Adaptation and acclimatization to formaldehyde in methylotrophs capable of high-concentration formaldehyde detoxification

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Formaldehyde is a highly toxic chemical common in industrial effluents, and it is also an intermediate in bacterial metabolism of one-carbon growth substrates, although its role as a bacterial growth substrate per se has not been extensively reported. This study investigated two highly formaldehyde-resistant formaldehyde utilizers, strains BIP and ROS1; the former strain has been used for industrial remediation of formaldehyde-containing effluents. The two strains were shown by means of 16S rRNA characterization to be closely related members of the genus Methylobacterium. Both strains were able to use formaldehyde, methanol and a range of multicarbon compounds as their principal growth substrate. Growth on formaldehyde was possible up to a concentration of at least 58 mM, and survival at up to 100 mM was possible after stepwise acclimatization by growth at increasing concentrations of formaldehyde. At such high concentrations of formaldehyde, the cultures underwent a period of formaldehyde removal without growth before the formaldehyde concentration fell below 60 mM, and growth could resume. Two-dimensional electrophoresis and MS characterization of formaldehyde-induced proteins in strain BIP revealed that the pathways of formaldehyde metabolism, and adaptations to methylotrophic growth, were very similar to those seen in the well-characterized methanol-utilizing methylotroph Methylobacterium extorquens AM1. Thus, it appears that many of the changes in protein expression that allow strain BIP to grow using high formaldehyde concentrations are associated with expression of the same enzymes used by M. extorquens AM1 to process formaldehyde as a metabolic intermediate during growth on methanol.

INTRODUCTION

Methylotrophic micro-organisms are distinguished by their ability to utilize reduced one-carbon compounds as sources of carbon and energy (Anthony, 1982). One of the most remarkable features of methylotrophic metabolism is the general use of formaldehyde, a highly reactive molecule and potent poison to living systems (Grafstrom et al., 1983), as a principal metabolic intermediate, and, in a few documented instances, as a growth substrate (Anthony, 1982). Hence, in addition to numerous other applications in biotechnology and bioremediation, methylotrophic organisms may be used for removal of the toxic and reactive pollutant formaldehyde.

The role of formaldehyde in methylotrophic metabolism has been most extensively studied in the pink-pigmented methylotroph Methylobacterium extorquens AM1, which is a facultative methanol utilizer and a member of the α-proteobacteria (Peel & Quayle, 1961; Anthony, 1982; Lidstrom, 1992; Bratina et al., 1992). Here, as in other methylotrophic bacteria, formaldehyde is an important branch point in metabolism, from which carbon can be dissimilated to carbon dioxide to produce energy, or assimilated into biomass (Anthony, 1982; Lidstrom, 1992). In M. extorquens AM1, there are at least two pathways for formaldehyde dissimilation, and one for its assimilation. Conjugation of formaldehyde to tetrahydromethanopterin (H4MPT) (Chistoserdova et al., 1998) and tetrahydrofolate (H4F) (Marison & Attwood, 1982) in the two major pathways for formaldehyde dissimilation minimizes the cellular
concentration of this toxic intermediate, and may trap the otherwise freely diffusible formaldehyde within the cell. The genes and enzymes of the H₂MPT- and H₂F-dependent pathways are now well characterized, as are most of the steps in the serine cycle for formaldehyde assimilation in M. extorquens AM1 (Chistoserdova et al., 2003), and a gapped genome sequence is available (http://www.integratedgenomics.com/genomereleases.html).

