JOURNAL OF BACTERIOLOGY, Sept. 2011, p. 4758–4765 Vol. 193, No. 18
0021-9193/11/$12.00 doi:10.1128/JB.05375-11
Copyright © 2011, American Society for Microbiology. All Rights Reserved.

An Integrated Proteomics/Transcriptomics Approach Points to Oxygen as the Main Electron Sink for Methanol Metabolism in *Methylotenera mobilis*▽†

David A. C. Beck,[1,2]‡ Erik L. Hendrickson,[1]‡ Alexey Vorobev,[1] Tiansong Wang,[3] Sujung Lim,[3] Marina G. Kalyuzhnaya,[3] Mary E. Lidstrom,[1,3] Murray Hackett,[1] and Ludmila Chistoserdova[1]*

Department of Chemical Engineering,[1] The eScience Institute,[2] and Department of Microbiology,[3] University of Washington, Box 355014, Seattle, Washington 98195

Received 23 May 2011/Accepted 5 July 2011

***Methylotenera* species, unlike their close relatives in the genera *Methylophilus*, *Methylobacillus*, and *Methylovorus*, neither exhibit the activity of methanol dehydrogenase nor possess *mxaFI* genes encoding this enzyme, yet they are able to grow on methanol. In this work, we integrated a genome-wide proteomics approach, shotgun proteomics, and a genome-wide transcriptomics approach, shotgun transcriptome sequencing (RNA-seq), of *Methylotenera mobilis* JLW8 to identify genes and enzymes potentially involved in methanol oxidation, with special attention to alternative nitrogen sources, to address the question of whether nitrate could play a role as an electron acceptor in place of oxygen. Both proteomics and transcriptomics identified a limited number of genes and enzymes specifically responding to methanol. This set includes genes involved in oxidative stress response systems, a number of oxidoreductases, including XoxF-type alcohol dehydrogenases, a type II secretion system, and proteins without a predicted function. Nitrate stimulated expression of some genes in assimilatory nitrate reduction and denitrification pathways, while ammonium downregulated some of the nitrogen metabolism genes. However, none of these genes appeared to respond to methanol, which suggests that oxygen may be the main electron sink during growth on methanol. This study identifies initial targets for future focused physiological studies, including mutant analysis, which will provide further details into this novel process.**

The *Methylotenera* genus is a recent addition to the family *Methylophilaceae* (16, 17). This genus represents methylotrophs that are not only sufficiently divergent in genomics terms from the formerly described *Methylophilaceae* but also differ from them by not possessing one of the hallmark enzymes of methylotrophy, methanol dehydrogenase (16, 19). However, *Methylotenera* species are still able, albeit weakly, to grow on methanol (16, 17). This capability must be enabled by an alternative enzyme system that remains unidentified. It has been proposed that methanol oxidation by *Methylotenera mobilis* JLW8 may be linked to (aerobic) denitrification, and XoxF, a protein homologous to the large subunit of methanol dehydrogenase (MxaF), was implicated in this process (18). The more recently described species *Methylotenera versatilis* 301 possesses the homologs of both XoxF proteins encoded in the genome of *M. mobilis* JLW8 but does not possess genes for denitrification (19). Thus, two questions remained unanswered with regard to methanol metabolism by *Methylotenera*: whether, at least in some cases, it is connected to denitrification and whether XoxF enzymes are involved. In order to approach these questions, in this study we integrated shotgun nanoflow scale liquid chromatography-tandem mass spectrometry (LC/MS/MS) proteomics with whole-transcriptome shotgun sequencing (RNA-seq), using *M. mobilis* JLW8 as a model to evaluate its physiological responses to methanol and nitrate.

MATERIALS AND METHODS

Cultivation and sample preparation for proteomics and RNA-seq. For proteome analysis, cultures of *M. mobilis* JLW8 (500 ml) were grown in minimal mineral medium MM2, consisting of 15 mM K_2HPO_4, 16 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 10 mM $NaNO_3$, and 1× Vishniac trace elements (described in reference 24) supplemented with 1 mM methanol, or in Hypho minimal medium (a medium differing from MM2 only by containing 10 mM NH_4SO_4 in place of $NaNO_3$ [24]) supplemented with 1 mM methanol and 10 mM $NaNO_3$ to optical densities at 600 nanometers (OD_{600}) of approximately 0.25 ± 0.03. Cells were harvested approximately 720 h after inoculation by centrifugation at 4,500 × *g* for 15 min at 4°C. The pellets were resuspended in cooled 20 mM Tris-HCl buffer (pH 8.0) and centrifuged again at 4,500 × *g* for 7 min and again for 4 min to remove all of the supernatant. The pellets were immediately frozen in liquid nitrogen and stored at −80°C until further use.

For RNA-seq analysis, cells were grown on methylamine (30 mM) in Hypho medium to an OD_{600} of approximately 0.45 ± 0.05, requiring approximately 44 h of growth after inoculation. Stop solution (5% buffer-equilibrated [pH 7.4] phenol in ethanol) was added, and cells were harvested by centrifugation as described above and immediately used for RNA extraction. For methanol-induced samples, cells were grown on methylamine as described above, centrifuged, washed with MM2 medium, and resuspended in either Hypho plus methanol (25 mM) or in MM2 plus methanol (25 mM, 50 ml total volume, in 250-ml flasks). After 1 h of incubation at 30°C, with shaking, cultures were pelleted as described above and immediately used for RNA extraction. Two biological replicates were used for each condition (i.e., a total of six samples were processed to collect the proteomics data and a total of six samples were processed to collect the transcriptomics data).

Proteomics. The proteome of methylamine-grown *M. mobilis* JLW8 was previously analyzed using a metagenomic scaffold for database matching of tryptic

* Corresponding author. Mailing address: Department of Chemical Engineering, University of Washington, Box 355014, Seattle, Washington 98195. Phone: (206) 616-1913. Fax: (206) 616-5721. E-mail: milachis@u.washington.edu.

‡ Both authors contributed equally.

† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 15 July 2011.

Downloaded from http://jb.asm.org/ on February 19, 2020 by guest

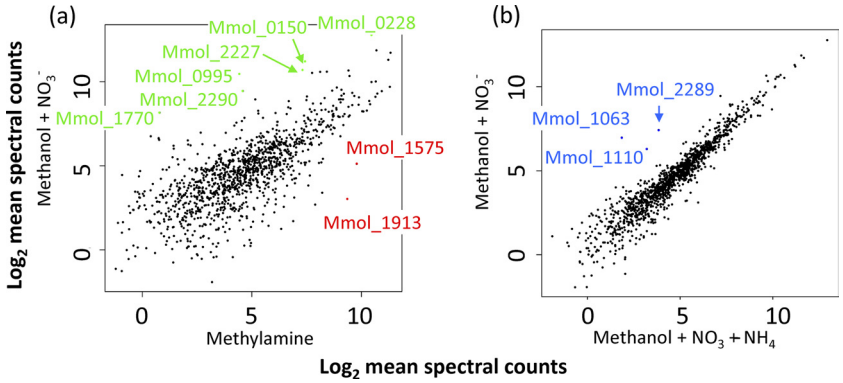

FIG. 1. Log_2-normalized protein abundances. (a) Methanol plus nitrate versus methylamine. Shown in red are select proteins detected at higher abundances under methylamine growth conditions (Mmol_1913, putative metal transporter component; Mmol_1575, methylamine dehydrogenase large subunit). Shown in green are select proteins detected at higher abundances in methanol under nitrate growth conditions (Mmol_1770, XoxF; Mmol_2290, nitrogen regulatory protein PII; Mmol_0995, PQQ biosynthesis protein C; Mmol_2227, alkyl hydroxyperoxide reductase; Mmol_0150, catalase/peroxidase; Mmol_0228, peroxiredoxin). (b) Methanol plus nitrate versus methanol plus nitrate plus ammonium. Shown in blue are proteins detected at higher abundances in methanol with nitrate (Mmol_2289, conserved hypothetical protein; Mmol_1110, DNA-binding protein; Mmol_1063, NO dioxygenase).

peptides (4). In this study, the data sets were reanalyzed using the *M. mobilis* JLW8 genomic scaffold (19). For methanol-grown cultures, cell lysis, trypsin digestion, linear ion trap-tandem mass spectrometry, Sequest database searching (11), and subsequent bioinformatics were carried out as described in reference 3, except that the lysis buffer contained 20 mM rather than 15 mM Tris-HCl (pH 8.5), the digested peptides were washed three times, once with 0.5% trifluoroacetic acid (TFA) and 5% acetonitrile, once with 0.5% TFA and 25% acetonitrile, and once with 0.5% TFA and 50% acetonitrile, and the peptides from high-pressure liquid chromatography (HPLC) prefractionation were collected in 5 rather than 10 fractions. Three unique peptides were required for positive identification. Reverse database peptide identification (10, 26) yielded false discovery rates of <1% at the protein level (13). Quantitation was done using spectral counts (12, 22) to calculate relative-abundance ratios and *q* values (27, 29) derived from *t* test scores with a *q*-value cutoff of 0.01, all calculated similarly to the method of Bosch et al. (3).

Transcriptomics. RNA extraction was performed as described before (14). The integrity of the RNA preparations was tested on a Bioanalyzer 2100 instrument (Agilent), using an Agilent RNA 6000 Nano kit as suggested by the manufacturer. The rRNA content was reduced using a MICROB*Express* Bacterial mRNA purification kit (Ambion). The RNA samples were submitted to a sequencing facility (the High-Throughput Genomics Unit, Department of Genome Sciences, University of Washington; http://www.htseq.org/index.html), where a sequencing platform-specific chemistry was utilized to produce cDNA and sequencing was carried out using platform-specific protocols, producing reads of 36 bp in length. Reads corresponding to each sample were aligned to the reference genome using the Burrows-Wheeler alignment tool (BWA [20]), using default parameters for small genomes. No corrections were applied to raw-data sets prior to this analysis. At the gene level, the reads mapped per one kilobase per one million reads (RPKM) (23) in the sample were normalized across all samples with quantile normalization (2) in preparation for differential-abundance analysis.

RESULTS

Proteomics, protein abundance, and proteins present at higher abundance on methanol. The proteomics experiments were designed to investigate the abundances of proteins in cultures grown on methanol (1 mM) in the presence of nitrate (10 mM) relative to those in cultures grown on methylamine (30 mM). This procedure enabled us to test for protein abundance patterns for enzymes predicted to be involved in methanol oxidation, assimilatory nitrate reduction, and denitrification. Protein relative abundances were also evaluated for cultures grown on methanol (1 mM) in the presence of both nitrate (10 mM) and ammonium (10 mM) to test whether any protein expressed in response to nitrate and/or methanol was controlled by the presence of ammonium.

A total of 734,906 peptide mass spectra were matched to the 2,338 proteins inferred from the *M. mobilis* JLW8 genome (best accessed via the Joint Genome Institute Genome Portal at http://genome.jgi-psf.org/) (19). Across all experimental conditions and biological replicates, we qualitatively identified 1,598 proteins, or 68% of the inferred proteome. More precisely, we detected 1,274 and 1,223 proteins in the two methylamine replicates, 1,304 and 1,361 proteins in the two methanol-plus-nitrate replicates, and 1,331 and 1,295 proteins in the two methanol-plus-nitrate-plus-ammonium replicates. (See FileS 1 in the supplemental material for summaries of all proteome comparisons. This file also contains qualitative coverage data for each detected protein, raw spectral counts, and other detailed information.) The three sets of two biological replicates for each condition were highly reproducible in terms of calculated abundance comparisons within each condition, with correlation coefficients of 0.92, 0.92, and 0.96, respectively, for methylamine, methanol with nitrate, and methanol with nitrate and ammonium (see FileS 2 in the supplemental material). The data sets representing the two latter conditions were virtually superimposable, with very few exceptions (Fig. 1 and Discussion), suggesting that ammonium had a very limited effect on protein expression during methylotrophic growth of *M. mobilis* JLW8. While the cultures grew much more slowly on methanol (doubling time, approximately 70 h) (18) than on methylamine (doubling time, approximately 8 h) (17), we did not note a significant growth rate response in terms of protein abundances, and these showed good correlation between the methanol and methylamine samples (Fig. 1).

