Insights into the physiology of *Methylothermobacter mobilis* as revealed by metagenome-based shotgun proteomic analysis

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While the shotgun proteomics approach is gaining momentum in understanding microbial physiology, it remains limited by the paucity of high-quality genomic data, especially when it comes to poorly characterized newly identified phyla. At the same time, large-scale metagenomic sequencing projects produce datasets representing genomes of a variety of environmental microbes, although with lower sequence coverage and sequence quality. In this work we tested the utility of a metagenomic dataset enriched in sequences of environmental strains of *Methylothermobacter mobilis*, to assess the protein profile of a laboratory-cultivated strain, *M. mobilis* JLW8, as a proof of principle. We demonstrate that a large portion of the proteome predicted from the metagenomic sequence (approx. 20 %) could be identified with high confidence (three or more peptide sequences), thus gaining insights into the physiology of this bacterium, which represents a new genus within the family *Methylophilaceae*.

INTRODUCTION

The number of microbial species whose genomes have been completely sequenced is steadily growing; however, these organisms still represent only a fraction of cultivated microbes. These in turn represent only a fraction of existent microbes (Rappé & Giovannoni, 2003), thus making the overall genomic coverage of microbial diversity minuscule. The recent surge in metagenomic sequencing, and downstream applications such as metatranscriptomics and metaproteomics (Ram *et al.*, 2005; Denef *et al.*, 2007; Frias-Lopez *et al.*, 2008; Gilbert *et al.*, 2008), are transforming the field of microbiology. However, due to the novelty of these approaches and to their inherent shortcomings such as low sequence coverage and low sampling depth (Tringe *et al.*, 2005; Rusch *et al.*, 2007), the utility and the potential applications of these datasets remain poorly explored. Recently, applicability of high-quality genomic information in the identification of peptides between related environmental strains has been explored, using a probability-based model and a random mutation simulation model (Denef *et al.*, 2007). Here we explore a reversed approach, using a recently generated metagenomic dataset representing multiple strains of a single bacterial species, *M. mobilis* (Kalyuzhnaya *et al.*,

2008b), for protein detection in the type strain of *Methylothermobacter mobilis*, JLW8, a laboratory-cultivated strain.

M. mobilis JLW8 is the only formally described representative of *Methylothermobacter*, a new genus within the family *Methylophilaceae* (Kalyuzhnaya *et al.*, 2006). All known members of this family are obligate methylophilic, i.e. they are specialized in degradation of organic compounds containing no carbon-carbon bonds (C₁ compounds), most prominently methanol and methylamine (Lidstrom, 2006). Based on culture-independent surveys, *Methylothermobacter* species appear to be ubiquitous, having been detected in marine, freshwater, acid mine drainage, polluted soil, sedimentary rock and glacier environments (according to the entries in the non-redundant database, <http://www.ncbi.nlm.nih.gov/>), consistent with an important role in global carbon cycling. However, the exact function of these bacteria remains unknown. *M. mobilis* JLW8 was isolated from a 63 m deep sampling site in Lake Washington sediment (Kalyuzhnaya *et al.*, 2006). Samples used for stable isotope probing-based enrichment of methylophilic communities that were also used for metagenomic sequencing were collected from the same site, but at a later date (Kalyuzhnaya *et al.*, 2008b). From metagenomic analysis conducted on these samples, the methylamine microcosm was dominated by *M. mobilis* strains, which allowed for extracting a composite genome of *M. mobilis* and conducting metabolic reconstruction for this species (Kalyuzhnaya *et al.*, 2008b). However, the dominant strains of *M. mobilis* represented in the

Abbreviations: GS/GOGAT, glutamine synthetase/glutamate synthase; RMP, ribulose monophosphate.

A supplementary table is available with the online version of this paper.

metagenome appear to be distinct from *M. mobilis* JLW8, based on 16S rRNA sequence comparisons. The study undertaken here pursued three main objectives: (1) to validate the metagenome-based sequence assembly and binning of *M. mobilis* sequences conducted in the previous study, (2) to test whether this metagenomic sequence provides a quality protein database for peptide identification in a strain without perfect matches in the metagenome and test the limitation of such a database, and (3) to obtain new insights into the physiology of *M. mobilis*.

