

1 Lanthanide transport, storage, and beyond: genes and processes contributing to XoxF function in  
2 *Methylobacterium extorquens* AM1

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14 Running Head: Lanthanide transport and storage

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23 **ABSTRACT**

24 Lanthanides are elements that have been recently recognized as “new life metals” in bacterial  
25 metabolism, yet much remains unknown regarding lanthanide acquisition, regulation, and use.  
26 The lanthanide-dependent methanol dehydrogenase XoxF produces formaldehyde, which is  
27 lethal to *Methylobacterium extorquens* AM1 if allowed to accumulate. This property enabled a  
28 transposon mutagenesis study to expand knowledge of the lanthanide-dependent metabolic  
29 network. Mutant strains were reconstructed and growth studies were conducted for over 40  
30 strains detailing the involvement of 8 novel genes in lanthanide-dependent and independent  
31 methanol growth, including a fused ABC-transporter, aminopeptidase, LysR-type transcriptional  
32 regulator, putative homospermidine synthase, *mxuD* homolog (*xoxD*), porin family protein, and  
33 genes of unknown function previously published as *orf6* and *orf7*. Using genetic and biochemical  
34 analyses, strains lacking individual genes in the lanthanide transport cluster were characterized  
35 and named *lut* for **l**anthanide **u**tilization and **t**ransport (*META1\_1778* to *META1\_1787*).  
36 Consistent with previous reports, we corroborated that a TonB-ABC transport system is required  
37 for lanthanide transport to the cytoplasm. However, an additional outer membrane transport  
38 mechanism became apparent after longer growth incubations. Additionally, suppressor mutations  
39 that rescued growth of the ABC-transporter mutants were identified. Transcriptional reporter  
40 fusions were used to show that like iron transport, expression from the TonB-dependent receptor  
41 promoter, *lutH*, is repressed when lanthanides are in excess. Energy dispersive X-ray  
42 spectroscopy analysis was used to visualize the localization of lanthanum in wild-type and  
43 TonB-ABC transport mutant strains and showed for the first time, that *M. extorquens* AM1  
44 stores cytoplasmic lanthanides in mineral form.

45

46 **IMPORTANCE**

47 Understanding the role of lanthanides in bacterial systems is an emergent field of study. Results  
48 from this work define mechanisms by which the methylotrophic bacterium, *M. extorquens* AM1,  
49 acquires and accumulates lanthanides. Lanthanides are critical metals for modern technologies  
50 and medical applications, yet lanthanide mining is cost-prohibitive and harmful to the  
51 environment. Methylotrophs are an attractive platform for the recovery of lanthanides from  
52 discarded electronics, mining leachate, and other waste streams as these microorganisms have  
53 been proven to sense and transport these metals for use in alcohol oxidation. Further,  
54 methylotrophs are effective biotechnological platforms for chemical productions and can use  
55 pollutants such as methane, and inexpensive feedstocks such as methanol. Defining the  
56 lanthanide acquisition, transport, and accumulation machinery is a step forward in designing a  
57 sustainable, clean platform to recover lanthanides in an efficient and less environmentally  
58 destructive manner.

## 59 INTRODUCTION

60 Lanthanide metals (Ln) impact the metabolism of many Gram-negative methylotrophic bacteria  
61 at both catalytic and transcriptional levels (1–3). Diverse types of pyrroloquinoline quinone  
62 (PQQ)-containing alcohol dehydrogenases (ADHs), specifically from the XoxF- and ExaF-  
63 families, have been shown to coordinate Ln in the active site (4–9) where they act as potent  
64 Lewis acids to facilitate a hydride transfer from the alcohol to PQQ, prompting alcohol oxidation  
65 (7, 10, 11). Some XoxF-methanol dehydrogenases (MeDHs) catalyze sequential oxidations to  
66 produce formate from methanol (12, 13), while formaldehyde is the product of other XoxF-  
67 MeDHs (8). Though much progress has been made regarding the catalytic role of Ln in ADH  
68 reactions, relatively little is known about how these Ln are acquired and incorporated into the  
69 enzymes that use them, and if they are stored intracellularly.

70         The role of Ln in metabolism is not exclusively catalytic. When Ln are transported into  
71 the cell, a transcriptional response occurs; this effect is commonly referred to as “the Ln-switch”  
72 or “rare earth-switch” (14–17). During the Ln-switch, the *mx*a operon is downregulated and  
73 transcript levels of the *xox* operon are upregulated (8, 15–19). In *M. extorquens* strains AM1 and  
74 PA1, the MxbDM two-component system and *xoxF* have been shown to be required for  
75 expression of the *mx*a genes and repression of the *xox* genes in the absence of Ln (20–22).  
76 However, suppressor mutations in the *mx*bD sensor kinase can arise which bypass the need for  
77 XoxF, presumably by constitutively activating the MxbM response regulator (3, 21, 22).

78         The machinery necessary for Ln transport is in the early stages of characterization and is  
79 predicted to be analogous to siderophore-mediated iron transport (22, 23). In *M. extorquens*  
80 AM1, ten genes predicted to be involved in Ln transport and utilization are clustered together in  
81 the genome (*META1\_1778 – META1\_1787*) and encode a TonB-dependent receptor, an ABC-

82 type transporter, four exported proteins of unknown function, lanmodulin which binds Ln, and an  
83 additional periplasmic-binding protein capable of Ln binding (*META1\_1781*) (23, 24). TonB-  
84 dependent receptors are commonly used for the transport of metal ions such as iron ( $\text{Fe}^{3+}$ ) and  
85 nickel ( $\text{Ni}^{2+}$ ) that form poorly soluble salts under physiological conditions (25, 26). To facilitate  
86 the uptake of insoluble  $\text{Fe}^{3+}$ , these receptors have high affinity for metal-siderophore complexes.  
87 Siderophores are small molecules (hydroxamates, catecholates, and carboxylates) that are  
88 excreted by many bacteria and fungi to chelate insoluble metals (27–29). TonB is anchored in the  
89 inner membrane and spans the periplasmic space to interact with TonB-dependent receptors in  
90 the outer membrane. Once the siderophore- $\text{Fe}^{3+}$  complex binds to the TonB-dependent receptor,  
91 it is transported through the receptor into the periplasm using the proton motive force harnessed  
92 by the TonB-ExbB-ExbD energy transducing system (30–32). In the periplasm, the siderophore-  
93  $\text{Fe}^{3+}$  complex binds to a periplasmic binding protein that facilitates its interaction with an ABC-  
94 type transporter to translocate the siderophore-bound  $\text{Fe}^{3+}$  to the cytoplasm (33, 34). Iron release  
95 from the siderophore complex involves a reduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ .

96 In *M. extorquens* strain PA1, genetic studies disrupting either the TonB-dependent  
97 receptor or deletions spanning multiple genes in the Ln transport cluster showed the requirement  
98 of some but not all genes in this cluster for Ln-dependent methanol growth (22). Accordingly,  
99 Mattocks et al. identified homologs of this system in *M. extorquens* AM1 and showed that a  
100 putative exported protein encoded by *META1\_1781* can efficiently bind Ln from lanthanum  
101 ( $\text{La}^{3+}$ ) to gadolinium. Additionally, they showed that the periplasmic binding component of the  
102 ABC transporter encoded by *META1\_1778* was unable to bind Ln (23), possibly because it  
103 instead binds the predicted siderophore-like molecule necessary to transport Ln into the cell.

104           Currently, the regulation of the Ln-transport cluster genes is not understood. Iron  
105 transport is highly regulated as Fe<sup>2+</sup> excess can promote Fenton chemistry and lead to the  
106 production of highly reactive radicals, which damage DNA, proteins, and other biomolecules  
107 (35). In bacteria such as *Escherichia coli*, the ferric uptake regulator (Fur) and the small RNA,  
108 *rhyB*, help to mediate iron homeostasis. When Fe<sup>2+</sup> accumulates, Fur binds to iron uptake  
109 operons including *fecABCDE* (36), *fhuACDB* (37), *cirA* (38), *tonB* (39) and *exbB-exbD* (37) to  
110 repress iron transport and prevent oxidative stress. Herein, we show that like the iron paradigm,  
111 expression from the Ln TonB-dependent receptor is repressed by Ln when they are in excess.

112           In this study, we describe new pieces necessary to complete the Ln puzzle: the  
113 identification of novel transcriptional regulators and genes of unknown function, and the  
114 discovery and visualization of Ln storage in *M. extorquens* AM1. Growth phenotypes are  
115 reported for over 40 strains in methanol media containing and lacking Ln and reveal  
116 requirements for novel genes in both Ln-dependent and Ln-independent methanol growth. A  
117 detailed phenotypic characterization for strains lacking each component of the first 8 genes in the  
118 Ln transport cluster is described, including the ability of these strains to mutate or acclimate to  
119 allow Ln transport through a predicted secondary mechanism. Ln uptake was also quantified for  
120 several transport mutant strains. Finally, we show that *M. extorquens* AM1 not only incorporates  
121 Ln into the cytoplasm, but also stores Ln as crystalline cytoplasmic deposits. To our knowledge,  
122 this is the first demonstration of this behavior by a methylotrophic microorganism known to use  
123 Ln for metabolism.

124 **RESULTS**

125 **XoxF produces formaldehyde *in vivo*.** While XoxF can produce formate directly from  
126 methanol *in vitro*, XoxF contributes to formaldehyde production *in vivo* in *M. extorquens* AM1  
127 (8). To expand these findings, strains lacking *fae* (encoding a formaldehyde-activating enzyme)  
128 and the different ADHs were constructed and tested for growth on solid La<sup>3+</sup> medium containing  
129 both succinate and methanol as carbon sources (Fig. 1, Table 1). To confirm that loss of the  
130 Ca<sup>2+</sup>-dependent MxaFI-MeDH does not affect growth of the *fae* mutant when Ln are present, an  
131 *fae mxaF* double deletion mutant strain was tested as a control. Loss of *xoxF1* alone allowed  
132 survival of the *fae* mutant strain, while additional loss of *xoxF2* allowed growth similar to the  
133 wild-type strain (Table 1). Loss of neither *exaF* nor *mxoF* conferred methanol resistance to the  
134 *fae* mutant strain, indicating that MxaFI and ExaF are not major contributors to formaldehyde  
135 production *in vivo* when Ln are present.

136

137 **Identification of gene products that contribute to Ln-dependent methanol growth.** A

138 transposon mutagenesis study was designed to take advantage of the *in vivo* formaldehyde  
139 production capability of XoxF to identify genes required for Ln-dependent methanol oxidation.  
140 The strain used to conduct the mutant hunt contained a mutation in *mxoF* to make cells  
141 dependent on Ln for formaldehyde production, and a second mutation in *fae*, which would result  
142 in formaldehyde accumulation and death of the cells when methanol was provided as a substrate  
143 (Fig. 1). Transposon mutants with disruptions in genes involved in processes required for Ln-  
144 dependent methanol oxidation were selected for on medium containing succinate, methanol,  
145 La<sup>3+</sup>, tetracycline (Tc), and rifamycin (Rif). Transposon insertions that reduced or eliminated  
146 formaldehyde production allowed survival and colony formation since methanol resistant strains

147 could use succinate for growth. In addition to genes required for methanol oxidation, this mutant  
148 hunt had the potential to isolate insertions in an unknown formaldehyde transport system, which  
149 would theoretically reduce formaldehyde levels in the cytoplasm (Fig. 1).

