Characterization of a phyllosphere methylotroph community isolated with lanthanides

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Running Title

Lanthanide-dependent methylotroph isolation

Competing Interests

The authors declare no competing financial interests.

Abstract

The influence of lanthanide biochemistry during methylotrophy demands a reassessment of how the composition and metabolic potential of methylotrophic phyllosphere communities are affected by the presence of lanthanides. To investigate this, methylotrophs – predominant members of the phyllosphere– were isolated from soybean leaves by selecting for bacteria capable of methanol oxidation with lanthanide cofactors. Strikingly, of the 344 pink-pigmented facultative methylotroph isolates, none were obligately lanthanide-dependent. Phylogenetic analyses revealed that all strains were nearly identical to each other and to model strains from the *extorquens* clade of *Methylobacterium*, with *rpoB* providing higher resolution than 16s *r*RNA for strain-specific identification. Despite low species diversity, the metabolic capabilities of the community diverged greatly. Strains encoding identical PQQ-dependent alcohol dehydrogenases displayed significantly different growth from each other on alcohols in the presence and absence of lanthanides. Additionally, 3% of our isolates were capable of growth on sugars and 23% were capable of growth on aromatic acids, expanding the range of multicarbon substrates utilized by *extorquens* clade members. Whole-genome sequences of eleven novel strains are reported. We hypothesize that the expansion of metabolic capabilities and differential lanthanide metabolisms among closely related strains point to evolution of niche partitioning strategies to promote colonization of the phyllosphere.

Introduction

The phyllosphere, the aerial portion of plants dominated by leaves[1], is colonized by a diverse community of microorganisms[2–4]. Despite the abundance of potential substrates provided

by plant leaves (carbohydrates, organic acids, amino acids, volatiles), microorganisms in the phyllosphere must overcome waxy leaf cuticles, UV light, temperature fluctuations, low water availability, and other biotic and abiotic impediments to colonization[2,3]. Cultivation-independent studies of the phyllosphere have only emerged within the last decade[3]. A proteogenomic study characterized the phyllosphere community of *Glycine max* (soybean) and showed high levels of proteins characteristic of the *Alphaproteobacteria* class and specifically, *Methylobacterium* genus. Methylotrophs of the *Methylobacterium* genus are so-named due to their capacity to utilize reduced one-carbon compounds (e.g., methanol, methylamine, formaldehyde, formate) as their sole source of carbon and energy. In the phyllosphere, methylotrophs are well-suited to take advantage of methanol released by pectin methylesterases during primary cell wall breakdown[4,5] and may even stimulate plants into releasing additional methanol by secreting the plant hormone cytokinin, which triggers plant cell division[2].

In Gram-negative methylotrophs, pyrroloquinoline quinone (PQQ) methanol dehydrogenases convert methanol to formaldehyde[6]. Although the first-identified methanol dehydrogenase from Gram-negative methylotrophs was the calcium (Ca^{2+}) -dependent MxaFI[6,7], recent studies have highlighted the importance of an alternative methanol dehydrogenase, XoxF, which coordinates a lanthanide atom in complex with PQQ, rather than Ca^{2+} [6,8,9]. XoxF is widespread in the environment[10,11] and phylogenetically ancestral to MxaFI[6]. Metaproteomics analysis of the soybean phyllosphere showed comparable protein levels of XoxF to MxaFI[12,13]. In addition to XoxF, ExaF is a lanthanide-dependent alcohol dehydrogenase with sub-nanomolar K_M towards ethanol[14]. Induction of XoxF can be exclusionary of MxaFI at lanthanide concentrations higher than 100 nM, but at lower concentrations (≤ 50 nM) both enzyme systems can be expressed simultaneously[23,24]. Although the importance of lanthanide-dependent alcohol

dehydrogenases in alcohol metabolism is clear[23,24], the catalytic functions and activities of this diverse set of enzymes are only just beginning to be understood[18,23].

Lanthanides have expanded our understanding of methylotrophic metabolism and ecology even beyond the phyllosphere. The addition of lanthanides to the isolation process of microbial communities has led to the discovery of new cultivable methylotrophs in coal slag and marine environments, but this phenomenon has yet to be tested on phyllosphere communities [15,16]. Lanthanides have also unlocked previously unknown metabolic capabilities of already characterized plant symbionts. For example, a *Bradyrhizobium* strain isolated from legumes displayed little to no growth on methanol, despite maintaining genes for methanol metabolism, until the addition of lanthanides to the growth media[17].

Methylotrophs can also take advantage of C1 compounds other than methanol that are present in the phyllosphere, such as methylamine. Methylotrophs have several mechanisms by which they can grow on methylamine. Methylamine dehydrogenases (*mauFBEDACJGLMN*) catalyze the oxidation of methylamine to formaldehyde, which can be further oxidized to formate for assimilation or dissimilation[8,9,11]. Alternatively, methylamine can be converted via the *N*-methylglutamate pathway (*mgdDCBA*, *mgsABC*, *gmaS*) to methylene-tetrahydrofolate for assimilation or dissimilation, although the extent of formaldehyde as an obligate intermediate in this pathway remains unknown[11]. The model organism *M. extorquens* AM1 encodes both pathways yet primarily uses methylamine dehydrogenase during growth on methylamine, whereas *M. extorquens* PA1 only encodes the N-methylglutamate pathways[11,12] and exhibits slower growth on methylamine than *M. extorquens* AM1[12].

Most methylotrophs isolated from the phyllosphere are pink-pigmented facultative methylotrophs (PPFMs)[4], meaning they can grow on C_1 substrates as well as multi-carbon

compounds such as acetate (C_2), pyruvate (C_3), and succinate (C_4). Recently, facultative methylotrophy has expanded to include species capable of growth on sugars[13] within the *extorquens* clade and species capable of growth on methoxylated aromatic acids within the *nodulans* and *aquaticums* clades of *Methylobacterium*[20]. Methylotrophic growth on the latter is predicted to occur via an initial demethoxylation of the methoxy group that is released as formaldehyde to generate protocatechuic acid; formaldehyde can be assimilated or dissimilated via methylotrophic pathways. The capability to utilize different carbon sources [9,10] may explain why methylotrophs are so well-suited to life in the phyllosphere[15,16] where metabolite availability changes rapidly[4.]

Despite extensive characterizations of the microbial community in the phyllosphere as well as recent advances in lanthanide-dependent metabolisms, the effect of lanthanides on methylotrophic community composition in the phyllosphere remains unknown. To investigate this, methylotrophs were isolated from the phyllosphere of soybean plants and selected for growth on methanol in the presence and absence of lanthanides, and then grown on different carbon substrates. Growth parameters were measured with and without the addition of lanthanum (La^{3+}). These strains were found to be phylogenetically similar but phenotypically distinct, with growth rates differentially affected by substrate identity and the presence or absence of lanthanum.