METHODS

Cultivation and harvesting. *M. mobilis* JLW8 was grown on methylamine, the only substrate identified so far that supports its growth in the laboratory, essentially as described previously (Kalyuzhnaya *et al.*, 2006). Per biological replicate, cells from a 2 l culture (OD₆₀₀ 0.2) were harvested by centrifugation at 4500 g for 8 min at 4 °C, briefly washed with cooled 20 mM Tris/HCl pH 8, flash-frozen in liquid nitrogen and stored at –80 °C.

Protein extraction and digestion. Frozen cell pellets were resuspended in 500 µl hot resuspension buffer (20 mM Tris/HCl pH 8.0, 5 mM DTT) and lysed by boiling for 2 min in a water bath, followed by cooling on ice for 10 min. For nucleic acid digestion, 10 units benzonase nuclease (Roche) was added after adjusting the suspension to 2 mM MgCl₂ (final concentration). After 15 min incubation at room temperature, 200 µl of ethanol- and buffer-washed glass beads (150 µm) was added, the sample volume was adjusted to 1.5 ml with resuspension buffer, and bead beating was performed for 4 min at 48 r.p.m. in a MiniBeadBeater (BioSpec Products). Total protein concentration was determined by Bradford protein assay (Bio-Rad); this was approximately 1 mg ml^{–1}. The homogenate was lyophilized to dryness. After resuspension in 500 µl digestion buffer (2 M urea, 5 % acetonitrile, 5 mM dithiothreitol, 0.1 % Rapigest), the sample was digested as previously described (Bosch *et al.*, 2008).

HPLC pre-fractionation and linear ion trap mass spectrometry. The soluble fraction after digestion was lyophilized to a volume of approximately 150 µl and centrifuged for 10 min at 14 000 r.p.m. in a tabletop centrifuge (Eppendorf). A 10–20 µl sample of the supernatant was applied to a PLRP-S reversed-phase column (2.1 mm i.d. × 150 mm, 300 Å, 5 µm; Polymer Laboratories) with mobile phases of 0.1 % trifluoroacetic acid in H₂O and acetonitrile. Peptides were eluted at 0.2 ml min^{–1} with a gradient of 2–60 % acetonitrile in 60 min and 60–90 % acetonitrile in 20 min and collected as five separate fractions. The fractions were each lyophilized to 20 µl, and after reconstitution to volumes of 120 µl with acetic acid and acetonitrile at final concentrations of 0.5 % and 5 % (v/v), respectively, subjected to LC/LC-MS/MS (two-dimensional capillary HPLC/linear ion trap tandem mass spectrometry) analysis as previously described (Bosch *et al.*, 2008).

SEQUEST database searching, DTaselect filtering and estimation of random false positive identification rate. Using the SEQUEST algorithm, raw data were searched against a concatenated FASTA database that consisted of polypeptide sequences derived from the *M. mobilis* composite genome (Kalyuzhnaya *et al.*, 2008b), the human subset of the non-redundant database (nrdb) depleted of all virus sequences, and reversed sequence (decoy) versions of both databases appended. The concatenated database comprised a total of 214 148 protein sequences, 12 951 of which represented the *M. mobilis* specific meta-database. The search results were filtered in DTaselect

applying the parameters described by Bosch *et al.* (2008). Sequest ΔCn/Xcorr values of 0.08/1.9, 0.08/2.2 and 0.08/3.3 were used for singly, doubly and triply charged peptides, respectively. Three peptides unique to a particular ORF were required for positive identification. Random false positive identifications were determined at the peptide level as a ratio of two times the number of reversed peptide identifications in the concatenated database to the total number of identified peptides (Elias & Gygi, 2007). By this means, the qualitative random false positive rate was estimated to be 1.5 % for the first biological replicate and 2.1 % for the second biological replicate.

Data processing and normalization. The in-house methods developed for estimating protein abundances from spectral counting values have been described by Xia *et al.* (2007a, b). In order to normalize the two biological replicates, the total sum of the average spectral counts for the second biological replicate was divided by the total sum of the average spectral counts for the first biological replicate, resulting in a normalization factor of 1.077. Each average spectral count value in the first biological replicate was multiplied by this normalization factor.

Reproducibility of biological replicates. Linear regression analysis demonstrated good data reproducibility for the two biological replicates ($R=0.86$ for the 1924 proteins common to both replicates; data not shown).