150 Over 600 transposon mutants were isolated, and their insertion locations mapped to the  
151 *M. extorquens* AM1 genome. A variety of colony sizes were evident indicating that in some  
152 mutants, formaldehyde production was eliminated while in others, it might only have been  
153 reduced. Since it is likely that a portion of these transposon mutants became methanol-resistant  
154 due to spontaneous second-site suppressor mutations and not a transposon insertion, only genes  
155 that were identified three or more times were considered for further analysis and are listed in  
156 Table 2. Among the genes identified were those with obvious roles in methanol oxidation such as  
157 PQQ biosynthesis and the *xoxFGJ* genes. Three different clusters predicted to function in  
158 cytochrome *c* biogenesis and heme export (*cyc* and *ccm* genes) (reviewed in (40)) were identified  
159 along with insertions in an *mxuD* homolog gene (41). Insertions were isolated in six of the Ln  
160 transport cluster genes including the ABC transporter components, the TonB-dependent receptor,  
161 and two putative exported proteins (Fig. 2A). Notably, insertions were not isolated in Ln-binding  
162 proteins, lanmodulin and *META1\_1781* (23, 24). Additional genes of unknown function were  
163 also discovered, including a LysR-type transcriptional regulator, a fused ABC transporter, a  
164 porin family protein, a putative homospermidine synthase, a cytosol aminopeptidase, and *orf6*  
165 and *orf7*, which are located downstream of the *pqqABCDE* operon (42). Based upon the work  
166 detailed within, we propose to name the Ln transport cluster genes as *lut* for **l**anthanide  
167 **u**tilization and **t**ransport (Fig. 2A).

168



169 **Identification of novel components involved in methanol growth independent of Ln.** To

170 assess if the identified genes are specifically involved in Ln-dependent methanol oxidation or  
171 general methanol oxidation, 24 genes were chosen for reconstruction in *mxoF* and/or wild-type  
172 strain backgrounds. Methanol growth was assessed in the presence and absence of  $\text{La}^{3+}$  for all  
173 strains. As expected, deletion of genes in either of the two *pqq* biosynthesis operons  
174 ( $\Delta pqqBCDE$ ;  $\Delta pqqF$ ) eliminated Ln-dependent and independent methanol growth as did  
175 mutations in each of the three identified cytochrome *c* biogenesis and heme export clusters:  
176 *cycK*, heme lyase; *ccmB*, heme exporter; and *ccmC*, heme exporter (Table 3).

177 Novel genes affecting methanol metabolism were identified (Table 3). First, a fused  
178 ABC-type transporter (*META1\_2359*) mutant was unable to grow in methanol medium with or  
179 without  $\text{La}^{3+}$ . After 85-100 h, second site suppressor mutations arose that allowed the strain to  
180 grow to similar culture densities as the wild-type strain but at a reduced growth rate. Second,  
181 *META1\_3908* encodes a putative leucyl aminopeptidase that shares 38% identity and 57%  
182 similarity with PepA from *E. coli* MG1655. Loss of *META1\_3908* resulted in a long growth lag  
183 (24 h without  $\text{La}^{3+}$ ; 15 h with  $\text{La}^{3+}$ ) and an approximate 60% decrease in growth rate in both  
184 conditions. The aminopeptidase appears to be in an operon based on overlap with *META1\_3909*,  
185 a putative membrane protein of unknown function. Deletion of *META1\_3909* did not result in a  
186 growth defect in methanol media with or without  $\text{La}^{3+}$  (data not shown). Third, loss of *hss*  
187 (*META1\_2024*) encoding a putative homospermidine synthase resulted in a relatively short lag  
188 and an approximate 35% reduction in growth rate in methanol medium containing or lacking  
189  $\text{La}^{3+}$ . Finally, while a LysR-type regulator (*META1\_0863*) was hit nine times in the transposon  
190 mutagenesis study, loss of this LysR-type regulator only resulted in an approximate 10%  
191 decrease in growth rate in liquid methanol media. However, on solid methanol media, loss of

192 *META1\_0863* had a more pronounced defect with colonies half the size of wild type (data not  
193 shown).

194  
195 **Identified genes specific to Ln-dependent methanol growth.** It was previously shown that  
196 *xoxF* is required for growth in methanol medium that lacks Ln as XoxF is required for expression  
197 of the MxaFI-MeDH (14, 21). Deletions were constructed in the *xoxG* and *xoxJ* genes to confirm  
198 that *xoxGJ* are required for La<sup>3+</sup>-dependent, but not La<sup>3+</sup>-independent methanol growth in *M.*  
199 *extorquens* AM1 as recently shown for *M. extorquens* PA1 (22). In the absence of La<sup>3+</sup>, loss of  
200 *xoxG* resulted in a growth reduction of 21% when compared to wild-type growth, while the *xoxJ*  
201 growth defect was subtle (7% reduction) but with a 21 h lag (Table 3.) However, in methanol  
202 La<sup>3+</sup> medium, loss of either *xoxG* or *xoxJ* was equivalent to loss of both *xoxF1* and *xoxF2*  
203 indicating that XoxGJ are essential for XoxF-dependent methanol oxidation as suggested by  
204 recent biochemical studies (43–45). Transcriptional reporter fusion studies to assess expression  
205 from the *mx*a promoter using a fluorescent reporter confirmed that unlike *xoxF*, neither *xoxG* nor  
206 *xoxJ* was required for expression of the *mx*a genes in succinate plus methanol medium (average  
207 relative fluorescence units (RFU)/OD<sub>600</sub>: wild type, 322.9 ± 62.5; *xoxF1 xoxF2*, 3.1 ± 1.3; *xoxG*,  
208 313.9 ± 24.0; *xoxJ*, 512.5 ± 54.9) with the *xoxJ* mutant resulting in a 1.9-fold upregulation from  
209 the *mx*a promoter. These data are consistent with XoxF presence, but not XoxF activity, being  
210 required for expression of the *mx*a genes as reported for *M. extorquens* PA1 (22).

211 The MxaD homolog, *META1\_1771*, shares 43% identity and 58% similarity to the *M.*  
212 *extorquens* AM1 MxaD protein, which has been suggested to facilitate interactions (directly or  
213 indirectly) between a MeDH and its cytochrome (41). Loss of the *mx*aD homolog did not cause a  
214 growth defect in methanol medium lacking La<sup>3+</sup> but displayed an ~30% reduction in growth rate

215 when  $\text{La}^{3+}$  was provided (Table 3). These data suggest an important but non-essential role for the  
216 MxaD homolog in Ln-dependent methanol oxidation.

217 Marker-less deletion strains lacking either *orf6* or *orf7* were constructed and methanol  
218 growth was assessed in the presence and absence of  $\text{La}^{3+}$ . In methanol medium with  $\text{La}^{3+}$ , growth  
219 of the *orf6* mutant strain mirrored that of the *xoxF1 xoxF2* double mutant while growth of the  
220 *orf7* mutant was faster and like that of the *xoxF1* single mutant (Table 3). Since these genes are  
221 downstream of the operon encoding the PQQ biosynthetic pathway (*pqqABCDE*), addition of  
222 PQQ was tested to see if it would rescue the growth defect seen for the *orf6* and *orf7* mutants.  
223 While 1  $\mu\text{M}$  PQQ was able to rescue growth of the *pqqBCDE* and *pqqF* mutant strains, PQQ did  
224 not rescue growth of the *orf6* or *orf7* mutants (data not shown).

225 Finally, an outer membrane porin-like gene (*META1\_5071*) was identified in the  
226 transposon mutant hunt. Insertions were constructed in wild-type and *mxoF* strain backgrounds  
227 and growth in the presence and absence of  $\text{La}^{3+}$  was assessed. A slight growth defect was  
228 observed in the wild-type background in methanol  $\text{La}^{3+}$  medium (~10% reduction). However, in  
229 the absence of *mxoF* when cells require Ln for growth, a further growth reduction was observed  
230 (~20% reduction) along with a growth lag of 21 h.

231  
232 **Genetic characterization of the lanthanide utilization and transport cluster.** The increasing  
233 evidence that Ln-dependent enzymes are widely distributed among different microbial taxa and  
234 environments leads to the parallel question of how these elements are scavenged for use in living  
235 systems. Our transposon mutagenesis studies demonstrate that genes encoding homologs of the  
236 TonB- and ABC-dependent  $\text{Fe}^{3+}$  scavenging pathways play a role in methanol metabolism when  
237 Ln are present. In addition to the transport system homologs, two of the four putative exported

238 proteins were identified as important for Ln-dependent growth. To facilitate a detailed genetic  
239 characterization of this gene cluster (Fig. 2A), mutations in individual genes from *lutA* through  
240 *lutH* were constructed in wild-type and *mxoF* mutant backgrounds and tested for growth in the  
241 presence and absence of  $\text{La}^{3+}$  (Table 3, Fig. 2C, Fig. S1). Mutants lacking individual transport  
242 cluster genes (*lutABEFG*) were complemented by expressing the respective gene in pCM62 (46)  
243 and growth similar to the wild-type strain was restored in each case (data not shown).

244 In the absence of *mxoF*, *M. extorquens* AM1 must obtain Ln for methanol growth (14)  
245 thus providing a condition to assess gene requirements for Ln utilization. Loss of the putative  
246 exported proteins that were not hit in the transposon mutant hunt either did not result in a growth  
247 defect (*mxoF lutD*) or decreased the growth rate by ~30% (*mxoF lutC*) (Table 3, Fig. 2C).  
248 Complementation of *lutC* was not tested so it is formally possible that the decrease in growth rate  
249 was due to polarity onto *lutEFG*. However, the *lutC* mutation was constructed such that adequate  
250 space for a *rut* site required for Rho-dependent polarity is not present (Table S1). In the *mxoF*  
251 strain background, loss of the remaining *lut* genes encoding putative exported or periplasmic  
252 components resulted in a significant growth defect. Specifically, loss of *lutA* (periplasmic  
253 binding component of ABC transporter) and the downstream putative exported protein encoded  
254 by *lutB* resulted in a ~88% reduction in growth rate, while strains lacking *lutG* displayed an  
255 ~81% reduction in growth rate (Fig. 2C, Table 3). The growth observed for these strains was not  
256 identified as second-site suppression or acclimation in our tests. These data show that while the  
257 periplasmic components of the Ln transport cluster are important to facilitate Ln utilization and  
258 transport, they are either not essential or have redundancy. This non-essentiality is in contrast  
259 with the requirement for the ATPase and membrane components of the ABC transporter (*LutE*  
260 and *LutF*, respectively), and the TonB-dependent receptor (*LutH*). Loss of *lutE* or *lutF* in the

261 *mxoF* strain background did not allow growth (Fig. 2C). However, after ~200 h and 150 h  
262 respectively, second-site suppressor mutations arose which facilitated growth albeit 88% slower  
263 than that of the wild-type strain (Fig. S1, Table 4). The *mxoF lutE* and *mxoF lutF* strains retained  
264 the ability to grow in methanol with  $\text{La}^{3+}$  after succinate passage, indicating a genotypic change.  
265 In contrast, after 90-120 h, growth of the *mxoF lutH* strain occurred in only ~60% of the cultures  
266 (Fig. S1, Table 4). If growth did occur, this strain could grow without a lag once inoculated into  
267 fresh methanol  $\text{La}^{3+}$  medium and exhibited wild-type colony sizes on solid methanol medium  
268 similar to the wild-type strain (data not shown). However, if the culture was instead first streaked  
269 onto minimal succinate medium to obtain isolated colonies, then inoculated into or onto fresh  
270 methanol  $\text{La}^{3+}$  media (liquid or solid), the strain lost the ability to grow indicating that the  
271 original growth was due to acclimation, not suppressor mutations.

272 As reported by Ochsner et al. (22), loss of *lutH* alone did not result in a growth defect in  
273 methanol  $\text{La}^{3+}$  medium consistent with the hypothesis that LutH is needed for transport of Ln  
274 into the cell and lack of Ln transport through the outer membrane enables growth with methanol  
275 via the  $\text{Ca}^{2+}$ -MxaFI MeDH. For all other strains disrupted in the *lut* gene cluster, growth with a  
276 functional *mxoF* gene was similar to growth of strains lacking *mxoF* (Table 3). Intriguingly, loss  
277 of the membrane and ATPase components of the ABC transporter eliminated growth in the wild-  
278 type background (Fig. 2C, Table 4). However, suppressor mutations arose in the *lutE* and *lutF*  
279 mutant strains after 75-91 h. A second transposon mutant hunt revealed that insertions which  
280 restored the ability of the *lutE* mutant to grow on methanol  $\text{La}^{3+}$  medium mapped to the TonB-  
281 dependent receptor, *lutH*, consistent with expression of *mxoF* when cells are unable to sense Ln.  
282 These data suggest that Ln must enter the cytoplasm in order to upregulate expression of the *xox*  
283 and *exaF* ADHs and that Ln uptake into the periplasmic space may be enough to repress *mxo*

284 expression. It is also possible that Ln must first enter the cytoplasm to be released from the  
285 predicted siderophore-like molecule (recently termed, lanthanophore (47)) to be incorporated  
286 into XoxF. One possibility does not exclude the other. These discoveries suggest new hypotheses  
287 to be tested regarding the Ln-switch.