Previous studies have shown that formaldehyde is toxic to succinate-grown M. extorquens AM1 at 1 mM (Marx et al., 2003), and that this strain is unable to utilize formaldehyde at 21 mM as a growth substrate (Peel & Quayle, 1961). In order to characterize how methylotrophic metabolism may be exploited to bioremediate formaldehyde, we have investigated two strains of pink-pigmented methylotrophs, BIP and ROS1, which can utilize formaldehyde at high concentrations as a growth substrate. Strain BIP is used commercially by BIP Ltd for remediation of formaldehyde at high concentrations as a growth substrate. Strain BIP was isolated during this study. Strain BIP was also grown in 250 or 1000 ml Quickfit flasks, respectively, sealed with

**METHODS**

**Bacterial strains and culture methods.** Two strains of pink-pigmented short Gram-negative rod-shaped methylotrophs, strains BIP and ROS1, were used during this study. Strain BIP was isolated from a waste-treatment bioreactor in the formaldehyde-processing plant of BIP Ltd (West Midlands, UK); the bioreactor contained methanol and formaldehyde partitioned between gas and liquid phases. Strain ROS1 is a strain from our laboratory collection that was obtained from a screening process for methanol and formaldehyde utilizers.

Strains BIP and ROS1 were grown at 30 °C on nitrate mineral salts (NMS) and ammonium mineral salts (AMS) liquid medium or agar plates (Whittenbury et al., 1970) with added growth substrate, as stated for each experiment. Formaldehyde stock solution (approx. 2 M) was prepared by heating aqueous paraformaldehyde in sealed glass tubes at 115 °C for 60 min; the concentration of formaldehyde was determined accurately before use. Formaldehyde stock solution and methanol were sterilized by filtration through disposable 0.2 µm pore-size disposable sterile filter units (Millipore) before adding to media; other carbon sources were added from stock solutions that had been sterilized by autoclaving. Liquid cultures of 50 or 200 ml were grown in 250 or 1000 ml Quickfit flasks, respectively, sealed with Suba-Seals (W. H. Freeman), and incubated with shaking at 180 r.p.m. Cell growth was monitored by measuring OD₅₅₀. Strain BIP was also grown in continuous culture in an LH 500 series fermenter (L. H. Engineering), with a working volume of 1-8 l. The pH was maintained at 6-8 by automatic addition of 0·5 M NaOH. At steady state during continuous operation, when the dilution rate was 0·03 h⁻¹ and the concentration of formaldehyde in the feedstock was 120 mM, the concentration of formaldehyde in the fermenter vessel (measured from the outflow) was <1 mM. Cells were harvested from the fermenter vessel by removing no more than one quarter of the working volume of the culture, and then the reactor was fed with formaldehyde-containing medium to resume steady state for at least 120 h before further collections.

**Formaldehyde and methanol determination.** Cells were removed from culture samples by centrifugation (7500 g, 4 °C, 5 min), and then the formaldehyde concentration in the supernatant was quantified using an assay based on the Hantzsch reaction (Nash, 1953), as follows. A sample (0·5 ml) was mixed with an equal volume of reagent B (2 M ammonium acetate, 0·05 M acetic acid and 0·02 M acetylacetone), and incubated at 58 °C for 5 min. Formaldehyde concentration was then determined colorimetrically at 412 nm as the formaldehyde adduct diacetylthiodihydrolutidine. The blank contained distilled water in place of the sample. The range of formaldehyde standards was linear from 0 to 1 mM; samples with concentrations beyond this range were diluted as appropriate. Methanol concentrations were estimated by GC analysis of 2 µl samples using a Series 104 gas chromatograph (Pye Unicam) fitted with a 4 mm i.d. × 1 m Porapak Q column (Waters). Nitrogen was used as a carrier gas at a flow rate of 25 ml min⁻¹. The column was maintained at 200 °C, the flame-ionization detector temperature was 220 °C, and the injection temperature was 200 °C. The quantification of peak areas was achieved using a 3390A integrator (Hewlett Packard). Methanol concentrations of samples were determined by reference to a standard curve constructed using solutions of methanol in the range 0–2 g l⁻¹.

