

ORIGINAL ARTICLE

Functioning *in situ*: gene expression in *Methylotenera mobilis* in its native environment as assessed through transcriptomics

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Methylotrophs, organisms able to gain energy and carbon from compounds containing no carbon-carbon bonds, such as methane, methanol and methylated amines, are widespread in nature. However, knowledge of their nutrient preference and their metabolism is mostly based on experiments with cultures grown in defined laboratory conditions. Here, we use transcriptomics to explore the activity of one methylotroph, *Methylotenera mobilis* in its natural environment, lake sediment from which it has been previously isolated. Cells encapsulated in incubation cassettes were exposed to sediment conditions, with or without supplementation with a carbon/energy source (methylamine), and gene-expression patterns were compared for those cells to patterns for cells incubated in a defined medium supplemented with methylamine. A few specific trends in gene expression were observed at *in situ* conditions that may be of environmental significance, as follows. Expression of genes for the linear formaldehyde oxidation pathway linked to tetrahydromethanopterin increased, suggesting an important role for this pathway *in situ*, in contrast to laboratory condition culture, in which the cyclic ribulose monophosphate pathway seemed to be the major route for formaldehyde oxidation. Along with the ribulose monophosphate cycle that is also a major pathway for assimilating C₁ units, the methylcitric acid cycle seemed to be important *in situ*, suggesting that multicarbon compounds may be the natural carbon and/or energy substrates for *M. mobilis*, challenging the notion of an obligately methylotrophic lifestyle for this bacterium. We also detected a major switch in expression of genes responsible for the mode of motility between different conditions: from flagellum-enabled motility in defined medium to *in situ* expression of pili known to be involved in twitching motility and adherence. Overall, this study offers a novel approach for gaining insights into the lifestyle of individual microbes in their native environments.

The ISME Journal (2010) 4, 388–398; doi:10.1038/ismej.2009.117; published online 19 November 2009

Subject Category: integrated genomics and post-genomics approaches in microbial ecology

Keywords: *Methylotenera mobilis*; methylotrophy; lake sediment; transcriptomics

Introduction

Methylotrophy, the ability to use single carbon (C₁) compounds as sources of carbon and energy, is widespread in nature and found in a variety of bacterial phyla. Some methylotrophs are also able to grow on multicarbon substrates (facultative methylotrophs), whereas several subtypes are known as obligate methylotrophs, that is they are restricted to usage of C₁ compounds, as determined by laboratory experiments. This physiological subtype includes most of the methanotrophic species (alpha- and

gammaproteobacteria and Verrucomicrobia), marine methylotrophs belonging to the *Methylophaga* genus and most of the species belonging to Methylophilaceae (Jenkins and Jones, 1987; Hanson and Hanson, 1996; Semrau *et al.*, 2008). Despite the limited substrate range, these species are abundant in nature, suggesting that obligate dependence on C₁ compounds may be an advantageous adaptation. Enzymatic lesions in carbohydrate metabolism and/or tricarboxylic acid cycle and a limited array of organic compound transporters have been suggested as the main potential causes of obligate methylotrophy (Shishkina and Trotsenko, 1982; Wood *et al.*, 2004). Indeed, such lesions have been confirmed through the genomic analysis of some obligate methylotrophs, such as *Methylobacillus flagellatus* (Chistoserdova *et al.*, 2007). However, genomes of other obligate methylotrophs, such as *Methylococcus*

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Received 3 August 2009; revised 28 September 2009; accepted 5 October 2009; published online 19 November 2009

capsulatus, were found to encode all the functions necessary for growth on multicarbon compounds (Ward *et al.*, 2004; Kelly *et al.*, 2005). This points to the fact that growth and behavior of such microbes as observed in the laboratory may not fully reflect their physiological potential encoded in the genome and that some of the genomic features must be only expressed under specific environmental conditions.

Here, we present an approach for characterizing physiological abilities of individual microbes *in situ*, through transcriptomic profiling, in an attempt to reveal functions specifically expressed in the environment that remain silent in the laboratory. We used *Methylotenera mobilis* as a model organism. In the laboratory, this bacterium is only able to grow on methylamine as a single source of carbon, nitrogen and energy and poorly on methanol when nitrogen is supplied as an additional terminal electron acceptor (Kalyuzhnaya *et al.*, 2006, 2009). However, the genomic analysis of natural populations of *Methylotenera* species has suggested a broader metabolic potential. For example, a complete set of genes encoding the methylcitric acid (MCA) cycle has been identified, pointing to the ability to metabolize propionate (Kalyuzhnaya *et al.*, 2008). In this study, we characterize gene expression in *M. mobilis* cells in its natural niche, lake sediment.

Materials and methods

Cultivation

M. mobilis JLW8 was routinely grown in a minimal mineral medium (Kalyuzhnaya *et al.*, 2006) supplemented with 30 mM methylamine. For testing alternative growth substrates, the same medium was used in which methylamine was replaced with one

of the followings (10 mM each): propionate, methylpropionate, methylcitrate, levulinic acid, benzoic acid, para-aminobenzoic acid, benzylamide, methoxyphenol, vanillin. Cultures were incubated in 250 ml flasks, with shaking (200 r.p.m.) at 28 °C.

Preparation of the incubation cassettes

Slide-A-Lyzer 3.5 K MWCO Dialysis Cassettes (12 ml Capacity, Thermo Fisher Scientific, Waltham, MA, USA) were re-hydrated in 100 ml of sterile double-distilled water (ddH₂O) for 10 min. To prevent possible contamination of the samples by the trace (in)organic compounds from the membrane material, each cassette was thoroughly rinsed with sterile water and incubated overnight in 0.5 l of sterile ddH₂O.