Materials & Methods

Methylotroph isolation

Environmental methylotrophic bacterial strains were isolated from soybean plants (*Glycine max*) growing on the Michigan State University Agronomy Farm (East Lansing,

Michigan, U.S.A. 42.6908, -84.4866). Four leaves were harvested from four different plants on September 7, 2018, and each leaf was placed into a sterile 50 mL conical tube. 50 ml of sterile 50 mM phosphate buffer (pH 7.3) was added to each tube, the tubes were vortexed for 5 minutes, and 100 µL of buffer was spread onto solidified (1.5% wt/vol agar) MP minimal salts media[30]. The isolation medium contained 125 mM methanol, RPMI 1640 Vitamins Solution (Sigma Aldrich, St. Louis, MO, USA) diluted to 1X, and 50 µg/mL cycloheximide. Isolation media was prepared with and without 2 µM $LaCl_3$. Isolation plates were grown at 30 °C for several days until bacterial colonies were visible. Pink colonies were selected from the plates and struck out for isolated colonies onto solidified media of the same composition to confirm growth. Single colonies of isolated strains were then inoculated into 650 µL of MP medium with 125 mM methanol and 1X RPMI 1640 Vitamins Solution in a 48-well microplate. All isolated strains were inoculated into medium with the same concentration of La^{3+} as they were isolated with, and then incubated shaking for 48 hours at 200 rpm on an Innova 2300 platform shaker (Eppendorf, Hamburg, Germany) at 30°C. Strains were frozen for future use by adding 25 µL of sterile dimethyl sulfoxide to 500 µL of late-exponential phase culture in a sterile screw-cap vial, flash-freezing in liquid nitrogen, and storing at -80°C.

Strains cultivation

Cultures used for growing and maintaining strains, extracting DNA, and subculturing for growth phenotypic analyses were prepared as follows: Freezer stocks of each strain were struck out onto MP agar supplemented with 15 mM succinate and 1X RPMI 1640 Vitamins Solution and incubated at 30°C until single colonies emerged (2-4 days). Single colonies of each strain were used to inoculate 3 mL cultures of MP with 15 mM succinate and 1X RPMI 1640 Vitamins Solution and grown overnight in round-bottom glass culture tubes (ThermoFisher Scientific,

Waltham, MA, USA) at 30°C, shaking at 200 rpm on an Innova S44i shaker (Eppendorf, Hamburg, Germany) to an OD_{600} of approximately 1.5.

Chromosomal DNA extraction for whole-genome and marker gene sequencing

Genomic DNA was extracted from 3 mL overnight cultures of each strain (see ***Strains Cultivation***) using the DNeasy PowerSoil Pro Kit from Qiagen (Hilden, Germany) per manufacturer's protocol. Genomic DNA was quantified using a Take3 Microvolume Plate Spectrophotometer (BioTek, Winooski, VT, USA). Whole-genome sequencing via PacBio Sequel II was performed by the Department of Energy's Joint Genome Institute (JGI, Walnut Creek, CA, USA). Genomes can be accessed through JGI Integrated Microbial Genomes & Microbiomes (IMG) (**Table 1**). The full-length *rpoB* sequences from each strain was obtained from IMG, aligned using MAFFT and MUSCLE, and a Maximum-Likelihood tree using a Tamura-Nei model and 100 bootstrap replications was constructed using MEGA. Average nucleotide identity scores were calculated through JGI IMG interface for pairwise genome comparisons using whole-genome sequences from the IMG database as inputs.

Growth phenotypic analyses

Isolate strains were cultivated as described in ***Strains Cultivation*** and were screened for their ability to grow on diverse substrates in the presence and absence of La^{3+}. To screen for the ability to grow on diverse substrates, overnight cultures of each strain were washed twice in MP media with no carbon source at 2000 x g for 10 minutes, resuspended in MP media to an OD_{600} of 0.1, and spotted (10 uL) onto MP agar plates supplemented with 1X 1640 RPMI Vitamins Solution, and either a soluble substrate (20 mM methanol, 34 mM ethanol, 15 mM methylamine, 5 mM potassium oxalate, 25 mM glucose, 25 mM fructose) or an insoluble substrate (5 mM vanillic acid); the insoluble substrates were added as non-sterilized powders directly to the

melted agar prior to pouring the plate. Agar plates were incubated at 30C for 3-5 days or until visible colonies formed.

To screen for La^{3+} effects during growth on methanol, overnight cultures of each strain were washed twice in MP media with no carbon source at 2000 x g for 10 minutes and inoculated at an OD600 of 0.1 into a transparent 96-well plate (Corning, Corning, NY, USA) containing 200 uL of MP media supplemented with 125 mM methanol, 1X RPMI 1640 Vitamins Solution, and +/- 2 μM $LaCl_3$ for endpoint growth assays. Strains with demonstrated growth on solid media of different substrates were additionally phenotyped in liquid media in the presence and absence of La^{3+}. Overnight cultures of strains of interest were washed twice in MP media with no carbon source at 2000 x g for 10 minutes and inoculated at an OD600 of 0.1 into a transparent 48-well plate (Corning, Corning, NY, USA) containing 650 uL of MP media supplemented with 1X 1640 RPMI Vitamins Solution, +/- 2 μM $LaCl_3$, and appropriate carbon source (20 mM methanol, 15 mM methylamine, 34 mM ethanol, 5 mM vanillic acid, 25 mM fructose). All endpoint assays and growth curves were carried out at 30°C and 548 rpm using a Synergy HTX plate reader (BioTek, Winooski, VT, USA) with OD600 readings measured every 30 minutes. Data was analyzed using Microsoft Excel. 2-4 replicates were run for each strain in each condition, and at least 10 exponential-phase data points were used for linear regression analysis to calculate the growth rates of each strain. Paired t-tests were performed to identify statistically significant ($p < 0.05$) differences between growth in the presence or absence of $LaCl_3$ for each strain. One-way ANOVA with post-hoc Tukey HSD was performed to identify statistically significant ($p < 0.05$) differences among all strains in the presence or absence of $LaCl_3$.

Results

The soybean phyllosphere methylotrophic community is not obligately lanthanide-dependent

Methylotrophic bacterial strains were isolated from the soybean phyllosphere by selection on minimal salts media with methanol as the sole carbon source. Previous studies have shown that the presence of lanthanides allows for the isolation of novel methylotrophic strains so lanthanum was also included in the isolation media. Of 344 total isolates, 158 strains were isolated with the addition of La^{3+} and 186 strains without the addition of La^{3+} to the selection medium. After isolating single colonies, strains were retested for growth on methanol in the presence and absence of La^{3+} to determine if lanthanides were necessary for growth. All strains were pink, showed growth on methanol in the presence and absence of La^{3+} in the growth medium, and showed growth on succinate; thus, all strains were classified as pink-pigmented facultative methylotrophs (PPFMs). Each strain was assigned a unique SLI (**S**oybean **L**eaf **I**solate) number, which is used to reference specific strains throughout this study (**Table 1**).

Methylotrophic communities from the soybean phyllosphere are phylogenetically similar

Using NCBI BLAST, *rpoB* sequences from each isolate were compared against the Joint Genome Institute's (JGI) Integrated Microbial Genomes (IMG) database to putatively assign species-level taxonomic classifications to each isolate. All isolates matched to *Methylobacterium* species within group B[18], with the closest species being *Methylobacterium extorquens* AM1, *Methylobacterium extorquens* PA1, *Methylobacterium extorquens* TK001, and *Methylobacterium zatmanii* 135. Untrimmed full-length *rpoB* sequences from each isolate, as well as from reference *Methylobacterium* and related species, were aligned using MUSCLE, and Maximum-Likelihood trees with 100 bootstrap replications were constructed using MEGA

(**Figure 1A**). The *rpoB*-based trees show a high degree of relatedness amongst all isolates, integrating SLI strains among other reference methylotroph strains within the *extorquens* clade.

