the product of the RuMP pathway (a three-carbon compound) and glyceraldehyde 3-phosphate (GAP) which enters the rearrangement part to regenerate Ru5P.

The ***fixation part*** (Figure 2) of the RuMP pathway involves two unique enzymes, 3-hexulose-6-phosphate synthase (HPS) and 6-phospho-3-hexuloisomerase (PHI). Three FA molecules undergo aldol condensation reactions with three molecules of Ru5P to form three H6P. One of the H6P molecules is further converted to F6P that can enter the cleavage part.

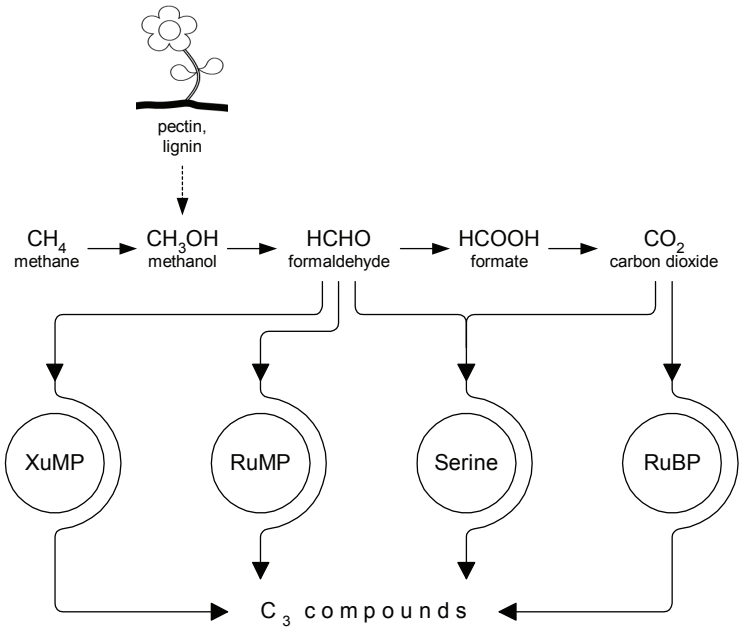


Figure 1. Four different pathways for one-carbon assimilation in methylotrophs. Natural carbon sources that can be degraded to methanol, such as pectin and lignin from plants, are indicated (41).

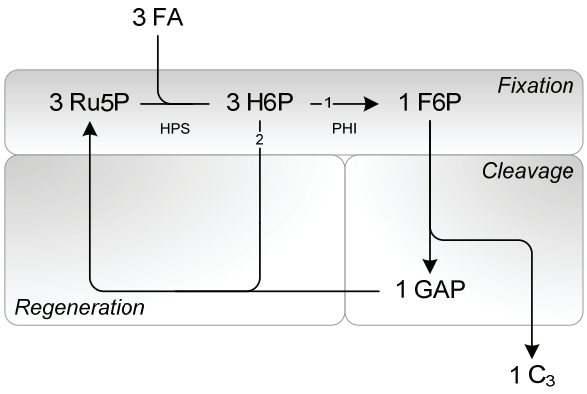


Figure 2. Outline of the RuMP pathway. Upon the completion of one cycle, three FA molecules are converted into one three-carbon molecule. The enzymes of the fixation part are indicated. The numbers on the reaction arrows relate to the stoichiometry of the reaction.

Abbreviations of intermediates: FA, formaldehyde; Ru5P, ribulose 5-phosphate; H6P, hexulose 6-phosphate; F6P, fructose 6-phosphate; GAP, glyceraldehyde phosphate.

Abbreviations of enzymes: HPS, 3-hexulose-6-phosphate synthase; PHI, 6-phospho-3-hexuloisomerase.

The ***cleavage part*** (Figure 3) exists as two variants that convert F6P to GAP and a three-carbon product of the RuMP pathway. The fate of F6P is the conversion to either fructose-1,6-bisphosphate (FBP) or 2-keto-3-deoxy-6-phosphogluconate (KDPG). In the ***FBP aldolase*** (***FBPA) variant***, F6P is converted by phosphofructokinase (PFK) to FBP at the expense of one ATP. FBP is then cleaved by FBPA to GAP and dihydroxyacetone phosphate (DHAP). DHAP can be converted by glycolytic steps into pyruvate, generating one NAD(P)H and two ATP. Alternatively, in the **KDPG aldolase (KDPGA) variant**, F6P is converted through three steps similar to the Entner-Doudoroff pathway to KDPG, generating one NAD(P)H, and subsequently cleaved by KDPG aldolase to GAP and pyruvate.

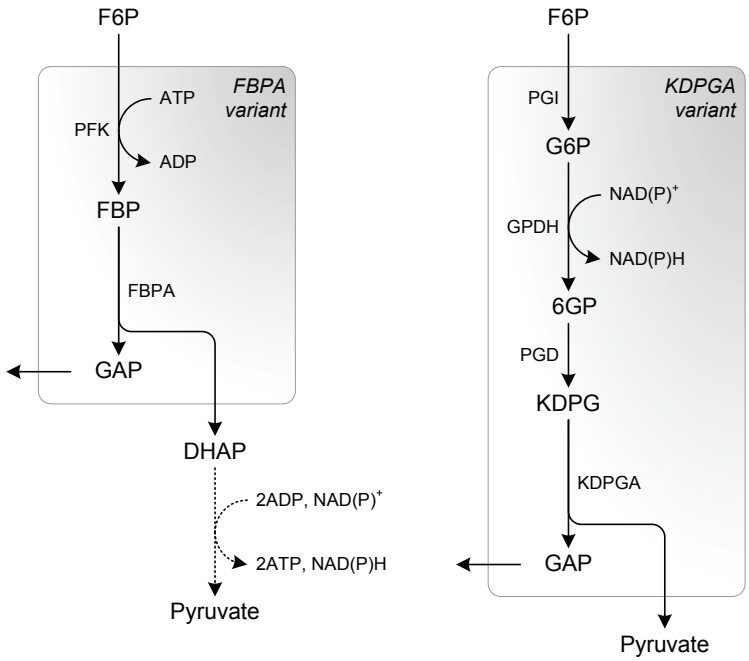


Figure 3. Two variants of the cleavage part of the RuMP pathway. The FBPA variant converts F6P to GAP and DHAP. Further conversion of DHAP to pyruvate by glycolytic enzymes is indicated. Alternatively, the KDPGA variant converts F6P to GAP and pyruvate using enzymes of the Entner-Doudoroff pathway.

Abbreviations of intermediates: F6P, fructose 6-phosphate; FBP, fructose-1,6-bisphosphate; GAP, glyceraldehyde phosphate; DHAP, dihydroxyacetone phosphate; G6P, glucose-6-phosphate; 6GP, 6-phospogluconate; KDPG, 2-keto-3-deoxy-6-phosphogluconate.

Abbreviations of enzymes: PFK, phosphofructokinase; FBPA, fructose-1,6-bisphosphate aldolase; PGI, phosphoglucose isomerase; GPDH, glucose-6-phosphate dehydrogenase; PGD, 6-phosphogluconate dehydratase; KDPGA, 2-keto-3-deoxy-6-phosphogluconate aldolase.

Regeneration of Ru5P is critical in order to maintain the activity of the RuMP cycle. In the ***rearrangement part*** (Figure 4), GAP from the cleavage part is rearranged with two H6P molecules from the fixation part to three molecules of Ru5P. Two variants of the rearrangement part exist and they share the three enzymes transketolase (TK), ribose-5-phosphate isomerase (RPI) and ribulose-5-phosphate 3-epimerase (RPE). The difference between the variants is the usage of transaldolase (***TA variant***) or sedoheptulose-1,7-bisphosphatase (***SBPase variant***).

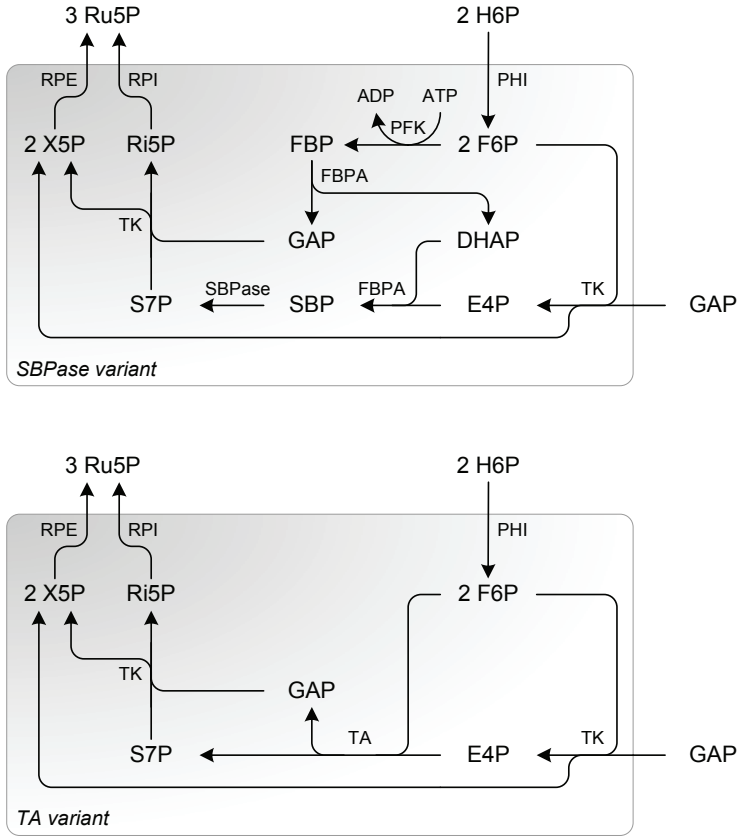


Figure 4. Two variants of the rearrangement part of the RuMP pathway.

Abbreviations of intermediates: Ru5P, ribulose 5-phosphate; H6P, hexulose 6-phosphate; X5P, xylulose 5-phosphate; Ri5P, ribose 5-phosphate; FBP, fructose-1,6-bisphosphate; F6P, fructose 6-phosphate; GAP, glyceraldehyde phosphate; DHAP, dihydroxyacetone phosphate; S7P, sedoheptulose-7-phosphate; SBP, sedoheptulose-1,7-bisphosphate; E4P, erythrose 4-phosphate.

Abbreviations of enzymes: RPE, ribulose-5-phosphate 3-epimerase; RPI, ribose-5-phosphate isomerase; PHI, 6-phospho-3-hexuloisomerase; PFK, phosphofructokinase; FBPA, fructose-1,6-bisphosphate aldolase; TK, transketolase; SBPase, sedoheptulose-1,7-bisphosphatase; TA, transaldolase.

Two possible cleavage variants and two possible rearrangement variants make up four theoretical ways to run the RuMP pathway. All variants produce one NAD(P)H per pyruvate generated. The most energetically favorable combination is the FBPA/TA variant, which in addition yields one ATP, while the least energetically favorable KDPGA/SBPase variant requires one ATP per pyruvate generated (41). All variants but the latter have been reported in methylotrophic bacteria.

1.2.5 Detoxification of formaldehyde is important in methylotrophs

Methanol is oxidized into formaldehyde which represents a key intermediate in methanol utilization. As for higher animals, rapid uptake of alcohol can put the cell into a toxic state. Methanol toxicity in methylotrophic bacteria is assumed to be due to accumulation of formaldehyde (2, 20, 21, 51, 101). This compound is unusually reactive; its mode of action is complex and includes a number of biological molecules, making it highly toxic to the cell (130). The earliest published use of formaldehyde as an antimicrobial agent dates back to 1886.

As formaldehyde is a key metabolite for methylotrophs, different strategies have evolved to handle this toxic compound. The compound may be assimilated into cell carbon by one of the four main assimilation routes outlined in Figure 1. Interestingly, RuMP pathways have recently been found in non-methylotrophic bacteria, and it has been speculated that they function to detoxify intracellular formaldehyde (101, 102, 127, 167). As an alternative to assimilation, methylotrophic bacteria may generate energy by oxidizing formaldehyde to CO_2 by dissimilatory pathways (Figure 5).

One of the dissimilatory pathways found in many methylotrophs is a linear oxidation of formaldehyde via formate to CO_2, generating reducing equivalents (41 and references therein). The reactions are catalyzed by formaldehyde dehydrogenase (FADH) and formate dehydrogenase (FDH), which are present in a large number of methylotrophs. The TCA cycle of organisms harboring such a pathway is suggested to play a minor role in energy metabolism during growth on one-carbon sources (141, 171).

