MINI-REVIEW

Bacillus methanolicus: a candidate for industrial production of amino acids from methanol at 50°C

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Abstract Amino acids are among the major products in biotechnology in both volume and value, and the global market is growing. Microbial fermentation is the dominant method used for industrial production, and today the most important microorganisms used are Corynebacteria utilizing sugars. For low-prize bulk amino acids, the possibility of using alternative substrates such as methanol has gained considerable interest. In this mini review, we highlight the unique genetics and favorable physiological traits of thermotolerant methylotroph Bacillus methanolicus, which makes it an interesting candidate for overproduction of amino acids from methanol. B. methanolicus genes involved in methanol consumption are plasmid-encoded and this bacterium has a high methanol conversion rate. Wild-type strains can secrete 58 g/l of L-glutamate in fed-batch cultures at 50°C and classical mutants secreting 37 g/l of L-lysine have been selected. The relative high growth temperature is an advantage with respect to both reactor cooling requirements and low contamination risks. Key genes in L-lysine and L-glutamate production have been cloned, high-cell density methanol fermentation technology established, and recently a gene delivery method was developed for this organism. We discuss how this new knowledge and technology may lead to the construction of improved L-lysine and L-glutamate producing strains by metabolic engineering.

Introduction

Amino acids are used as food and feed supplements, pharmaceuticals, cosmetics, polymer materials, and agricultural chemicals (Ikeda 2003; Faurie and Thommel 2003; Marx et al. 2006). The most important industrial amino acid producer today is the bacterium Corynebacterium glutamicum, which produces about 2 million tons of amino acids per year, above 1 and 0.6 million tons of L-glutamate and L-lysine, respectively (Eggeling and Bott 2005). Several decades of extensive research has resulted in detailed data revealing C. glutamicum metabolism and physiology, and its genome sequence was recently published (Ikeda and Nakagawa 2003; Kalinowski et al. 2003), and also that of the closely related and more thermotolerant Corynebacterium efficiens (Nishio et al. 2003). Genetic tools are well-developed, and this knowledge and technology has been used to generate efficient C. glutamicum amino acid overproducers by metabolic engineering (see Ohnishi et al. 2002; Eggeling and Bott 2005 and references therein). The substrates for C. glutamicum fermentation are generally sugar from agricultural crops.

Methanol is an alternative substrate to sugar for the production of chemical intermediates and commodity microbial products

There is a growing global demand for amino acids and the possibilities to utilize alternative substrates as feedstock in

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fermentation have therefore gained considerable interest. One-carbon (C_1) compounds occur abundantly throughout nature, and methane and methanol are two of the most important C_1 compounds from a biotechnological and a bulk chemical point of view (Linton and Niekus 1987; Olah et al. 2006). Compared to molasses, for example, methanol is a pure raw material that can be completely utilized during bacterial fermentations. Today, almost all methanol worldwide is produced from synthesis gas (syn-gas, a mixture of CO and H_2) obtained from the incomplete combustion of natural gas. However, new ways for its production directly from natural gas without going through syn-gas and by hydrogenative chemical recycling of CO_2 (e.g., from industrial exhausts of fossil fuel burning plants) are being developed. Methanol can also be prepared from biomass, but these processes play only a minor role (Olah et al. 2006). As for any other commodities, methanol prices vary depending on supply and demand, and the gas price is linked to the price of crude oil. Since 1975, the average wholesale price for methanol has fluctuated between 100 and 350 USD per ton (Olah et al. 2006). The supply and, thus, the price of sugar will vary depending on the weather conditions in the major sugar producing regions, as well as agricultural politics. Data collected from the United States Department of Agriculture (http://www.ers.usda.gov/Briefing/Sugar/data.htm) and from Methanex (http://www.methanex.com/products/methanolprice.html) show that over the last 6 years, the price of methanol has been similar to that of raw sugar (Fig. 1). However, mega-methanol production facilities (5,000 tons/day) are now being constructed in regions rich in natural gas, such as the Caribbean, Latin America, and the Middle East. The production costs for methanol in mega-methanol plants have been estimated to be well below 100 USD per ton, and they are expected to allow the price of methanol to remain at a relatively low level as long as natural gas reserves are available. In regions rich in coal such as USA and China, large-scale production of methanol from coal may provide an alterna-tive domestic route for methanol (Olah et al. 2006). The price of molasses as a substrate varies both with geographical location and with the sugar crop and is typically between 50 and 100% of the price of raw sugar on a fermentable sugar basis.

Amino acid production from methanol by methylotrophs

Methylotrophs comprise the large number of both aerobic and anaerobic microorganisms that can grow on reduced compounds lacking C–C bonds, such as methane and methanol (Anthony 1982; Large and Bamforth 1988). Obligate methylotrophs can exclusively utilize C_1 compounds as a sole carbon and energy source, while facultative methylotrophs can utilize both C_1 and multicarbon compounds. Genetic tools for many methylotrophs have been established, and engineering of methylotrophs leading to overproduction of different amino acids are reported including L-serine (Izumi et al. 1993; Hagishita et al. 1996), L-threonine (Motoyama et al. 1994), L-glutamate (Motoyama et al. 1993), and L-lysine (Motayama et al. 2001). Representative L-lysine and L-glutamate producing methylotrophs reported in the literature are listed in Table 1. For example, in the Gram-negative obligate methylotroph *Methylophilus methylotrophus*, the expression of a mutant gene encoding dihydrodipicolinate synthase deregulated in L-lysine inhibition caused increased L-lysine synthesis to about 1 g/l at 37°C (Tsujimoto et al. 2006). By co-expressing a mutant gene encoding an L-lysine transporter they obtained a recombinant strain, strain AS1 (pSEA10), secreting 11.3 g/l of L-lysine from methanol (Gunji and Yasueda 2006). A recombinant mutant, AL119 (pDYOM4-2), of the Gram-negative obligate methylotroph *Methylobacillus glycogenes*, overexpressing a dihydrodipicolinate synthase partly deregulated in L-lysine inhibition was reported to produce about 8 g/l of L-lysine and 37 g/l of L-glutamate from methanol at 37°C (Motayama et al. 2001). To our knowledge, no commercial methanol-based