We sought to augment our understanding of the SLI community composition at the taxonomic level by employing pairwise average nucleotide identity (ANI) comparisons of all orthologous genes shared among all assembled SLI genomes and representative methylotrophic genomes using IMG database. Results from the complete pairwise ANI comparisons can be found in the **Table S1**, but have been condensed to include only SLI strains and representative species from the *extorquens, aquaticum,* and *nodulans* clades in **Figure 1B**. Based on ANI comparisons, all SLI strains were most similar to *Methylobacterium* species from the *extorquens* clade (93-99% ANI) when compared to the *nodulans* (79% ANI) and *aquaticum* clades (78% ANI), indicating that our community likely belongs to the *extorquens* clade. Based on the 95% standard species cutoff[19], all SLI strains except for SLI 516, 575, and 576 are genetically identical to the model organisms *Methylobacterium extorquens* AM1/PA1. Additionally, SLI 231, 233, 274, 285, 499, and 505 appear to be nearly 100% genetically identical to each other; yet, as will be discussed in detail later, they exhibit distinct metabolic differences (see **Table S2** for full comparison of genomes).

Methylotroph isolates possess broad metabolic capabilities independent of lanthanide availability

Although phyllosphere isolates were phylogenetically similar to research strains such as *Methylobacterium extorquens* AM1 and PA1, we hypothesized that environmental methylotrophs might possess expanded substrate repertoires and broader metabolic capabilities. To test this, all isolates were screened for growth on C_1 substrates (methanol, methylamine, dimethylsulfide), C_2 substrates (ethanol, oxalate), sugars (fructose, glucose), aromatic acids

(vanillic acid), and complex insoluble substrates (lignin, cellulose, tyrosine) **(Table 2)**. Lanthanides were also added to growth media, but this addition did not unlock novel substrate utilization capabilities for any SLI tested. All strains exhibited growth on methanol, methylamine, and ethanol. No strains grew on dimethylsulfide, oxalate, glucose, lignin, cellulose, or tyrosine. Notably, 10 strains exhibited growth on fructose and 78 strains grew on vanillic acid.

A subset of SLI strains (SLI 223, 231, 233, 274, 285, 384, 499, 505, 516, 575, 576) that were shown to have broad metabolic capabilities from the screen described above were spotted onto solid media containing five different carbon sources without La^{3+}, with growth of *M. extorquens* PA1 serving as a comparison (**Figure 2A),** to visualize relative differences in metabolic capabilities. From this subset, all grew on methanol, confirming the initial growth phenotype, and all strains also grew on ethanol and methylamine. SLI 231, 233, 499, 505, and 575 grew on vanillic acid. Isolates that grew on vanillic acid also grew more robustly on methylamine, as evidenced by larger and more opaque colony phenotypes. SLI 516, 575, and 576 were the only strains of this subset capable of growth on fructose. Interestingly, these three strains also exhibited biofilm formation during growth in liquid cultures (**Figure 2B-C**) and had heterogeneous colony morphology compared to all other strains (**Figure 2A**). This initial screen revealed that aromatic acid catabolism and fructose utilization are more widespread within the *extorquens* clade than previously assumed[20].

Whole-genome sequencing of representative SLI strains reveals new* extorquens *strains

The genomes of SLI strains 223, 231, 233, 274, 285, 384, 499, 505, 516, 575, 576 were sequenced through the Joint Genome Institute and annotated using the IMG Annotation Pipeline v.5.0.22. Summaries of the genome information for each strain compared to *M. extorquens* AM1

and PA1 are represented in **Table 1**. Notably, all strains but SLI 223 encoded at least one plasmid in addition to the chromosome, with most strains (SLI 231, 233, 274, 285, 499, 505, 516, 575, 576) encoding at least three additional plasmids. The size, % GC, and number of genes per chromosome and per plasmid were very similar for SLI 231, 233, 274, 285, 499, and 505 and for SLI 516, 575, 576; this underscores the high degree of ANI between the strains (**Figure 1B)**. SLI 516, 575, and 576 – which are the least similar by ANI to *M. extorquens* AM1 and PA1 (**Figure 1B**) – also have the smallest chromosome sizes despite having the broadest metabolic capabilities (**Figure 2A**). Interestingly, none of the strains encoded the megaplasmid (1261460 bp) found in *M. extorquens* AM1, and the plasmid sizes of the SLI strains were very different from the plasmid sizes of *M. extorquens* AM1.

All SLI genomes encode calcium-dependent methanol dehydrogenases and cognate cytochromes (*mxaFIG*) and lanthanide-dependent methanol dehydrogenases and cognate cytochromes (*xoxFG*) indicating that all strains are capable of lanthanide-independent and lanthanide-dependent alcohol metabolism. However, all SLI strains but SLI 516, 575, and 576 encode lanthanide-dependent alcohol dehydrogenases and cognate cytochromes (*exaFG*), indicating differences in the influence of lanthanides on metabolism. In addition, all strains encode the complete *lut* cluster involved in lanthanide utilization and transport[21], except for SLI 516, 575, and 576 which lack the gene for the lanthanide-binding protein, lanmodulin. Additionally, all genomes encode tetrahydromethanopterin and tetrahydrofolate-dependent pathways for C_1 oxidation and marker genes for the serine cycle for C_1 assimilation, indicating that all strains are Type II methylotrophs. All strains also encode the *N*-methylglutamate pathway for methylamine utilization (*mgdDCBA*, *mgsABC*, *gmaS*). Strains SLI 231, 233, 274, 285, 499, 505, 516, 575, and 576 encode a previously identified gene island that confers the ability to grow

on methoxylated aromatics[20]. A complete summary of all relevant genes found in the SLI strains is included in **Table S2**.

Addition of lanthanides influences the catabolism of alcohols

Based on our initial screen for substrate utilization on solid media, six representative isolates (SLI 231, 233, 384, 499, 505, 575) were chosen as a test cohort for downstream growth analyses because they displayed expanded metabolic capabilities compared to AM1 or PA1, which cannot grow on sugars or aromatic acids. To further investigate the impact of La^{3+} on this community, the growth rates and total biomass yields of the test cohort strains were compared during growth on methanol and ethanol as lanthanides have been shown to impact the oxidation of both alcohols in methylotrophic bacteria[8].

Growth rates (**Figure 3A, 3C**) and yields (**Figure 3B, 3D**) on 20 mM methanol and 34 mM ethanol, both with and without La^{3+} in the growth medium, were measured. Growth rates on methanol were significantly higher in the presence of La^{3+} for SLI 231, 233, 384, 499, and 505 (**Figure 3A**). Final yields were significantly lower in the presence of La^{3+} for SLI 384 but significantly higher in the presence of La^{3+} for SLI 499 and SLI 505; thus, lanthanides can have variable effects on growth yields despite similar trends in growth rates. Even amongst strains encoding identical lanthanide-independent and -dependent methanol dehydrogenases, there was significant variability in the final yields achieved in the presence of La^{3+} between SLI 575 and SLI 231, SLI 233, SLI 499, and SLI 505 and in the absence of La^{3+} between SLI 233 and SLI 575. Growth rates on ethanol were marginally higher in the presence of La^{3+} for SLI 231, SLI 233, SLI 384, SLI 499, and SLI 505. Although SLI 231, SLI 233, SLI 384, SLI 499, and SLI 505 possess high degrees of genomic similarity (**Figure 1B**) and all strains encode identical lanthanide-dependent enzymes, SLI 384 exhibits significantly higher growth rates than the other

strains in the presence of La^{3} (p-values for significance of all strains from one-way ANOVA in **Table S3**). Unlike the phenotypes with methanol (**Figure 3A-B**), the presence of La^{3+} in the growth medium does not appear to have significant effects on the final yields obtained, except for SLI 505 which exhibited significantly lower yields with La^{3+}.

For most strains, the presence of La^{3+} increases growth rate in methanol but the effect on yields during growth on methanol and ethanol appear to be strain-dependent. Thus, addition of lanthanides did not result in a universal phenotypic change during growth on alcohols despite most strains possessing nearly identical lanthanide-dependent enzymes.

