

1 **Functional role of lanthanides in enzymatic activity and transcriptional**
2 **regulation of PQQ-dependent alcohol dehydrogenases in *Pseudomonas putida***
3 **KT2440**

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19 **ABSTRACT**

20 The oxidation of alcohols and aldehydes is crucial for detoxification and efficient catabolism of
21 various volatile organic compounds (VOCs). Thus, many Gram-negative bacteria have evolved
22 periplasmic oxidation systems, based on pyrroloquinoline quinone-dependent alcohol
23 dehydrogenases (PQQ-ADHs), which are often functionally redundant. Using purified enzymes
24 from the soil-dwelling model organism *Pseudomonas putida* KT2440, the present study reports
25 the first description and characterization of a lanthanide-dependent PQQ-ADH (PedH) in a non-
26 methylotrophic bacterium. PedH exhibits enzyme activity on a similar substrate range as its
27 Ca²⁺-dependent counterpart PedE, including linear and aromatic primary and secondary
28 alcohols as well as aldehydes, however, only in the presence of lanthanide ions including La³⁺,
29 Ce³⁺, Pr³⁺, Sm³⁺ or Nd³⁺. Reporter assays revealed that PedH not only has a catalytic function,
30 but is also involved in the transcriptional regulation of *pedE* and *pedH*, most likely acting as a
31 sensory module. Notably, the underlying regulatory network is responsive to as little as 1 – 10
32 nM of lanthanum, a concentration assumed to be of ecological relevance. The present study
33 further demonstrates that the PQQ-dependent oxidation system is crucial for efficient growth
34 with a variety of volatile alcohols. From these results we conclude that functional redundancy
35 and inverse regulation of PedE and PedH represents an adaptive strategy of *P. putida* KT2440 to
36 optimize growth with volatile alcohols in response to different lanthanide availability.

37 **IMPORTANCE**

38 Due to their low bioavailability, lanthanides have long been considered as biologically inert. In
39 recent years however, the identification of lanthanides as a cofactor in methylotrophic bacteria
40 has attracted tremendous interest among various biological fields. The present study reveals
41 that one of the two PQQ-ADHs produced by the model organism *P. putida* KT2440 also utilizes
42 lanthanides as a cofactor, thus expanding the scope of lanthanide employing bacteria beyond
43 the methylotrophs. Similar to methylotrophic bacteria, a complex regulatory network is
44 involved in the lanthanide-responsive switch between the two PQQ-ADHs encoded by *P. putida*
45 KT2440. We further show that functional production of at least one of the enzymes is crucial for
46 efficient growth with several volatile alcohols. Overall, our study provides a novel understanding
47 for the redundancy of PQQ-ADHs observed in many organisms and further highlights the
48 importance of lanthanides for bacterial metabolism, particularly in soil environments.

49 INTRODUCTION

50 As a soil-dwelling organism, *Pseudomonas putida* can encounter a large diversity of volatile
51 organic compounds (VOCs) from different sources (1–3). The ecological role of many VOCs is not
52 clearly defined but the number of known specific functions is rapidly increasing. These functions
53 include the growth promotion of plants, anti-herbivore, -bacterial, and -fungal activities, and
54 signaling both within the same and between different species (4–7). Among many other
55 chemicals, VOCs include cyclic, acyclic, aromatic, and terpenoid structures with alcohol and
56 aldehyde moieties, which are mainly derived from the metabolism of bacterial, yeast, fungal, or
57 plant species. Beside their specific molecular function, they can also serve as carbon and energy
58 sources for a wide range of microorganisms. To efficiently use volatile alcohols and aldehydes, it
59 is advantageous if their metabolism is initiated by pyrroloquinoline quinone (PQQ)-dependent
60 alcohol dehydrogenases (PQQ-ADHs) for at least two different reasons. Firstly, by using a
61 periplasmic oxidation system, the organism is able to rapidly detoxify the often harmful
62 chemicals without prior transport into the cytoplasm (8, 9). Secondly, the periplasmic location
63 of the enzymes allows the rapid capture of a volatile carbon source by the conversion to and
64 accumulation of acidic products with decreased volatility.

65 The reaction mechanism of PQQ-ADHs is still not completely resolved, but most likely proceeds
66 *via* an ion-assisted direct hydride transfer from the substrate to the C5 of the non-covalently
67 bound cofactor PQQ (10–12). PQQ-ADHs can be divided into different subclasses depending
68 either on their molecular composition (quinoproteins or quinohemoproteins), or whether they
69 are membrane bound or freely soluble within the periplasm (13). Many organisms express
70 different classes or even multiple PQQ-ADHs of the same type, indicating the importance of

71 these enzymes (14–16). The genome of *P. putida* KT2440 encodes two PQQ-ADHs, namely
72 PP_2674 (*pedE*) and PP_2679 (*pedH*), which have been shown to be involved in the metabolism
73 of different substrates (17–19). PedE is a homolog of ExaA (QEDH) from *Pseudomonas*
74 *aeruginosa*, which represents the most intensively studied member of the class of soluble
75 ethanol dehydrogenases (20–24). ExaA and homologs thereof accept a wide variety of
76 substrates and rely on a Ca²⁺ ion in the active site, in addition to the PQQ cofactor, for the
77 oxidation of primary and secondary alcohols, as well as aldehydes (18, 24). Despite their broad
78 substrate range, ExaA-like enzymes show only very poor conversion of methanol. Not
79 surprisingly, methano- and methylotrophic bacteria, which can use methane and methanol as a
80 source of carbon and energy, encode a different type of PQQ-dependent enzyme, the MxaF-
81 type of methanol dehydrogenases (MxaF-MDH) (25, 26). These enzymes display high substrate
82 specificity for methanol and formaldehyde and also depend on a Ca²⁺ as cofactor (27).
83 Interestingly, methano- and methylotrophic bacteria encode an additional type of PQQ-
84 dependent methanol dehydrogenases, the XoxF-MDH type, which utilizes rare earth metals
85 (REM) of the lanthanide series as cofactor instead of calcium (28–30).

86 Since their discovery, several XoxF-type MDHs from different methano- and methylotrophs have
87 been identified and characterized (31–33). Phylogenetic analysis of available sequence
88 information suggests that lanthanide dependency is an ancestral feature of PQQ-ADHs, and that
89 these enzymes are more abundant than their Ca²⁺-dependent counterparts (15, 34). In addition,
90 a very recent publication described the first lanthanide-dependent ethanol dehydrogenase in *M.*
91 *extorquens* AM1 (16). As a consequence, REM-dependent enzymes and the microorganisms
92 which produce them have sparked a lot of academic and commercial interest, as they might be

93 exploited in a broad variety of biotechnological fields (35, 36). Potential applications range from
94 the development of new biocatalysts and biosensors to the use of the associated
95 microorganisms in biomining, bioleaching, and recycling processes for REMs. However, so far
96 lanthanide-dependent PQQ-ADHs have been limited to methano- and methylotrophic bacteria.

