Unusual Sulfur Requirements During Laboratory Growth of *Leutibacter*

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Abstract

Many terrestrial bacteria are assumed to utilize sulfate transport and metabolism as a means for fulfulling cellular sulfur requirements. As such, many types of defined minimal media for bacterial growth under laboratory conditions contain sulfate as their sulfur source. Herein, an exception to this assumption is described as sulfate transport capabilities have been lost at least (and maybe twice) in lineages of plant and fungi associated *Leutibacter.* However, *Leutibacter* sp. 9143 can grow in minimal media when sulfur is supplemented as parts of both organic (cysteine and methionine) as well as inorganic (thiosulfate) compounds, and when co-cultured with its original fungal host. Furthermore, an independent strain of *Leutibacter* (UNC366Tsa5.1) potentially posseses an even narrower sulfur requirement than *Luteibacter* sp. 9143. These results highlight both the suprising sulfur requirements *Luteibacter* strains, which may be illustrative of close ties between these strains and associated eukaryotes, as well as a need for caution when interpreting novel auxotrophies based off of differential growth in minimal versus rich media.

Introduction

Since sulfur fundamentally enables critical physiological and chemical interactions within and between cells, it is an essential nutrient required across all life(Sekowska, Kung, and Danchin 2000). Given the importance of sulfur for various biochemical processes, it is of no surprise that proteobacteria have evolved a variety of mechanisms to obtain this sometimes limiting element. While these pathways range from scavenging sulfur from inorganic (e.g sulfates and sulfides) or organic (e.g. cysteine, methionine, and sulfonates) sources, many proteobacterial species are thought to rely on sulfate transporters CysAUW and CysP to internalize this element (Sekowska, Kung, and Danchin 2000; Kertesz 2001). The prevalence of sulfate transport and metabolism has led this compound to form the basis of sulfur supplementation for a variety of different types of defined growth media for aerobic bacteria (e.g. M9 and MOPS).

Luteibacter is a bacterial genus, relatively closely related to Xanthomonads, often found as members of plant and fungal microbiomes (Lundberg et al. 2012; Hoffman and Arnold 2010). Although little is known about their precise ecological functions, *Luteibacter* strains have been demonstrated to act as plant growth promoting bacteria and may do so by producing phytohormones in partnership with endophytic fungi (Hoffman et al. 2013). *Luteibacter* sp. 9143 was originally isolated as an endohyphal bacterial strain found within an isolate of the Ascomycete *Pestaliotiopsis (Arendt et al. 2016)*. In characterizing growth patterns across different media types for *Luteibacter* sp. 9143, I observed that this and closely related strains grew within rich media and when co-cultured with its fungal host in M9 media but failed to grow in minimal media when grown by itself, suggestive of auxotrophy. A combination of selective supplementation, as well as screening of Biolog plates, demonstrates that this strain is not an auxotroph in the traditional sense but simply cannot utilize sulfate as a sulfur source. Further experiments show that *Luteibacter* sp. 9143 can grow in M9 media if supplemented with either

cysteine, methionine, or high levels of thiosulfate and also when co-cultured with its fungal host. Lastly, the requirement for sulfate-independent sulfur supplementation appears to be widespread across *Luteibacter* but that some strains possess more limited sulfur scavenging capabilities than *Luteibacter* sp. 9143. These results suggest that strains of *Luteibacter* have evolved a unique metabolic niche for proteobacteria, potentially reliant on associations with other hosts, in order to obtain an essential element for growth.

Methods

Strains. The focal *Luteibacter* strain used across experiments in this study is DBL564, a rifampicin resistant isolate of *Luteibacter* sp. 9143 described in Arendt et. al 2016(Arendt et al. 2016). DBL966 is a rifampicin resistant isolate of *Luteibacter* sp. UNCMF366Tsu5.1 first described in Lundeberg et. al (Lundberg et al. 2012)and obtained from the Dangl Lab.

