Formaldehyde-Responsive Proteins TtmR and EfgA Reveal a Trade-off between Formaldehyde Resistance and Efficient Transition to Methyлотrophy in *Methyлотrubrum extorquens*

Jannell V. Bazurto,^{a,b,c,d,e,f} Eric L. Bruger,^{a,b,c} Jessica A. Lee,^{a,b,c,g} Leah B. Lambert,^a Christopher J. Marx^{a,b,c}

^aDepartment of Biological Sciences, University of Idaho, Moscow, Idaho, USA

^bInstitute for Modeling Collaboration and Innovation, University of Idaho, Moscow, Idaho, USA

^cInstitute for Bioinformatics and Evolutionary Studies, University of Idaho, Moscow, Idaho, USA

^dDepartment of Plant and Microbial Biology, University of Minnesota, Twin Cities, Minnesota, USA

^eMicrobial and Plant Genomics Institute, University of Minnesota, Twin Cities, Minnesota, USA

^fBiotechnology Institute, University of Minnesota, Twin Cities, Minnesota, USA

^gNASA Ames Research Center, Moffett Field, California, USA

ABSTRACT For bacteria to thrive, they must be well adapted to their environmental niche, which may involve specialized metabolism, timely adaptation to shifting environments, and/or the ability to mitigate numerous stressors. These attributes are highly dependent on cellular machinery that can sense both the external and intracellular environment. *Methyлотrubrum extorquens* is an extensively studied facultative methyлотroph, an organism that can use single-carbon compounds as its sole source of carbon and energy. In methyлотrophic metabolism, carbon flows through formaldehyde as a central metabolite; thus, formaldehyde is both an obligate metabolite and a metabolic stressor. Via the one-carbon dissimilation pathway, free formaldehyde is rapidly incorporated by formaldehyde activating enzyme (Fae), which is constitutively expressed at high levels. In the presence of elevated formaldehyde levels, a recently identified formaldehyde-sensing protein, EfgA, induces growth arrest. Here, we describe TtmR, a formaldehyde-responsive transcription factor that, like EfgA, modulates formaldehyde resistance. TtmR is a member of the MarR family of transcription factors and impacts the expression of 75 genes distributed throughout the genome, of which many encode transcription factors and/or are involved in stress response, including *efgA*. Notably, when *M. extorquens* is adapting its metabolic network during the transition to methyлотrophy, *efgA* and *ttmR* mutants experience an imbalance in formaldehyde production and a notable growth delay. Although methyлотrophy necessitates that *M. extorquens* maintains a relatively high level of formaldehyde tolerance, this work reveals a trade-off between formaldehyde resistance and the efficient transition to methyлотrophic growth and suggests that TtmR and EfgA play a pivotal role in maintaining this balance.

IMPORTANCE All organisms produce formaldehyde as a by-product of enzymatic reactions and as a degradation product of metabolites. The ubiquity of formaldehyde in cellular biology suggests that all organisms have evolved mechanisms of mitigating formaldehyde toxicity. However, formaldehyde sensing is poorly described, and the prevention of formaldehyde-induced damage is understood primarily in the context of detoxification. Here, we used an organism that is regularly exposed to elevated intracellular formaldehyde concentrations through high-flux one-carbon utilization pathways to gain insight into the role of formaldehyde-responsive proteins that modulate formaldehyde resistance. Using a combination of genetic and transcriptomic analyses, we identified dozens of genes putatively involved in formaldehyde resistance, determined the relationship between two different formaldehyde response systems, and

Citation Bazurto JV, Bruger EL, Lee JA, Lambert LB, Marx CJ. 2021. Formaldehyde-responsive proteins TtmR and EfgA reveal a trade-off between formaldehyde resistance and efficient transition to methyлотrophy in *Methyлотrubrum extorquens*. J Bacteriol 203:e00589-20. <https://doi.org/10.1128/JB.00589-20>.

Editor Anke Becker, Philipps University Marburg

Copyright © 2021 American Society for Microbiology. All Rights Reserved.

Address correspondence to Christopher J. Marx, cm Marx@uidaho.edu.

Received 29 October 2020

Accepted 30 January 2021

Accepted manuscript posted online

22 February 2021

Published 8 April 2021

identified an inherent trade-off between formaldehyde resistance and optimal transition to methylotrophic metabolism.

KEYWORDS methylotrophy, formaldehyde, stress response, adaptation, trade-offs, enhanced formaldehyde growth protein A (EfgA), MarR transcription factor

Methylotrophy, a trait found in all domains of life, is the unique metabolic ability of organisms to use one- or multicarbon compounds lacking carbon-carbon bonds as sole sources of carbon and energy. Growth substrates for methylotrophs include compounds such as methane (methanotrophs), methanol, and methylamines (mono-, di-, and tri-) and methylated sulfur species. A key feature of methylotrophic metabolism is that each carbon of the growth substrate flows through the potent toxin formaldehyde as a central intermediate. Formaldehyde is harmful to all cell types because of its reactivity with electrophiles, such as free amines and thiols. It can form adducts with or cross-link a number of biological molecules, such as DNA and proteins. Despite the centrality of formaldehyde in methylotrophic metabolism, until very recently, formaldehyde-specific stress responses had not been described in methylotrophs.

In *Methylobacterium* (formerly *Methylobacterium*) *extorquens* AM1, the most extensively studied facultative methylotroph, the intracellular formaldehyde concentration is estimated at ~1 mM during growth on methanol (1). Methanol is directly oxidized to formaldehyde in the periplasm by methanol dehydrogenase (MDH). Free endogenous formaldehyde is kept relatively low in the cytoplasm by formaldehyde activating enzyme (Fae), which condenses formaldehyde with the C₁ carrier dephosphotetrahydromethanopterin (dH₄MPT); this is the first of three reactions in the pterin-dependent dissimilatory pathway that oxidizes formaldehyde to formate (2–4). Previous work has demonstrated that strains defective in formaldehyde dissimilation by disruption of pathway enzymes (*fae*) or synthesis of the dH₄MPT C₁ carrier (*mptG*) are sensitive to methanol (4). Formate is the branchpoint metabolite and can be further oxidized to CO₂ for energy production or shunted toward a tetrahydrofolate-dependent assimilation pathway for ultimate incorporation into biomass via the serine cycle (5, 6).

Very recently, experimental evolution of *M. extorquens* PA1 for growth on formaldehyde as a sole source of carbon and energy revealed multiple loci that could increase formaldehyde resistance (7). Although dissimilation by the dH₄MPT pathway is the primary mechanism for removing free formaldehyde from the cytoplasm (3, 4), none of the beneficial mutations were in these genes. Instead, this work found that single mutations in one of three genetic loci (*efgA*, *def*, or *Mext_0925*) could independently confer the ability to use formaldehyde as a sole source of carbon and energy. The most frequent class of mutations (nearly 80%) were loss-of-function mutations in enhanced formaldehyde growth protein A (EfgA), a protein that directly binds formaldehyde and halts translation in the presence of excess formaldehyde by an unknown mechanism. Although strains lacking EfgA can grow in the presence of higher formaldehyde concentrations than can the wild type (WT), the absence of EfgA increases methanol sensitivity in strains that are vulnerable to formaldehyde stress (*fae* and *mptG*), suggesting that the physiological role of EfgA is to decrease formaldehyde-induced stress. Peptide deformylase (PDF; encoded by *def*), is a ribosomally associated enzyme that is essential for translation and involved in protein quality control (8). Selection for mutations in *def* provided further evidence for the importance of translational regulation in response to formaldehyde stress.

Mext_0925 is a homolog of the MarR transcription factor (7). This family of regulators is found in bacteria and archaea and was originally characterized in *Escherichia coli* (9–12). In *E. coli*, MarR is encoded by the multiple antibiotic resistance (*mar*) operon. It is a ligand-sensing repressor that binds the operator of the *marRAB* operon in the absence of ligand and is thus self-regulating (13, 14). A number of structurally distinct compounds, such as tetracycline, chloramphenicol, and salicylate, can bind MarR and induce conformational changes that lead to DNA release (15). Additionally, the *marRAB*

operon can be activated by aromatic amino acid metabolites directly and indirectly (16). Subsequently, *marRAB* expression gives rise to the transcription factor MarA, a member of the AraC/XylS family, which in turn upregulates dozens of genes that contribute to resistance to multiple antibiotics, oxidative stress, and organic solvent stress (17–19). Broader examination of MarR homologs in a variety of organisms show that homologs (i) can be repressors, activators, or both; (ii) have highly variable regulon sizes; and (iii) control a variety of cellular processes involved in various stress responses and metabolic pathways (15).

In *Bacillus subtilis*, the MarR homolog HxlR has a role in formaldehyde stress response (20, 21). *hxlR* is divergently transcribed from the *hxlAB* operon that HxlR positively regulates. The *hxlAB* operon encodes two key proteins of the ribulose monophosphate pathway of formaldehyde detoxification and is induced by exogenous formaldehyde (20, 22). *In vitro*, formaldehyde does not impact binding of HxlR, and as is the case with several MarR homologs, the mechanism of activation has remained elusive. In some instances, however, the mechanism of activation of MarR homologs is clear. For example, in organisms such as *Acinetobacter baylyi* and *Streptomyces coelicolor*, where MarR homologs regulate operons encoding enzymes required for the catabolism of aromatic lignin-derivatives such as ferulate and protocatechuate, respectively, ligand binding leads to the release of cognate DNA and the operon is derepressed (15, 23–25). This leads to expression of the relevant enzymes, and the ligand/carbon substrates are utilized.