97 In the present study, we report the first description and detailed characterization of a
98 lanthanide-dependent PQQ-ADH (PedH) in the non-methylotrophic bacterium *Pseudomonas*
99 *putida* KT2440, which represents a model organism for industrial and environmental
100 applications (37–40). We demonstrate that PedH exhibits enzymatic activity only in the
101 presence of lanthanides, including but not limited to lanthanum, praseodymium and cerium,
102 and show that this enzyme has a similar substrate range as PedE, the recently characterized
103 Ca²⁺-dependent PQQ-ADH from KT2440 (18). By the use of deletion mutants and transcriptional
104 reporter fusions, we provide evidence that the functional redundancy of the PQQ-ADHs reflects
105 the variable availability of lanthanides in the natural environment of *P. putida* KT2440 and show
106 that these enzymes are crucial for efficient growth with a variety of volatile alcohols. Finally, we
107 reveal that PedH plays an important role in the regulatory switch between *pedH* and *pedE*
108 transcription, most likely acting as a sensory module. From these data, we conclude that KT2440
109 responds to lanthanide availability with the inverse transcriptional regulation of the two PQQ-
110 ADHs to optimize growth with volatile alcoholic and aldehyde substrates.

111 RESULTS

112 **Biochemical characterization of PedE and PedH.** Like many other organisms, *Pseudomonas*
113 *putida* KT2440 harbors more than one gene annotated as a PQQ-ADH, namely PP_2674
114 (PedE/QedH; GI: 26989393) and PP_2679 (PedH; GI: 26989398). To study the rationale for this
115 redundancy, we purified and characterized the corresponding enzymes. A one-step affinity
116 chromatography method produced soluble C-terminally His-tagged PedE and PedH to visible
117 purity (**Fig. S1**) from cell lysates of *E. coli* BL21 (DE3). Under optimized reaction conditions,
118 which include the presence of 1 mM Ca²⁺, the specific activities of purified PedE with a variety of
119 substrates were determined (**Table 1**). For all linear primary alcohols and aldehydes,
120 comparably high enzyme activities ranging from 1.9 ± 0.2 U mg⁻¹ to 6.7 ± 0.9 U mg⁻¹ were found.
121 Similarly, 2-phenylethanol, the secondary alcohol 2-butanol, cinnamyl alcohol, and the acyclic
122 sesquiterpene farnesol were efficiently converted with specific activities ranging from 6.7 ± 1.1
123 U mg⁻¹ to 2.0 ± 0.3 U mg⁻¹. Methanol, 2,3-butanediol, and ethanolamine were poor substrates
124 for the enzyme with about 10-fold decrease in specific activity compared to ethanol or 2-
125 phenylethanol. From all substrates tested, cinnamyl aldehyde was the only compound with
126 which no activity was detected for PedE.

127 When we assayed purified PedH under the optimized reaction conditions used for PedE, no
128 activity for any of the tested substrates was observed (data not shown). Comparison of the
129 active sites of both enzymes, using homology models based on the crystal structure of the
130 ethanol dehydrogenase ExaA of *P. aeruginosa* (PDB: 1FLG) revealed that, similar to other
131 characterized representatives of the PQQ-dependent ethanol dehydrogenase type, the PedE
132 protein harbors a serine residue at amino acid position 295, which is involved in the

133 coordination of the Ca^{2+} ion (**Fig. 1A**). In contrast, in PedH this residue is exchanged to an
134 aspartate (**Fig. 1B**). As this aspartate residue has recently been associated with the coordination
135 of trivalent lanthanide ions in the active site of PQQ-dependent methanol and ethanol
136 dehydrogenases in methylotrophs (15, 16), we tested PedH for activity with ethanol in the
137 presence of a variety of rare earth metals (**Fig. 2**). From these experiments we found that PedH
138 showed no activity when 1 μM of Er^{3+} , Sc^{3+} , Y^{3+} or Yb^{3+} was added to the reaction mixture.
139 However, in the presence of 1 μM of the lanthanides La^{3+} , Ce^{3+} , Pr^{3+} , Nd^{3+} , Sm^{3+} , Gd^{3+} or Tb^{3+}
140 enzymatic activities were detected, with maximal specific activities observed with Pr^{3+} and Nd^{3+} ,
141 and only very low activities with Gd^{3+} or Tb^{3+} .

142 Under optimized conditions, which include the supplementation with 1 μM Pr^{3+} instead of Ca^{2+} ,
143 PedH showed a similar activity pattern as PedE (**Table 1**), but exhibited about 2-fold higher
144 specific activities. Further, the functional concentration range of the cation cofactor differed
145 dramatically for the two enzymes (**Fig. S2A**). While PedE showed enzyme activity at
146 concentrations from 10 μM to 10 mM CaCl_2 with a peak in activity at 1 mM, PedH activity was
147 found with lanthanide concentrations as little as 10 nM and up to 100 μM with a peak in activity
148 at 1 μM . From these data we calculated the dissociation constants (K_D) for various metals to the
149 corresponding enzyme and found that PedH has an 850- to 2500-fold higher binding affinity for
150 lanthanides ($K_D = 25 - 75$ nM; **Fig. S2B2**) as PedE does for Ca^{2+} ($K_D = 64$ μM ; **Fig. S2B1**). The
151 subsequent determination of kinetic parameters with ethanol showed that V_{max} of PedH was
152 approximately 1.7-fold higher than that observed for PedE (10.6 U mg^{-1} vs. 6.1 U mg^{-1} ; **Fig. 3**).
153 However, the corresponding K_M was 2-fold lower for PedE compared to PedH (85 μM vs. 177
154 μM). A similar pattern was found with acetaldehyde and 2-phenylethanol, but with

155 approximately 1.6-fold (2-phenylethanol) and 10–15-fold (acetaldehyde) lower catalytic
156 efficiencies compared to those measured with ethanol. Statistical analysis (two-tailed t -test; $\alpha =$
157 0.05; $N = 3$; GraphPad Prism, version 7.03) revealed that all maximal velocities (V_{max}) and
158 binding constants (K_M) except for the K_M with ethanol were significantly different ($P < 0.05$)
159 between PedE and PedH, however no significant differences could be observed in the catalytic
160 efficiencies (k_{cat}/K_M).

161 ***Growth with volatile alcohols in the presence and absence of lanthanides.*** In a next step,
162 individual ($\Delta pedE$, $\Delta pedH$, Δpqq) and combinatorial ($\Delta pedE\Delta pedH$) deletion mutants were tested
163 for growth with several VOCs using an agar plate assay in the presence and absence of 20 μM
164 lanthanum (**Fig. 4A**). Strains KT2440 (type strain), KT2440* (Δupp strain used as the parental
165 strain for knockout mutants) and $\Delta pedH$ grew efficiently with ethanol, 1-butanol, and 2-
166 phenylethanol in the absence of La^{3+} . Strain $\Delta pedE$ displayed no growth under this condition.
167 Even more interestingly, the addition of 20 μM of La^{3+} to the agar medium not only resulted in
168 growth of the $\Delta pedE$ strain, but also restricted the growth of $\Delta pedH$. The double mutant
169 $\Delta pedE\Delta pedH$ and the Δpqq mutant, which is deficient in PQQ biosynthesis, showed no growth
170 under both conditions. These experiments revealed that efficient growth with all tested
171 alcohols, except the microbial fermentation product 2,3-butanediol, was dependent on the
172 functional expression of PedE or PedH.

