Dissimilation of [^{13}C]methanol by continuous cultures of *Bacillus methanolicus* MGA3 at 50 °C studied by ^{13}C NMR and isotope-ratio mass spectrometry

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Using a continuous culture of *Bacillus methanolicus* MGA3 limited by 100 mM methanol in the feed and growing at a dilution rate $D=0.25\text{ h}^{-1}$, transients in dissolved methanol were studied to determine the effects of methanol toxicity and the pathway of methanol dissimilation to CO_2 . Steady-state cultures were disturbed by pulses of methanol resulting in a rapid change in concentration of 6.4–12.8 mM. *B. methanolicus* MGA3 responded to a sudden increase in available methanol by a transient decline in the biomass concentration in the reactor. In most cases the culture returned to steady state between 4 and 12 h after pulse addition. However, at a methanol pulse of 12.8 mM, complete biomass washout occurred and the culture did not return to steady state. Integrating the response curves of the dry biomass concentration over a 12 h time period showed that a methanol pulse can cause an average transient decline in the biomass yield of up to 22%. ^{13}C NMR experiments using labelled methanol indicated that the transient partial or complete biomass washout was probably caused by toxic accumulation of formaldehyde in the culture. These experiments also showed accumulation of formate, indicating that *B. methanolicus* possesses formaldehyde dehydrogenase and formate dehydrogenase activity resulting in a methanol dissimilation pathway via formate to CO_2 . Studies using isotope-ratio mass spectrometry provided further evidence of a methanol dissimilation pathway via formate. *B. methanolicus* MGA3, growing continuously under methanol limitation, consumed added formate at a rate of approximately $0.85\text{ mmol l}^{-1}\text{ h}^{-1}$. Furthermore, significant accumulation of $^{13}\text{CO}_2$ in the reactor exhaust gas was measured in response to a pulse addition of [^{13}C]formic acid to the bioreactor. This indicates that *B. methanolicus* dissimilates methanol carbon to CO_2 in order to detoxify formaldehyde by both a linear pathway to formate and a cyclic mechanism as part of the RuMP pathway.

Keywords: Gram-positive methylotroph, ribulose monophosphate cycle, formaldehyde oxidation, methanol pulse, formaldehyde toxicity

INTRODUCTION

Restrictive facultative strains of the Gram-positive thermotolerant methylotroph *Bacillus methanolicus* (Arfman *et al.*, 1992) may be useful for the production of amino acids and microbial protein at 50 °C (Schendel *et*

al., 1990; Pluschkell, 1999). Wild-type *B. methanolicus* MGA3 (ATCC 53907) and homoserine dehydrogenase mutants have been shown to secrete substantial quantities of L-glutamic acid and L-lysine when grown at 50 °C in fed-batch cultures on methanol as the sole source of carbon and energy (Hanson *et al.*, 1996; Lee *et al.*, 1996). In contrast to Gram-negative methylotrophs such as *Methylobacillus flagellatus* (Marchenko *et al.*, 1999) that also have been reported to secrete amino acids and vitamins, the single cell membrane of *B. methano-*

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Abbreviations: RuMP, ribulose monophosphate.

licus should be an advantage for rapid secretion of high levels of amino acids. To maximize the yield of amino acids, the fraction of methanol carbon dissimilated as CO₂ must be minimized. Supplying excess methanol to fed-batch cultures of *Bacillus methanolicus* can result in an increase in CO₂ evolution (Lee *et al.*, 1996). Therefore, understanding the physiological response of *B. methanolicus* growing at 50 °C to fluctuating methanol levels and the pathways that detoxify intracellular formaldehyde is important for the design and control of fed-batch methanol feeding strategies for large-volume fermentations where the concentration of methanol may not be uniform.

B. methanolicus is capable of rapid growth on methanol or mannitol in minimal media. Growth on glucose as a sole source of carbon and energy is very slow. Maximum growth rates on methanol occur at 50–53 °C (Schendel *et al.*, 1990). Endospore formation is minimal at 50 °C, but more extensive when cultures are shifted from 50 to 37 °C during exponential growth (Schendel *et al.*, 1990).

Aerobic methylotrophic proteobacteria possess tetrahydromethanopterin (H₄MPT)-dependent enzymes for methanol assimilation (Vorholdt *et al.*, 1999). Such enzymes have so far not been found in Gram-positive methylotrophs. *B. methanolicus* has been shown to contain an NAD-dependent methanol dehydrogenase for the oxidation of methanol to formaldehyde (De Vries *et al.*, 1992). Formaldehyde is assimilated by the ribulose monophosphate (RuMP) pathway (Arfman *et al.*, 1989, 1991; Dijkhuizen *et al.*, 1988; Vonk *et al.*, 1991). This pathway may function to detoxify accumulating formaldehyde by assimilation into RuMP and may also dissimilate methanol carbon as CO₂ in a cyclic version of the RuMP pathway similar to that in *Methylobacillus flagellatus* KT (Chistoserdova *et al.*, 2000). In addition to methanol dehydrogenase, crude cell extracts of *B. methanolicus* MGA3 grown on methanol contain a high specific activity of hexulose-6-phosphate synthase [3.72–6.27 mmol formaldehyde (mg protein)⁻¹ min⁻¹; Schendel *et al.*, 1990]. However, little else is known about the pathways of formaldehyde assimilation or dissimilation (as CO₂) that may play a role in detoxifying formaldehyde.

