An aerobic link between lignin degradation and C₁ metabolism: growth on methoxylated aromatic compounds by members of the genus *Methylobacterium*

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Running title

Methylobacterium growth on methoxylated aromatics

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Abstract

Microorganisms faces many barriers in the degradation of the polycyclic aromatic polymer lignin, one of which is an abundance of methoxy substituents. Demethoxylation of lignin-derived aromatic monomers in aerobic environments releases formaldehyde, a potent cellular toxin that organisms must eliminate in order to further degrade the aromatic ring. Here we provide the first comprehensive description of the ecology and evolution of the catabolism of methoxylated aromatics in the genus *Methylobacterium*, a plant-associated genus of methylotrophs capable of using formaldehyde for growth. Using comparative genomics, we found that the capacity for aromatic catabolism is ancestral to two clades, but has also been acquired horizontally by other members of the genus. Through laboratory growth assays, we demonstrated that several *Methylobacterium* strains can grow on *p*-hydroxybenzoate, protocatechuate, vanillate, and ferulate; furthermore, whereas non-methylotrophs excrete formaldehyde as a byproduct during growth on vanillate, *Methylobacterium* do not. Finally, we surveyed published metagenome data to find that vanillate-degrading *Methylobacterium* can be found in many soil and rhizosphere ecosystems but is disproportionately prominent in the phyllosphere, and the most highly represented clade in the environment (the root-nodulating species *M. nodulans*) is one with few cultured representatives.

Introduction

Microbial processes for degrading lignin and lignin-derived aromatics are of intense interest in a diversity of fields, ranging from bioenergy engineering—in which the recalcitrance of lignin is a major hurdle in the processing of plant biomass [1]—to the fossil fuel industry—which seeks to understand microbial transformations of coal [2]. Lignin, which comprises approximately 20% of the carbon fixed by photosynthesis on land [3], is an exceptionally complex, irregular, polycyclic aromatic polymer, in which many of the constituent aromatic rings are heavily substituted with methoxy (-OCH₃) groups [4].

In the aerobic microbial degradation of lignin-derived aromatic monomers such as vanillate, the degradation of the aromatic ring proceeds by the protocatechuate branch of the beta-ketoadipate pathway. The gene cluster encoding this pathway is widely distributed among soil microorganisms, and has a complex evolutionary history resulting in diverse patterns of gene organization and regulation [5–7]. In the case of methoxylated aromatics, ring cleavage must be preceded by the removal of the methoxy group; in most aerobic organisms, this occurs via vanillate monooxygenase, a Rieske [2Fe-2S] enzyme that demethylates vanillate to generate protocatechuate and formaldehyde [8–12] (Fig. 1). Formaldehyde is a potent electrophile, and toxic to microorganisms due to its reactivity with DNA and proteins [13]. Elimination of this toxin is therefore essential to lignin degradation. Multiple studies have demonstrated experimentally, through either engineered disruption or constitutive expression of formaldehyde oxidation capacity in *Bradyrhizobium diazoefficiens* [11], *Pseudomonas putida* [14], *Burkholderia cepacia* [9], and *Corynebacterium glutamicum* [15], that demethoxylation of vanillate is dependent on the activity of an functional formaldehyde detoxification system, and formaldehyde removal may be the rate-limiting step to the degradation of lignin-derived methoxylated aromatics.

Detoxification pathways in lignin-degrading organisms are diverse, and in some organisms include more than one mechanism. In some cases formaldehyde is oxidized

to CO₂ via formate, such as in the thiol-dependent systems in *C. glutamicum* and *B. japonicum* or the zinc-dependent dehydrogenase in *P. putida* [16]; in others it may be incorporated into biomass via the RuMP pathway, as in *B. cepacia* [17]. Furthermore, it has recently been discovered that methanogens of the genus *Methermicoccus* are capable of using the methoxy groups of coal-derived aromatic compounds for the production of methane through a novel metabolic pathway ("methoxydotrophic methanogenesis"), illuminating a previously-unknown link between coal degradation and methane production via C₁ metabolism [18].

Methylotrophs, organisms that use reduced C₁ compounds for growth, therefore make appealing candidates for efficient degradation of lignin-derived methoxylated aromatics. Members of the genus *Methylobacterium*, and particularly the model organism *M. extorquens*, have long been studied for their metabolism of simple C₁ compounds such as methanol, formate, methylamine, and halogenated methanes [19]. Formaldehyde is a central intermediate in the metabolism of many of these substrates, raising the possibility that, if *Methylobacterium* were capable of demethoxylating aromatic compounds, they could also use the resulting formaldehyde as a growth substrate. It has previously been documented that two members of the genus are capable of growth on aromatic compounds [20–22]; however, the prevalence of this trait among *Methylobacterium* and the organization and evolutionary history of the genes involved has not been described. Furthermore, to our knowledge, it has not been shown whether these organisms are capable of methoxydotrophy: growth on methoxy groups from aromatic compounds.

Here we report novel findings on the ecology and evolution of methoxydotrophic growth and catabolism of aromatic compounds by members of the genus *Methylobacterium*. We explored the genomic capacity of *Methylobacterium* species by searching published genomes, verified growth and formaldehyde production/consumption on aromatic compounds in the laboratory, and surveyed published metagenome data to assess the distribution and prevalence of aromatic-degrading *Methylobacterium* in the environment. For a thorough description of aerobic microbial degradation of lignin-derived aromatic

compounds, we direct the reader to the excellent reviews published previously [5, 23]; a simplified diagram of the pathway and the four genes of interest to this study (*vanA*, *pcaG*, *pobA*, *ech*) is given in Figure 1. Because we were especially interested in organisms that could use the methoxy group of vanillate, our metagenomic analysis focused especially on *vanA* (KO:K03862), the gene encoding the alpha subunit of vanillate monooxygenase.