Fig. 1 Comparison of the recent monthly price fluctuations of methanol (*solid line*) and raw sugar (*dashed line*). Data for the US Gulf Coast from Methanex and from the United States Department of Agriculture (see text)
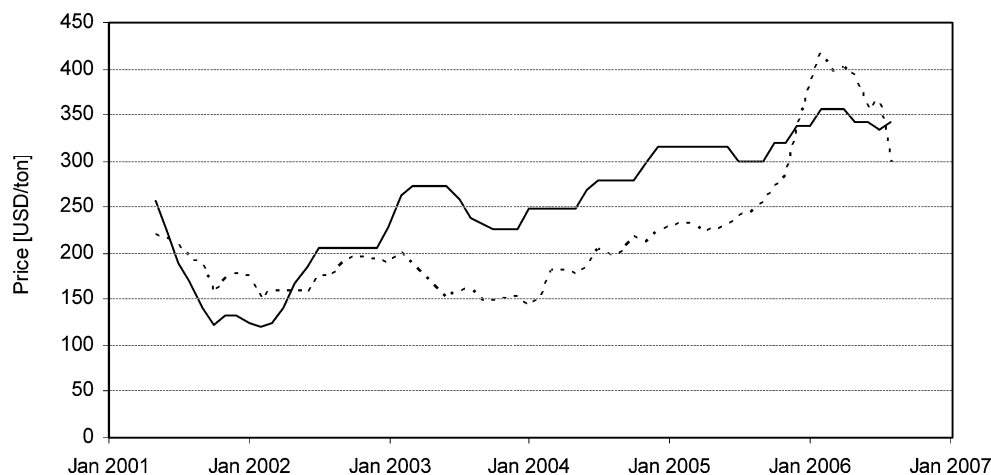
Table 1 Methanol-utilizing overproducers of L-lysine and L-glutamate

Species	Strain	Description	L-lysine (g/l)	L-glutamate (g/l)	Biomass[a]	Time (h)	Reference
Bacillus methanolicus	MGA3	Wild type	ND	58[b]	33 g/l	NR	Schendel et al. (2000), Brautaset et al. (2003)
B. methanolicus	NOA2	Wild type	<1	27	24 g/l	47	Brautaset et al. (2003)
B. methanolicus	NOA2 L20#1HNV#3	Classical mutant[c]	19	NR	20 g/l	32	Schendel et al. (1990)
B. methanolicus	NOA2#13A52-8A66	Classical mutant[c]	37[d]	17	19 g/l	64	Hanson et al. (1996), Lee et al. (1996)
Methanomonas methylovora	M16-8	Wild type	NR	11	18 (OD$_{610}$)	48	Kono et al. (1972)
Methylobacillus glycogenes	RV3	Classical mutant[e]	NR	39	24 (OD$_{660}$)	84	Motoyama et al. (1993)
M. glycogenes	AL119(pDYOM4-2)	Recombinant strain[f]	8	37	36 (OD$_{660}$)	72	Motoyama et al. (2001)
Methylophilus methylotrophus	AS1(pSEA10)	Recombinant strain[g]	11	<1	36 (OD$_{660}$)	72	Gunji and Yasueda (2006)
Pseudomonas insueta	K-038	Classical mutant[h]	NR	33	NR	58	Nakayama et al. (1976)
Protaminobacter candidus[i]	M89-3A	Wild type	NR	5	15 (OD$_{610}$)	48	Kono et al. (1972)
Protaminobacter thiaminophagus[i]	M135-7	Wild type	NR	9	18 (OD$_{610}$)	60	Kono et al. (1972)

Some values are estimated from plots. *ND* not detected, *NR*, not reported

[a] Biomass in grams per liter is dry cell weight. OD$_{660}$ is the optical density at 660 nm. OD$_{610}$ is the optical density at 610 nm.

[b] Average productivity reported as 2.8 g L-glutamate l^{-1} h^{-1}. Yield of L-glutamate per methanol reported as 0.36 g/g.

[c] NOA2 L20#1 HNV#3 and NOA2#13A52-8A66 are homoserine auxotrophs, resistant to amino acid analogs. Both are derived from strain NOA2 by several rounds of mutagenesis.

[d] Both references report L-lysine and L-glutamate production from strain NOA2#13A52-8A66. The L-lysine concentration given here is from Hanson et al. (1996).

[e] RV3 is a phenylalanine auxotrophic revertant.

[f] AL119 is an L-threonine overproducer, which also secretes large amounts of L-glutamate. Plasmid pDYOM4-2 expresses dihydrodipicolinate synthase partially insensitive to the feedback inhibition by L-lysine.

[g] AS1 is a wild type. Plasmid pSEA10 expresses dihydrodipicolinate synthase desensitized from feedback inhibition by L-lysine and a mutated L-lysine/L-arginine exporter.

[h] K-038 requires L-isoleucine (leaky type)

[i] Later reclassified as *Methylobacterium* sp.

industrial production process of any amino acids exists; however, this may change in the future based on the success of engineering efficient methylotrophic amino acid-producing strains and also based on cost and supply of methanol vs sugar substrates.

The candidate bacterium *Bacillus methanolicus*

The methylotrophic bacterium *B. methanolicus* has many unique traits that make it an important candidate for the bioconversion of methanol into amino acids. In this mini review, we will focus on scientific progress that has been made leading to a better understanding of *B. methanolicus* physiology and biology, particularly related to the pathway of methanol assimilation, methanol and formaldehyde tolerance, and metabolic routes for production of the amino acids L-lysine and L-glutamate. The ability to use metabolic engineering to create recombinant strains with improved

production properties was delayed mainly due to the lack of functional genetic tools. However, a method for constructing recombinant *B. methanolicus* strains that retained the ability to grow on methanol was recently established, and the current progress and strategies for generating L-lysine and L-glutamate overproducers by metabolic engineering of *B. methanolicus* is discussed.