**Molecular genetics methods.** DNA of strains BIP and ROS1 was isolated by a modification of the method of Oakley & Murrell (1988), as follows. Cells harvested from a 50 ml culture were resuspended in 2 ml SET buffer (20 %, w/v, sucrose, 50 mM EDTA and 50 mM Tris/HCl, pH 8·0) plus 100 µl lysozyme solution (5 mg lysozyme ml⁻¹, 10 mM NaCl, 50 mM EDTA, 50 mM Tris/HCl, pH 8·0) and incubated at 37 °C for 1 h. A 100 µl volume of 20 % (v/v) SDS, 50 µl 20 mg ml⁻¹ protease K in 50 mM EDTA, and 50 mM Tris/HCl (pH 8·0) was added, and the mixture was incubated at 55–60 °C for 1 h. The lysate was stored at −20 °C until subjected to phenol/chloroform extraction, and nucleic acid was subsequently recovered by ethanol precipitation, as described by Sambrook et al. (1989).

Amplification of 16S rRNA genes was achieved using eubacterial universal primers 27F (5'-AGAGTTTGATCCTGCTTG-3') and 1492R (5'-AAGGAGGTATCCAGCAGC-3'), designed by Edwards et al. (1989). PCR products were purified using an OmniGene thermal cycler (Hybaid). Each reaction (100 µl) contained 1·5 mM MgCl₂, 20 mM Tris/HCl (pH 8·4), 50 mM KCl, 0·05 % (v/v) detergent W-1, 200 µM each dNTP, 1 ng µl⁻¹ each primer, 2·5 units Taq DNA polymerase (Gibco-BRL) and 0·1 ng µl⁻¹ template DNA. Reaction conditions were as follows: denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min, polymerization at 72 °C for 1 min 30 s; and a final extension at 72 °C for 5 min.

The 1·5 kb PCR products were cloned into the pCR II vector using the TOPO TA Cloning Kit (Invitrogen), and the resulting plasmids were purified by using QIAprep Spin Miniprep kits (Qiagen). The DNA sequence was determined by cycle sequencing with the Dye Terminator Kit (PE Applied Biosystems).

**Two-dimensional electrophoresis and MS analysis.** The sample preparation procedure before two-dimensional electrophoresis (2DE) depended on the gel staining method to be used. Gels that were to be used to compare levels of protein expression, and for MS identification of some protein spots, would eventually be silver stained; the samples to be loaded on such gels were prepared as follows. Cells were harvested, resuspended in 25 mM MOPT (pH 7·5) containing 1 mM benzamidine, broken by passing three times through a French pressure cell (American Instrument Company; 110 MPa, 4 °C) and centrifuged (49000 g, 30 min, 4 °C) to remove unbroken cells. The supernatant was used as the crude extract, and, where necessary, was separated into soluble and membrane components by centrifugation (150000 g, 90 min, 4 °C). The
new supernatant was kept as the soluble fraction, and the membrane-containing pellet was washed with 25 mM MOPS buffer (pH 7.5) containing 10 mM EDTA and 1 mM benzamidine before centrifuging again (150,000 g, 60 min, 4 °C) to pellet the particulate material. Before 2DE, crude extract and soluble samples were denatured using lysis solution I (9 M urea, 2 M thiourea, 4% CHAPS and 30 mM Tris base), whereas membrane samples were solubilized and denatured in lysis solution II [5 M urea, 2 M thiourea, 2% CHAPS, 40 mM Tris base and 60 mM N-decyl-N,N,N-dimethyl-3-ammonio-1-propanesulfonate (SB3-10)]. MS identification of some abundant proteins was achieved using two-dimensional gels stained with Coomassie blue; samples were prepared in the same way as for silver-stained gels, except that the cells were suspended in lysis buffer I before breaking in the French pressure cell.