Materials and Methods

Phylogenetic analysis

Genetic potential of *Methylobacterium* species [24] (Table S1) for the degradation of aromatic compounds was assessed through analysis of genomes available on the IMG/M database [25]. We should note that Green and Ardley [26] recently proposed a restructuring of the genus *Methylobacterium*, including a renaming of the genus of the *M. extorquens* clade from *Methylobacterium* to *Methylorubrum*, and a suggestion to reclassify the *M. aquaticum* and *M. nodulans* clades into different genera entirely. However, because the present study is concerned with the four clades as a coherent group of evolutionarily related taxa that are still known as *Methylobacterium* by many research groups, and all are currently still listed within that genus by the List of Prokaryotic Names with Standing in Nomenclature [27], in this manuscript we continue to refer to all organisms as *Methylobacterium*.

Genes of interest were identified by KEGG Orthology ID: *vanA* (vanillate monooxygenase alpha subunit, KO:K03862); *pcaG* (protocatechuate 3,4-dioxygenase alpha subunit, KO:K00448); *pobA* (*p*-hydroxybenzoate 3-monooxygenase, KO:K00481); and *ech* (*trans*-feruloyl-CoA hydratase/vanillin synthase, KO:K18383; we searched also for IMG Term Object ID 8865, vanillin synthase). Known lignin-degrading organisms were added to the phylogenies as reference organisms, and *Prochlorococcus marinus* NATL2A and archaeal species were used as outgroups (Table S2). The genes used for the phylogeny in Figure 1 were 16S rRNA (the RNA component of the small ribosomal

subunit), *rpoB* (DNA-directed RNA polymerase subunit beta, KO:K03043), *atpD* (ATP Synthase F1 complex beta subunit, KO:K02112), and *recA* (recombination protein RecA, KO:K03553), all of which have been shown previously to be informative for phylogenetic analysis in *Methylobacterium* and closely related organisms [28–30].

All gene sequences were aligned using Clustal Omega v.1.2.3 [31], using four combined HMM/guide tree iterations and default settings for all other parameters. Phylogenetic trees were generated using IQ-Tree v.1.6.1 [32] on the CIPRES Science Gateway [33], with one partition, outgroups given in Table S2, ultrafast bootstrapping with 1000 replicates, and SH-like Single Branch test [34] with 1000 replicates; for all other parameters, the default settings were used.

Genomic context of aromatic catabolism genes

To assess synteny in the gene neighborhoods surrounding the *vanA* and *pcaH* genes in *Methylobacterium* species, we aligned scaffolds, or segments of assembled genomes, using MAUVE v.2.4.0 with the progressiveMauve algorithm and default parameter settings [35, 36]. Figures were generated using a GenoPlotR v.0.8.7 [37] for R v.3.4.3 [38] in RStudio v.1.1.423 [39].

GC content in the *Methylobacterium sp.* AMS5 genome was calculated in R using the alphabetFrequency function from Biostrings v.2.46.0 [40]; for the plot in Fig. S1, a sliding window of 5 kb was used for the full genome and 500 bp for the catabolic island region. Genome signature difference was calculated using a custom script in R, using the formula for the δ^* difference [41] with tetranucleotides rather than dinucleotides.

Growth assays on aromatic substrates

All organisms were grown on MPIPES medium [42] with the addition of 1x Wolfe's Vitamins [43], 25 μ M LaCl_3 (as has been found to facilitate growth in some *Methylobacterium spp.* [44]), and carbon substrates as specified. All conditions contained 16 mM of carbon (4 mM succinate, 2.67 mM glucose, 2.29 mM benzoate, 2.29 mM PHBA, 2.29 mM PCA, 2 mM vanillate, 1.6 mM ferulate, 0.4 g/L Kraft lignin).

Methylobacterium sp. 4-46 was also tested on methanol without LaCl₃. For each assay, one colony was inoculated from a culture plate into 5 mL MPIPES with 3.5 mM disodium succinate and grown 24 hours to obtain a stationary-phase culture, then diluted 1:64, and grown again until stationary. Using that inoculum, the growth experiment was conducted as follows: each replicate was diluted 1:64 into MPIPES and aliquoted into several wells of a Costar 48-well tissue culture-treated plate (product #3548, Corning Inc., Corning, NY) for non-volatile substrates, or into Balch-type glass culture tubes for vanillin, along with carbon substrate provided at concentrations such that all conditions were equimolar in carbon (16 mM). Total volume was 640 μ L per well in culture plates or 5 mL per tube in Balch tubes. Each strain was tested in biological triplicate (3 separate colonies). Balch tubes were sealed with butyl rubber serum stoppers to prevent escape of vanillin.