Culture Conditions. For all growth curve experiments, populations of each *Leutibacter* strain were streaked from frozen stocks to Lysogeny Broth (LB) agar plates supplemented with rifampicin at 50 ng/uL and grown at 27oC. Prior to beginning the growth curve, a single colony was picked to 2mL liquid LB media containing rifampicin and grown overnight. All liquid cultures were grown at 27oC on a rotating shaker (200rpm). For fungal co-culture experiments, an isolate of *Pestalotiopsis* 9143 was grown for 1 week in 3mL M9 media on a rotating shaker at 27oC and 200rpm. The fungal mass was then extracted from this culture into 500uL M9, macerated by shaking with glass beads, after which 500uL of additional M9 was added to this tube. A 100uL volume of this resuspension was then added to 10mL M9 with *Luteibacter* sp. 9143 and 2mL aliquots from these master cultures were aliquoted to 4 test tubes for each treatment. Fungal co-culture experiments were carried out twice independently, with negative controls treated as the experimental cultures except without the addition of fungi. For bacterial growth curve experiments, after overnight growth in LB media, 1mL of each culture was pelleted by centrifuge, washed twice in 10Mm MgCl2, resuspended in 1mL 10Mm MgCl2, and a subset of this resuspension was used to inoculate a master culture for each experiment as described below. All growth curves were performed in standard M9 media with 4% glucose (hereafter M9), grown at 27oC on a rotating shaker (200rpm). All M9 media used therefore contained MgSO4 in addition to other sulfur compounds. This base M9 media was independently supplemented with additional chemicals where necessary. To begin a growth curve, 10mL of M9 media with appropriate supplementation was inoculated with 100uL of *Leutibacter* and was aliquoted in 2mL increments to test tubes. Bacterial count at the start of the experiment was sampled by plating a dilution series of this inoculum on LB agar plates supplemented with rifampicin. All cultures were grown for at least 10 days, and cell counts measured accordingly by sampling a small volume (2uL) of each culture and plating a dilution series on LB agar plates supplemented with rifampicin. For Biolog assays, strain DBL564 was sent to Biolog and assayed by their in-house technicians.

Genomewide Sulfur Pathway Comparisons

The complete genome sequence for *Luteibacter* sp. 9143 was described in a previous publication (Baltrus et al. 2017), and all genomic comparisons took place through the Integrated Microbial Genomes (IMG) platform (http://img.jgi.doe.gov) developed by the JGI (Markowitz et al. 2009). Briefly, the genome sequences and annotations for all strains mentioned within Figure 3 were queried for annotations involving all KEGG pathways relevant for sulfur metabolism. All pathways that showed at least one entry for any of these genomes are represented in Figure 3.

Results and Discussion

Co-culture with host fungus enables growth of Luteibacter sp. 9143 in M9 media without supplementation

As part of experiments to establish a co-culture system for *Luteibacter* sp. 9143 and its original host fungus (*Pestalotiopsis* sp. 9143), it was observed that the supernatant of fungal-bacterial cultures in M9 media grew turbid over time only under co-culture conditions but not when bacterial cultures were grown in the same media *sans* fungus (data not shown). More detailed growth curve experiments (Figure 1A), demonstrated that *Luteibacter* sp. 9143 viable cell counts in supernatants increased over time during fungal co-culture but not when the same bacteria were grown in M9 alone. These observations strongly suggested that co-culture with its host fungus (*Pestalotiopsis* sp. 9143) could supplement one or more missing nutrients for *Luteibacter* sp. 9143 growth in M9 media.

Luteibacter species grow in M9 media supplemented with additional sulfur sources

Further growth curve experiments confirmed that *Luteibacter* sp. 9143 was unable to grow in base M9 media, but that this strain could grow if M9 was supplemented with Casamino acids, suggesting that this strain was auxotrophic for at least one amino acid (Figure 1B). Supplementation of M9 cultures with combinations of each amino acid found within Casamino acids (data not shown), suggested that the addition of either methionine or cysteine could support growth of *Leutibacter* sp. 9143 in M9. Full growth curves following these early experimental attempts, as one can see in Figure 1B, clearly show that *Luteibacter* can grow in M9 media if it is supplemented with either 100uM cysteine or methionine. Although this strain reliably grows after supplementation with both 10Mm and 100uM methionine, growth patterns in cysteine are more sporadic likely due to the reactive nature of this amino acid (Supplemental Figure 1). Since growth is apparent after 10 days in each of these experiments regardless of treatment, this time period was chosen as a setpoint going forward for further supplementation assays.