DNA amplification, sequencing and analysis. DNA of *M. mobilis* JLW8 was isolated using a QIAamp DNA minikit (Qiagen). Semi-randomly selected fragments of DNA, as shown in Table 1, were amplified by PCR, cloned and sequenced essentially as described by Kalyuzhnaya *et al.* (2008a). Oligonucleotide primers for amplification were designed based on the metagenomic sequence, as follows. First, a gene to be amplified was chosen (genes involved in methylotrophy, central pathways or hypothetical genes). All the homologues representing different *M. mobilis* strains in the metagenome were then aligned using tools available as part of the Joint Genome Institute IMG/M package (<http://img.jgi.doe.gov/cgi-bin/m/main.-cgi>), and consensus sequences were chosen with no or few mismatches.

Peptide mass predictions. Peptide mass predictions were done using the PeptideMass program available at the ExPASy Server (Gasteiger *et al.*, 2005).

Enzyme assays. Cells were grown on methylamine as above, to OD₆₀₀ 0.8, pelleted by centrifugation, as above, resuspended in Tris/HCl pH 8.0 buffer and disrupted by passage through a French pressure cell at 1.2×10^8 Pa. Cell extracts were centrifuged at 14 000 r.p.m. for 25 min at 4 °C to remove cell debris. The activity of glutamine synthetase was assayed as described by Murrell & Dalton (1983), the activity of glutamate synthase was assayed as described by Bravo & Mora (1988), and the activity of glutamate dehydrogenase was assayed as described by Yarrison *et al.* (1972).

RESULTS AND DISCUSSION

Protein divergence between *M. mobilis* JLW8 and environmental *M. mobilis*

In the composite genome of *M. mobilis* that is part of the recently described metagenomic dataset (Kalyuzhnaya *et al.*, 2008b), there are three matches to the 16S rRNA gene of *M. mobilis* JLW8. One of these is a small fragment (291 nt) that seems to represent a very conserved part of

Table 1. Comparison of proteins translated from DNA fragments of *M. mobilis* JLW8 with homologous proteins translated from the composite genome

ID, identity. Proteins (sequences of oligonucleotide primers used for PCR amplification are shown in parentheses, first forward and then reverse): MtdB, methylene H₄MPT dehydrogenase (atggaaaagctagcattt, ttaagagtgaacttttaatt); Fae, formaldehyde activating enzyme (gtgtttgggttaaccatc, cagcacaacgcggtgttg); FdhA, large subunit of formate dehydrogenase (gattgccttactgtgccac, ctgaatacgggttaactcg [1]; atgaagattgggaagtcacg, cgcttactaaatgtcttg [2]); Fdh4A, large subunit of formate dehydrogenase (gaagactccatgagtagtg, gattatcaccgggatagac); Mdh, malate dehydrogenase (gagattgactgcgattgatg, aaggttaccggtgtaataccagtcg); AceE, component E1 of pyruvate dehydrogenase complex (gtggcttgatgctttaatg, ctaatgattcaggtcatc [1]; ctgcaaccattccaaattg, cggttcagccacttttagc [2]); GltA, GltB, large and small subunits of glutamate synthase (cgtgattcgtaaacgtcttg, ccatcattacgagtcac; acaagatccagaattgcgtaaac, gcgcctgagttacgcacacag); MauN, polyferredoxin involved in methylamine oxidation (ctgatcggggcgc-cattgtg, tatcacgcccccttgctccgc); PrpB, methylisocitrate lyase (cggtgccatcaacgcttac, cgcagagagtggtatagc); TR, predicted transcriptional regulator (gagattgactgcgattgatg, ggtgtaataccagtcg); Hp, hypothetical protein (tgcaaccattccaaattg, ggcttcagccacttttagc [1]; gttattaagccataccatc, gaggggtgattgctgcag [2]).