Here, we probed the physiological role of Mext_0925, which is implicated in modulating formaldehyde resistance. Unlike many described MarR homologs, Mext_0925 is not in an operon or divergently transcribed from an operon; thus, there is no genomic context for discerning its regulon. In the absence of obvious genetic associations, we opted to use transcriptomic analyses to identify differentially expressed genes in a Δ Mext_0925 mutant. By coupling this approach with *in vivo* genetic studies, we found that Mext_0925 is specifically responsive to formaldehyde stress. Our results define the relationship between Mext_0925 and EfgA, the only characterized formaldehyde stress response system in *M. extorquens*, and demonstrate that the ability of *M. extorquens* to respond to formaldehyde stress at the transcriptional and translational levels is critical for the optimal transition to methylotrophy. Based on our findings, we conclude that Mext_0925 represents a second formaldehyde-specific response system in *M. extorquens* and suggest the name TtmR for a regulator of the transition to methylotrophy.

RESULTS

Loss of a MarR-family regulator confers formaldehyde resistance. To test whether the previously identified *ttmR*^{EV0} allele (7) is a loss-of-function allele, a precise deletion of the coding region *ttmR* was constructed by allelic exchange in the wild type, and its growth was characterized. The 1-base-pair deletion in *ttmR*^{EV0} was in the open reading frame; the frameshift at nucleotide (nt) 140/588 yielded a truncated protein with 73 amino acids, of which only 46 were wild type. The evolved isolate with *ttmR*^{EV0} and the Δ *ttmR* strain could each use 4 mM formaldehyde as a sole carbon source, reaching a modest absorbance of $\sim$ 0.10 to 0.13 when 4 mM formaldehyde was provided. At 6 mM formaldehyde, both the *ttmR*^{EV0} and Δ *ttmR* mutants failed to grow after 48 h, unlike the Δ *efgA* strain (Fig. 1A and 2A). In both the Δ *ttmR* and Δ *efgA* mutants, the maximum cell densities often trended upward as formaldehyde increased until formaldehyde toxicity became inhibitory and prevented growth. For the Δ *ttmR* mutant, growth in the presence of 4 but not 6 mM formaldehyde was also seen when formaldehyde was added to an additional carbon source, either succinate or methanol (Figure 1B and C). In the absence of exogenous formaldehyde (i.e., during growth on succinate or methanol alone), however, the Δ *ttmR* strain did not display any differences in lag time, growth rate, or maximum cell density compared with wild type (Fig. 2B and C; see Table S2 in the supplemental material). Phenotypic comparison of *ttmR* and Δ *efgA* mutants demonstrate that although the Δ *efgA* mutant was more formaldehyde

FIG 1 Formaldehyde-evolved isolates have increased formaldehyde resistance. Wild-type (CM2730) and mutant strains harboring loss-of-function mutations in Δ *efgA* (CM3745) or *ttmR^{EVO}* (CM3919) were grown in liquid MP medium with 0, 2, 4, 6, 8, or 10 mM exogenous formaldehyde. Maximum cell densities are shown when formaldehyde was provided as a sole source of carbon and energy (A) or as a stressor when methanol (B) or succinate (C) was provided as the primary carbon source. Error bars represent the standard error of the mean of biological replicates.

resistant, the mutations are comparable in that they (i) allowed formaldehyde growth, (ii) did not impact growth in the absence of formaldehyde stress, and (iii) conferred formaldehyde resistance during the consumption of alternative carbon sources (Fig. 1 and 2) (7).

TtmR is not involved in a general stress response. The exact nature of formaldehyde cytotoxicity in *M. extorquens* is unknown; however, in various organisms, previous work has identified numerous forms of formaldehyde-induced DNA damage (26, 27), and more recent work has implicated formaldehyde-induced protein damage in human cell cytotoxicity (28). In considering possible mechanisms of increased formaldehyde resistance, we recognized that cellular processes that are likely to mitigate formaldehyde-induced damage, such as DNA repair and protein quality control, might

FIG 2 Deletion of *ttmR* recapitulates increased formaldehyde resistance. Wild type (CM2730) and the constructed Δ *ttmR* mutant (CM4732) were grown in liquid MP medium containing 0, 4, or 6 mM exogenous formaldehyde. Absorbance up to 48 hours was measured to assess growth when formaldehyde was provided as a sole source of carbon and energy (A) or as a stressor when methanol (B) or succinate (C) was provided as the primary carbon source. Error bars represent the standard error of the mean of biological replicates.

be involved in generalized stress responses or formaldehyde-specific stress responses. To address whether the loss of TtmR led to a formaldehyde-specific response, the *ttmR^{EVO}* strain was exposed to a variety of other stressors. Specifically, we chose stressors that addressed whether the *ttmR^{EVO}* mutant might (i) be broadly resistant to aldehydes, (ii) confer multidrug resistance (as a homolog of MarR), or (iii) mediate a generalized stress response.

Growth assays in media supplemented with various C₂ aldehydes (glyoxal and acetaldehyde), as well as a few other aldehydes (butyraldehyde, propionaldehyde, and glutaraldehyde) representing potential metabolic intermediates, indicated TtmR appears to be specific to formaldehyde stress. In both succinate- and methanol-based media, the *ttmR^{EVO}* mutant showed comparable defects in growth to WT other than a very subtle growth improvement when either glyoxal or acetaldehyde was present (see Fig. S1 in the supplemental material).

To evaluate antibiotic resistance, disk diffusion assays with different antibiotics were performed with WT or Δ *ttmR* mutant soft agar overlays. The wild type was resistant to 9 of the 14 antibiotics tested (i.e., no zone of inhibition) (see Table S3 in the supplemental material). Of the five remaining antibiotics, the Δ *ttmR* mutant did not display any increased resistance (i.e., smaller zone), allowing us to conclude that although there may be differences in specific antibiotic resistances, there was no broadly acquired antibiotic resistance (Table S3).

Finally, the *ttmR^{EVO}* strain was screened for its increased resistance to heat shock (55°C, treated 24 h after entry into stationary phase in liquid medium), oxidative stress (H₂O₂, soft agar overlays), and alcohol stress (ethanol, in solid medium). The *ttmR^{EVO}* mutant strain behaved similar to wild type when exposed to each of the stressors, other than an increase in survival during heat shock, which may indicate an elevated protein stress response (Table S3). Collectively, these data suggest that TtmR is not a general stress response protein but rather its activity is specific to modulating stress induced by formaldehyde. However, it is also possible that TtmR has a more general role in protein stress that would be revealed under different experimental conditions.

TtmR modulates the expression of genes involved in an array of cellular functions.

To understand the impact of TtmR on the transcriptome, we performed RNA sequencing analysis on wild type and the Δ *ttmR* mutant during early exponential growth (optical density [OD] 0.2) in minimal medium with 15 mM methanol. Specifically, we aimed to identify differentially expressed genes (DEGs) that would explain the increased resistance to formaldehyde.

A pairwise comparison of log-transformed counts of wild type and the Δ *ttmR* mutant was performed, and DEGs were identified by imposing a 2-fold change cutoff ($\log_2$ fold change [$\log_2$ FC], >1.0) with an adjusted *P* value (*P*_{adj}) less than 0.05. In total, we identified 75 DEGs in the Δ *ttmR* mutant, of which 61 (81%) were upregulated and 14 (19%) were downregulated (Table 1; Fig. 3; see Fig. S2 in the supplemental material). The expression differences observed in the upregulated genes were quite large; 34 had fold changes of ≥ 4 ($\log_2$ FC, >2.0) and six had fold changes of ≥ 10 ($\log_2$ FC, ≥ 3.3). In contrast, all downregulated genes had ≤ 4.6 fold change. Notably, several of the most upregulated DEGs (e.g., 5 of the 6 with fold changes of ≥ 10) were clustered in the chromosome in operons or multiple adjacent operons (Fig. 3).

Using their RefSeq annotations and Pfam domains, the gene functionality of all of the DEGs were categorized into 14 groups which included 12 groups of genes with inferred functionality (*n*=58), one group that encoded hypothetical proteins (*n*=11), and one group of singletons (*n*=6), whose functionality was only represented by a single DEG (Fig. S3). Of the 58 DEGs with a proposed function, the largest categories were regulatory (*n*=12), stress (*n*=10), signaling (*n*=8), and transport (*n*=7). Genes classified as regulatory were comprised of a variety of transcription factors, sigma factors, or antisigma factors. Of the regulatory DEGs, three were Crp homologs, predicted to bind cyclic nucleotides, and three were predicted to have roles in stress response (oxidative stress, cold shock, and generalized stress response, respectively). As many of the DEGs are themselves regulatory, the