288

289 **Quantification of lanthanide uptake.** The Arsenazo dye-based assay (48) was used to quantify  
290  $\text{La}^{3+}$  levels in culture supernatants (spent media) from wild type; *lutA*, *lutE*, *lutF* (ABC  
291 transporter); and *lutH* (TonB-dependent receptor) mutant strains during growth (Fig, 3A). Strains  
292 were grown in methanol medium containing 2  $\mu\text{M}$   $\text{La}^{3+}$  and limiting succinate (3.75 mM) as  
293 *lutA*, *lutE*, and *lutF* mutant strains are unable to grow or grow poorly with methanol as a sole  
294 carbon source (Table 3). Under these conditions, no significant decreases in  $\text{La}^{3+}$  levels were  
295 observed for any strain during early- to mid-exponential phase ( $\text{OD}_{600} = 0.4$ ) (Fig. 3B). As  
296 cultures continued to grow and succinate was likely depleted, significant differences in  $\text{La}^{3+}$   
297 levels became apparent. At an  $\text{OD}_{600}$  of  $\sim 0.7$ , the supernatants of wild-type cultures contained  
298  $1.0 \pm 0.1 \mu\text{M}$   $\text{La}^{3+}$  (Fig. 3B). Similar levels were measured for the *lutA*, *lutE*, and *lutF* mutant  
299 strains ( $1.1 \pm 0.1 \mu\text{M}$   $\text{La}^{3+}$ ) suggesting Ln are still transported through the outer membrane. The  
300 *lutA*, *lutE*, *lutF* mutant strains were unable to grow to an OD higher than 0.68 (Fig. 3A), but after  
301 8 h in stationary phase, the  $\text{La}^{3+}$  concentration in the supernatant continued to decrease to  $0.7 \pm$   
302  $0.1 \mu\text{M}$  (data not shown). Levels of  $\text{La}^{3+}$  in the supernatants from *lutH* mutants grown to the  
303 same OD were significantly higher ( $1.5 \pm 0.1 \mu\text{M}$ , Fig. 3B). Although a *lutH* mutant strain can  
304 grow to a similar density as the wild-type strain, it is hypothesized that unlike wild type, the *lutH*  
305 mutant grows using MxaFI for methanol consumption regardless of Ln presence, and therefore,  
306 differences in  $\text{La}^{3+}$  levels in the supernatant are expected. In stationary phase,  $\text{La}^{3+}$  levels in the

307 supernatants from wild-type cultures were half of those found for the *lutH* mutant cultures ( $0.6 \pm$   
308  $0.1 \mu\text{M}$  and  $1.3 \pm 0.1 \mu\text{M}$ , respectively) (Fig. 3B). Decrease of  $\text{La}^{3+}$  in the *lutH* culture  
309 supernatants may reflect adsorption of  $\text{La}^{3+}$  onto the surface of the cells or interaction of  $\text{La}^{3+}$   
310 with lipopolysaccharide, a phenomenon observed for other metals in different bacterial species  
311 (49). Additionally, the growth and acclimation data for the *mxoF lutH* strain suggests a  
312 secondary mechanism for Ln transport through the outer membrane (Table 3, Fig. S1).

313  
314 **Visualization of  $\text{La}^{3+}$  accumulation in *lut* transporter mutants.** To further investigate the  
315 roles for the components of the TonB-ABC transport system, location of  $\text{La}^{3+}$  was assessed by  
316 transmission electron microscopy (TEM) in the *lutA*, *lutE*, *lutF*, *lutH* mutant strains. TEM  
317 coupled with energy dispersive X-ray spectroscopy (EDS) has been used to determine the  
318 elemental composition of cellular inclusions (50, 51) while  $\text{La}^{3+}$  has been widely used as an  
319 intracellular and periplasmic stain for electron microscopy (52–54). Here, we show that  $\text{La}^{3+}$  can  
320 be directly identified by TEM if accumulated inside *M. extorquens* AM1 cells. Strains were  
321 grown in methanol medium containing  $20 \mu\text{M}$   $\text{La}^{3+}$  and limiting succinate ( $3.75 \text{ mM}$ ). Samples  
322 with post-fixation by  $\text{OsO}_4$  and staining with 2% uranyl acetate allowed the outer and inner  
323 membrane of the bacterial cells to be distinguished (Fig. 4A-E left subpanels), while  
324 visualization without fixation and staining enabled metal content analysis by removing the  
325 interaction between  $\text{Os}^{8+}$  and phosphate, which interferes with  $\text{La}^{3+}$  measurements (Fig. 4A-E  
326 right subpanels). Mutants lacking the TonB-dependent receptor (*lutH*) did not display  $\text{La}^{3+}$   
327 deposits (Fig. 4A). In contrast, localized  $\text{La}^{3+}$  deposits were visualized in the periplasmic space  
328 in mutant strains lacking the ABC-transporter components (*lutA*, *lutE*, and *lutF*) as shown in Fig.  
329 4B-E. EDS microanalyses confirmed these electron dense periplasmic deposition areas contained

330  $\text{La}^{3+}$  (Fig. 4F). Electron-dense deposits were also observed in the cytoplasm from wild-type cells  
331 grown with exogenous  $\text{La}^{3+}$  (Fig 4G). Taken together, these directly demonstrate the  
332 requirements for the TonB-dependent receptor and ABC transporter in Ln transport.

333

334 **Expression of *lutH* is repressed by  $\text{La}^{3+}$ .** To test if expression of the of Ln uptake genes is  
335 regulated in a similar manner to  $\text{Fe}^{3+}$  uptake genes, a fluorescent transcriptional reporter fusion  
336 was used to monitor expression from the *lutH* promoter in methanol media. Addition of  
337 exogenous  $\text{La}^{3+}$  repressed expression 4-fold (RFU/ $\text{OD}_{600}$  =  $210 \pm 7$  without  $\text{La}^{3+}$ ; RFU/ $\text{OD}_{600}$  =  
338  $47 \pm 5$  with  $\text{La}^{3+}$ ) suggesting that when Ln are in excess, transport is down-regulated. This is  
339 consistent with the mechanism of control for iron homeostasis (37, 39).

340

341 **Ln are stored as cytoplasmic crystalline deposits.** To determine if *M. extorquens* AM1 stores  
342 Ln, an *mxoF* mutant was grown in methanol medium with 10-times the standard concentration of  
343  $\text{La}^{3+}$  (20  $\mu\text{M}$   $\text{LaCl}_3$ ) resulting in a growth rate of  $0.15 \pm 0.00 \text{ h}^{-1}$ . Six hours post stationary phase,  
344 cells were washed four times and sub-cultured into methanol medium lacking  $\text{La}^{3+}$  using Ln-  
345 depleted tubes (14). To ensure growth was not due to contamination, strains were streaked onto  
346 methanol solid medium without  $\text{La}^{3+}$ . As shown in Fig. 5, a similar growth rate to that observed  
347 in the presence of  $\text{La}^{3+}$  was obtained (subculture #1,  $0.16 \pm 0.00 \text{ h}^{-1}$ ) until the culture reached an  
348  $\text{OD}_{600}$  of  $\sim 1.0$ . A slower growth rate was noted as growth continued to an OD of  $\sim 1.5$ . This  
349 culture was then washed and sub-cultured into fresh methanol medium lacking Ln as described  
350 above. Cells continued to grow but at a very impaired rate ( $0.01 \pm 0.0 \text{ h}^{-1}$ ) until they reached a  
351 peak OD of  $\sim 1.3$ . The use of Ln-depleted glassware reduces Ln levels such that growth of an  
352 *mxoF* mutant strain appears negligible for 100 h when grown in media lacking Ln (14). As these



353 storage growth curves were carried out for >600 h, a control experiment was done to determine  
354 growth due to either background levels of non-MxaF ADH activity or due to leeching of residual  
355 Ln from the glassware. Growth of an *mxoF* mutant strain that had not been previously grown  
356 with Ln had slower growth than subculture #2 with a growth rate of  $0.003 \pm 0.000 \text{ h}^{-1}$  (data not  
357 shown).

358 To visualize Ln storage within the cell, cultures of the wild-type strain were grown in the  
359 presence and absence of  $20 \mu\text{M LaCl}_3$ , harvested at mid-exponential phase, and immediately  
360 fixed with glutaraldehyde. Samples without post-fixation and staining were analyzed for both  
361 visualization and metal content. Electron-dense deposits were observed in the cytoplasm from  
362 cells grown with exogenous  $\text{La}^{3+}$  (Fig. 6A-B). Samples were analyzed using EDS and  
363 corroborated that the dense deposits contained  $\text{La}^{3+}$  (Fig. 6C). When grown without  $\text{La}^{3+}$ , only a  
364 few cells showed smaller electron dense areas (Fig. 6E-F); however, EDS analysis did not detect  
365  $\text{La}^{3+}$  in these cases (Fig. 6G). These data demonstrate that  $\text{La}^{3+}$  can be stored in *M. extorquens* as  
366 metal deposits. Moreover, EDS analysis of electron dense areas from the wild-type strain grown  
367 with  $\text{La}^{3+}$  (Fig S2) determined a content of La ( $22.2 \pm 1.0\%$ ), P ( $15.1 \pm 2.1\%$ ), and oxygen (O)  
368 ( $51.1 \pm 1.9\%$ ) suggesting Ln are complexed with phosphate. Traces of chloride (3.0%), calcium  
369 (2.2%), and aluminum (3.4%) ions were also detected. The copper, carbon, and silicon ion  
370 content from the support grids and embedding medium were not considered for metal content  
371 calculations. High-resolution transmission electron microscopy (HRTEM) images of the La  
372 deposits showed an atomic lattice (Moire fringes/pattern) indicating a crystalline nature (Fig.  
373 6D). These results suggest that La is embedded in inorganic phosphate crystals which form the  
374 electron dense deposits observed in the cytoplasm.

375 **DISCUSSION**

376           The Ln-dependent XoxF-MeDH has been shown to produce formaldehyde *in vivo* (8).  
377 Here we show that formaldehyde accumulation due to disruption of *fae* is lethal when tested on  
378 methanol and La<sup>3+</sup> solid medium, which enabled a genetic selection to identify genes required  
379 for or involved in methanol oxidation by XoxF. Additionally, our phenotypic data are consistent  
380 with *in vitro* and *in vivo* work that suggests formate is the product of ExaF-mediated methanol  
381 oxidation (5, 8) and with our previous work that shows expression from the *mxo* promoter is  
382 repressed when Ln are present (8, 14).

383           The mutational analysis presented expands the identification of gene products  
384 contributing to methanol metabolism, Ln transport and utilization, and the characterization of  
385 genes with known or predicted roles in Ln metabolism. Using growth and transcriptional reporter  
386 fusion studies, we show that unlike *xoxF*, *xoxG* and *xoxJ* are not required for expression of the  
387 *mxo* genes but are likely required for XoxF activity as loss of either *xoxG* or *xoxJ* resulted in  
388 growth that mirrored the *xoxF1 xoxF2* double mutant strain. Recent biochemical and structural  
389 analyses of XoxG suggest that this cytochrome is tuned specifically for light Ln, while XoxJ  
390 interacts with and may activate XoxF (44). In the methanotroph *Methylomonas* sp. strain LW13,  
391 loss of *xoxG* also results in a growth defect in methanol medium lacking Ln, suggesting an  
392 unknown role in metabolism in addition to functioning as a cytochrome for XoxF-mediated  
393 methanol oxidation (43). Our studies are consistent with an unknown role for XoxGJ in *M.*  
394 *extorquens* AM1 as strains lacking *xoxG* or *xoxJ* display a growth lag or reduction in growth  
395 rate. These data are in contrast to previous reports for *M. extorquens* PA1 and AM1, where loss  
396 of *xoxGJ* did not result in a growth phenotype in methanol medium lacking Ln (22, 42). Notably,

397 the previous AM1 studies were carried out on agar plates where subtle growth defects may not  
398 be apparent and both published studies were conducted using a different growth medium.