While considering the most abundant (i.e., most frequently detected) proteins, we separated these into two categories: proteins typically detected at high abundances in bacterial cultures previously investigated using the proteomics approach (3, 5, 13) and constituting core proteins involved in essential cell functions (ribosomal proteins, RNA polymerase, ATP syn-

Downloaded from http://jb.asm.org/ on February 19, 2020 by guest

TABLE 1. Most abundant proteins (excluding core proteins) and respective transcript abundances[a]

Function	Protein	Count for indicated peptide		Count for indicated transcript	
		Methylamine	Methanol plus NO_3	Methylamine	Methanol plus NO_3
Reactive oxygen detoxification					
Peroxiredoxin	Mmol_2228	2569.4	10341.7 (Up)	2157.4	10220.4 (Up)
Catalase	Mmol_0150	346.4	4431.4 (Up)		1015.9 (Up)
Alkyl hydroxyperoxide reductase	Mmol_2227	381.9	4098.6 (Up)	258.0	1180.8 (Up)
Superoxide dismutase	Mmol_2197	235.0	1675.5 (Up)	197.5	139.2 (Same)
Thiol peroxidase	Mmol_1296	3323.0	6988.0 (Up)	394.0	61.0 (Down)
Methylotrophy					
Fae (2)	Mmol_2056	78.3	575.1 (Up)	182.7	2405.9 (Up)
PQQ biosynthesis protein D	Mmol_0996	0	448.6 (Up)	45.1	50.3 (Same)
PQQ biosynthesis protein B	Mmol_0994	36.9	316.8 (Up)	38.5	7.0 (Down)
PQQ biosynthesis protein C	Mmol_0995	42.9	2631.9 (Up)	146.2	33.5 (Down)
Hexulose phosphate isomerase	Mmol_1337	368.7	1049.0 (Up)	142.8	38.4 (Down)
Transketolase	Mmol_1980	929.0	1501.8 (Same)	176.3	205.6 (Same)
Hexulose phosphate synthase (2)	Mmol_0313	1653.8	1071.6 (Same)	548.8	311.7 (Same)
Methylene-H_4MPT dehydrogenase	Mmol_1346	1158.3	476.3 (Same)	147.3	113.2 (Same)
Fae (1)	Mmol_1253	2844.0	1909.8 (Same)	525.1	274.9 (Same)
Phosphoenolpyruvate synthase	Mmol_1348	337.1	595.3 (Same)	116.6	185.9 (Same)
Transaldolase	Mmol_1339	3082.0	1849.5 (Same)	344.7	160.1 (Same)
6-Phosphogluconate dehydrogenase	Mmol_0143	597.7	363.0 (Same)	243.2	109.6 (Same)
Ribose 5-phosphate isomerase	Mmol_0287	699.8	312.8 (Same)	104.4	97.9 (Same)
Hexulose phosphate synthase (1)	Mmol_1338	5208.0	6388.8 (Same)	3136.0	920.4 (Down)
Enolase	Mmol_1550	919.2	643.8 (Same)	97.0	31.1 (Down)
Fae2	Mmol_0024	870.3	279.9 (Down)	333.3	150.0 (Same)
Methylamine dehydrogenase large subunit	Mmol_1575	1796.7	66.3 (Down)	184.9	66.1 (Down)
Putative metal transport protein	Mmol_1913	1296.0	15.0 (Down)	602.9	13.2 (Down)
Oxidoreductases and accessory proteins					
Aldehyde dehydrogenase (NAD)	Mmol_0791	899.3	2942.0 (Up)	135.4	117.7 (Same)
XoxF (copy 1)	Mmol_1770	3.5	538.3 (Up)	320.5	620.0 (Same)
XoxG	Mmol_2045	819.0	571.0 (Same)	415.0	520.7 (Same)
XoxJ	Mmol_2047	500.0	508.0 (Same)	234.7	341.8 (Same)
XoxF (copy 2)	Mmol_2048	306.1	155.3 (Same)	222.9	150.6 (Same)
NADH:flavin oxidoreductase	Mmol_0733	731.5	551.4 (Same)	93.1	31.6 (Down)
Nitrogen metabolism					
Nitrogen regulatory protein PII	Mmol_2290	48.0	1330.5 (282.0) (Up)	34.4	18.5 (13.3) (Same)
Glutamine synthetase	Mmol_2138	1182.1	1179.6 (Same)	254.5	144.5 (Same)
Other					
Outer membrane protein	Mmol_2143	434.2	2805.0 (Up)	2529.0	1074.5 (Down)
Cold shock protein	Mmol_0596	2752.5	767.5 (Down)	336.2	2926.7 (Up)
Beta-propeller protein	Mmol_0285	1116.8	1402.3 (Same)	142.9	164.9 (Same)
DNA binding protein	Mmol_1318	1380.1	3072.9 (Same)	1045.0	1468.7 (Same)

[a] Sums of peptide counts from two biological replicates are shown, normalized per 333 amino acid residues (corresponding to 1 kb of sequence). Sums of transcript counts from two biological replicates are shown, normalized per kilobase of sequence per 1 million reads. In parentheses, data are shown for methanol plus nitrate plus ammonium conditions (peptides) or for methanol plus nitrate conditions (transcripts). "Up" indicates a trend for higher abundances of peptides/transcripts in response to methanol conditions; "Down" indicates a trend for lower abundances of peptides/transcripts in response to methanol conditions; "Same" indicates no change. Data from only methylamine conditions and methanol-plus-nitrate conditions are shown.

thase, etc.) (5) and proteins that may be specific to the metabolism of C_1 compounds by *M. mobilis* JLW8. The latter were investigated in detail and are listed in Table 1 with spectral counts normalized by protein size. Based on their predicted functions, they fell into the following major classes: (i) reactive oxygen detoxification enzymes, (ii) known methylotrophy enzymes, (iii) oxidoreductases, (iv) nitrogen metabolism proteins, and (v) others.