The first-dimension separation (which was IEF) was effected by applying protein samples (50 μg for silver staining, or 500 μg for Coomassie-blue staining) to immobilized pH gradient (IPG) strips (linear pH gradient 4–7, 18 cm in length; Amersham Pharmacia). The IPG strips were next soaked in rehydration solution [9 M urea, 2 M thiourea, 2% CHAPS, trace of Bromophenol blue, 2-8 mg DTT ml⁻¹ and 0-5% IPG buffer (Amersham Pharmacia)] for 12 h at 20 °C, according to the manufacturer’s instructions. IEF of proteins was carried out at 500 V (1 h), 1000 V (1 h) and then 8000 V (11 h) by means of an IPGphor IEF system (Amersham Pharmacia). After IEF, the IPG strips were equilibrated with 10 ml SDS equilibrium buffer [50 mM Tris/HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and a trace of Bromophenol blue] containing 100 mg DTT for 15 min, and then the buffer was replaced with the same volume of equilibration buffer containing 250 mg iodoacetamide for a further 15 min, according to the manufacturer’s instructions. Second-dimension separation was performed in a precast SDS-12.5% (w/v) PAGE gel at 30 mA (30 min) and then 50 mA (80 min), using a Multiphor II flatbed system (Amersham Pharmacia). Molecular masses were estimated by reference to Dalton Mark VII-L markers (Sigma) run alongside the experimental sample during SDS-PAGE. Proteins were visualized by silver staining according to the method of Heukeshoven & Dernick (1989), or by using Coomassie blue.

Protein spots of interest were excised from the gel, and a blank piece of gel was also excised to serve as a control. The gel pieces were washed and then the gel pieces were incubated at 37 °C for 20 h. Peptides were extracted from the gel with 3 x 20 μl 50% (v/v) acetonitrile containing 5% (v/v) formic acid (Shevchenko et al., 1996). The extracts were pooled, evaporated to dryness using a vacuum centrifuge, and dissolved in 10 μl 0-1% (v/v) trifluoroacetic acid in Milli-Q water. The peptide-containing extracts were then concentrated and desalted by reverse-phase chromatography using ZipTip C₁₈ spin columns (Millipore), as described by Regula et al. (2000).

The desalted samples were analysed using a Q-TOF mass spectrometer (Micromass) fitted with a nanoflow infusion capillary inlet. The potentials of the nanoflow capillary and cone were set at 1000 and 40 V, respectively. MS data were acquired over the range of 400–2400 m/z during a 2-4 s scan by means of the MassLynx data system version 3.4 (Waters). Ions with multiple charges were subject to low-energy tandem mass spectrometry (MS/MS). Precursor ions were selected using the first quadrupole, and subjected to collision-induced dissociation with argon gas, and with the collision energy between 18 and 45 eV. MS/MS data were acquired over the range of 50–2400 m/z during a 2-4 s scan.

MS/MS spectra obtained from the tandem mass spectrometry were exported as sequest files, and were searched against the NCBI non-redundant protein database using the Mascot MS/MS ion search from Matrix Science (http://www.matrixscience.com). Variable amino acid modifications of carbamidomethylcysteine, and oxidation of methionine, were allowed, since these modifications commonly occur as a result of protein separation by 2DE. The peptide mass tolerance was set to ±2 0 Da, and the fragment mass tolerance to ±0 8 Da. Data were reported with individual ion scores. When a peptide ion produced a significant match via a Mascot search, the sequence suggested by Mascot was checked by eye against the MS data to ensure that the assignment of the sequence was an accurate fit to the data. In some cases, short amino acid sequences could be obtained from peptide ions with no significant matches to the database. Such sequences were searched against the translated M. extorquens AM1 ORF database (http://pedant.gsf.de). Any proteins that were not identified after these searches were processed by the MaxEnt 3 algorithm, which converts multiply charged peaks onto a singly charged axis. Amino acid sequences were then manually assigned, and the derived sequences were subjected to the same database searches.