TABLE 1 Genes differentially expressed in a *ΔttmR* mutant^a

Locus	Annotation (RefSeq)	Functional category	Log ₂ FC	P _{adj}
Mext_0044	Hypothetical protein	Hypothetical	1.015	3.00E−3
Mext_0191	Chemotaxis sensory transducer	Chemotaxis	−2.189	2.81E−129
Mext_0349	Flagellin domain protein	Motility	1.121	8.86E−17
Mext_0432	ROSMUCR transcriptional regulator	Regulatory	−1.166	8.85E−44
Mext_0438	Hypothetical protein	Signaling	2.335	1.36E−143
Mext_0439	Hypothetical protein	Regulatory	1.652	1.24E−25
Mext_0493	Hypothetical protein	Inclusion bodies	−1.235	2.44E−16
Mext_0563	Acetate kinase	Central carbon metabolism	2.647	1.15E−124
Mext_0564	Secretion protein HlyD family protein	Transport	3.610	0.00
Mext_0565	ABC transporter related	Transport	3.068	6.09E−291
Mext_0566	ABC-2 type transporter	Transport	2.898	1.08E−144
Mext_0567	Phosphoketolase	Central carbon metabolism	2.076	4.73E−148
Mext_0580	Pseudogene	Pseudogene	−1.234	8.45E−38
Mext_0596	Response regulator receiver	Regulatory	1.472	3.29E−81
Mext_0608	Helix-turn-helix domain-containing protein AraC type	Regulatory	1.501	1.27E−44
Mext_0687	Flagellar FlbT family protein	Motility	1.274	3.95E−35
Mext_0869	Hypothetical protein	Stress (oxidative)	1.149	5.60E−10
Mext_0875	PAS sensor protein	Signaling (heat shock)	1.131	5.67E−37
Mext_0925	MarR-like regulatory protein	TtmR	−9.624	1.38E−95
Mext_1031	Glycerone kinase	Central carbon metabolism	1.002	6.93E−21
Mext_1190	PAS sensor protein	Signaling	1.304	1.92E−64
Mext_1191	Membrane protein of unknown function UCP014873	Membrane DUF	3.307	0.00
Mext_1192	67-kDa myosin-cross-reactive antigen family protein	Stress (FA metabolism)	2.395	1.81E−143
Mext_1355	UspA domain protein	Stress	3.064	0.00
Mext_1356	Cytochrome <i>c</i> class I	Cytochrome metabolism	3.227	0.00
Mext_1357	Cyclic nucleotide-binding	Regulatory (cNMP)	2.086	6.65E−176
Mext_1358	ABC transporter related	Transport	2.837	2.01E−249
Mext_1359	ABC transporter related	Transport	2.131	9.30E−160
Mext_1360	Cytochrome <i>bd</i> ubiquinol oxidase subunit I	Cytochrome metabolism	2.607	1.66E−216
Mext_1361	Cytochrome <i>d</i> ubiquinol oxidase, subunit II	Cytochrome metabolism	2.728	2.92E−238
Mext_1362	<i>cyd</i> operon protein YbgT	Cytochrome metabolism	3.150	2.69E−256
Mext_1363	Regulatory protein DeoR	Regulatory (sugar)	3.069	0.00
Mext_1364	Amidase	Translation (tRNA charging)	2.218	7.97E−68
Mext_1755	Aminoglycoside phosphotransferase	Signaling (phosphotransferase)	−1.098	7.26E−27
Mext_1761	Methionine- <i>S</i> -sulfoxide reductase	Stress (oxidative), regulatory (PTM)	1.598	2.08E−37
Mext_1965	Response regulator receiver	Signaling	−1.037	8.82E−35
Mext_1978	Diguanylate cyclase	Signaling (c-di-GMP)	1.230	1.73E−37
Mext_1979	Hypothetical protein	Hypothetical	1.160	1.62E−08
Mext_2100	Hypothetical protein	Hypothetical (transport)	−1.145	4.33E−38
Mext_2114	Hypothetical protein	Hypothetical	1.186	6.88E−3
Mext_2488	OsmC family protein	Stress (oxidative)	2.837	2.28E−184
Mext_2494	Conserved hypothetical membrane spanning protein	Hypothetical (membrane)	1.035	2.75E−10
Mext_2495	CsbD family protein	Stress	1.568	2.67E−19
Mext_2904	Hypothetical protein	Regulatory (stress)	1.055	2.33E−10
Mext_3311	Hypothetical protein	Hypothetical	2.342	4.15E−117
Mext_3387	Hypothetical protein	Hypothetical	−2.109	2.83E−46
Mext_3402	Hypothetical protein	Hypothetical	2.275	2.38E−121
Mext_3407	ABC transporter related	Transport	1.319	3.12E−06
Mext_3498	Heat shock protein 20	Chaperone/heat shock	3.861	0.00
Mext_3499	Putative transcriptional regulatory protein, Crp/Fnr family	Regulatory (cNMP)	4.117	0.00
Mext_3500	UspA domain protein	Stress (universal)	3.573	0.00
Mext_3501	Hypothetical protein	Translation (ribosomal conflict)	2.779	1.99E−260
Mext_3502	Transport associated	Transport	3.045	0.00
Mext_3503	Hypothetical protein	Hypothetical	2.599	1.25E−148
Mext_3504	UspA domain protein	Stress (universal)	3.249	0.00
Mext_3505	Regulatory protein Crp	Regulatory (cNMP)	3.699	0.00
Mext_3506	Response regulator receiver	Regulatory	1.557	9.75E−27
Mext_3508	UspA domain protein	Stress (universal)	3.401	0.00
Mext_3509	Metal-dependent phosphohydrolase HD subdomain	Signaling	2.707	2.11E−242
Mext_3523	Hemolysin-type calcium-binding region	C-N hydrolase	−2.042	8.32E−29
Mext_3821	Choloylglycine hydrolase	C-N hydrolase	2.101	1.03E−99
Mext_3833	Cytochrome <i>d</i> ubiquinol oxidase, subunit II	Cytochrome metabolism	1.073	7.21E−17

(Continued on next page)

TABLE 1 (Continued)

Locus	Annotation (RefSeq)	Functional category	Log ₂ FC	P _{adj}
Mext_3834	Cytochrome <i>bd</i> ubiquinol oxidase subunit I	Cytochrome metabolism	1.358	2.35E−32
Mext_4099	ATP-binding region ATPase domain protein	Signaling	3.238	0.00
Mext_4100	Carbonic anhydrase	pH	1.323	4.67E−28
Mext_4158	EfgA	Stress	−1.041	5.94E−35
Mext_4283	Hypothetical protein	Hypothetical	1.306	6.29E−15
Mext_4421	Hypothetical protein	Hypothetical	1.492	4.11E−24
Mext_4514	Autoinducer-binding domain protein	Regulatory (quorum sensing)	1.280	2.40E−39
Mext_4556	Heat shock protein 20	Chaperone/heat shock	−1.055	8.33E−33
Mext_4599	Flavin reductase domain protein FMN-binding	Redox reaction	1.809	8.86E−10
Mext_4618	Nucleotide sugar dehydrogenase	Sugar metabolism	1.325	7.69E−05
Mext_4620	NAD-dependent epimerase/dehydratase	Sugar metabolism	1.145	6.81E−13
Mext_4700	Integrase, catalytic region	Phage	−1.009	1.14E−05
Mext_4782	Chaperonin GroEL	Chaperone/heat shock	−1.477	6.20E−74
Mext_4783	Chaperonin Cpn10	Chaperone/heat shock	−1.385	4.09E−57

RNA sequencing analysis was performed on wild type and the *ΔttmR* mutant with cells in the early exponential phase of growth on methanol. The log₂ fold change (Log₂FC) in gene expression of the *ΔttmR* mutant was calculated relative to that of the wild type. Adjusted *P* values (*P*_{adj}) were also calculated. Genes detailed here have a Log₂FC of >1.0 and *P*_{adj} of <0.05. Abbreviations: cNMP, cyclic nucleotide monophosphate; PTM, posttranslational modification; FA, fatty acid; DUF, domain of unknown function.

full array of DEGs identified in the *ΔttmR* mutant likely represent loci that are directly controlled by TtmR and indirectly controlled by TtmR, through other regulators. The signaling group included genes that encoded one or more PAS sensor domains and/or predicted members of two-component regulatory systems, such as histidine kinases or response regulator receivers (single domain or paired with diguanylate cyclase domain). DEGs of the stress group encoded universal stress proteins, whose mechanisms are highly variable, and proteins predicted to be involved in oxidative stress and solvent stress. Genes in the transport grouping were involved in a type I secretion system (T1SS) and ABC transporters. There was little indication about the molecules that might be transported, with the exception

FIG 3 Differentially expressed genes in the *ΔttmR* mutant. RNA sequencing analysis was performed on the wild type and the *ΔttmR* mutant with cells in the early exponential phase of growth on methanol. The log₂ fold change (Log₂FC) in gene expression of the *ΔttmR* mutant was calculated relative to wild type. Adjusted *P* values were also calculated. Differentially expressed genes are defined as having a Log₂FC of >1.0 and *P*_{adj} of <0.05; each bar represents an individual gene. Horizontal bars over clusters of genes indicate two or more adjacent genes with the number of genes clustered indicated above the bar. Gene annotations are provided in Table 1.

of an annotated sulfonate transporter. The remaining DEGs were involved in carbon metabolism, cytochrome metabolism, C-N hydrolase and chaperone/heat shock.

The DEGs that were downregulated in the $\Delta ttmR$ mutant strain were scattered across functional categories with the exception of three of the four DEGs encoding chaperonin/heat shock proteins. One possibility for the downregulated DEGs is that they are positively regulated by TtmR, when it is present.

Interestingly, we noticed inconsistency in the directionality of expression changes for chaperone genes. Chaperonin GroEL, chaperonin Cpn10, and heat shock protein 20 (Hsp20) were downregulated while a second Hsp20 gene homolog, *Mext_3498*, was significantly upregulated (14.5 $\times$; Log₂FC, 3.9). *Mext_3498* was part of the highly upregulated 12-gene cluster and appears to be the first gene in a two-gene operon that also encoded a Crp homolog with a cyclic nucleotide-binding motif. These nonuniform changes may reflect the distinct roles of chaperones involved in housekeeping protein quality control versus those which are stress related. The considerable upregulation of the Hsp20 homolog *Mext_3498* may prevent formaldehyde-induced protein damage even in the midst of downregulation of chaperonin GroEL, chaperonin Cpn10, and a second homolog of Hsp20.