Protein	Length (aa)	No. of matches†	Coverage range (×)	ID range (%)	ID for most covered protein (%)
MtdB*	307	2 (2)	2.8–3.9	93	93
Fae*	187	5 (4)	2.2–4.5	94–98	94
Fdh4A	241	6 (3)	1.4–4.3	90–91	91
Mdh	224	6 (3)	1.5–3.8	92–96	93
AceE [1]‡	224	7 (3)	1.5–3.1	95–99	99
AceE [2]‡	205	7 (3)	1.8–4.4	97–98	97
FdhA [1]‡	222	6 (1)	1.5–3.8	94–96	96
FdhA [2]‡	198	10 (1)	1.2–4.2	95–100	97
GltA	197	8 (1)	1.2–3.6	95–100	100
GltB	220	8 (1)	1.6–3.6	94–97	94
MauN	180	4 (3)	1.6–5.1	94–96	95
PrpB	171	4 (3)	1.4–3.2	98–99	98
TR	155	5 (3)	1.5–3.8	83–98	96
HP [1]‡	141	8 (6)	1.2–5.1	91–99	92
HP [2]‡	103	4 (1)	1.3–2.1	79–81	79

*Complete sequences of MtdB and Fae were obtained. All other sequences are partial.

†The number of full-length sequences is shown in parentheses.

‡In the cases of AceE and FdhA, two different fragments of the same protein were obtained. In the case of HP, two different proteins are discussed.

the gene, with 100 % homology to the *M. mobilis* JLW8 16S rRNA gene, at low sequence coverage ($1.8 \times$; Kalyuzhnaya *et al.*, 2008b). Two others are nearly complete genes with 97 and 98 % homology, respectively, present at much higher coverage ($13.2 \times$ and $20.4 \times$, respectively). Typically, the level of divergence for protein-coding genes between different strains is higher than for 16S rRNA genes (Konstantinidis & Tiedje, 2005). To obtain an estimate of divergence between proteins of *M. mobilis* JLW8 and proteins of *M. mobilis* represented in the metagenome, we amplified 15 DNA fragments, as described in Methods, translated polypeptide sequences from these, and compared them to the metagenome-based *M. mobilis* protein database. Each query peptide had a few hits in the database, in accordance with the composite nature of this database. Few 100 % matches were observed. While most environmental sequences were highly similar (93–98 %) to the *M. mobilis* JLW8 sequences, some sequences diverged as much as 21 % (Table 1). The divergence from the sequences present in the metagenome at the highest coverage (i.e. the most well-assembled sequences) ranged from 0 to 21 %. Complete sequences for two proteins,

methylene tetrahydromethanopterin dehydrogenase (MtdB) and formaldehyde-activating enzyme (Fae), both involved in formaldehyde metabolism (Chistoserdova *et al.*, 2003), were obtained, and these were compared to the homologues translated from the composite genome in terms of predicted tryptic peptide masses. Only two full-sequence homologues were found for MtdB, both at 93 % amino acid identity (Table 1). Out of the 20 predicted tryptic peptides, 8 (40 %) had perfect matches in the database. Four full-sequence homologues were detected for Fae, with amino acid identities ranging from 94 to 98 %. Out of the 12 predicted peptides, 10 (83 %) had perfect matches in the database. These results suggested that for most proteins, a large portion of the peptides of strain JLW8 obtained by tryptic digestion were expected to have identical matches in the protein database translated from the metagenome.

Qualitative proteome coverage

Spectral counts as used here are summations of the number of redundant peptides (i.e. including repeated measure-

ments of the same peptide at different stages of the HPLC separation) observed that map uniquely to a particular protein or protein grouping as defined by the user (Bosch *et al.*, 2008). While the precise relationship between observed spectral counts and abundance within a given proteome is complex, for microbial systems high counts usually correlate with high abundance, as validated by global transcription measurements and other means (Bosch *et al.*, 2008). However, low counts do not necessarily imply low abundance, due to detectability differences among peptides. Data for two biological replicates were collected and analysed. The *M. mobilis* specific protein database utilized for peptide identification was represented by polypeptide sequences translated from the 12 951 gene sequences that constituted the *M. mobilis* composite genome (covering approximately five closely related *M. mobilis* genomes; Kalyuzhnaya *et al.*, 2008b), downloaded from the IMG/M database, along with their automated annotations. These annotations were manually curated for proteins with high spectral counts and thus inferred high relative abundance (see Supplementary Table S1, available with the online version of this paper). A total of 2353 and 2336 polypeptides in this database were matched with the peptides identified in the first and the second biological replicate samples, respectively (Table S1), with a positive detection cutoff of at least three peptide matches per protein (at a cutoff of two peptide matches, 2767 and 2787 peptides were identified, respectively; data not shown). This identification rate (approx. 20 % of total proteins in the database) is, as expected, in a lower range compared to the identification rate typical of proteins matched to a database specific for a given organism (up to 60 %, for example, in *M. extorquens* AM1; Bosch *et al.*, 2008), but within the range of the previously conducted metagenome-based proteomic analyses (Ram *et al.*, 2005) and in agreement with the recent models for protein detection efficiency as a function of sequence divergence (Denef *et al.*, 2007). An additional factor affecting the peptide detection was the partial nature of many of the polypeptides translated from the *M. mobilis* metagenome, due to low sequence assembly (Kalyuzhnaya *et al.*, 2008b).