Differential abilities of Luteibacter sp. 9143 *to utilize organic and inorganic sulfur sources*

Given that *Luteibacter* sp. 9143 growth in M9 could be supplemented with either sulfur containing amino acid, the focal strain is not truly an auxotroph in the traditional sense. This set of data rather suggests that, unlike what is commonly assumed for strains of proteobacteria, *Luteibacter* sp. 9143 instead fails to use sulfate as a sulfur source. Following up on these experiments, a variety of other sulphur sources were tested for their ability to supplement *Luteibacter* growth in M9 media. Serine was also included in these assays because its structure is similar to cysteine. None of the assayed compounds, other than methionine and cysteine, could support growth of *Luteibacter* at 100uM (Figure 2A). However, it appears as though sodium thiosulphate can additionally support growth of this strain when supplemented at 10Mm (Figure 2B). These data suggest that *Luteibacter* sp. 9143 can utilize organic sulfur sources like cysteine or methionine for growth under natural conditions, but can use thiosulphate if concentrations are high enough.

The ability of *Luteibacter* sp. 9143 to utilize a variety of sulfur sources was independently assayed using Biolog phenotype array plates (Table 1 and Supplemental file 1). Data from two independent Biolog assays supported growth curve data in that they showed *Luteibacter* sp. 9143 could utilize both cysteine and thiosulfate as sulfur sources and additionally suggested that this strain could utilize Djenkolic acid, lipoamide, and lanthionine. In contrast to repeatable growth curve data, Biolog results do not reliably support the ability of *Luteibacter* sp. 9143 to utilize methionine as a sulfur source.

Genomic Comparisons Suggest that a Clade of Luteibacter *Has Lost the Ability to Import Sulfate*

JGI's IMG server was used to perform pathway function analysis across *Luteibacter* genomes with genomes for both *Dyella* and *Xanthomonas* included as outgroups for these comparisons. As shown in Figure 3, the main genes responsible for sulfate transport in proteobacteria (*cysAUW* and *cysP/sbp*) are present in both outgroups and a subset of *Luteibacter* species, but are absent from the genomes of all strains found as fungal endophytes. These genes are additionally absent from a clade of *Luteibacter* strains isolated from Arabidopsis roots. The most parsimonious interpretation of this pattern is that *cysAUWP* were either independently lost from both clades of *Luteibacter*, or lost in an ancestor of *Luteibacter* and reacquired by a subset of strains including *L. rhizoxinicus*. Either of these scenarios would require two independent events, either two losses of *cysAUWP* or one loss and one gain of *cysAUWP* by *L. rhizoxinicus*.

Genome comparisons suggested that other *Luteibacter* strains would also require a sulfur source other than sulfate for growth, so the growth requirements of *Luteibacter* sp. UNCMF366Tsu5.1 were assayed in M9 media alone and supplemented with sulfur sources shown to promote the growth of *Luteibacter* sp. 9143. Surprisingly, although *Luteibacter* sp. UNCMF366Tsu5.1 was able to grow in M9 supplemented with methionine, it was not able to grow in M9 supplemented with either cysteine or thiosulfate (Figure 4). Two alternate, and not necessarily mutually exclusive, explanations could account for this differential growth behavior: 1) Transport and sulfur utilization for methionine occurs independently from that of cysteine and thiosulfate for these strains and the latter is missing from *Luteibacter* sp. UNCMF366Tsu5.1 2) *Luteibacter* sp.