RESULTS

The formaldehyde-degrading strains BIP and ROS-1 are α-proteobacteria closely related to M. extorquens

In order to confirm the similarity of the strains BIP and ROS1 to previously characterized pink-pigmented methylotrophs, the 16S rRNA genes were cloned and sequenced, and phylogenetic analysis was performed. BLASTN searches (Altschul et al., 1997) of the GenBank database revealed that the 16S rRNA sequences of BIP and ROS-1 were most similar to 16S rRNA genes from the genus Methylobacterium. The sequence from strain BIP showed 98-02% sequence identity with the 16S rRNA gene from Methylobacterium thiocyanatum, while that from strain ROS-1 showed 98-85% identity with the 16S rRNA from the closely related Methylobacterium sp. GK118, and the sequences from strains BIP and ROS-1 shared 98-09% identity with each other. The 16S rRNA sequences from strains BIP and ROS1 were aligned with a selection of related 16S sequences, including that from Escherichia coli as an outgroup, using the program CLUSTALW (Higgins et al., 1994). Evolutionary distance values were calculated for the 1360 positions that could be aligned, and a phylogenetic tree was reconstructed by the neighbour-joining method using the PHYLIP software (http://evolution.genetics.washington.edu/phylip.html). Strains ROS-1 and BIP were therefore found to be closely related to the pink-pigmented facultative methylotrophs within the α-subdivision of the proteobacteria, and somewhat less closely related to the non-pigmented class II methanotrophs represented by Methylosinus trichosporium and Methylocystis parvus. The primary sequence identity between strains ROS1 and BIP, and other Methylobacterium species, was comparable to the interspecies similarity level within the genus Methylobacterium (94-5-99-1%) reported by Doronina.
et al. (2000), on the basis of which strains BIP and ROS1 were classified as *Methylobacterium* strains. The 16S rRNA genes from strains BIP and ROS1 shared 92.7 and 91.8% identity, respectively, with the corresponding sequence from the well-characterized *M. extorquens* AM1 (Tsuji et al., 1990).

**Strains BIP and ROS-1 are facultative methylotrophs that can remove and grow on formaldehyde over a wide range of conditions**

BIP and ROS1 formed colonies when cultivated on plates containing methanol as the sole source of carbon and energy, and could also grow using formaldehyde as the growth substrate. As with other α-proteobacterial pink-pigmented methylotrophs (Anthony, 1982), the methylotrophic lifestyle of BIP and ROS1 was facultative, and the strains were not able to use methane as the growth substrate. Growth of both strains was observed on plates containing succinate or pyruvate as the sole carbon and energy source. Growth was also observed on plates containing glucose, sucrose, citrate or acetate, although the colonies were small, showing that these compounds were not good growth substrates for strain BIP; indeed the possibility of growth due to scavenging of nutrients from impurities in the agar cannot be excluded with these poorer growth substrates. These results are consistent with those previously obtained using *M. extorquens* AM1, except that the latter organism did not grow on formaldehyde or acetate (Peel & Quayle, 1961).

Optical density and formaldehyde concentrations were monitored during growth of strains BIP and ROS1 in AMS medium containing formaldehyde, at a range of concentrations, as the only source of carbon and energy. Growth and efficient removal of formaldehyde to ≥10 μM were observed with cultures incubated with starting concentrations of formaldehyde up to 61 mM (Fig. 1). The growth rate of the cells did not vary greatly with formaldehyde concentrations within the range tested, and there was a positive correlation between the amount of formaldehyde added and the time taken to remove it completely (Fig. 1).

The kinetics of growth of strain BIP were investigated in duplicate at eight concentrations of formaldehyde between 0.5 and 100 mM, and tested for fit to the Michaelis–Menten kinetic model by means of a Hanes plot (Cornish-Bowden, 1995). The maximum specific growth rate (μ_max, which is analogous to V_max) was 0.13 h⁻¹, and the apparent K_s was ≤0.65 mM. Growth of BIP was also investigated as a function of methanol concentration between 19 and 310 mM, with methanol as the sole source of carbon and energy. The observed μ_max (0.26 h⁻¹) was achieved at 31 mM methanol. Methanol concentrations greater than this resulted in slower growth. The maximum biomass attained (as judged by the maximum optical density) was achieved at 160 mM methanol (data not shown).