The isolation of thermotolerant methylotrophic bacilli later designated *B. methanolicus*

A mixed culture growing on methanol containing spore-forming bacteria was reported in the 1970s (Snedecor and Cooney 1974), and several reports on isolation of thermotolerant and methylotrophic bacilli appeared in the scientific literature in the late 1980s (Al-Awadhi et al. 1988, 1989; Dijkhuizen et al. 1988; Brooke et al. 1989). Some of

these strains were initially classified as *Bacillus brevis*, but were later reclassified as *B. methanolicus* (Arfman et al. 1992a). *B. methanolicus* is a Gram-positive aerobic methylotroph growing at temperatures from 35 to 60°C. These strains can readily be isolated from soil samples, wastewater treatment systems, and volcanic hot springs. The cells are rod-shaped, and they may form oval endospores, but *B. methanolicus* cells typically sporulate poorly in liquid growth medium at 50°C (Schendel et al. 1990). Most strains of *B. methanolicus* can grow in 2% but not in 5% NaCl (Arfman et al. 1992a), and wild-type strains such as MGA3 and NOA2 require biotin and vitamin B_{12} for growth (Schendel et al. 1990). Adaptation to rapid growth in artificial or natural seawater-based media has been reported (Komives et al. 2005), indicating that this bacterium has low cost requirements regarding growth components. Some wild-type strains with high salt tolerance (DFS2, HEN9) that can grow in the presence of 8% ammonium glutamate have been isolated from marsh soil (Brautaset et al. 2004). *B. methanolicus* strains are restricted methylotrophs, meaning that they can utilize few alternative carbon sources, indicating that methylotrophy is an important metabolic trait of this organism in nature (Arfman et al. 1992a,b).

B. methanolicus uses the ribulose monophosphate pathway for C_1 assimilation

In the cells methanol is converted into formaldehyde catalyzed by a methanol dehydrogenase (see below). Among methylotrophs four different routes for formaldehyde assimilation are known (Anthony 1982, 1991; Large and Bamforth 1988; Dijkhuizen et al. 1992). Common to these pathways is that C_1 compounds are fixed to molecules that are regenerated by the completion of one cycle; the product is one three-carbon compound synthesized from three C_1 compounds. *B. methanolicus* assimilates formaldehyde by the ribulose monophosphate (RuMP) pathway (De Vries et al. 1990; Kato et al. 2006), which can be divided into three parts (Fig. 2). In the fixation part (Fig. 2a), three formaldehyde molecules are condensed with three molecules of ribulose 5-phosphate (Ru5P) and catalyzed by 3-hexulose-6-phosphate synthase (HPS) to make three hexulose 6-phosphate (H6P) molecules. One of the H6P molecules is further converted to fructose 6-phosphate (F6P) catalyzed by 6-phospho-3-hexuloisomerase (PHI). Two variants of the cleavage part exist, and *B. methanolicus* uses the fructose-1,6-bisphosphate aldolase (FBPA) variant, where F6P is converted by phosphofructokinase (PFK) to fructose-1,6-bisphosphate (FBP) at the expense of one ATP (Fig. 2b). FBP is then cleaved by

FBPA to glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP). DHAP can be converted by glycolytic steps into pyruvate, generating one NAD(P)H and two ATP. GAP, together with two H6P molecules, enters the regeneration part of the pathway, where three Ru5P molecules are regenerated. Two variants of the regeneration part exist (Fig. 2c), and they share the three enzymes transketolase, ribose-5-phosphate isomerase, and ribulose-5-phosphate 3-epimerase (RPE). The difference between the variants is the usage of transaldolase (TA variant) or a sedoheptulose-1,7-bisphosphatase (SBPase variant). Energetically, all four possible variants of the RuMP pathway produce one NAD(P)H per pyruvate generated, and the most favorable combination is the FBPA/TA variant, which in addition yields one ATP.

Methanol oxidation and formaldehyde assimilation by *B. methanolicus* involves the concerted induction of chromosomal and plasmid-encoded genes

B. methanolicus uses an NAD-linked methanol dehydrogenase (MDH; Arfman et al. 1989) to oxidize methanol into formaldehyde, and the *B. methanolicus* C1 *mdh* gene has been cloned by De Vries et al. (1992). Interestingly, 12 years later (Brautaset et al. 2004), it was shown that *mdh*, together with five RuMP pathway genes, are plasmid-borne in *B. methanolicus* (see below). The *mdh* gene encodes a 385-amino acid polypeptide representing a type III NAD-dependent alcohol dehydrogenase. MDH activity involves an activator protein denoted ACT, and the genetic and biochemical properties of ACT and MDH have been well-characterized (Arfman et al. 1997; Hektor et al. 2002; Kloosterman et al. 2002). In contrast to *mdh*, the *act* gene has a chromosomal origin in *B. methanolicus* (Brautaset et al. 2004). The MDH protein is a decameric nicotinoprotein, and each subunit contains a bound NAD(H) cofactor, one Zn^{2+} ion and one or two Mg^{2+} ions. The in vitro MDH activity is about 40 times higher when purified ACT protein is added; ACT activation involves hydrolytic removal of the nicotinamide mononucleotide moiety of NAD(H) (Kloosterman et al. 2002). Increased NADH/NAD ratios in the cells reduce ACT-mediated activation of MDH, presumably acting as a control mechanism for preventing accumulation of toxic formaldehyde in the cells. The *mdh* gene is transcribed at very high levels in wild-type *B. methanolicus* MGA3 cells growing both methylotrophically on methanol or non-methylotrophically on mannitol (Jakobsen et al. 2006). MDH can constitute up to 30% of total protein in the cells (Arfman et al. 1989). These findings indicate that this organism retains a high capacity to oxidize methanol under different nutritional conditions.
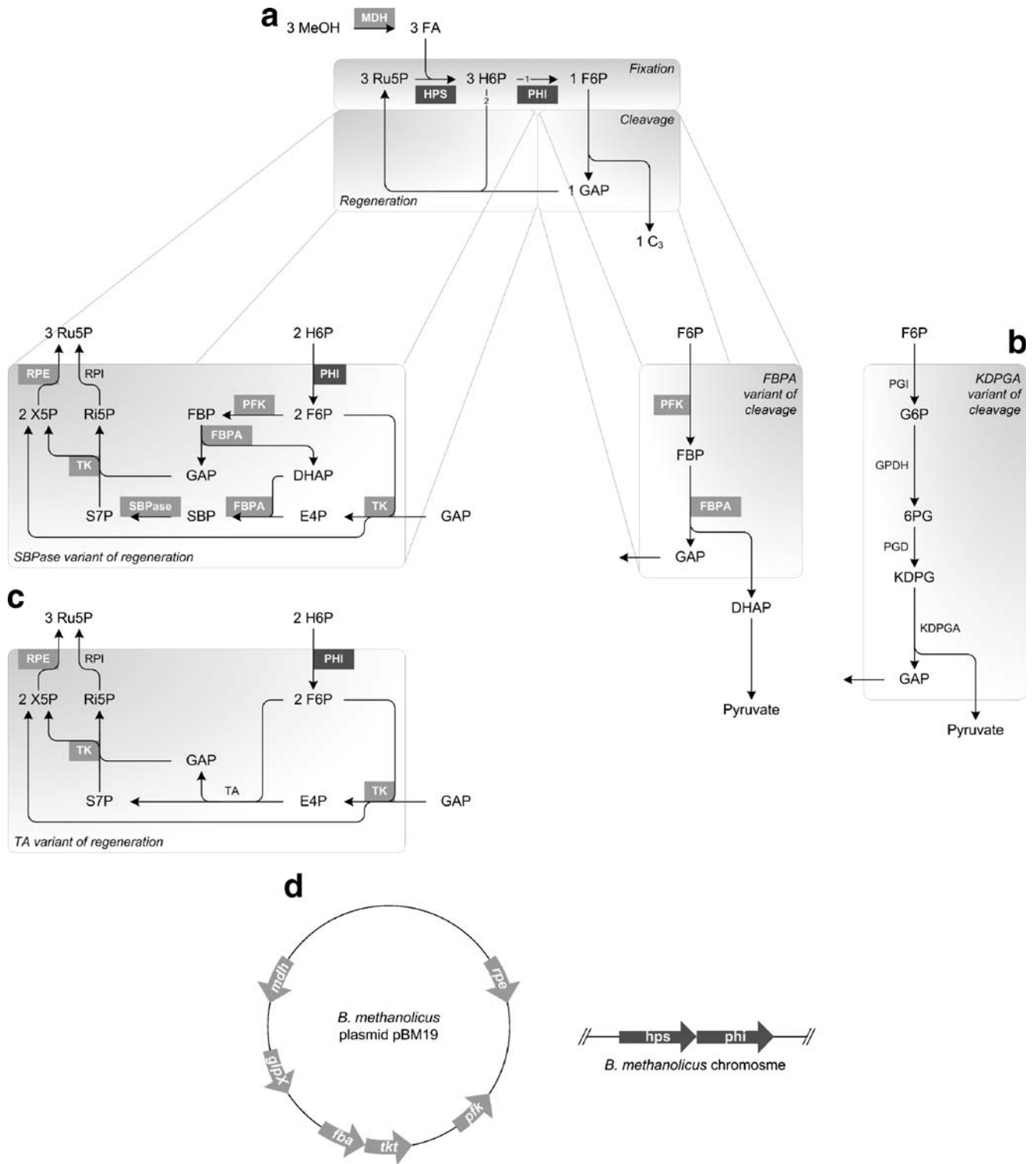
B. methanolicus plasmid pBM19 carries mdh and five RuMP pathway genes