Of the 75 DEGs identified here, 20 of them were identified in the 33 previously associated with the transcriptome of the phenotypically formaldehyde-tolerant subpopulation of wild-type *M. extorquens* described elsewhere (29). We find that the directionalities, but not the magnitudes, of expression changes are comparable to those found in the formaldehyde-tolerant subpopulation (Fig. 3; Table 1). In all 20 instances, gene expression differences were more pronounced in the $\Delta ttmR$ mutant than in the subpopulation. The genes of the methanol utilization pathways in the $\Delta ttmR$ mutant did not meet our conservative criteria for being identified as DEGs. However, closer examination showed modest decreases ($\sim$ 1.3- to 1.6-fold changes; $P_{\text{adj}} < 0.05$) in the expression of *mxoF*, which encodes the large subunit of methanol dehydrogenase (30, 31), as well as genes involved in formate assimilation and oxidation (see Table S4 in the supplemental material).

Overall, our data suggest that TtmR elimination allows formaldehyde resistance by tuning the expression of a number of loci that collectively launch a formaldehyde-specific physiological response that may include downstream regulation, cyclic nucleotide signaling, transport, and general stress responses. Notably, the DEGs identified were not involved in cellular functions most often associated with formaldehyde stress, such as DNA repair or formaldehyde detoxification by methylotrophic pathways or alcohol/aldehyde dehydrogenases.

TtmR can modulate formaldehyde resistance independent of EfgA. EfgA is a predicted formaldehyde sensor that modulates formaldehyde resistance by halting translation in the presence of elevated formaldehyde (7). Like TtmR, the absence of EfgA confers *M. extorquens* with the ability to grow on formaldehyde as a sole source of carbon and energy (Fig. 1A), presumably because formaldehyde-mediated translational pausing has been eliminated. Our transcriptomic analysis showed that *efgA* was $\sim$ 2.0 $\times$ downregulated in the $\Delta ttmR$ mutant. Thus, our data suggested it was possible that TtmR mediates formaldehyde resistance through regulating *efgA* expression.

To test this hypothesis, we constructed a $\Delta ttmR \Delta efgA$ double mutant by successive allelic exchange. We then compared the growth of the double mutant to that of each of the single mutants with variable concentrations of formaldehyde provided in the media. In the absence of exogenous formaldehyde, growth of the double mutant was indistinguishable from either of the single mutants (data not shown). In contrast, when 6 mM formaldehyde was added to the growth medium, the $\Delta ttmR$ mutant failed to grow after 24 h, while the $\Delta ttmR \Delta efgA$ double mutant was more fit than the $\Delta efgA$ single mutant (Fig. 4). These data demonstrated that TtmR has some effects that extend beyond regulating EfgA, although the transcriptomic data suggest that the formaldehyde resistance of *ttmR* mutants might be partially mediated by decreased *efgA* expression.

FIG 4 The $\Delta ttmR \Delta efgA$ double mutant has increased formaldehyde resistance. Growth of the $\Delta ttmR$ (CM4732), $\Delta efgA$ mutant (CM3745), and $\Delta ttmR \Delta efgA$ double mutant (CM4733) was quantified in liquid MP medium (succinate) containing 6 mM formaldehyde. Error bars represent the standard error of mean of biological replicates.

TtmR and EfgA are required for optimal transition to methylotrophy. To understand when TtmR and EfgA would be beneficial to *M. extorquens*, we sought to identify growth conditions that induced formaldehyde stress in a physiologically relevant context (i.e., from methanol utilization). Previous work has demonstrated that during the switch from multicarbon to single-carbon growth, *M. extorquens* AM1 experiences a transient formaldehyde imbalance, such that formaldehyde is secreted into the growth medium (32). Researchers suggested that imbalance occurs as a result of nontranscriptional regulation preventing flux through methylotrophic pathways where toxic metabolites (formaldehyde, glycine, and glyoxylate) are produced.

We assayed the growth of wild-type, $\Delta ttmR$, and $\Delta efgA$ strains during the transition to methylotrophy by subculturing stationary-phase, succinate-grown cells into fresh medium containing methanol as the sole carbon source. Both mutant strains displayed lag times that were comparable to each other but were significantly longer (by ~6 h) than those observed in the wild type (Table 2; Fig. 5A). However, the growth rates and maximum cell densities of all strains were indistinguishable (Table 2). Cell viability assays uniformly showed what may be a small dip in viability in both mutants, but when they were considered for each strain individually, neither decrease reached statistical significance (WT versus $\Delta ttmR$, $P = 0.223$; WT versus $\Delta efgA$, $P = 0.215$) during the apparent lag phases. These data suggested that mutant strains take longer to enter exponential growth, rather than experiencing death (Fig. 5C). The ability of strains to transition between distinct modes of metabolism was further probed in an analogous experiment, where methanol-grown cells (Fig. 5A) were subcultured into fresh medium containing succinate as the sole carbon source; no growth defect was observed (Fig. 5B).

To further characterize the defect in the transition to methylotrophy of the $\Delta ttmR$ and $\Delta efgA$ strains, we monitored formaldehyde accumulation in the culture superna-

TABLE 2 Growth during carbon substrate transitions^a

Switch	Strain	Lag (h)	Maximum cell density (A_{600})	<i>P</i> value	Growth rate (μ)	<i>P</i> value
S → M	WT	8	0.98 ± 0.028		0.2139 ± 0.002	
	$\Delta ttmR$ strain	14	0.84 ± 0.014	0.090	0.2295 ± 0.011	0.031
	$\Delta efgA$ strain	14	0.96 ± 0.000	0.493	0.2077 ± 0.001	0.575
M → S	WT	8	1.13 ± 0.000		0.2333 ± 0.018	
	$\Delta ttmR$ strain	8	1.13 ± 0.000	0.500	0.2491 ± 0.003	0.471
	$\Delta efgA$ strain	8	1.12 ± 0.014	NV	0.2325 ± 0.018	0.960

^aGrowth of succinate (S)- or methanol (M)-grown stationary-phase cells subcultured into fresh medium with 15 mM methanol or 3.5 mM succinate, respectively, was monitored. Error shown represents standard error of the mean from biological replicates. Error was not provided for lag time, as resolution at low cell densities was not sensitive enough to quantify. A two-tailed Student's *t* test was performed to identify statistically significant differences in maximum cell densities or growth rates between WT and mutant strains in a given switch condition ($P < 0.05$). NV, no value.

FIG 5 The $\Delta ttmR$ and $\Delta efgA$ mutants are defective in the transition to methylotrophy. The wild type (CM2730), $\Delta ttmR$ mutant (CM4732), and $\Delta efgA$ mutant (CM3745) were first acclimated to succinate in liquid MP medium. Their growth was then assayed upon their inoculation to methanol-based medium by absorbance (A) and cell viability (C). Formaldehyde concentrations in the growth medium was measured by a colorimetric assay (D). After reaching stationary phase, the methanol-grown cultures were subcultured to succinate-based medium and assayed for growth by absorbance (B). Error bars represent the standard error of mean from biological replicates. In panel D, individual values are shown and the mean is indicated.

tants. In wild type, formaldehyde accumulated in the supernatant and peaked at $\sim 100 \mu\text{M}$ upon entry into exponential growth, which was comparable to previously described results in *M. extorquens* AM1 (32). In the supernatants of the $\Delta ttmR$ and $\Delta efgA$ mutants, formaldehyde accumulated to nearly $3\times$ to $4\times$ the levels observed in wild type (~ 275 and $\sim 400 \mu\text{M}$, respectively), demonstrating that formaldehyde-resistant mutants have perturbed formaldehyde metabolism (Fig. 5D).

Collectively, these data show that during the transition to methylotrophy, formaldehyde imbalance is exacerbated in the absence of TtmR or EfgA and suggest that both proteins are required for formaldehyde homeostasis. Therefore, in mutant strains, there is a clear trade-off between acquiring formaldehyde resistance and the ability to adapt to methylotrophic growth.

Phenotypic heterogeneity during the transition to methylotrophy. It has been previously observed that bacterial stressors can provoke phenotypic heterogeneity, and recently, phenotypic heterogeneity in formaldehyde tolerance was demonstrated in wild-type populations of unstressed *M. extorquens* PA1 (29). Here, in the presence of elevated formaldehyde, individual cells experience a binary outcome and either tolerate formaldehyde from the onset of exposure and grow or succumb to formaldehyde stress and die. As the transition to methylotrophy leads to formaldehyde imbalance,

FIG 6 Heterogeneity during the transition to methylotrophic metabolism. Following acclimation to growth on either single- or multicarbon sources, stationary-phase cells were labeled with a fluorescent dye that intercalates into the cell membrane and thereby allows tracking of cell division through dilution of the starting signal. Patterns of growth were tracked in continued growth in methanol (left column) or during shifts from succinate to methanol (right column) by measuring fluorescence of individual cells within the populations using flow cytometry at time points over the course of population growth. The resulting data are displayed as distributions of fluorescence intensity that demonstrate how a population does or does not migrate (grow) over time. During continued growth on methanol, there is a uniform decrease in the fluorescence distribution for the whole population for each of the three strains (left column). In contrast, a distinct shoulder or even a second peak of the distribution representing cells that maintain high fluorescence indicates that not all cells have initiated growth simultaneously, if at all. This second mode of the distribution is seen, at least transiently, for all genotypes but is most pronounced in magnitude and time that it remains a part of the population for the $\Delta efgA$ strain. Data are plotted as histograms of per-cell fluorescence intensity (measured at the emission wavelength of 525 nm, fluorescein isothiocyanate [FITC] channel) of cell populations at different time points across growth. Each time point in a panel represents cell events surveyed within an equivalent volume of growing culture volume. Each panel depicts a single representative biological replicate.

we wanted to determine if the extended lag times of the $\Delta ttmR$ and $\Delta efgA$ mutants during this transition were due to increased variance of individuals entering exponential growth (i.e., some cells initiating growth after a longer time or not at all) or were indicative of a change in the mean behavior of individuals without increased variance.