399 Our transposon mutagenesis and growth studies lay the foundation for future work  
400 characterizing the roles for new methylotrophic gene products. A fused ABC-transporter  
401 (*META1\_2359*) was found to be essential for methanol growth independent of Ln presence.  
402 Some possible functions for *META1\_2359* may include formaldehyde transport or PQQ export  
403 into the periplasm for incorporation into the ADHs. Leucyl aminopeptidases like *META1\_3908*  
404 are often involved in protein processing or turnover, yet some have been shown to exhibit DNA  
405 binding activity and facilitate regulation or site-specific recombination (55). Homospermidine  
406 synthases (*hss*) function in polyamine biosynthesis and their products (e.g. putrescine,  
407 spermidine) and have been implicated in roles as diverse as host pathogen interactions, biofilm  
408 formation, siderophore production, acid resistance, and free radical scavenging (56, 57). LysR-  
409 type regulators are members of a large family of transcriptional regulators that can be both  
410 activators and repressors of a wide variety of genes involved in diverse cellular processes (58).  
411 More work is required to identify specific roles for these genes in the metabolism of *M.*  
412 *extorquens* AM1.

413 Loss of the *mxuD* homolog, *META1\_1771*, resulted in a growth defect only if  $\text{La}^{3+}$  was  
414 included in the medium. Based on its sequence similarity to MxD, localization in the genome,  
415 and growth phenotypes, we propose to name *META1\_1771* as *xoxD*. Interestingly, upstream of  
416 *xoxD* is another *mxuD* homolog (*META1\_1772*) and downstream is an *mxuE* homolog  
417 (*META1\_1770*). These genes were not identified in the transposon mutant hunt and were not  
418 characterized in this study.

419 Before the existence of Ln-dependent MeDHs were known, mutations were constructed  
420 in *orf6* and *orf7* due to their proximity to C<sub>1</sub> genes and it was concluded that these genes likely  
421 did not have a role in C<sub>1</sub>-metabolism as they grew on methanol medium in the absence of Ln  
422 (42). Results here clearly suggest that *orf6* and *orf7* are important for Ln-dependent  
423 methylotrophy and more work is needed to determine their specific roles.

424 Characterization of the *lut* operon is consistent with the observations recently reported for  
425 *M. extorquens* PA1 (22) where a TonB-ABC transport system contributes to Ln transport into the  
426 cytoplasm. It has been proposed that Ln may be acquired via lanthanophores (47), which enter  
427 through a TonB-dependent receptor (22, 23). It may be that secondary mechanisms exist to take  
428 up Ln chelated by phosphates or citrate, compounds which are present in *Methylobacterium*  
429 PIPES (MP) medium (59). It is possible that these complexes pass through the outer membrane  
430 via a porin, though that is speculation at this point. Of interest, *META1\_5017* is a porin family  
431 protein that was identified in our transposon mutagenesis studies, and loss of this gene resulted in  
432 a 21 h lag and an approximate 20% reduction in growth rate only in the *mxoF* mutant  
433 background. This phenotype would be consistent with a reduction in Ln import. It may be that in  
434 the *lutH* mutant, non-siderophore bound Ln slowly enter through *META1\_5017* porin and once a  
435 threshold is reached, trigger expression and function of the Ln-dependent ADHs. This may  
436 explain the acclimation of the *mxoF lutH* strain after ~100 h but more work is needed to confirm  
437 this hypothesis. Since transposon mutants disrupted in the genes encoding lanmodulin or the Ln-  
438 binding protein downstream of lanmodulin were not isolated, our observations are consistent  
439 with the findings in *M. extorquens* PA1 that suggest a non-essential or redundant role for these  
440 genes in Ln metabolism (22). Notably, the *mxoF fae* transposon mutagenesis study did not  
441 identify obvious gene candidates for lanthanophore biosynthesis though over 600 insertions were

442 mapped to the genome. This may indicate that more than one lanthanophore is produced by the  
443 cell or that the lanthanophore has an essential role that is not yet understood. Additionally, we  
444 show that like the paradigm for regulation of siderophore mediated  $\text{Fe}^{3+}$  uptake, expression from  
445 the TonB-dependent receptor promoter, *lutH*, is repressed by Ln.

446 Our transport, TEM, and EDS analyses are consistent with LutH facilitating Ln transport  
447 into the periplasm and the ABC transport system facilitating Ln transport into the cytoplasm.  
448  $\text{La}^{3+}$  concentrations found in the supernatant from strain variants lacking transport system  
449 components suggest that once in the periplasm, significant concentrations of  $\text{La}^{3+}$  do not go back  
450 outside of the cell as ABC transporter mutants showed uptake of  $\text{La}^{3+}$  from the medium similar  
451 to the wild-type strain. Intriguingly, TEM and EDS studies with ABC transporter mutant strains,  
452 demonstrated localized accumulation of Ln in the periplasmic space. It is not yet clear how or  
453 why Ln accumulate in specific areas rather than appear diffused throughout the periplasm. Taken  
454 together, our phenotypic growth, transport, and visualization studies suggest that Ln must enter  
455 the cytoplasm for the *xox* and *exaF* ADHs to be expressed and that Ln uptake into the  
456 periplasmic space may be enough to repress *mxo* expression. This work will facilitate new  
457 exploration of the Ln-switch.

458 It has been observed that bacteria such as *Bacillus licheniformis* (60), *Myxococcus*  
459 *xanthus* (54), and *Pseudomonas aeruginosa* (61) can effectively adsorb  $\text{La}^{3+}$  in mineral form  
460 onto their cell surface when Ln are at high concentrations (mM). Here, TEM and EDS analyses  
461 demonstrate that *M. extorquens* AM1 stores Ln in the cytoplasm in crystal form. This is  
462 consistent with recent studies for the non-methylotroph *Thermus scotoductus* SA-01 which  
463 showed that  $\text{Eu}^{3+}$  can accumulate in the cytoplasm. However, these studies were conducted using  
464 very high  $\text{Eu}^{3+}$  concentrations that are not typically found in nature (51). Further, a role for  $\text{Eu}^{3+}$

465 in the metabolism of *T. scotoeductus* SA-01 remains to be defined. For many bacteria,  
466 biomineralization is a mechanism used to cope with toxicity of different metals, manage waste  
467 products, sense and change orientations in accordance with geomagnetic fields, and store  
468 important cations for growth (62–67). It has been reported that some microorganisms store  
469 polyphosphate as volutin or metachromatic granules, and these granules are often complexed  
470 with cations like  $Mg^{2+}$  and  $Ca^{2+}$  (68–71). It is not yet known if *M. extorquens* AM1 stores Ln  
471 complexed to polyphosphate, however, the ratios of P, O, and La detected in our studies are  
472 consistent with Ln phosphates. Detailed studies are necessary to define the exact chemical  
473 structure of Ln storage deposits in *M. extorquens* AM1. Our current findings bring exciting  
474 implications for bacterial metabolism and cell biology, and for the development of  
475 bioremediation and biometallurgy strategies for Ln recovery.

476 **MATERIALS AND METHODS**

477 **Bacterial strains and cultivation.** Strains and plasmids used in this study are listed in Table S2.

478 *E. coli* strains were cultivated in Lysogeny Broth (LB) medium (72) (BD, Franklin Lakes, NJ) at

479 37°C. *M. extorquens* AM1 strains were grown in *Methylobacterium* PIPES [piperazine-*N,N'*-

480 bis(2-ethanesulfonic acid)] (MP) media (59) supplemented with succinate (15 mM) and/or

481 methanol (125 mM) as described (14) unless otherwise stated. Conjugations took place on Difco

482 Nutrient Agar (Thermo Fisher Scientific, Waltham, MA). Cultures were grown in round-bottom

483 polypropylene or borosilicate glass culture tubes, or 250 mL polypropylene Erlenmeyer flasks

484 (Thermo Fisher Scientific, Waltham, MA). If glass tubes or flasks were used to culture bacteria,

485 they were pretreated to remove Ln as previously described (14). Liquid cultures were grown at

486 29°C and shaken at 200 and 180 rpm in Innova 2300 and Excella E25 shaking incubators

487 (Eppendorf, Hamburg, Germany), respectively. LaCl<sub>3</sub> was supplemented to a final concentration

488 of 2 or 20 µM when indicated. To prepare PQQ, methoxatin disodium salt (Santa Cruz

489 Biotechnology, Dallas, Tx) was dissolved in deionized water at pH 12-13 and filter sterilized.

490 When necessary, antibiotics were added at the following concentrations: rifamycin (Rif, 50

491 µg/mL), tetracycline (Tc, 10 µg/mL for LB, 5 µg/mL for MP or 10 µg/mL when used together

492 with Rif), kanamycin (Km, 50 µg/mL), ampicillin (Ap, 50 µg/mL).

493

494 **Plasmid and strain construction.** Primers used for plasmid and strain construction are listed in

495 Table S1. The allelic exchange plasmid pHV2 was constructed by cloning the *sacB* gene from

496 pCM433 (73) into the PscI site of pCM184 (74) in the same orientation as the Tc resistant gene.

497 Insertion and orientation of *sacB* was confirmed by colony PCR. The *lutH* transcriptional

498 reporter fusion was constructed by cloning the promoter region of *lutH* into the AclI and EcoRI

499 sites upstream of a promoter-less *venus* gene in pAP5 (21). To create overexpression constructs  
500 for complementation studies, individual genes in the *lut* operon (*lutA*, *lutB*, *lutE*, *lutF*, and *lutG*)  
501 were cloned into the KpnI and SacI sites downstream of a  $P_{lac}$  promoter in pCM62 (46).  
502 Diagnostic PCR was used to confirm successful integration of inserts. Plasmids were maintained  
503 in *E. coli* TOP10 (Invitrogen, Carlsbad, CA).

504 Gene deletions were constructed using pCM184 or pHV2 as previously described (14)  
505 except 5% sucrose was added for counter selection against single crossovers (73) when using  
506 pHV2. Plasmids were conjugated into *M. extorquens* AM1 via biparental mating using *E. coli*  
507 S17-1 (75) or triparental mating using *E. coli* TOP10 (Invitrogen, Carlsbad, CA) and *E. coli*  
508 harboring the conjugative plasmid pRK2013 as described (14). When indicated, the Km  
509 resistance cassette was resolved using pCM157 to achieve marker-less deletions (74).

510

511 **Transposon mutagenesis.** Suicide vector pCM639 carrying a mini transposon IS*phoA*/hah-Tc  
512 (76) was conjugated into *mxoF fae* and *lutE* strain backgrounds via triparental mating as  
513 described (14, 77). Dilutions of the mating mixtures were plated onto MP succinate (15 mM)  
514 plus methanol (50 mM)  $La^{3+}$  medium for the *mxoF fae* strain background and MP methanol (125  
515 mM)  $La^{3+}$  medium for the *lutE* strain background. Media contained 10  $\mu$ g/mL Tc to select for  
516 successful integration of the mini transposon into *M. extorquens* AM1 chromosome and 50  
517  $\mu$ g/mL Rif to counter select against *E. coli* strains bearing pCM639 or pRK2013. Plates were  
518 incubated for 5-7 days at 29°C. Transposon mutant colonies were streaked onto MP succinate  
519 plus methanol  $La^{3+}$  Tc (*mxoF fae*) or MP methanol  $La^{3+}$  (*lutE*) medium for downstream studies.