The 19,167-bp and medium-copy number plasmid pBM19 in *B. methanolicus* MGA3 carries *mdh* and five RuMP pathway genes (*glpX*, class II SBPase; *fba*, class II FBPA; *tkt*, TKT; *pfk*, PFK; *rpe*, RPE; Fig. 2d). Genetic character-

ization of 13 different wild-type strains indicated that pBM19-like plasmids are common among *B. methanolicus* species (Brautaset et al. 2004). Two more genes representing the fixation part of the RuMP pathway, *hps* (encoding HPS activity) and *phi* (encoding PHI activity), were found on the chromosome organized as an operon (Fig. 2d). By using quantitative polymerase chain reaction, it was

◀ **Fig. 2** Outline of the RuMP pathway and illustration of the plasmid-dependent methylotrophy in *B. methanolicus*. **a** Upon the completion of one cycle, three formaldehyde (*FA*) molecules are converted into one three-carbon molecule. The enzymes of the fixation part (see text) are indicated. **b** 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 2-keto-3-deoxy-6-phosphogluconate aldolase (*KDPGA*) variant converts F6P to GAP and pyruvate using enzymes of the Entner–Doudoroff pathway. **c** Two variants of the regeneration part of the RuMP pathway (see text). **d** Genetic organization of *mdh* and the totally seven different RuMP pathway genes identified in *B. methanolicus* MGA3. *B. methanolicus* genes and gene products of plasmid (*grey background*) and chromosomal (*black background*) origin are shown. Genes: *mdh* MDH, *glpX* SBPase, *fba* FBPA, *tkt* TKT, *pfk* PFK, *rpe* RPE, *hps* HPS, *phi* PHI. Enzymes: *PGI* Phosphoglucose isomerase, *GPDH* glucose-6-phosphate dehydrogenase, *PGD* 6-Phosphogluconate dehydratase. Abbreviations: *X5P* xylulose-5-phosphate, *Ri5P* ribose-5-phosphate, *S7P* sedoheptulose-7-phosphate, *SBP* sedoheptulose-1,7-bisphosphate, *E4P* erythrose-4-phosphate, *6PG* 6-phosphogluconate, *G6P* glucose-6-phosphate, *KDPG* 2-keto-3-deoxy-6-phosphogluconate. For other abbreviations, see text

demonstrated that these plasmid and chromosomal RuMP pathway genes are 6- to 40-fold upregulated in cells growing on methanol compared to those growing on mannitol (Jakobsen et al. 2006), and curing of pBM19 resulted in loss of the ability to grow on methanol. This represents experimental evidence that *B. methanolicus* methylotrophy is plasmid-linked. Based on enzyme activity measurements in crude extracts, *B. methanolicus* was originally reported to use the TA regeneration variant of the RuMP pathway (Arfman et al. 1989; Dijkhuizen et al. 1992). However, considering the predicted functions of the identified RuMP pathway genes, it seems likely that *B. methanolicus* may use the SBPase regeneration variant (Fig. 2c). The induced state of the RuMP pathway genes is important for the methanol and formaldehyde tolerance level of the cells (see below), and cells pregrown on mannitol or in any other rich media lacking methanol must be gradually adapted to increasing methanol concentrations to avoid cell death (Jakobsen et al. 2006).

pBM19 is crucial for methylotrophic growth and a burden upon non-methylotrophic growth

Comparative growth studies using the pBM19-cured *B. methanolicus* strain MGA3C-A6 (Brautaset et al. 2004) and its parental wild-type strain, unravelled new and interesting physiological traits of *B. methanolicus*. MGA3C-A6 cannot grow on methanol, while it has a significantly higher growth rate on mannitol compared to the wild-type (0.37 h^{-1} vs 0.30 h^{-1} in shake flasks), indicating that pBM19 represents a burden for cells under growth conditions where its genes are not needed (Jakobsen et al. 2006). The latter assumption was confirmed by 140 generations of continuous growth of wild-type cells in

mannitol medium resulting in loss of pBM19 from the culture. Mutant MGA3C-A6 growing on mannitol can tolerate the addition of about tenfold higher concentration of methanol and only half the concentration of formaldehyde compared to the wild-type grown under similar conditions. The high methanol tolerance was reduced to wild-type levels in recombinant MGA3C-A6 cells expressing MDH activity (Jakobsen et al. 2006). This indicates that methanol sensitivity of *B. methanolicus* is mainly caused by toxic accumulation of formaldehyde generated by the high MDH activity and that the RuMP pathway plays an important role also for the formaldehyde tolerance of the cells. By growing wild-type cells in a mixture of mannitol and methanol, the majority of the total carbon consumed derives from methanol. Under such conditions, the RuMP pathway genes are transcriptionally induced (Jakobsen et al. 2006).