Sample collection and experimental setup

Sediment cores and water samples were collected as described before (Kalyuzhnaya *et al.*, 2004). The samples were delivered to the lab on ice and used immediately in the incubation experiments. Lake water used for incubations was filtered through 0.22 μm filters (Millipore, Billerica, MA, USA). *M. mobilis* JLW8 was grown in methylamine-supplemented minimal medium as above. Cells were collected in the middle exponential phase of growth (optical density, OD₆₀₀ = 0.4 ± 0.1) by centrifugation at 5000 r.p.m. for 15 min at room temperature. Each of the two biological replicates (RA and RB in Figure 1) was divided into five sub-samples. One of these was washed with 30 ml mineral medium (RA1 and RB1) and the rest of the sub-samples were washed with the filtered lake water. Cells were collected by centrifugation as above and re-suspended in 11 ml of minimal medium (RA1 and RB1) or filtered lake water (the rest of the samples) to a final

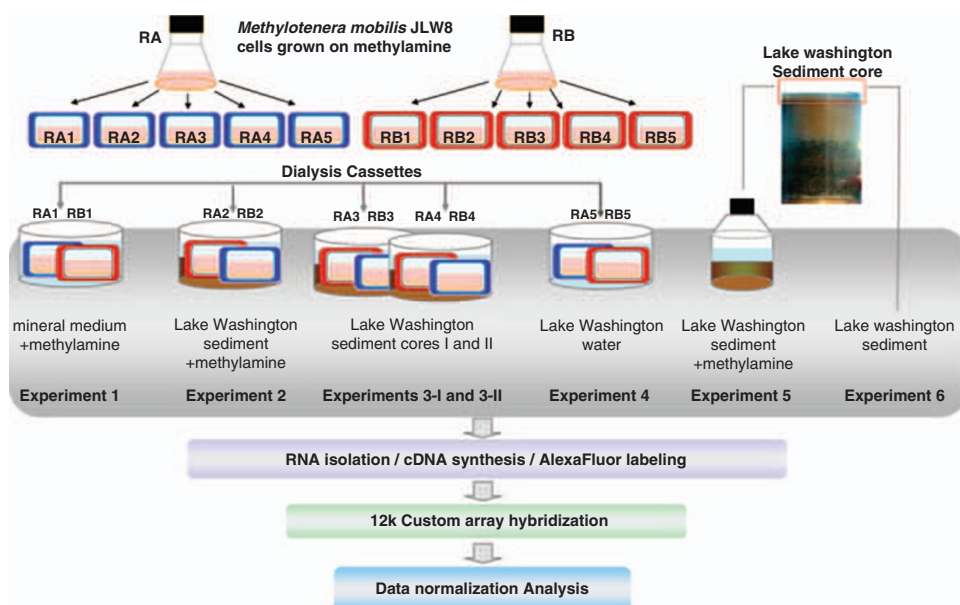


Figure 1 Overview of the experimental setup.

$OD_{600} = 0.3 \pm 0.02$; 10 ml samples were injected into the incubation cassettes, and these were implanted into different media as follows: samples RA1 and RB1 were placed into a 0.5 l jar filled with 250 ml minimal medium supplemented with 10 mM methylamine (Experiment 1); samples RA2 and RB2 were placed into a 0.5 l jar filled with 250 ml of a semi-liquid sediment collected from the top of a core and supplemented with 10 mM methylamine (Experiment 2); samples RA3, RB3, RA4 and RB4 were directly implanted into the top (1 cm) of two different sediment cores (biological replicates, Experiments 3-I and 3-II); and samples RA5 and RB5 were placed into a 0.5 l jar with 250 ml sterile lake water (Experiment 4). In each case the incubation cassettes were fully covered by liquid (medium or lake water). Samples were incubated at 10 °C (ambient temperature) for 10 days. As the filtering procedure used to purify the lake water may have not resulted in sterile water, because of the potential presence of ultramicroorganisms (Torrella and Morita, 1981; Fischer and Velimirov, 2000), the purity of the culture before and after incubation in cassettes was tested by microscopic observations, restriction fragment length polymorphism profiling of the 16S rRNA gene fragments and by plating on permissive (minimal medium supplemented with methylamine) and restrictive media (tryptic soy agar, TSA; nutrient agar, NA and Luria-Bertani agar, LA; all from BD, Franklin Lakes, NJ, USA). In two additional experiments (Experiments 5 and 6) native populations of *M. mobilis* were tested, after incubation with added methylamine essentially as described before (Kalyuzhnaya *et al.*, 2008) and with no prior treatment, respectively.

RNA extraction

Cassettes were removed from respective media and cells were transferred into 15 ml tubes containing 0.5 ml of 'stop solution' (5% buffer equilibrated phenol (pH 7.4) in ethanol) and collected by centrifugation at 4500 g for 15 min at 10 °C; 50 ml of the top layer of lake sediment and 50 ml of the methylamine microcosm were fixed by 2.5 ml of 'stop solution' and collected by centrifugation at 4500 g for 15 min at 10 °C. RNA extraction was carried out as described earlier (Necessian *et al.*, 2005) with the following modification: cell pellets (or sediment samples) were re-suspended in 0.75 ml (5 ml for sediment samples) of RNA extraction buffer (0.15 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.5; 2.5% CTAB, 0.4 M NaCl, 2% SDS; 2% *N*-lauroylsarcosine sodium salt). Two rounds of DNase I treatment were carried out, using the DNasefree kit (Life Technologies, Carlsbad, CA, USA), in accordance with manufacturer's instructions. The resulting RNA was further purified using the RNeasy columns (Invitrogen, Carlsbad, CA, USA). The integrity of RNA preparations was tested using the Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA) and the Agilent RNA 6000 nano kit, as suggested by the manufacturer.

cDNA synthesis and labeling

First-strand cDNA synthesis and labeling was performed using the SuperScriptindirect cDNA labeling system (Invitrogen) and Alexa Fluor 555 and Alexa Fluor 647 fluorescent dyes (Invitrogen). Purification of labeled cDNA was carried out in accordance with the manufacturer's instructions with one modification: the QIAquick spin columns (Qiagen, Valencia, CA, USA) were used. The quality of labeling was tested using a nanoDrop spectrophotometer (Thermo Fisher Scientific). Only samples with dye/nucleotide ratios $> 200 \text{ pmol } \mu\text{g}^{-1}$ were used for hybridization.