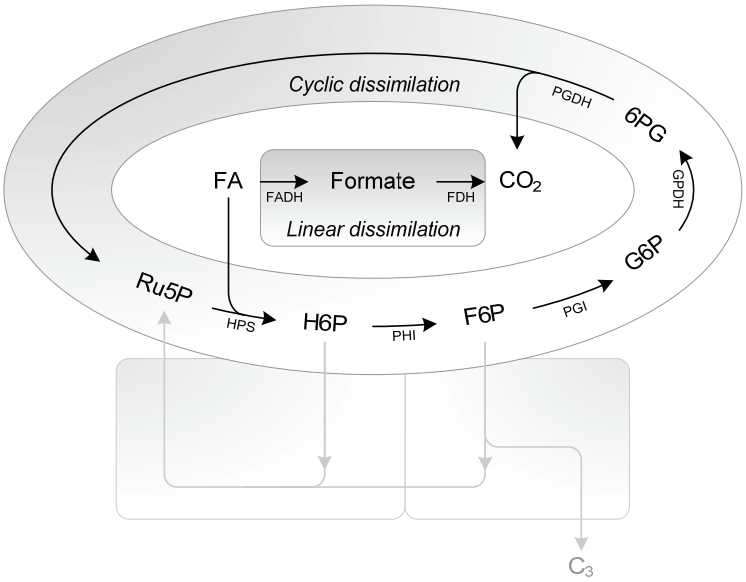


Figure 5. Linear and cyclic pathways for dissimilation of formaldehyde to CO_2. Both pathways produce reducing equivalents. The linear dissimilatory pathway converts formaldehyde to CO_2 by the action of two distinct enzymes. The cyclic dissimilatory pathway is a combination of the fixation part of the RuMP pathway and the pentose phosphate pathway. The remaining parts of the RuMP pathway for assimilation of formaldehyde (Figure 2) are indicated in gray.

Abbreviations of intermediates: 6PG, 6-phosphogluconate; FA, formaldehyde; Ru5P, ribulose 5-phosphate; H6P, hexulose 6-phosphate; F6P, fructose 6-phosphate; G6P, glucose-6-phosphate.

Abbreviations of enzymes: PGDH, 6-phosphogluconate dehydrogenase; FADH, formaldehyde dehydrogenase; FDH, formate dehydrogenase; GPDH, glucose-6-phosphate dehydrogenase; HPS, 3-hexulose-6-phosphate synthase; PHI, 6-phospho-3-hexuloisomerase; PGI, phosphoglucose isomerase.

An alternative route for oxidation of formaldehyde to CO_2 by methylotrophs was earlier reported (30, 147). This cyclic dissimilatory pathway has been termed dissimilatory RuMP cycle as it shares the enzymes HPS and PHI with the RuMP pathway (Figure 5). The cycle is essentially a combination of the fixation part of the RuMP pathway (HPS and PHI) and the pentose phosphate pathway. Carbon enters the dissimilatory cycle by the action of phosphoglucose isomerase (PGI), converting F6P into glucose 6-phosphate (G6P). Reducing equivalents are generated by oxidation of G6P to 6-phosphogluconate (6PG) and subsequently to Ru5P by G6P dehydrogenase (GPDH) and 6PG dehydrogenase (PGDH), respectively. Ru5P is thereby regenerated and can again participate in formaldehyde fixation.

1.3 Microbial production of lysine

1.3.1 Lysine is a product of the branched aspartate pathway

The carbon for lysine synthesis is derived from oxaloacetate of the central carbon metabolism. Oxaloacetate is converted into aspartate, the precursor for the so-called aspartate pathway (Figure 6) which includes the biosynthetic pathways for lysine, methionine and threonine (119). The two first enzymatic reactions of the lysine biosynthetic pathway are common for the biosynthesis of all the end products of the aspartate pathway. Threonine is a precursor for isoleucine while methionine is converted to S-adenosylmethionine, a common methyl donor (119). *meso*-diaminopimelate is a constituent of the bacterial cell wall peptidoglycan, and is a component of the spore cortex for sporulating Gram-positive bacteria (98, 119). In addition, dipicolinate is an important component of spores. Thus, a complex regulation of shared and unique enzymatic steps must allow biosynthesis of intermediates, side products and end products in a balanced manner according to the changing requirements of the cell.

1.3.2 Prokaryotes harbor different biosynthetic pathways for lysine

Three variants of the lysine biosynthetic pathway are recognized in prokaryotes (15, 77, 139, 148, 163). At the level of piperideine-2,6-dicarboxylate, the carbon flow can follow (i) the succinylase variant involving four enzymatic conversions via succinylated intermediates to *meso*-diaminopimelate, (ii) the similar four-step acetylase variant involving acetylated intermediates, and (iii) the dehydrogenase variant in which piperideine-2,6-dicarboxylate is converted to *meso*-diaminopimelate in one enzymatic step. *Bacillus subtilis* and most other *Bacillus* species use the acetylase variant (119), while *E. coli* is reported to use the succinylase variant (162). The dehydrogenase variant operates in a few *Bacillus* species such as *B. sphaericus*, *B. globisporus* and *B. pasteurii* (119, 163). Lysine biosynthesis in the commercial producer *C. glutamicum* is reported to be mediated by a combination of the succinylase variant and the dehydrogenase variant (143, 161). The flux distribution between these two pathways is variable; at the start of a batch cultivation about 75% of the lysine is made via the dehydrogenase variant, while at

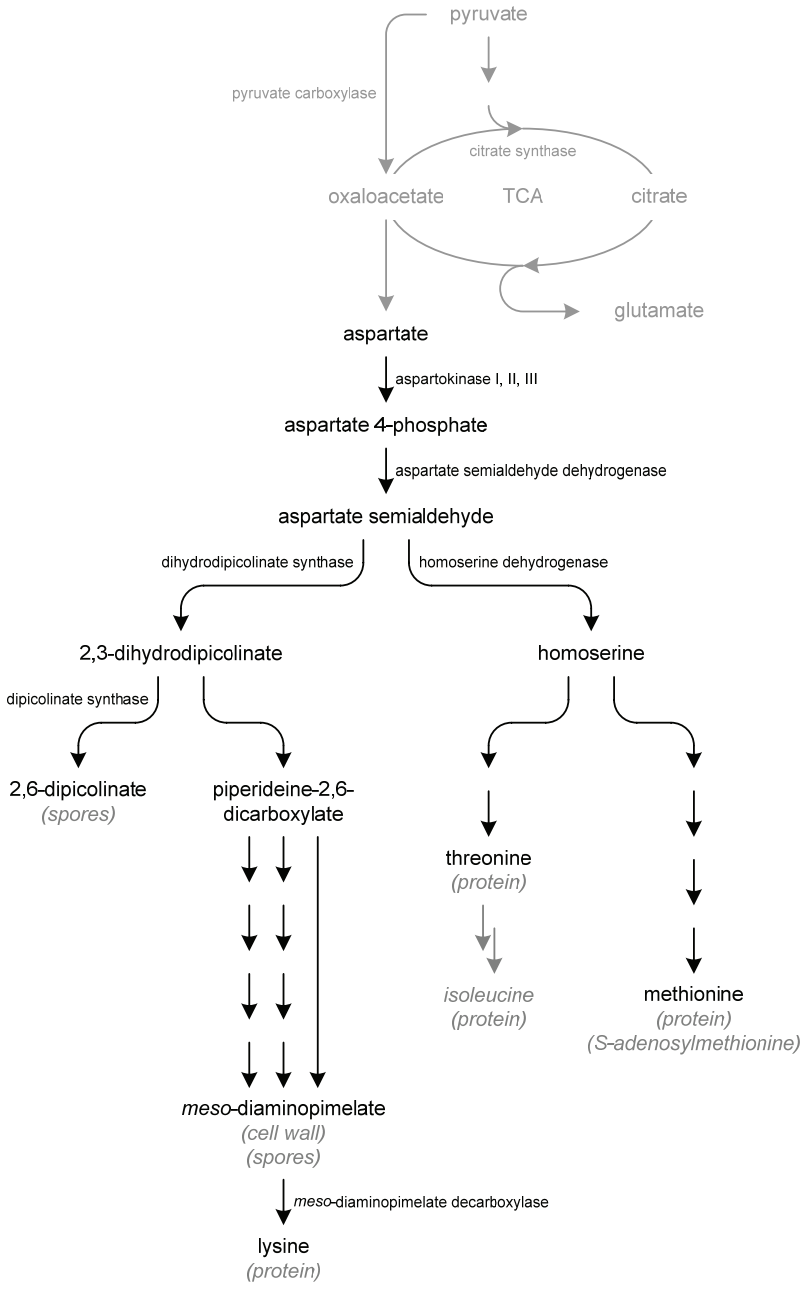


Figure 6. General overview of the aspartate pathway (in black). Three routes from piperideine-2,6-dicarboxylate to meso-diaminopimelate are known in prokaryotes (see text). The major metabolic functions of the end products are indicated in parentheses. The multi-step conversion of threonine to isoleucine is indicated in gray. Likewise is the conversion of pyruvate to the aspartate precursor oxaloacetate indicated in gray, represented by the TCA cycle and the anaplerotic reaction catalyzed by pyruvate carboxylase.

the end of the process lysine is almost exclusively made via the succinylase route (44). The use of different pathways has been suggested to facilitate adaptation to changing ammonium concentrations. Ammonium is used a substrate in the dehydrogenase variant, and at low ammonium concentrations, typically at the end of the fermentation phase, the succinylase variant is favored, in which a transaminase incorporates an amino group from glutamate.

1.3.3 Aspartokinase controls the flow into the aspartate pathway

The flux from aspartate towards the end products of the aspartate pathway is controlled by aspartokinase (ATP:4-L-aspartate-4-phosphotransferase, EC 2.7.2.4). *C. glutamicum* harbors one aspartokinase enzyme, while multiple aspartokinase isozymes exist in the well characterized strains *B. subtilis* and *E. coli*. In general, aspartokinase enzymes are highly regulated and both enzyme activity and synthesis are controlled by different products of the aspartate pathway.

The three aspartokinase isozymes of *B. subtilis* have been characterized, and all isozymes are regulated in a distinct manner (52, 108, 129). Aspartokinase I is inhibited by *meso*-diaminopimelate, aspartokinase II is inhibited by lysine, while aspartokinase III is regulated by concerted feedback inhibition by lysine and threonine. In addition, lysine and threonine act as corepressors for aspartokinase II and III synthesis, respectively. Aspartokinase I cannot substitute for aspartokinase II and III, and the same conclusion is valid for the opposite situation under most conditions (119, 172, 173), indicating a functional specialization among the aspartokinase isozymes. Aspartokinase I is believed to mainly support biosynthesis of *meso*-diaminopimelate and dipicolinate for use in cell walls and spores. Aspartokinase II and III, however, are thought to be responsible for supplying aspartate phosphate for the subsequent synthesis of the amino acid end products of the aspartate pathway, and the functions of these isozymes are believed to be redundant (26, 119, 172, 173). Aspartokinase isozymes resembling those of *B. subtilis* have been identified in several other *Bacillus* species. Although not completely characterized, strains such as *B. stearothermophilus*, *B. cereus*, *B. licheniformis* and *B. brevis* have been reported to harbor multiple aspartokinase isozymes with similar regulation as in *B. subtilis* (119 and references within).

For comparison, the single aspartokinase of coryneform bacteria is controlled by concerted feedback inhibition by threonine and lysine (33, 42), similar to the aspartokinase III of *B. subtilis*. The three aspartokinase isozymes of *E. coli* display considerable similarity with *B. subtilis* aspartokinase II (24, 119), but both function and regulation differ compared to *B. subtilis*. Each isozyme is individually controlled by one of the end products of the aspartate pathway, lysine, methionine and threonine (29). Compared to *B. subtilis*, no provisions are made for ensuring the synthesis of diaminopimelate for the cell wall at conditions of excess amino acids as in rich media. Two of the *E. coli* aspartokinases are bifunctional proteins that also have homoserine dehydrogenase activity (29), while in *B. subtilis* such activity is mediated by a single monofunctional enzyme (119).

1.3.4 Aspartokinase is a key target for improvement of microbial lysine production

Bacteria do not normally excrete amino acids in significant amounts due to regulatory mechanisms that allow amino acid synthesis only to meet the cell's requirement for use in protein synthesis. Deregulation of aspartokinase has been reported to be the most important step in the development of commercial lysine producing strains (42, 122, 134). Historically, *C. glutamicum* has been the bacterium of choice for industrial production of lysine, and the first era of strain development was mainly based on classical mutagenesis followed by selection of mutants with desired characteristics. Resistance towards the lysine analogue S-(2-aminoethyl) cysteine (AEC) is a key property of classical *C. glutamicum* lysine-producing mutants (131). Several of these mutants have been shown to carry aspartokinases deregulated from the original concerted feedback inhibition by lysine and threonine (42, 122).