A number of predicted oxidative stress response enzymes, including the alkyl hydroxyperoxide reductase/peroxiredoxin pair, catalase/peroxidase, superoxide dismutase, and thiol peroxidase, were some of the most abundant proteins detected via proteomics. In addition, all of these proteins were overexpressed during growth on methanol (Table 1 and Fig. 1). The predicted protein Mmol_0150, which is annotated as a catalase/peroxidase, was one of the most differentially abundant of the oxygen stress proteins, being 13-fold more abundant on methanol than on methylamine. It is noteworthy that this type of catalase/peroxidase (KatG) is unique to *M. mobilis* JLW8 and is not present in the genomes of other *Methylophilaceae*, including the closely related *Methylotenera versatilis* 301 (19).

In the category of predicted methylotrophy-specific proteins, the most frequently detected proteins were enzymes known to be involved in methylotrophy by *Methylophilaceae*. These included hexulose phosphate synthase (HPS; both homologs, Mmol_0313 and Mmol_1338) and hexulose phosphate isomer-

Downloaded from http://jb.asm.org/ on February 19, 2020 by guest

ase, the key enzymes of the ribulose monophosphate (RuMP) cycle; formaldehyde-activating enzyme (Fae), which catalyzes condensation of formaldehyde with tetrahydromethanopterin (H_4MPT; both homologs, Mmol_1253 and Mmol_2056); the Fae homolog known as Fae2 (Mmol_0024), so far without a well-defined function; enzymes involved in the regeneration reactions that are parts of the RuMP cycle (transaldolase, transketolase, ribose 5-phosphate isomerase); and enzymes involved in interconversions of C_3 compounds (enolase and phosphoenolpyruvate synthase). The enzyme essential for formaldehyde oxidation via the oxidative RuMP cycle, 6-phosphogluconate dehydrogenase (GndB), was also highly expressed. Of these enzymes, one of the Fae enzymes (Mmol_2056) had the most pronounced differential abundance (7-fold increase on methanol) based on the spectral counts. Some of the proteins involved in biosynthesis of the cofactor pyrroloquinoline quinone (PQQ) were highly expressed in the methanol-grown cultures. PQQ is essential for the function of a number of dehydrogenases, including methanol dehydrogenase (1), suggesting that a PQQ-linked enzyme may be involved in methanol oxidation. Conversely, the large subunit of methylamine dehydrogenase (MauB; Mmol_1575) was detected with high spectral counts in the methylamine-grown culture but had very low counts in the methanol-grown cultures.

In the oxidoreductase category that includes enzymes with a potential for oxidation of methanol, we noted high abundances of both XoxF proteins and predicted accessory Xox proteins (i.e., proteins translated from genes immediately downstream from *xoxF* genes and coregulated with *xoxF* genes). One of these enzymes (Mmol_1770) was significantly more abundant on methanol (150-fold), while the other (Mmol_2048) was present at similar, relatively high abundances on both methanol and methylamine (Table 1). A high level of expression of XoxF proteins in *M. mobilis* JLW8 provides additional evidence for a role in methanol oxidation in this organism. Other highly abundant oxidoreductases included a NAD-dependent aldehyde dehydrogenase of unknown specificity (Mmol_0791), similarly abundant in a related species, *Methylobacillus flagellatus* KT, previously investigated using shotgun proteomics (13), and a NADH:flavin oxidoreductase (Mmol_0733) that also has unknown substrate specificity. The former was more abundant on methanol, while the latter was present at similar levels on both substrates.

In the predicted nitrate metabolism category, we found a small nitrogen regulatory protein (PII; Mmol_2290) at high spectral counts in cells grown on methanol plus nitrate. This protein was present at low spectral counts in cells grown either on methylamine or on methanol in the presence of ammonium, suggesting that this protein is regulated by alternative sources of nitrogen. The second highly abundant protein in this category was glutamine synthetase, which was similarly abundant on both substrates. A function in recycling the ammonium that is a product of the methylamine dehydrogenase (MADH) reaction along with formaldehyde was previously proposed for this enzyme (4).

In the "others" category, a hypothetical protein predicted to have a transmembrane domain was detected at high abundances under both conditions, though it was more abundant during growth on methanol. Also detected at high abundances was a predicted cold shock protein that exhibited increased abundance on methylamine. Two more proteins with strong signals were a predicted DNA binding protein and a hypothetical protein predicted to possess a beta-propeller structure, which were found at similar abundances on both substrates (Table 1).

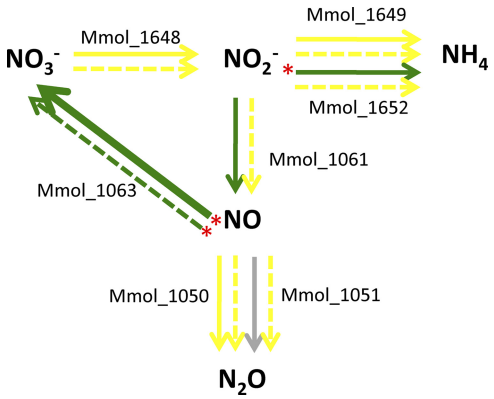

FIG. 2. Expression of nitrate metabolism genes and abundances of nitrate metabolism enzymes in *M. mobilis*. Solid arrows represent protein abundances and dashed lines represent transcription levels (the widths of the arrows correlate with levels of transcription/protein abundance). Green reflects induction during growth/transition to methanol in the presence of nitrate. Yellow reflects no significant change in transcription/protein abundance. Red asterisks denote regulation by nitrogen source (nitrate or ammonium; see text). Gray indicates lack of detection. Mmol identifiers correspond to respective enzymes/subunits.