Microarray hybridization

A custom microarray was designed and manufactured by CombiMatrix Corporation (Seattle, WA, USA) using the CustomArray 12K platform as described earlier (Kalyuzhnaya *et al.*, 2008). Briefly, array design was based on the composite genomic sequence of *M. mobilis* representing a few closely related strains (Kalyuzhnaya *et al.*, 2008), and oligonucleotide (35–40 bp in length) probes were designed to target 7195 specific genes and 2403 gene clusters (at 90% sequence similarity cutoff; Kalyuzhnaya *et al.*, 2008). With the genome size of *M. mobilis* predicted to be 2.5 Mb (Kalyuzhnaya *et al.*, 2008), on the average over five probes were present on the array to target every gene. However, not all of them were expected to be perfect matches. Hybridization experiments were carried out with two biological replicates for each set of conditions. In addition, two technical replicate experiments were carried out for each biological replicate from Experiments 3-I and 3-II. Arrays were re-hydrated in nuclease-free water at 65 °C for 10 min, and incubated in pre-hybridization buffer (4 × SSC, 1% SDS, $10 \mu\text{g ml}^{-1}$ salmon sperm DNA, 0.5% BSA) at 58 °C for 30 min. The hybridization mixture contained in 100 μl : 4 × SSC solution (0.06 M trisodium citrate and 0.6 M sodium chloride), 1% SDS, $10 \mu\text{g ml}^{-1}$ salmon sperm DNA, 20% formamide, and up to 4 μg (500 pmol dye) of labeled cDNA. Hybridizations were carried out in a rotisserie oven at 58 °C for 16 h. Arrays were washed with 2 × SSC, 0.1% SDS for 30 min at 58 °C, followed up by two additional washes in 1 × SSC, and 0.1 × SSC for 10 min each. Washed arrays were covered with the Imaging solution and the LifterSlip coverslip (CombiMatrix) and loaded into the Genepix 4000B microarray scanner (Axon Instruments, Union City, CA, USA). Data from image files were extracted using the CombiMatrix Microarray Imager Software (<https://webapps.combimatrix.com/software/microarrayimager/MIprogramHome.jsp>). Raw data were imported into the R program (The R Project for Statistical Computing, <http://www.r-project.org/>), transformed into the logarithmic scale (base 2) and normalized using the quantile method as implemented in the LIMMA package (Smyth, 2005). With the exception of Experiments 5 and 6, the significance

of differential expression between the two samples was determined using the empirical Bayes method (Smyth, 2004), to shrink the gene-wise sample variances toward a common value. The resulting statistics were corrected for multiple hypotheses testing using Benjamini and Hochberg false discovery rates (Benjamini and Hochberg, 1995), and significant changes in gene expression were identified at $P < 0.05$. These data were imported into the Microsoft Access Database format for further comparative analysis. For Experiments 5 and 6, the SAM software (Tusher *et al.*, 2001) was used to perform statistical analysis with the significance testing as above and with the false discovery rates capped at 5%. Annotations for the differentially expressed genes were extracted from the Integrated Microbial Genome and Metagenome (IMG/M) database (<http://img.jgi.doe.gov/cgi-bin/m/main.cgi>) and manually verified. Microarray data have been deposited in the NCBI.

Results and discussion

Experimental setup

The experimental setup (Figure 1) was designed to compare gene expression *in situ* (lake sediment, Experiments 3-I and 3-II) to gene expression in laboratory conditions (minimal medium with methylamine as a single energy/carbon source, Experiment 1), for a single strain of *M. mobilis*, strain JLW8, a bacterium isolated from this site (Kalyuzhnaya *et al.*, 2006) that has been shown to be significant in C_1 cycling (Kalyuzhnaya *et al.*, 2008, 2009). To further discriminate between the specific response to the *in situ* conditions and the methylamine-induced response, comparisons were made between the culture incubated in the sediment supplemented with methylamine (Experiment 2) and the culture in typical laboratory conditions (Experiment 1). As an extra control, we also used a culture incubated in Lake Washington water that is assumed to be very poor in nutrient content, to define trends in gene expression in response to starvation. In two separate experiments (Experiments 5 and 6) we assessed response of the natural population of *Methylotenera* strains inhabiting Lake Washington sediment to the addition of methylamine, mimicking a temporary increase in the availability of this model substrate.

Growth of *M. mobilis* in the incubation cassettes

Microscopic observation of the samples post incubation (Experiments 1–4) revealed that a single type of rod-shaped cells, typical of *M. mobilis* JLW8 (Kalyuzhnaya *et al.*, 2006), was present. Plating of serial dilutions of the samples on minimal medium supplemented with methylamine resulted in formation of a single type of colonies, typical of *M. mobilis* JLW8, whereas no growth was observed on TSA, LB or NA media (Kalyuzhnaya *et al.*, 2006). The restriction fragment length polymorphism profiles

of the 16S rRNA gene fragments from all samples were identical to the restriction fragment length polymorphism profile of the *M. mobilis* JLW8 16S rRNA gene. These findings show that the cultures planted into the incubation cassettes remained pure over the course of the experiment.

We measured the post-incubation OD_{600} of cells to determine whether the incubation conditions supported growth of *M. mobilis*. Of all samples, the samples incubated in minimal medium with methylamine showed the highest final OD (0.9 ± 0.08 ; triple compared with the start $OD_{600} = 0.3 \pm 0.02$), whereas the OD of the cell cultures incubated in the lake water slightly decreased (0.25 ± 0.02). The final OD of cultures incubated in the top layer of sediment cores was 0.68 ± 0.05 , and a similar OD (0.7 ± 0.05) was observed for samples exposed to the sediment supplemented with methylamine, suggesting that growth of *M. mobilis* in sediment was not likely to be limited by the carbon/energy source.