By genetic engineering, lysine producing strains have been developed by introduction of feedback resistant key enzymes. Introduction of a feedback deregulated aspartokinase in *C. glutamicum* ATCC 13032 increased lysine production from 0 to 55 g/l (116). A number of favorable mutations causing feedback deregulation have been localized in the gene encoding *C. glutamicum*'s aspartokinase, and all the major lysine companies such as Ajinomoto, Archer Daniels Midland, BASF, Degussa and Kyowa Hakko have claimed patents related to this enzyme (42).

AEC-resistant lysine excreting mutants were early isolated also among other bacterial strains such as *B. subtilis, B. licheniformis* and *B. brevis* (23, 54, 66, 103, 156). For example, AEC-resistant *B. subtilis* BAEC29 was reported to produce 16 g/l of lysine (23), although the responsible mutation(s) were not identified. Mutations have been mapped to aspartokinase genes for several other *B. subtilis* AEC-resistant strains investigated (81, 96, 97, 121, 168), but lysine production exceeding 1 g/l has not been reported for these strains.

1.3.5 Strain development of C. glutamicum towards high-level production of lysine

From the 50-year experience with *C. glutamicum* strain development, a number of key properties of efficient production strains can be highlighted. The original *C. glutamicum* mutants used for large scale production of lysine were amino acid auxotrophs. A first generation homoserine auxotrophic mutant yielding 44 g/l of lysine was patented already in 1961 (122). Further development of production strains was mediated by additional auxotrophies. For example, *C. glutamicum* ATCC 21513 requires homoserine, leucine, biotin, thiamin and pantothenic acid (135). However, as the number of growth requirements increased upon further mutagenesis, strains acquired sensitivity towards temperature and unfavorable pH, and became affected by limitations of vitamins and micronutrients. Amino acid auxotrophies resulted in increased raw material costs or the use of complex raw materials that increase process variation and impair purification. Development of so-called leaky strains reduced these problems, as mutants harbor functional but inefficient biosynthetic pathways (42). The mutants are able to synthesize components required for growth, while intracellular concentrations are kept low to avoid inhibition or repression of key enzymes for lysine synthesis (42). For example, reduced activity of homoserine dehydrogenase (responsible for the biosynthesis of homoserine, a precursor for threonine; Figure 6) increased lysine production up to 20 g/l in prototrophic revertants from homoserine auxotrophic *C. glutamicum* strains (65, 140).

The strategy of classical mutagenesis and selection of coryneform bacteria proved to be successful. Classical strains accumulate well above 100 gram lysine per liter with conversion yields above 0.5 g lysine per g glucose (see 42, 84, 91, 135 for overviews). However, along with the development of modern techniques for genetic engineering of *C. glutamicum* (73, 79), considerable effort has been put down to

introduce specific genetic alterations for further optimization of lysine production. Three major competitors in the lysine industry – BASF, Kyowa Hakko and Degussa – launched independent *C. glutamicum* genome projects and the genome sequence was published in 2003 (69, 74). Today, essentially all the genes in the lysine biosynthetic pathway of this organism have – more or less successfully - been targeted for mutations in the development of lysine producing mutants.

In *C. glutamicum,* the dihydrodipicolinate synthase is believed to act as a barrier to control the flux of aspartate semialdehyde towards lysine (Figure 6). Enhancement of this activity has shown promising results on lysine production by means of regulating the branch point at which carbon flows either towards lysine, or towards methionine and threonine (43, 56, 85). Overexpression of genes of the succinylase branch of the lysine biosynthetic pathway has also demonstrated increased lysine production (59).

Identification of the lysine export system in *C. glutamicum* opened up possibilities to decrease intracellular lysine concentration in the cell. Lysine export is required when *C. glutamicum* grows on lysine-containing peptides as carbon source, since the bacterium lacks enzymes for lysine degradation (45). Lysine is excreted against the membrane potential by a 2 OH^-/lysine symport, and is dependent on the electron motive force (19). Strains with different lysine productivities have been shown to possess different excretion systems, and lysine excretion was enhanced fivefold to the wild type by overexpressing the export system (14, 19, 22, 67, 85, 158).

Optimization of central carbon metabolism including anaplerotic reactions to increase the availability of oxaloacetate and aspartate has given promising results (42 and references within). Pyruvate carboxylase, as a supplier of oxaloacetate, has been reported to be a limiting enzymatic step for high lysine production in *C. glutamicum* (116, 120). NADPH, required for several steps within lysine biosynthesis, has been considered as another possible limitation to efficient lysine production in *C. glutamicum* (84). A close correlation of lysine yield and the extent of flux through the NADPH-generating pentose phosphate pathway as well as the extent of anaplerotic flux was observed in a study comparing strains with different lysine yield (164).

Recently, modern flux analysis and whole-cell approaches have been employed towards further optimization of lysine production (60, 115, 142). Improving the tolerance for favorable process conditions such as high operating

temperature (to decrease process cooling costs) has also been an important contribution to the research on microbial lysine production (114).

Ohnishi et al (116) demonstrated that introduction of a minimal number of specific mutations in a wild type background could yield high lysine production. Simultaneous introduction of mutant aspartokinase, pyruvate carboxylase and homoserine dehydrogenase (Figure 6), lead to a production of 80 gram lysine per liter with a productivity of 3.0 g/(l·h).

1.3.6 Microbial production of lysine from methanol

As described in section 1.1.6, carbohydrates of cane molasses, beet molasses and starch hydrolysates from corn and cassava are the carbon sources of choice for today's amino acid industry. However, methanol has become of interest as an alternative raw material for industrial biotechnological production (section 1.1.6), and several reports on methanol-utilizing bacteria have been presented in the scientific literature. Reported microbial methanol-based amino acid production includes glutamate (109), lysine (111) and threonine (110), all from *Methylobacillus glycogenes*, and serine (55, 71) from *Hyphomicrobium methylovorum* and *Methylobacterium* sp.

In regards of lysine-production, co-expression of mutant genes encoding a lysine-exporter and dihydrodipicolinate synthase in the Gram-negative obligate methylotroph *Methylophilus methylotrophus* resulted in a lysine production of 11 g/l at 37ºC (53, 152). A recombinant mutant of the Gram-negative obligate methylotroph *Methylobacillus glycogenes* overexpressing a dihydrodipicolinate synthase partly released from lysine inhibition produced 8 g/l of lysine and 37 g/l of glutamate at 37ºC (111). As summarized in Table 1 in Brautaset et al. (17) (attached as Paper III in this thesis) listing representative methanol-utilizing lysine and glutamate overproducers, the methylotroph with the highest volumetric lysine production reported in the literature is *Bacillus methanolicus* (section 1.4).

1.4 *Bacillus methanolicus and its potential for amino acid production*

1.4.1 B. methanolicus is a thermotolerant methylotroph

B. methanolicus is a Gram-positive aerobic thermotolerant methylotroph of which several wild type strains have been isolated (1, 9, 21, 40, 136). The bacterium can grow at temperatures from 35 to 60°C, with an optimum of 50°C (136). Brautaset et al. (17) (attached as paper III in this thesis) presents a mini-review of this bacterium as a candidate for industrial production of amino acids from methanol, and therefore only a brief description of the bacterium is given in this section.

B. methanolicus oxidizes methanol to formaldehyde by an NAD-dependent MDH (9-11, 37, 39, 157). Formaldehyde can then be assimilated into cell carbon via the RuMP pathway (section 1.2.4) (38, 40, 76). Thermotolerant methylotrophic *Bacillus* bacteria has been reported to use the FBPA/TA variant of the RuMP pathway (11, 41). In *B. methanolicus* C1, SBPase activity could not be detected, and together with the presence of a low TA activity in cell extracts, the TA variant of the rearrangement part of the RuMP pathway was assumed.

Formaldehyde not assimilated through the RuMP pathway may be dissimilated to CO_2 for energy generation and possible detoxification (section 1.2.5 and Figure 5). Similar to other methylotrophs, *B. methanolicus* is reported to be highly sensitive to methanol (123). Methanol pulses of less than 10 mM have been reported to severely affect the growth rate of cultures under methanol limitation, presumably due to formaldehyde accumulation. Activities of all the enzymes of a dissimilatory RuMP cycle have been measured in *B. methanolicus* (11). In fact, as no FADH or FDH activity was measured, early reports on *B. methanolicus* assumed that the dissimilatory RuMP cycle was responsible for energy generation in *B. methanolicus* (11). However, later, a linear dissimilatory pathway was proposed for *B. methanolicus* as researchers observed accumulation of formate upon methanol pulses, formate consumption, and detection of labeled CO_2 upon labeled formate addition (123). In sum, these findings suggest that both routes for formaldehyde dissimilation may be operative in *B. methanolicus*.

1.4.2 Background for genetic engineering of B. methanolicus

Compared to the industrial amino acid producer *C. glutamicum*, the knowledge and available tools for genetic engineering of *B. methanolicus* have been limited. The genome sequences of related species such as *B. subtilis* (86), *Bacillus halodurans* (149), *Bacillus licheniformis* (128, 155), *Bacillus anthracis* (125) and *Bacillus cereus* (70) have been published and offer possibilities for gene mining and *in silico* studies of these related genomes. Nevertheless, lack of the complete *B. methanolicus* genome sequence (or single key genes) has conferred limitations on the possibilities for rational metabolic engineering of *B. methanolicus*.

Three *B. methanolicus* genes have previously been cloned, sequenced and reported in the literature, two of those encoding enzymes of the aspartate pathway. Schendel and Flickinger (138) reported the cloning of *lysC* encoding aspartokinase II. The enzyme was similar to that of *B. subtilis* in respect to both primary structure and feedback inhibition. Inhibition studies of total aspartokinase activity in *B. methanolicus* wild type strain MGA3 suggested the presence of two additional aspartokinase isozymes, with all three isozymes inhibited in a similar manner as those of *B. subtilis*. However, the predicted genes for aspartokinase I and III were not cloned.

The gene encoding *meso*-diaminopimelate decarboxylase, *lysA*, was cloned and sequenced from *B. methanolicus* MGA3 by Mills and Flickinger (105). This enzyme, catalyzing the last step in the lysine biosynthetic pathway, was shown to be inhibited by lysine and was suggested as a possible limiting step for lysine biosynthesis in lysine-overproducing strains of *B. methanolicus*. Furthermore, the cloning and sequencing of *B. methanolicus citY* encoding citrate synthase II was published together with studies on its role in glutamate production (18) (section 1.4.3).

In 1996, a restriction modification system of *B. methanolicus* was reported (35). A gene delivery method for this bacterium using protoplast transformation was published a year later (34). This report demonstrated the successful introduction of empty shuttle vectors into *B. methanolicus* and represented the first techniques for recombinant manipulation of *B. methanolicus*. However, although genetic constructions for gene overexpression later become available, the report's authors were not able to introduce them into *B. methanolicus* (M. C. Flickinger, personal

communication). Indeed, no reports of recombinant *B. methanolicus* strains were published until 2004 (16, attached as Paper I in this thesis).

1.4.3 B. methanolicus is a candidate for amino acid production

As reviewed in Brautaset et al. (17) (attached as Paper III in this thesis), *B. methanolicus* strains may overproduce lysine and glutamate. The bacterium's potential for amino acid production is illustrated by the wild type MGA3 which is reported to produce 58 g/l of glutamate when grown on methanol (18, 137). With the lack of genetic engineering tools for this organism, previous work on strain improvement has relied on random chemical mutagenesis and screening procedures. These efforts have resulted in the creation of several lysine-producing strains (57, 90, 136), for example the *B. methanolicus* mutant strain 13A52-8A66. This mutant is a homoserine auxotrophic mutant that requires the addition of both threonine and methionine to the growth medium. It is resistant to AEC and the meso-diaminopimelate analogue diaminobutyrate, and has been reported to produce 37 g/l lysine (57). This is to date the highest lysine production reported by any methylotrophic bacterium.

Increased oxygen consumption of a methanol-based process compared to that of a sugar based process increases heat generation and represents increased cost of reactor cooling. The thermotolerant property of *B. methanolicus*, allowing rapid growth at 50°C compensates for this increased oxygen consumption. At about 200 m^3 reactor liquid volume, the cooling water requirements for an organism growing on glucose at 35°C and an organism growing on methanol at 50°C are reported to be similar (83). Furthermore, at 50°C, *B. methanolicus* is reported to sporulate poorly in growth medium (136), an important property since lysine production is dependent on vegetative cells throughout the fermentation process.