173 To validate these findings, growth experiments in liquid M9 medium with 2-phenylethanol as
174 sole carbon and energy source in the presence and absence of 20 μM La^{3+} were performed (**Fig.**
175 **4B1-4**). For this, plastic Erlenmeyer flasks were used to avoid potential contaminations of rare
176 earth metals (REM) from the glassware (33). Growth of the liquid cultures followed a similar

177 pattern to that observed in the agar plate assay. While strain KT2440* (**Fig. 4B1**) showed growth
178 after an 18–20 h lag phase and a peak in optical density at about 35 h in both conditions, the
179 absence and the presence of lanthanum, strain $\Delta pedE\Delta pedH$ (**Fig. 4B4**) did not display growth in
180 either condition. Growth of $\Delta pedE$ (**Fig. 4B3**) was observed exclusively in the presence of
181 lanthanum. Lastly, strain $\Delta pedH$ (**Fig. 4B2**) showed growth similar to that of the KT2440*
182 wildtype in the absence of lanthanum, but no growth was detected when 20 μM La^{3+} were
183 supplemented.

184 **Transcriptional regulation of *pedE* and *pedH* determines growth with alcoholic volatiles.** The
185 previous experiments proved that for efficient growth with various VOCs the functional
186 expression of at least one of the PQQ-ADHs is essential. The growth inhibition of the $\Delta pedH$
187 strain in the presence of lanthanum indicated a potential repression of the *pedE* gene in the
188 presence of lanthanides, similar to recent reports in different methylotrophic bacteria (41–43).
189 To proof this hypothesis, we constructed two reporter strains suitable for probing *pedE* and
190 *pedH* promoter activities in KT2440*. When these strains were tested with 1 mM of 2-
191 phenylethanol in M9 medium (**Fig. 5A**), the addition of up to 10 nM La^{3+} did not affect *pedE*
192 promoter activity compared to the condition in the absence of lanthanum. In contrast, the
193 presence of 100 nM – 100 μM of La^{3+} resulted in reduced *pedE* promoter activity. An inverse
194 pattern was found for the *pedH* promoter. Here, very low activities were detected in the
195 presence of up to 10 nM La^{3+} . Upon addition of 100 nM or more lanthanum, expression from
196 *pedE* promoter was induced with a peak at 10 μM .

197 The importance of transcriptional regulation of *pedE* and *pedH* was further tested by growth
198 experiments with 2-phenylethanol in liquid M9 medium (**Fig. 5B**). Growth of KT2440* was not

199 affected by the addition of up to 100 μM of lanthanum. On the other hand, strain $\Delta pedH$
200 showed a linear decrease in growth in the presence of increasing La^{3+} concentrations up to 1
201 μM , and no measurable growth when 10 μM or more La^{3+} was present in the medium. In
202 contrast, growth of strain $\Delta pedE$ was only observed in the presence of 10 μM or more La^{3+} .
203 Strain $\Delta pedE\Delta pedH$ did not grow under any of the tested conditions. A similar correlation
204 between growth and promoter activity of $pedE$ and $pedH$ was observed for Ce^{3+} , Pr^{3+} , Nd^{3+} , and
205 Sm^{3+} (**Fig. S3**).

206 These results demonstrate that KT2440 inversely regulates $pedE$ and $pedH$ promoter activity in
207 response to varying lanthanide concentrations and suggests that this regulation represents the
208 primary determinant for growth. However, in comparison to earlier studies with *M. extorquens*
209 AM1, the effective lanthanide concentration needed for growth was much higher (10 μM vs. 5
210 nM)(41). As lanthanides are known to form very poorly soluble complexes with phosphate and
211 hydroxide ions, we wondered whether this difference was caused by the minimal medium used
212 for growth (MP vs. M9). When the experiments were repeated with MP medium, the same
213 general trend and correlation of promoter activity and growth was found as described for M9
214 medium (**Fig. 6AB**). However, one difference was that the effective lanthanum concentration to
215 trigger a transcriptional response and growth was considerably lower (1 nM and 10 nM).
216 Another difference was that at a concentration of 10 nM La^{3+} minimal growth of both single
217 mutant strains was observed. The latter observation indicated that environmental conditions
218 might exist in which PedE and PedH are both functionally produced. To further test this
219 hypothesis, an additional growth experiment was performed and indeed showed growth of

220 both single mutants in a concentration range of 1 – 15 nM La³⁺ after prolonged (48 h) incubation
221 (Fig. S9).

222 **Impact of *PedE* and *PedH* on transcriptional regulation.** In *M. extorquens* AM1, the
223 transcription of methanol dehydrogenases is regulated, at least partially, by the PQQ-dependent
224 enzymes themselves (44). To test whether a similar outside-in signaling is also present in *P.*
225 *putida* KT2440, expression from the *pedE* and *pedH* promoter were quantified during growth
226 with 2-phenylethanol in MP medium (Fig. 7). In the absence of lanthanum, the $\Delta pedH$ strain
227 showed a 4-fold induction of *pedE* promoter activity, whereas the $\Delta pedE$ strain exhibited a slight
228 decrease (0.5-fold) in expression from the *pedE* promoter compared to KT2440* (Fig. 7A). The
229 presence of 10 nM La³⁺ resulted in a strong reduction of *pedE* promoter activity in all strains (21-
230 fold for KT2440*, 6-fold for $\Delta pedE$, 127-fold for $\Delta pedH$) compared to the control without
231 lanthanum. In comparison to *pedE*, expression from the *pedH* promoter was considerably lower
232 for all strains in the absence of lanthanum (Fig. 7B). However, when lanthanum was present, a
233 strong induction of *pedH* promoter activity in strain KT2440* (37-fold) and $\Delta pedE$ (29-fold) was
234 detected. Notably, the expression from the *pedH* promoter was dramatically reduced (2-fold vs.
235 37-fold) in the $\Delta pedH$ strain in comparison to the strains capable of producing a functional PedH
236 protein.

237 DISCUSSION

238 Lanthanide-dependent enzymes have so far been found exclusively within methylotrophic
239 organisms (16, 28–30, 32, 33). Using purified enzymes, we uncover that PedH, one of the two
240 PQQ-dependent ADHs (PQQ-ADHs) produced by the non-methylotrophic model organism *P.*
241 *putida* KT2440, is also a lanthanide-dependent enzyme, which utilizes La³⁺, Ce³⁺, Pr³⁺, Nd³⁺, Sm³⁺,
242 Gd³⁺ and Tb³⁺ as metal cofactor. The highest catalytic rates were observed with Pr³⁺ and Nd³⁺.
243 Notably, with lanthanides of higher atomic masses than Nd³⁺ the specific activity decreased
244 gradually, eventually resulting in no detectable activity with the heaviest lanthanides tested
245 (Er³⁺ and Yb³⁺). An analogous effect was previously reported by Pol *et al.*, who investigated the
246 impact of different lanthanides on growth rates of *Methylacidiphilum fumariolicum* SolV (33). A
247 possible explanation for these observations is that the decreased atomic radius, which is a
248 consequence of the lanthanide contraction, limits the more heavy lanthanides from being
249 functionally incorporated into the active site of PedH (45).