Protein-based reconstruction of methylotrophy-specific metabolic pathways

In most cases, peptides of *M. mobilis* JLW8 were matched to a number of homologous proteins in the database, representing protein clusters, similarly to the results represented in Table 1. Those homologues with positive peptide matches were grouped manually into clusters, and peptide spectral counts within each cluster were summed. Some of the inferred proteins with the highest summed spectral counts observed in both biological replicates were the ones usually found highly expressed in bacteria, such as the translation elongation factor, chaperonins GroEL and DnaK, subunits of ATP synthase, DNA-directed RNA polymerase, ribosomal proteins, etc. (Table S1) that can be defined as core proteins (Callister *et al.*, 2008). However, of

most interest to us were the peptides representing major enzymes and pathways specifically related to methylamine metabolism in *M. mobilis*. One of the most highly expressed metabolic enzymes was hexulosephosphate synthase (Hps, phylotype 1; Table 2, Table S1 and Fig. 1), the first enzyme in the ribulose monophosphate (RMP) cycle operating in formaldehyde assimilation/oxidation in *Methylophilaceae* (Lidstrom, 2006). The gene for this enzyme is part of a methylotrophy gene island in *M. mobilis* (Kalyuzhnaya *et al.*, 2008b) that is very similar to an island also found in *Methylobacillus flagellatus* (Chistoserdova *et al.*, 2007b). Interestingly, both *Methylobacillus mobilis* and *Methylobacillus flagellatus* encode a second copy of Hps (phylotype 2, approximately 89 % identity with phylotype 1 in *M. mobilis*). This enzyme is also detectable in the *M. mobilis* proteome, but at a much lower level of total counts (Table 1). Other enzymes that are parts of the RMP cycle, both assimilatory and dissimilatory branches, were also identified at relatively high spectral counts, the most abundant being transaldolase and transketolase, which are involved in RMP regeneration (Fig. 1). Peptides for the catalytic subunits of methylamine dehydrogenase (MauAB) were found at high counts, along with the proposed novel electron-accepting cytochrome (MauO; Kalyuzhnaya *et al.*, 2008b), suggesting that this is the main primary oxidation system. Interestingly, MauD, whose function has been proposed as being in small-subunit maturation (van der Palen *et al.*, 1997), was found at relatively high counts, unlike the proteins involved in cofactor biosynthesis (for example, MauG; Li *et al.*, 2008), suggesting a possible role as a chaperone. Polypeptides were detected at high counts forming the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. While not typically considered as part of the methylamine utilization pathway, the presence of these enzymes suggests a mechanism for utilizing the ammonia that results from methylamine oxidation. In this respect, the incomplete tricarboxylic acid cycle (Kalyuzhnaya *et al.*, 2008b), in addition to its anaplerotic function in providing central metabolism intermediates (Lidstrom, 2006), must play another important role, in providing the α -ketoglutarate that is an essential intermediate in the GS/GOGAT cycle. The presence of the activities of methylamine dehydrogenase and the hexulosephosphate synthase/isomerase pair in *M. mobilis* JLW8 has been previously reported, supporting the analysis conducted here (Kalyuzhnaya *et al.*, 2006). To confirm identification of the GS/GOGAT cycle, we measured the activities of the respective enzymes and found them at high levels (95 ± 3 and 174 ± 5 mU, respectively). Glutamate dehydrogenase (GDH), another enzyme known to assimilate ammonia, was undetectable in cell extracts, in agreement with the lack of a gene homologue encoding GDH in the *M. mobilis* genomic database.