Plates were incubated at 30 °C in a LPX44 Plate Hotel (LiCONiC, Woburn, MA) with shaking at 250 RPM. Optical density was assessed using a Wallac 1420 Victor2 Microplate Reader (Perkin Elmer, Waltham, MA), reading OD₆₀₀ for 0.4 seconds at intervals of between 2 and 5 hours. Culture tubes were incubated in a TC-7 tissue culture roller drum (New Brunswick Scientific, Edison, NJ) at a speed setting of 7, and OD₆₀₀ was measured in the culture tubes using a Spectronic 200 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Outputs from the Wallac 1420 software were collated using Curve Fitter [42]; data cleaning, analysis, and plotting were then conducted using custom scripts in R (Supplemental Files S1, S2). Because many of the strains formed clumps, maximum OD₆₀₀ was used as a semi-quantitative metric for growth. Non-growth was defined as no OD₆₀₀ above 0.025.

Formaldehyde production during growth on vanillate

Growth of *Methylobacterium* strains on vanillate was initiated with stationary-phase MPIPES-succinate cultures as described above; these were diluted 1:64 into 5 mL MPIPES with 2 mM vanillate and grown 2 days until stationary phase. This inoculum was diluted 1:32 into culture flasks containing 20 mL of MPIPES with 2 mM vanillate and incubated, shaking, at 30 °C. Cultures were sampled every 4 hours: OD₆₀₀ was

measured in a SmartSpec Plus Spectrophotometer (Bio-Rad Laboratories, Hercules, CA), and the supernatant of 500 μ L of centrifuged culture was used for the measurement of formaldehyde using a colorimetric assay [45]. All strains were tested in biological triplicate.

Formaldehyde production of non-methylotrophs during vanillate growth was assayed similarly. Stationary-phase vanillate-grown cultures of *Pseudomonas putida* KT2440 and *Rhodococcus jostii* RHA1 were diluted 1:64 into 5 mL MPIPES medium in culture tubes, with sampling every 2 hours. Vanillate was provided at 4 mM; although this was greater than in the *Methylobacterium* experiment, we have found that vanillate concentration has little effect on the resulting concentration of formaldehyde in the medium (Fig. S3).

Analysis of *Methylobacterium vanA* genes in published metagenomes

To assess the abundance of *Methylobacterium*-encoded *vanA* genes in the environment, we searched all published metagenomes available through the IMG/M portal on December 20, 2017. The "Function Search" tool was used to identify all metagenomes containing genes annotated with KO:K03862 (*vanA*). To increase our chances of observing meaningful ecological patterns, we restricted our analysis to metagenomes containing >100 *vanA* genes. Genes on scaffolds with a phylogenetic lineage assignment of *Methylobacterium* were designated "*Methylobacterium vanA*." and the ecological context was taken from the Ecosystem Type field in the sample metadata associated with each gene. Because it was unclear whether all researchers used consistent definitions, we combined phylloplane with phyllosphere, and rhizoplane with rhizosphere.

To provide a tree-based assessment of *vanA* diversity in addition to the USEARCH-based IMG/M phylogenetic lineage assignment [46], we downloaded the *Methylobacterium vanA* gene sequences and placed them in the reference *vanA* phylogeny (described above). Pplacer v.1.1 [47] was used with HMMer v.3.1b.2 [48], RAxML v.8.2 [49], and taxtastic v.0.8.8 for Python 3. The resulting tree was paired with metadata from sequence scaffolds in Phyloseq v.1.22.3 for R [50], to generate the plot

in Fig. 8.

The relative abundances of all *vanA* or all *Methylobacterium* reads within each metagenome were found using the Statistical Analysis tool in IMG [25]. For each Ecosystem Type, the average and standard error among metagenomes was calculated manually, in order to omit datasets with "NA" values due to unassembled genes that impeded the Statistical Analysis tool. Because the *Methylobacterium vanA* genes analyzed here originated from only the subset of metagenomes having >100 *vanA* genes, the ratio of *Methylobacterium vanA* to all *vanA* was calculated using only the metagenomes analyzed in that part of the study.

Results

Genetic capacity for metabolism of methoxylated aromatic compounds is present primarily in two clades of *Methylobacterium*

We searched the published genomes of 26 *Methylobacterium* strains for the presence of *vanA*, *pcaG*, *pobA*, and *ech*. We found that all four genes are indeed present within the genus *Methylobacterium*. They are primarily limited to two closely related clades, the *aquaticum* clade and the *nodulans* clade; furthermore, they are present in nearly all organisms in these clades (Fig. 1). However, there are exceptions. One member each of the *extorquens* clade (*Methylobacterium* sp. AMS5) and the *radiotolerans* clade (*M. pseudosasiacola*) also carry some aromatic catabolism genes (Fig. 1). Two members of the *nodulans* clade (sp. 4-46 and sp. WSM2598) appear to lack *vanA*, though *Methylobacterium* sp. 4-46 carries *ech* (Fig. 1) and *vdh* (not shown), conferring the capacity to produce vanillate from ferulate, suggesting that the organism may have previously had the capacity to utilize the product of these reactions but has since lost *vanA*. Among the closest relatives to *Methylobacterium*, *Enterovirga rhinocerotis* has none of the four genes of interest, and *Microvirga flocculans* has only *pobA* and *pcaH*.

Taken together, these patterns suggest that the capacity to metabolize several lignin-derived aromatic compounds was acquired by the *aquaticum/nodulans* clade early in its divergence within the *Methylobacterium*, but members of other clades have acquired the genes more recently by horizontal gene transfer. To investigate this evolutionary story further, we analyzed the phylogenies of the genes themselves, and also examined the genomic contexts of *vanA* and *pcaG*.