Dissimilatory pathways for formaldehyde oxidation to CO_2

Measurements in crude extracts of *B. methanolicus* have demonstrated phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase activities (Fig. 3), indicating a cyclic dissimilatory pathway for oxidation of formaldehyde into CO_2 (Arfman et al. 1989). Later, indication of an alternative pathway for formaldehyde dissimilation has also been reported using [^{13}C] nuclear magnetic resonance by feeding labeled methanol ($^{13}CH_3OH$) to *B. methanolicus* (Pluschkell and Flickinger 2002). To verify the activity of formate dehydrogenase (FDH), pulses of ^{13}C formate were added to a
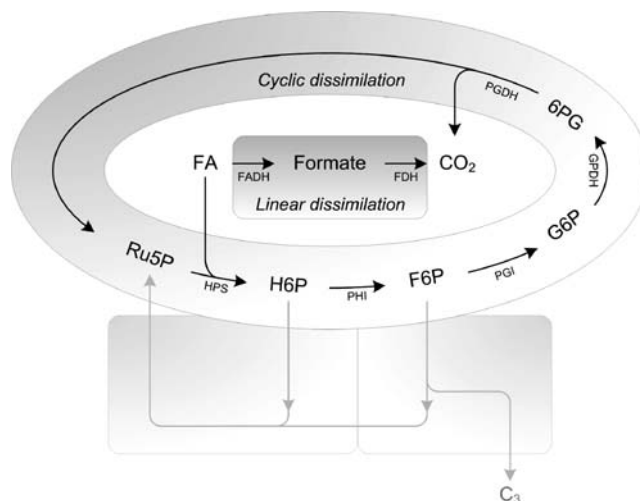
Fig. 3 Outline of the linear and cyclic dissimilatory pathways for formaldehyde. The linear dissimilatory pathway converts formaldehyde (*FA*) to CO_2 by the action of FADH and FDH. The cyclic dissimilatory pathway is a combination of the fixation part of the RuMP pathway and the pentose phosphate pathway. The remaining parts of the RuMP pathway are here indicated in *grey*. Enzymes: *PGDH* 6-Phosphogluconate dehydrogenase. For other enzymes and abbreviations, see text and legend under Fig. 2

continuous culture at steady state and $^{13}CO_2$ enrichment was monitored in the exhaust gas by isotope ratio mass spectroscopy (Pluschkell and Flickinger 2002). These data indicate that formaldehyde can also be oxidized to CO_2 by a linear pathway via formate (Fig. 3), presumably catalyzed by formaldehyde dehydrogenase and FDH. In addition to generating reducing power, the latter pathway is assumed to play a role in regulating intracellular formaldehyde levels in the cells upon methanol growth. To date, no genes representing any of these two alternative dissimilatory pathways have been cloned from *B. methanolicus*.

Methanol consumption rate and theoretical product yield

High substrate consumption rates and high product yields are desirable for all microbial production processes. Based on reported data from a 14-l fed-batch fermentation with *B. methanolicus* MGA3 in which 50 g dry cell weight per liter was achieved (Schendel et al. 1990), a mean methanol consumption rate of at least 7 g/h can be calculated. Theoretical product yields can be estimated based on assumed biochemical pathways of the organism. However, the dissimilatory metabolism of methanol is not fully elucidated, and as for L-lysine in particular, three different biosynthesis pathways are recognized in prokaryotes (Kindler and Gilvarg 1960; Sundharadas and Gilvarg 1967; White 1983; Berges et al. 1986). Comparisons of theoretical L-lysine yields from methanol by *B. methanolicus* and from glucose by *C. glutamicum* are given in Table 2. Most *Bacillus* species are reported to use the acetylase variant of the L-lysine biosynthesis pathway (Paulus 1993). Although all three variants of the pathway have been suggested to exist in *C. glutamicum* (Weinberger and Gilvarg 1970; Tosaka and Takinami 1978; Schrumpf et al. 1991), the succinylase and the dehydrogenase variants appear to be the most important (Sonntag et al. 1993; Wehrmann et al. 1998). A comparison of stoichiometric conversions of methanol or glucose to L-lysine is shown in Fig. 4. Table 2 and Fig. 4 show that the yield of L-lysine

from methanol by *B. methanolicus* is similar (0.71 and 0.81 g L-lysine-HCl/g methanol) to the yield from glucose by *C. glutamicum* (0.68 and 0.82 g L-lysine·HCl/g glucose). Thus, *B. methanolicus*, with a high methanol consumption rate combined with high theoretical product yields should be a potentially efficient biocatalyst for the production of amino acids from methanol.