A high methanol consumption rate and high theoretical product yields by *B. methanolicus* indicate that this organism has the potential of becoming an efficient biocatalyst for the production of lysine from methanol. These issues are further discussed in Brautaset et al. (17).

As discussed in section 1.2.5, the TCA cycle of organisms harboring a linear dissimilatory pathway is suggested to play a minor role in energy metabolism during growth on one-carbon sources. Glutamate production was abolished in a *B.*

methanolicus mutant deficient in citrate synthase II, a key enzyme of the TCA cycle (18). Upon glutamate addition, this mutant grew well in minimal medium. Possibly, reduced citrate synthase activity (lowering or abolishing glutamate production) may be a key to exclusive lysine overproduction by *B. methanolicus* mutants deregulated in the lysine biosynthetic pathway, enabling a high yield of lysine from methanol.

In sum, properties discussed in this section indicate a possible future process alternative for the large scale production of lysine from methanol by *B. methanolicus*.

2 AIMS OF THE STUDY

B. methanolicus may be regarded as an alternative industrial producer of lysine from methanol. Although it has a number of valuable qualities for industrial exploitation, several properties such as lysine productivity must be greatly improved in order to represent a genuine alternative to today's industrial production of lysine from sugar by *C. glutamicum*. Additionally, several aspects of the *B. methanolicus* biology, especially its methylotrophic property, are of interest in a basic science perspective, and findings within this area could contribute to a better understanding of microbial methylotrophy in general.

The first aim was to establish a reliable protocol for gene delivery in *B. methanolicus* as a foundation for metabolic engineering of this organism.

As other methylotrophs, *B. methanolicus* has been shown to be sensitive to high concentrations of its substrate, methanol (section 1.2.5 and 1.4.1). Such sensitivity may pose a problem for industrial use of this organism in large bioreactors with incomplete mixing. Additionally, as for any biotechnological production, a high substrate uptake rate is a prerequisite for high productivity. An aim for this project was therefore to expand the understanding of carbon fixation and assimilation in this organism, and possibly use such generated knowledge to improve industrially valuable characteristics of this bacterium by genetic engineering.

Due to the high degree of regulation of microbial lysine biosynthesis pathways, manipulation of key enzymes is normally essential for lysine overproduction in any organism. As deregulation of aspartokinase historically has been the most important step in the development of industrial lysine producing strains, this key enzymatic step was a natural starting point for metabolic engineering towards the generation of lysine overproducing recombinant strains of *B. methanolicus*. This study was limited to transcriptional deregulation and did not focus on alteration of putative feedback inhibition of the *B. methanolicus* aspartokinases.

3 SUMMARY OF RESULTS AND DISCUSSION

3.1 Theoretical considerations

(Paper III)

3.1.1 Stoichiometric conversion of methanol to lysine

Equations for the stoichiometric conversion of methanol to lysine are the basis for evaluation of the *B. methanolicus* lysine production potential in terms of product yield. However, different dissimilation mechanism of formaldehyde to CO_2, characterized by the generation of none, one or two NAD(P)H per formaldehyde molecule are reported (88), and the preferred mechanism in *B. methanolicus* is not known. Furthermore, as described in section 1.3.2, three variants of the lysine biosynthesis pathway are recognized in prokaryotes, and evidence of which variant(s) are active in *B. methanolicus* is yet not know.

Most *Bacillus* species are reported to use the acetylase variant of the lysine biosynthesis pathway (119). By assuming this variant, two equations for the stoichiometric conversion of methanol to lysine are presented in Equation 1, based on the generation of either one or two NAD(P)H per formaldehyde molecule.

$$7\ CH_3OH + 2\ NH_3 + 3.5\ O_2 \rightarrow C_6H_{14}O_2N_2 + CO_2 + 10\ H_2O \quad \text{(alternative A)}$$

$$8\ CH_3OH + 2\ NH_3 + 5\ O_2 \rightarrow C_6H_{14}O_2N_2 + 2\ CO_2 + 12\ H_2O \quad \text{(alternative B)}$$

Equation 1. Stoichiometric conversion of methanol (CH_3OH) to lysine ($C_6H_{14}O_2N_2$) by *B. methanolicus*. The acetylase variant of the lysine biosynthetic pathway is assumed. Alternatives A and B indicate different NAD(P)H-yield dependent on the dissimilation pathway (two and one NAD(P)H per formaldehyde molecule, respectively) (88). The synthesis of one lysine molecule requires two molecules of NAD(P)H.

As a comparison to the equations describing lysine production from methanol by *B. methanolicus* (Equation 1), theoretical equations were calculated for lysine production by the industrially preferred organism *C. glutamicum* from glucose. All three variants of the lysine biosynthetic pathways have been suggested to exist in *C. glutamicum* (139, 151, 162), although the succinylase and the dehydrogenase variants have been reported to be the most important (143, 161). Equations assuming the two latter variants are given in Equation 2.

$$1.5\ C_6H_{12}O_6 + 2\ NH_3 + 2\ O_2 \rightarrow C_6H_{14}O_2N_2 + 3\ CO_2 + 11\ H_2O \quad \text{(succinylase variant)}$$

$$1.23\ C_6H_{12}O_6 + 2\ NH_3 + 0.38\ O_2 \rightarrow C_6H_{14}O_2N_2 + 1.38\ CO_2 + 3.38\ H_2O \quad \text{(dehydrogenase variant)}$$

Equation 2. Stoichiometric conversion of glucose ($C_6H_{12}O_6$) to lysine ($C_6H_{14}O_2N_2$) by *C. glutamicum*, assuming the use of the succinylase or the dehydrogenase variant of the lysine biosynthetic pathway. The synthesis of one lysine molecule by the dehydrogenase variant requires two NAD(P)H, while synthesis via the succinylase variant is carbon-limited and requires no additional NAD(P)H.

3.1.2 Theoretical product yield

Theoretical maximum lysine yields can be calculated based on the stoichiometric equations given in section 3.1.1. For comparison, theoretical maximum lysine yields can similarly be calculated for the other variants of the lysine biosynthetic pathway. Table 1 compares maximum theoretical lysine yields from methanol by *B. methanolicus* to those from glucose by *C. glutamicum*.

Table 1. Theoretical maximum yields of lysine (grams of lysine-HCl per gram of methanol or glucose)

Lysine biosynthesis variant used	**Acetylase variant**	**Succinylase Variant**	**Dehydrogenase variant**
Yield of lysine from methanol by *B. methanolicus*	0.71 / 0.81 [1)]	0.63	0.71 / 0.81 [1)]
Yield of lysine from glucose by *C. glutamicum*	0.78	0.68	0.82

1) Values are dependent on the dissimilation pathway: The two values reported are based on the generation of one or two NAD(P)H per formaldehyde molecule.

As productivity is a function of substrate uptake rate and product yield, the methanol consumption rate of *B. methanolicus* is an important factor for evaluation of the lysine production potential of this organism. Based on reported data from a 14 l fed-batch fermentation with *B. methanolicus* MGA3 (50 g/l dry cell weight in 15 h) (136), a mean methanol consumption rate of at least 7 g/(l·h) can be calculated. This value is similar to glucose consumption rates reported for *C. glutamicum* (7).

Thus, based on high reported methanol conversion rates, theoretical maximum lysine yields comparable to those of *C. glutamicum* and comparable sugar-

and methanol-prices (section 1.1.6), *B. methanolicus* may be a potentially efficient biocatalyst for the production of lysine from methanol.

3.2 Establishment of protocols for gene delivery and high cell density cultivations of *B. methanolicus*

(Papers I, II and IV, and work not described in a manuscript)

3.2.1 Protoplast transformation

A protoplast transformation protocol for *B. methanolicus* classical AEC-resistant mutant NOA2-13A5-2 was published in 1997 (34). This was the first report of a genetic transformation system, shuttle vectors and integrative vectors for *B. methanolicus*. Using a polyethylene glycol (PEG) mediated transformation protocol, the authors reported high transformation efficiencies, demonstrating a presumably reliable tool for genetic modification. The amount of DNA used or the actual number of true transformants from these experiments was not stated (34). Later attempts by the authors to establish *B. methanolicus* transformants using the reported protocol and various genetic constructs for recombinant gene expression in *B. methanolicus* failed (M. C. Flickinger, unpublished results). In fact, no recombinant *B. methanolicus* strains were published by any laboratory in the following years. Our initial attempts of obtaining recombinant *B. methanolicus* by using the published protocol failed. These attempts included the use of several *B. methanolicus* wild type strains and various shuttle vectors such as pDQ507 (34), pDQ508 (34) and pTB1.9 (see below).

As a gene delivery protocol is a critical tool for metabolic engineering of *B. methanolicus*, substantial effort was devoted to the development of improved and new genetic tools for this organism. In brief, the previously published protocol (34) is based on lysozyme treatment of exponentially growing cells for the generation of protoplasts, PEG-mediated transformation of the protoplasts with plasmid DNA, followed by selection and regeneration of transformants on solid medium. Variations of the protocol of Cue et al were evaluated in order to develop a more reliable protocol for gene delivery. The results from these experiments with *B. methanolicus* wild type strain MGA3 were characterized by poor reproducibility, in accordance with the observations of Cue et al (34). Due to the low reproducibility, only general observations are summarized in Table 2.

Table 2. General observations from the optimization of a protoplast transformation protocol for *B. methanolicus* MGA3. The final protocol is given in Appendix 1.

Establishment of recombinant strains is dependent on:

1) generation of protoplasts
2) transformation of protoplasts with plasmid DNA
3) regeneration of protoplasts

Experiments repeatedly demonstrated that the success-rate of point 2) and 3) above were in reverse ratio. Intensified lysozyme treatment of the cells (by means of increased lysozyme concentration or incubation period) improved point 2) as observed by the number of true transformants per number of regenerated cells (without antibiotic selection). However, at the same time this worsened point 3) as observed by the total number of regenerated cells (without antibiotic selection) per number of cells entering the protocol.

The number of surviving transformants was found to increase by:

- Increasing the amount of plasmid DNA (up to 10 µg plasmid DNA to 10^9 lysozyme-treated cells).
- Gentle handling of protoplasts (by means of centrifugation conditions, constant temperature (25°C) during all experimental stages, low shear in all mixing steps).
- Decreased PEG-concentration during PEG treatment (down to 25% PEG_{8000}).
- Dilution of the mixture of protoplast, DNA and PEG after PEG-treatment to facilitate centrifugation.

The highest protoplast transformation frequency reported by Cue et al (34) was obtained with the *E. coli – B. methanolicus* shuttle vector pDQ508 (8.4 kb), constructed by cloning an endogenous *B. methanolicus* DNA fragment into an *E. coli* vector (34, 35). Upon sequencing of the naturally occurring *B. methanolicus* MGA3 plasmid pBM19 (section 3.3.1), pDQ508 was found to harbor a 4 kb region of pBM19, presumably carrying replication elements, but not including the complete *repB* gene. An alternative *E. coli – B. methanolicus* shuttle vector, pTB1.9 (6.0 kb), was constructed, and included a 2 kb pBM19 region covering the putative origin of replication and the complete *repB* gene of pBM19. In various protoplast transformation experiments, transformation with pTB1.9 gave 1.5 to 4-fold higher number of transformants than with pDQ508. Whether the improved transformation efficiency was due to decreased vector size, insertion of the complete *repB* gene, or some unidentified factor is unknown.

After protoplast transformation of *B. methanolicus* wild type MGA3 with pDQ508 or pTB1.9 (both plasmids carrying a neomycin resistance marker), a number of candidates (up to 90% in certain experiments) selected on solid regeneration medium with neomycin selection (5 µg/ml) were found not to grow after transfer to

new, selective medium (solid or liquid). Plating of MGA3 onto solid regeneration medium with neomycin did not produce colonies; neither did plating of cultures obtained by the completion of the protoplast transformation protocol without plasmid DNA. Additionally, transformants that proved to retain their neomycin resistance upon transfer to new selective medium did not grow in methanol medium and were cured for pBM19 (section 3.3.2). In sum, these results indicate incompatibility between pBM19 and pTB1.9 / pDQ508, due to the sharing of one or more elements of the plasmid replication or partitioning systems, as has been observed in several microbial systems (104, 113).

Optimization of the protoplast protocol led to the development of an alternative protocol (Appendix 1) that was successfully used for the establishment of several recombinant *B. methanolicus* strains such as MGA3(pTB1.9mdh) and MGA3(pTB1.9mdhL) (16). However, the reproducibility of the results obtained from this protocol remained poor, and the actual number of viable recombinant strains using MGA3 and pTB1.9-based vectors was normally less than 10 colonies. Transformation using pHP13 and pHP13-derived vectors did not produce a higher number of recombinant strains than using pTB1.9, in agreement with the results of Cue et al (34).