Phylogeny of aromatic catabolism genes supports ancestral origins in *M. aquaticum* and *M. nodulans* clades and horizontal acquisition by two other species

The phylogenies of *pobA*, *pcaG*, and *vanA* were largely congruent with the phylogeny of conserved housekeeping genes within the *Methylobacterium* but not between *Methylobacterium* and their closest relatives, supporting the hypothesis that the four genes are ancestral to the *aquaticum/nodulans* clades but were not necessarily acquired together (Fig. 2). The *ech* phylogeny was the most difficult to interpret, as the *Methylobacterium* separated into three non-monophyletic clades (Fig. 2); it is possible that not all gene homologs in the phylogeny code for enzymes specific to ferulate. Notably, *Methylobacterium* sp. AMS5 and *M. pseudosasiacola* were more closely related to each other than to any other species in both their *pobA* and *pcaG* sequences, despite their phylogenetic distance from each other at the genome level. And while the *pobA* sequences of those two strains fell within those of other *Methylobacterium*, their *pcaG* sequences clustered with the *Sphingomonas*, further support not only for the hypothesis that these two disparate species both acquired the beta-ketoadipate pathway via horizontal gene transfer, but that they may have acquired it from the same donor or that one served as the donor to the other.

Gene synteny in *pcaG* and *vanA* neighborhoods is consistent with phylogeny

We further examined the relationships of *vanA* and *pcaG* across species by comparing patterns of synteny within the genome neighborhood for each. In all strains with *pcaG*, the gene was located within a cluster of beta-ketoadipate pathway genes, and five of these genes were present in all strains in the same order. Almost all strains also

contained a gene annotated as an uncharacterized conserved protein, DUF849 (COG3246, beta-keto acid cleavage enzyme), between *pcaG* and *pcaB* (Fig. 3). We found no genes annotated as *pcaI* and *pcaJ* (beta-ketoadipate:succinyl-CoA transferase) or *pcaF* (encoding beta-ketoadipyl-CoA thiolase) within these operons, which are necessary for the final steps of the beta-ketoadipate pathway [5, 7]. All *Methylobacterium* strains with *vanA* also had the other two genes required to confer the ability for the demethoxylation of vanillate: *vanB* (encoding the other subunit of vanillate monooxygenase) and *vanR* (an AraC family transcriptional regulator). Some strains also had genes encoding a Major Facilitator Superfamily transporter specific to protocatechuate (*pcaK*) or vanillate (*vanK*).

The genomic contexts of *pcaG* and *vanA* among strains exhibited patterns that agreed overall with the phylogenies described above (Fig. 3). Closely related strains shared common genes in the regions near *pcaG* and *vanA*, with differences among the strains increasing with increasing distance from the genes of interest. Also consistent with phylogeny, *Methylobacterium* sp. AMS5 and *M. pseudosasiacola* shared no commonalities with the *M. aquaticum* clades or with each other in the neighborhoods surrounding the *pca* genes or the *van* genes. Notably, in most previously described organisms, the *pca* gene is not co-located with the other genes of interest in this study (Tables S1, S2). However, the reaction product of the enzyme encoded by *pobA* (PCA) is the substrate for the gene encoded by *pcaGH*; the proximity of the genes therefore suggests that if these two organisms gained the *pca* genes via horizontal transfer, the *pob* genes may have been transferred simultaneously as part of a catabolic island. We found further support for the catabolic island hypothesis in the proximity of *vanA* and *ech* to the *pca* genes in *Methylobacterium* sp. AMS5.

***Methylobacterium* sp. AMS5 carries a catabolic island conferring the ability to degrade lignin-derived aromatic compounds, in an *M. extorquens*-like genome**

The co-localization of all four aromatic catabolism genes of interest in the genome of *Methylobacterium* sp. AMS5 prompted us to examine the region more closely. AMS5 was originally isolated in 2011 from the stem of a hypernodulating strain of soybean [51]

but is very closely related model organism *M. extorquens* PA1 [52, 53] (Fig. 1). We aligned the two genomes, as well as that of another clade member, *M. zatmanii* PSBB041, at the region where the aromatic catabolism genes are located (Fig. 4). The comparison revealed that the region appears to be prone to the insertion and excision of mobile genetic elements.

At the locus where *M. zatmanii* carries a gene encoding a putative IS5-type transposase (possibly nonfunctional due to a frameshift with an internal stop codon), AMS5 contained an additional 22-kb region: a catabolic island containing genes that appear to confer the full ability to degrade ferulate, vanillate, vanillin, *p*-hydroxybenzoate, and protocatechuate, flanked by two different transposase genes from the MULE superfamily. On the 3' side of this region, the two strains share 11 genes (14 kb), many related to nucleotide sugar metabolism, that are also not present in PA1 (Fig. 4), or in any of the other *Methylobacterium* species surveyed in this study. The corresponding locus in the PA1 genome carries only a putative site-specific integrase/recombinase, which is present only in the other *M. extorquens* strains (AM1, PA1, DM4; and *M. chloromethanicum*, *M. populi*, which have previously been identified as *M. extorquens* strains [54]). The gene adjacent to the integrase encodes UDP-*N*-acetylglucosamine 2-epimerase (*wecB*); the sequence appears to be complete in *M. zatmanii* (1,137bp) but truncated in PA1 (missing 912 bp from the 3' end). In all three genomes, the region of variability lies immediately at the 3' end of the genes for tRNA-Pro and tRNA-Arg.