SLI strains encode identical methylamine oxidation genes but display different growth phenotypes

Similar to *M. extorquens* PA1, all SLI genomes encode the *N*-methylglutamate pathway for methylamine utilization. To determine if the growth of SLI strains on methylamine is influenced by lanthanides, SLI strains were grown in 15 mM methylamine in the presence and absence of $LaCl_3$. Growth rates (**Figure 4A**) and final yields (**Figure 4B**) are reported. Addition of $LaCl_3$ did not significantly impact the growth rate or yield for any strain tested. Interestingly, SLI 575 had significantly lower yields than SLI 231, 233, 499, and 509, despite having a similar growth rate to all four strains; this trend was not lanthanide-dependent. Also of note, SLI 384 had nearly triple the lag time and half the growth rate and final yield as other SLI strains despite encoding identical *N*-methylglutamate pathway genes, pointing to additional details about methylamine catabolism in SLI strains beyond the *N*-methylglutamate pathway that result in the diminished growth phenotypes observed.

SLI strains in the extorquens clade have novel multi-carbon substrate utilization capacities

Genes that confer the ability to grow on methoxylated aromatic acids (e.g., ferulic acid, vanillic acid, protocatechuic acid, *p*-hydroxybenzoic acid) have been identified recently in methylotrophs primarily from the *aquaticum* and *nodulans* clades[20]. These genes exist as a genetic island that is notably absent from the *extorquens* clade, save for their presence in *Methylobacterium sp.* AMS5[20]. Our screen isolated 78 strains capable of growth on the methoxylated aromatic acid, vanillic acid. Of the SLI strains with assembled genomes, those capable of growth on vanillic acid encode gene islands identical to each other (**Table S2**). Aromatic acid gene islands in SLI strains include all the genes found in the aromatic acid gene island in *Methylobacterium sp.* AMS5 although specific regulatory genes are in a different order[20]. SLI strains from the test cohort were grown on 5 mM vanillic acid in the presence and absence of $LaCl_3$ and their growth was compared to that of *Methylobacterium sp.* AMS5 (**Figure 5**). SLI 384 does not encode genes for vanillic acid catabolism and therefore does not grow on vanillic acid (**Figure 2**). Despite encoding nearly identical aromatic acid gene islands, the SLI strains have significantly faster growth rates than *Methylobacterium sp.* AMS5 and all but SLI 575 have significantly higher final yields, both in the presence and absence of lanthanides (p-values for significance of all strains from one-way ANOVA in **Table S3**). Lanthanides have little effect on growth rates (except for SLI 505) but variable effects on final yields in SLI 231, SLI 233, SLI 499, and SLI 505. The substantial growth achieved by SLI strains on vanillic acid demonstrates that the ability to grow on methoxylated aromatic acids is widely distributed among members of the *extorquens* clade and not restricted to members of the *nodulans* and *aquaticum* clades, as was previously hypothesized[20].

Most methylotrophs, including *M. extorquens* AM1 and PA1, encode genes necessary for sugar oxidation but lack sugar-specific transporters and assimilatory pathways required for their utilization as a primary substrate[22]. Notably, complete sugar catabolism has only been demonstrated in endophytic *extorquens* clade members[23]. Thus, it was striking to isolate ten strains capable of robust growth on fructose as the sole carbon source (**Figure 2**). Of the strains with assembled genomes, SLI 516, SLI 575, and SLI 576 were capable of growth on fructose. Interestingly, these three SLI strains also displayed the lowest sequence similarity to other SLI strains and to *M. extorquens* AM1 and PA1 based on ANI (**Figure 1B**), hinting that the differences in genomes might be due in part to unknown genes specific to sugar transport and catabolism. To quantify growth on fructose, SLI 575 was grown on 25 mM fructose in the presence and absence of $LaCl_3$ (**Figure 6**). Growth rates and yields in both conditions were nearly identical. To our knowledge, SLI 516, 575, and 576 are the first identified epiphytic *extorquens* strains capable of growth on a sugar substrate.

Strain designation of SLI community

Based on the expanded metabolic capabilities of our SLI strains as compared to their genetically-similar *M. extorquens* AM1 and PA1 counterparts, we propose that these are novel *Methylobacterium extorquens* strains. We have named the strains using the nomenclature *M. extorquens* PS for **P**hyllosphere **S**oybean, inspired by *M. extorquens* PA1 for **P**hyllosphere **A**rabidopsis (**Table 1**).

Discussion

Methylotrophs that inhabit the phyllosphere influence both the plants that they colonize and global biogeochemical processes[1,4]. Yet, studies investigating the physiology, biodiversity,

and ecology of methylotrophic communities of the phyllosphere have only emerged within the last decade[3,24] and do not take into account the role of lanthanides in influencing their community composition. Furthermore, despite the abundance of methylotrophs in the phyllosphere, the bulk of phylogenomic studies on *Methylobacterium* species use genomes isolated from non-phyllosphere environments. Here, we describe the first community-level characterization of the role of lanthanides on natural methylotrophic populations in soybean plants, revealing novel insights into methylotrophic biodiversity, metabolic capabilities, and lanthanide-dependent metabolisms.

Marker gene sequencing for assigning taxonomic groups has proven difficult in *Methylobacterium*, as16s *r*RNA has poor phylogenetic resolution in *Methylobacterium* [18]. We characterized our soybean methylotroph community composition using *rpoB* (**Figure 1A**), a highly polymorphic single-copy gene with sub-species resolution with *Methylobacterium*[18]. Our phylogenetic analysis revealed two insights: 1) all of our isolates are closely related to each other (**Figure 1A**) and fall within the *extorquens* clade of *Methylobacterium* (**Figure 1A-B**), and 2) even amongst nearly identical strains (**Figure 1B**), there is broad diversity in terms of the possession of lanthanide-related genes and metabolic capabilities, and that these differences are reflected in how strains group based on *rpoB* sequences (**Figure 7**). Overall, our results substantiate *rpoB* as a superior marker gene for phylogenetic analysis of *Methylobacterium* species.

Crops, such as soybeans, can sequester lanthanides from the soil through repeated cycles of harvest limiting lanthanide availability in the soil and affecting lanthanide availability for methylotrophs in the phyllosphere. Thus, it is not surprising that our microbial isolation from soybean plants did not reveal any novel lanthanide-dependent phenotypes despite previous

studies identifying organisms that have increased heterogeneity in the presence of lanthanides [37] or that are strictly dependent on lanthanides for growth[22-24]. The maintenance of both calcium-dependent and lanthanide-dependent methanol dehydrogenase systems in our SLI strains could suggest that 1) lanthanides are not always present or bioavailable in the phyllosphere and therefore MxaFI remains essential for methylotrophic growth; 2) lanthanides are present but extensively competed for, so calcium-dependent methanol oxidation systems offer an alternative for methylotrophic growth; and/or 3) lanthanide-dependent enzymes have additional non-metabolic or non-lanthanide-related functions that necessitate their maintenance. These hypotheses could be examined further by repeating our isolations in non-domesticated phyllosphere environments where lanthanides are more prevalent or bioavailable[13] and where the isolation of obligately lanthanide-dependent methylotrophs might be more likely.

Notably, SLI 575 does not encode ExaF or lanmodulin and is the only strain that does not exhibit significant lanthanide-dependent growth phenotypes on alcohols (**Figure 3**). As lanmodulin is reported to be one of the most highly expressed peptides in the phyllosphere[31], has been proposed to function as a lanthanide biosensor, and has been shown to bind lanthanides[25,26] it is possible that SLI 575 is lacking the sensor required to coordinate lanthanide availability with metabolic processes and the role of lanmodulin in the phyllosphere specifically remains unknown. SLI 575 also lacks annotated PQQ-dependent ethanol dehydrogenases, but grows on ethanol (**Figure 3C-D**) but MxaF and XoxF have previously been demonstrated to be the predominant enzymes for ethanol oxidation *in vivo*[14].