This study utilized continuous, steady-state methanol-limited cultures of *B. methanolicus* MGA3 growing at 50 °C to investigate the growth-inhibitory effects of pulses of methanol that perturb steady-state methanol concentrations. Using the methods described here, the degree of methanol toxicity could be quantified, and the physiological response elucidated using ¹³C NMR and isotope-ratio mass spectrometry. In recent years, NMR and GC-MS techniques have become well established as powerful tools in identifying novel metabolic pathways and flux patterns by unambiguous *in vivo* detection of intermediates (Bacher *et al.*, 1998; Shimizu, 2000). Representative examples are the investigation of the metabolism of cosubstrates in *Penicillium chrysogenum* (Christensen & Nielsen, 2002), the identification of pathway intermediates in fluoranthene metabolism in

mycobacteria (Rehmann *et al.*, 2001), and the *in situ* monitoring of biotransformations (Breckner & Ribbons, 2000; Breckner *et al.*, 2000). In the case of *B. methanolicus* MGA3, *in vivo* evidence supporting a pathway of methanol dissimilation via formate to CO₂ was found using ¹³C NMR and isotope-ratio mass spectrometry that has previously not been reported in Gram-positive methylotrophic bacilli.

METHODS

Bacterial strain and culture medium. All investigations were carried out using the wild-type strain *B. methanolicus* MGA3 (ATCC 5390, Schendel *et al.*, 1990). Continuous-culture medium consisted of a minimal salts solution (MS) containing (g l⁻¹): K₂HPO₄·3H₂O, 6.47; NaH₂PO₄·H₂O, 1.89; (NH₄)₂SO₄, 3.6; and MgSO₄·7H₂O, 0.25. This was supplemented with 100 mmol methanol l⁻¹, 20 µg biotin l⁻¹, 1 µg vitamin B₁₂ l⁻¹, 0.01 % (v/v) antifoam MAZU DF 204 (PPG Industries), 0.2 mmol citric acid l⁻¹ and 1.0 ml l⁻¹ of a concentrated trace metal solution (FeCl₂·4H₂O, 20 mM; ZnCl₂, 1 mM; MnCl₂·4 H₂O, 50 mM; CaCl₂·2H₂O, 50 mM; CuCl₂·2H₂O, 160 µM; CoCl₂·6H₂O, 170 µM; Na₂MoO₄·2H₂O, 200 µM; H₃BO₃, 490 µM). When a methanol feed concentration higher than 100 mM was used, all compounds in the medium, except the phosphate buffer, were increased proportionately. Citric acid is not a carbon or energy source for *B. methanolicus*, and is added only to prevent precipitation of trace metals. MY medium used for ¹³C NMR experiments was composed of MS supplemented with 1.0 ml l⁻¹ of the concentrated trace metal solution, 20 µg biotin l⁻¹, 1 µg vitamin B₁₂ l⁻¹ and 0.5 g yeast extract (Difco) l⁻¹.

Continuous-culture growth conditions. *B. methanolicus* MGA3 was grown continuously in a stirred-tank, 2 litre jacketed glass bioreactor (Applikon Dependable Instruments), which was operated with a constant liquid volume of 1000 ml. Containers with sterile feed medium were placed on a top-loading electronic balance (Mettler PE16), which was interfaced with a microprocessor-controlled pump (Scilog Expert CP-120). A mass-flow control program was used so that the feed rate was constant and independent of tubing fatigue. A pressurized feed port was designed (as described by Evans *et al.*, 1970) in order to reduce back-growth contamination from the reactor into the feed carboy. A sample port constructed in the feed line allowed sampling from the medium under sterile conditions for determination of the bioreactor inlet methanol concentration. The pH of the culture was controlled at 6.7 ± 0.05 (Metrohm) using 8 M potassium hydroxide. The dissolved O₂ tension was monitored with a galvanic oxygen probe (Johnson *et al.*, 1964). Both the pH and the dissolved O₂ tension were recorded on a PC. The temperature in the reactor was controlled at 50 ± 0.5 °C. The entire apparatus including the media feed tanks was enclosed in a walk-in incubator. The agitation speed was 600 r.p.m. The culture was oxygenated with air at a rate of 1 litre min⁻¹ (at standard conditions: 10⁵ Pa, 21 °C) to maintain a dissolved O₂ tension of approximately 7 kPa. About 2 % (20 ml min⁻¹) of the total gas flow into the reactor was used to create a jet in the pressurized feed port. The flow meter measuring the total gas flow was calibrated for an operating pressure of 1.3 atm (1 atm = 10⁵ Pa) in the reactor. The inlet and outlet gas streams were analysed on-line using a quadropole mass spectrometer (Questor High Speed Process Analyser, Extrel Corporation). Calibration was carried out once a week for nitrogen, O₂, CO₂, argon, water, ammonia and methanol. The total scan time required to

measure all components in a sample stream was 6 s. The lag time between a change in the concentration in the reactor and the mass spectrometer response was 1.5 min. The analysis of the inlet and exit gas streams was used to determine on-line the O_2 uptake rate, the CO_2 evolution rate and the respiratory quotient of the continuously growing culture of *B. methanolicus* MGA3.

Using this apparatus, a methanol-limited continuous culture could be maintained for several weeks. Negligible wall growth above the liquid level in the reactor occurred after 2 weeks. Direct gravimetric measurements of the vessel wall growth dry biomass showed that this fraction of the biomass never exceeded 1% of the total biomass generated during the continuous-culture experiment.

Determination of residual methanol, cell yield and formic acid. Residual methanol concentration in the reactor and culture samples was measured by immediately filtering reactor samples (Acrodisc, 0.2 μ m pore size, low-protein-binding HT Tuffryn membrane) followed by analysis by gas chromatography (Hewlett Packard, model 5890) using a flame-ionization detector and a 30 m DB-WAX column (Chrom Tech). The dry biomass concentration in the reactor was determined directly by drying and weighing. A 25 ml sample of culture suspension was centrifuged for 10 min at 4 °C and 17000 g in a Beckman centrifuge (model J2-21, JA 20 rotor). The supernatant was discarded, the cell pellet resuspended in 12 ml minimal salts solution (MS), and centrifuged again for 10 min. The cell pellet was resuspended in 12 ml fresh MS and dried at 80 °C in aluminium cups to a constant mass. Two 12 ml samples of MS were also dried to correct for salt mass. MS was used instead of distilled water in order to prevent any cell lysis during centrifugation or resuspension. The dry biomass concentration ($g\ l^{-1}$) was calculated as the mean of four replicate determinations.