UNCMF366Tsu5.1 is more sensitive to reactive effects of cysteine and high levels of thiosulfate. Differential growth of both *Luteibacter* strains also argues argues against contamination as the reason *Luteibacter* sp. 9143 could grow in 10Mm but not 100uM thiosulfate. It should be noted that, if *Luteibacter* sp. UNCMF366Tsu5.1 was assayed in the way that *Luteibacter* sp. 9143 was assayed, a natural conclusion from the data would be that this strain was a methionine auxotroph. While it's possible that this is actually the case, given how related strains behave under laboratory conditions, a more likely possibility is potentially that this strain has a more limited sulfur scavenging capability than *Luteibacter* sp. 9143.

Sulfur Acquisition by Luteibacter *Under Natural Growth Conditions*

At a minimum, sulfur is required for growth of all known bacteria under all known conditions because it is a main component of an amino acid (methionine) present in all translated proteins (Sekowska, Kung, and Danchin 2000). Sulfate is one of the most widely available and utilized sulfur sources for terrestrial bacteria, which is highlighted by conservation of the sulfate transport pathway genes *cysAUWP* across many proteobacterial strains including xanthomonads (Pereira et al. 2015). That certain clades of *Luteibacter* have altered abilities to metabolize sulfate compared to closely related outgroups like Xanthomonas, speaks to a difference in ecological niche for these strains compared to their more widely studied relatives.

For both *Luteibacter* sp. 9143 and UNC366Tsa5.1, it is possible that sulfur is naturally acquired through close relationships with fungi and/or plants rather than acquired from other environmental sources. While sulfur concentrations are thought to be relatively low inside of plants compared to the surrounding environment (Sekowska, Kung, and Danchin 2000), it is possible that these strains could be adapted to scavenging for free amino acids or other organic sulfur containing compounds provided by either/both types of eukaryotic hosts. Indeed, while *Luteibacter* sp. UNCMF366Tsa5.1 was isolated from the rhizosphere of Arabidopsis, it remains a possibility that this strain was closely associated with fungi in proximity to these plant roots (Lundberg et al. 2012). Also worth mentioning is that sulpholipids are a main component of plant cells and could be used as a sulfur source by these bacteria.

Alteration of sulfur metabolism and acquisition pathways due to availability of sources other than sulfate has actually been demonstrated multiple times in aquatic bacterial clades (Tripp et al. 2008; Smith et al. 2016; González, Kiene, and Moran 1999). It is hypothesized that an abundance of dimethylsulphoniopropionate (DMSP), supplied by other marine microbes, enabled the loss of sulfur transporters (Reisch, Moran, and Whitman 2011; Moran et al. 2012). In some cases, it has even been shown that DMSP can be supplied by eukaryotes (the diatom *Thalassiosira pseudonana)* forming close relationships with these marine bacteria Ruegeria pomeroyi (Durham et al. 2015). Although not necessarily abundant under terrestrial conditions, tests reported above show that *Leutibacter* sp. 9143 fails to use DMSP as a sulfur source under lab conditions. That sulfate transport has been lost under such a variety of conditions speaks to potentially significant evolutionary or metabolic costs associated with maintenance of sulfate assimilation pathways.

Conclusions

The experiments presented herein clearly demonstrate that a subset of *Luteibacter* strains have lost commonly found pathways that enable utilization of sulfate as a sulfur source Sulfur acquisition by these strains under natural conditions therefore potentially requires access to organic sulfur sources, such as those provided by host organisms, which further suggests that association with host organisms is a key component of the ecological niche for this subset of strains. These results also suggest that one should be cautious when interpreting differential growth of bacteria in rich compared to minimal media as auxotrophy, because it is also possible that the strains of interest have different growth requirements than closely related strains.

Acknowledgements

I thank Kayla Arendt, Betsy Arnold, Kevin Hockett, and Paul Carini for advice with experiments and interpretation. Financial support was provided by the National Science Foundation (NSF IOS-1354219 to D. A. B., Betsy Arnold, and Rachel E. Gallery).