Fed-batch high cell density cultivation methods at 50°C for *B. methanolicus*

As stated above, MDH levels in *B. methanolicus* are high under any conditions indicating that methanol, when available, will be rapidly converted into formaldehyde in the cells. Therefore, to control in vivo accumulation of toxic formaldehyde and achieve high *B. methanolicus* cell density, continuous feeding of methanol is required when cultivating this organism in a bioreactor. Online methanol analysis is used to keep the methanol level constant at a limited concentration (Schendel et al. 1990; Lee et al. 1996). Too rapid feeding of methanol results not only in formaldehyde toxicity, but also in a substantial fraction of the methanol carbon being dissimilated to CO_2 (see Fig. 3), accompanied with an increase in oxygen demand (Pluschkell and Flickinger 2002). Methanol is more reduced than sugars, and methanol fermentations are therefore characterized by high oxygen demands. As heat evolution increases with oxygen consumption, cooling requirements are high for fermentations using methanol compared to sugar. Due to the thermotolerant phenotype of *B. methanolicus*, a dramatic reduction in cooling water is a major cost benefit of employing this organism compared to using methylotrophic bacteria growing at 30 to 37°C (Motayama et al. 2001; Tsujimoto et al. 2006). 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 (Komives et al. 2005). Another effect of increased process temperature is lowered oxygen solubility in the medium. At atmospheric pressure, increasing the temperature from 35 to
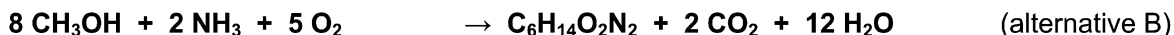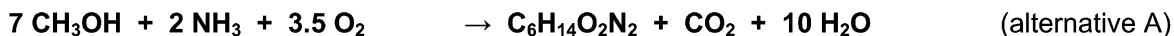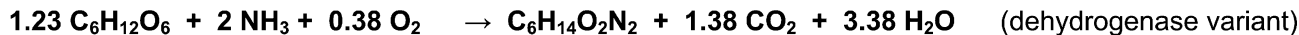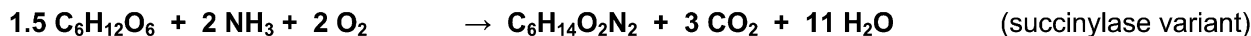
Table 2 Theoretical maximum yields of lysine (grams of L-lysine-HCl/gram of methanol or glucose)

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

For comparison, yields were calculated for all possible variants of the lysine biosynthesis pathway, although the dehydrogenase variant, to our knowledge, has not been reported for *Bacillus*. The values in bold represent the most likely pathways (see text).

Theoretical yields have been based on known biochemical pathways and the following assumptions: (1) Oxidation of NAD(P)H in the respiratory chain yields 2 ATP, while oxidation of other components (FAD, PQQ, etc.) yields 1 ATP. (2) NAD(P)H can be synthesized from NADH by transhydrogenase. (3) Uptake and assimilation of ammonia require 1 NAD(P)H and 1 ATP.

[a] Oxidation of formaldehyde to formate may or may not yield NAD(P)H (Large and Bamforth 1988), and the theoretical yeild varies accordingly.

L-lysine production from methanol by B. methanolicus:

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

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

L-lysine production from glucose by C. glutamicum:

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

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

Fig. 4 Comparison of stoichiometric conversion of methanol (CH_3OH) or glucose ($C_6H_{12}O_6$) to L-lysine ($C_6H_{14}O_2N_2$) by *B. methanolicus* and *C. glutamicum*, respectively. For *B. methanolicus*, the acetylase variant of the L-lysine biosynthesis pathway is assumed, while the succinylase and the dehydrogenase variants are assumed for *C. glutamicum* (see text). Different conversion mechanisms of formaldehyde to formate [distinguished by NAD(P)H generation] are known (Large and Bamforth 1988), giving rise to two possible conversion alternatives for L-lysine from methanol, indicated here by alternatives A and B. The synthesis of one L-lysine molecule from methanol by *B. methanolicus* or from glucose by *C. glutamicum* (using the dehydrogenase variant) require two molecules NAD(P)H, while the conversion of glucose to L-lysine by *C. glutamicum* using the succinylase variant is carbon-limited and yields NAD(P)H in surplus

50°C decreases the solubility of oxygen about 20%; therefore, there is an increased demand for oxygen supply in the fermentors under such growth conditions. Both laboratory scale fed-batch cultures and chemostats of *B. methanolicus* have been investigated, and dry cell mass of up to 50 g/l has been achieved (Schendel et al. 1990; Lee et al. 1996; Pluschkell and Flickinger 2002; Brautaset et al. 2003). Optimization of fermentation media composition, inoculum, online monitoring, and feeding strategies are critical to obtaining high and reproducible cell and amino acid yields in bioreactors. Due to the requirements to avoid formaldehyde toxicity and to supply sufficient oxygen, special attention must be paid to aeration and methanol feeding strategies when bioreactor volume increases to the scale used in industrial amino acid production (several hundred cubic meters). The design of large bioreactors should prevent non-homogeneous mixing and poor oxygen mass transfer resulting in localized formaldehyde toxicity and oxygen starvation.

L-Glutamate production by *B. methanolicus* strains

The flow of methanol-derived carbon from oxaloacetate (OAA) is split between transamination to aspartate and formation of citrate (Fig. 5). The citrate cycle (TCA cycle) of organisms harboring the linear dissimilation pathway of formaldehyde (see above) is assumed to play a minor role in energy metabolism during methylotrophic growth (Zatman 1981; Shishkina and Trotsenko 1982). In wild-type *B. methanolicus* MGA3, intracellular levels of L-glutamate are significant, ~90 mM, and in fed-batch culture this strain can secrete up to 58 g/l of L-glutamate (Schendel et al. 2000; Brautaset et al. 2003; Table 1). This indicates that a substantial fraction of pyruvate enters the first reactions of the TCA cycle, leading to formation of 2-oxoglutarate (Fig. 5). The *B. methanolicus citY* gene, encoding an active citrate synthase (CS) II protein, has been cloned and sequenced, and chromosomal inactivation of this gene demonstrated that this bacterium has several forms of CS (Brautaset et al. 2003). 2-Oxoglutarate can be transaminated to L-glutamate or alternatively oxidized by 2-oxoglutarate dehydrogenase (ODHC) of the TCA cycle. *Bacillus subtilis* has no assimilatory glutamate dehydrogenase activity; ammonium assimilation occurs solely by the glutamine synthetase/glutamate synthase pathway (Fisher 1999). It remains unknown which of these two alternative mechanisms *B. methanolicus* uses for converting 2-oxaloacetate into L-glutamate. Interestingly, wild-type *B. methanolicus* MGA3 has low ODHC activity (Brautaset et al. 2003), which is likely one major reason for the high L-glutamate production by this organism. In *C. glutamicum*, effective L-glutamate export can be achieved by using Tween 40 (polyethylene sorbitan monooleate) or biotin limitations in the growth medium. In a screen of different surfactants only Tween 80 stimulated L-glutamate secretion in *B. methanolicus* (Hanson et al. 2000; Schendel et al. 2000), and biotin limitation does not increase L-glutamate secretion in this bacterium; it is also required for cell growth.