Tracking the entry into growth in populations by dilution of a membrane-intercalating dye in individual cells revealed heterogeneous initiation of growth in $\Delta ttmR$ and $\Delta efgA$ strains. When cells were transferred from one methanol-grown culture to fresh medium with methanol, all strains displayed a gradual and uniform lessening in the distributions of per-cell fluorescence, indicating uniform growth of cells within these populations (Fig. 6; see Fig. S4 in the supplemental material). In contrast, following the transition to methylotrophic growth, a notable skew, or split, in the distribution was observable by 9 h. Most pronounced in the $\Delta efgA$ mutant, this result suggests the existence of a subpopulation of nongrowing cells in the $\Delta efgA$ lineages, which is not

effectively transitioning to growth on methanol. The $\Delta ttmR$ lineages experience a similar delay; however, in this genotype, the effect is seen as a rightward skew of the distribution rather than as two distinct peaks, suggesting that not all cells survive the transition. This effect was dramatically diminished in the wild-type strain, suggesting that it experiences more uniformity and less growth inhibition during the transition between growth on different carbon substrates across members of the population. This result is also consistent with bulk growth trends observed in the methylotrophic switch (Fig. 5).

DISCUSSION

During methylotrophic growth, all carbon flows through formaldehyde, a cytotoxic compound that can potentially damage a myriad of biomolecules. Accordingly, methylotrophs are more resistant to formaldehyde than nonmethylotrophs (29). In large part, formaldehyde resistance can be attributed to the constitutively high expression and activity of Fae, which catalyzes the first step of formaldehyde oxidation and eliminates free formaldehyde from the cytoplasm. Despite this, formaldehyde pools in *M. extorquens* are elevated in comparison to nonmethylotrophs (1, 34–37), and the mechanisms by which these greater pool sizes are tolerated and regulated are largely unknown, as are specific mechanisms of averting formaldehyde-induced cellular damage. Recent work uncovered the role of EfgA in directly sensing formaldehyde and acting to inhibit translation (7). The current work indicates that TtmR, a MarR-like transcription factor, is a second regulator in the formaldehyde stress response predicted to act at the level of transcription. TtmR regulates transcription of *efgA*, but the growth phenotypes of double mutants also indicate that TtmR regulates other targets beyond this. TtmR and EfgA each contribute to formaldehyde homeostasis and are particularly critical during the transition to methylotrophic metabolism. The combination of these distinct systems suggests that formaldehyde metabolism is monitored and managed across transcriptional and translational regulatory processes.

Without TtmR, *M. extorquens* does not display any obvious growth defects during methylotrophic growth but, instead, displays increased formaldehyde resistance. Increased formaldehyde resistance, as measured by growth in the presence of increasing formaldehyde concentrations, can be achieved by alleviating formaldehyde damage or by relaxing regulatory mechanisms that would otherwise prevent growth under potentially harmful conditions. Characterized formaldehyde stress response systems in bacteria prevent formaldehyde damage by formaldehyde detoxification and are analogous to formaldehyde oxidation systems used by a variety of methylotrophs for C_1 utilization (21). In many organisms where formaldehyde stress has been investigated, formaldehyde-induced DNA damage is often identified and cell damage and death are attributed to genotoxicity (26, 27, 38). More recently however, formaldehyde-induced proteotoxicity has been implicated in cytotoxicity in some human tissues (28), in synthetic methylotrophs (39), and has been suggested by our own work in *M. extorquens* (7, 29). Therefore, other mechanisms of formaldehyde resistance might activate appropriate DNA repair systems and/or heat shock proteins to mitigate DNA and/or protein damage.

Surprisingly, in the transcriptome of the $\Delta ttmR$ mutant, there are no DEGs that are obviously linked to methylotrophic pathways, independent formaldehyde detoxifying enzymatic activities, or DNA repair. Furthermore, of the four genes grouped into chaperone/heat shock, three of them are downregulated in the absence of TtmR. Functional groupings showed that the majority of DEGs encode regulators, signaling proteins, stress response proteins, hypothetical proteins, and transporters whose functions and physiological roles are unknown. This variety of DEGs suggests that formaldehyde resistance may be the result of cumulative, coordinated responses from many uncharacterized mechanisms.

Although increased formaldehyde resistance that emerges in strains lacking TtmR or EfgA could be an advantageous trait in a methylotroph, we have demonstrated that a physiological and ecological benefit of TtmR and EfgA is to speed the transition to

methylophony when endogenous formaldehyde is known to accumulate. Many *Methylobacterium/Methylobacterium* bacteria are plant associated and can use methanol derived from plants by pectin metabolism, among other compounds (40–45). Some, such as *M. extorquens* PA1, which was isolated from *Arabidopsis thaliana*, are epiphytes and live on plant leaf surfaces (40). On leaf surfaces, methanol is released from the stomata, small pores in plant tissues that allow gas exchange (46). Methanol availability, therefore, fluctuates as the stomata open and close and is most abundant in the morning, upon the first stomatal opening of the day (47, 48). To take advantage of this nutritional niche, *M. extorquens* must quickly transition to methylophytic metabolism, which necessarily includes production of a metabolic stressor, formaldehyde (1, 32). Therefore, during this transition, the physiological state of *M. extorquens* must also be prepared for formaldehyde stress to occur. Here, TtmR and EfgA were shown to be required for the optimal transition to methylophytic growth, and furthermore, in their absence, excess formaldehyde accumulation was observed, demonstrating dysregulation of formaldehyde metabolism. On the phylloplane, the organism's native environment, where methanol emissions are transient and come in bursts, loss of TtmR or EfgA would likely be disadvantageous and render *M. extorquens* less competitive within its ecological niche where methanol capture is important.

The transition to methylophony in *M. extorquens* AM1 has been previously investigated with systems biology and genetic approaches but did not identify either the *ttmR* or *efgA* locus (1, 5, 6, 32). There, investigators found that upon transitioning from the multicarbon growth substrate succinate to the single-carbon growth substrate methanol, cells have a lag in carbon assimilation and experience a transient imbalance in formaldehyde metabolism, with formaldehyde accumulating such that it was secreted into the growth medium (32). Further investigation revealed that in adapting cells, carbon flux restriction was mediated by methenyl-dH₄MPT, an intermediate in the formaldehyde dissimilation that directly inhibits MtdA, the enzyme that generates the substrate for entry into the serine cycle assimilation pathway and may regulate Fae activity by an unknown mechanism (1). These authors propose carbon flux restriction is an adaptive strategy that minimizes the accumulation of toxic metabolites generated in methylophytic pathways, including formaldehyde. As strains defective in dH₄MPT biosynthesis (Δ *mpg*) were previously shown to be more methanol (i.e., formaldehyde) sensitive in the absence of EfgA (7), we conclude that EfgA-mediated formaldehyde homeostasis does not involve methenyl-dH₄MPT.

All of the phenotypes of the Δ *ttmR* mutant were mirrored in the Δ *efgA* mutant; therefore, we considered the possibility that TtmR and EfgA were interconnected. Specifically, we considered whether TtmR was a positive regulator of *efgA* expression. The transcriptomic data initially seemed to support this possibility, as *efgA* expression was 2-fold lower in the Δ *ttmR* mutant. However, genetic analysis showed that TtmR and EfgA elimination could cause formaldehyde resistance and a defective switch to methylophony independent of one another. Thus, this work has identified TtmR as a second formaldehyde stress response protein, which can act independently of EfgA and a role for TtmR and EfgA in formaldehyde tolerance and homeostasis. Given the phenotypic overlap of these proteins, it is still possible, and perhaps quite likely, that they independently impact a common cellular function that results in the formaldehyde-associated phenotypes observed. Alternatively, the overlap may represent that *efgA* expression is just one of multiple mechanisms that TtmR exerts its effects on formaldehyde homeostasis.