520



521 **Location of transposon insertions.** To identify the transposon insertion sites, genomic DNA  
522 was isolated using Qiagen's DNeasy UltraClean microbial kit (Qiagen, Germantown, MD).  
523 Degenerate nested PCR was performed as described (77, 78) with the following exceptions: PCR  
524 reactions contained 1  $\mu$ M of each primer, 0.05 U/ $\mu$ L Dream Taq (Thermo Fisher Scientific,  
525 Waltham, MA), and 5% dimethyl sulfoxide. Modifications to the PCR amplification parameters  
526 included 2 minutes for the initial denaturation at 95°C, 6 cycles of annealing at 40°C followed by  
527 25 cycles of annealing at 65°C for the first PCR reaction, and 30 cycles of annealing at 65°C for  
528 the second PCR reaction. PCR products were purified using a Qiagen PCR purification kit  
529 (Germantown, MD). Sequence analysis was performed using TransMapper, a Python-based  
530 software developed in-house to identify transposon insertion locations and map them to the *M.*  
531 *extorquens* AM1 genome. Insertion locations were visualized using the SnapGene Viewer (GSL  
532 Biotech LLC, Chicago, IL).

533

534 **Phenotypic analyses.** Growth phenotypes were determined on solid or in liquid MP media using  
535 a minimum of three biological replicates. On solid media, colony size was scored after four days.  
536 Growth curve analysis was conducted at 29°C in an Excella E25 shaking incubator (New  
537 Brunswick Scientific, Edison, NJ) using a custom-built angled tube rack holder as previously  
538 described (14). Optical density (OD<sub>600</sub>) was measured at 600 nm using a Spectronic 20D  
539 spectrophotometer (Milton Roy Company, Warminster, PA). For strains with extended growth  
540 lags, suppression and acclimation was assessed. Strains from the growth curves were streaked  
541 onto methanol La<sup>3+</sup> medium after they reached stationary phase. If the parent stock strain did not  
542 grow on methanol La<sup>3+</sup> and the strain post-growth curve grew, acclimation versus suppression  
543 was tested. Strains were passaged from the methanol medium plate to a succinate medium plate.

544 After colonies grew on succinate medium, they were streaked back onto methanol  $\text{La}^{3+}$  medium.  
545 If strains retained the ability to grow on methanol medium, it was concluded that growth was due  
546 to a suppressor mutation. If strains lost the ability to grow on methanol medium after succinate  
547 passage, it was concluded growth was due to acclimation and not a genetic change.

548 For Ln storage growth experiments, *mxoF* deletion strains were grown with 20  $\mu\text{M}$   $\text{LaCl}_3$   
549 until six hours after entrance into stationary phase. Cells were centrifuged and washed four times  
550 with MP medium lacking  $\text{La}^{3+}$  and a carbon source, and sub-cultured into fresh MP methanol  
551 medium without  $\text{La}^{3+}$ . Six hours post stationary phase, the cultures were streaked onto MP  
552 methanol medium with and without  $\text{La}^{3+}$  to check for contamination. Cultures were again  
553 washed and sub-cultured as described above. This process was repeated for two rounds of  
554 growth without  $\text{La}^{3+}$ .

555  
556 **Transcriptional reporter fusion assays.** *M. extorquens* AM1 strains carrying *mxo* and *lutH*  
557 transcriptional reporter fusions (Table S2) which use *venus* (79) as a fluorescent reporter were  
558 grown in MP medium supplemented with methanol only or methanol and succinate with and  
559 without  $\text{La}^{3+}$  as indicated in the text. Expression was measured as relative fluorescent units  
560 (RFU) using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA) and normalized  
561 to  $\text{OD}_{600}$  as previously described (14).

562  
563  **$\text{La}^{3+}$  depletion during *M. extorquens* AM1 growth.** Overnight cultures of wild type, *lutA*, *lutE*,  
564 *lutF*, and *lutH* mutant strains were inoculated 1:50 into 250 mL polycarbonate flasks (Corning  
565 Inc., Corning, NY) containing 75 mL of MP medium (59). Succinate (3.75 mM) and methanol  
566 (125 mM) were added as carbon sources with 2  $\mu\text{M}$   $\text{LaCl}_3$ . Flasks were incubated at 28°C at 200

567 rpm in Innova 2300 shaking incubators (Eppendorf, Hauppauge, NY) for 44 h. To monitor  $\text{La}^{3+}$   
568 depletion during *M. extorquens* AM1 cultivation, the Arsenazo III assay was used as previously  
569 described (48). 5 mL samples were collected at 4 different time points ( $\text{OD}_{600}$  of 0.04, 0.4, 0.7,  
570 and 1.5). The concentration of  $\text{La}^{3+}$  remaining in the supernatant was calculated using the  
571 calibration curve prepared as previously described (48). A control of 3 uninoculated flasks  
572 containing MP medium with 2  $\mu\text{M}$   $\text{LaCl}_3$  were considered to determine  $\text{La}^{3+}$  adsorption by the  
573 flasks which was subtracted from the culture measurements. The initial concentration of  $\text{La}^{3+}$  in  
574 the media (before growth) was measured using the Arsenazo III assay in the same way as  
575 described above. Significant differences between depletion of  $\text{La}^{3+}$  by different strains were  
576 calculated using One-way ANOVA followed by a T-test.

577

578 **Cellular locations of Ln visualized using Transmission Electron Microscopy (TEM).** Sample  
579 preparation for TEM: wild-type, *lutA*, *lutE*, *lutF*, and *lutH* mutant strains were grown in MP  
580 medium containing 125 mM methanol and 3.75 mM succinate as carbon sources with or without  
581 the addition of 20  $\mu\text{M}$   $\text{LaCl}_3$  until they reached an OD of  $\sim 0.6$ . 3 mL of cells were harvested by  
582 centrifugation for 3 min at  $1500 \times g$  at room temperature and fixed for 30 min in 1 mL of 2.5%  
583 (v/v) glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M cacodylate buffer  
584 (Electron Microscopy Sciences, Hatfield, PA) at room temperature. After fixation, cells were  
585 pelleted by centrifugation for 3 min at  $1500 \times g$  at room temperature and washed with 1 mL of  
586 0.1 M cacodylate buffer. Cell pellets were embedded in 2% (w/v) agarose and washed three  
587 times with 0.1 M cacodylate buffer. When indicated, pellets in agarose blocks were post-fixed  
588 for 30 min in 1% osmium tetroxide in 0.1 M cacodylate buffer. Samples were washed three times  
589 with 0.1 M cacodylate buffer, dehydrated in acetone, and embedded in Spurr resin (Electron

590 Microscopy Sciences, Hatfield, PA). Blocks were polymerized at 60°C for 48 h. 70 nm sections  
591 were obtained with a Power Tome XL ultramicrotome (RMC Boeckeler Instruments, Tucson  
592 AZ), deposited on 200 mesh carbon coated grids and stained with 2% uranyl acetate (Electron  
593 Microscopy Sciences, Hatfield, PA). To assess the presence of La<sup>3+</sup> by EDS, sections were left  
594 unstained. To image the distribution of cellular La, a TEM JOEL 1400 Flash (Japan Electron  
595 Optics Laboratory, Japan) was used. Detection of La in the cells and high-resolution imaging  
596 were done with a JEOL 2200FS (Japan Electron Optics Laboratory, Japan) operated at 200kV.  
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599

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617

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628 **TABLES**

629 Table 1. Ability of ADH mutations to confer methanol resistance to an *fae* mutant strain on  
630 succinate and methanol solid medium with La<sup>3+</sup>.

Strain	Succinate + MeOH + La <sup>3+</sup>
Wild type	++
<i>fae</i>	-
<i>fae xoxF1</i>	+
<i>fae xoxF1 xoxF2</i>	++
<i>fae exaF</i>	-
<i>fae mxaF</i>	-

631  
632 Methanol is abbreviated as MeOH. No growth is represented by a - sign; growth is represented  
633 with a + sign. A single + indicates that colony size is approximately half of the wild-type colony  
634 size.

635 Table 2. Genes identified three or more times via transposon mutagenesis.

Gene Designation	Gene Name	Putative Function
<i>META1_0863</i>		LysR-type regulator
<i>META1_1292</i>	<i>cycL</i>	<i>c</i> -type cytochrome biogenesis
<i>META1_1293</i>	<i>cycK</i>	Heme lyase
<i>META1_1294</i>	<i>cycJ</i>	Periplasmic heme chaperone
<i>META1_1740</i>	<i>xoxF1</i>	Lanthanide-dependent methanol dehydrogenase
<i>META1_1741</i>	<i>xoxG</i>	Cytochrome <i>c</i>
<i>META1_1742</i>	<i>xoxJ</i>	Periplasmic binding protein
<i>META1_1746</i>	<i>orf6</i>	Unknown
<i>META1_1747</i>	<i>orf7</i>	Unknown
<i>META1_1748</i>	<i>pqqE</i>	PQQ biosynthesis
<i>META1_1749</i>	<i>pqqCD</i>	PQQ biosynthesis
<i>META1_1750</i>	<i>pqqB</i>	PQQ biosynthesis
<i>META1_1771</i>	<i>xoxD</i>	MxaD homolog
<i>META1_1778</i>	<i>lutA</i>	ABC transporter-periplasmic binding component
<i>META1_1779</i>	<i>lutB</i>	Exported protein
<i>META1_1782</i>	<i>lutE</i>	ABC transporter-ATP binding component
<i>META1_1783</i>	<i>lutF</i>	ABC transporter-membrane component
<i>META1_1784</i>	<i>lutG</i>	Exported protein
<i>META1_1785</i>	<i>lutH</i>	TonB-dependent receptor
<i>META1_2024</i>	<i>hss</i>	Homospermidine synthase
<i>META1_2330</i>	<i>pqqF</i>	Protease
<i>META1_2331</i>	<i>pqqG</i>	Protease
<i>META1_2359</i>		Fused ABC transporter
<i>META1_2732</i>	<i>ccmC</i>	Heme export
<i>META1_2734</i>	<i>ccmG</i>	<i>c</i> -type cytochrome biogenesis
<i>META1_2825</i>	<i>ccmB</i>	Heme export
<i>META1_2826</i>	<i>ccmA</i>	Heme export
<i>META1_3908</i>		Leucyl aminopeptidase
<i>META1_5017</i>		Porin family protein

636

637

638 Table 3. Growth parameters for strains grown in methanol medium with and without La<sup>3+</sup>.

Strain	Growth Rate (h <sup>-1</sup> ) <sup>‡</sup>	
	MeOH	MeOH La <sup>3+</sup>
<b>Control strains</b>		
Wild type	0.14 ± 0.01	0.16 ± 0.01
<i>mxoF</i>	NG	0.16 ± 0.01
<i>xoxF1</i>	30 h lag, 0.09 ± 0.01	6-9 h lag, 0.07 ± 0.00
<i>xoxF1 xoxF2</i>	NG*	6 h lag, 0.04 ± 0.01
<i>mxoF xoxF1 xoxF2</i>	NG	6 h lag, 0.04 ± 0.00
<b>Genes involved in general methanol oxidation</b>		
<i>pqqBCDE</i>	NG	NG
<i>pqqF</i>	NG	NG
<i>cycK</i>	NG	NG
<i>ccmB</i>	NG	NG
<i>ccmC</i>	NG	NG
<i>META1_2359</i>	NG*	NG*
<i>mxoF META1_2359</i>	NG	NG*
<i>META1_3908</i>	24 h lag, 0.05 ± 0.00	15 h lag, 0.07 ± 0.00
<i>mxoF META1_3908</i>	NG	15 h lag, 0.07 ± 0.01
<i>hss</i>	9 h lag, 0.09 ± 0.01	6 h lag, 0.10 ± 0.01
<i>mxoF hss</i>	NG	0.11 ± 0.01
<i>META1_0863</i>	0.13 ± 0.00	0.14 ± 0.00
<b>Genes involved in Ln-dependent methanol oxidation</b>		
<i>xoxG</i>	3 h lag, 0.11 ± 0.01	0.04 ± 0.00
<i>xoxJ</i>	21 h lag, 0.13 ± 0.00	0.04 ± 0.01
<i>xoxD</i>	0.14 ± 0.01	0.11 ± 0.01
<i>mxoF xoxD</i>	NG	6 h lag, 0.11 ± 0.01
<i>orf6</i>	9 h lag, 0.10 ± 0.01	0.03 ± 0.00
<i>orf7</i>	6 h lag, 0.14 ± 0.00	9 h lag, 0.08 ± 0.01
<i>META1_5017</i>	0.15 ± 0.00	0.14 ± 0.01
<i>mxoF META1_5017</i>	NG	21 h lag, 0.13 ± 0.01
<i>lutA</i>	0.15 ± 0.01	0.02 ± 0.01, 96 h; 0.08 ± 0.01 <sup>#</sup>
<i>mxoF lutA</i>	NG	0.02 ± 0.00
<i>lutB</i>	0.15 ± 0.01	0.02 ± 0.00, 87 h; 0.03 ± 0.01 <sup>#</sup>
<i>mxoF lutB</i>	NG	0.02 ± 0.00
<i>lutC</i>	0.13 ± 0.01	9 h lag, 0.11 ± 0.01
<i>mxoF lutC</i>	NG	9 h lag, 0.11 ± 0.01