3.2.2 Electroporation

Transformation by means of electroporation was evaluated in order to achieve a more reliable and less laborious protocol for gene delivery than the optimized protoplast transformation protocol (section 3.2.1 and Appendix 1). Initial attempts were based on electroporation-protocols developed for related bacteria (e.g. 99, 146). However, in agreement with previous attempts (M. C. Flickinger, personal communication), recombinant *B. methanolicus* strains were not established by using these protocols and identified the need for a customized protocol in order to use electroporation as means for gene delivery in this organism.

A breakthrough in the development of a protocol for electroporation of *B. methanolicus* was extensive incubation in non-selective liquid medium after electroporation, followed by selection of recombinant strains in liquid medium: The electroporated culture was allowed to grow in rich, non-selective liquid medium over night before the culture was transferred to liquid, rich medium with antibiotic

selection. After six to eight hours incubation in selective medium, results were scored on a growth / no-growth basis. Cultures showing growth in liquid, selective medium were plated on selective solid medium in order to obtain single colonies.

Further optimization of the electroporation protocol was performed based on acquired knowledge on the importance of induced transcription levels of *mdh* and RuMP genes for improved methanol tolerance (section 3.3.5). As a consequence, recombinant strains were adapted to methanol by transferring cells from rich medium to a liquid medium with low methanol content (30 mM) before the methanol concentration in the medium was increased to normal levels (150 to 200 mM).

As the developed electroporation protocol (Appendix 2) includes an extensive incubation period prior to selection and isolation of recombinant strains, this method is not compatible with the generation of numerous unique recombinant strains from a single electroporation, such as the establishment of a mutant library. However, the protocol has proven to be a reliable method for gene delivery that is routinely used for various *B. methanolicus* strains and shuttle vectors, including the preferred cloning vector pHP13. Based on the number of colony-forming units upon plating on selective, solid medium and the number of generations during the liquid incubation periods, the number of unique transformants obtained by one electroporation experiment is estimated to normally vary between 1 and 100. This number is typically generated based on 10^9 cells and 1 µg plasmid DNA, and the variation in the transformation efficiency is highly dependent on the plasmid of choice.

3.2.3 High cell density fermentation

Existing infrastructure at SINTEF Materials and Chemistry, Department of Biotechnology offered a potential for running controlled laboratory-scale high cell density fermentations in an efficient manner. Operating conditions such as dissolved oxygen level, pH, temperature and air-flow is controlled and monitored online together with CO_2 evolution rate.

However, in order to run fermentation trials with methanol medium, control of the methanol concentration in each fermentor was critical. An infrastructure as outlined in Figure 7 was built in order to run multiple parallel fermentors with online monitoring and individual control of the methanol level. Headspace gas from each fermentor is analyzed by a mass spectrometer to determine the methanol

concentration in the headspace which is assumed to be in equilibrium with the liquid culture. In order to analyze head space gas from multiple fermentors, computer controlled valves were installed to multiplex between the headspace gases of the different fermentors. Headspace gas is carried from the fermentor, through valves for multiplexing and a flow-meter, to the mass spectrometer in stainless steel tubing with the overpressure inside the fermentor vessel as the driving force. All steel tubings, multiplexing valves and flow-meter are heated to 55 or 65°C and insulated in order to prevent condensation of methanol in the headspace gas. Custom software handles calibration and analysis of the ion-signals from the mass spectrometer in order to report online methanol concentrations for each fermentor. Additional custom software with control engineering algorithms evaluates online and historic methanol concentrations in the individual fermentors. This software then finally controls pumps for feeding of a methanol feed-solution to each fermentor to maintain a desired methanol level in the fermentation broth.

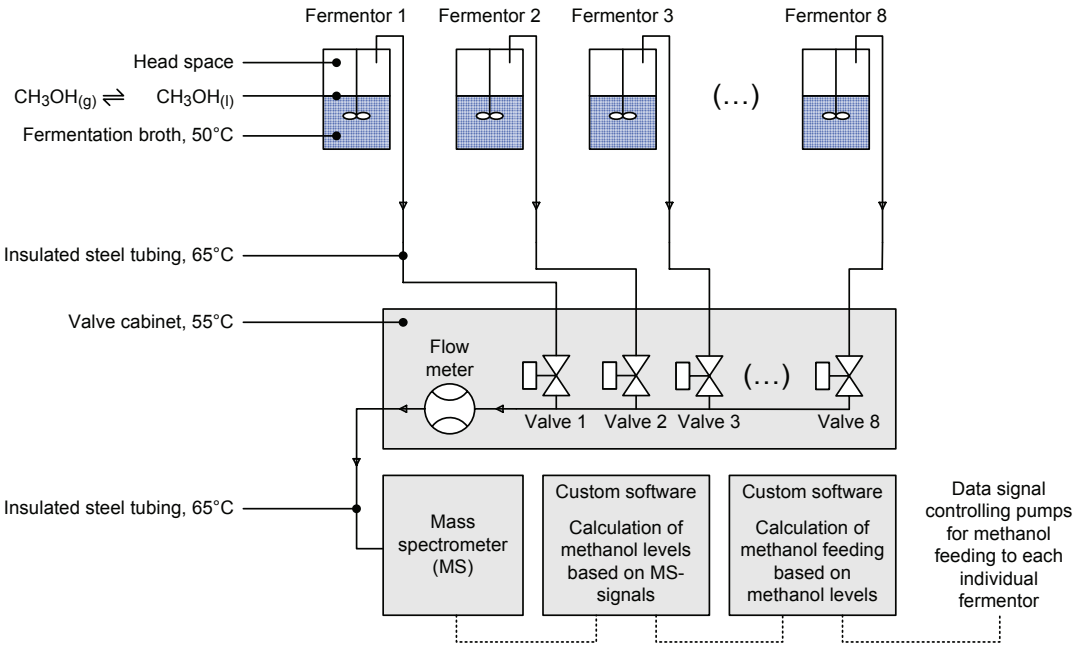


Figure 7. Outline of system for online monitoring of methanol levels and individual control of methanol feeding. Computer-controlled multiplexing of the headspace gases from each fermentor allows online analysis of multiple fermentors with one mass spectrometer.

3.3 Study and engineering of *B. methanolicus* methanol metabolism

(Papers I and II)

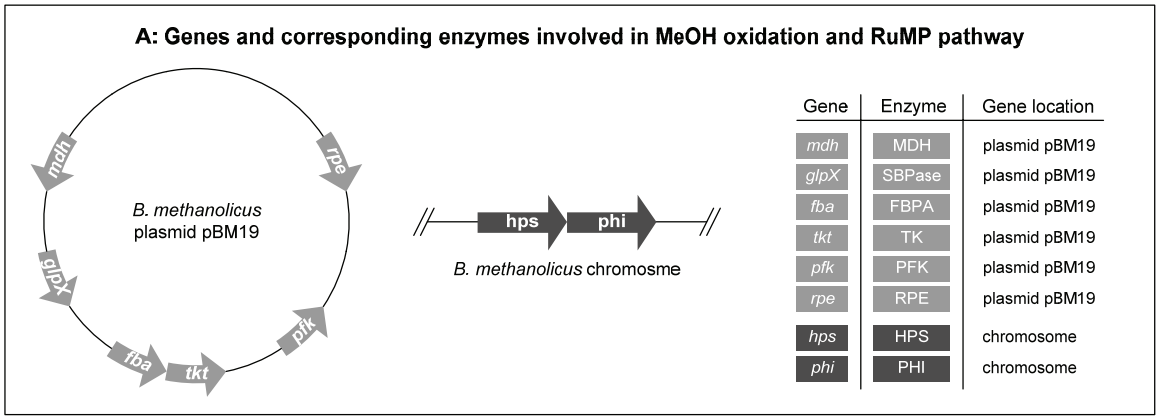
3.3.1 Natural plasmid pBM19 harbors key enzymes for methanol oxidation and assimilation

A 19 kb plasmid was isolated from wild type *B. methanolicus* MGA3 and designated pBM19. Surprisingly, sequencing of pBM19 unraveled the presence of key genes for methanol metabolism, including *mdh* encoding methanol dehydrogenase (Figure 8). In addition to *mdh*, pBM19 was found to carry genes for the putative enzymes sedoheptulose-1,7-bisphosphatase (SBPase; encoded by *glpX*), fructose-1,6-bisphosphate aldolase (FBPA; *fba*), transketolase (TK; *tkt*), 6-phosphofructokinase (PFK; *pfk*) and ribulose-5-phosphate 3-epimerase (RPE; *rpe*), all with putative roles in the RuMP pathway (section 1.2.4 and Figure 8).

B. methanolicus has previously been assumed to use the TA variant of the RuMP rearrangement part (section 1.4.1) as a low TA activity but no SBPase activity could be detected in cell extracts (Figure 4). It is however, not known whether TA activity is crucial for methanol consumption in *B. methanolicus*. The presence of the *glpX* gene encoding SBPase on pBM19 suggests that the SBPase variant of the RuMP rearrangement part may function in *B. methanolicus* (Figure 8).

Figure 8 (next page). Illustration of plasmid dependent methylotrophy in *B. methanolicus*. Genes and gene products of plasmid origin are on gray background while genes and gene products of chromosomal origin are on black background. A: Natural plasmid pBM19 with genes and corresponding enzymes involved in methanol oxidation and the RuMP pathway (left), and chromosomal genes involved in the RuMP pathway (center). B: Methanol oxidation and overview of the RuMP pathway. C: Fixation part of the RuMP pathway. D: Two possible variants of the regeneration part of the RuMP pathway. E: Two possible variants of the cleavage part of the RuMP pathway.

Abbreviations: *mdh*, MDH, methanol dehydrogenase; *glpX*, SBPase, sedoheptulose-1,7-bisphosphatase; *fba*, FBPA, fructose-1,6-bisphosphate aldolase; *tkt*, TK, transketolase; *pfk*, PFK, phosphofructokinase; *rpe*, RPE, ribulose-5-phosphate; *hps*, HPS, 3-hexulose-6-phosphate synthase; *phi*, PHI, 6-phospho-3-hexuloisomerase; RPI, ribose-5-phosphate; TA, transaldolase; PGI, phosphoglucose isomerase; GPDH, glucose-6-phosphate dehydrogenase; PGD, 6-phosphogluconate dehydratase; KDPGA, 2-keto-3-deoxy-6-phosphogluconate aldolase.