Here, we demonstrate that achieving formaldehyde resistance through the loss of TtmR or EfgA comes at a cost, as increased formaldehyde resistance simultaneously limits the adaptability of *M. extorquens* to methylophytic metabolism. The formaldehyde accumulation observed during the transition to methylophony suggests that strains lacking TtmR and EfgA achieve formaldehyde resistance by losing their ability to sense and respond to perturbations in formaldehyde homeostasis, rather than by simply improving cellular processes that reduce formaldehyde damage. Interestingly,

this trade-off can be avoided, as mutations that permitted growth on formaldehyde that occurred in other loci, *def* and *efgB*, did not cause a defect in the transition to methylotrophy (see Fig. S5 in the supplemental material). Therefore, the transcriptomic profile observed in strains lacking TtmR likely reflects relaxing a combination of cellular components that typically govern formaldehyde balance as well as those that are responding to the resulting formaldehyde perturbation. The variety of DEGs, which includes many regulators, suggest that TtmR is part of a larger gene regulatory network. Additionally, it appears that both TtmR and EfgA are required for *M. extorquens* to rapidly shift from growth on multicarbon substrates to growth on one-carbon substrates and may be of general importance for facultative methylotrophs.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. Bacterial strains used in this study (see Table S1 in the supplemental material) are derivatives of *Methylobacterium extorquens* PA1 (formerly *Methylobacterium*) (40, 49, 50) where genes for cellulose synthesis were deleted to prevent aggregation and optimize liquid growth measurements (51). Therefore, the genotype referred to herein as wild-type (CM2730) is more accurately Δ *celABC*. The Δ *efgA* mutant (CM3745) additionally has a markerless deletion that eliminated 404 bp from the open reading frame (ORF) of *Mext_4158* (21 to 424/435 bp) (7), and the Δ *tmmR* mutant (CM4732) has a markerless deletion of the entire coding region (588 bp) of *Mext_0925*. All growth experiments with liquid medium were performed with *Methylobacterium* piperazine-*N,N'*-bis(2-ethanesulfonic acid) (MP) medium (51) with 3.5 mM succinate; 15 mM methanol; or 2, 4, 6, 8, or 10 mM formaldehyde as a carbon source. For growth on solid MP medium, Bacto Agar (15 g/liter; BD Diagnostics) was added and carbon source concentrations were elevated (15 mM succinate and 125 mM methanol). Formaldehyde stock solutions (1 M) were prepared by boiling a mixture of 0.3 g paraformaldehyde and 10 ml of 0.05 N NaOH in a sealed tube for 20 min; stock solutions were kept at room temperature and made fresh weekly. When present in the media, kanamycin was used at a final concentration of 50 μ g/ml.

Genetic approaches. Markerless deletions were generated by allelic exchange as previously described using either pCM433 (52) or pPS04 (7). Vectors were designed using SnapGene software. The HiFi assembly kit from New England BioLabs was used to construct vectors from linearized vector backbone (restriction enzyme-digested) and PCR-generated inserts.

Growth quantitation. To initiate liquid growth, individual colonies were used to inoculate 2 ml MP medium containing 3.5 mM succinate or 15 mM methanol in biological triplicate. Cultures were shaken (250 rpm) during incubation at 30°C for 24 h (succinate) or 36 h (methanol) and then subcultured (1/64) into 5 ml of identical medium for further acclimation. After this second 24- to 36-h (succinate) or 36- to 48-h (methanol) incubation, the stationary-phase acclimation cultures were again subcultured (1/64) into relevant media for growth measurements. Cell density was determined by monitoring absorbance with a Spectronic 200 (Thermo Scientific) or a SmartSpec Plus (Bio-Rad) at 600 nm. Maximum cell density is defined as the absorbance reached upon entry into stationary phase. Cell viability was determined by harvesting cells from a 100- μ l aliquot of culture by centrifugation, discarding supernatant and resuspending the cell pellet into an equal volume of MP medium (no carbon). Cell suspensions were then serially diluted (1/10 dilutions, 200- μ l total volume) in 96-well polystyrene plates with MP medium (no carbon), and 10- μ l aliquots of each dilution were spotted to MP medium plates (15 mM succinate) using technical triplicates. Plates were inverted and incubated at 30°C until colony formation was apparent (4 to 6 days), at which point colonies were counted. Technical triplicates were averaged for each sample; biological replicates were averaged.

Formaldehyde quantification. Formaldehyde concentrations in the culture media were measured as previously described (53). Supernatant from a 100- μ l aliquot of culture was isolated by centrifugation (14,000 $\times$ g). In technical triplicates, 10 μ l of the supernatant or 100 μ l of 0.1 $\times$ supernatant (diluted with MP medium, no carbon) was combined with 190 or 100 μ l Nash reagent B (2 M ammonium acetate, 50 mM glacial acetic acid, and 20 mM acetylacetone), respectively, in 96-well polystyrene plates. Reaction plates were incubated (60°C and 10 min) and cooled to room temperature (5 min), and absorbance was read at 432 nm on a Wallac 1420 Victor multilabel reader (Perkin Elmer). Formaldehyde standards were prepared daily from 1 M formaldehyde stock solutions, and a standard curve was read alongside all sample measurements.

RNA sequencing analysis. The WT (CM2730) and the Δ *tmmR* mutant (CM4732) were grown in biological triplicate in MP medium with 15 mM methanol as described above. The final growth vessel was a 250-ml flask, with a culture volume of 100 ml. When the optical density at 600 nm (OD_{600}) reached 0.2 to 0.3, cells from 50 ml of culture were harvested by centrifugation in the Eppendorf centrifuge 5810R (5 min, 4,000 $\times$ g, and 4°C). The supernatant was decanted, and cells were washed with ice cold MP medium (no carbon) and centrifuged once more to remove wash supernatant. Pellets were immediately frozen by submerging tubes in liquid nitrogen and stored at -80°C .

Nucleic acid extraction and molecular manipulation (RNA extraction, cDNA generation, and library preparation) and sequencing were conducted by Genewiz (South Plainfield, NJ). The total RNA was extracted with the RNeasy Plus minikit (Qiagen); rRNA was depleted with the Ribo-Zero rRNA removal kit (Illumina); the quality of the resulting RNA samples was determined with an Agilent 2100 bioanalyzer

and Qubit assay. Once the cDNA library was generated, it was sequenced on an Illumina HiSeq instrument (2 × 150 bp). The raw data (FASTQ format) was provided to investigators for further analysis.

An analysis pipeline developed by the University of Minnesota Genomics Center and the Research Informatics Solutions (RIS) group at the University of Minnesota Supercomputing Institute was used for data analysis. The pipeline uses FastQC (54) to assess the quality of the sequencing data, Trimmomatic (55) to remove the low-quality bases and adapter sequences, HISAT2 (56) to align the curated reads to the *M. extorquens* PA1 genome (GenBank accession [CP000908.1](#)), Cuffquant and Cuffnorm from the Cufflinks package (33) to generate fragments per kilobase per million (FPKM) expression values, and featureCounts from the Rsubread R package (57) to generate raw read counts. DESeq2 was used to convert raw count data to normalized counts and all further statistical analyses were carried out in R (58). Differentially expressed genes in the *ΔttmR* mutant were identified by a pairwise comparison to the WT strain and had a log₂ fold change of >1 and a false discovery rate (FDR)-adjusted *P* value of <0.05.

Stress tests. For all tests, cultures of WT (CM2730) and the *ttmR*^{EVO} mutant (CM3919) were grown in biological triplicate in MP medium (succinate), as described above.

Alternative aldehyde growth assays. Growth was quantified in liquid media as described above with succinate or methanol as the primary carbon source. Wild type (CM2730) was subjected to variable glyoxal, acetaldehyde, glutaraldehyde, butyraldehyde, and propionaldehyde concentrations (up to 10 mM) to identify a concentration that would lead to a growth defect but allow growth within a 24-h period. Once the working concentrations were identified (1.25 mM for acetaldehyde and glyoxal and 2.5 mM for the remaining aldehydes), the *ttmR*^{EVO} (CM3919) and *ΔefgA* (CM3745) mutants were grown under identical conditions, alongside the wild-type control.

Antibiotic resistance assays. Stationary-phase cultures (100 μl) in MP medium (succinate) were used to inoculate 3.5 ml of soft agar (0.8%) that was previously melted and then cooled to ~50°C. Inoculated soft agar was agitated by vortexing (~10 sec) and then was overlaid to solid MP medium (succinate). Soft agar overlays were allowed to solidify at room temperature for 1 to 2 h. Antibiotic discs (VWR) saturated with absolute amounts of individual antibiotics (novobiocin [5 μg], tetracycline [30 μg], streptomycin [10 μg], chloramphenicol [30 μg], colistin [10 μg], cefoxitin [30 μg], gentamicin [10 μg], erythromycin [15 μg], rifampicin [5 μg], ciprofloxacin [10 μg], vancomycin [30 μg], kanamycin [30 μg], nalidixic acid [30 μg], ampicillin [10 μg]) were placed on top of agar plates with sterile tweezers. Plates were incubated at 30°C for ~24 h and then scored for diameter of resulting zones of inhibition.

Oxidative stress. Stationary-phase cultures were used to prepare soft agar overlays as described for antibiotic resistance assays. After solidification, 5 μl of 30% hydrogen peroxide (59) was spotted onto the center of the plate. Plates were incubated at 30°C for 4 to 6 days and scored for diameter of resulting zones of inhibition.

Alcohol stress. Stationary-phase cultures were serially diluted with MP medium (no carbon), plated to solid medium containing 2% ethanol (59), incubated at 30°C for 4 to 6 days, and scored for indication of stress (colony size, small or variable) and viability.

Heat shock. Mid-exponential-phase cultures were placed in a 55°C water bath for 5 or 10 min (59). Cells were then transferred to room temperature and serially diluted with MP medium (no growth carbon source) and plated onto solid medium (succinate). After incubation at 30°C for 4 to 6 days, they were scored for viability (survival).