Table 3. (continued)

Strain	Growth Rate (h <sup>-1</sup> ) <sup>‡</sup>	
	MeOH	MeOH La <sup>3+</sup>
Genes involved in Ln-dependent methanol oxidation		
<i>lutD</i>	0.16 ± 0.01	0.18 ± 0.02
<i>mxoF lutD</i>	NG	0.16 ± 0.03
<i>lutE</i>	3 h lag, 0.12 ± 0.02	NG*
<i>mxoF lutE</i>	NG	NG*
<i>lutF</i>	0.12 ± 0.02	NG*
<i>mxoF lutF</i>	NG	NG*
<i>lutG</i>	12 h lag, 0.15 ± 0.01	12 h lag, 0.03 ± 0.00
<i>mxoF lutG</i>	NG	12 h lag, 0.03 ± 0.00
<i>lutH</i>	0.14 ± 0.01	0.16 ± 0.00
<i>mxoF lutH</i>	NG	NG*

639 <sup>‡</sup>Data for a minimum of three biological replicates are reported.

640 \* indicates if a suppressor mutation or acclimation event allowed eventual growth of the strain  
641 (detailed in Table 4).

642 # indicates two growth rates were observed with an initial growth rate lasting for the time  
643 indicated, followed by a final growth rate. The first growth rate visually appeared as a lag except  
644 strains slowly grew during this time.

645 NG indicates no growth. Methanol is abbreviated as MeOH.

646 Table 4. Growth parameters of suppressor and acclimation events.

Strain	MeOH		MeOH La <sup>3+</sup>	
	Time of S/A (h)	S/A Growth Rate (h <sup>-1</sup> ) <sup>‡</sup>	Time of S/A (h)	S/A Growth Rate (h <sup>-1</sup> ) <sup>‡</sup>
<i>xoxF1 xoxF2</i>	S: 108-144	0.10 ± 0.01		
<i>META1_2359</i>	S: 85-100	0.11 ± 0.02	S: 85	0.10 ± 0.01
<i>mxoF META1_2359</i>			S: 120-140	0.07 ± 0.02
<i>lutE</i>			S: 75-90	0.07 ± 0.01
<i>mxoF lutE</i>			S: 200-220	0.02 ± 0.00
<i>lutF</i>			S: 79-91	0.09 ± 0.02
<i>mxoF lutF</i>			S: 145-157	0.02 ± 0.01
<i>mxoF lutH</i>			A: 90-120	0.14 ± 0.02

647 <sup>‡</sup>Data for a minimum of three biological replicates is reported.

648 Abbreviations are as follows: methanol is abbreviated as MeOH, suppression as S, acclimation  
649 as A.

650 **FIGURE LEGENDS**

651 **Fig 1. Schematic representation of the metabolic processes relevant for the *mxoF fae***

652 **transposon mutagenesis study.** When coordinated to Ln, the XoxF methanol dehydrogenase is  
653 known to produce formaldehyde *in vivo*. When *M. extorquens* AM1 lacks *fae* as indicated by a  
654 red X, formaldehyde accumulates to toxic levels rendering strains unable to grow on solid media  
655 even in the presence of alternative substrates such as succinate. In the absence of *mxoF*, if a  
656 process required for XoxF function is disrupted by a transposon insertion, formaldehyde is  
657 reduced or eliminated, and growth can occur using succinate as a carbon and energy source.  
658 Dashed lines are used to represent the formaldehyde transporter as this function has not been  
659 demonstrated.

660

661 **Fig 2. Characterization of the Ln transport cluster.** A) Genomic map of the genes contained

662 in the Ln transport cluster (*META1\_1778* to *META1\_1787*). Purple, genes encoding the ABC  
663 transport system; green, gene encoding the TonB-dependent transporter; blue, genes encoding  
664 putative exported proteins identified by transposon mutagenesis; gray, lanmodulin and additional  
665 exported genes in the Ln transport cluster not identified by transposon mutagenesis. B)

666 Schematic representation of the proteins predicted to be involved in Ln transport. The three-  
667 dimensional structures of monomers of LutH and LutA were predicted using homology modeling  
668 (HHpredserver and MODELLER) (80) and homodimers of the ABC transporter were predicted  
669 using GalaxyHomomer (81). C) Growth of wild type and *lut* mutant strains grown in 125 mM  
670 methanol medium with 2  $\mu$ M LaCl<sub>3</sub>. Graphs depict representative data from three biological  
671 replicates. Variation between replicates was < 5% except for *mxoF lutC* and *mxoF lutD* which  
672 had growth variances of 9 and 20% respectively as reported in Table 3.

673

674 **Fig 3. Measurement of Ln uptake.** A) Growth analysis of wild type (red circles), *lutH* encoding  
675 the TonB receptor (dark blue triangles), *lutA* encoding the periplasmic binding protein  
676 component of the ABC transporter (bright blue squares), *lutE* encoding the ATP binding  
677 component of the ABC transporter (light blue diamonds), and *lutF* encoding the transmembrane  
678 component of the ABC transporter (light blue circles), grown in the presence of limiting  
679 succinate (3.75 mM), methanol (125 mM), and 2  $\mu\text{M}$   $\text{LaCl}_3$ . Data represent the average of three  
680 biological replicates with variances < 5%. B)  $\text{La}^{3+}$  concentration ( $\mu\text{M}$ ) present in the supernatant  
681 at different optical densities ( $\text{OD}_{600}$ ) for wild type (red), *lutH* (dark blue), and *lutA*, *lutE*, *lutF*  
682 (gradient of light blue respectively). Each bar represents the average of three biological replicates  
683 with error bars showing the standard deviation. One-way analysis of variance (ANOVA)  
684 followed by a T-test was used to represent statistical significance. \*The *p*-value is <0.005.

685

686 **Fig 4. Transmission and electron microscopy for visualization of Ln.** Thin sections of  
687 A) *lutH*, B) *lutE* C) *lutF*, D) *lutA*, and G) wild-type strains growth in minimal medium with 3.75  
688 mM succinate, 125 mM methanol, and 20  $\mu\text{M}$   $\text{LaCl}_3$ . White arrows indicate depositions of  
689 electron scattering material in the periplasm. Each panel depicts cells that were fixed with 2.5%  
690 glutaraldehyde, either post-fixed with  $\text{OsO}_4$  and stained with uranyl acetate to detect cell  
691 membranes (left subpanel), or without post-fixation and staining for elemental analysis (right  
692 subpanel). E) Magnification of the La-deposits localized in the periplasmic space from the *lutA*  
693 mutant strain; black arrows show the boundaries of the outer membrane and inner membrane. F)  
694 Energy-dispersive X-ray analysis of the electron-dense deposits observed inside the periplasm.

695

696 **Fig 5. Assessment of the ability of an *mxoF* mutant strain to store Ln for methanol growth.**

697 Optical density (OD<sub>600</sub>) measurements of cultures grown with excess LaCl<sub>3</sub> (20 μM) (gray  
698 circles) until six hours post stationary phase. Cells were washed and reinoculated into La<sup>3+</sup> free  
699 methanol medium in La<sup>3+</sup> tubes (subculture #1, dark blue circles). After 6 hours in stationary  
700 phase, subculture #1 was washed and reinoculated into methanol medium without La<sup>3+</sup>  
701 (subculture #2, light blue circles). Representative data from three biological replicates is shown.  
702 Growth variance was <5%.

703

704 **Fig 6. Visualization of Ln storage.** TEM of ultrathin sections of wild-type cells grown with (A-

705 B) and without (E-F) 20 μM La<sup>3+</sup> in minimal medium with 3.75 mM succinate and 125 mM  
706 methanol. C) and G) Elemental analysis of electron dense deposits was conducted using EDS on  
707 unstained ultrathin sections from cells grown with (C) and without (G) La<sup>3+</sup>. D) High-resolution  
708 transmission electron microscopy analysis of the wild-type strain revealing the presence of an  
709 atomic lattice in electron-dense areas that suggests La<sup>3+</sup> is embedded in inorganic crystals inside  
710 the cell.

711

712

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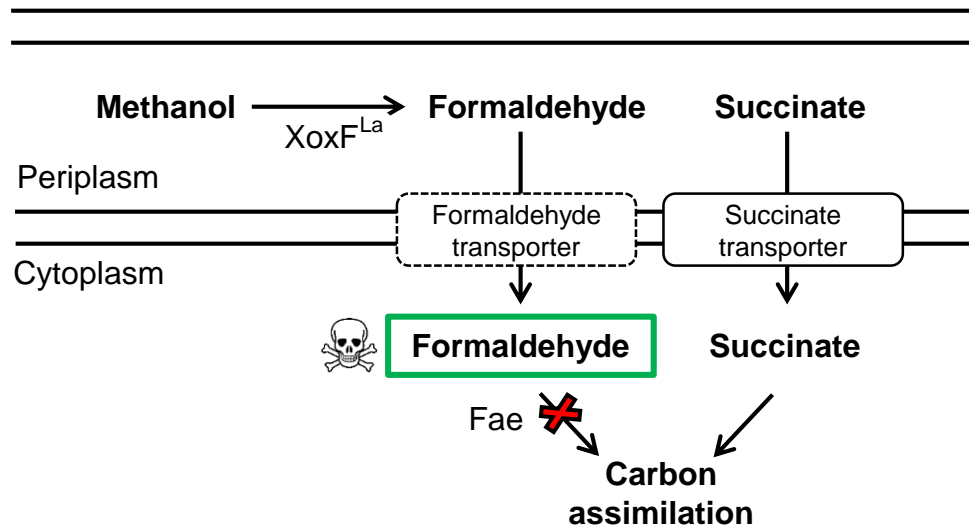