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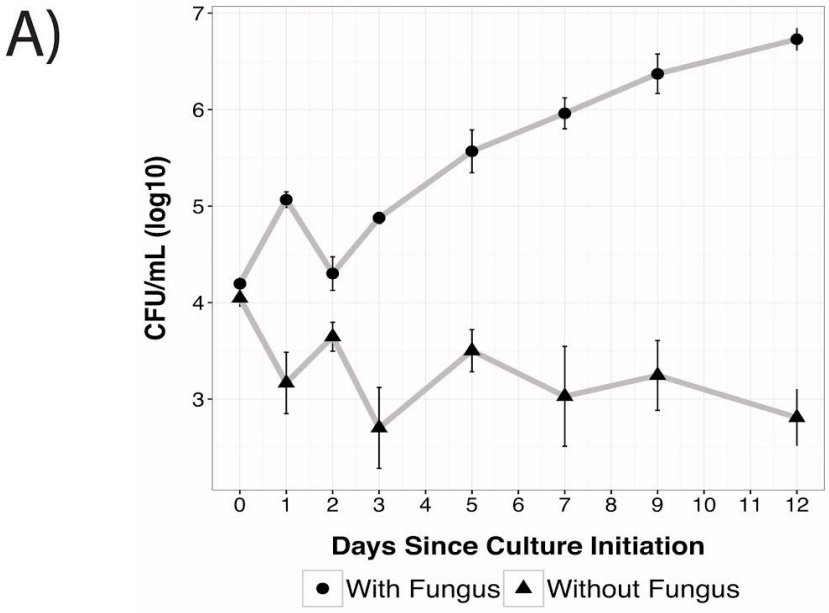
Figure 1. *Luteibacter* sp. 9143 Requires Sulfur Sources Other than Sulphate During Laboratory Growth. A) Representative growth curves of *Luteibacter* sp. 9143 in M9 (triangles) or Co-cultured with the fungus *Pestalotiopsis neglecta* 9143 (circles). Four cultures were grown in each case, and bacterial population sizes in the supernatant were sampled at times indicated. Points represent the average of four samples. B) Growth curves of *Luteibacter* sp. 9143 in M9 media alone (circles), or supplemented with 100uM Methionine (pluses), 100uM Cysteine (squares), Casamino Acids (triangles). Each plotted point shows the average from 8 replicates (over two different experiments) for each treatment combination. In all cases, error bars represent +/- one standard error.