Formic acid was measured by the combined use of gas chromatography and isotope-ratio mass spectrometry (Kratos MS25 GC/MS) equipped with a 30 m DB-WAX capillary column (Chrom Tech). Ionization was accomplished by electron impact at 70 eV. A 100 mM aqueous solution of [^{13}C]formic acid (99% ^{13}C , Cambridge Isotope Laboratories) was diluted tenfold in 0.3 M H_2SO_4 and then mixed 1:1 with samples of culture supernatant. Calibration was performed by using aqueous solutions with known concentrations of [^{12}C]formic acid (Mallinckrodt, analytical reagent) between 0 and 20 mM. One millilitre was injected into the gas chromatograph. The oven temperature was held at 80 °C for 1 min, and then increased to 200 °C at a rate of 25 °C min^{-1} , with a holding time of 5 min. The ratio of the ion intensities measured at mass 46 and mass 47 was used as the measure of the [^{12}C]formic acid concentration in the sample. Samples were measured in triplicate and the results averaged.

Transient changes in methanol concentration. The effect of transient changes in the concentration of methanol in the reactor were investigated at a dilution rate of 0.25 h^{-1} , limited by 100 mM methanol in the feed. Steady-state conditions were disturbed by injecting 6–13 mmol methanol through a septum directly into the reactor. The amount of methanol injected was varied in order to study the effect of different initial methanol concentrations on the culture's response. One sample of the culture was taken immediately after methanol addition, rapidly sterile-filtered, and the initial concentration of methanol measured by gas chromatography. Subsequent samples were taken to follow the time-course of the dry biomass and the methanol concentration in the reactor in response to each methanol pulse. Mass spectrometry was used to monitor the effect of the transient increase in methanol on

the O_2 uptake rate and the CO_2 evolution rate of the perturbed (non-steady-state) culture.

^{13}C NMR methods. *In vivo* ^{13}C NMR experiments were performed on oxygenated suspensions of *B. methanolicus* MGA3 taken from a continuous culture growing at a dilution rate (D) of 0.1 h^{-1} . Methanol was the limiting substrate at a feed concentration of 300 mM. A sample volume of 25 ml was taken from the chemostat and immediately centrifuged for 10 min at 4 °C and 17000 g in a centrifuge (Beckman model J2-21). The cell pellet was then resuspended in 5 ml fresh, chilled (approx. 4 °C) MY medium that did not contain methanol. A cell density of about 20 $g\ l^{-1}$ was needed to achieve sufficient NMR signal intensity. This concentrated cell suspension was kept cold on ice for no more than 10 min. Just before use, the suspension was warmed to 50 °C in a water bath. Measurements were made using 4 ml culture in a 12 mm NMR tube (514A-7PP, Wilmad) equipped with a glass capillary to allow *in situ* aeration (see below); 75.46 MHz ^{13}C NMR spectra were obtained with a Nicolet NT300WB spectrometer. 3-Trimethylsilylpropionate (TMSP) was used to calibrate the chemical shift prior to the experiment. Generally, 132 transients were acquired every 5 min, using a 70° pulse, and 8096 data points over 18180 Hz spectral width. High-level (2 W) proton irradiation was used during the pulse and acquisition to ensure good proton decoupling. Before Fourier transformation, 15 Hz line broadening was applied to the signal to improve the signal-to-noise ratio.

^{13}C NMR investigations were carried out to determine the flow of carbon in *B. methanolicus* MGA3 when the cells were exposed to a pulse of methanol. During these NMR experiments, the temperature was maintained at 50 °C. *In situ* aeration (approx. 20 $ml\ min^{-1}$; 40% O_2 in air) was achieved with a small glass capillary fixed in place inside the NMR tube and connected to a supply of pressurized air and O_2 . Maintaining aerobic conditions and controlling the temperature at 50 °C was necessary, because otherwise neither significant methanol consumption nor accumulation of metabolites was observed. After establishing a baseline for 5 min, a 40 μ l pulse of methanol (99% ^{13}C , Cambridge Isotope Laboratories) was added to the aerated cell suspension, resulting in an initial concentration of 250 mM methanol. The flux of carbon was monitored by continuing to acquire and superimpose 132 spectra every 5 min for a total time period of 3 h.

The chemical shifts of formaldehyde (82 p.p.m.) and formate (split peak at 170 p.p.m., 172 p.p.m.) were confirmed by acquiring and superimposing approximately 1600 scans of aqueous solutions of these compounds (natural ^{13}C enrichment) for 1 h. For formaldehyde, 3 ml water was mixed with 1 ml of a 30 $g\ l^{-1}$ solution of formaldehyde freshly prepared from paraformaldehyde (Sigma) and 200 μ l methanol (49 p.p.m.) as a reference. For formate, 4 ml of a 30 $g\ l^{-1}$ solution of sodium formate (Mallinckrodt, analytical reagent) was mixed with 200 μ l methanol.

Detection of conversion of formic acid to CO_2 . Experiments were designed to investigate whether *B. methanolicus* MGA3 was able to metabolize formic acid to CO_2 by growing the strain in continuous culture at a dilution rate of 0.25 h^{-1} under methanol-limiting conditions (100 mM feed concentration). At steady state, 250 ml of reactor exhaust gas was collected and sealed in a glass bomb (Fisher). A pulse of [^{13}C]formic acid (99% ^{13}C , Cambridge Isotope Laboratories) was then applied to the cells by injection through a septum in the reactor head plate, resulting in an initial concentration of approximately 2 mM. After this pulse, a second sample of exhaust gas was collected. From both gas samples, the CO_2

was purified cryogenically by first applying a strong vacuum (0.13 Pa) at -79°C , and then freezing the CO_2 and sealing it in a glass tube cooled to liquid nitrogen temperature. These CO_2 samples were subsequently analysed using a dual-inlet isotope-ratio mass spectrometer (Finnigan MAT Delta E).