Assessing population heterogeneity. Isolated colonies were inoculated into 2 ml of liquid MP media with either 3.5 mM succinate or 15 mM methanol. Upon growth, cultures were diluted 1/64 into 5 ml of the same liquid media in sealed Balch tubes and grown for 24 h (succinate) or 30 h (methanol) to allow all cultures to reach stationary phase. After completing this acclimation step, 1 ml of each culture was collected and centrifuged for 1 minute at 10,000 × *g*. The supernatant was removed; and the cell pellet was resuspended in 500 μl of diluent C containing 2 μl PKH67 dye (PKH67 green fluorescent cell linker kit; Sigma-Aldrich), incubated for 5 minutes at room temperature, and quenched by adding 500-μl 1% bovine serum albumin (BSA) solution. Samples were pelleted for 1 minute at 10,000 × *g* and subsequently washed in equal volumes of 1% BSA (once) followed by MP medium (twice). After a final resuspension in an equal volume of MP medium, the final mixes were diluted 1/64 into 5-ml MP media containing 15 mM methanol to initiate experimental cultures, and the remainder was mixed with dimethyl sulfoxide (DMSO) to 8% and stored at -80°C. Samples taken during the experimental growth period were also mixed with DMSO to 8% and stored at -80°C. Signal intensity of samples was assessed via flow cytometry analysis (Beckman Coulter Cytoflex S). After excitation at 488 nm, dye intensity was measured via emission detected with a 525-nm bandpass filter. Cell gating was determined by the distribution of red fluorescence detection from the mCherry-tagged strain CM3839.

Data availability. Raw Illumina read data and processed files of read counts per gene and normalized expression levels per gene have been deposited in the NCBI GEO database (accession no. [GSE159711](#)).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.5 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.7 MB.

ACKNOWLEDGMENTS

We thank Juan E. Abrahante of the University of Minnesota Informatics Institute (UMII) for assistance with the pipeline for transcriptome sequencing (RNA-seq) data analysis and Siavash Riazi for assistance with modifying R scripts. We thank members of the Marx laboratory, Bazurto laboratory, and Lon Chubiz for critical reading of the manuscript. We thank Chandler Hellenbrand for assistance in conducting experiments. The flow cytometry was carried out at the IBEST Optical Imaging Core at the University of Idaho (IBEST is supported in part by NIH COBRE grant P30GM103324).

This work was supported by funding from an Army Research Office MURI subaward to C.J.M. (W911NF-12-1-0390), a CMCI pilot grant to C.J.M. (parent NIH award P20GM104420), an INBRE Undergraduate Research Fellowship to L.B.L. (parent NIH award P20GM103408), and a Beacon Center for Evolution in Action pilot grants to J.V.B. and E.L.B. (NSF cooperative agreement DBI-0939454).

J.V.B. contributed conceptualization, funding acquisition, investigation, methodology, visualization, writing – original draft preparation, writing – review & editing; E.L.B. contributed conceptualization, funding acquisition, investigation, methodology, visualization, writing – original draft preparation, writing – review & editing; J.A.L. contributed conceptualization, investigation, methodology, writing – original draft preparation, writing – review & editing; L.B.L. contributed funding acquisition, investigation; C.J.M. contributed conceptualization, funding acquisition, project administration, resources, supervision, writing – original draft preparation, writing – review & editing.

REFERENCES

- Martinez-Gomez NC, Good NM, Lidstrom ME. 2015. Methenyl-dephosphotetrahydromethanopterin is a regulatory signal for acclimation to changes in substrate availability in *Methylobacterium extorquens* AM1. *J Bacteriol* 197:2020–2026. <https://doi.org/10.1128/JB.02595-14>.
- Vorholt JA, Marx CJ, Lidstrom ME, Thauer RK. 2000. Novel formaldehyde-activating enzyme in *Methylobacterium extorquens* AM1 required for growth on methanol. *J Bacteriol* 182:6645–6650. <https://doi.org/10.1128/jb.182.23.6645-6650.2000>.
- Chistoserdova L, Vorholt JA, Thauer RK, Lidstrom ME. 1998. C1 transfer enzymes and coenzymes linking methylotrophic bacteria and methanogenic Archaea. *Science* 281:99–102. <https://doi.org/10.1126/science.281.5373.99>.
- Marx CJ, Chistoserdova L, Lidstrom ME. 2003. Formaldehyde-detoxifying role of the tetrahydromethanopterin-linked pathway in *Methylobacterium extorquens* AM1. *J Bacteriol* 185:7160–7168. <https://doi.org/10.1128/jb.185.23.7160-7168.2003>.
- Marx CJ, Van Dien SJ, Lidstrom ME. 2005. Flux analysis uncovers key role of functional redundancy in formaldehyde metabolism. *PLoS Biol* 3:e16. <https://doi.org/10.1371/journal.pbio.0030016>.
- Crowther GJ, Kosály G, Lidstrom ME. 2008. Formate as the main branch point for methylotrophic metabolism in *Methylobacterium extorquens* AM1. *J Bacteriol* 190:5057–5062. <https://doi.org/10.1128/JB.00228-08>.
- Bazurto JV, Nayak DD, Ticak T, Davlieva M, Lambert LB, Quates CJ, Patel JS, Ytreberg FM, Shamoo Y, Marx CJ. 2020. EfgA halts bacterial translation in response to intracellular formaldehyde. *bioRxiv* <https://doi.org/10.1101/2020.10.16.343392>.
- Piatkov KI, Vu TTM, Hwang C-S, Varshavsky A. 2015. Formyl-methionine as a degradation signal at the N-termini of bacterial proteins. *Microb Cell* 2:376–393. <https://doi.org/10.15698/mic2015.10.231>.
- George AM, Levy SB. 1983. Gene in the major cotransduction gap of the *Escherichia coli* K-12 linkage map required for the expression of chromosomal resistance to tetracycline and other antibiotics. *J Bacteriol* 155:541–548. <https://doi.org/10.1128/JB.155.2.541-548.1983>.
- George AM, Levy SB. 1983. Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: involvement of a non-plasmid-determined efflux of tetracycline. *J Bacteriol* 155:531–540. <https://doi.org/10.1128/JB.155.2.531-540.1983>.
- Cohen SP, Yan W, Levy SB. 1993. A multidrug resistance regulatory chromosomal locus is widespread among enteric bacteria. *J Infect Dis* 168:484–488. <https://doi.org/10.1093/infdis/168.2.484>.
- Cohen SP, Hächler H, Levy SB. 1993. Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. *J Bacteriol* 175:1484–1492. <https://doi.org/10.1128/jb.175.5.1484-1492.1993>.
- Martin RG, Nyantakyi PS, Rosner JL. 1995. Regulation of the multiple antibiotic resistance (*mar*) regulon by *mar*ORA sequences in *Escherichia coli*. *J Bacteriol* 177:4176–4178. <https://doi.org/10.1128/jb.177.14.4176-4178.1995>.
- Martin RG, Rosner JL. 1995. Binding of purified multiple antibiotic-resistance repressor protein (MarR) to *mar* operator sequences. *Proc Natl Acad Sci U S A* 92:5456–5460. <https://doi.org/10.1073/pnas.92.12.5456>.
- Grove A. 2017. Regulation of metabolic pathways by MarR family transcription factors. *Comput Struct Biotechnol J* 15:366–371. <https://doi.org/10.1016/j.csbj.2017.06.001>.
- Chubiz LM, Rao CV. 2010. Aromatic acid metabolites of *Escherichia coli* K-12 can induce the *mar*RAB operon. *J Bacteriol* 192:4786–4789. <https://doi.org/10.1128/JB.00371-10>.
- Seoane AS, Levy SB. 1995. Identification of new genes regulated by the *mar*RAB operon in *Escherichia coli*. *J Bacteriol* 177:530–535. <https://doi.org/10.1128/jb.177.3.530-535.1995>.
- Seoane AS, Levy SB. 1995. Characterization of MarR, the repressor of the multiple antibiotic resistance (*mar*) operon in *Escherichia coli*. *J Bacteriol* 177:3414–3419. <https://doi.org/10.1128/jb.177.12.3414-3419.1995>.
- Sharma P, Haycocks JRJ, Middlemiss AD, Kettles RA, Sellars LE, Ricci V, Piddock LJV, Grainger DC. 2017. The multiple antibiotic resistance operon of enteric bacteria controls DNA repair and outer membrane integrity. *Nat Commun* 8:1444. <https://doi.org/10.1038/s41467-017-01405-7>.
- Yurimoto H, Hirai R, Matsuno N, Yasueda H, Kato N, Sakai Y. 2005. HxlR, a member of the DUF24 protein family, is a DNA-binding protein that acts as a positive regulator of the formaldehyde-inducible *hxl*AB operon in *Bacillus subtilis*. *Mol Microbiol* 57:511–519. <https://doi.org/10.1111/j.1365-2958.2005.04702.x>.
- Chen NH, Djoko KY, Veyrier FJ, McEwan AG. 2016. Formaldehyde stress responses in bacterial pathogens. *Front Microbiol* 7:257. <https://doi.org/10.3389/fmicb.2016.00257>.
- Yasueda H, Kawahara Y, Sugimoto S. 1999. *Bacillus subtilis* *yckG* and *yckF* encode two key enzymes of the ribulose monophosphate pathway used by methylotrophs, and *yckH* is required for their expression. *J Bacteriol* 181:7154–7160. <https://doi.org/10.1128/JB.181.23.7154-7160.1999>.
- Parke D, Ornston LN. 2003. Hydroxycinnamate (*hca*) catabolic genes from *Acinetobacter* sp. strain ADP1 are repressed by HcaR and are induced by hydroxycinnamoyl-coenzyme A thioesters. *Appl Environ Microbiol* 69:5398–5409. <https://doi.org/10.1128/aem.69.9.5398-5409.2003>.