L-Lysine production by *B. methanolicus* strains

Generation of classical *B. methanolicus* mutants overproducing L-lysine

Researchers at the University of Minnesota constructed a series of classical mutants that overproduce various levels

Fig. 5 Overview of biochemical pathways from pyruvate to L-glutamate and L-lysine in *B. methanolicus*, with relevant enzymes and intermediates indicated (see text). The conversion of aspartate semi-aldehyde to L-lysine is indicated by the seven enzymatic steps of the acetylase/succinylase variants of the L-lysine biosynthetic pathway. It is not known which mechanism, glutamate dehydrogenase or glutamine synthase/ glutamate synthetase, is employed by *B. methanolicus* (see text)
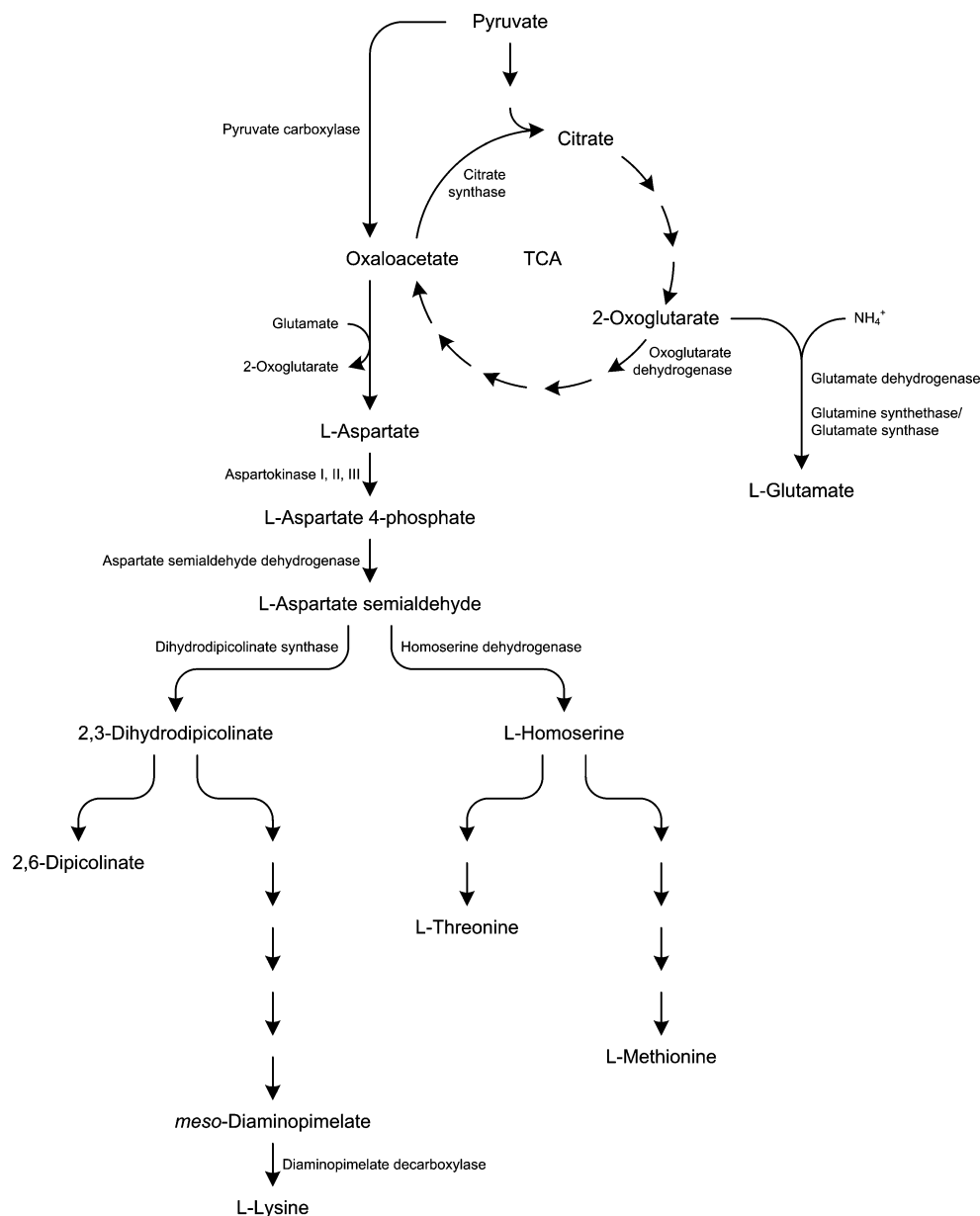
of L-lysine during the 1990s. For example, by using repetitive cycles of random mutagenesis and selection of *B. methanolicus* wild-type strain NOA2, mutant NOA2#13A52-8A66 was obtained. This homoserine auxotrophic mutant is resistant to the L-lysine analogue *S*-2-aminoethyl cysteine (AEC) and the diaminopimelic acid (DAP) analogue diaminobutyrate, and it requires supply of both threonine and methionine in the growth medium for rapid growth. Mutant NOA2#13A52-8A66 was capable of secreting 37 g/l L-lysine and 17 g/l L-glutamate in 11-l fed-batch methanol cultures (Lee et al. 1996; Hanson et al. 1996); to date, this is still the highest L-lysine production reported by any methylotrophic bacterium (Table 1).

CS and pyruvate carboxylase play important roles in C-flow from the key intermediate pyruvate

To achieve high L-lysine production, it is important that a high fraction of pyruvate is converted into aspartate (Fig. 5). Reducing CS levels by inactivation of *citY*, which encodes CSII, has been shown to strongly reduce L-glutamate production in *B. methanolicus* (Brautaset et al. 2003). Interestingly, the *citY* mutant strain could grow well on methanol medium as long as supplied with excess L-glutamate in the growth medium (Brautaset et al. 2003). This manipulation, however, had no positive effect on production rate or yield, most probably due to lack of deregulation of key enzymes in the L-lysine biosynthetic pathway. We

believe that the *citY*-deficient mutant should be an interesting host for additional mutations aiming at constructing L-lysine-overproducing *B. methanolicus* strains that do not simultaneously co-secrete L-glutamate. Pyruvate carboxylase (PC) activity has been reported to be one major bottleneck for high L-lysine production in *C. glutamicum* to supply OAA (Peters-Wendish et al. 2001; Ohnishi et al. 2002). PC activity was significantly elevated in the L-lysine-overproducing *B. methanolicus* mutant NOA2#13A52-8A66 compared to in the wild-type strain. Both the wild type *B. methanolicus* and L-lysine-overproducing mutant NOA2#13A52-8A66 have high intracellular levels of OAA, about 450 mM, indicating a high activity of PC in this bacterium (Brautaset et al. 2003). Also, genetic analysis of mutant NOA2#13A52-8A66 has demonstrated that it has no mutations in the *citY* gene. Thus, it seems plausible to suggest that metabolic engineering to further improve L-lysine production of *B. methanolicus* should focus on deregulating key enzymes in the L-lysine biosynthetic pathway downstream of PC and OAA.