RESULTS

Response of *B. methanolicus* to methanol transients in continuous culture

The effect of transients in the ambient methanol concentration on the growth of *B. methanolicus* MGA3 in continuous culture was investigated in a series of experiments in which a pulse of methanol was applied to the steady-state culture as described in Methods. It was expected that the additional supply of methanol to a methanol-limited culture would result in a transient increase in the O_2 uptake rate, CO_2 evolution rate, and dry biomass concentration. In this investigation, the amount of additional carbon provided by the pulse was small, such that an increase in dry biomass concentration would be limited to $0.1\text{--}0.2\text{ g l}^{-1}$.

Fig. 1(a, b, c) shows examples of how *B. methanolicus* MGA3, growing at steady state under methanol-limited conditions, responded to a pulse of methanol which abruptly increased the ambient methanol concentration. The steady-state culture had a dry biomass concentration of $1.59 \pm 0.03\text{ g l}^{-1}$, an O_2 uptake rate of $17.3 \pm 0.37\text{ mmol l}^{-1}\text{ h}^{-1}$ and a CO_2 evolution rate of $6.68 \pm 0.10\text{ mmol l}^{-1}\text{ h}^{-1}$. The resulting respiratory quotient was 0.39.

The data acquired when the methanol pulse was increased to an initial concentration of 8.9 mM in the reactor are summarized in Fig. 1(a). *B. methanolicus* MGA3 responded to the pulse by an immediate, transient increase in the O_2 uptake rate, which remained above the steady-state value for approximately 25 min. Since the effect on the CO_2 evolution rate was only very small, the respiratory quotient was transiently reduced below its steady-state value. During this time, the residual methanol concentration decreased to approximately 2 mM . Interestingly, the dry biomass concentration did not increase but transiently declined in response to the sudden increase in methanol availability. The dry biomass concentration reached a minimum of 1.21 g l^{-1} (24% less than the steady-state value) after approximately 3.25 h. This reduction in the dry biomass concentration in the reactor caused the residual methanol concentration to increase temporarily from 2.0 to 4.5 mM during the time period of 1.0 to 3.25 h, indicating that the cells were unable to consume the methanol fed to the reactor as they had been under steady-state conditions. *B. methanolicus* MGA3 subsequently 'recovered' from the disturbance, as indicated by the increasing biomass concentration in the reactor. During this period (time = 3 to 6 h), both the O_2 uptake rate and the CO_2 evolution rate increased as a result of more rapid cell growth (specific growth rate $\mu > D$). Nine hours after application of the pulse, the culture returned to its steady state.

Fig. 1. Response of a continuous culture of *B. methanolicus* MGA3 (limited by 100 mM methanol in the feed) to a methanol pulse resulting in an increase in the dissolved methanol concentration to 8.9 mM (a), 10.9 mM (b) or 12.8 mM (c). $\blacktriangle$, Residual methanol concentration; ----, O_2 uptake rate (OUR); —, CO_2 evolution rate (CER); $\cdots$, respiratory quotient (RQ); $\blacktriangledown$, dry biomass.

The data acquired in an experiment in which the methanol pulse was increased to an initial concentration of 10.9 mM in the reactor are summarized in Fig. 1(b). The characteristics of the response were analogous to those described above for a pulse of 8.9 mM , but more strongly pronounced. Within the first hour after application of the additional methanol, the residual concentration was reduced to 3.5 mM concomitantly with a transient rise in the O_2 uptake rate and a decrease in the respiratory quotient. The biomass was partially washed out of the reactor and reached its minimum

Table 1. Effect of pulse additions of methanol on a continuous culture of *B. methanolicus* MGA3 growing at steady state under methanol limitation (100 mM methanol in the feed, $D=0.25\text{ h}^{-1}$)

The data are representative of a 12 h time period beginning with the time of methanol injection. Steady-state data are included for comparison.

	CH ₃ OH added in the pulse			
	Steady state	6.4 mmol	8.9 mmol	10.9 mmol
CH ₃ OH supplied (mmol)	300.0	306.4	308.9	310.9
CH ₃ OH consumed (mmol)	298.5	303.9	303.5	277.2
CO ₂ produced (mmol)	80.1	81.7	79.0	70.6
O ₂ consumed (mmol)	208.0	211.4	208.8	191.8
Biomass produced (g)	4.8	4.7	4.5	3.5
$Y_{X/S}$ (g g ⁻¹)	0.50	0.49	0.46	0.39
Y_{X/CO_2} (g mol ⁻¹)	59.9	57.5	57.0	49.6
Y_{X/O_2} (g mol ⁻¹)	23.1	22.2	21.6	18.2
Mean specific rate of methanol consumption (mmol g ⁻¹ h ⁻¹)	15.5	16.2	16.9	19.8
Carbon recovered (%)	96.2	93.2	90.0	80.0

concentration of 0.8 g l⁻¹ (50 % of the steady-state value) at approximately 4.5 h. Simultaneously, the remaining cells were unable to consume all the methanol entering the reactor, causing the residual concentration to rise to a maximum of almost 20 mM at 6.5 h. This increase occurred gradually over a time period of 5 h and is therefore distinctly different from the sudden pulse to the methanol-limited culture at the beginning. It took 12 h for the culture to return to steady state.

Fig. 1(c) shows how the continuous, methanol-limited culture of *B. methanolicus* MGA3 responded to a methanol pulse resulting in a change in concentration to 12.8 mM in the reactor. Contrary to the previous cases described, the culture was now unable to return to steady state. Instead, after 14 h, the biomass was almost completely washed out of the reactor.