Methylotrophy and methylcitric acid cycle. Spectral counts for known methylotrophy proteins not listed in the high-abundance category are listed in FileS 3 in the supplemental material. With the exception of the abundances of proteins encoded by the methylamine utilization gene cluster that were induced on methylamine (Mmol_1567 to Mmol_1577), there were no significant changes in abundance recorded. Spectral counts for the enzymes involved in the methylcitric acid cycle are also listed in FileS 3 in the supplemental material. While it remains unknown whether this cycle contributes to methylotrophy metabolism, the presence of this cycle is a signature of *Methylotenera* species (19), and expression of this cycle during methylotrophic growth has been previously reported (4). The abundances of the enzymes involved in this cycle were comparable with the abundances of the RuMP enzymes, and these did not significantly change among samples representing different growth conditions.

Denitrification. Of special interest were the enzymes involved in denitrification as well as in assimilatory nitrate reduction pathways. We have previously identified candidate genes for these functions (18). Most of the proteins implicated in these functions, including proteins predicted to be involved in nitrate transport and proteins without established functions found in the nitrate metabolism gene clusters (see FileS 3 in the supplemental material), were detected at low to moderate peptide counts, with the exception of the B subunit of NO reductase, which remained undetected, likely due to its transmembrane nature (Fig. 2; see also FileS 3 in the supplemental material). No significant changes in the abundances of nitrate

Downloaded from http://jb.asm.org/ on February 19, 2020 by guest

reductase (Mmol_1648), the small subunit of the assimilatory (NADP-linked) nitrite reductase (Mmol_1649), and the C subunit of NO reductase were observed under the methylamine and the two methanol conditions. In contrast, the large subunit of assimilatory nitrite reductase (Mmol_1652) and the dissimilatory nitrite reductase (Mmol_1061) were both more abundant in the methanol-plus-nitrate cultures. However, this is likely a response to nitrate and not to methanol. In the methanol-plus-nitrate-plus-ammonium culture, the level of Mmol_1652 went down, suggesting that this protein may be regulated by ammonium. One of the most highly detected and most differentially expressed enzymes involved in nitrogen species interconversions was NO dioxygenase (Mmol_1063), a detoxification enzyme that converts the toxic NO into nitrate with the use of reducing power. This enzyme also appeared to be regulated (repressed) by the presence of ammonium.

Other differentially abundant proteins. Some proteins were detected with low to moderate abundances but with pronounced differential abundances, with proteins more abundant on methanol falling into the following categories: predicted regulatory proteins (Mmol_1374, 56-fold increase; Mmol_1740 to Mmol_1742, 3- to 55-fold increase; Mmol_1925, 12-fold increase), predicted Fe-S cluster assembly proteins (Mmol_1123 to Mmol_1129, up to a 15-fold increase), and other proteins, including those of unknown function (Mmol_0888 and Mmol_2013, 13-fold increase). These likely represent systems specifically involved in regulating either methanol oxidation or nitrate metabolism genes. None of these proteins were regulated by ammonium. The induction of the Fe-S cluster assembly proteins may be a response to the production of NO, which is known to attack Fe-S clusters (30) that are essential to a number of methylotrophy (formate dehydrogenase, glucose 6-phosphate dehydrogenase) and nitrate metabolism (periplasmic nitrate reductase, NO dioxygenase) enzymes. The specific functions of the remaining methanol-induced proteins will need to be addressed in the future via focused physiological studies, including mutant analysis, as well as via in-depth biochemical studies.

For the methylamine-specific proteins other than the characterized methylamine oxidation proteins (see FileS 3 in the supplemental material), we detected several gene clusters of interest. They encoded proteins that form a putative metal transporter (Mmol_1912 to Mmol_1914), proteins that form an efflux system (Mmol_2293 to Mmol_2295), and proteins that compose a signal transduction/regulation system (Mmol_0126 to Mmol_0131). Determination of the exact functions of these proteins will also require further physiological studies.

Transcriptomics, transcript abundance, and transcriptional response to alternative substrates. The transcriptomics experiments were designed to probe slightly different processes than the proteomics experiments. Specifically, we aimed to address changes in gene expression during the transition from methylamine to methanol as opposed to gene expression during steady-state growth on methanol, potentially detecting transcripts and systems that have a transient response. A 1-h time point was chosen after the switchover of the substrates (methylamine to methanol), based on prior work that investigated induction of methylotrophy functions (28) and on our observations of *Methylotenera* species (A. Vorobev and L. Chistoserdova, unpublished data). Similar switchover experiments were conducted in the presence of nitrate to further discriminate between methanol-specific and nitrogen source-specific responses (see Materials and Methods).

As the RNA-seq method results in data sets of significant size for each sample, typically, complete genome coverage is achieved (9, 25). Here, we collected Illumina-based RNA-seq data for two biological replicates for each of the three conditions, ranging from 9.34 to 29.30 million 36-base reads, for a total of 4.23 Gb of sequence. Statistics for the RNA-seq data sets are shown in FileS 4 in the supplemental material. Despite the implementation of mRNA enrichment steps, RNA preparations were dominated by rRNA (87 to 98%). However, the numbers of reads matching protein coding regions of the genome were sufficient for semiquantitative analysis of transcript abundance, expressed as RPKM (see FileS 4 in the supplemental material). Close correlation was observed between biological replicates for each experimental condition, with R^2 between replicates ranging from 0.71 to 0.94 (see FileS 2 in the supplemental material). When the genes that had the highest numbers of matched transcripts (interpreted as the most highly expressed genes) were considered, a significant overlap with the genes corresponding to the most highly detected proteins as described above was noted. In addition to a number of core metabolism genes not described here (for details, see FileS 5 in the supplemental material), key methylotrophy genes and some oxidative stress genes were identified (Table 1). Notably, the genes for peroxiredoxin (Mmol_2228), HPS (Mmol_1338), and Fae (Mmol_2056) were some of the most highly transcribed genes (Table 1). Both *xoxF* genes (and their accessory genes) were transcribed at relatively high levels, but no immediate significant response to methanol was observed (Table 1).