Genes for the tetrahydromethanopterin (H₄MPT)-linked C₁ transfer pathway are found in most methylotrophs (Vorholt *et al.*, 2000; Kalyuzhnaya *et al.*, 2005). In some of

Table 2. Major enzymes detected in *M. mobilis* JLW8 with high spectral counts

KDPG, 2-keto-3-deoxy-6-phosphogluconate; MADH, methylamine dehydrogenase; RFAP, ribofuranosylaminobenzene 5'-phosphate.

Protein/function	Total spectral counts*		Normalized per amino acid†
	Replicate 1	Replicate 2	
Hexulosephosphate synthase (Hps [1])	2216	1759	17.7
Formaldehyde activating enzyme (Fae)	1434	608	10.9
Transaldolase (Tal)	1482	1933	10.1
Glutamine synthetase (GlnA)	1079	898	4.2
MADH large subunit (MauB)	821	709	4.1
Transketolase (Tkt)	1335	967	3.5
Enolase (Eno)	593	912	3.5
Homologue of formaldehyde-activating enzyme	308	316	3.4
Essential for small subunit maturation (MauD)	203	480	3.3
Hexulosephosphate synthase (Hps [2])	159	503	2.9
Ribose 5-phosphate isomerase (Ppi)	201	309	2.3
Aconitate hydratase B (AcnB)	414	398	2.2
Ribulosephosphate 3-epimerase (Rpe)	204	297	2.1
6-Phosphogluconate dehydrogenase (GndB)	653	354	2.0
β -RFAP synthase (MptG)	147	277	1.9
Glyceraldehyde phosphate dehydrogenase (Gpd)	257	331	1.7
Cytochrome associated with XoxF (XoxG)	103	276	1.7
Hexulosephosphate isomerase (Hpi)	114	134	1.4
Electron acceptor from MADH (MauO)	86	76	1.3
Putative dehydrogenase (XoxF)	295	268	0.9
Isocitrate dehydrogenase (Idh)	220	159	0.9
PEP synthase (Pps)	294	396	0.9
Phosphoglycerate kinase (Pkg)	135	196	0.8
MADH small subunit (MauA)	59	79	0.7
Glutamate synthase large subunit (GltA)	517	545	0.7
2-KDPG aldolase (Eda)	61	78	0.7
Glucose-6-phosphate dehydrogenase (Zwf)	171	162	0.7
Glutamate synthase small subunit (GltB)	169	150	0.6
Methylisocitrate lyase (PrpB)	96	94	0.6
Pyruvate dehydrogenase component E1 (AceE)	269	229	0.6
Fhc complex subunit D (FhcD)	74	44	0.4
Pyruvate dehydrogenase component E3 (LpdE)	86	133	0.4
Succinate dehydrogenase α subunit (SucC)	82	31	0.4
Succinate dehydrogenase β subunit (SucD)	51	90	0.4

*Number of peptides observed for each entry or group of closely related entries in the metagenomic inferred protein database, including redundant measurements of the same peptide.

†This value is intended as a means for comparison of abundance of proteins of different length.

them, such as *Methylobacterium extorquens*, this is an essential pathway in both formaldehyde detoxification and energy metabolism (Chistoserdova *et al.*, 1998, 2003), while in others, such as *Methylobacillus flagellatus*, this pathway has been demonstrated to be non-essential, the cyclic RMP pathway playing the major role (Chistoserdova *et al.*, 2000). Low abundances of most of the H₄MPT-linked enzymes and of the formate dehydrogenases suggest a secondary role for this linear oxidative pathway in *Methylothermobacter mobilis* grown on methylamine. However, Fae (formaldehyde-activating enzyme; phylotype 1), the first enzyme in this pathway, was found to be one of the

most abundant proteins. The demonstrated function of this enzyme is in directly binding formaldehyde (Vorholt *et al.*, 2000). However, the unusual location of the *fae* gene in the *M. mobilis* genome has prompted a hypothesis of its having a secondary role, as a sensor or signal transducer (Kalyuzhnaya *et al.*, 2008b). A second phylotype of Fae (56 % amino acid identity with phylotype 1 Fae) was also detected in the proteome, at a much lower level. Interestingly, a Fae homologue named Fae2, of unknown function (Chistoserdova *et al.*, 2003; Kalyuzhnaya *et al.*, 2008a), was also observed at high counts, suggesting an important if not yet demonstrated role in C₁ metabolism.