The presence of the truncated gene in PA1 suggests that the region of sugar metabolism genes may originally have been present but were excised, whereas the arrangement of genes in the catabolic island suggest it was acquired into a *M. zatmanii*-like genomic background. The origins of the catabolic island itself are uncertain; as shown above (Fig. 2), there appear to be diverse phylogenetic origins represented among the different genes within the pathway, and BLAST search for the entire region in the NCBI Nucleotide database found no other organisms containing all genes in the same order. We could detect no genes in the region relating to replication or conjugation as might be expected in an integrative and conjugative element (ICE) [55].

Furthermore, no significant difference in tetranucleotide composition or in GC content was found between the inserted region (GC% = 71.4) and the full genome (GC% = 71.1) (Fig. S1), indicating that the catabolic island has likely been present in the genome long enough for amelioration [56].

Genome content predicts ability to grow on aromatic compounds in most *Methylobacterium* strains

We next wanted to assess whether *Methylobacterium* strains carrying genes for the catabolism of methoxylated aromatic compounds could indeed use those compounds as growth substrates. We chose 8 species from the *M. aquaticum/nodulans* clades, shown in Figure 5 (and Table S1). All of these strains carry all four genes of interest except *Methylobacterium* sp. 4-46, which lacks *vanA* and so was predicted not to grow on vanillate or ferulate (Fig. 1). We conducted a series of growth experiments on defined mineral medium with substrates of each of the four gene products of interest, as well as succinate (a known growth substrate for all the strains tested), glucose (used by only some *Methylobacterium* species), benzoic acid (another aromatic acid with a degradation pathway separate from the beta-ketoadipate pathway [57]), vanillin, and Kraft lignin (a soluble form of polymeric lignin). All strains grew on succinate and none on lignin; growth on PHBA, PCA, vanillate, and ferulate was as expected for all strains given their genome content, with the exceptions that *M. variabile* did not grow on vanillate and *M. nodulans* did not grow on ferulate (Fig. 5, Fig. S2, Supplemental File S2). No growth was observed on vanillin at the concentration tested; however, this may be due in part to the toxicity of vanillin [58], as we have observed growth on vanillin by *M. nodulans* when using lower concentrations in previous experiments.

Non-methylotrophs excrete formaldehyde during vanillate growth, whereas *Methylobacterium* do not

We were especially interested to ask whether methylotrophs are different from non-methylotrophs in their ability to cope with the formaldehyde produced during vanillate metabolism. Although, as mentioned above, it is recognized that the formaldehyde released by vanillate monooxygenase is a burden to organisms growing on vanillate, to

our knowledge no attempts have been made to measure the dynamics of accumulation of the toxin during growth. We therefore measured formaldehyde in the medium during the growth of two well-studied non-methylotrophic lignin degraders, *Pseudomonas putida* KT2440 and *Rhodococcus jostii* RHA1: we assayed each strain on vanillate as well as on PCA as a control compound, as PCA is the direct product of vanillate demethylation and therefore involves the same metabolism except for the effect of formaldehyde. In both strains, when growth occurred on vanillate, formaldehyde accumulated in the medium concomitant with the increase in optical density, peaking at concentrations of 0.94 mM (Fig. 6A, Table S4). When cultures entered stationary phase (approximately 10 hours for *P. putida*, 15 hours for *R. jostii*), formaldehyde concentrations began to decrease, ultimately returning to below the detection limit. No formaldehyde was detected at any time during growth on PCA. Moreover, growth on PCA was faster: stationary phase was reached at 7 hours for *P. putida* and 10 hours for *R. jostii*. These results suggest that while both *P. putida* and *R. jostii* can oxidize formaldehyde, removal is slower than production.

We conducted a similar experiment on the five strains of *Methylobacterium* showing the greatest growth on vanillate in our laboratory conditions: *M. aquaticum*, *M. nodulans*, *M. tarhaniae*, *Methylobacterium* sp. AMS5, and *M. aquaticum* MA-22A. We incubated all cultures with vanillate for 33 hours, long enough for AMS5 to show marked growth and all other species to consume all the substrate and reach stationary phase. No detectable formaldehyde was produced at any time (Fig. 6B, Table S4). These results suggest that *Methylobacterium* species are able to consume the formaldehyde generated from vanillate demethylation as rapidly as it is produced, likely via the same pathways by which the formaldehyde generated from methanol is used for energy generation and biosynthesis.

***Methylobacterium*-derived *vanA* reads in published metagenomes are predominantly from the *M. nodulans* cluster, with highest relative abundance in the phyllosphere**

Aside from the environments in which they were isolated (Table S1), there exists scant information on the ecological niches of the aromatic-degrading *Methylobacterium* clades—for instance, whether they are more likely than the other *Methylobacterium* to inhabit ecosystems rich in lignin, such as soil or the rhizosphere. We therefore sought to learn more about the prevalence and abundance of *vanA*-carrying *Methylobacterium* species in the environment by querying the publicly available metagenome datasets on the JGI IMG/M database. The distinctness of the *Methylobacterium* genes within the phylogeny of known *vanA* sequences (Fig. 2) makes it possible to deduce phylogeny from DNA sequence. *vanA* is present as a single copy in most genomes in which it is found.