Previous studies have reported that the model organism *M. extorquens* PA1 has an average growth rate of 0.042 h^{-1} and an average final yield of OD_{600} 0.712 on 15 mM methylamine[27,28]. While our SLI strains exhibit similar growth rates regardless of $LaCl_3$ addition

(**Figure 4A**), the final yields are nearly half of what is achieved by PA1 (**Figure 4B**); this suggests metabolic bottlenecks towards efficient methylamine assimilation that are not immediately obvious from a preliminary genomic analysis of C1 assimilation genes. One possibility is that methylamine that is incorporated via the N-methylglutamate pathway preferentially serves as a nitrogen source rather than a carbon source[27,28] but future studies are required to validate this hypothesis in our SLI strains

Although the ability to utilize methanol as a substrate has provided a competitive advantage for methylotrophs in the phyllosphere, sugars and aromatic acid are abundant enough to support the growth of diverse microorganisms[2,20,29]. It is therefore advantageous for microbes to be able to utilize both multicarbon and one-carbon sources when available. Detailed investigations into methylotrophic aromatic acid catabolism in the presence and absence of lanthanides is necessary to understand how this metabolism functions in carbon cycling of lignin by-products in natural environments. In addition, genomic analyses for fructose transporters or assimilatory pathways in SLI 516, 575, or 576 (SLI strains with assembled genomes capable of growth on fructose) identified genes encoding for phosphofructokinase that were absent in all other SLI strains. Phosphofructokinase is a key regulatory enzyme of glycolysis, responsible for the phosphorylation of fructose to fructose-6-phosphate. The end-product of this reaction is also an intermediate of the assimilatory RuMP cycle; however, none of our SLI strains employ the RuMP cycle for assimilation.

Taken together, the methylotrophic community in the phyllosphere of soybeans is metabolically diverse and heavily influenced by lanthanide availability when consuming alcohols. We hypothesize that the capacity to expand substrate repertoires might also be important drivers of efficient colonization. Whether the selection for low species diversity is at

the level of the plant or arises through natural selection within methylotrophic communities, this finding points to the evolution of niche partitioning strategies among a single species to maximize resources in shared habitats. Results from this study pave the way for future work to investigate 1) expanded metabolic capabilities in *extorquens* clade methylotrophs, 2) tradeoffs associated with lanthanide-dependent and -independent alcohol metabolism, and 3) niche partitioning among genetically identical strains during plant colonization.

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Competing Interests

The authors declare no competing financial interests.

Data Availability

The data generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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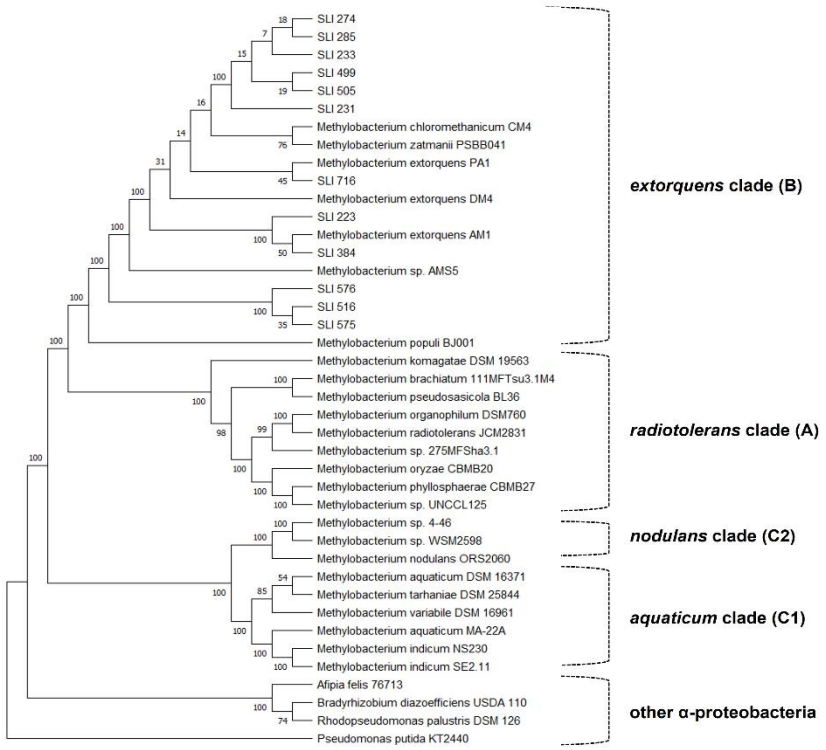
Figures and Tables

Table 1. Summary of SLI strain genomes. Whole-genome sequencing performed by Joint Genome Institute and annotations obtained using the IMG Annotation Pipeline v.5.0.22

Strain Information			Chromosome			Plasmid		
Strain	**Proposed Name**	**IMG Genome ID**	**Size (bp)**	**% GC**	**No. of genes**	**Size (bp)**	**% GC**	**No. of genes**
M. extorquens AM1	-	644736386	5511322	68.71	5029	1261460 44195 37858 24943	67.65 67.93 65.25 66.94	1168 33 34 30
M. extorquens PA1	-	641228497	5471154	68.18	4939	-	-	-
SLI 223	*M. extorquens* PS 223	2917523761	5504362	68.23	5222	-	-	-
SLI 231	*M. extorquens* PS 231	2918956041	5373034	68.29	5165	166358 27292 29644	67.47 61.51 58.78	176 25 27
SLI 233	*M. extorquens* PS 233	2932223180	5373474	68.29	5287	166399 27296 29645	67.47 61.51 58.79	181 28 27
SLI 274	*M. extorquens* PS 274	2947147188	5374052	68.29	5202	166386 27292 29646	67.47 61.51 58.79	178 26 27
SLI 285	*M. extorquens* PS 285	2932228704	5375017	68.30	5295	166399 27292 29655	67.47 61.51 58.78	182 25 28
SLI 384	*M. extorquens* PS 384	2918961435	5504365	68.23	5233	28462	67.45	48
SLI 499	*M. extorquens* PS 499	2947152626	5373409	68.29	5218	166383 27292 29645	67.47 61.51 58.79	178 25 27
SLI 505	*M. extorquens* PS 505	2918966707	5372985	68.29	5170	166379 27292 29645	67.47 61.51 58.79	176 26 27
SLI 516	*M. extorquens* PS 516	2918972107	5009907	68.66	4762	199519 28884 20194	67.26 66.85 60.93	215 39 30
SLI 575	*M. extorquens* PS 575	2918977154	5009893	68.66	4764	199519 28890 20194	67.26 66.84 60.93	214 39 30
SLI 576	*M. extorquens* PS 576	2918982202	5009856	68.66	4762	199518 28890 20194	67.26 66.84 60.93	215 39 31