A computational analysis was carried out in order to compare steady-state characteristics with the cases in which the culture was disturbed by a transient increase in methanol concentration. A 12 h time period, beginning with the application of the methanol pulse, was chosen as the basis for comparison. Only those cases were considered in which the culture was able to return to steady state, i.e. the size of the pulse was ≤ 10.9 mmol methanol. Integration of the CO₂ evolution rate, the O₂ uptake rate, the residual methanol concentration and the dry biomass concentration was performed for all cases. Under the given conditions, the non-steady-state mass balances for methanol and biomass simplified to:

$$\text{CH}_3\text{OH}_{\text{consumed}}(\text{mmol}) = F \int_{t=0\text{ h}}^{t=12\text{ h}} (c_{\text{in}} - c) dt + \text{CH}_3\text{OH}_{\text{pulse}}$$

$$\text{Biomass}_{\text{produced}}(\text{g}) = F \int_{t=0\text{ h}}^{t=12\text{ h}} x dt$$

where F is the feed rate (0.25 l h⁻¹), c_{in} the methanol concentration in the feed (100 mM), c the methanol

concentration in the reactor (mM), $\text{CH}_3\text{OH}_{\text{pulse}}$ the methanol added with the pulse (mmol), t time (h) and x the biomass concentration in the reactor (g l⁻¹).

The trapezoidal rule was applied to integrate the discrete data. O₂ consumption and CO₂ production were calculated similarly using the on-line data. The results of the analysis are summarized in Table 1. In comparison to steady-state conditions, the biomass production was reduced by 2 % (6.4 mmol methanol pulse) to 27 % (10.9 mmol methanol pulse). Similarly, the biomass yield on methanol decreased by 2 % to 22 % with increasing pulse size, which is also reflected in the increase of the mean specific rate of methanol consumption. Furthermore, the sudden transient increase in the ambient methanol concentration caused an overall increase in the amount of CO₂ produced and O₂ consumed during the production of a fixed amount of dry biomass. CO₂ production increased by 4 % to 21 % over steady-state production, reflected in an equivalent decrease in the biomass yield on evolved CO₂, Y_{X/CO_2} (g mol⁻¹). Simultaneously, O₂ consumption rose by 4 % to 27 %, expressed in the decline of the biomass yield on consumed O₂, Y_{X/O_2} (g mol⁻¹). Carbon balances were calculated for all cases, using a biomass composition of 51.8 % (w/w) carbon (Pluschkell, 1999). For steady-state conditions, >95 % of all carbon is recovered in biomass, CO₂ and residual methanol. With increasing strength of the methanol pulse, carbon recovery declined from approximately 94 % to 80 %, indicating that metabolites that were not accounted for probably accumulated intra- or extracellularly as the size of the methanol pulse was raised.

¹³C NMR analysis

¹³C NMR investigations were carried out to investigate the initial steps in the pathway of carbon assimilation in *B. methanolicus* MGA3. The goal was to determine if

Fig. 2. ^{13}C NMR analysis of the response of continuously grown cells of *B. methanolicus* MGA3 (limited by 300 mM methanol in the feed, $D=0.1\text{ h}^{-1}$) to a pulse of ^{13}C methanol.

sudden increases in the dissolved methanol concentration can cause the accumulation of toxic levels of formaldehyde, the first intermediate of methanol assimilation. In addition, the studies evaluated whether *B. methanolicus* was able to dissimilate formaldehyde via formate to CO_2 . The experiments were conducted as described in Methods and repeated twice with nearly identical results.

Fig. 2 summarizes the response of *B. methanolicus* MGA3, taken from a methanol-limited continuous culture, to a pulse of ^{13}C methanol (initial concentration 250 mM). Each spectrum shown represents 132 scans superimposed over a time period of 5 min. The methanol pulse was applied after acquiring a baseline for the first 5 min. The experiment was completed after 3 h (36 spectra). The decline in the intensity of the methanol signal (chemical shift 49 p.p.m.) with time indicates consumption by the cells. Cell-free experiments in the aerated NMR tube showed that the rate of loss of methanol due to evaporation was negligible over this same time period. Assuming a linear relationship between the methanol concentration and the NMR signal, the data were integrated to determine the time-course of the methanol concentration and to estimate the specific rate of methanol consumption at a cell density of 20 g l^{-1} (Fig. 3). The steady-state specific rate of methanol consumption in the chemostat was approximately $7.5\text{ mmol g}^{-1}\text{ h}^{-1}$ using a 300 mM methanol feed and $D=0.1\text{ h}^{-1}$. The initial rate in the NMR

Fig. 3. Time-course of the methanol concentration (■) and estimate of the specific rate of methanol consumption (●) during *in vivo* ^{13}C NMR experiments. The steady-state specific consumption rate is shown for the conditions under which the cells were grown in the chemostat (300 mM methanol in the feed, $D=0.1\text{ h}^{-1}$).

experiment was significantly higher at approximately $18.5\text{ mmol g}^{-1}\text{ h}^{-1}$, approaching the steady-state rate at about 0.5 h, and gradually declining as methanol was being consumed. After 2.5 h, methanol was almost completely metabolized. This increased rate of methanol consumption in the beginning of the experiment was expected from the results of the transient chemostat studies, where immediately after a methanol pulse, the initial specific rate of methanol consumption was $25\text{ mmol g}^{-1}\text{ h}^{-1}$. Simultaneous with methanol consumption was the appearance of three peaks identified as metabolites. Two of them were formaldehyde (82 p.p.m.) and formate (171 p.p.m.). The third compound (65 p.p.m.) was more difficult to identify conclusively because the ^{13}C spectra of many phosphorylated monosaccharides display signals of similar chemical shift. When methanol is assimilated into the RuMP pathway, it is first incorporated into hexulose 6-phosphate as the C-1 carbon atom, and then becomes the C-1 atom of fructose 6-phosphate in the next reaction (Anthony, 1982). ^{13}C NMR data support the hypothesis that the signal at 65 p.p.m. may be caused by 1- ^{13}C fructose 6-phosphate (Levy *et al.*, 1980). The ^{13}C spectrum of hexulose 6-phosphate is not reported. This signal may be a 1- ^{13}C hexose phosphate; however, further evidence is required for a more conclusive identification. The signal is not glucose 6-phosphate since the C-1 carbon atom of α -D-glucose in water has a much higher chemical shift of approximately 94 p.p.m. (Levy *et al.*, 1980). Glucose 6-phosphate is the product of fructose 6-phosphate isomerization in the dissimilatory RuMP cycle proposed by Arfman *et al.* (1989).