Our study set comprised 1,651 metagenomes, with a total assembled gene count of 5.60×10^9 ; we retrieved 317,816 scaffolds carrying *vanA*, of which 348 had *Methylobacterium* as their IMG phylogenetic lineage assignment (a frequency of 0.11%) (Tables S5, S6, S7). However, our phylogenetic placement using pplacer found that only 182 of these genes actually fell within the *Methylobacterium* clades, whereas 31 clustered more closely with *Azospirillum halopraeferens*, and the remaining 135 were distributed among more distantly related organisms (Fig. S4). We therefore focused only on the genes identified by both IMG and pplacer as *Methylobacterium* for the remainder of our analyses.

In absolute numbers, *vanA* genes belonging to *Methylobacterium* were highest in samples from the soil (43% of *Methylobacterium vanA*) and rhizosphere/rhizoplane (40%), the ecosystems in which *vanA* is most abundant generally (Fig. 7). However, we were especially interested in environments where *Methylobacterium* are most prominent within the community of vanillate degraders (high relative abundance of *Methylobacterium vanA* genes among total *vanA*). We found that there were many ecosystem types in which *vanA* was found but *Methylobacterium vanA* was not, including engineered ecosystems and those associated with animal hosts (Table S5). Where *Methylobacterium* were found, they made up the largest proportion of *vanA* in phyllosphere/phylloplane samples ($0.28 \pm 0.13\%$ of *vanA* genes) (Fig. 7). Relative

abundances in the other ecosystem types were one order of magnitude lower (freshwater: $0.024 \pm 0.016\%$; rhizoplane/rhizosphere: $0.022 \pm 0.010\%$; peat moss: 0.017% ; soil: $0.014 \pm 0.005\%$; sediment: $0.011 \pm 0.005\%$, where $\pm$ denotes standard error; only 1 peat moss genome contained *Methylobacterium vanA*). This distribution across ecosystem types was qualitatively similar to that of total *Methylobacterium* reads in all metagenomes (Fig. 7).

The metagenome sequences identified as *Methylobacterium vanA* fell into three phylogenetic clusters (Fig. 8). The majority (114 genes, 63%) were most closely related to *M. nodulans*; 17% (31 genes) clustered with *Methylobacterium* sp. AMS5, and 20% (37 genes) clustered with the remaining *vanA*-carrying species, in the *M. aquaticum* clade (Fig. 8). These three clades were distributed approximately evenly across all ecosystem types.

Discussion

We have provided the first comprehensive description of growth of *Methylobacterium* on lignin-derived methoxylated aromatic acids. *Methylobacterium* species with this capability can use vanillate as a sole carbon substrate without the transient formaldehyde accumulation in the environment that is observed with non-methylotrophic vanillate degraders. The genes encoding aromatic catabolism are found almost exclusively in all members of the *M. aquaticum* and *M. nodulans* clades, but acquisition by horizontal gene transfer has been observed in other *Methylobacterium* species. These findings shed new light on the ecology of the genus.

Although there are important implications for our understanding of the bacterial lignin-degrading community, we are far from proposing that *Methylobacterium* be classified as lignin degraders: the species we studied were not found to grow on Kraft lignin, nor is there evidence that *Methylobacterium* can produce the peroxidases or laccases necessary for lignin depolymerization. In the environment, these organisms may depend

upon aromatic acids released by the action of other lignin-degrading organisms, which have been found to be a prominent component of leaf litter leachate [59]. Alternatively, *Methylobacterium* may encounter these compounds primarily in plant root exudates [60]. In addition to acting as a growth substrate, aromatic acids play an important role in plant-microbe signaling: vanillate, ferulate, *p*-hydroxybenzoate, and protocatechuate all influence on the process and productivity of root nodulation by other members of the *Rhizobiales* [61, 62]. This is significant in light of the fact that the members of the *M. nodulans* clade were, as the species name suggests, isolated from root and stem nodules of their plant hosts [21, 63]; and that we found this clade to be the most abundant among the *Methylobacterium vanA* genes found in the environment. Furthermore, *Methylobacterium sp.* AMS5 was isolated in a study on soybean epiphytes that are particularly responsive to host nodulation phenotype [51]. It is likely that at least part of the importance of aromatic catabolism in the *Methylobacterium* is to facilitate the relationships of these plant-associated organisms with their hosts. A link between aromatic catabolism and plant-microbe symbioses could help to explain our finding that organisms from the *M. nodulans* and *Methylobacterium sp.* AMS5 clades are dominant among the *Methylobacterium vanA* sequences found in the environment, despite the fact that there are few genome-sequenced representatives.

Perhaps the most compelling results from this study are the new insights into the evolution of the genus *Methylobacterium*. One element is the absence of the *vanA* gene in *Methylobacterium sp.* 4-46 and *sp.* WSM298, the only two species in the *M. nodulans*/ *M. aquaticum* clades to lack the capacity for methoxytrophic growth. These species do carry genes for transforming ferulate to vanillin (*ech*) and vanillin to vanillate (*vdh*), which may be remnants from predecessors that were able to metabolize vanillate but lost the capability. Notably, *Methylobacterium sp.* 4-46 is also one of very few *Methylobacterium* species reported to be unable to grow on methanol [64]; our lab has found that it can indeed use methanol as a growth substrate but only in the presence of LaCl₃ (Fig. S5, Table S8), suggesting the involvement of a XoxF-type methanol dehydrogenase [44], and possibly a different role for methanol oxidation in this organism's ecology. Given our hypothesis that methanol oxidation and vanillate

demethylation require the same pathways for metabolizing the formaldehyde produced, it is possible that the loss of *vanA* in *Methylobacterium* sp. 4-46 might be related to its different style of methylotrophy. We have also observed that this and several non-*Methylobacterium* lignin-degrading species possess the genes to use PCA (the aromatic product of vanillate demethylation) but not the methoxy group (Fig. 1). Yet we have found no species in which the reverse is true, although it would theoretically be possible for a methylotroph with *van* genes but no *pca* genes to carry out methoxydotrophic growth by utilizing only the methoxy group of vanillate.