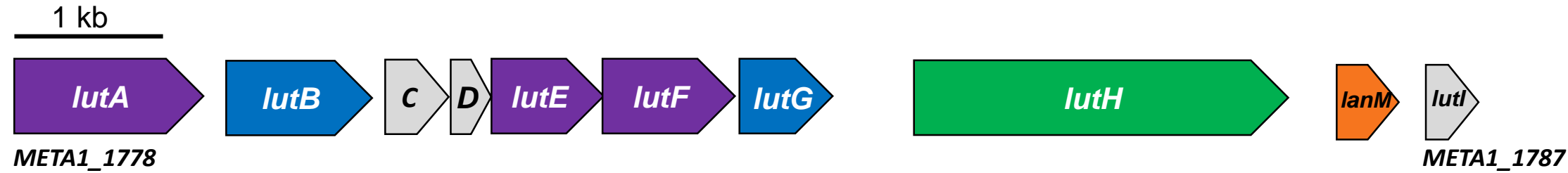
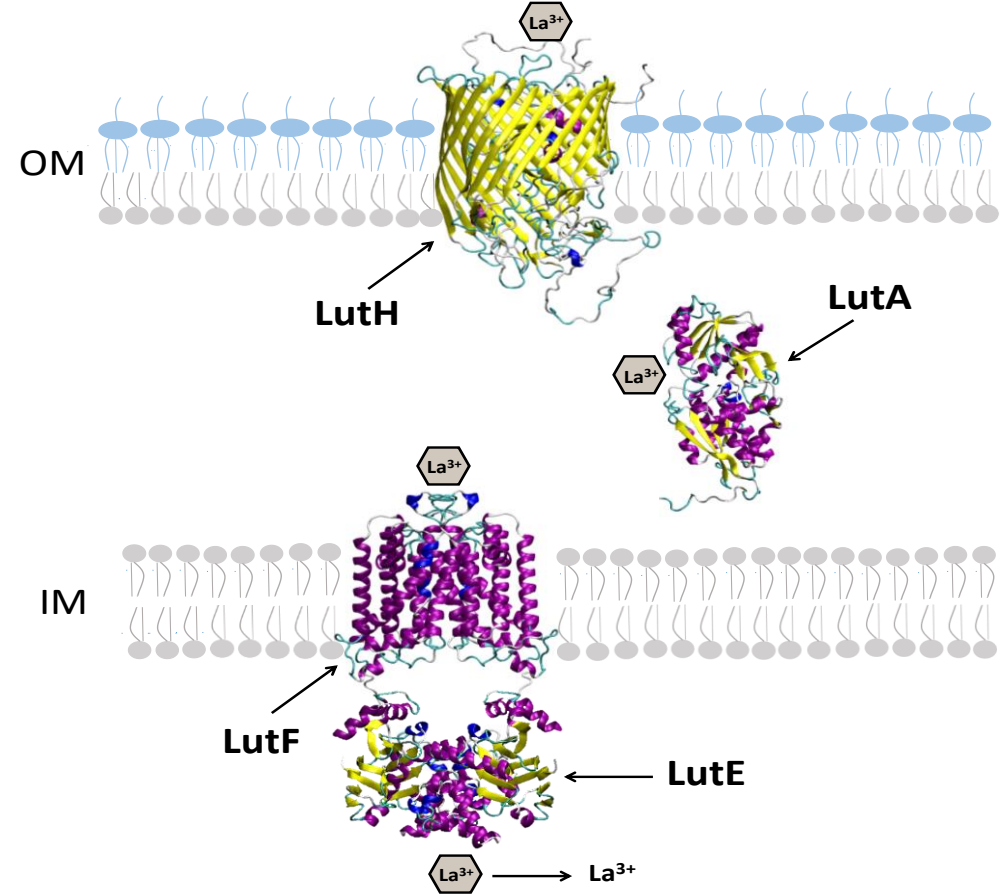
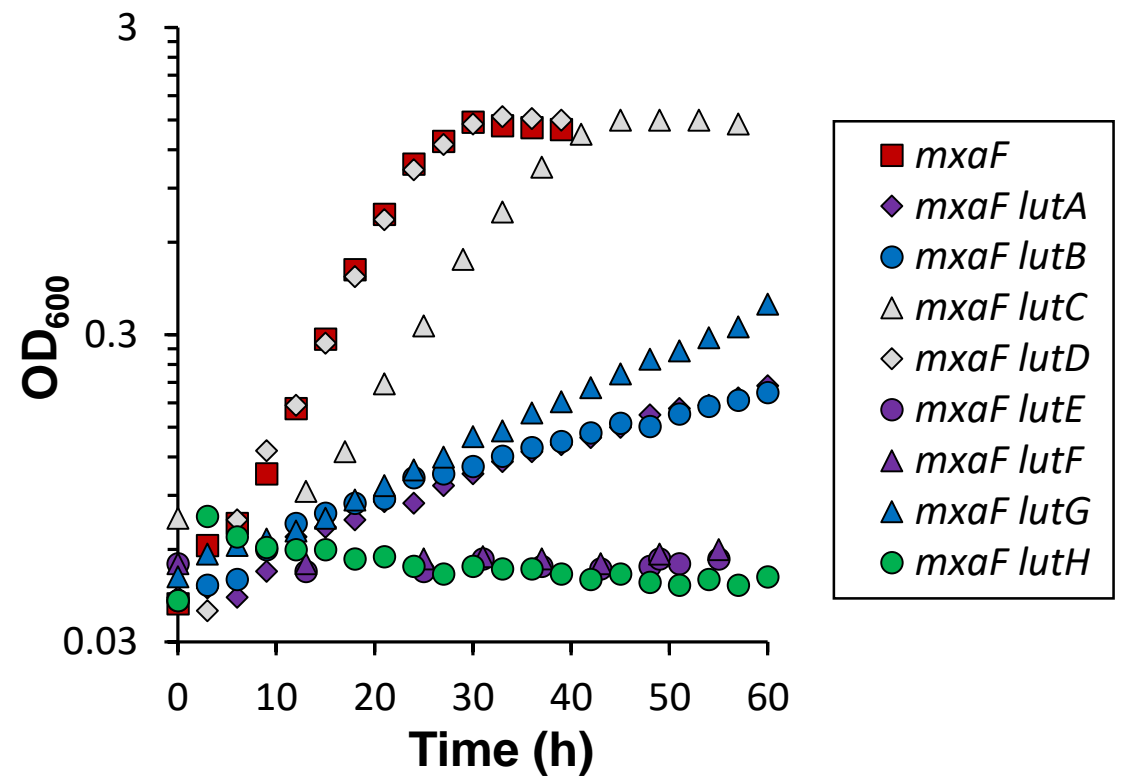
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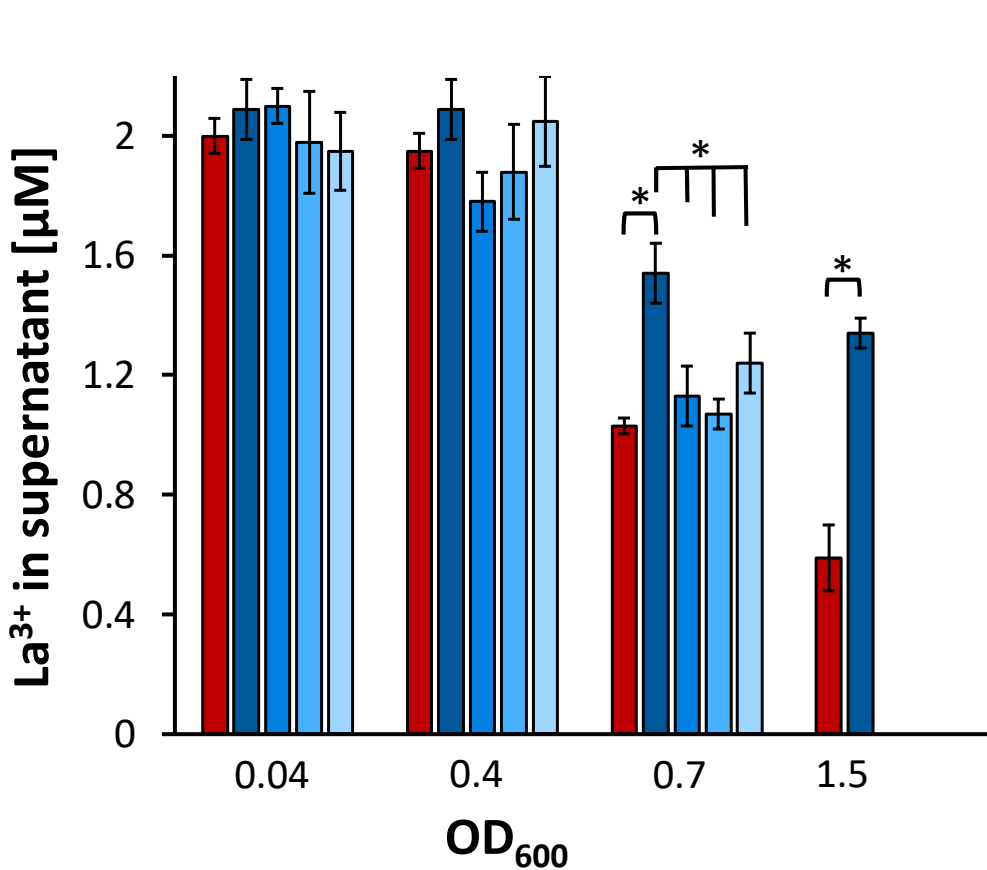
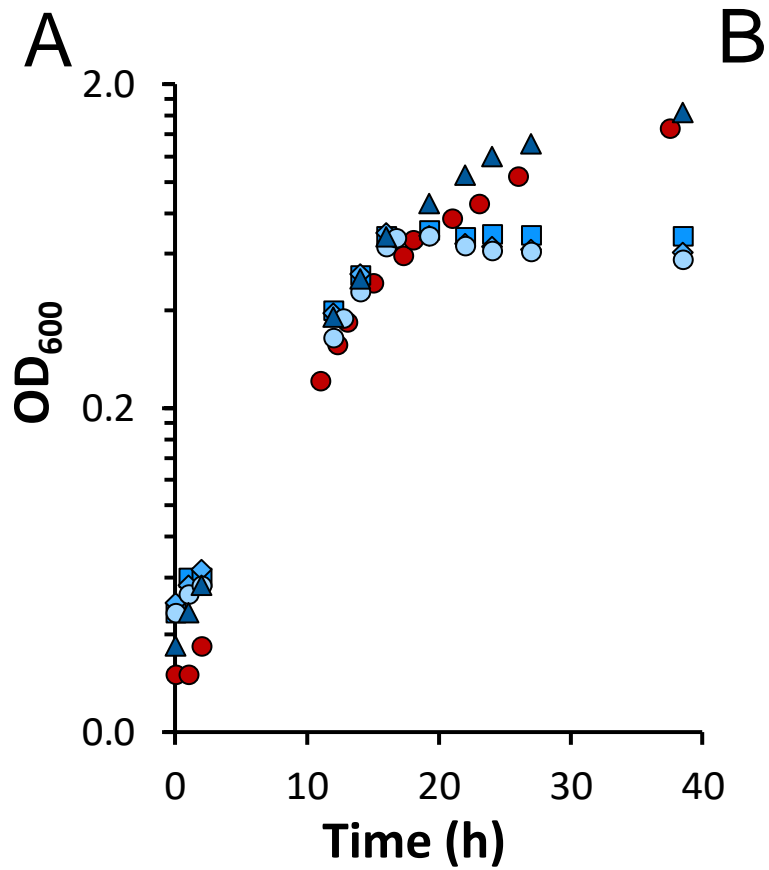