250 Kinetic parameters determined with Pr³⁺ and the three model substrates ethanol, acetaldehyde
251 and 2-phenylethanol revealed that V_{max} of PedH is about twofold higher compared to its Ca²⁺-
252 dependent counterpart PedE. It has been proposed that the increased activity can be explained
253 by the higher Lewis acidity of the lanthanides in comparison to calcium (46). Interestingly, we
254 found that the K_M values of the lanthanide-dependent enzyme PedH with acetaldehyde or 2-
255 phenylethanol are significantly smaller than those of the Ca²⁺-dependent protein PedE. One
256 possible explanation for this result is the higher polarity that arises in the active pocket of the
257 trivalent cation coordinating PedH compared to the divalent cation coordinating PedE. Another
258 explanation could be a smaller catalytic pocket, due to the higher atomic radius of Pr³⁺

259 compared to Ca^{2+} . The latter argument was proposed in an earlier study with the *mxoF*-type
260 MDH of *M. extorquens* AM1 as a reason for decreased activities when Ca^{2+} was replaced with
261 Ba^{2+} (47). In any case, apart from the approximately 2-fold increased specific activity of PedH
262 compared to PedE, the substrate scope and catalytic efficiencies (k_{cat}/K_M) of both enzymes were
263 found to be similar, which suggests that both enzymes are functionally redundant and only
264 differ in their cofactor dependency.

265 Functional redundancy is a well-known mechanism to improve robustness in complex systems
266 (48). The fact that many organisms express multiple PQQ-ADHs can be interpreted as an
267 adaptation to maintain an important function under variable environmental conditions or in
268 different microhabitats. Our study is supportive of such a hypothesis, as we demonstrate that
269 under conditions of high lanthanide availability, efficient growth of cells with various naturally
270 occurring alcoholic VOCs relies on the functional production of the lanthanide-dependent ADH
271 PedH. Similarly, for growth in the absence of lanthanides, functional production of the calcium-
272 dependent ADH PedE is mandatory. In this context it is important to note that growth in the
273 agar plate assay used in this study is restrictive, as it depends on diffusion and evaporation of
274 the volatile substrates. Thus, the assay most likely cannot discriminate between substrates for
275 which PedE or PedH function are essential and substrates for which other but less efficient
276 catabolic routes exist. We found that growth on ethanol (unpublished data) and 1-butanol (19),
277 but not 2-phenylethanol (current study) is possible, but less efficient for a strain lacking PedE
278 and PedH. From our and published data from a previous study (49), we therefore conclude that
279 beside the fact that PedE and PedH is not essential for growth with short chain aliphatic alcohols

280 (C2 to C5), both enzymes provide rapid conversion of these substrates, which is crucial for
281 efficient growth under restrictive conditions.

282 We further show that growth phenotypes strongly correlate with inverse transcriptional
283 regulation of *pedE* and *pedH*. Similar results have been reported for several methylotrophic
284 bacteria (41, 42). When cells of *P. putida* KT2440 were grown in liquid MP medium, the addition
285 of as little as 10 nM of lanthanides was sufficient to trigger *pedE* repression and a strong (20-
286 fold) concomitant induction of *pedH*. The transcription of *pedH* was found to be strongly
287 influenced by the PedH protein itself, implying a role for PedH as a lanthanide sensory module.
288 In *M. extorquens* AM1 the transcription of the calcium-dependent methanol dehydrogenase
289 *mxoF* strictly relies on the presence of XoxF proteins (41, 44). Our data demonstrate that this
290 regulation is different in *P. putida*, as *pedE* is only partially repressed by PedH. The fact that
291 *pedE* repression in the presence of lanthanum is still observed in the $\Delta pedH$ mutant strain,
292 together with the notion that the induction of *pedH* is not fully mediated by PedH, strongly
293 suggests the existence of at least one additional lanthanum-responsive regulatory module.
294 Notably, a very recent study identified that the transmembrane associated sensory histidine
295 kinase MxaY mediates the lanthanide-responsive switch of the PQQ-dependent MDHs in
296 *Methylomicrobium buryatense* (43). In *P. putida* KT2440 three different membrane associated
297 sensory histidine kinases, PedS1 (PP_2664), PedS2 (PP_2671) and PP_2683, are encoded within
298 close proximity of *pedE* and *pedH* as part of the predicted ErbR (AgmR) regulon (17). Whether
299 one of these sensor kinases serves a similar function as MxaY needs to be determined in future
300 studies.

301 From an ecological point of view, it is interesting that growth and inverse regulation occur even
302 in presence of high Ca^{2+} concentrations (100 μM) when only nanomoles of lanthanum are
303 supplied. From these data we conclude, similar to previous studies with *M. buryatense* or *M.*
304 *extorquens* (32, 41), that the lanthanide-dependent enzyme PedH is the preferred PQQ-ADH
305 when both metal cofactors are simultaneously accessible. Nevertheless, it was also
306 demonstrated that under certain low REM concentrations, specific conditions exist in which
307 both single mutants can grow. This suggests that the inverse regulation of the two enzymes is
308 not a strict *on-off* switch, but rather operates by strongly shifting the transcription in favor of
309 one of the enzymes depending on the REM concentration.

310 REM utilizing PQQ-ADHs have been suggested to be ancestral and more widespread than their
311 calcium-dependent homologues (15, 50). This might indicate that calcium-dependent enzymes
312 have evolved to colonize different and/or additional environmental niches in which lanthanide
313 availability is less pronounced. Compared to soil environments, especially the rhizosphere,
314 lanthanide concentrations in the phyllosphere and endosphere, as well as in other non-plant
315 higher organisms are comparably low (51–54). It is thus tempting to speculate, that Ca^{2+} -
316 dependent enzymes are of particular relevance for interactions with multicellular organisms
317 outside of soil environments.

318 Metabolic interdependencies have been proposed as driving force for species co-occurrence
319 and the emergence of mutualism in diverse microbial communities impacting their robustness,
320 structure, and function (55–57). This is of particular interest in the context of periplasmic PQQ-
321 ADHs, as organic alcohols and the corresponding oxidation products are not only crucial
322 intermediates of the global carbon cycle, but can also exhibit additional functions including

323 signaling and growth inhibition (4–7, 58). A recent study reported that regulation of the MxaF-
324 and XoxF-type MDHs in a methanotrophic bacterium can be influenced by the presence of a
325 non-methanotrophic methylotroph in co-culture experiments (59). The authors nicely
326 demonstrate that during co-cultivation in the presence of methane and lanthanides, the
327 methanotrophic bacterium shifts its gene expression from the *xoxF*- to the *mxαF*-type MDH. As
328 a result of this change, leakage of methanol from the methanotroph was observed, which
329 subsequently served as growth substrate for the non-methanotrophic partner. Although the
330 mechanism of this phenomenon is not yet resolved, it indicates that different types of PQQ-
331 ADHs might not only be important for potential interactions with higher organisms as discussed
332 above, but also within microbial communities. Based on our data, one can speculate that similar
333 interactions are not limited to methano- and methylotrophic bacteria, but are relevant in a
334 much broader ecological context.