The L-lysine biosynthetic pathway from aspartate

Deregulation of aspartate kinase (AK) has been the most important step in the development of L-lysine-overproducing strains (Pfefferle et al. 2003), and screening for AEC resistance has led to the development of AK-deregulated and L-lysine-overproducing mutants of *B. subtilis* (Chaudhuri et al. 1983). *C. glutamicum* has one AK while bacilli typically have three AK isoenzymes that are regulated in a distinct manner (Paulus 1993; Belitsky 2002 and references therein). The *B. methanolicus* genes *lysC* (encoding AKII) and *lysA* (encoding DAP decarboxylase), representing the first and the last step in the L-lysine biosynthetic pathway (Fig. 5), respectively, have been cloned and characterized (Schendel and Flickinger 1992; Mills and Flickinger 1993). Biochemical characterizations of the *lysA* gene product confirmed that the protein is also inhibited by L-lysine (Mills and Flickinger 1993), and both *lysC* and *lysA* may represent interesting targets for genetic manipulations to improve L-lysine production by *B. methanolicus*.

Establishment of a gene delivery system and its application to improve methanol consumption in *B. methanolicus*

The characterization of the restriction modification system (Cue et al. 1996) and the reporting of a gene delivery method by using protoplast transformation (Cue et al. 1997) were the first reports of molecular biology techniques for recombinant manipulation of *B. methanolicus*. However, due to incompatibility problems with the constructed

shuttle vectors and very poor transformation efficiencies (Brautaset et al. 2004), no recombinant *B. methanolicus* strains were published using these methodologies. The characterization of the natural *B. methanolicus* MGA3 plasmid pBM19 (see above) gave some biological explanations to these problems. The *Escherichia coli–B. methanolicus* shuttle vector pDQ508 constructed by Cue et al. (1997) contains replication elements of pBM19, and selecting for pDQ508 caused loss of pBM19 and concomitant lost ability of recombinant cells to grow on methanol (Brautaset et al. 2004). However, a new electroporation method for gene delivery by using the *E. coli–B. subtilis* shuttle plasmid pHP13 was recently established by Jakobsen et al. (2006). This method involves selection in liquid medium and implements an adaptation strategy when transferring recombinant cells from rich to defined methanol media, to avoid lethal formaldehyde accumulation and death of recombinant cells. By using this method, recombinant *B. methanolicus* strains that retain the ability to grow on methanol can be constructed (Jakobsen et al. 2006). This has resulted in new possibilities to use metabolic engineering strategies to improve production properties of this organism.

Improved methanol consumption and methanol tolerance level of *B. methanolicus* by metabolic engineering

The unique genetic organization of *B. methanolicus* RuMP pathway genes (see above) may lead to new possibilities to improve the methanol assimilation rate of this bacterium by genetic engineering. To achieve high amino acid production, an efficient assimilation of the methanol in favor of wasteful dissimilation into CO_2 is desirable (see Fig. 3). In contrast to the pBM19-borne *RuMP* genes, which all appear in multiple copies, the operon encoding *hps* and *phi* (Fig. 2d) is present in a single copy in the cells (Brautaset et al. 2004). Therefore, in an attempt to increase the RuMP pathway activity in the cells, this chromosomal operon was cloned on the medium copy number vector pHP13 and transformed into *B. methanolicus* wild-type MGA3 by electroporation (see above). The resulting recombinant strain displayed about threefold increased coupled HPS + PHI activity and had a significantly improved growth rate on a range of high methanol concentrations (up to 1,440 mM) compared to the wild-type (Jakobsen et al. 2006). The recombinant cells also displayed significantly higher tolerance level to high methanol concentrations in the growth media before cell growth was impaired. Together, these data demonstrate that the coupled HPS + PHI activity of the RuMP pathway (Fig. 2a) plays an important role in regulating the methanol assimilation rate and the methanol tolerance level in the cells (Jakobsen et al. 2006).

Conclusions and perspectives

During the recent years considerable scientific progress has resulted in an increased understanding of the unique genetics and physiology of thermotolerant *B. methanolicus*. In particular, the metabolic routes for methanol consumption are well-characterized and this knowledge has led to better cultivation strategies, new and useful insight into the mechanisms for formaldehyde and methanol sensitivity, and has also been valuable for the development of recombinant methodologies for *B. methanolicus* strains. The usefulness of this acquired biological knowledge and new technology has been demonstrated by the construction of recombinant *B. methanolicus* mutants with improved methanol consumption rate and increased formaldehyde tolerance level. Key genes involved in both L-glutamate and L-lysine production have been cloned and characterized, and the construction and characterization of classical mutants overproducing L-lysine has gained important insight into the intermediary methanol metabolism related to high amino acid production of *B. methanolicus*. Such mutants should also represent valuable sources useful for inverse metabolic engineering strategies involving mapping of beneficial mutations resulting in improved amino acid production. By using the genetic tools, mutant genes can be reintroduced into a defined genetic background to obtain recombinant strains with improved amino acid production. Such methods have been successfully used to generate effective L-lysine overproducers of *C. glutamicum* (Ohnishi et al. 2002).

As we see it, the major limitations for constructing industrial competitive producer strains of *B. methanolicus* at present are the lack of a genome sequence and also a genetic toolbox that should be further expanded. The total number of *B. methanolicus* genes sequenced is so far relatively small (De Vries et al. 1992; Schendel and Flickinger 1992; Mills and Flickinger 1993; Brautaset et al. 2003, 2004). A genome sequence is required for global analyses (transcriptome, metabolome, proteome) of methanol metabolism in *B. methanolicus* and its amino acid overproducing mutants and for more efficient targeting of key enzymes important for high amino acid production. By also increasing the genetic toolbox of this bacterium, e.g., to include suicide vectors for homologous recombination, adjustable expression systems, and obtain higher transformation efficiencies, the potential for manipulating genes encoding key enzymes involved in conversion of methanol into L-lysine and L-glutamate could be fully explored. The documented high methanol consumption rate and the high capability of *B. methanolicus* strains to overproduce both L-glutamate and L-lysine from methanol at elevated temperature should make the new era of using metabolic engineering to improve these properties exciting from a scientific and from an applied point of view.

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