Insights into the lifestyle of *M. mobilis* JLW8 *in situ*, as judged by differential gene expression

Two different lake sediment cores were used to mimic *in situ* conditions for *M. mobilis* cells (biological replicates; Figure 1). Expression profiles of *M. mobilis* cells incubated in these conditions were compared with the profile of cells grown in minimal medium supplemented with methylamine (Experiment 1). To determine general trends of gene expression *in situ* versus laboratory conditions, we looked for genes with significant expression changes (> 2 -fold; $P < 0.05$) in both biological replicates. A strong correlation was observed (0.95) between probe ratios of genes with significantly different expression between Experiments 1 and 3. This suggests that the environmental factors influencing gene expression in *M. mobilis* were uniform in both sediment cores. Expression of a total of 295 genes was found significantly changed *in situ*. Out of these, 164 genes were downregulated and 131 genes were upregulated (Supplementary Table 1; Figure 2a). Comparing gene expression in cells exposed to methylamine-supplemented sediment to methylamine-grown cells identified a total of 236 genes with altered expression (> 2 -fold; $P < 0.05$), among which 123 genes were downregulated, and 113 were upregulated (Supplementary Table 1; Figure 2b). In cells incubated with lake water, downregulation was observed for the majority of the genes, including genes encoding cell replication, transcription and translation. A total of 1194 genes were downregulated more than twofold, whereas no increase in expression above twofold was observed for any gene. The gene-expression profile and the reduction in cell density over the incubation period in this experiment indicated a profound metabolic shut-down, likely as a manifestation of nutrient deficiency. This latter data set was not included in further comparisons.

General cell functions

Analysis of the set of genes showing significant difference in expression in both *in situ*-mimicking

experiments (the unsupplemented sediment incubations, Experiments 3-I and 3-II, and incubations in the sediment supplemented with methylamine,

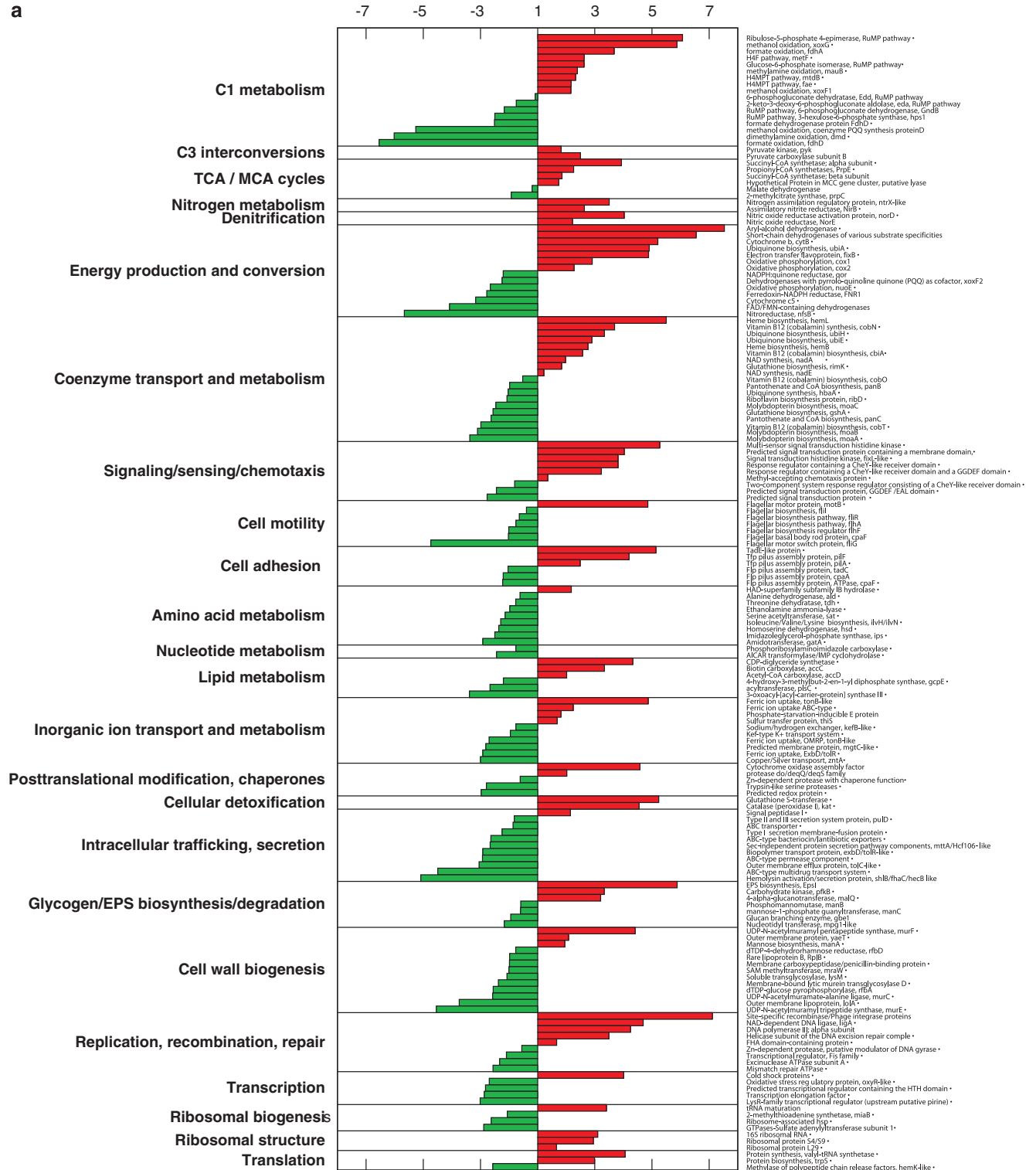


Figure 2 Comparison of the expression profiles of *M. mobilis* cells incubated in (a) lake sediment cores (Experiment 3) and (b) in lake sediment supplemented with 10 mM methylamine (Experiment 2) to the expression profile of cells incubated in laboratory conditions (minimal medium supplemented with methylamine, Experiment 1). Each bar represents fold change in fluorescence intensity of a gene probe. Data from multiple probes were averaged. Differentially expressed genes with *P*-values <0.05 are noted by asterisks. Only genes relevant to the discussion are shown. For the complete set of differentially expressed genes see Supplementary Table 1.