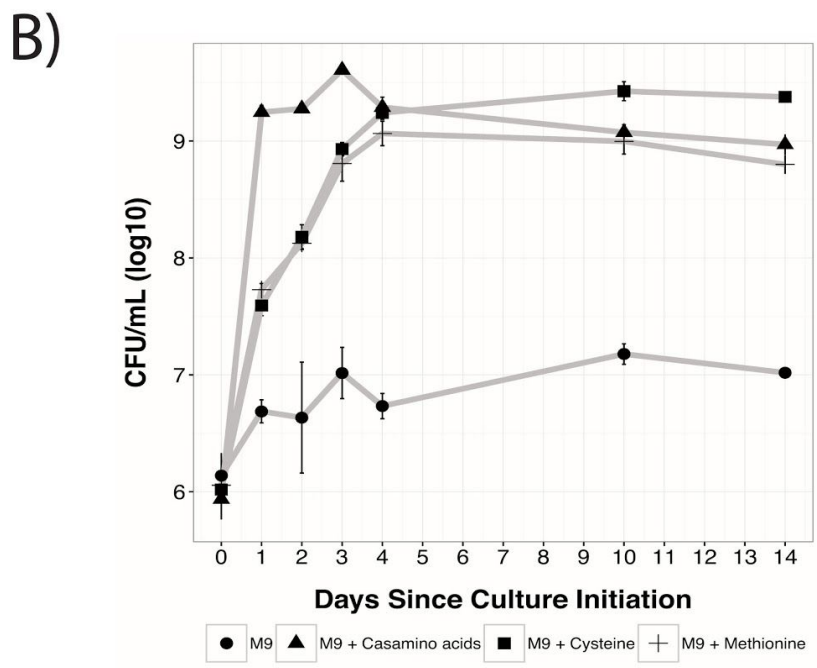


Figure 2. Cysteine, Methionine, and Thiosulphate Can Be Utilized as Sulphur Sources by *Luteibacter* sp. 9143. Plots show each data point for day 0 (white plot) and day 10 (grey plot) sampling of growth of *Luteibacter* sp. 9143 in M9 supplemented with a variety of sulphur sources or other controls. In each case, population sizes were sampled for each culture on both days. A) Cultures supplemented at 100uM with 0.1x Casamino Acids (Cas), Cysteine (Cys), DMSP, Thiosulfate (Thio), Serine (Ser), or unsupplemented (M9). B) Cultures supplemented at 10Mm, with the only difference in treatment being Methionine (Met) instead of cysteine. In all cases, error bars represent +/- one standard error.