A



B

	Methylobacterium aquaticum MA-22A	Methylobacterium extorquens AM1	Methylobacterium extorquens PA1	Methylobacterium nodulans ORS 2060	SLI 223	SLI 231	SLI 233	SLI 274	SLI 285	SLI 384	SLI 499	SLI 505	SLI 516	SLI 575	SLI 576	SLI 716
Methylobacterium aquaticum MA-22A	100.0	79.5	79.3	82.5	79.3	79.4	79.4	79.4	79.4	79.3	79.4	79.4	79.2	79.2	79.2	79.3
Methylobacterium extorquens AM1	79.4	100.0	97.3	78.8	99.8	97.4	97.3	97.4	97.4	99.8	97.4	97.4	93.3	93.3	93.3	97.3
Methylobacterium extorquens PA1	79.3	97.3	100.0	78.9	97.3	97.4	97.4	97.4	97.4	97.3	97.4	97.4	93.3	93.3	93.3	97.4
Methylobacterium nodulans ORS 2060	82.5	78.9	78.9	100.0	78.9	79.0	79.0	79.0	79.0	78.9	79.0	79.0	78.9	78.9	78.9	78.9
SLI 223	79.3	99.8	97.3	78.8	100.0	97.4	97.4	97.4	97.4	100.0	97.4	97.4	93.4	93.4	93.4	97.4
SLI 231	79.3	97.4	97.4	78.9	97.4	100.0	100.0	100.0	100.0	97.4	100.0	100.0	93.5	93.5	93.5	97.4
SLI 233	79.3	97.3	97.4	78.9	97.4	100.0	100.0	100.0	100.0	97.4	100.0	100.0	93.5	93.5	93.5	97.4
SLI 274	79.3	97.4	97.4	78.9	97.4	100.0	100.0	100.0	100.0	97.4	100.0	100.0	93.6	93.6	93.6	97.4
SLI 285	79.4	97.4	97.4	78.9	97.4	100.0	100.0	100.0	100.0	97.4	100.0	100.0	93.5	93.5	93.5	97.4
SLI 384	79.3	99.8	97.3	78.8	100.0	97.4	97.4	97.4	97.4	100.0	97.4	97.4	93.4	93.4	93.4	97.4
SLI 499	79.4	97.4	97.4	78.9	97.4	100.0	100.0	100.0	100.0	97.4	100.0	100.0	93.5	93.5	93.5	97.4
SLI 505	79.3	97.4	97.4	78.9	97.4	100.0	100.0	100.0	100.0	97.4	100.0	100.0	93.5	93.5	93.5	97.4
SLI 516	79.2	93.3	93.3	78.9	93.4	93.5	93.5	93.5	93.5	93.4	93.5	93.5	100.0	100.0	100.0	93.4
SLI 575	79.2	93.3	93.3	78.9	93.4	93.5	93.5	93.5	93.5	93.4	93.5	93.5	100.0	100.0	100.0	93.4
SLI 576	79.2	93.3	93.3	78.9	93.4	93.5	93.5	93.5	93.5	93.4	93.5	93.5	100.0	100.0	100.0	93.4
SLI 716	79.3	97.3	97.4	78.9	97.4	97.4	97.4	97.4	97.4	97.4	97.4	97.4	93.4	93.4	93.4	100.0

Figure 1. Phylogenetic and taxonomic characterization of a methylotrophic community isolated from the soybean phyllosphere. (**A**) Phylogenetic tree based on *rpoB* sequences. Untrimmed *rpoB* sequences from each isolate as well as from reference *Methylobacterium* and related species were obtained from IMG, aligned using MUSCLE, and the Maximum-Likelihood trees were constructed with 100 bootstrap replications using MEGA. Groupings indicate clade identity for each strain[18] (**B**) Average nucleotide identity of all SLI strains compared to representative species from the *extorquens*, *nodulans*, and *aquaticum* clades. All pairwise comparison ANIs found in **Table S1**

Table 2. Distribution of substrate-utilization capabilities of SLI community. All 344 isolates were grown in liquid minimal salts media containing either C1 (20 mM methanol, 15 mM methylamine, 1 mM dimethylsulfide), C2 (34 mM ethanol, 5 mM potassium oxalate), sugar (25 mM fructose, 25 mM glucose), or complex insoluble substrates (vanillic acid, cellulose, lignin, tyrosine)

	Substrate	**No. of SLI strains**
C_1 Substrates	Methanol	344
	Methylamine	344
	Dimethylsulfide	0
C_2 Substrates	Ethanol	344
	Potassium Oxalate	0
Sugars	Glucose	0
	Fructose	10
Insoluble C_{2+} Substrates	Vanillic Acid	78
	Lignin	0
	Cellulose	0
	Tyrosine	0

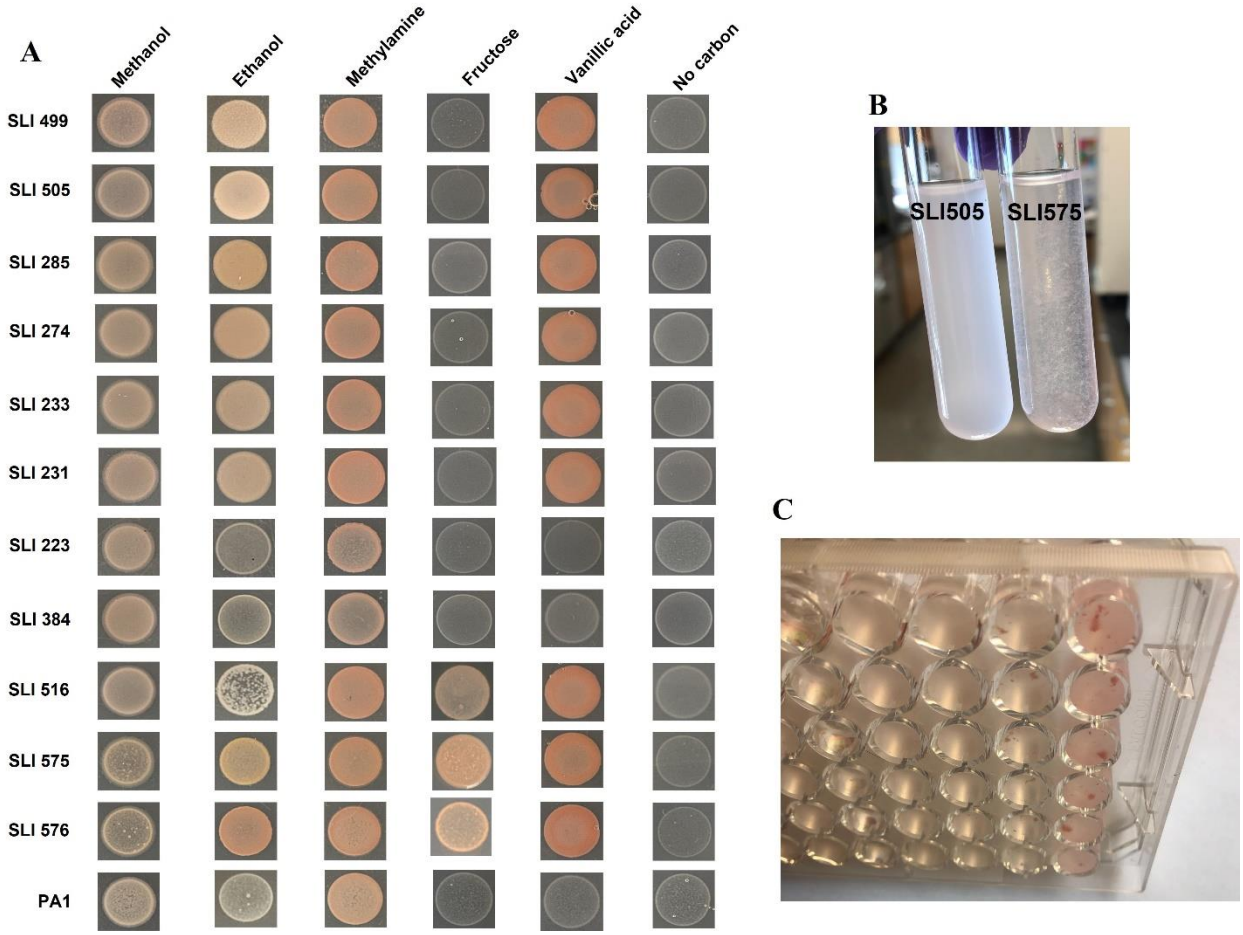


Figure 2. (A) Spotting of a subset of SLI strains on various substrates to demonstrate differential metabolic capabilities of SLI test cohort. All strains were spotted on minimal agar supplemented with either 20 mM methanol, 34 mM methanol, 15 mM methylamine, 5 mM vanillic acid, 25 mM fructose, or no carbon. *Methylobacterium extorquens* PA1Δ*cel* as a comparison of known substrate utilization capabilities in a model *extorquens* clade strain. Biofilm formation of SLI 575 in liquid culture **(B**) and in microplates (**C**).