Formaldehyde and formate identification were based on comparison with the ^{13}C spectra of cell-free, aqueous

Fig. 4. (a) Identification of formaldehyde. The top spectrum is a mixture of methanol and freshly prepared formaldehyde in water; the bottom spectrum was observed after a pulse of methanol was applied to an aerated suspension of *B. methanolicus* MGA3 (A, methanol; B, C and D are from formaldehyde). (b) Identification of formate. The top spectrum is a mixture of methanol and sodium formate in water; the bottom spectrum was observed after a pulse of methanol was applied to an aerated suspension of *B. methanolicus* MGA3 (A, methanol; the split peaks B and C are from formate).

solutions of these compounds (natural ^{13}C enrichment), using methanol as a reference. Fig. 4(a) compares the spectrum of formaldehyde with the fourth spectrum of Fig. 2, which represents the scans acquired and superimposed between 15 and 20 min of the experiment. Formaldehyde, freshly prepared from paraformaldehyde, showed three separate signals in the ^{13}C spectrum. The signal at 82 p.p.m. (C) was the strongest. Two smaller peaks appeared at 55 p.p.m. (B) and at 90 p.p.m. (D). The spectrum from the experiment with *B. methanolicus* MGA3 also showed signals at identical chemical shifts, with the signal at 82 p.p.m. dominating in size. Methanol generated a single peak at 49 p.p.m. (A), as confirmed by analysing a sample of pure methanol in water (not shown). The fact that formaldehyde pro-

duced multiple signals may be due to incomplete depolymerization of paraformaldehyde. Formaldehyde can polymerize in aqueous solutions by an anionic mechanism, resulting in chains or in cyclic trimers such as trioxymethylene or paraldehyde (2,4,6-trimethyl-1,3,5-trioxane) (Beyer & Walter, 1988). Furthermore, an autocatalytic condensation of two molecules of formaldehyde to form glycolaldehyde can occur in dilute solutions of formaldehyde (Beyer & Walter, 1988). Other investigators of formaldehyde metabolism by *Escherichia coli* (Hunter *et al.*, 1984), or methanol oxidation by the obligate methanotroph *Methylosinus trichosporium* (Cornish *et al.*, 1984), have reported a single chemical shift for hydrated ^{13}C formaldehyde at approximately 83 p.p.m.

Fig. 4(b) compares the spectrum of sodium formate with the fifth spectrum of Fig. 2, representing the scans acquired and superimposed between 20 and 25 min of the experiment. Formate was identified as a split signal with the chemical shifts of 170 (B) and 172 p.p.m. (C). The spectrum of the methanol-pulse experiment with *B. methanolicus* MGA3 also showed a split peak at the same chemical shifts. This provides evidence that *B. methanolicus* MGA3 can synthesize formate from methanol. Since the presence of cells and gas bubbles in the NMR tube were the cause of some peak broadening relative to cell-free non-aerated solutions, the split signal of formate often collapsed into one broader signal at approximately 171 p.p.m.

Within 5 min after the application of the methanol pulse, formaldehyde began to accumulate. A small signal for a hexose phosphate was also detected, whereas no formate had yet accumulated to a detectable concentration (in the order of 1 mM). Within 15 min, the formaldehyde signal increased to its maximum intensity. Formate was first detected 10 min after application of the methanol pulse. Over the 3 h time period, the methanol signal continued to decline in intensity and almost vanished by the end of the experiment. The formaldehyde peak decreased slowly after 15 min, but never completely disappeared. The signals corresponding to formate and hexose phosphate followed a parallel time-course, increasing for approximately the first 45 min and then remaining nearly constant.

Oxidation of formate by *B. methanolicus*: detection by isotope-ratio mass spectrometry

The ^{13}C NMR investigation described above demonstrated that, under certain conditions, methanol metabolism by *B. methanolicus* MGA3 results in the biosynthesis of formate. The next step was to determine whether formate could be further oxidized to CO_2 by an enzyme such as an NAD-dependent formate dehydrogenase (Anthony, 1978). Dissolved $^{13}\text{CO}_2$ or bicarbonate is usually too dilute to be detected in ^{13}C NMR studies of aerated cell suspensions, or may only generate a very low-intensity signal (Cornish *et al.*, 1984). It was therefore necessary to find a different approach to

Fig. 5. Response of a continuous culture of *B. methanolicus* MGA3 ($D=0.25\text{ h}^{-1}$, limited by 100 mM methanol in the feed) to a sodium formate pulse of 15.7 mM at 0 h. $\blacktriangle$, Measured formate concentration; ----, predicted formate concentration in washout assuming no formate uptake by the cells; $\square$, dry biomass; $\circ$, specific CO_2 evolution rate (CER); $\cdots$, respiratory quotient (RQ).

investigate potential CO_2 production from formate. The method chosen was to measure $^{13}\text{CO}_2$ enrichment in the gas phase of a continuous culture of *B. methanolicus* MGA3 in response to a pulse of ^{13}C formic acid using isotope-ratio mass spectrometry. An enrichment as low as 0.01% can be detected.