Fig. 1. Central metabolic pathways reconstructed from the composite genome of *M. mobilis* (Kalyuzhnaya *et al.*, 2008b). Proteins shown in red were found at over 10 peptides normalized per amino acid (see explanation for Table 2; large font) or at over 1 peptide per amino acid (small font). Proteins shown in orange were found at over 0.1 peptide per amino acid, and proteins shown in green were found at less than 0.1 peptides per amino acid. Proteins shown in black were not detected. Enzyme descriptions can be found in Table 1 and in Supplementary Table S1. Abbreviations: H₄MPT, tetrahydromethanopterin; MFR, methanofuran; H6P, hexulose 6-phosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; 6PGL, 6-phosphogluconolactone; 6PG, 6-phosphogluconate; Ru5P, ribulose 5-phosphate; E4P, erythrose 4-phosphate; S7P, sedoheptulose 7-phosphate; Xu5P, xylulose 5-phosphate; GAP, glyceraldehyde phosphate; DHAP, dihydroxyacetone phosphate; DPG, diphosphoglycerate; PG, phosphoglycerate; PEP, phosphoenolpyruvate; Ac-CoA, acetyl-CoA; α -KG, α -ketoglutarate.

One metabolic feature that distinguishes *Methylobacterium mobilis* from *Methylobacillus flagellatus*, as predicted from genome–genome comparisons, is the presence of the genes for a complete methylcitric acid (MCA) cycle. All the proteins constituting this pathway were positively identified in this study (Table 2, Supplementary Table S1 and Fig. 1). The role of this pathway in *M. mobilis* remains unknown. Its involvement in metabolism of methylamine is not likely as all the essential metabolites are produced in the RMP cycle in combination with the enzymes interconverting C₃ metabolites and the partial tricarboxylic acid cycle (Fig. 1). More likely, the presence of the specific MCA cycle proteins during growth on methylamine is due to the peculiarities of regulation of this metabolic pathway. It has been previously suggested that this pathway may play a role in utilization of methylated compounds whose degradation results in propionate as a product (Kalyuzhnaya *et al.*, 2008b). While this hypothesis is a subject for experimental testing, the present study demonstrates expression of the entire set of the genes involved, thus supporting functionality of this pathway.

Curiously, XoxF and XoxG, homologues of, respectively, the large subunit of methanol dehydrogenase and an associated cytochrome, were found at high abundance. The potential role of the Xox system has been attracting attention recently, implicating it in C₁ metabolism, but so far without a well-defined function (Wilson *et al.*, 2008; Kalyuzhnaya *et al.*, 2008a). Another highly abundant enzyme was a putative aldehyde dehydrogenase (Adh) whose function also remains unknown.

Conclusions

We utilized a metagenomic sequence of *M. mobilis* for a strain-specific proteomic analysis of the closely related *M. mobilis* JLW8 grown on methylamine. To address the first specific objective (see Introduction), we provided an experimental validation of sequence assembly and *M. mobilis*-specific sequence binning conducted in our previous study (Kalyuzhnaya *et al.*, 2008b), by detecting proteins involved in major metabolic pathways predicted to operate in metabolism of methylamine. To address the second specific objective, we demonstrated that a metagenomic dataset of inherently lower quality compared to completed genomic sequences is usable for cross-strain protein detection, by identifying a large portion of polypeptides predicted to be encoded in the genome of *M. mobilis* JLW8, with high confidence. To address the third specific objective, we obtained new insights into the metabolism of methylamine by *M. mobilis*, including expression of multiple pathways for formaldehyde oxidation (linear versus cyclic) and multiple enzyme paralogues (Hps, Fae), suggesting redundancy of key methylotrophy functions previously noted for other organisms (Chistoserdova *et al.*, 2000, 2007a). In addition, we established a potential link between methylamine oxidation and ammonia utilization via the GS/GOGAT pathway. This

pathway is likely to play a role in the removal of ammonia produced by the methylamine dehydrogenase reaction to prevent its accumulation to toxic levels. We also demonstrated that all the proteins involved in the proposed MCA cycle were expressed, suggesting a central role for this cycle in *M. mobilis* metabolism. This work is a prelude to future applications of this metagenomic dataset for protein identification in other *M. mobilis* strains as well as in natural microbial populations (metagenome-based meta-proteomics).

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