A: Genes and corresponding enzymes involved in MeOH oxidation and RuMP pathway
mdh
rpe
glpX
pfk
fba
tkt
B. methanolicus
plasmid pBM19
hps
phi
B. methanolicus chromosme
Gene
Enzyme
Gene location
mdh
MDH
plasmid pBM19
glpX
SBPase
plasmid pBM19
fba
FBPA
plasmid pBM19
tkt
TK
plasmid pBM19
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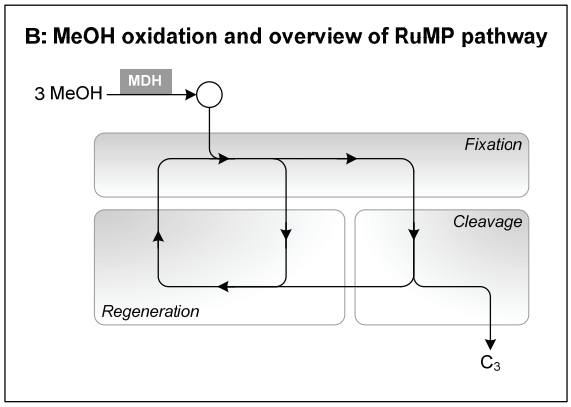
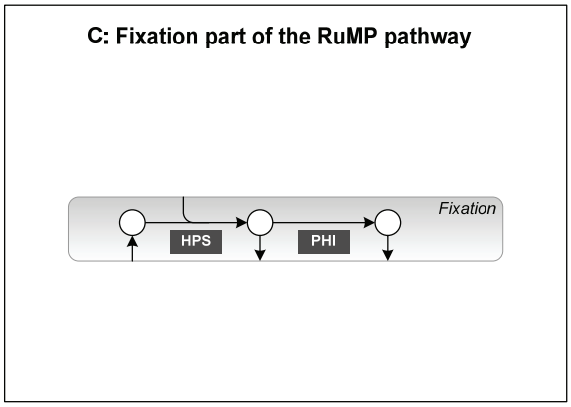
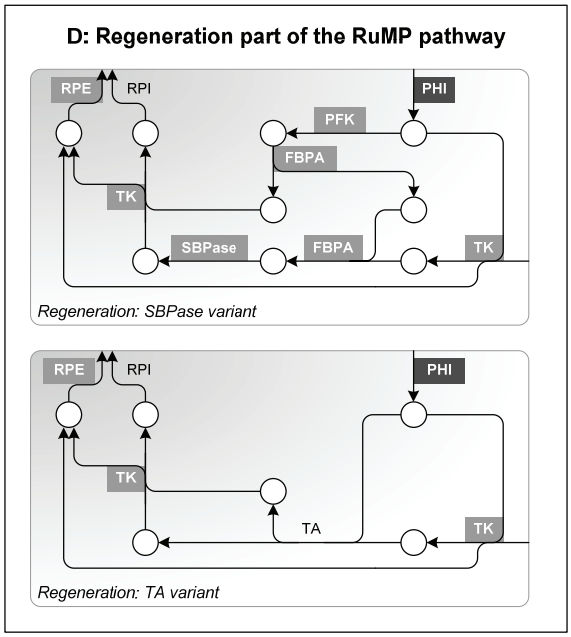
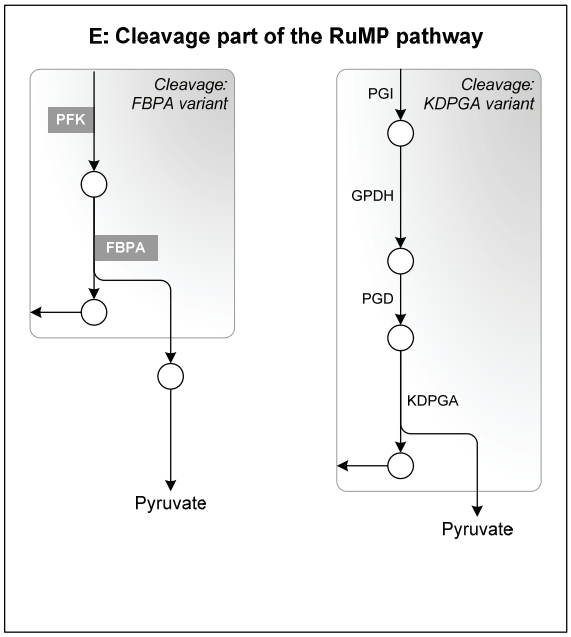
hps
HPS
chromosome
phi
PHI
chromosome


B: MeOH oxidation and overview of RuMP pathway
3 MeOH
MDH
Fixation
Cleavage
Regeneration
C3


C: Fixation part of the RuMP pathway
Fixation
HPS
PHI


D: Regeneration part of the RuMP pathway
RPE
RPI
PHI
PFK
FBPA
TK
SBPase
FBPA
TK
Regeneration: SBPase variant
RPE
RPI
PHI
TK
TA
TK
Regeneration: TA variant


E: Cleavage part of the RuMP pathway
Cleavage:
FBPA variant
PFK
FBPA
Pyruvate
PGI
Cleavage:
KDPGA variant
GPDH
PGD
KDPGA
Pyruvate

Southern hybridization experiments indicated that no chromosomal copies of pBM19-genes exist, and the copy number of pBM19 was estimated to be 10 to 16 copies per chromosome. Screening of an additionally 13 different *B. methanolicus* wild type strains demonstrated that they all harbor plasmids carrying *mdh* and RuMP genes, with restriction patterns similar but not identical to pBM19.

3.3.2 pBM19 is critical for methylotrophic growth, but represents a metabolic burden for B. methanolicus when growing on mannitol

After successful transformation of MGA3 with the pBM19-based shuttle vector pTB1.9 (section 3.2.1), southern hybridization experiments and PCR analysis confirmed loss of the natural plasmid pBM19, probably caused by plasmid incompatibility (104, 113). Upon prolonged cultivation of MGA3(pTB1.9) in non-selective medium, a pTB1.9-cured strain designated MGA3C-A6 was isolated. Neither MGA3(pTB1.9) nor MGA3C-A6 could grow on methanol, demonstrating that pBM19 is critical for methylotrophic growth by this organism. Introduction of the *B. methanolicus mdh* gene returned *mdh* transcription to wild type levels, but was not sufficient to restore methanol growth of MGA3C-A6, indicating that pBM19 genes besides *mdh* are required for methanol consumption in this organism. To our knowledge, plasmid-linked methylotrophy has previously not been reported in any organism. Although large plasmids are commonly present in methylotrophic bacteria (38, 92), the understanding of the biological significance of these replicons is limited. Based on the characterization of pBM19, its function in *B. methanolicus* and the fact that methylotrophy does not correlate well with traditional methods of bacterial classification (5, 88), it is tempting to speculate that methylotrophy might be a transferable metabolic trait in nature.

As mannitol is the only sugar that has been reported to support rapid growth of *B. methanolicus* (16, 136), the pBM19-cured strains were characterized in a defined mannitol medium. Interestingly, while the specific growth rates of wild type MGA3 in shake flasks cultures on mannitol and methanol medium were similar (0.30 h^{-1} and 0.32 h^{-1}, respectively), the pBM19-cured strain MGA3C-A6 showed a higher specific growth rate on mannitol (0.37 h^{-1}) (Table 3). These data indicate that the maintenance of pBM19 represents a metabolic burden for *B. methanolicus* growing on mannitol. This was supported by the characterization of a resulting

population after prolonged growth of the wild type MGA3 in liquid defined mannitol medium: After 140 generations, 80% of the colonies tested were cured of pBM19 and could no longer grow on methanol. Mannitol may be taken up by the cells as fructose-6-phosphate, similar to other *Bacillus* species (160), indicating that *B. methanolicus* may have isozymes of both PFK and FBPA to metabolize fructose-6-phosphate (Figure 3).

3.3.3 Methanol metabolism relies on both chromosomal and plasmid-borne genes

The *hps* and *phi* genes encoding 3-hexulose-6-phosphate synthase (HPS) and 6-phospho-3-hexuloisomerase (PHI), respectively, were PCR amplified from wild type MGA3 total DNA. The genes are critical for the fixation phase of the RuMP pathway, and operons including *hps* and *phi* have previously been cloned from both methylotrophs utilizing the RuMP pathway (107, 132, 170), and non-methylotrophs such as *B. subtilis* (167). The finding of *hps* and *phi* being located on the *B. methanolicus* chromosome suggests that methanol oxidation is governed by the concerted action of both chromosomally and plasmid-borne genes in this organism. This notion is supported by pBM19's lack of the gene encoding the *B. methanolicus* MDH activator protein ACT (61, 80).

3.3.4 Genes for methanol metabolism are transcriptionally upregulated upon methylotrophic growth

To further investigate the biological function of pBM19 for methylotrophic growth, transcriptional regulation of pBM19-genes in MGA3 grown in mannitol and methanol media was analyzed by RT-PCR. The results of these analyses showed that the *mdh* gene was induced about threefold in cells growing on methanol compared to those growing in mannitol medium. Also the putative RuMP-genes *glpX*, *fba*, *tkt*, *pfk* and *rpe* were induced upon methanol growth, supporting the assumed roles of these genes for methanol metabolism in *B. methanolicus*. Induced expression of genes for the metabolism of one-carbon compounds have been reported in both autotrophic (154) and methylotrophic (3, 11, 75, 89, 170) bacteria. The induction of the pBM19-

Table 3. Summary of specific growth rates on methanol and mannitol-based minimal medium, and methanol and formaldehyde tolerance of wild type *B. methanolicus* MGA3 and recombinant strains.

Strain name	Description	Presence of genes for methanol oxidation and assimilation [1]								Specific growth rate [h^{-1}] [2]		Methanol tolerance [3]		Formaldehyde tolerance [3]
		mdh	*glpX*	*fba*	*tkt*	*pfk*	*rpe*	*hps*	*phi*	Methanol medium	Mannitol medium	Methanol medium	Mannitol medium	Mannitol medium
MGA3	Wild type and reference strain	☑	☑	☑	☑	☑	☑	☑	☑	0.32	0.30	★★	★	★★
MGA3C-A6	MGA3 without pBM19	☐	☐	☐	☐	☐	☐	☑	☑	Cannot grow	0.37	NA	★★★	★
MGA3C-A6(pTB1.9mdhL)	MGA3C-A6 with *mdh* on plasmid pTB1.9	☑	☐	☐	☐	☐	☐	☑	☑	Cannot grow	0.26	NA	★	NT
MGA3(pHP13)	Wild type carrying empty shuttle vector	☑	☑	☑	☑	☑	☑	☑	☑	0.32	0.26	★★	NT	NT
MGA3(pHP13hps+phi)	Wild type with *hps* and *phi* on plasmid pHP13	☑	☑	☑	☑	☑	☑	**☑**	**☑**	0.37	0.25	★★★	NT	NT

1) Bold face indicate overexpression of gene
2) Specific growth rate measured in defined methanol ($MeOH_{200}$) or mannitol ($Mann_{10}$) medium in shakeflasks
3) Methanol and formaldehyde tolerance indicated by stars. Increasing number of stars imply increasing methanol- and formaldehyde tolerance, measured as specific growth rate in minimal medium after methanol (60 – 2880 mM) or formaldehyde (0.2 – 5.4 mM) addition. NA, not applicable; NT, not tested

genes assumed to be involved in methanol metabolism varied from 3-fold (*mdh*) to 40-fold (*pfk*). A low *pfk* transcription in mannitol medium and a corresponding strong induction upon methylotrophic growth may indicate that the *pfk* gene product is only needed during carbon assimilation by the RuMP pathway. This is similar to previously reported data on an ATP-dependent PFK activity induced by methanol, whereas a pyrophosphate-dependent PFK enzyme is expressed upon growth on sugar (3).

The chromosomal genes *hps* and *phi* involved in the fixation phase of the RuMP cycle were similarly shown to be induced about 6-fold upon methanol growth compared to growth on mannitol. In accordance with these data, the corresponding increase in *in vitro* activity of HPS+PHI in MGA3 was measured to be about two-fold.

To investigate which one-carbon compound (methanol or formaldehyde) induces the RuMP genes in *B. methanolicus*, HPS+PHI activity was measured in mannitol-grown MGA3 and MGA3C-A6 pulsed with either methanol or formaldehyde. The *in vitro* HPS+PHI activity of MGA3 cells grown in methanol was, as stated above, about twofold higher than that of mannitol-grown MGA3 cells. We achieved a similar twofold increase in HPS+PHI activity when mannitol-grown MGA3 cells were pulsed with methanol before cell harvesting. However, in the pBM19-cured strain MGA3C-A6 lacking *mdh*, no increase in HPS+PHI activity could be detected upon pulsing of methanol to a mannitol culture. However, both MGA3 and MGA3C-A6 displayed a 1.5 to 2-fold increase in HPS+PHI upon pulsing of formaldehyde to a mannitol-culture. These data confirm that formaldehyde, and not methanol, is the inducer of the RuMP pathway genes *hps* and *phi*, and that this induction is independent of any pBM19 function. These findings are consistent with induced expression of HPS and PHI upon small formaldehyde additions in non-methylotrophic bacteria (106, 167), and earlier suggestions that formaldehyde can induce HPS activity in *B. methanolicus* C1 (8).

3.3.5 Transcription levels of mdh and RuMP pathway genes modulate the cell's methanol tolerance

To investigate the cell's methanol tolerance, wild type strain MGA3 was pulsed with methanol in defined methanol and mannitol medium, while growth rate was monitored. These experiments showed that cells already growing on methanol tolerated pulsing of about 10-fold higher methanol additions than cells growing on mannitol (Table 3). Together with the RT-PCR data displaying methanol-induced

transcription of *mdh* and RuMP pathway genes (section 3.3.4), these results indicate that the transcription level of *mdh* and RuMP pathway genes control the *B. methanolicus* methanol tolerance. Previous reports have shown that *B. methanolicus* is highly sensitive to methanol pulses under methanol limitation (Pluschkell and Flick 2002). Our new data contributes to the understanding of physiological responses to fluctuations in methanol levels and should be of high relevance for industrial large-scale and high-cell-density fed-batch fermentations in which the methanol concentrations inside the fermentors may not be uniform.

In an MGA3 culture grown in a medium containing both methanol and mannitol, about 75 % of the carbon utilized was derived from methanol. The methanol tolerance of this culture was similar to that of the culture growing exclusively on methanol, suggesting that *B. methanolicus* can induce its RuMP pathway genes as long as methanol is present.