335 Lastly, the discovery of lanthanides as a cofactor in biotechnological important organisms other
336 than methylotrophic bacteria expands the possible applications one can envision for biomining,
337 bioleaching, and recycling processes of rare earth metals (60–65). As such, we believe that
338 future research about lanthanide-utilizing enzymes and organisms will improve our
339 understanding of natural and synthetic microbial communities and could provide a basis for
340 novel biotechnological tools and processes.

341 MATERIAL AND METHODS

342 **Bacterial strains, plasmids, and culture conditions.** Strains and plasmids used in this study
343 (**Table S1**) as well as a detailed description of their construction (**Text S1**) can be found as
344 supplementary material. Unless otherwise noted, *Pseudomonas putida* KT2440 and *Escherichia*
345 *coli* strains were maintained on solidified (1.5% agar [w/v]) Lysogeny Broth (LB, Maniatis *et al.*,
346 1982). Strains were routinely cultured in liquid LB medium, a modified M9 salt medium
347 containing 74 mM phosphate buffer (pH 7), 18.6 mM NH₄Cl, 8.6 mM NaCl, 2 mM MgSO₄, 100
348 μM CaCl₂ with a trace element solution containing Na₃-citrate 51 μM, ZnSO₄ 7 μM, MnCl₂ 5 μM,
349 CuSO₄ 4 μM, FeSO₄ 36 μM, H₃BO₃ 5 μM, NaMoO₄ 137 nM, NiCl₂ 84 nM or a modified MP
350 medium (67) containing 100 μM CaCl₂ instead of 20 μM CaCl₂ supplemented with succinate or 2-
351 phenylethanol as sole source of carbon and energy at 30°C with shaking. Where indicated, 40 μg
352 mL⁻¹ kanamycin or 15 μg mL⁻¹ gentamycin for *E. coli* and 40 μg mL⁻¹ kanamycin, 20 μg mL⁻¹ 5-
353 fluoro uracil or 30 μg mL⁻¹ gentamycin for *P. putida* strains was added to the medium for
354 maintenance and selection, respectively.

355 **Growth experiments in liquid medium.** All liquid growth experiments were carried out using a
356 modified M9 minimal salt medium or MP medium (see above) supplemented with 25 mM
357 succinate or 5 mM 2-phenylethanol as carbon and energy source. To avoid potential lanthanide
358 contaminations from glassware, all growth experiments were carried out in 125 mL
359 polycarbonate vessels (Corning) or in polypropylene 96 well 2 ml deep well plates (Carl Roth). If
360 not stated otherwise, precultures were grown in 5 ml minimal medium (15 mL Falcon tubes)
361 supplemented with succinate at 30 °C and 180 rpm using a rotary shaker (HT Minitron, Infors).
362 The next day, cultures were washed three times in fresh minimal medium without a carbon and

363 energy source and used to inoculate 1 ml (for 2 ml deep well plates) or 25 ml (for 125 ml
364 polycarbonate vessels) of fresh medium to an initial optical density at 600 nm (OD_{600}) of 0.01.
365 Subsequently, cultures were supplemented with the carbon and energy source as well as
366 varying concentrations of lanthanides and incubated at 30°C and 180 rpm (for 125 ml
367 polycarbonate vessels) or 800 rpm (for 2 ml deep well plates). For experiments in 125 ml
368 polycarbonate vessels, growth was monitored by measuring the OD_{600} at regular intervals using
369 a photometer (BioPhotometer, Eppendorf). For experiments carried out in 2 ml deep well
370 plates, OD_{600} was determined after 24 h or 48 h by measuring 200 μ L of cell culture transferred
371 to a microtiter plate (Greiner bio-one) in a microplate reader (POLARstar Omega, BMG Labtech).
372 All data are presented as the mean value of biological triplicates with error bars representing
373 the corresponding standard deviation.

374 **Agar plates assay.** For growth on solidified medium (1.5% agar [w/v]) with different substrates,
375 M9 medium plates without addition of a carbon source and trace element solution were freshly
376 prepared with or without the addition of 20 μ M lanthanum chloride. Cell mass of the strains
377 was obtained from LB agar plates, suspended in M9 medium without carbon and energy source,
378 and adjusted to an optical density of 0.5. After drying the plates for 20 minutes in a laminar flow
379 cabinet, 10 μ L of each cell suspension was dropped onto the same plate and distributed using
380 an inoculation loop on about 1/6 of the plate's surface. When all strains were distributed, a 10
381 μ L drop of a 1:1 mixture (v/v) of ethanol, 1-butanol, 2-3 butanediol, 1-octanol, or 2-
382 phenylethanol in dimethyl sulfoxide (DMSO) was placed in the middle of the plate.
383 Subsequently, the plates were sealed in plastic bags and incubated at room temperature. After
384 48 h, growth was quantified with a digital imaging system (Vilber Lourmat, QUANTUM ST4)

385 using the standard fluorescence settings with combined white light and UV illumination (ex. 254
386 nm) for 1 sec, an aperture of 11 and using the preinstalled F590 nm filter. All individual pictures
387 were subsequently sized, isolated from the background, and corrected for sharpness (+ 50%),
388 brightness (+ 20%), and contrast (+ 40%) using the graphic formatting function in Microsoft
389 PowerPoint.

390 **Transcriptional reporter assays.** For transcriptional reporter assays, *P. putida* harboring either
391 Tn7-based *pedE-lux* and *pedH-lux* transcriptional reporter fusion were grown overnight in a
392 modified MP medium with succinate, washed three times in MP medium with no added carbon
393 source and finally suspended in MP medium or M9 medium with 1 mM 2-phenylethanol to an
394 OD₆₀₀ of 0.1. For luminescence measurements, 180 µl of cell suspension were added to 20 µl of
395 tenfold concentrated metal salt solution in white 96-well plates with clear bottom (µClear,
396 Greiner Bio-One). Microtiter plates were placed in a humid box to prevent evaporation,
397 incubated at 30°C with continuous agitation (200 rpm) and light emission as well as OD₆₀₀ were
398 recorded at regular intervals in a FLX-Xenius plate reader (SAFAS, Monaco) for up to 6 h. For
399 both parameters, background provided by the MP medium was subtracted, and the
400 luminescence was normalized to the corresponding OD₆₀₀. Experiments were performed in
401 biological triplicates and data are presented as the mean value with error bars representing the
402 corresponding standard deviation.