24. Tropel D, van der Meer JR. 2004. Bacterial transcriptional regulators for degradation pathways of aromatic compounds. *Microbiol Mol Biol Rev* 68:474–500. <https://doi.org/10.1128/MMBR.68.3.474-500.2004>.
25. Davis JR, Brown BL, Page R, Sello JK. 2013. Study of PcaV from *Streptomyces coelicolor* yields new insights into ligand-responsive MarR family transcription factors. *Nucleic Acids Res* 41:3888–3900. <https://doi.org/10.1093/nar/gkt009>.
26. Kawanishi M, Matsuda T, Yagi T. 2014. Genotoxicity of formaldehyde: molecular basis of DNA damage and mutation. *Front Environ Sci Eng China* 2. <https://doi.org/10.3389/fenvs.2014.00036>.
27. Grafstrom RC, Fornace AJ, Jr., Autrup H, Lechner JF, Harris CC. 1983. Formaldehyde damage to DNA and inhibition of DNA repair in human bronchial cells. *Science* 220:216–218. <https://doi.org/10.1126/science.6828890>.
28. Ortega-Atienza S, Rubis B, McCarthy C, Zhitkovich A. 2016. Formaldehyde is a potent proteotoxic stressor causing rapid heat shock transcription factor 1 activation and Lys48-linked polyubiquitination of proteins. *Am J Pathol* 186:2857–2868. <https://doi.org/10.1016/j.ajpath.2016.06.022>.
29. Lee JA, Riazzi S, Nemati S, Bazurto JV, Vasdekis AE, Ridenhour BJ, Remien CH, Marx CJ. 2019. Microbial phenotypic heterogeneity in response to a metabolic toxin: continuous, dynamically shifting distribution of formaldehyde tolerance in *Methylobacterium extorquens* populations. *PLoS Genet* 15:e1008458. <https://doi.org/10.1371/journal.pgen.1008458>.
30. Nunn DN, Lidstrom ME. 1986. Isolation and complementation analysis of 10 methanol oxidation mutant classes and identification of the methanol dehydrogenase structural gene of *Methylobacterium* sp. strain AM1. *J Bacteriol* 166:581–590. <https://doi.org/10.1128/jb.166.2.581-590.1986>.
31. Anderson DJ, Lidstrom ME. 1988. The *maxFG* region encodes four polypeptides in the methanol-oxidizing bacterium *Methylobacterium* sp. strain AM1. *J Bacteriol* 170:2254–2262. <https://doi.org/10.1128/jb.170.5.2254-2262.1988>.
32. Skovran E, Crowther GJ, Guo X, Yang S, Lidstrom ME. 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. <https://doi.org/10.1371/journal.pone.0014091>.
33. Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL, Rinn JL, Pachter L. 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc* 7:562–578. <https://doi.org/10.1038/nprot.2012.016>.
34. Müller JEN, Meyer F, Litsanov B, Kiefer P, Potthoff E, Heux S, Quax WJ, Wendisch VF, Brautaset T, Portais J-C, Vorholt JA. 2015. Engineering *Escherichia coli* for methanol conversion. *Metab Eng* 28:190–201. <https://doi.org/10.1016/j.jmben.2014.12.008>.
35. Heck HD, Casanova-Schmitz M, Dodd PB, Schachter EN, Witek TJ, Tosun T. 1985. Formaldehyde (CH₂O) concentrations in the blood of humans and Fischer-344 rats exposed to CH₂O under controlled conditions. *Am Ind Hyg Assoc J* 46:1–3. <https://doi.org/10.1080/15298668591394275>.
36. Luo W, Li H, Zhang Y, Ang CYW. 2001. Determination of formaldehyde in blood plasma by high-performance liquid chromatography with fluorescence detection. *J Chromatogr B Biomed Sci Appl* 753:253–257. [https://doi.org/10.1016/S0378-4347\(00\)00552-1](https://doi.org/10.1016/S0378-4347(00)00552-1).
37. Nagy K, Pollreis F, Takáts Z, Vékey K. 2004. Atmospheric pressure chemical ionization mass spectrometry of aldehydes in biological matrices. *Rapid Commun Mass Spectrom* 18:2473–2478. <https://doi.org/10.1002/rcm.1648>.
38. Takahashi K, Morita T, Kawazoe Y. 1985. Mutagenic characteristics of formaldehyde on bacterial systems. *Mutat Res* 156:153–161. [https://doi.org/10.1016/0165-1218\(85\)90058-8](https://doi.org/10.1016/0165-1218(85)90058-8).
39. Chen FY-H, Jung H-W, Tsuei C-Y, Liao JC. 2020. Converting *Escherichia coli* to a synthetic methylophil growing solely on methanol. *Cell* 182:933–946. <https://doi.org/10.1016/j.cell.2020.07.010>.
40. Knief C, Frances L, Vorholt JA. 2010. Competitiveness of diverse *Methylobacterium* strains in the phyllosphere of *Arabidopsis thaliana* and identification of representative models, including *M. extorquens* PA1. *Microb Ecol* 60:440–452. <https://doi.org/10.1007/s00248-010-9725-3>.
41. Anthony C. 1982. The biochemistry of methylophil. Academic Press, London, United Kingdom.
42. Corpe WA, Rheem S. 1989. Ecology of the methylophilic bacteria on living leaf surfaces. *FEMS Microbiol Ecol* 5:243–249. <https://doi.org/10.1111/j.1574-6968.1989.tb03698.x>.
43. Van Dien SJ, Okubo Y, Hough MT, Korotkova N, Taitano T, Lidstrom ME. 2003. Reconstruction of C(3) and C(4) metabolism in *Methylobacterium extorquens* AM1 using transposon mutagenesis. *Microbiology (Reading)* 149:601–609. <https://doi.org/10.1099/mic.0.25955-0>.
44. Sy A, Timmers ACJ, Knief C, Vorholt JA. 2005. Methylophilic metabolism is advantageous for *Methylobacterium extorquens* during colonization of *Medicago truncatula* under competitive conditions. *Appl Environ Microbiol* 71:7245–7252. <https://doi.org/10.1128/AEM.71.11.7245-7252.2005>.
45. Müller DB, Vogel C, Bai Y, Vorholt JA. 2016. The plant microbiota: systems-level insights and perspectives. *Annu Rev Genet* 50:211–234. <https://doi.org/10.1146/annurev-genet-120215-034952>.
46. Abanda-Nkwatt D, Müsch M, Tschiersch J, Boettner M, Schwab W. 2006. Molecular interaction between *Methylobacterium extorquens* and seedlings: growth promotion, methanol consumption, and localization of the methanol emission site. *J Exp Bot* 57:4025–4032. <https://doi.org/10.1093/jxb/erl173>.
47. Nemecek-Marshall M, MacDonald RC, Franzen JJ, Wojciechowski CL, Fall R. 1995. Methanol emission from leaves (enzymatic detection of gas-phase methanol and relation of methanol fluxes to stomatal conductance and leaf development). *Plant Physiol* 108:1359–1368. <https://doi.org/10.1104/pp.108.4.1359>.
48. Hüve K, Christ MM, Kleist E, Uerlings R, Niinemets U, Walter A, Wildt J. 2007. Simultaneous growth and emission measurements demonstrate an interactive control of methanol release by leaf expansion and stomata. *J Exp Bot* 58:1783–1793. <https://doi.org/10.1093/jxb/erm038>.
49. Marx CJ, Bringle F, Chistoserdova L, Moulin L, Farhan Ul Haque M, Fleischman DE, Gruffaz C, Jourand P, Knief C, Lee M-C, Muller EEL, Nadalig T, Peyraud R, Roselli S, Russ L, Goodwin LA, Ivanova N, Kyrpides N, Lajus A, Land ML, Médigue C, Mikhailova N, Nolan M, Woyke T, Stolyar S, Vorholt JA, Vuilleumier S. 2012. Complete genome sequences of six strains of the genus *Methylobacterium*. *J Bacteriol* 194:4746–4748. <https://doi.org/10.1128/JB.01009-12>.
50. Green PN, Ardley JK. 2018. Review of the genus *Methylobacterium* and closely related organisms: a proposal that some *Methylobacterium* species be reclassified into a new genus, *Methylorubrum* gen. nov. *Int J Syst Evol Microbiol* 68:2727–2748. <https://doi.org/10.1099/ijsem.0.002856>.
51. Delaney NF, Kaczmarek ME, Ward LM, Swanson PK, Lee M-C, Marx CJ. 2013. Development of an optimized medium, strain and high-throughput culturing methods for *Methylobacterium extorquens*. *PLoS One* 8:e62957. <https://doi.org/10.1371/journal.pone.0062957>.
52. Marx CJ. 2008. Development of a broad-host-range *sacB*-based vector for unmarked allelic exchange. *BMC Res Notes* 1:1. <https://doi.org/10.1186/1756-0500-1-1>.
53. Nash T. 1953. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem J* 55:416–421. <https://doi.org/10.1042/bj0550416>.
54. Andrews S. 2010. FastQC: a quality control tool for high throughput sequencing data. Babraham Bioinformatics. <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>.
55. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30:2114–2120. <https://doi.org/10.1093/bioinformatics/btu170>.
56. Kim D, Langmead B, Salzberg SL. 2015. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods* 12:357–360. <https://doi.org/10.1038/nmeth.3317>.
57. Liao Y, Smyth GK, Shi W. 2019. The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. *Nucleic Acids Res* 47:e47. <https://doi.org/10.1093/nar/gkz114>.
58. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15:550. <https://doi.org/10.1186/s13059-014-0550-8>.
59. Gourion B, Francez-Charlot A, Vorholt JA. 2008. PhyR is involved in the general stress response of *Methylobacterium extorquens* AM1. *J Bacteriol* 190:1027–1035. <https://doi.org/10.1128/JB.01483-07>.