3.3.6 Methanol sensitivity is caused by formaldehyde accumulation

To investigate the cause of the toxic effects of methanol pulsing, we grew the wild type MGA3 and the pBM19-cured strain MGA3C-A6 in mannitol medium and observed the effect of methanol pulsing on growth rate. Interestingly, MGA3C-A6 cells lacking the *mdh* gene could tolerate about a 10-fold higher methanol concentration than wild type MGA3 (Table 3). Reintroduction of the homologous *mdh* gene into MGA3C-A6 reduced the methanol tolerance down to wild type levels, suggesting that the toxic effect in response to methanol addition is largely due to accumulation of formaldehyde produced by the MDH-mediated conversion of methanol.

Furthermore, phenotypic responses of formaldehyde-pulsing to mannitol-grown cultures revealed that the tolerance of MGA3C-A6 for formaldehyde is significantly lower than that of the wild type strain (Table 3). These data support the hypothesis that *mdh* causes the formation of formaldehyde when methanol is pulsed into the growth medium and in the absence of a functional RuMP pathway, formaldehyde may accumulate to toxic levels.

A linear pathway for dissimilation of formaldehyde via formate to CO_2 was previously reported in *B. methanolicus* (123) (Figure 5). Additionally, activities of the enzymes involved in the cyclic formaldehyde dissimilation pathway via glucose-6-

phosphate has been measured in *B. methanolicus* (11). It is plausible to assume that both the RuMP pathway, the linear dissimilatory pathway and the cyclic dissimilatory pathway play a role in formaldehyde detoxification by *B. methanolicus* (Figure 5).

3.3.7 Methanol tolerance and specific growth rate on methanol can be improved by overexpression of hps and phi

In contrast to the chromosomally located RuMP pathway genes *hps* and *phi*, the genes *mdh*, *glpX*, *fba*, *tkt*, *pfk* and *rpe* are located on pBM19 with an estimated copy number of 10 to 16 per chromosome. By introduction of a shuttle vector containing *hps* and *phi* into *B. methanolicus*, the *in vitro* HPS+PHI activity of methanol-grown cells increased about three-fold. Interestingly, the specific growth rate in methanol medium increased from 0.32 h^{-1} to 0.37 h^{-1} upon over-expression of *hps* and *phi*. Furthermore, the recombinant strain's methanol tolerance improved, as demonstrated by improved growth rate after methanol pulsing (up to 1440 mM) to cultures growing in methanol medium (originally containing 200 mM methanol). These data indicate that methanol assimilation efficiency and methanol tolerance are closely connected in *B. methanolicus* and both traits can be improved by increasing the gene dosage of *hps* and *phi*.

3.4 Metabolic engineering of the *B. methanolicus* aspartate pathway by means of overexpressing aspartokinase

(Paper IV and results not described in a manuscript)

3.4.1 B. methanolicus harbors three genes encoding aspartokinase activity

The presence of three different aspartokinase isozymes in *B. methanolicus* inhibited in a similar way as those of *B. subtilis* was previously predicted based on inhibition studies (138). However, only *lysC* encoding aspartokinase II was previously cloned and sequenced. In order to study the role of the *B. methanolicus* aspartokinase isozymes on lysine production, the genes for aspartokinase I (*dapG*) and III (*yclM*) were cloned based on previously reported DNA sequences of related species.

dapG encoding aspartokinase I was shown to be located inside a putative operon, similar to what has been found in *B. subtilis* and other bacteria (25, 64, 153). Partial sequencing of the putative *B. methanolicus dap* operon revealed putative genes encoding aspartate semialdehyde dehydrogenase, aspartokinase I and dihydrodipicolinate synthase, in addition to parts of an upstream putative gene encoding dipicolinate synthase, all enzymes of the aspartate pathway (Figure 6).

3.4.2 Individual overexpression of aspartokinase-encoding genes increases lysine production in B. methanolicus

The three genes *dapG*, *lysC* and *yclM* encoding aspartokinase I, II and III, respectively, were overexpressed in *B. methanolicus* by using a cassette cloning and expression vector system. The three coding regions were coupled to the *mdh* promoter and ribosome binding site and introduced into *B. methanolicus* wild type MGA3. The resulting recombinant strains MGA3(pHP13mp-dapG), MGA3(pHP13mp-lysC) and MGA3(pHP13mp-yclM) all displayed increased *in vitro* aspartokinase activity (4, 42 and 5-fold, respectively). Interestingly, all recombinant strains overexpressing individual aspartokinase genes displayed increased lysine production in high cell density fermentation trials on methanol, with similar specific growth rates as the wild type and the control strain MGA3(pHP13) harboring empty expression vector. The wild type MGA3 reached a maximum dry cell weight of 58 g/l in 23 hours with an initial specific growth rate of 0.49 h^{-1} and a final lysine concentration of 0.18 g/l in the growth medium (Table 4). The most dramatic increase in lysine production was observed with the recombinant strain overexpressing *yclM*, encoding aspartokinase III, which produced over 60-fold more lysine (11 g/l; volume corrected value) than the control strain. The recombinant strains overexpressing *dapG* and *lysC* displayed a 2-fold and 10-fold increase in lysine production, respectively (Table 4). The glutamate production by all strains was similar (48 – 52 g/l), and the production of the other end products of the aspartate pathway remained low (methionine < 0.5 g/l; threonine < 0.1 g/l) in all recombinant strains. The lysine overproducing recombinant strains showed elevated intracellular lysine concentrations, while the intracellular concentration of threonine remained low in all strains (<0.5 mM).

Aspartokinase activity is known to be controlled by efficient allosteric feedback inhibition in several species, and the reduction of susceptibility of

aspartokinase to feedback inhibition has been the most important step in the development of L-lysine producing strains. Numerous examples of increased lysine production upon allosteric feedback deregulation of aspartokinase exist, especially in *C. glutamicum* (e.g. 32). The 60-fold lysine overproduction of MGA3(pHP13mp-yclM) overexpressing wild type aspartokinase III therefore seems surprising, and similar lysine overproduction has not previously been reported in the literature. Increasing the copy number of a wild type aspartokinase gene in a *Brevibacterium flavum* mutant resulted in a 33% increase in lysine production (from 23.7 to 28.7 g/l) (95), while overexpression of wild type aspartokinase in *C. glutamicum* was detrimental to growth in defined medium (32, 82).

Table 4. Initial specific growth rate, maximum dry cell weight and final lysine production of *B. methanolicus* wild type MGA3, MGA3 mutant M168-20, and recombinant strains overexpressing *dapG*, *lysC* and *yclM*. "+M" denotes methionine-feeding throughout the fermentation trial.

Strain	Specific growth rate [h^{-1}]	Dry cell weight [g/l] 1)	Lysine production [g/l] 1)	Lysine in growth medium [g/l] 2)
MGA3	0.49	58	0.18	0.12
MGA3(pHP13)	0.49	56	0.18	0.12
MGA3(pHP13mp-dapG)	0.50	62	0.38	0.23
MGA3(pHP13mp-lysC)	0.46	61	1.8	1.1
MGA3(pHP13mp-yclM)	0.50	54	11	7.0
M168-20 +M	0.40	69	14	7.8
M168-20(pHP13) +M	0.39	73	15	8.3
M168-20(mp-yclM) +M	0.33	58	14	9.0
MGA3(pHP13) +M	0.53	71	0.22	0.15

1) Reported biomass and lysine production are corrected for dilution caused by feeding throughout the fermentation in order to compare results from different bioreactor trials.
2) Actual lysine concentration measured in growth medium.

Although the reason for the exclusive overproduction of lysine by the recombinant MGA3-strains is unknown, the resulting low intracellular threonine concentration may partly explain the high potential for lysine production by MGA3(pHP13mp-yclM). Aspartokinase III is synergistically feedback regulated by lysine and threonine in *B. subtilis*, and regulation of the different aspartokinases is suggested to be similar in *B. methanolicus* (138). Aspartokinase III of *B. subtilis* retained more than 25% of the activity upon addition of 20 mM lysine *in vitro* (52); inhibition was not efficient unless both lysine and threonine were present (>1 mM).

3.4.3 AEC-resistant mutant M168-20 overproduces lysine and harbors no mutations in aspartokinase-encoding genes (results not described in a manuscript)

After one round of mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine, an AEC-resistant mutant of MGA3 was selected and designated M168-20 (Rick Dillingham, unpublished results). This mutant required addition of methionine for growth in defined methanol-medium. In a high cell density fermentation trial, M168-20 produced significant amounts of lysine, reaching a maximum of 14 g/l in 30 hours, with an initial specific growth rate of 0.40 h^{-1} (Table 4). No accumulation of threonine or methionine could be detected in the growth medium. The final glutamate production (50 g/l) was similar to that of MGA3 (49 g/l).

The final biomass concentration of M168-20 was higher than that of MGA3 (69 g/l and 58 g/l, respectively), suggesting a possible effect of methionine feeding on cell growth. To investigate potential effects of methionine feeding, fermentation trials of MGA3(pHP13) with and without addition of methionine were compared. Indeed, the final biomass concentration of MGA3(pHP13) with methionine feeding was higher than without (73 g/l and 58 g/l, respectively) and comparable to that of M168-20 (69 g/l) (Table 4). Also the initial specific growth rate increased upon methionine feeding of MGA3(pHP13) (from 0.49 to 0.53 h^{-1}), indicating that methionine is beneficial for growth of *B. methanolicus* in defined methanol medium. Methionine feeding caused no major effect on lysine production by MGA3(pHP13) (Table 4).

Remarkably, genetic characterization of M168-20 revealed no mutations in the coding regions of *dapG, lysC* and *yclM*. Similarly, no mutations were found in regions of 350, 594 and 489 bp upstream of *asd*, *lysC* and *yclM* coding regions,

respectively. Additionally, *in vitro* aspartokinase activity of crude extract of M168-20 was similar to that of MGA3 (both 0.05 U/mg protein). Together, these results indicate that mutant M168-20 carries wild type aspartokinase isozymes regulated in the same manner as for MGA3. Several studies on both *C. glutamicum* and *B. subtilis* report AEC resistance being mediated by altered transcription regulation of aspartokinase (81, 96, 97, 121) or amino acid substitutions in aspartokinase (e.g. 116). However, also another lysine overproducing *B. methanolicus* AEC-resistant mutant was previously reported to carry a wild-type aspartokinase II gene (138), suggesting that aspartokinase may not be a primary target for AEC-resistance in wild type *B. methanolicus*.

To investigate if the mutation(s) in M168-20 was located in other genes encoding aspartate pathway enzymes, M168-20 was further genetically characterized. Interestingly, these results demonstrated no mutations in the key genes *asd*, *dapA* and *lysA* (both coding and upstream regions). The remaining genes of the aspartate pathway were not investigated due to unavailable genetic sequences. The methionine requirement of M168-20 may suggest that it is affected in homoserine dehydrogenase, similar to previously reported homoserine auxotrophs and AEC-resistant mutants of *B. methanolicus* (136), and an AEC resistant mutant of *B. flavum* (95). This could explain the methionine requirement and a potentially lower intracellular threonine concentration which would decrease a presumably concerted feedback inhibition of aspartokinase III by lysine and threonine. This is in agreement with RT-PCR data of shakeflask cultures demonstrating increased *yclM* transcription in M168-20 compared to that of MGA3 (Roman Netzer and Trygve Brautaset, unpublished results), as *yclM* expression is known to be repressed by threonine in related organisms such as *Bacillus sphaericus* and *B. subtilis* (13, 119).

3.4.4 Overexpression of yclM does not increase lysine production in M168-20 during high cell density fermentation trials (results not described in a manuscript)

Recombinant M168-20 strains individually overexpressing *dapG*, *lysC* and *yclM* were established. Similar to the MGA3 series of recombinant strains, crude extracts of the M168-20 recombinant strains overexpressing individual aspartokinase-encoding genes displayed increased aspartokinase activities (4-fold, 39-fold and 3-fold, respectively). Unlike for the MGA3 series of recombinant strains, the lysine

production of M168-20(pHP13mp-yclM) overexpressing *yclM* was similar at all times during the fermentation trial to that of a control strain carrying an empty expression vector. The resulting final lysine production were 14 and 15 g/l, respectively (Table 4), indicating that under these conditions, potential positive effects of increased AK activity on L-lysine production may be suppressed by another, unknown limiting factor. Analysis of the growth medium throughout the fermentation trial verified no nitrogen limitation (1.7 g nitrogen/l still available at the end of the fermentation trial).

Shakeflask studies of the M168-20 recombinant strains overexpressing *dapG*, *lysC* and *yclM* demonstrated that they all produced more L-lysine than the control strain (Ingemar Nærdal and Trygve Brautaset, unpublished results), verifying the genetic construction of these strains and illustrating that overexpression of wild type aspartokinase-encoding genes may be interesting even in lysine overproducing strains. However, the reason for M168-20(pHP13mp-yclM) not yielding increased L-lysine production under the high cell density fermentations trials remains unknown and illustrates the need for further understanding of the control mechanisms of the lysine biosynthesis pathway in *B. methanolicus*.