403 **Enzymatic assays.** Details about the expression and purification procedure for PedE and PedH
404 can be found as supplementary material (**Text S1**). Enzyme activities of purified PedE and PedH
405 were measured using a dye-linked colorimetric assay in 96 well microtiter plates (Greiner bio-
406 one) based on previous studies (24, 68). Under optimized conditions (**Fig. S4-S8**), one well

407 contained a total volume of 250 μL of assay solution supplemented with: 100 mM Tris HCl pH 8;
408 500 μM PMS; 150 μM 2,6-dichlorophenol indophenol (DCPIP); 25 mM imidazole; 1 mM CaCl_2 for
409 PedE or 1 μM PrCl_3 for PedH; 1 μM PQQ for PedE or 50 μM PQQ for PedH; 12.5 μL substrate and
410 2.5–20 $\mu\text{g}/\text{ml}$ enzyme. The reaction was started by addition of the substrate to the reaction
411 mixture and the activity was calculated based on the change of OD_{600} within the first minute
412 upon substrate addition. The molar extinction coefficient of DCPIP was experimentally
413 determined to be $24.1 \text{ cm}^{-1}\text{M}^{-1}$ at pH 8 (**Fig. S2**). Due to a substrate independent background
414 activity, the assay solution without substrate was incubated for 45 minutes at 30°C prior to
415 enzyme activity measurements. As activities were between 8- and 12-fold higher when using
416 imidazole compared to ammonium chloride or ethylamine, imidazole was used in all
417 experiments (**Fig. S3**). Negative control reactions, including the potential effect of BSA or assay
418 mixture without the addition of enzyme, did not show any reduction of DCPIP under the
419 conditions used (data not shown). All assays were performed in three replicates and data are
420 presented as the mean value with error bars representing the corresponding standard
421 deviation.

422 ***Metal dependency of the enzymes.*** To test the metal dependency of PedE and PedH, a similar
423 set-up as described above was used omitting CaCl_2 for PedE or PrCl_3 for PedH in the assay
424 solution. 1 μM of different rare earth metals was added prior to incubation at 30°C . These
425 included LaCl_3 , CeCl_3 , PrCl_3 , NdCl_3 , SmCl_3 , GdCl_3 , ErCl_3 , YbCl_3 , ScCl_3 and YCl_3 . Activities were
426 determined in triplicates as described above.

427 ***Enzyme kinetics.*** The kinetic constants of the enzyme substrate combinations were determined
428 using the enzyme assay described above with various substrate concentrations measured in

429 triplicates. The resulting activity constants were calculated by fitting the enzyme activities by
430 nonlinear regression to the Michaelis-Menten equation using the 'Michaelis-Menten' least-
431 square fit method with no constraints in GraphPad Prism (GraphPad Software, version 7.03).

432 **Homology models.** The PedE and PedH homology models were built with Swiss-Model (69). As
433 ExaA has the highest sequence similarity with both PedE (60%) and PedH (49%) of all available
434 crystal structures in the Swiss-Model template library, the crystal structure of the PQQ
435 dependent ADH ExaA of *P. aeruginosa* (1FLG) was used as a template for model construction
436 (23). Visualization of the models was carried out using PyMOL (70).

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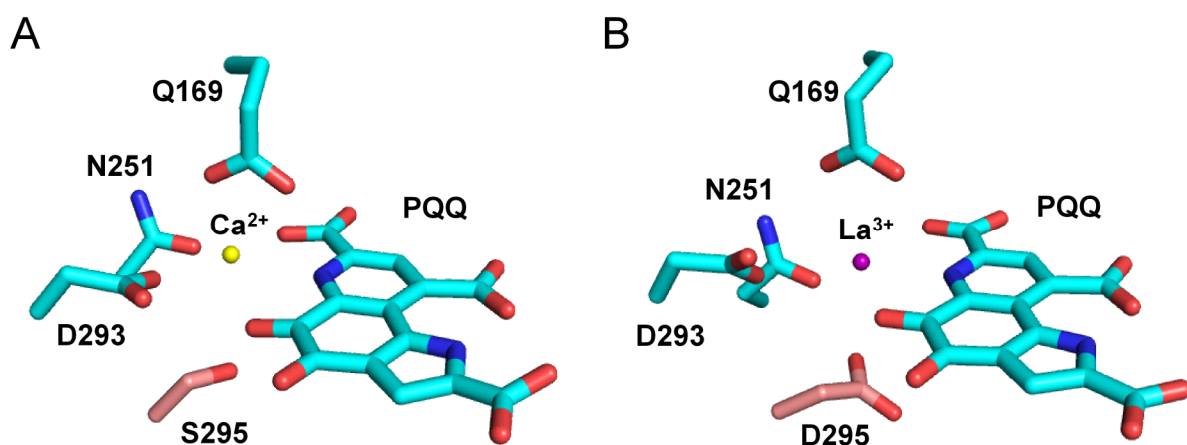
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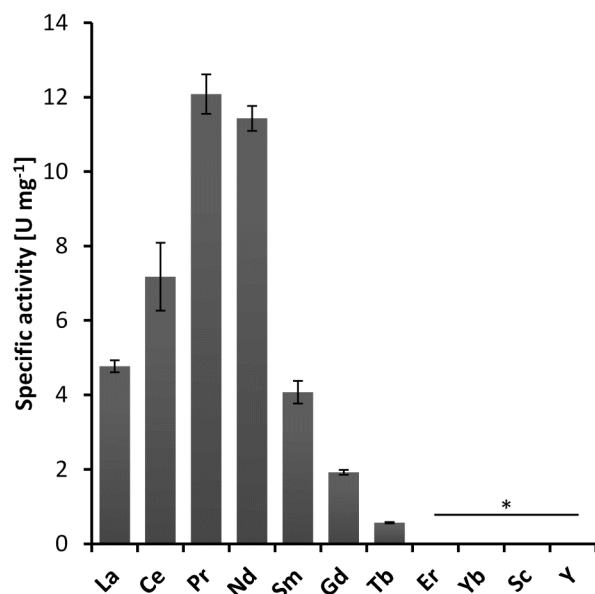
663 **FIGURES**