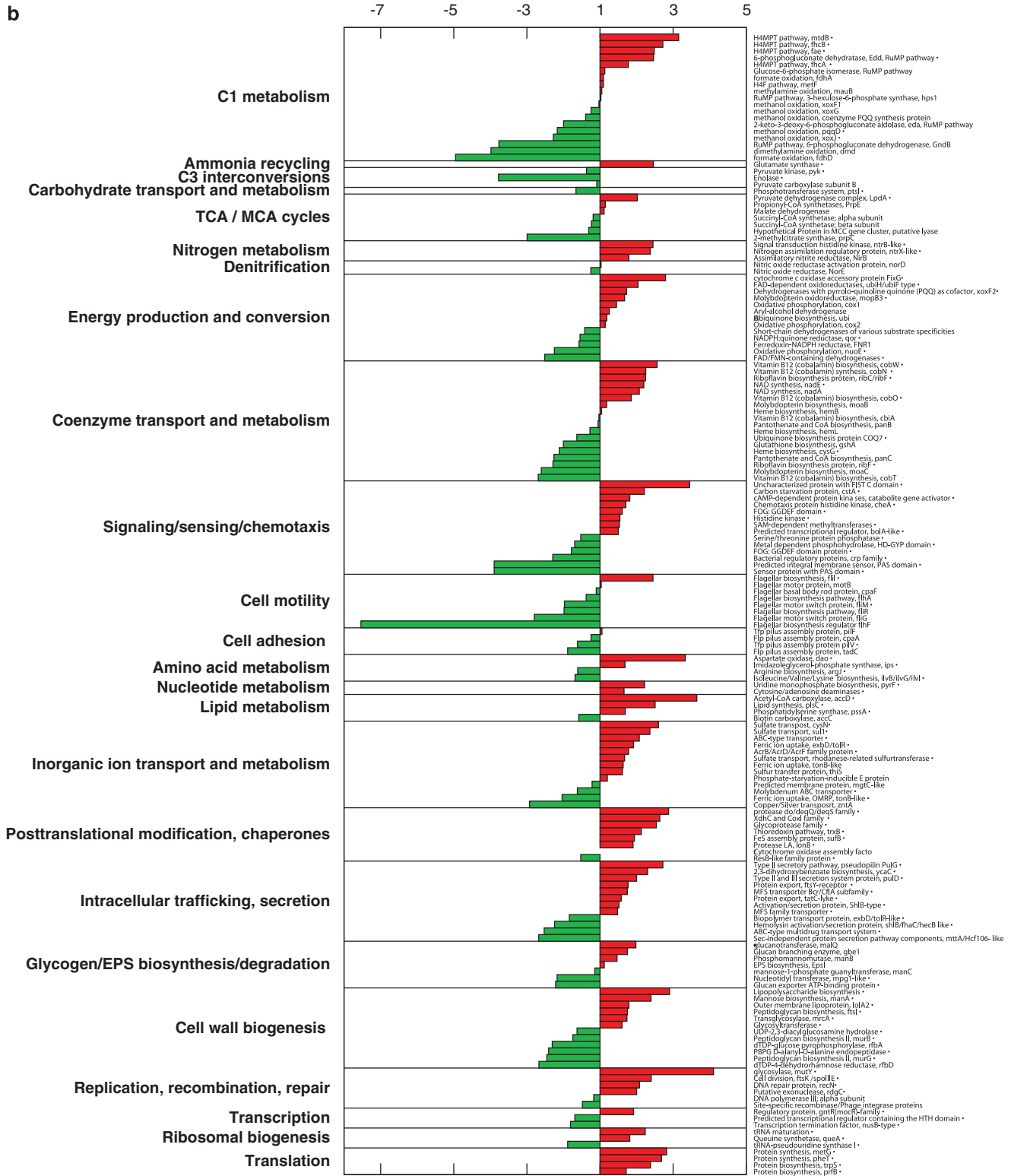


Figure 2 Continued.

Experiment 2) compared to laboratory conditions (Experiment 1) identified a total of 107 genes displaying a similar regulation pattern. As this regulation pattern was not influenced by the presence of the carbon/energy source (methyla-

mine), these genes likely represent metabolic systems and pathways regulated by sediment conditions, rather than carbon/energy source. On the basis of function prediction these genes could be divided into the following major categories: with

diminished expression (90 genes): cell division and replication (*polA*, *mutS*), cell wall biogenesis, cell motility (flagellum biosynthesis), intracellular trafficking and secretions (ABC-type transport systems, efflux pumps, secretion proteins, *sec*-independent translocation proteins), and exopolysaccharide (EPS) biosynthesis; with elevated expression (17 genes): ribosomal biogenesis, maturation and structure (*rumA*), DNA repair (*mutY*), protein biosynthesis, nitrogen assimilation, sulfur uptake, sensing (*cheY*), and vitamin biosynthesis. Of the genes known to be involved in C_1 metabolism, expression of genes for oxidation of formaldehyde (*fae*, *mtdB*, *fhc*, *fdh*) was elevated in both Experiments 2 and 3 compared with Experiment 1 (laboratory conditions). This is consistent with the presence of alternative sources of carbon in the sediment whose oxidation must produce formaldehyde as an intermediate.