4 SUGGESTIONS FOR FURTHER WORK

The development of protocols for gene delivery by means of protoplast transformation and electroporation offered the possibility of constructing recombinant *B. methanolicus* strains. However, future metabolic engineering of this organism will highly benefit from, and to a certain extent be dependent on further development of the genetic toolbox. Protocols for *B. methanolicus* gene knockout and gene replacement by homologous recombination (e.g. by means of suicide vectors or temperature-sensitive vectors) would make possible the construction of valuable recombinant strains. An extended library of useful promoters (both inducible and constitutive promoters of various strengths) would offer the possibility of tuning gene expression and preventing potential toxic cell conditions.

The major importance of aspartokinase in regards to *B. methanolicus* lysine biosynthesis was illustrated by the increase in lysine production upon overexpression of aspartokinase isozymes. To improve the knowledge of the aspartokinase properties and to better predict possible effects of manipulations of the aspartokinase isozymes, a biochemical characterization of these enzymes would be valuable. Such information in combination with the measurement of intracellular metabolites such as intermediates and end products of the aspartate pathway should have a significant impact on the future strategies for metabolic engineering of *B. methanolicus* towards efficient production of lysine. Furthermore, investigations of the effects of deregulated aspartokinase isozymes are motivated by the considerable impacts of such approaches in other lysine-producing bacteria such as *C. glutamicum*. In addition, a characterization of the available and dominating lysine biosynthetic pathway(s) would improve the understanding of the *B. methanolicus* lysine biosynthesis and serve as a basis for further metabolic engineering of this organism.

As discussed in this thesis, several *B. methanolicus* pathways are believed to exist for formaldehyde detoxification. Insight into the split at which formaldehyde is being assimilated or dissimilated by various pathways should be valuable for improved understanding of methylotrophy in *B. methanolicus*, and may be an important contribution to future genetic engineering towards more efficient bioproduction by this organism. Such studies may include flux analysis of the assimilation and dissimilation pathways and engineering of both available and newly

cloned genes (e.g. formaldehyde and formate dehydrogenase for which gene cloning and sequencing has been initiated (Matt Smith, unpublished results)).

In the process of cloning and sequencing of *yclM*, other genes encoding aspartate pathway enzymes were fully or partly sequenced. Especially *asd* and *dapA* are highly relevant candidates for further genetic engineering and evaluation of potential effects on lysine biosynthesis in *B. methanolicus*, possibly in combination with overexpression of *yclM*.

As a library of classical lysine overproducing mutants exists (M. C. Flickinger, unpublished results), selected genes assumed relevant for lysine biosynthesis can be sequenced in such mutants to possibly indicate additional limiting enzymatic steps for lysine biosynthesis. Such studies may be supplemented by the analysis of relevant intracellular metabolite levels, to generate a broad hypothesis-generating data-pool.

Engineering of the lysine export mechanism in *C. glutamicum* proved to be an efficient approach to increase lysine excretion rate in this organism. Identification and future improvement of a similar system in *B. methanolicus* may prove to be a key approach for obtaining economically competitive lysine production rates and levels also in this organism.

Improved osmotolerance of *B. methanolicus* may be required to maintain or increase the lysine production rate at high osmotic pressure caused by e.g. high lysine levels. Experiments have indicated that *B. methanolicus* does not harbor uptake systems for common osmoprotectants (Anders Øverby, Øyvind M. Jakobsen, unpublished results). Work has been initiated on introduction of heterologous uptake systems and biosynthesis routes for osmoprotectants.

It is tempting to speculate if methylotrophy as a fundamental physiological property can be transferred to other bacteria by the introduction of pBM19-like plasmids. Such an approach would be based on the finding of *mdh* and critical RuMP-pathway genes on the natural plasmid pBM19, the fact that several non-methylotrophs already carry the *hps* and *phi* genes, and the knowledge that methylotrophy does not correlate well with traditional methods of bacterial classification. Attempts have been made to engineer *C. glutamicum* to produce lysine from methanol (150), but an organism that can utilize methanol as the sole source of carbon and energy, obtained by genetic engineering from a non-methylotroph has not been reported. Such a result should have impacts far beyond those of basic

science, as selected processes within microbial industrial production could potentially be converted from sugar-based to methanol-based processes.

B. methanolicus is a natural producer of glutamate. In addition to the focus on lysine production, current research in our laboratories is also directed towards the use of *B. methanolicus* for industrial production of glutamate from methanol.

5 CONCLUSIONS

Improved and new tools for *B. methanolicus* gene delivery by means of protoplast transformation and electroporation were developed. These tools served as the basis for the first demonstrations of recombinant gene expression in any *B. methanolicus* strain, and represent today a reliable protocol for establishing recombinant *B. methanolicus* mutants.

The naturally occurring plasmid pBM19 of the thermotolerant methylotroph *B. methanolicus* MGA3 was shown to carry the key gene *mdh* encoding methanol dehydrogenase which is responsible for methanol oxidation and critical for methanol utilization in this organism. Additionally, pBM19 harbors five genes involved in the RuMP pathway for the assimilation of methanol-derived carbon. While pBM19 represented a metabolic burden when growing on mannitol, *B. methanolicus* cells cured of this plasmid could no longer grow on methanol, demonstrating the first example of plasmid-linked methylotrophy in any organism. Transcription of both pBM19-borne and chromosomally located genes for methanol oxidation and assimilation are induced upon methylotrophic growth, with formaldehyde as the inducing compound. Transcriptional upregulation of *mdh* and RuMP pathway genes increases the cell's methanol tolerance, and an upregulated RuMP pathway is believed to improve the cell's ability to detoxify intracellular formaldehyde. Increased activity of the chromosomally located genes *hps* and *phi* encoding 3-hexulose-6-phosphate synthase and 6-phospho-3-hexuloisomerase, respectively, improved the specific growth rate and the cell's methanol tolerance, indicating HPS+PHI activity as a limiting factor for assimilation and detoxification of formaldehyde.

dapG and *yclM* encoding aspartokinase I and III respectively, were cloned and sequenced from *B. methanolicus* MGA3, and represents, together with the previously known *lysC* encoding aspartokinase II, a set of three aspartokinase isozymes in this organism. Individual overexpression of these three wild type aspartokinase-encoding genes demonstrated improved lysine production. Upon overexpression of aspartokinase III in the wild type strain MGA3, the lysine production was improved more than 60-fold to 11 g/l in high cell density fermentation trials, while the production of the other end products of the aspartate pathway, methionine and threonine, remained low. A low intracellular threonine concentration may partly explain the high lysine production potential of the mutant

overexpressing aspartokinase III, as efficient feedback regulation of this enzyme may require the presence of appreciable concentrations of both intracellular lysine and threonine. A similar increase in lysine production upon overexpression of wild type aspartokinase has not previously been demonstrated in any organism.

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Appendix 1.

Protocol for protoplast transformation of *B. methanolicus*

Starting material

1. 1 ml ampoules of *B. methanolicus* are made from SOBsuc cultures (72) which are harvested at OD_{600} = 1.0 - 1.5 and added 15% (v/v) glycerol before freezing at -80°C.

Cultivation

2. Inoculate 100 µl thawed *B. methanolicus* culture from frozen ampoule in 100 ml SOBsuc medium. Grow in 500 ml baffled shakeflask for 16 hours at 50°C, 200 rpm.
3. Transfer 1 ml culture to 100 ml SOBsuc medium (pre-warmed to 50°C) in 500 ml baffled shakeflask and continue to grow at 50°C, 200 rpm to OD_{600} = 0.15 - 0.20.

Protoplast generation

4. Transfer 35 ml culture to a 50 ml centrifugation tube. Centrifuge 10 min, 2000 g, 25°C.
5. Pour off supernatant and resuspend in 3.5 ml 1 µg/ml lysozyme in SMMCB (34) by pipetting up and down 4 - 5 times.
6. Incubate for 60 min at 42°C, horizontally, 150 rpm.
7. Centrifuge 10 min, 1500 g, 25°C, pour off supernatant and resuspend in 3.5 ml SMMCB by pipetting up and down 4 - 5 times.
8. Centrifuge 10 min, 1500 g, 25°C, pour off supernatant and resuspend in 0.7 ml SMMCB. Pipette up and down about 25 times to resuspend.

Protoplast transformation

9. Mix 0.6 ml protoplast suspension with 3 µg plasmid (0.5 – 1 µg/µl DNA) in a 50 ml centrifugation tube.
10. Add 1.0 ml 40% PEG_{8000} in SMMC (34) with a pipette with an opening of approximately 4 mm in diameter, mix by pipetting up and down about 10 times and incubate at room temperature for 3 min.
11. Add 10 ml SMMCB.
12. Centrifuge 10 min, 2000 g, 25°C, pour off supernatant and resuspend in 5 ml SMMCB by pipetting up and down about 20 times.
12. Centrifuge 10 min, 2000 g, 25°C and pour off supernatant.

Protoplast regeneration

13. Resuspend the protoplast pellet in the 50 ml centrifugation tube in 0.4 ml SMMCB by pipetting up and down about 20 times.
14. Incubate at 50°C for 2 hours, 175 rpm. Pipette up and down about 20 times before plating.
15. Carefully plate 500 µL on a 13 cm Regeneration agar plate (34) with appropriate antibiotic pressure (e.g. 5 µg/ml neomycin).
16. Incubate the plate in plastic at 50°C for 2-3 days.

Appendix 2.
Protocol for electroporation of *B. methanolicus*

Starting material

1. 1 ml ampoules of *B. methanolicus* are made from SOBsuc cultures (72) which are harvested at OD_{600} = 1.0 - 1.5 and added 15% (v/v) glycerol before freezing at -80°C.

Cultivation and generation of electrocompetent cells

2. Inoculate 200 µl thawed *B. methanolicus* culture from frozen ampoule in 100 ml SOBsuc (72). Grow in 500 ml baffled shakeflask for 16 hrs at 50°C, 200 rpm.
3. Transfer 1 ml culture to 100 ml SOBsuc medium (pre-warmed to 50°C) in 500 ml baffled shakeflask and continue to grow at 50°C, 200 rpm to OD_{600} = 0.25.
4. Transfer 35 ml culture to a 50 ml centrifugation tube.
5. Centrifuge 5 min, 3200 g, 25°C. Pour off the supernatant and resuspend in 4.5 ml EP buffer (72) by pipetting up and down.
6. Centrifuge 10 min, 3200 g, 25°C. Pour off the supernatant and resuspend in 4.5 ml EP buffer by pipetting up and down.
7. Centrifuge 15 min, 3200 g, 25°C. Pour off the supernatant and resuspend in 200 µl EP buffer by pipetting up and down.
8. Aliquot into sterile tubes (100 µl per tube) and freeze at -80°C.

Electroporation

9. Mix 100 µl thawed electrocompetent cell suspension with 1 µg plasmid (0.5 – 1 µg/µl DNA) in a 1.5 ml tube. Incubate on ice for 15 - 40 min.
10. Transfer into cold electroporation cuvette (0.2 cm gap) and electroporate, 200 Ω, 25 µF and 12.5 kV/cm.
11. Immediately add 2 ml SOBsuc carefully into the electroporation cuvette and invert four times to mix.
12. Transfer the cells into a 50 ml centrifugation tube and add 3 ml SOBsuc.
13. Incubate at 50°C, 200 rpm, for about 16 hrs.

Cultivation and selection

14. Transfer the electroporated culture to 100 ml SOBsuc (pre-warmed to 50°C) with appropriate antibiotic pressure (e.g. 5 µg/ml chloramphenicol). Incubate for 6 hours.
15. Plate 100 µl on Regeneration agar plate (34) with appropriate antibiotic pressure (e.g. 5 µg/ml chloramphenicol).
16. Additionally, centrifuge 7 ml electroporated culture (3200 g, 5 min, 25°C), pour off the supernatant, resuspend the cells in 100 µl SOBsuc and plate out the concentrated culture on another Regeneration agar plate with appropriate antibiotic pressure.
17. Incubate the plates in plastic at 50°C over night.
18. Transfer cells from a colony into 100 ml $MeOH_{30}$ medium (72) with appropriate antibiotic pressure (e.g. 5 µg/ml chloramphenicol). Grow in 500 ml baffled shakeflask for 6 – 8 hrs at 50°C, 200 rpm.
19. Increase the methanol concentration to 200 mM and grow over night.

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