A preliminary experiment was performed to determine whether *B. methanolicus* MGA3 growing continuously under methanol limitation is able to take up formate from the culture supernatant. Fig. 5 summarizes the response of *B. methanolicus* MGA3 to a pulse of sodium formate (Mallinckrodt, analytical reagent) resulting in an initial concentration of 15.7 mM at time zero. Prior to adding the pulse, the culture was at steady state, growing at a dilution rate $D=0.25\text{ h}^{-1}$ with 100 mM methanol in the feed. The concentration of formate in samples of culture supernatant was measured by combined gas chromatography and isotope-ratio mass spectrometry (see above). The formate pulse caused a transient decrease in the dry biomass concentration. This was expected from batch-culture experiments, which showed that formate pulse additions can reduce the growth rate of *B. methanolicus* MGA3 by up to 30% in the presence of methanol (Pluschkell, 1999). In this continuous-culture experiment, the cells washed out at an initial rate of approximately 0.22 h^{-1} , i.e. they grew only at a mean rate of 0.03 h^{-1} . The minimum dry biomass concentration of 1.14 g l^{-1} – almost 30% less than at steady state – was reached after approximately 3 h. The culture's biomass level then returned to steady state. Interestingly, the formate pulse provoked a transient increase in the specific CO_2 evolution rate and the respiratory quotient. The specific CO_2 evolution rate reached a maximum of approximately $5.2\text{ mmol g}^{-1}\text{ h}^{-1}$ 2 h after the pulse, a value 20% higher than at steady state. This temporary increase may have been caused

either by an increase in the specific rate of methanol dissimilation or by CO_2 production from formate. In Fig. 5, the time-course of the measured residual formate concentration in the culture supernatant is compared with the concentration profile predicted for simple washout of formate, i.e. the case of no uptake of formate by *B. methanolicus* MGA3. The concentration declined more quickly with time than could be explained by washout alone. If a constant rate of formate uptake by the culture is assumed, this rate can be estimated by:

$$\frac{dc}{dt} = -D \cdot c - q$$

where c is the concentration of formate (mM), t is time (h), D is dilution rate (h^{-1}), and q is formate uptake rate ($\text{mmol l}^{-1}\text{ h}^{-1}$).

Integration from c_0 (at $t=0$) to c (at t) under the assumption that q is constant, gives:

$$c = \left(c_0 + \frac{q}{D} \right) \exp(-D \cdot t) - \frac{q}{D}$$

Thus, the formate uptake rate q can be estimated from a linear regression of c versus $\exp(-Dt)$. Using the measured concentration data for the time period of 0 to 4 h, the following result was obtained:

$$\frac{dc}{d[\exp(-Dt)]} = (19.1 \pm 0.60)\text{ mM}$$

and with $D=0.25\text{ h}^{-1}$ and $c_0=15.7\text{ mM}$:

$$q = (0.85 \pm 0.15)\text{ mmol l}^{-1}\text{ h}^{-1}$$

Formate uptake by continuously growing, methanol-limited *B. methanolicus* MGA3 was significant. The observed volumetric uptake rate of $0.85\text{ mmol l}^{-1}\text{ h}^{-1}$ translates into a specific uptake rate in this experiment of approximately $0.65\text{--}0.77\text{ mmol g}^{-1}\text{ h}^{-1}$. If all of the formate taken up was oxidized to CO_2 at the same rate, it would approximately account for the transient increase in the specific CO_2 evolution rate by $0.62\text{--}0.86\text{ mmol g}^{-1}\text{ h}^{-1}$ above the steady-state value.

With these data indicating that *B. methanolicus* may be able to consume formate, an experiment was designed to measure $^{13}\text{CO}_2$ production in response to a pulse of ^{13}C formic acid. A continuous culture of *B. methanolicus* MGA3 was grown at steady state and limited by 100 mM methanol in the feed, when ^{13}C formic acid was injected to yield an initial concentration of approximately 2 mM in the reactor. Samples of exhaust gas were collected before and after the pulse, the CO_2 was purified and then analysed for the fraction of the ^{13}C isotope by dual-inlet isotope-ratio mass spectrometry. Before ^{13}C formic acid addition, a fraction of 1.02% of the ^{13}C isotope was found in the CO_2 sample, which is equivalent to natural abundance. After ^{13}C formic acid addition, the fraction of $^{13}\text{CO}_2$ was increased to 2.15%. With the sensitivity of the method being in the order of

0.01%, these data indicate a significant enrichment of $^{13}\text{CO}_2$ in the sample taken after application of the ^{13}C formic acid pulse. This result is in agreement with all other observations and provides further evidence that *B. methanolicus* MGA3 is able to oxidize formic acid to CO_2 .

DISCUSSION

Effect of transient fluctuation of dissolved methanol concentration on *B. methanolicus*

The effect of transients in the ambient methanol concentration was investigated by pulse additions of methanol to a continuous culture of *B. methanolicus* MGA3, which at steady state was limited by 100 mM methanol in the feed and growing at $D=0.25\text{ h}^{-1}$. The culture responded to a sudden increase in available methanol of less than 10 mM by transient washout of biomass from the reactor. When the methanol concentration rapidly increased to more than 12 mM, the result was complete biomass washout at a specific rate $\mu < D = 0.25\text{ h}^{-1}$. Integrating the response curves of the dry biomass concentration over a 12 h time period showed that a methanol pulse can cause an average transient decline in the biomass yield of up to 22%. These data indicate that, in order to maximize the biomass yield from methanol in a large-volume bioreactor where the dissolved methanol levels may not be uniform, methanol must be fed to the reactor such that gradients in the local dissolved methanol concentration are less than 10 mM. This requires both a suitable algorithm for the control of the methanol concentration in the reactor and a distributed methanol-feeding mechanism. For industrial-scale manufacture of single-cell proteins by the Gram-negative bacterium *Methylophilus methylotrophus*, methanol was supplied to the reactor through thousands of nozzles throughout the reactor wall instead of by a single 'drip-in' mechanism (Leak, 1992, 1999). The perturbation of steady-state data presented here for *B. methanolicus* MGA3 also indicates that transients in the dissolved methanol concentration must be avoided in order to minimize transient decreases in cell yield and to minimize transient increases in the specific rates of CO_2 evolution and O_2 consumption. Minimizing transient increases in the specific rate of O_2 consumption is important since it is directly related to controlling the amount of heat generated per g dry biomass produced. These findings have significant implications for the design and operation of large-volume bioreactors for the growth of *B. methanolicus* strains for the industrial production of amino acids.