Interestingly, a number of highly transcribed genes for which no respective proteins were detected via the proteomics approach or for which proteins were detected at low spectral counts were identified. Examples of these are listed in FileS 6 in the supplemental material. Such highly expressed genes may correspond to proteins that are difficult to detect directly using mass spectrometry due to poor extraction efficiencies and/or small numbers of tryptic fragments. Indeed, many of these genes are predicted to encode small or transmembrane proteins that are known to have lower levels of detectability with the shotgun proteomics approach (13). Alternatively, some of the genes for proteins annotated as hypothetical proteins (with no homology to known proteins) may encode small RNA molecules. This possibility will be addressed in our future studies.

A number of the highly expressed genes appeared to be overexpressed in response to methanol (2.5- to 590-fold) (see FileS 6 in the supplemental material). One of the most differentially expressed genes, *mmol_1404*, was part of a unique (i.e., not present in other known *Methylophilaceae*) gene island encoding functions of type II secretion (*mmol_1387* to *mmol_1408*) (8, 19). Of the 22 genes that are parts of this cluster, 20 showed significantly increased expression during the transition to methanol (see FileS 5 in the supplemental material). This pattern was matched by the proteomics data, with 14 proteins being overexpressed on methanol and 8 proteins remaining undetected in all conditions (most of these are predicted transmembranous proteins located in either inner or outer membranes and forming a pore; see FileS 1 in the sup-

Downloaded from http://jb.asm.org/ on February 19, 2020 by guest

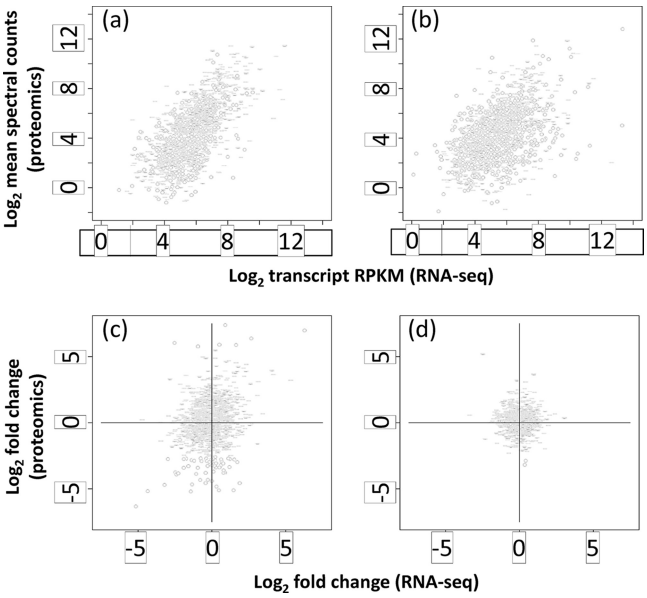

FIG. 3. Comparison of protein and transcript abundances and protein and transcript fold changes. (a and b) Protein and transcript abundances are shown for methylamine (a) and methanol plus nitrate (b). Correlations of proteomics and transcriptomics results for panels a and b are 0.64 and 0.5, respectively. (c and d) Comparisons of fold changes are shown for methanol plus nitrate versus methylamine (c) and methanol plus nitrate versus methanol plus ammonium (transcriptomics) or methanol plus nitrate plus ammonium (proteomics) (d). The dots were made semitransparent in order to provide an indication of density.

plemental material). Two other gene clusters were noted for increased transcription in response to methanol, *mmol_1459* to *mmol_1465* and *mmol_1910* to *mmol_1911*, which encode proteins with no predicted functions. Both of these were also located within the previously detected genomic islands unique to *M. mobilis* JLW8 (18).

A small number of transcripts appeared to be more abundant in methylamine cultures than in methanol-grown cultures, and they represented gene clusters for which the products were detected via proteomics (Table 1; see also FileS 1 in the supplemental material). Increases in transcription in response to nitrate were noted for only a handful of genes, including the genes for NO dioxygenase (*mmol_1063*), an assumed regulatory gene (*mmol_1062*), a gene annotated as a homologue of *soxZ* (*mmol_0068*; a total of three *soxZ* homologues are present in the genome) and the associated gene annotated as a beta-lactamase family protein (*mmol_0067*), and for a gene for Mmol_1470 (putative uncharacterized protein). While the regulation pattern for NO dioxygenase agrees with a pattern observed via proteomics, the roles and the significances of other genes remain to be identified.

Data integration. Overall, between the two complementary technologies, proteomics and transcriptomics, close correlations were found between detected proteins and detected transcripts in terms of protein and transcript abundances (Fig. 3). Despite the different experimental conditions for proteomics and RNA-seq, the correlation of methylamine protein abundance to transcript abundance was 0.64 and of methanol with nitrate, 0.5. When fold change was considered, in the case of methylamine versus methanol with nitrate, of the 1,355 open reading frames (ORFs) with detected proteins and transcripts, 772 followed the same trend in two experiments (57%). For the methanol with nitrate versus methanol with ammonium comparison, in which the differentially abundant transcripts were few and the overall magnitude of fold changes was quite small, noise dominated the fold change calculations and the results of the two methods were less close in agreement (676 of 1,398 detected genes, 48%).

DISCUSSION

In this work, we integrated Illumina-based transcriptomic analysis with shotgun proteomics to obtain insights into two metabolic processes that the model methylotroph *M. mobilis* JLW8 carries out but whose genetic determinants and gene regulation remained poorly understood. In our hands, the two experimental approaches produced compatible results showing reasonably close correlation between the two types of data sets. Similar trends were observed for both the most frequently detected and the most highly differentially expressed proteins and transcripts. Both approaches were successful in identifying the genes and proteins known to be important in methylotrophy metabolism in *M. mobilis* JLW8 (i.e., the established methylotrophy genes and their protein products), and clear trends were observed for transcripts and proteins known to represent regulated metabolic pathways, such as methylamine utilization. The genes responsible for metabolism downstream of the primary oxidation reactions indicated few changes in expression, as assessed by either proteomics or transcriptomics, supporting the assumption of common downstream metabolism for both conditions (i.e., oxidation and assimilation of the formaldehyde produced) (21).