![Graph](image-url)

**Fig. 1.** Growth and formaldehyde removal by strains BIP and ROS1 in AMS medium containing formaldehyde at initial concentrations of 11 mM (continuous line, ■), 24 mM (dashed line, ■), 36 mM (continuous line, ◇) and 61 mM (dashed line, ◇). In all cases, the standard errors of four replicates were <5% of the respective means.
Strains BIP and ROS1 show acclimatization to growth at very high formaldehyde concentrations

Direct cultivation of succinate-grown BIP and ROS1 cells was found to be impossible in liquid cultures at concentrations of formaldehyde greater than 58 mM (data not shown). However, stepwise acclimatization of the culture at increasing concentrations of formaldehyde (typically 20, 40, 60, 72 and then 100 mM, with about 7 day intervals between successive increases in concentration) allowed the cells to survive at formaldehyde concentrations up to 100 mM. Upon exposure to the high concentration of formaldehyde, growth continued for about one generation, and then cell density reached a plateau, whilst formaldehyde removal proceeded at a roughly uniform rate until the residual formaldehyde concentration had fallen to about 50 mM, whereafter growth resumed (Fig. 2).

Protein analysis of formaldehyde- and succinate-grown cells of strain BIP

Proteins produced by strain BIP were investigated by 2DE of samples from separate cultures grown using formaldehyde (20 mM) and succinate as carbon and energy source, and a total of 14 spots were positively identified by MS analysis (Table 1). Representative two-dimensional gels of the various protein samples are included as Supplementary Figures S1–S3 with the online version of this paper. Continuous culture (Table 1; Supplementary Figs S1 and S2), suggesting some form of posttranslational modification of the enzymes that changed the overall charge, but had little effect on the molecular mass.

Effect of formaldehyde concentration on expression profile of strain BIP

Data were also collected from cells grown in continuous culture, which allowed large amounts of biomass to be produced at a lower concentration of formaldehyde (≤1 mM) than would have been possible in batch culture. From this formaldehyde-limited culture, a total of four spots were identified by 2DE that varied significantly in intensity between expression at low and high formaldehyde concentrations (Table 1), although two of these could not be assigned owing to their low expression level, and the remaining two (which were induced by growth at low formaldehyde concentration in continuous culture) had deduced functions with no clear relationship to methylotrophic metabolism. Hence, any changes in gene expression between growth at the two concentrations tested appear to be relatively minor.

DISCUSSION

The results presented here clearly show that Methyllobacterium sp. strains BIP and ROS1 are highly formaldehyde-resistant formaldehyde utilizers. They can also detoxify formaldehyde at concentrations that are too high to support growth if they are first acclimatized at more modest formaldehyde levels, and they can then resume growth using formaldehyde when the concentration has been reduced sufficiently. M. thiocyanatum, which is the closest relative of strains ROS1 and BIP that the phylogenetic analysis identified, can grow on formaldehyde but at a maximum concentration of 1-25 mM (Wood et al., 1998). Thus the highest concentration of formaldehyde that supports growth of M. thiocyanatum is almost 50-fold lower than the maximum concentration that supports growth of strain BIP, and almost two orders of magnitude less than the 100 mM formaldehyde that strain BIP can effectively remediate. It is evident that strains BIP and ROS1 have adaptations that set them apart from related methylotrophs. These adaptations may have evolved or been acquired during long-term exposure to high concentrations of formaldehyde.