664

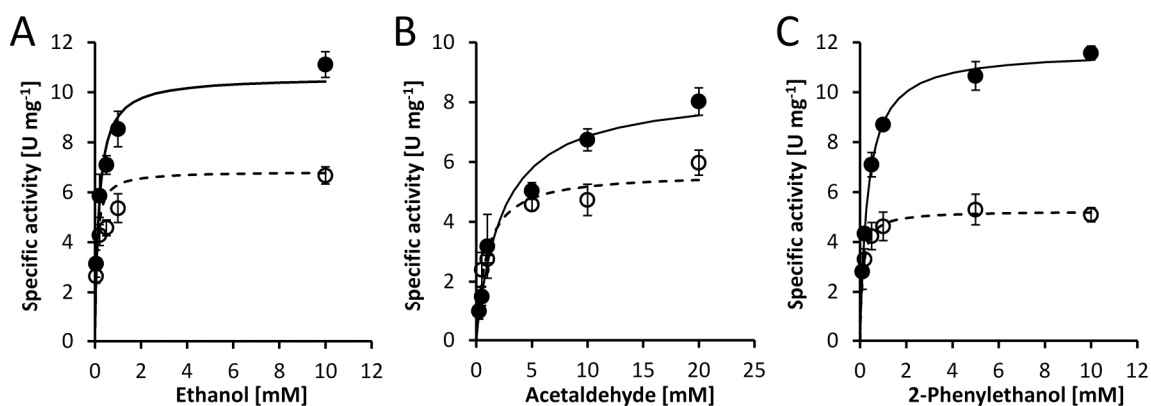
665 **FIG 1:** Homology models of PedE (A) (GI: 26989393) and PedH (B) (GI: 26989398) generated
666 with SWISS-MODEL based on the crystal structure of ExaA from *Pseudomonas aeruginosa* (PDB:
667 1FLG) using Pymol (70). The catalytic cation (*yellow or violet sphere*) coordinating amino acids
668 and the PQQ cofactor are shown as sticks using an element color code (C = cyan, O = red, N =
669 blue). The amino acid position 295 in PedE and PedH is highlighted by using a different color
670 code (C = light red).

671



672
 673 **Fig 2:** Specific activities of PedH in the presence of 1 μM of various rare earth metal ions with
 674 10 mM ethanol as substrate. Activities below detection limit are indicated (*). Data are
 675 presented as the mean value of three replicates and error bars represent the corresponding
 676 standard deviation.

677



D

Substrate	V_{max} [U mg ⁻¹]	K_M [μM]	k_{cat}/K_M [mM s ⁻¹]
Ethanol	6.1 ± 0.3	85 ± 18	81 ± 18
Acetaldehyde	5.6 ± 0.2	899 ± 155	7.0 ± 1.2
2-Phenylethanol	5.2 ± 0.2	115 ± 22	51 ± 10

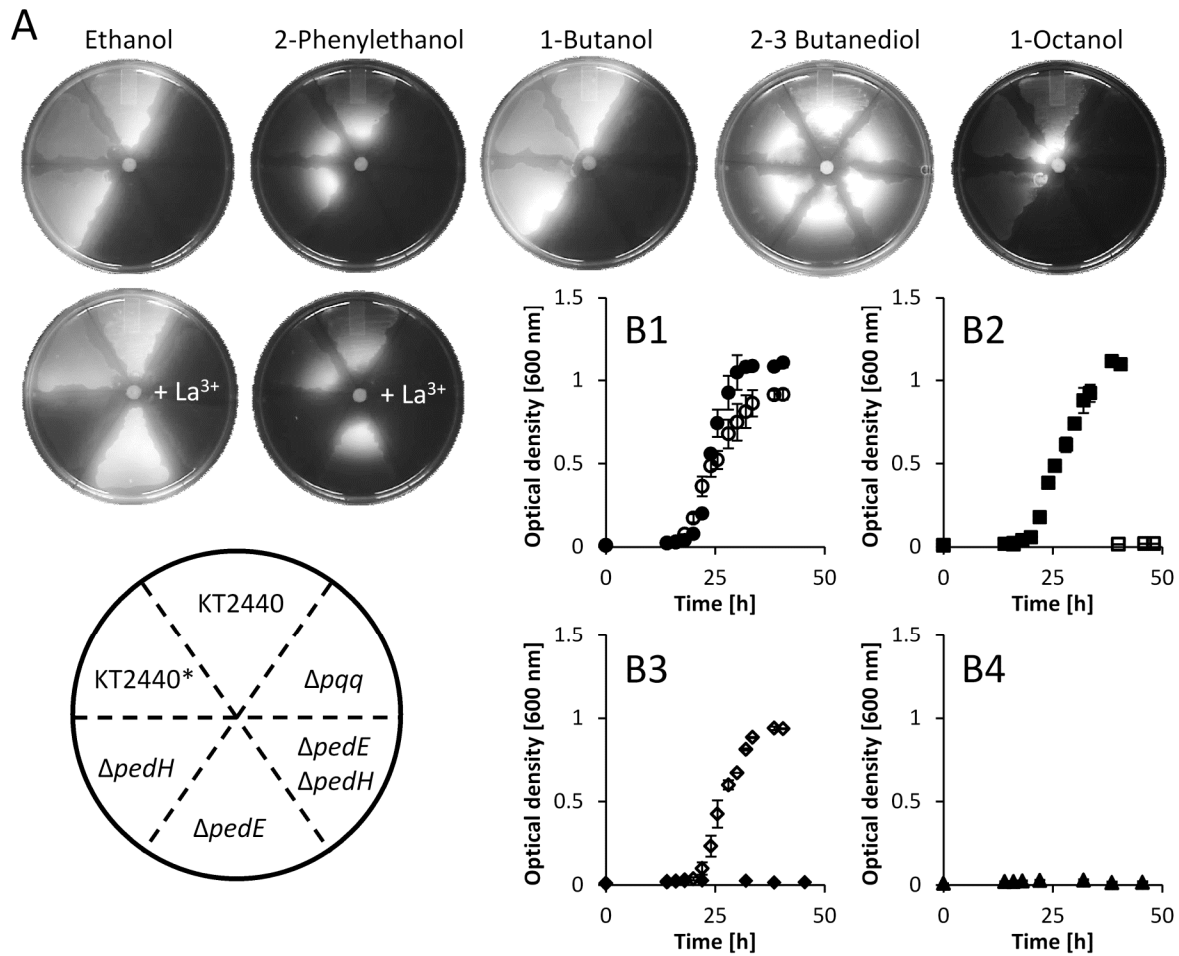
E

Substrate	V_{max} [U mg ⁻¹]	K_M [μM]	k_{cat}/K_M [mM s ⁻¹]
Ethanol	10.6 ± 0.4	177 ± 31	66 ± 12
Acetaldehyde	8.4 ± 0.5	2261 ± 457	4.1 ± 0.8
2-Phenylethanol	11.8 ± 0.2	329 ± 22	38 ± 3

678

679 **FIG 3:** Kinetic parameter determination. **A-C)** Michaelis-Menten plot showing specific enzyme
680 activities of PedE (*black circles*) and PedH (*white circles*) over a varying concentration of ethanol
681 (**A**), acetaldehyde (**B**), and 2-phenylethanol (**C**). For PedE, 1 mM of CaCl₂ and 50 μM PQQ was
682 used, while for PedH, 1 μM PrCl₃ and 1 μM PQQ was used in the reaction mixture. The data are
683 given as mean value of triplicate measurements with error bars representing the standard
684 deviation. Maximum velocity (V_{max}), substrate affinity (K_M) and the catalytic efficiency (k_{cat}/K_M ,
685 where k_{cat} is the turnover frequency per cofactor molecule) of PedE (**D**) and PedH (**E**) for different
686 substrates are derived from **A-C** using nonlinear regression to a Michaelis-Menten model
687 (*continuous lines* for PedH and *dashed lines* for PedE). Kinetic constants are represented as best
688 fit values ± standard error.