The other unexpected evolutionary finding relates to the acquisition of aromatic catabolism genes by horizontal transfer in two *Methylobacterium* species from outside of the *M. nodulans/aquaticum* clades. The discovery of a catabolic island in *Methylobacterium* sp. AMS5 is itself not unusual; *Methylobacterium* species have long been recognized to carry an abundance of IS (insertion sequence) elements, and it has been postulated that the associated genome rearrangements and horizontal gene transfer associated are important mechanisms of evolution in the genus [65–67]. Relevant to the present study is the prior observation that diverse features of the genomic background—and not necessarily those predicted by phylogeny—influence whether a newly introduced set of genes are immediately useful to the recipient organism [68]. Are there particular features of the *M. pseudosasiacola* and *Methylobacterium* sp. AMS5 genomes that allowed them to acquire the capacity for the degradation of methoxylated aromatics when no other known members of their clades did, or is the maintenance of this genomic capability the result of selective pressure specific to their ecological niche? Further work on the ecology of AMS5 and the biology of aromatic catabolism in *Methylobacterium* is necessary to address these questions.

This study has benefited from the wealth of knowledge that already exists on microbial degradation pathways for the degradation of lignin-derived aromatics in other organisms, and on methylotrophic metabolism in *Methylobacterium*, to deduce the likely fate of the methoxy group from vanillate. We found that in most cases, the gene annotations in IMG enabled us to correctly predict the substrates each strain could grow

on (the two exceptions were both cases of no growth, and we cannot rule out the possibility that growth could occur under different conditions). However, we did find some novel features of the *Methylobacterium* pathways: almost all the strains we studied appear have no homologs of *pcaI*, *pcaJ*, or *pcaF* encoded within the *pca* gene cluster. It is possible that the functions of these genes are carried out by homologs located elsewhere in the genome, as has been found in some other organisms [6]. A second possibility is raised by a previous study that carried out enzymatic screening and active site modeling on the DUF849 family of genes [69]: several of the DUF849 genes found in these *Methylobacterium* gene clusters were classified as beta-keto acid cleavage enzymes (G4 BKACE) predicted to act on betaketoadipate, raising the possibility that they might carry out the *pcaI/pcaJ* function and thus constitute a novel variant of the already diverse family of beta-ketoadipate pathway configurations [6, 7].

Likewise, our work benefited from the abundance of metagenome data that are publicly available, as a way of achieving a broad overview of the ecology our organisms of interest. However, *Methylobacterium* likely make up ~0.01-0.2% of organisms carrying *vanA* in environments where vanillate degraders are found; this low abundance means that even searching the entire set of available data on IMG/M yielded only 182 gene sequences of interest, and the majority of metagenomes with *Methylobacterium vanA* genes contained only one such gene, making statistical analysis difficult. Furthermore, the very environment where *Methylobacterium* make up a greater part of the *vanA* population is that where total *vanA* reads are low and therefore poorly represented in our dataset: the phyllosphere. Taken together, these caveats mean that the broad-scale ecological patterns observed in this initial survey should be interpreted with caution. However, as the first such survey carried out, our work provides a dataset on which to base more targeted assays to obtain a deeper understanding of the diversity and distribution of aromatic-degrading *Methylobacterium* in the environment.

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Figures and legends

Figure 1. Genes associated with aromatic catabolism are present in two clusters of closely-related *Methylobacterium* species, with some exceptions.

Genomes of *Methylobacterium* species and known aromatic-degrading bacteria were searched for four genes involved in different steps of the degradation of lignin-derived methoxylated aromatic compounds (upper left). Colored squares indicate the presence of each gene. Among the reference organisms (not *Methylobacterium*), all species that do not have *pcaG* do have the genes for protocatechuate 4,5-dioxygenase, indicating an alternative ring cleavage mechanism and different pathway for the catabolism of PCA. The phylogeny was composed using a concatenated alignment of four housekeeping genes (*atpD*, *recA*, *rpob*, and 16S rRNA), with the cyanobacterium species *Prochlorococcus marinus* as an outgroup (not shown). Branch labels indicate SH-aLRT branch support/ UltraFast bootstrap values; all unlabeled branches have values of 100/100. Accession numbers and published references are given in Tables S1 and S2.

Figure 2. Phylogenies of aromatic catabolism genes suggest all four are ancestral to the *M. aquaticum*/*M. nodulans* clades, and have been horizontally acquired by two other *Methylobacterium* species.