Activation of the protein biosynthesis machinery could be seen as an adaptation to environmental conditions with limited and heterogeneous sources of carbon that would enable successful competition for nutrients. Activation of vitamin biosynthesis pathways (heme, ubiquinone, NAD, cobalamin) as well as activation of iron and sulfur uptake systems (Figure 2a and b) would suggest that primary oxidation and energy generation systems active *in situ* would require specific cofactors. Activation of the multiple cytochrome systems (*cytbb*, *norE*, *cytcb*, *xoxG*) and of the oxidative phosphorylation pathway (*cox1*, *cox2*) in cells exposed to lake sediment could be seen as another example of adaptation to limiting and changing substrate availability.

*C*₁ metabolism

The genetics of C_1 metabolism in *M. mobilis* is well understood. An entire suite of genes encoding enzymes for methylamine oxidation, formaldehyde oxidation and assimilation, and for formate oxidation have been identified in the composite genome of *M. mobilis* (Kalyuzhnaya et al., 2008), and the proteomic analysis of cells grown on methylamine confirmed expression of most these genes (Bosch et al., 2009). As in other representatives of Methylophilaceae, the RuMP cycle seems to have a key role in both assimilation and dissimilation of formaldehyde (cyclic formaldehyde oxidation), whereas the H_4 MPT-linked pathway (linear formaldehyde oxidation) seems to have a secondary role (Chistoserdova et al., 2007; Bosch et al., 2009). In cells exposed to the *in situ* conditions, we observed a major switch for the linear versus cyclic oxidation of formaldehyde: the H_4 MPT-linked pathway (*mtdB*, *fae*, *fdhA*) seemed to be upregulated, whereas the entry into the RuMP cycle through hexulose phosphate synthase (*hps1*) seemed to be downregulated (Supplementary Table 1; Figure 2a). In addition to the H_4 MPT pathway genes, genes for an alternative

C_1 transfer pathway, the one linked to tetrahydrofolate (H_4F , *metF*, *purU*) was upregulated. The function of this pathway in methylotrophy by *Methylophilaceae* is not fully understood, but our data suggest that this pathway may serve as an additional route for C_1 oxidation. In the RuMP cycle, the partitioning of flux between oxidation (through 6-phosphogluconate dehydrogenase; *gndB*) and assimilation (through 6-phosphogluconate dehydratase; *edd*) was also altered, with the former being downregulated and the latter being upregulated. The branch of the RuMP cycle involved in regeneration of ribulose-5-phosphate also seemed to be upregulated. It is noteworthy that the switch down of a major NADPH-producing reaction (catalyzed by GndB) correlated with downregulation of a major electron acceptor from NADPH, NADPH:quinone reductase (Qor). Overall, our data suggest that, in contrast to the evidence from laboratory-grown cultures, cells growing *in situ* operate the RuMP pathway mostly in the assimilatory mode and use linear pathway(s) rather than the cyclic pathway for formaldehyde oxidation (Figure 3).

*C*₁ oxidation linked to denitrification

The recently conducted stable isotope probing experiments with Lake Washington sediment have implicated *Methylotenera*-like species in playing a role in methanol-linked denitrification, and this ability has been further confirmed by growth experiments with *M. mobilis* JLW8 as well as by measuring its denitrification activity (Kalyuzhnaya et al., 2009). Although the exact mechanism of the linkage between denitrification and methanol oxidation remains unknown, one protein has been found over-expressed in denitrifying conditions, encoded by the *xoxF* gene, a homolog of the *mxoA* gene encoding the large subunit of methanol dehydrogenase (Kalyuzhnaya et al., 2009). We found that *xoxF* and an associated gene *xoxG* (encoding a cytochrome) were upregulated in cells exposed to the *in situ* conditions (Figure 2a). However, in the sediment supplemented with methylamine, expression of these genes was significantly repressed (Figure 2b). These data further support the involvement of *xoxFG* in a metabolism alternative to methylamine oxidation.

No significant changes in the expression of genes for a periplasmic nitrate reductase, or assimilatory or dissimilatory nitrite reductases were observed under any conditions tested. However, expression of the heme/copper-type cytochrome *c* oxidase (*norE*) associated with nitric oxide reductase as well as of the nitric oxide reductase activating protein (*norD*) was elevated in cells exposed to the *in situ* conditions, suggesting activation of the denitrification pathway. Regulation of the denitrification pathway by microaerobic conditions is unlikely in this case as in the methylamine-supplemented sediment samples no changes in the expression of