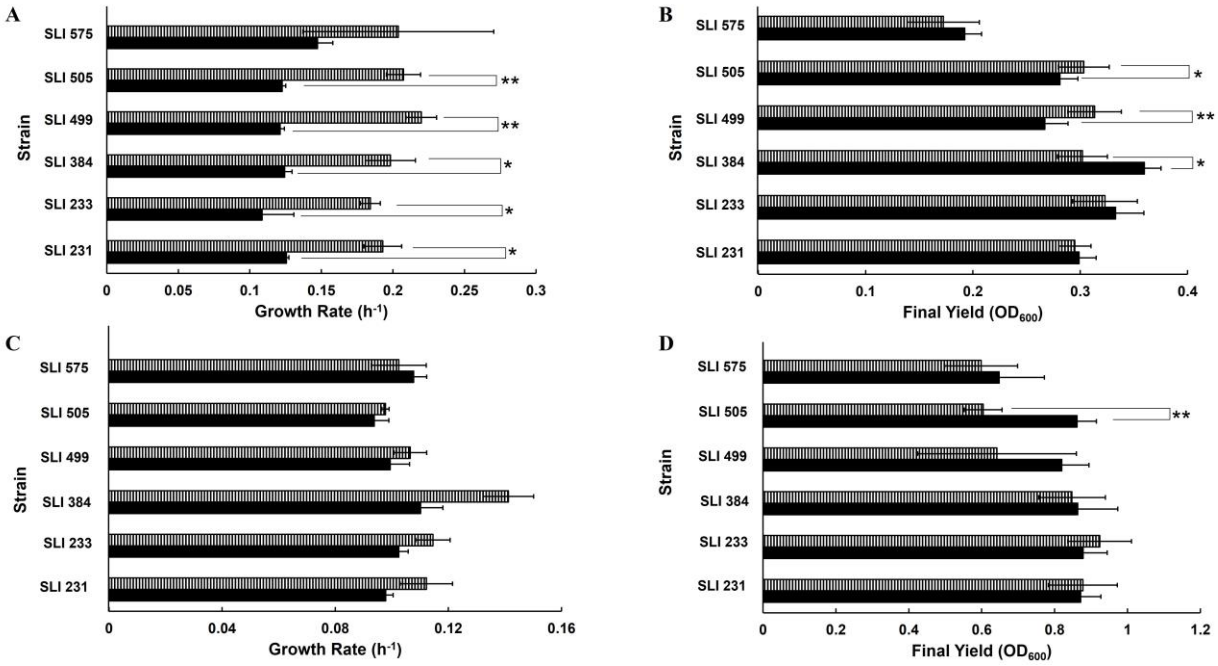


Figure 3. Growth phenotypes of SLI test cohort on alcohols in the presence and absence of La^{3+}. (**A**) Growth rates on 20 mM methanol +/- 2 µM $LaCl_3$ (**B**) Final yields on 20 mM methanol +/- 2 µM $LaCl_3$ (**C**) Growth rates on 34 mM ethanol +/-2 µM $LaLaCl_3$ (**D**) Final yields on 34 mM ethanol +/- 2 µM $LaCl_3$. Solid bars (bottom) represent cultures grown in the absence of $LaCl_3$; hatched bars (top) represent cultures grown in the presence of $LaCl_3$. N=3. Error bars represent standard deviation. Significant differences for each strain +/- $LaCl_3$ determined by Student's paired t-test (*, $p<0.05$; **, $p<0.01$). Significant differences between all strains +/- $LaCl_3$ determined using one-way ANOVA with post-hoc Tukey HSD (**Table S3**)

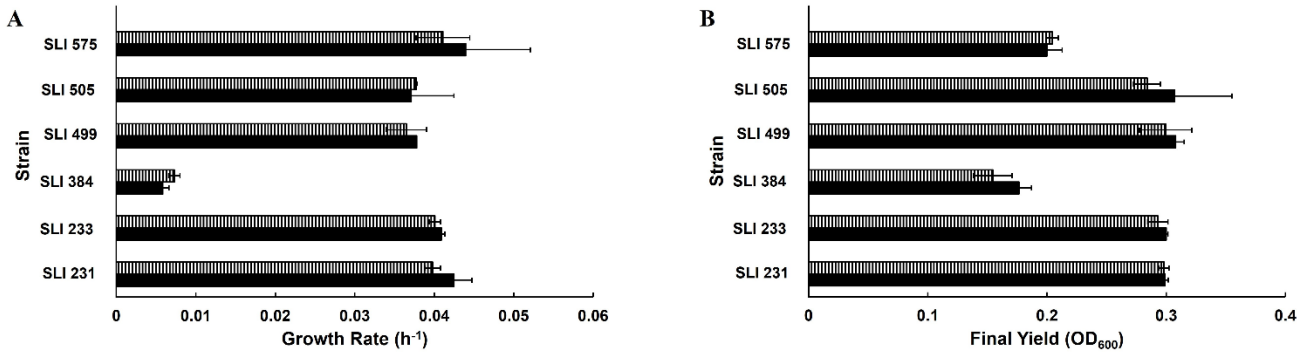


Figure 4. Growth phenotypes of SLI test cohort on methylamine in the presence and absence of La^{3+}. (**A**) Growth rates on 15 mM methylamine +/- 2 μM $LaCl_3$ (**B**) Final yields on 15 mM methylamine +/- 2 μM $LaCl_3$. Solid bars (bottom) represent cultures grown in the absence of $LaCl_3$; hatched bars (top) represent cultures grown in the presence of $LaCl_3$. N=3. Error bars represent standard deviation. Significant differences for each strain +/- $LaCl_3$ determined by Student's paired t-test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$). Significant differences between all strains +/- $LaCl_3$3 determined using one-way ANOVA with post-hoc Tukey HSD (**Table S3**)