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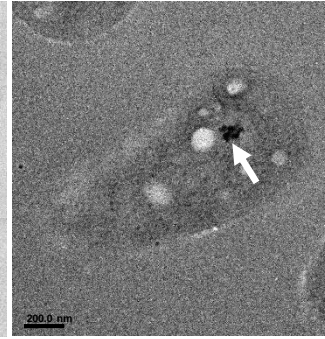
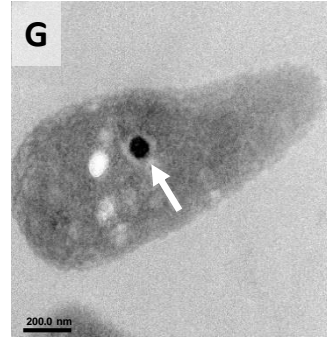
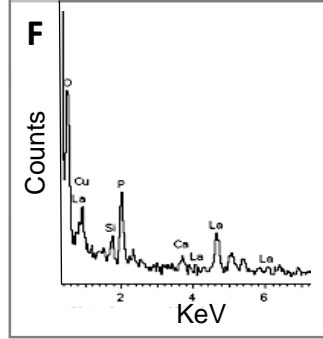
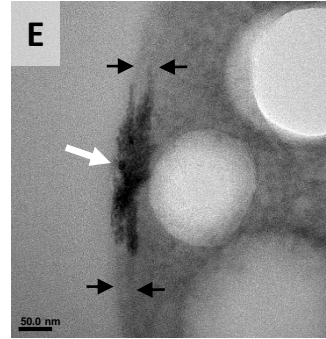
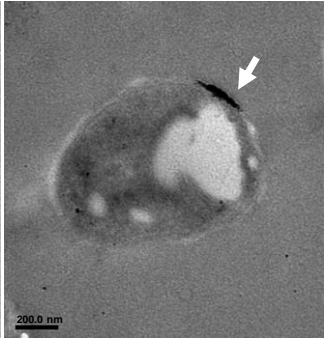
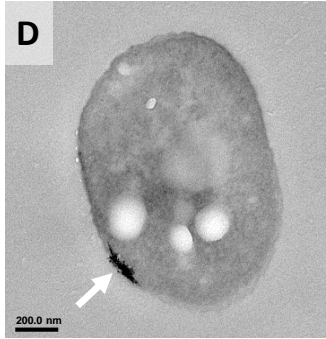
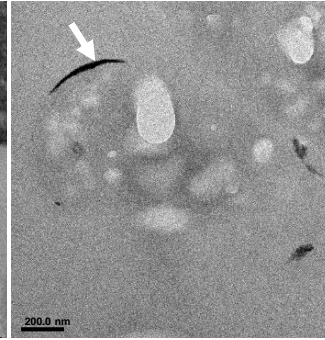
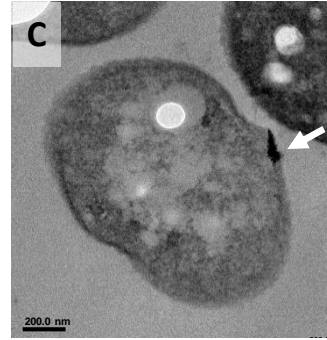
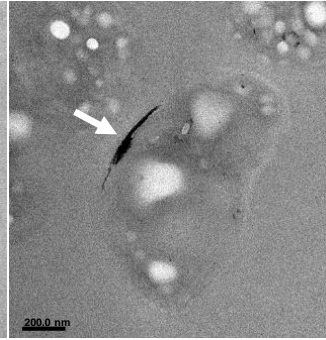
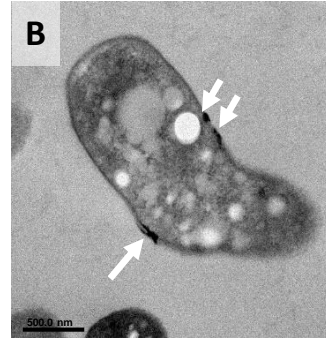
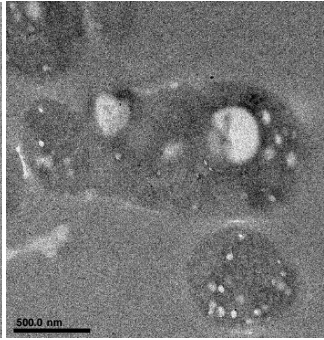
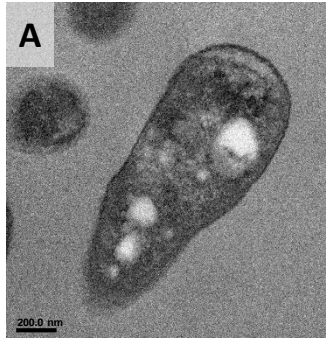
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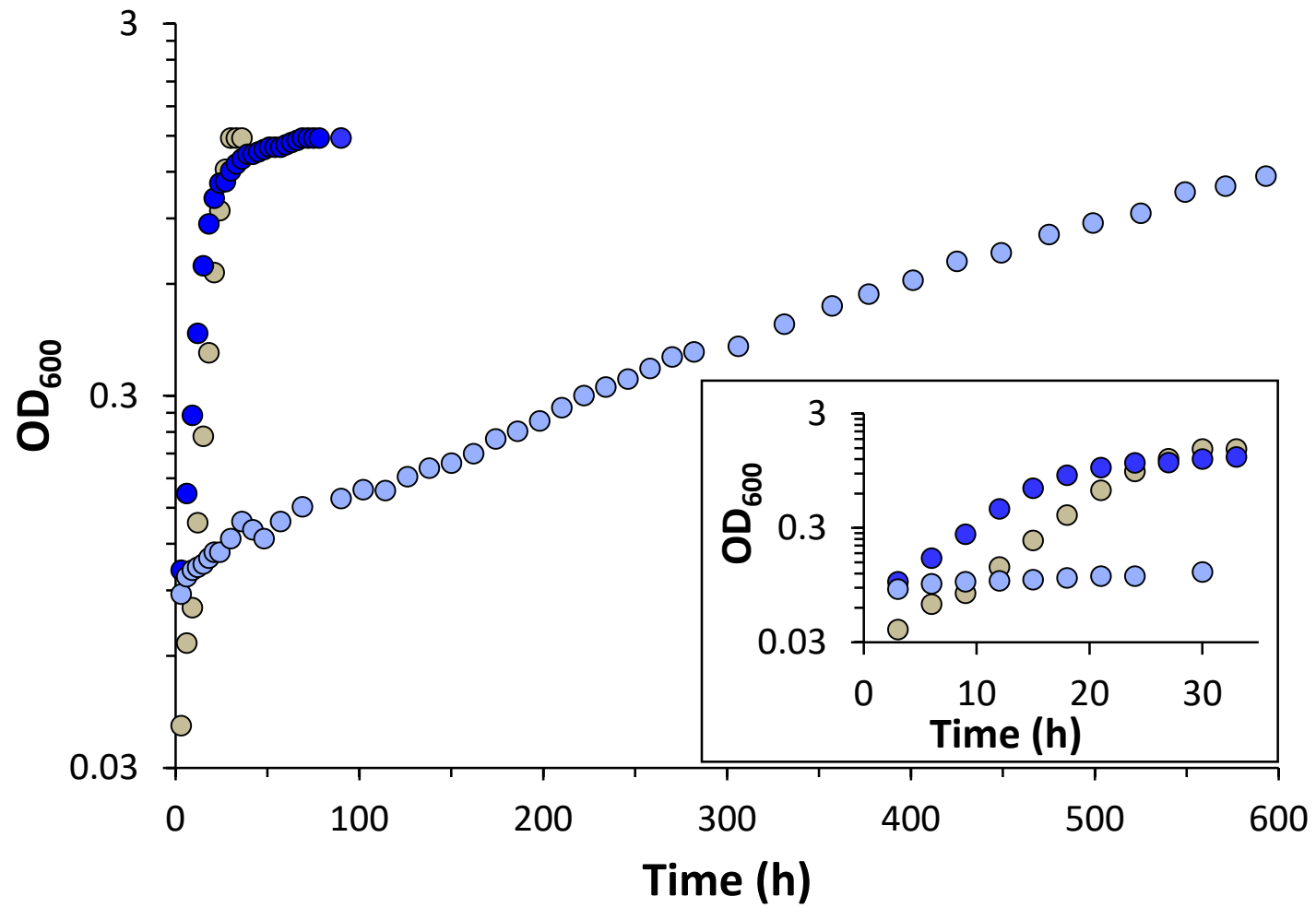


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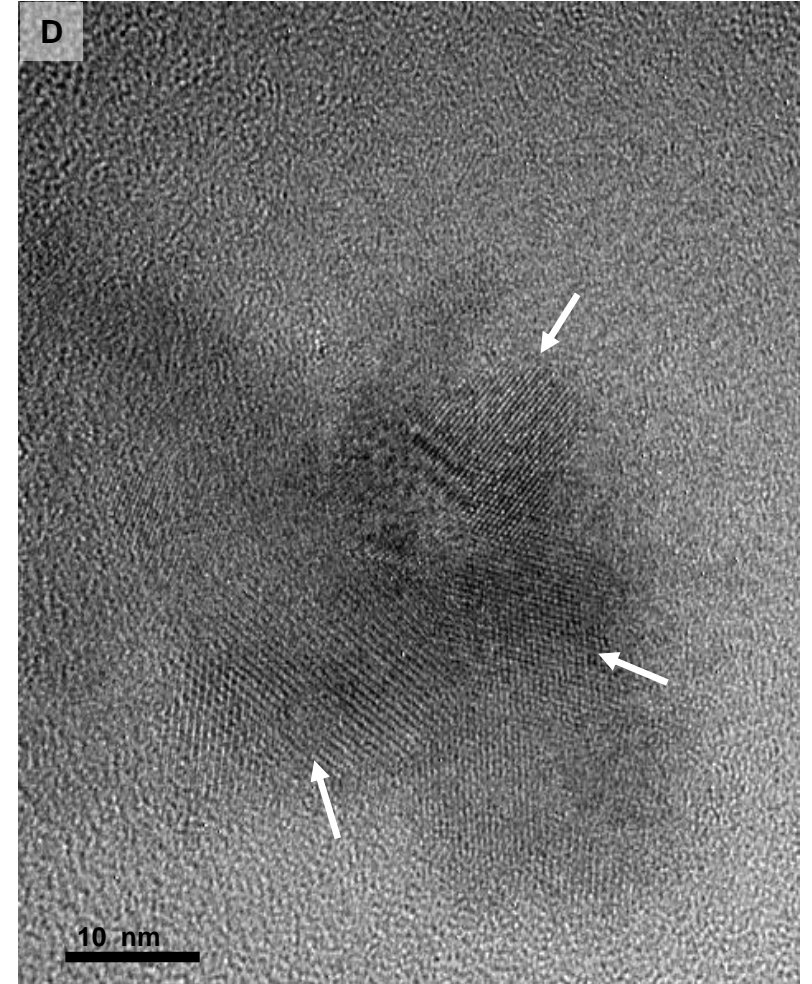
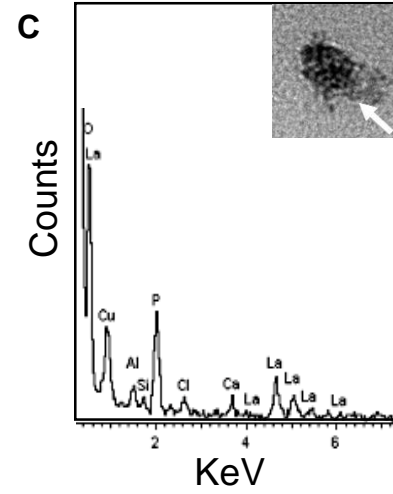
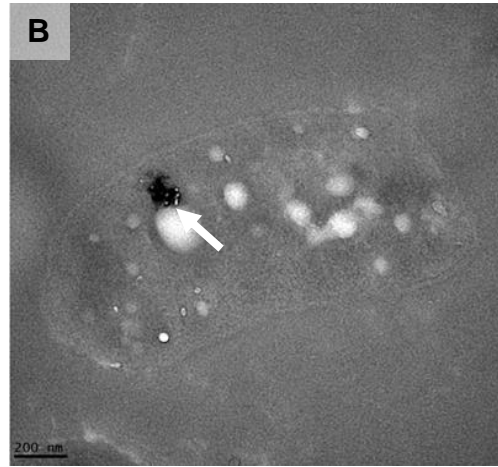
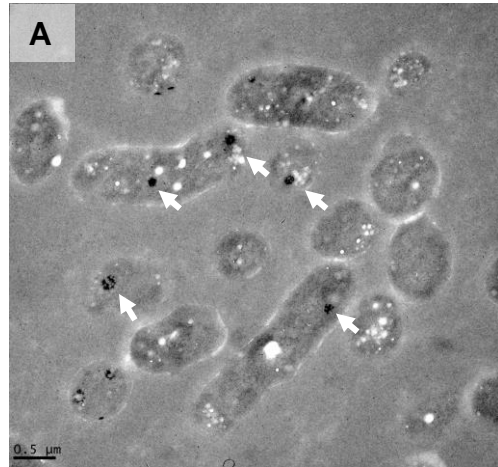








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