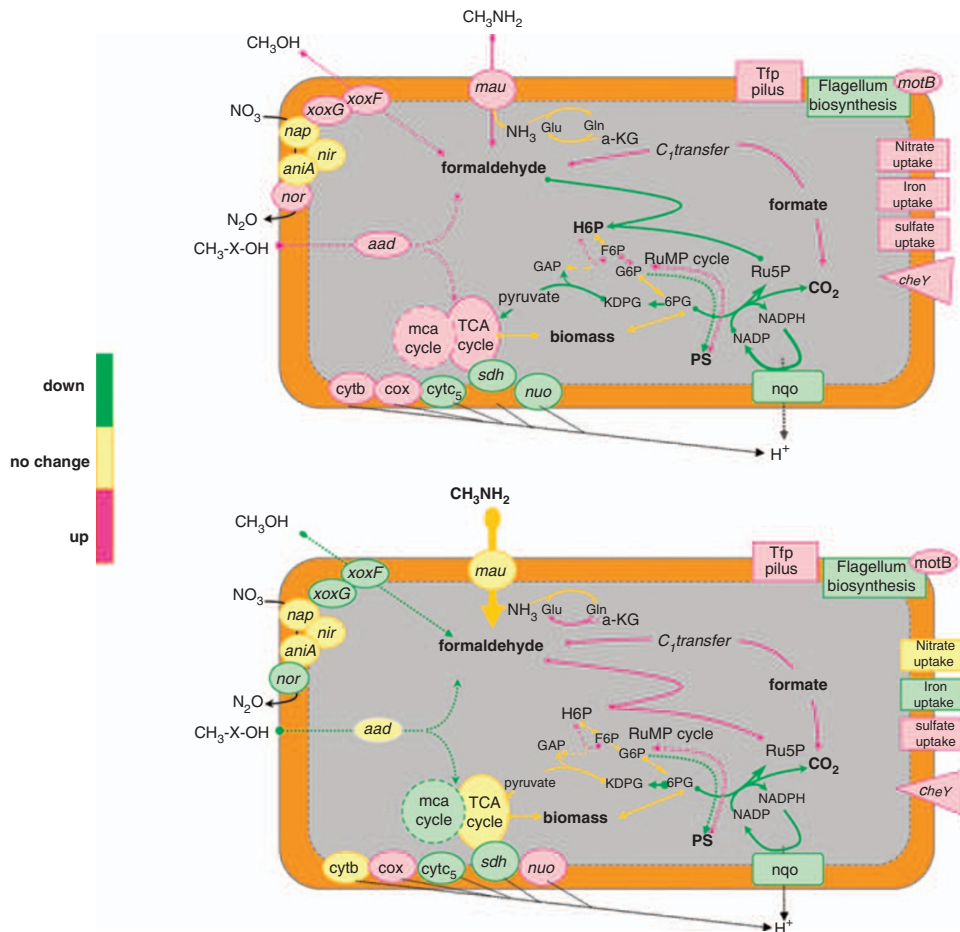


Figure 3 Major metabolic pathways reconstructed from the transcriptomic profiles of *M. mobilis* cells (top) incubated in lake sediment cores and (bottom) in lake sediment supplemented with 10 mM methylamine, in comparison with the profile of cells incubated in laboratory conditions (minimal medium supplemented with methylamine). Connections shown in red indicate upregulation for at least one gene in the pathway, connections shown in yellow indicate no change in expression and connections shown in green indicate downregulation for at least one gene in the pathway. Dashed lines indicate hypothetical pathways proposed based on the genomic and transcriptomic data. Enzyme descriptions can be found in Supplementary Table 1. H6P, hexulose 6-phosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; Ru5P, ribulose 5-phosphate; GAP, glyceraldehyde phosphate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; Glu, glutamine; Gln, glutamate; α -KG, α -ketoglutarate; PS, polysaccharide.

norE were observed, and expression of *norD* was slightly downregulated. Thus, the activation of denitrification pathway *in situ*, in the absence of methylamine, seems to signal a switch into a different metabolic mode, possibly involving endogenous methanol.

Other sources of carbon

Analysis of the composite genome of *M. mobilis* has revealed the presence of the genes for a complete MCA cycle, a feature that differentiates *M. mobilis* from the closely related *M. flagellatus* (Kalyuzhnaya et al., 2008), and expression of this pathway has been demonstrated earlier through proteomic analysis of methylamine-grown cells (Bosch et al., 2009). The confirmed function of this pathway (in other organisms) is in propionate and levulinic acid degradation (Vagelos, 1959; Brämer and Steinbüchel, 2001). We tested for growth of *M. mobilis* on propionate,

methyl-propionate, MCA or levulinic acid, as described in ‘Materials and methods’, but obtained negative results. The lack of growth on the intermediates of the pathway may be due to the inefficient transport of these compounds. Previously, we hypothesized that the pathway may be involved in degradation of other, yet unidentified substrates, likely through de-methylation, whose degradation results in propionate (Kalyuzhnaya et al., 2008). Indeed, we found that *prpE* and another gene in the MCA cycle gene cluster, encoding an unknown lyase were upregulated at the *in situ* conditions, suggesting that the pathway may be specifically activated in these conditions. In addition to genes of the MCA cycle, transcription of genes involved in reactions interconverting C₃ compounds (pyruvate kinase, pyruvate carboxylase) was elevated. These reactions are likely used as an additional source of oxaloacetate in the MCA cycle.

One of the most highly upregulated genes *in situ* was an oxidoreductase related to aryl-alcohol dehydrogenases (gene ID 2006719259). This gene belongs to the aldo-keto reductase family that includes related monomeric NADPH-dependent oxidoreductases. The specific function of aryl-alcohol dehydrogenases has not yet been established. An activity has been demonstrated in reduction of the carboxyl groups of aromatic acids resulting in the respective aldehydes (Gross and Zenk, 1969). The regulation pattern of this gene prompted us to test for growth of *M. mobilis* on aromatic compounds as sources of carbon and energy. The aromatic compounds tested as substrates are listed in the 'Materials and methods' section. Of all the substrates, slow growth (doubling time 172 h) was observed only on 2-methoxyphenol, a compound that serves as a model lignin-derived compound (Dorrestijn and Mulder, 1999). Controls with no added substrate showed no growth. The maximum OD reached by the culture (OD₆₀₀) was 0.26, suggesting that the pure culture of *M. mobilis* was poorly equipped to grow on this substrate. However, *in situ*, as part of a community, *M. mobilis* may be more successful in this metabolism. Despite the slow growth, this finding is significant, showing that bacteria designated as obligate methylotrophs based on standard laboratory growth tests indeed are capable of multicarbon metabolism, and this type of metabolism may be an important part of their *in situ* activity.