Concerning the enzymes implicated in methanol oxidation, both proteomics and transcriptomics approaches continue to suggest that the XoxF proteins are the main candidates for methanol oxidation, even though the expression patterns for the two homologs appeared to be different, with Mmol_1770 showing significant induction by methanol and Mmol_2048 being present at relatively high levels independently of the substrate. Such an expression pattern differentiates *M. mobilis* JLW8 from a close relative, *M. flagellatus* KT, whose physiology has been previously analyzed using the same shotgun proteomics approach (13). In the latter, the large subunit of methanol dehydrogenase was detected at high abundances in both methanol-grown and methylamine-grown cells, while all four XoxF homologs were found at very low spectral counts (13). While evidence for XoxF fulfilling the role of methanol dehydrogenase in organisms not possessing the classic, *mxaFI*-encoded enzyme is mounting (for a discussion, see reference 7), no ultimate proof, such as null mutants in which both growth and enzyme activity phenotypes confirm the direct role, has yet been obtained. Alternative functions could be envisioned, for instance, in stress response.

Of particular interest in this study was the difference in physiological processes that takes place during growth on methanol versus growth on methylamine or during switchover between the two substrates with reference to the presence or absence of alternative sources of nitrogen (ammonium, nitrate, or a combination of both). One surprising finding in this re-

Downloaded from http://jb.asm.org/ on February 19, 2020 by guest

spect was high levels of expression of oxidative stress enzymes, especially pronounced in cells growing on methanol (Table 1). This pattern, again, differentiates *M. mobilis* JLW8 from *M. flagellatus* KT. While some of the oxidative stress enzymes were detected at relatively high abundances in the latter organism, no significant difference in abundance was noted between methanol and methylamine cultures (13). The inferred oxidative stress does not appear to be nitrate induced, as this effect is also observed in the absence of nitrate. Thus, this effect is either directly connected to the mode of methanol oxidation by *M. mobilis* JLW8 or is a manifestation of a starvation mode of metabolism (caused by very slow growth). This phenomenon also differentiates *M. mobilis* JLW8 from *M. flagellatus* KT, which uses the classic methanol dehydrogenase to oxidize methanol and grows at similar rates on both methanol and methylamine.

With respect to the denitrification pathways, neither proteomics nor transcriptomics supported a significant role for denitrification. This conclusion is based on relatively low protein and transcript abundances overall and relatively modest responses to the addition of methanol and/or nitrate. The lone exception was NO dioxygenase, the NO-detoxifying enzyme. Pronounced response of this enzyme to the presence of nitrate, at both transcript and protein levels, likely suggests that rather than generating energy and serving as an electron sink, as occurs with NO reduction to N_2O, NO is used to regenerate NO_3^- with the use of reduced nicotinamide adenine dinucleotide phosphate [NAD(P)H], thus resulting in a futile cycle (Fig. 2). Another intriguing possibility is that NO dioxygenase is involved in the oxygen stress response, thus detoxifying both NO and oxygen. In either case, induction of this enzyme may constitute an additional aspect of a stressed metabolic state. Overall, the expression patterns for nitrogen metabolism enzymes suggest that denitrification is unlikely to be a major metabolic process or a significant source of energy during aerobic growth on methanol. However, it may be an essential pathway in microaerobic conditions that *Methylotenera* strains may face *in situ*.

Another pronounced response to methanol conditions was the expression of a gene cluster encoding type II secretion functions. This type of secretion system has been shown to participate in the transport of a variety of proteins across both inner and outer membranes to the cell exterior; genes encoding this system have been identified in the genomes of many environmental microbes and, in some cases, have been connected to a specific function or a specific phenotype (6). Increased expression of this gene cluster has been noted before, when the response of *M. mobilis* JLW8 to environmental conditions (unamended Lake Washington sediment) was compared to its response to laboratory growth conditions (15). The exact function of this system in *M. mobilis* JLW8 remains unknown. However, its pronounced response to the transition from methylamine to methanol as well as to environmental conditions (lake sediment) may indicate that it functions as a stress response. Alternatively, it may be involved in adhesion function as previously suggested (15). It is rather unlikely that this type II secretion system is directly involved in methanol oxidation, as the gene cluster responsible is unique to *M. mobilis* JLW8 and is not present in its close relatives, including *M. versatilis* 301 (19).

In conclusion, a number of genes and enzymes that are specifically induced during growth on methanol were identified in this study as candidates for involvement in the utilization of methanol. The patterns of expression for these genes and enzymes show little overlap with the patterns previously observed for a related organism, *M. flagellatus* KT. Moreover, some of the highly and/or differentially expressed genes appear to be unique to *M. mobilis* JLW8. These data further suggest that *M. mobilis* JLW8 and potentially other organisms not encoding the classic methanol dehydrogenase must utilize a novel mechanism for methanol oxidation. This study thus identified initial targets for future physiological studies, including mutant analysis, which will elucidate further details of this novel process.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation (grants MCB-0604269 and MCB-0950183). This work was facilitated through the use of the advanced computational, storage, and networking infrastructure of the Hyak supercomputer system, supported in part by the University of Washington eScience Institute.

We thank Fred Taub for database programming, bioinformatics, and IT support.