Evidence for the toxicity of transient accumulation of formaldehyde in response to fluctuating methanol concentration

The ^{13}C NMR experiments indicate that the transient partial and the complete biomass washout are probably caused by a transient toxic accumulation of formal-

dehyde, the first intermediate of methanol metabolism. This agrees with previous investigations of methylotrophic bacteria (Al-Awadhi *et al.*, 1988, 1990; Brooke *et al.*, 1989, 1990; Gírio *et al.*, 1995). Formaldehyde accumulation in response to a methanol pulse may be a sign that *B. methanolicus* MGA3 is unable to rapidly increase formaldehyde detoxification when a sudden increase in the rate of methanol oxidation occurs by methanol dehydrogenase. The rate of formaldehyde consumption appears to be slow, with formaldehyde still being detected 3 h after addition of the pulse (Fig. 2). This may be caused either by a decrease in *B. methanolicus* MGA3 cell viability due to *in vivo* reaction of free formaldehyde, or by the *in vivo* kinetics of the activity of either hexulose-6-phosphate synthase or formaldehyde dehydrogenase. The initial rate of methanol utilization by the cells was similar in the NMR studies and the transient chemostat experiments (18.5 and 25 mmol g $^{-1}$ h $^{-1}$, respectively), indicating that formaldehyde probably accumulated transiently in the chemostat as well. According to Arfman *et al.* (1989, 1991), the syntheses of methanol dehydrogenase and hexulose-6-phosphate synthase are not under coordinate control in thermotolerant, methylotroph bacilli. In addition, methanol dehydrogenase may constitute up to 22% of total soluble protein in the strain *B. methanolicus* C1 growing in continuous culture under methanol limitation (Arfman *et al.*, 1989). This implies that growth of *B. methanolicus* strains may generally be very sensitive to sudden changes in the dissolved methanol concentration due to the risk of formaldehyde accumulation.

Evidence for formate accumulation in *B. methanolicus* in response to fluctuating methanol concentration

The ^{13}C NMR data show accumulation of formate, indicating that *B. methanolicus* contains formaldehyde dehydrogenase activity. In addition, the studies using isotope-ratio mass spectrometry provided further evidence for the continuation of this pathway via formate dehydrogenase, resulting in a methanol dissimilation pathway via formate to CO_2 . *B. methanolicus* MGA3, growing continuously under methanol limitation, consumed formate pulsed into the culture at a rate of approximately 0.85 mmol l $^{-1}$ h $^{-1}$. Furthermore, significant accumulation of $^{13}\text{CO}_2$ in the reactor exhaust gas was measured in response to a pulse of ^{13}C formic acid to the culture. The biosynthesis of formate in the dissimilation of methanol by a Gram-positive methylotrophic *Bacillus* has not to our knowledge been reported before. Arfman *et al.* (1989, 1992) investigated similar thermotolerant, methylotrophic *Bacillus* strains, among them *B. methanolicus* PB1, whose 16S rRNA sequences match that of MGA3 to 99%. Using enzyme activity assays, this group concluded that formaldehyde dissimilation is by way of the dissimilatory RuMP cycle. However, the conditions of the cell extract preparation and the assay itself may not have been sufficiently sensitive to detect formaldehyde and formate dehydrogenase activity.

Fig. 6. Proposed pathway of ^{13}C -labelled carbon from methanol into dissimilatory and assimilatory pathways in *B. methanolicus* MGA3.

Distribution of ^{13}C -labelled methanol carbon in *B. methanolicus*

Fig. 6 shows the proposed flow of ^{13}C -labelled carbon in *B. methanolicus* MGA3 from methanol into the dissimilatory and assimilatory pathways. [^{13}C]Methanol dissimilation to formaldehyde, formic acid and CO_2 has been shown by our investigations using ^{13}C NMR techniques and isotope-ratio mass spectrometry. The 65 p.p.m. signal detected in the ^{13}C NMR studies is likely to be the C-1 atom of hexulose 6-phosphate, fructose 6-phosphate, or fructose 1,6-bisphosphate (Levy *et al.*, 1980). These three compounds are the first intermediates in all variants of the assimilatory RuMP cycle (Anthony, 1982). Activities of the enzymes involved in this cycle have been detected in thermotolerant, methylotrophic *Bacillus* strains similar to *B. methanolicus* MGA3 (Arfman *et al.*, 1989). Arfman *et al.* (1989) also found enzyme activities in these strains for dissimilation of methanol via glucose 6-phosphate in the dissimilatory RuMP cycle (Anthony, 1982). The activity of glucose-6-phosphate isomerase catalysing the initial reaction that produces glucose 6-phosphate from fructose 6-phosphate has been detected in extracts of methanol-grown *Bacillus* sp. C1 to be approximately $1.4 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ (Arfman *et al.*, 1989). The ^{13}C flux investigations carried out with *B. methanolicus* MGA3 suggest the activity of both the linear and the cyclic dissimilation pathways. The pathway via formate may play a specific role as a detoxifying reaction, and may be undetectable in cultures that have not been exposed to rapid transients in the dissolved methanol concentration. Similar observations were made by Jones & Bellion (1991), who applied an *in vivo* ^{13}C NMR method to cultures of the methylotrophic yeast *Hansenula polymorpha*. Only cells exposed to high methanol concentrations oxidized the substrate very rapidly, resulting in formaldehyde accumulation.

In our *B. methanolicus* transient chemostat experiments,

we observed a strong initial increase in the O_2 uptake rate immediately after addition of the methanol pulse. This may have been due to rapid oxidation of NADH resulting from the fast initial conversion of methanol to formaldehyde and possibly to formate, indicating a change in respiratory chain activity and possibly pathway utilization. The oxidation of formaldehyde to formate generates an additional NAD(P)H, while in cyclic RuMP dissimilation four enzymes are involved before additional NAD(P)H is produced. Compared to the steady-state data, approximately 3–15% of methanol carbon consumed in the methanol-pulse experiments could not be accounted for. It is possible that this loss reflects the carbon accumulated in intra- or extra-cellular formaldehyde and formate; however, further quantitative NMR studies are needed to investigate the percentage of methanol carbon that may be dissimilated via the linear pathway under various conditions.

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