Mobility and adhesion functions

Motility has an essential role in physiological adaptation to environmental conditions. A motile cell can quickly reach a favorable niche in response to external stimuli and thus can successfully compete for a nutrient. However, synthesis and maintenance of the motility systems are energetically costly for the cell, and the motility functions are known to be tightly regulated by the availability of essential nutrients (Zhao et al., 2007; Christen et al., 2007). *M. mobilis* cells have been shown to be motile when grown in standard laboratory conditions (Kalyuzhnaya et al., 2006). Genes for flagellum biosynthesis, motility and accessory genes have been identified in the composite genome of *M. mobilis* (Kalyuzhnaya et al., 2008). In this study, we observed that genes encoding motility functions (flagellin, capping protein, basal body, hook, export functions) were downregulated in the *in situ* conditions (Figure 2a). The flagellar motor protein gene (*motB*) was the only gene that was slightly upregulated. The expression of *motB* did not change significantly in the presence of methylamine in the sediment. However, expression of *fliL* was activated in the presence of methylamine (Figure 2b). Although the product of *fliL* is essential for flagellar rotation, it is not considered a part of the hierarchy of the flagellum structure (Jenal et al., 1994). Instead, it has been suggested to have a role in controlling the

switch between swimming and surface-associated lifestyles (Christen et al., 2007). Upregulation of *fliL* in the presence of methylamine thus may reflect a mechanism of activation of the flagellum machinery in response to the availability of a carbon source. Indeed, in these conditions (Experiment 3), expression of other flagellum functions (*fliL*, *fliG*, *fliA*, *cpaF*) was also upregulated (Supplementary Table 1).

Analysis of the composite genome of *M. mobilis* has revealed the presence of a type IV pilus assembly and secretion functions (Kalyuzhnaya et al., 2008). This type of pili is important in a number of bacterial processes, such as flagellum-independent 'twitching' motility, DNA uptake, adherence to surfaces, and biofilm formation (Graupner et al., 2000; Mattick, 2002; Tomich et al., 2007; Boyd et al., 2008). It has been speculated before that in *M. mobilis*, this pilus may serve as a specific phage receptor (Kalyuzhnaya et al., 2008). The expression pattern observed for the pilus biogenesis genes was reversed compared to the flagellum functions expression: it was upregulated in *in situ* conditions (Figure 2a), whereas no significant change was observed in the presence of methylamine. This result suggests that in conditions of scarce supply of nutrients, cells of *M. mobilis* may tend to switch from a planktonic lifestyle to the attached state, using pili for attachment, biofilm formation and/or twitching motility.

Environmental transcriptomics of *M. mobilis*

M. mobilis LW8 is so far the only cultivated representative of a large group of *Methylotenera* species that are part of the active C₁-using population in Lake Washington sediment (Kalyuzhnaya et al., 2008). We were thus interested to test for gene expression directly in the sediment sample, and compare that to a sediment community stimulated with methylamine as a model compound. For these experiments, RNA was isolated directly from the sediment samples. In these preparations, the *Methylotenera* RNA and the resulting *Methylotenera* cDNA were only a (minor) part of the communal RNA/cDNA. Hybridization with these preparations resulted in much higher background levels compared to hybridization with cDNA of pure cultures of *M. mobilis* (see above). Therefore, we searched for genes with elevated expression in response to methylamine using a high-stringency analysis algorithm, Significance Analysis of Microarrays (Tusher et al., 2001). A total of 38 genes showed significantly elevated expression (>2.5-fold; Supplementary Table 2), the most highly expressed being the 16S and 23S rRNA genes of *M. mobilis* (159- and 30-fold change, respectively), thus confirming that indeed *Methylotenera* species are the most active responders to methylamine in Lake Washington sediment. Other genes with elevated expression included potential methylotrophy genes (PQQ biosynthesis, NAD-dependent formate dehydrogenase, putative

dimethylamine dehydrogenase, anaerobic molybdopterin oxidoreductase) and genes of unknown functions. Of the latter, the gene for an uncharacterized aldehyde dehydrogenase was one of the most highly expressed genes (38-fold change). The product of this gene was previously identified with high spectral counts in methylamine-grown cells of *M. mobilis* JLW8 (Bosch *et al.*, 2009). However, the potential role of this protein in C₁ metabolism remains unknown. Among other functions stimulated by the addition of methylamine, over-expression of a phage protein (7-fold change) should be mentioned. Sequences of novel phages have been assembled as part of the methylamine microcosm metagenomic sequence, and these phages were implicated in having a role in controlling the population dynamics of *Methylotenera* (Kalyuzhnaya *et al.*, 2008). The increased abundance of phage RNA in the methylamine-stimulated community corroborates the proposed association between the phage and the methylamine-using *Methylotenera*. Other functions stimulated by the presence of methylamine included replication, cell wall biogenesis, coenzyme metabolism and adherence/twitching motility functions (Supplementary Table 2). In addition, a number of hypothetical genes showed elevated expression.

Concluding remarks

Currently, predictions for the environmental function of a specific microbial species, including methylotrophic species, are almost entirely based on the knowledge of the physiology of this or a related species in laboratory conditions. However, the function and the lifestyle of a specific microbe is a subject to many factors in a natural environment that are also prone to changes, which sometimes occur rapidly. In this work, we used transcriptomics to characterize physiology of a typical methylotroph, *M. mobilis*, in its native environment, lake sediment. We noted significant changes in gene expression in this organism in response to the *in situ* conditions, compared to laboratory conditions, suggesting that trends such as redirection of carbon flow between alternative formaldehyde oxidation pathways, activation of an additional assimilatory pathway, the MCA cycle, and switching from flagellum-enabled motility to twitching motility/adherence may be taking place. This work also further shifts the paradigm of obligate methylotrophy toward a scenario in which multi-carbon compounds may be metabolized or co-metabolized along with methylated compounds. Overall, this study offers a method by which other organisms can be studied in their natural environments.

Acknowledgements

This work was funded by the National Science Foundation as part of the Microbial Observatories Program (MCB-0131957). We are grateful to the crew of R/V *Clifford Barnes* for help with sample acquisition.

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Supplementary Information accompanies the paper on the ISME Journal website (<http://www.nature.com/ismej>)