For all organisms in Fig. 1, the sequences of *pobA* (*p*-hydroxybenzoate 3-monooxygenase), *pcaH* (protocatechuate 3,4-dioxygenase beta subunit), *vanA* (vanillate monooxygenase subunit A), and *ech* (*trans*-feruloyl-CoA hydratase/vanillin synthase) were aligned and phylogenies constructed using maximum likelihood, with homologs from archaeal organisms as outgroups (not shown). Accession numbers for all genes are given in Tables S1 and S2. Branch labels indicate SH-aLRT branch support/UltraFast bootstrap values; all unlabeled branches have values of 100/100. Comparison of these phylogenies and that in Fig. 1 suggest that all four genes are restricted to and prevalent in the *M. aquaticum* and *M. nodulans* clades, with a few losses (*vanA* by *Methylobacterium* sp. 4-46 and WSM2598) and some gains by horizontal gene transfer (in *M. pseudosasiacola* and *Methylobacterium* sp. AMS-5).

Figure 3. Genomic context of *vanA* and *pcaG* genes in *Methylobacterium* species supports a common evolutionary origin within the *M. aquaticum/nodulans* clades and separate origin in *M. pseudosasiicola* and *Methylobacterium* sp. AMS-5.

Genomic regions (from fully assembled genomes) or scaffolds (from partially assembled genomes) are shown with A) all *pcaG* genes aligned or B) all *vanA* genes aligned. Gene phylogenies are excerpted from Fig. 2. Genes related to the beta-ketoadipate pathway (in A) and to vanillate demethylation (in B) are shown in white. Homologous regions shared across multiple genomes are shown in the same color to facilitate comparison among genomes. Gene labels are abbreviations based on functional annotations; full information on all genes is given in Table S3. Members of the *M. aquaticum* and *M. nodulans* clades share some synteny in the regions surrounding the *van* and *pca* gene clusters, with the degree of synteny roughly paralleling their phylogenetic distance; as an exception, *Methylobacterium* sp. AMS-5 and *M. pseudosasiicola* differ markedly from the other species.

Figure 4. Alignment of three genomes from the *M. extorquens* clade reveals that *Methylobacterium* sp. AMS-5 harbors a catabolic island conferring the ability to degrade several aromatic substrates, in a genomic region prone to insertions and deletions.

The genome of *Methylobacterium* sp. AMS5 is shown aligned with that of two close relatives, *M. zatmanii* PSBB041 and *M. extorquens* PA1. Genes are color-coded to indicate function or commonality between genomes, as indicated by the key. Light gray shading between pairs of species connects regions shared by both. See Table S3 for full annotations corresponding to the short gene codes.

Figure 5. Growth of *Methylobacterium* species on aromatic compounds is predicted by genetic potential in most cases.

Methylobacterium species were grown in defined mineral medium with a single carbon substrate; all conditions contained 16 mM of carbon. Substrates corresponding to the four degradation pathways featured in this study are in bold; all species tested have genetic capacity for all four aromatic substrates except in the case of *Methylobacterium sp.* 4-46 and vanillate. Shading indicates maximum optical density (OD) at 600 nm measured during 80 hours of incubation, the average of three biological replicates. Original growth curves are shown in Fig. S2.

Figure 6. Non-methylotrophic lignin degraders *Pseudomonas putida* and *Rhodococcus jostii* produce formaldehyde when growing on methoxy-substituted aromatic compounds such as vanillate; *Methylobacterium* do not.

All organisms were grown in defined mineral medium with vanillate or protocatechuate (PCA) as a sole carbon source; growth was assayed by optical density at 600 nm (gray symbols and dashed lines) and formaldehyde in the medium was measured by a colorimetric assay (red symbols and solid lines). Each line represents one biological replicate. A) *P. putida* and *R. jostii* accumulate formaldehyde transiently in the medium when growing on vanillate, but not on the non-methoxylated compound PCA, and growth is slower on vanillate. B) All *Methylobacterium* species tested grew on vanillate without producing measurable formaldehyde.

Figure 7. Organisms carrying *vanA* are most abundant in peat moss, rhizosphere, and soil; *Methylobacterium* are most abundant in the phyllosphere both overall and among *vanA*-carrying organisms.

Relative abundances of A) *vanA* genes and B) *Methylobacterium* genes as a percentage of all genes in each metagenome in the IMG/M database at the time of study. The numbers above each bar give the number of metagenomes searched in each ecosystem type (all metagenomes contained both *vanA* genes and *Methylobacterium* genes). C) Relative abundance of *Methylobacterium vanA* genes as a percentage of all *vanA* genes. The fraction above each bar denotes the number of metagenomes in which *Methylobacterium vanA* genes were found over the number of metagenomes searched for that ecosystem type (metagenomes were only searched if they had >100 *vanA* genes). Error bars denote the standard error among all metagenomes in the set.

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Figure 8. Among *Methylobacterium vanA* sequences in published metagenomes, the *M. nodulans* clade is most abundant.

Sequences of 182 *Methylobacterium*-associated *vanA* gene fragments found in published metagenomes were added using pplacer [47] to a phylogeny of full-length *vanA* genes sequenced from reference genomes. 166 genes that were classified by IMG as *Methylobacterium* but clustered outside of the genus are not shown. Colored dots indicate the ecosystem type from which the metagenome sample originated, with size scaled to the likelihood weight ratio of the pplacer classification as a measure of confidence in the placement. 114 genes (63%) are classified as *M. nodulans*; 31 genes (17%) as *Methylobacterium* sp. AMS5, and 37 genes (20%) as *M. aquaticum*. Inset bar plot: abundance of *Methylobacterium vanA* genes, shaded by clade, in each genome in which they were found. Each bar represents one genome (genome IDs not shown).