Metabolism at high concentrations of formaldehyde showed evidence of the uncoupling of catabolism from anabolism (Tempest & Neijssel, 1981), since no increase in biomass was observed during the plateau period before...
### Table 1. Proteins identified by 2DE and MS/MS analysis

<table>
<thead>
<tr>
<th>Protein spot (RMQ number)*</th>
<th>Observed Mass (kDa)</th>
<th>pI</th>
<th>Theoretical Mass (Da)</th>
<th>pI</th>
<th>Sequence experimentally determined by MS/MS (positions along amino acid sequence of identified <em>M. extorquens</em> protein)</th>
<th>No. of residues identified</th>
<th>Protein identified</th>
<th>Role in methylotrophy</th>
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<tbody>
<tr>
<td><strong>Proteins expressed at similar levels on succinate and formaldehyde</strong></td>
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<tr>
<td>SF1 (05670)</td>
<td>35–36</td>
<td>5·0</td>
<td>33 283</td>
<td>4·90</td>
<td>AVV (aa 125–127) TNDAILYGGR (aa 233–242) DGAFLSPAEAVTSVK (aa 288–303)</td>
<td>30</td>
<td>Methenyl H₄MPT cyclohydrolase</td>
<td>Formaldehyde dissimilation</td>
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<td>SF2 (01528)</td>
<td>35–36</td>
<td>4·9</td>
<td>32 454</td>
<td>4·91</td>
<td>AAVDAGYAPNDWQVQQTGK (aa 231–249) IINEPTAAAL (aa 174–183)</td>
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<td>Electron transfer flavoprotein</td>
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<tr>
<td>SF3 (06982)</td>
<td>&gt; 66</td>
<td>5·13</td>
<td>68 540</td>
<td>5·18</td>
<td>DGALFSPAEAVTSVK (aa 288–303)</td>
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<td>Heat-shock protein, HSP70</td>
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<tr>
<td>SF4 (09682)</td>
<td>18–20</td>
<td>6·3</td>
<td>18 091</td>
<td>5·68</td>
<td>GSPAEAFCGNLVMNK (aa 53–68)</td>
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<td>6·0</td>
<td>18 091</td>
<td>5·68</td>
<td>GSPAEAFCGNLVMNK (aa 53–68)</td>
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<tr>
<td>SF6 (06958)</td>
<td>36–37</td>
<td>5·4</td>
<td>35 451</td>
<td>5·22</td>
<td>SFTLIQQATPR (aa 25–35) DVVDIVEACPR (aa 111–121)</td>
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<td>Malyl-CoA lyase</td>
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<td>MF1 (11455)</td>
<td>60–64</td>
<td>5·3</td>
<td>56 988</td>
<td>5·04</td>
<td>ITVE (aa 175–178) NAGVEGSIVV (aa 456–466)</td>
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<td>MF2 (05966)</td>
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<td>6·2</td>
<td>68 434</td>
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<td>LDGNVAALNAETGETV (aa 156–171) SGAELGV (aa 201–207)</td>
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<td>Methanol dehydrogenase z-subunit</td>
<td>Methanol oxidation</td>
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<tr>
<td>MF3 (05966)</td>
<td>&gt; 66</td>
<td>6·05</td>
<td>68 434</td>
<td>5·87</td>
<td>LTIAPYV (aa 185–191) VIIGSS (aa 196–201)</td>
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<td>Methanol dehydrogenase z-subunit</td>
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<td>21 726</td>
<td>4·72</td>
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<td>Methenyl H₄F cyclohydrolase</td>
<td>Formaldehyde dissimilation</td>
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<td>MF4 (00044)</td>
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<td>6·0</td>
<td>29 929</td>
<td>6·08†</td>
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<td>21</td>
<td>MxaJ</td>
<td>MDH assembly (Amaratunga et al., 1997)</td>
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<td><strong>Proteins specific to growth at low (≤1 mM) formaldehyde concentration relative to high concentration of formaldehyde</strong></td>
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<tr>
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<td>40–43</td>
<td>5·9</td>
<td>43 106</td>
<td>5·35</td>
<td>YDFPQDDIPITK (aa 161–172) LLIQGQAGDNGVGLR (aa 267–282)</td>
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<td>Translation elongation factor</td>
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<td>LVPGYEAP (aa 407–414) IPTVCGLSRL (aa 61–69)</td>
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<td>Glutamine synthetase</td>
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<td>MF8</td>
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<td>SA(I/L)(F/oM)</td>
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<td>No significant match</td>
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<td><strong>Proteins specific to growth at high (20 mM) formaldehyde concentration relative to low concentration of formaldehyde</strong></td>
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<td>MF9</td>
<td>~35</td>
<td>5·8</td>
<td>YDYVVVEE(I/L)(I/L)(F/oM)(I/L)(I/L)(I/L)K</td>
<td>17</td>
<td>No significant match</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Proteins designated SFx were found predominantly in the soluble fraction; those designated MFx were found predominantly in the particulate fraction. The RMQ numbers are the identifier numbers of the corresponding ORFs in the *M. extorquens* AM1 genome.
†The two sequence tags obtained for spot MF7 fell in ORFs MFQ12431 and RMQ12430. We presume that these ORFs are non-overlapping fragments of a single protein.
‡Data refer to the mature protein.
the acclimatized cells resumed growth (Fig. 2). Here, it may be to the cells’ advantage to sacrifice biomass yield to the removal of a growth substrate that is at a dangerously high concentration. A similar decrease in growth yield at very high methanol concentrations has been observed in *M. extorquens* (Harrison, 1976). Enhanced formaldehyde efflux from the cells may also contribute to survival at high concentrations of formaldehyde.