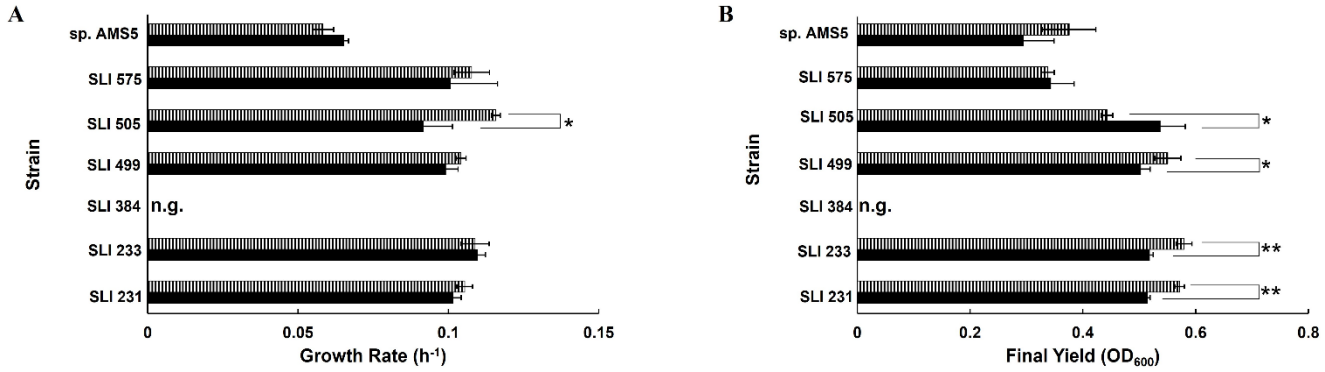


Figure 5. Growth phenotypes of SLI test cohort and *Methylobacterium sp.* AMS5 on vanillic acid in the presence and absence of La^{3+}. (**A**) Growth rates on 5 mM vanillic acid +/- 2 µM $LaCl_3$ (**B**) Final yields on 5 mM vanillic acid +/- 2 µM $LaCl_3$. n.g., no growth. Solid bars (bottom) represent cultures grown in the absence of $LaCl_3$; hatched bars (top) represent cultures grown in the presence of $LaCl_3$. N=3. Error bars represent standard deviation. Significant differences for each strain +/- $LaCl_3$ determined by Student's paired t-test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$). Significant differences between all strains +/- $LaCl_3$ determined using one-way ANOVA with post-hoc Tukey HSD (**Table S3**)

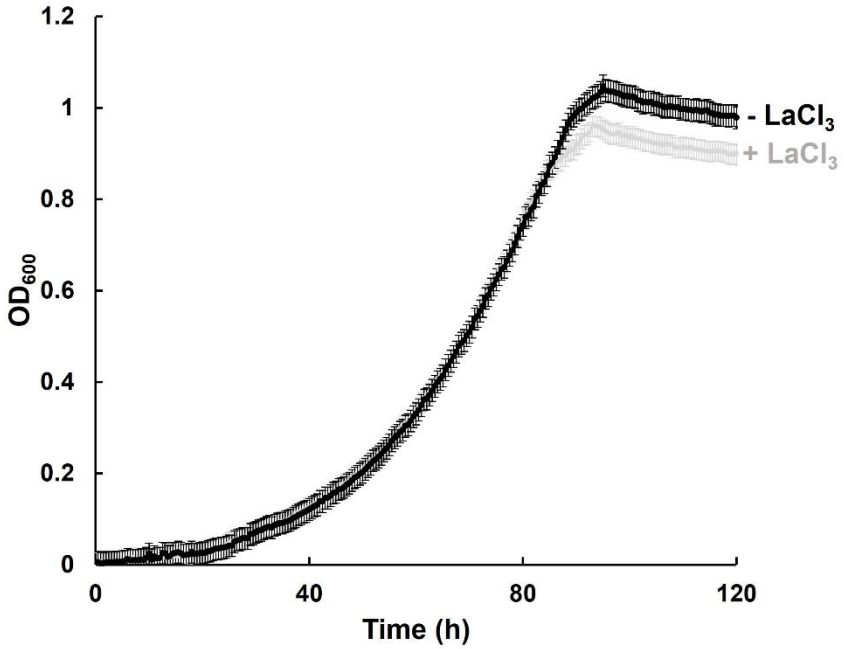


Figure 6. Growth curves of SLI 575 on 25 mM fructose +/- 2 μM $LaCl_3$. Black curves represent cultures grown in the absence of $LaCl_3$; grey curves represent cultures grown in the presence of $LaCl_3$. N=4. Error bars represent standard deviation.

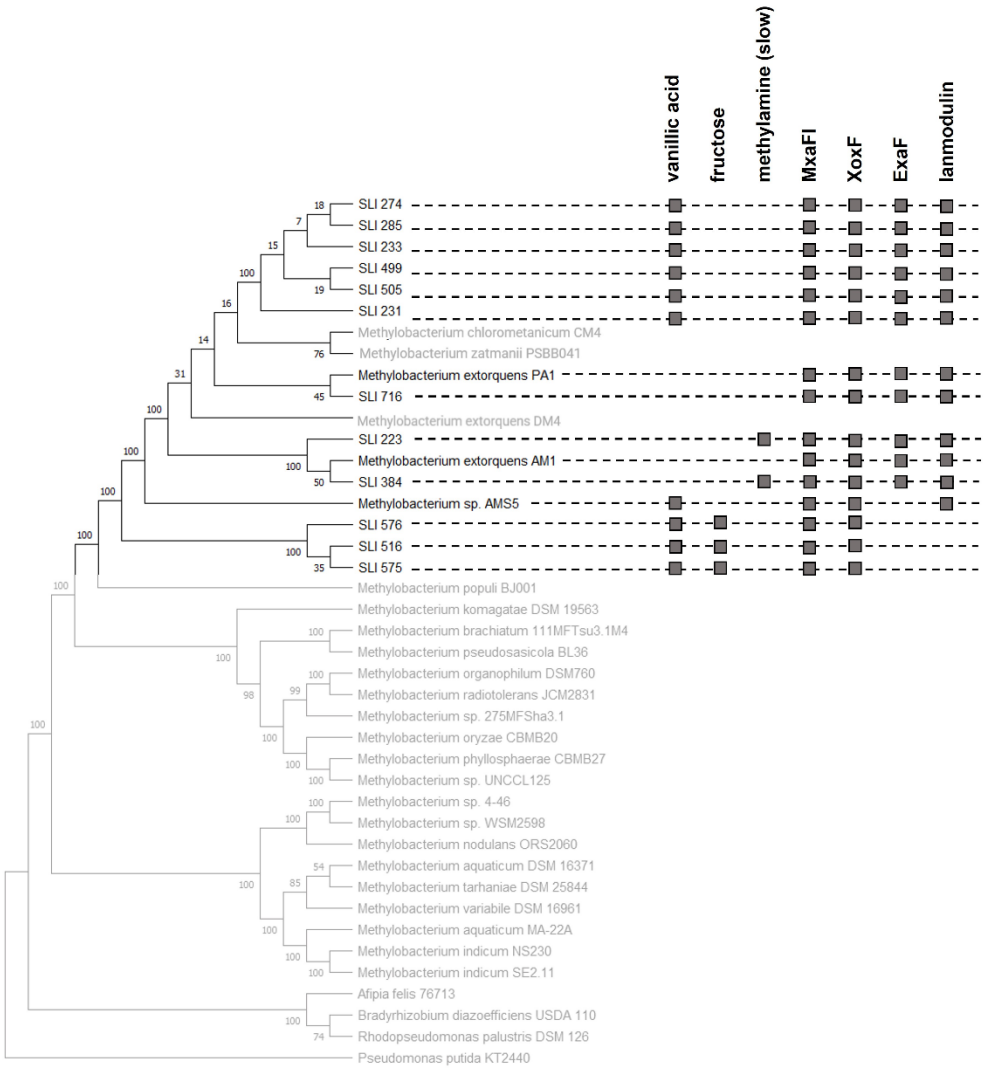


Figure 7. Metabolic capabilities (growth on vanillic acid, growth on fructose, slow growth on methylamine) and lanthanide-related gene products (MxaFI, XoxF, ExaF, lanmodulin) present in SLI strains and model *extorquens* clade species referenced in this study (*M. extorquens* AM1, *M. extorquens* PA1, *M. sp.* AMS5). Phylogenetic tree modified from **Figure 1A.**