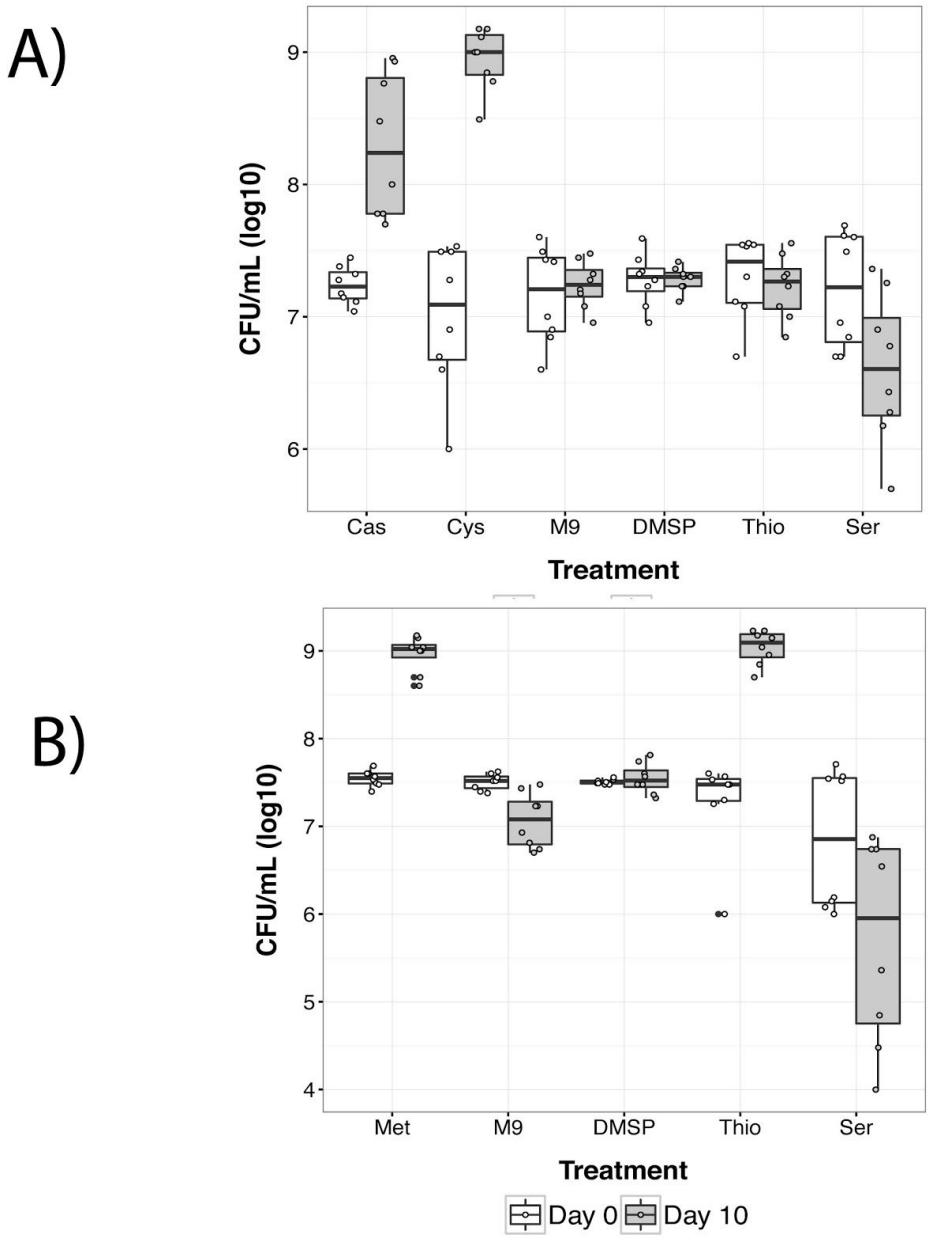


Figure 3. Multiple *Luteibacer* Strains Have Lost Ability to Transport Sulphate. A maximum likelihood phylogeny built using whole genomes sequences from *Luteibacter* strains and related species, with underlying data and methods explained in XX. Phylogeny was built in RealPHY using genomes with an "*" as references. Box on right shows presence (grey) or absence (white) of a variety of genes involved in sulfur utilization from the listed genomes. KEGG classifiers for each gene are shown at top of each column while gene names are shown at bottom. Numbers inside boxes indicate that multiple copies of this gene(s) are found within the genome.

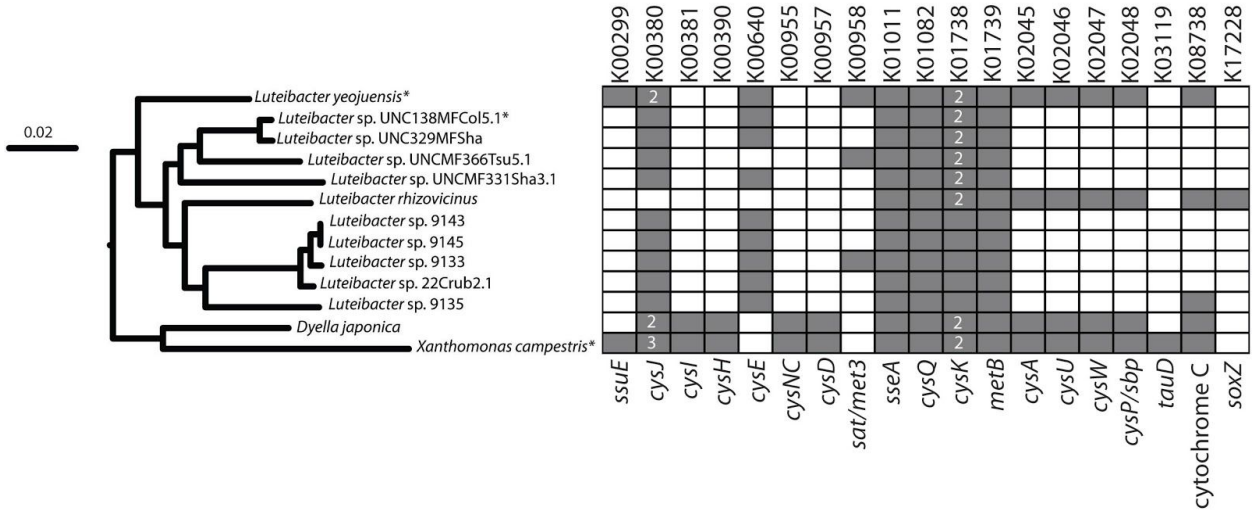


Figure 4. Phenotypic Diversity of Sulphur Utilization Across *Luteibacter* Strains Plots show each data point for day 0 (white plot) and day 10 (grey plot) sampling of growth of *Luteibacter* sp. 9143 (564) and *Luteibacter* sp. UNCMF366Tsu5.1 (966) in M9 supplemented with a variety of sulphur sources or other controls. Methionine and cysteine were supplemented at 100uM while thiosulfate was supplemented at 10Mm. In all cases, error bars represent +/- one standard error.