The results from the 2DE protein expression analysis are remarkably consistent with those found in a recent study, published during the preparation of this manuscript, which identified 229 proteins from *M. extorquens* AM1, 68 of which increased in expression during growth on methanol (Laukel et al., 2004). The multiple isoforms of the methanol dehydrogenase α-subunit and formaldehyde-activating enzyme are similar to multiple forms that were observed after 2DE of *M. extorquens* AM1 samples (Laukel et al., 2004). These may be due to in vivo posttranslational changes that alter the pI of the protein; alternatively, they may be due to partial carboxylation of lysyl residues during sample preparation (McCarthy et al., 2003). There were no formaldehyde-induced proteins identified in strain BIP that were not induced by methanol in *M. extorquens* AM1, although the 50S ribosomal protein L9 was not identified in the *M. extorquens* AM1 study (Laukel et al., 2004), and methenyl H₄MPT cyclohydrolase was constitutive in strain BIP, but is known to be increased in level of expression during growth on methanol in *M. extorquens* AM1 (Laukel et al., 2004; Chistoserdova et al., 1998; Vorholt et al., 1999). It is interesting that methanol dehydrogenase, which is induced by methanol (Laukel et al., 2004), is also induced during growth of strain BIP on formaldehyde. Whilst this may be incidental to formaldehyde oxidation, and result from the induction during formaldehyde growth of the proteins that are specific to growth on methanol, it is possible that methanol dehydrogenase participates in formaldehyde oxidation, since formaldehyde is a substrate for this enzyme (Heptinstall & Quayle, 1969; Anthony, 1982).

The evidence currently available suggests that many of the changes in protein expression that allow strain BIP to grow using formaldehyde at 20 mM are associated with expression of the same formaldehyde oxidation and assimilation enzyme that *M. extorquens* AM1 possesses to process formaldehyde as a metabolic intermediate during growth on methanol. This is consistent with the observation that strain BIP is itself able to utilize methanol, and that the formaldehyde and methanol resistance of succinate-grown *M. extorquens* AM1 is diminished in mutants that are defective in the H₄MPT pathway (Marx et al., 2003). Nonetheless, the fact that strain BIP is able to grow at concentrations of formaldehyde (≥20 mM) that support no growth in *M. extorquens* AM1 (Peel & Quayle, 1961) indicates that BIP possesses adaptations to life at high formaldehyde concentrations that *M. extorquens* AM1 does not.

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**REFERENCES**