REFERENCES

1. **Anthony, C.** 2004. The quinoprotein dehydrogenases for methanol and glucose. Arch. Biochem. Biophys. **428:**2–9.
2. **Bolstad, B. M., R. A. Irizarry, M. Astrand, and T. P. Speed.** 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics **19:**185–193.
3. **Bosch, G., et al.** 2008. Comprehensive proteomics of *Methylobacterium extorquens* AM1 metabolism under single carbon and nonmethylotrophic conditions. Proteomics **8:**3494–3505.
4. **Bosch, G., et al.** 2009. Insights into the physiology of *Methylotenera mobilis* as revealed by metagenome-based shotgun proteomic analysis. Microbiology **155:**1103–1110.
5. **Callister, S. J., et al.** 2008. Comparative bacterial proteomics: analysis of the core genome concept. PLoS One **3:**e1542.
6. **Cianciotto, N. P.** 2005. Type II secretion: a protein secretion system for all seasons. Trends Microbiol. **13:**581–588.
7. **Chistoserdova, L.** 2011. Modularity of methylotrophy, revisited. Environ. Microbiol. [Epub ahead of print.] doi:10.1111/j.1462–2920.2011.02464.x.
8. **Chistoserdova, L., et al.** 2007. Genome of *Methylobacillus flagellatus*, molecular basis for obligate methylotrophy, and polyphyletic origin of methylotrophy. J. Bacteriol. **189:**4020–4027.
9. **Croucher, N. J., and N. R. Thomson.** 2010. Studying bacterial transcriptomes using RNA-seq. Curr. Opin. Microbiol. **13:**619–624.
10. **Elias, J. E., F. D. Gibbons, O. D. King, F. P. Roth, and S. P. Gygi.** 2004. Intensity-based protein identification by machine learning from a library of tandem mass spectra. Nat. Biotechnol. **22:**214–219.
11. **Eng., J. K., A. L. McCormack, and J. R. Yates III.** 1994. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J. Am. Soc. Mass Spectrom. **5:**976–989.
12. **Gao, J., G. J. Opiteck, M. S. Friedrichs, A. R. Dongre, and S. A. Hefta.** 2003. Changes in the protein expression of yeast as a function of carbon source. J. Proteome Res. **2:**643–649.
13. **Hendrickson, E. L., et al.** 2010. Expressed genome of *Methylobacillus flagellatus* as defined through comprehensive proteomics and new insights into methylotrophy. J. Bacteriol. **192:**4859–4867.
14. **Kalyuzhnaya, M. G., D. A. C. Beck, and L. Chistoserdova.** 2011. Functional metagenomics of methylotrophs. Methods Enzymol. **495:**81–98.
15. **Kalyuzhnaya, M. G., et al.** 2010. Functioning *in situ*: gene expression in *Methylotenera mobilis* in its native environment as assessed through transcriptomics. ISME J. **4:**388–398.
16. **Kalyuzhnaya, M. G., et al.** 18 February 2011. Novel methylotrophic isolates from Lake Washington sediment and description of a new species in the genus *Methylotenera*, *Methylotenera versatilis* sp. nov. Int. J. Syst. Evol. Microbiol.]Epub ahead of print.] doi:10.1099/ijs.0.029165-0.
17. **Kalyuzhnaya, M. G., S. Bowerman, M. E. Lidstrom, and L. Chistoserdova.** 2006. *Methylotenera mobilis*, gen. nov., sp. nov., an obligately methylamine-utilizing bacterium within the family *Methylophilaceae*. Int. J. Syst. Evol. Microbiol. **56:**2819–2823.
18. **Kalyuzhnaya, M. G., et al.** 2009. *Methylophilaceae* link methanol oxidation to denitrification in freshwater lake sediment as suggested by stable isotope probing and pure culture analysis. Environ. Microbiol. Rep. **1:**385–392.

Downloaded from http://jb.asm.org/ on February 19, 2020 by guest

19. **Lapidus, A., et al.** 2011. Genomes of three methylotrophs from a single niche reveal the genetic and metabolic divergence of *Methylophilaceae*. J. Bacteriol. **15:**3757–3764.
20. **Li, H., and R. Durbin.** 2009. Fast and accurate short read alignment with Burrows Wheeler transform. Bioinformatics **25:**1754–1760.
21. **Lidstrom, M. E.** 2006. Aerobic methylotrophic prokaryotes, p. 618–634. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, vol. 2. Springer-Verlag, New York, NY.
22. **Liu, H., R. G. Sadygov, and J. R. Yates III.** 2004. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal. Chem. **76:**4193–4201.
23. **Mortazavi, A., B. A. Williams, K. McCue, L. Schaeffer, and B. Wold.** 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods **5:**621–628.
24. **Okubo, Y., E. Skovran, X. Guo, D. Sivam, and M. E. Lidstrom.** 2007. Implementation of microarrays for *Methylobacterium extorquens* AM1. OMICS **11:**325–340.
25. **Ozsolak, F., and P. M. Milos.** 2011. RNA sequencing: advances, challenges and opportunities. Nat. Rev. Genet. **12:**87–98.
26. **Peng, J., J. E. Elias, C. C. Thoreen, L. J. Licklider, and S. P. Gygi.** 2003. Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: the yeast proteome. J. Proteome Res. **2:**43–50.
27. **Porteus, B., C. Kocharunchitt, R. E. Nilsson, T. Ross, and J. P. Bowman.** 2011. Utility of gel-free, label-free shotgun proteomics approaches to investigate microorganisms. Appl. Microbiol. Biotechnol. **90:**407–416.
28. **Skovran, E., G. J. Crowther, X. Guo, S. Yang, and M. E. Lidstrom.** 2010. A systems biology approach uncovers cellular strategies used by *Methylobacterium extorquens* AM1 during the switch from multi- to single-carbon growth. PLoS One **5:**e14091.
29. **Storey, J. D., and R. R. Tibshirani.** 2003. Statistical significance for genome-wide studies. Proc. Natl. Acad. Sci. U. S. A. **100:**9440–9445.
30. **Tinberg, C. E., et al.** 2010. Characterization of iron dinitrosyl species formed in the reaction of nitric oxide with a biological Rieske center. J. Am. Chem. Soc. **132:**18168–18176.

Downloaded from http://jb.asm.org/ on February 19, 2020 by guest