689

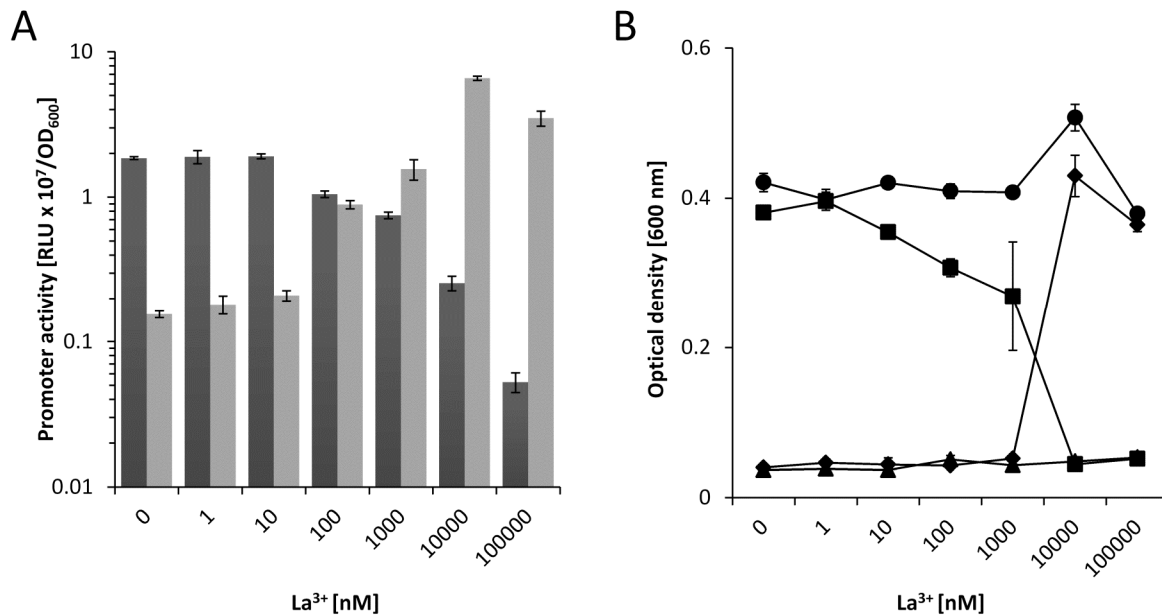


690

691 **FIG 4: A)** Growth with various substrates (10 μ L drop of a 1:1 mix with DMSO) on M9 agar
 692 plates. Growth was quantified with a digital imaging system after 48 h using combined white
 693 light and UV illumination (ex. 254 nm). All pictures were sized, isolated from the background,
 694 and corrected for sharpness (+ 50%), brightness (+ 20%), and contrast (+ 40%). **B1-4)** Growth of
 695 KT2440* (Δ *upp* strain used as the parental strain for knockout mutants; **B1**, circles), Δ *pedE* (**B2**,
 696 diamonds), Δ *pedH* (**B3**, squares), and Δ *pedE* Δ *pedH* (**B4**, triangles) in M9 medium with 5 mM 2-
 697 phenylethanol in the absence (black symbols) or presence of 20 μ M La³⁺ (open symbols). Growth
 698 was performed in 25 mL (125 mL plastic Erlenmeyer) at 30°C and 180 rpm shaking (Multifors)
 699 and quantified by optical density measurements at 600 nm. Data represent the mean of two

700 individual cultures with error bars representing the corresponding range. All error bars are
701 depicted but might not be visible due to the size of the corresponding symbol used for the mean
702 value.

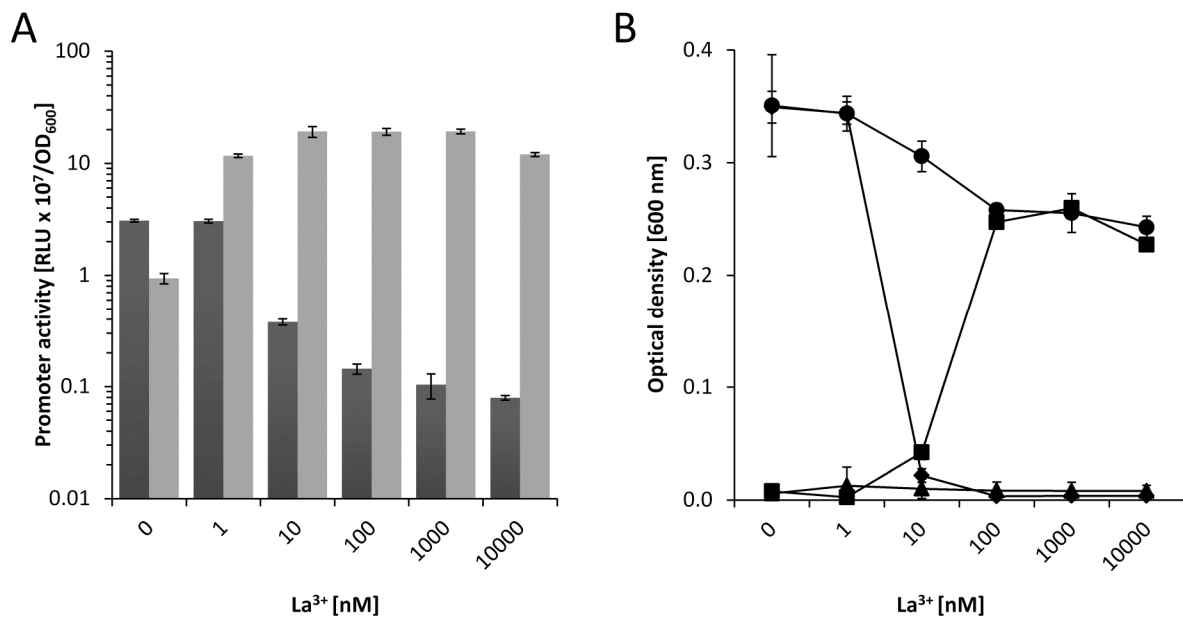
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704

705 **FIG 5: A)** Activities of the *pedE* (dark grey bars) and *pedH* (light grey bars) promoters in strain
706 KT2440* during incubation in liquid M9 medium (A) supplemented with 1 mM of 2-
707 phenylethanol in the presence of varying concentrations of La³⁺. Promoter activities are
708 presented as relative light units (RLU × 10⁷) normalized to OD₆₀₀. **B)** Growth of KT2440* (black
709 circles), ΔuppΔpedE (black diamonds), ΔuppΔpedH (black squares) and ΔuppΔpedEΔpedH (black
710 triangles) in liquid M9 medium with 5 mM of 2-phenylethanol in the presence of different
711 concentrations of La³⁺. Growth was determined as the optical density at 600 nm after
712 incubation at 30°C for 24 h. Data are presented as mean values from biological triplicates and
713 error bars represent the corresponding standard deviation.

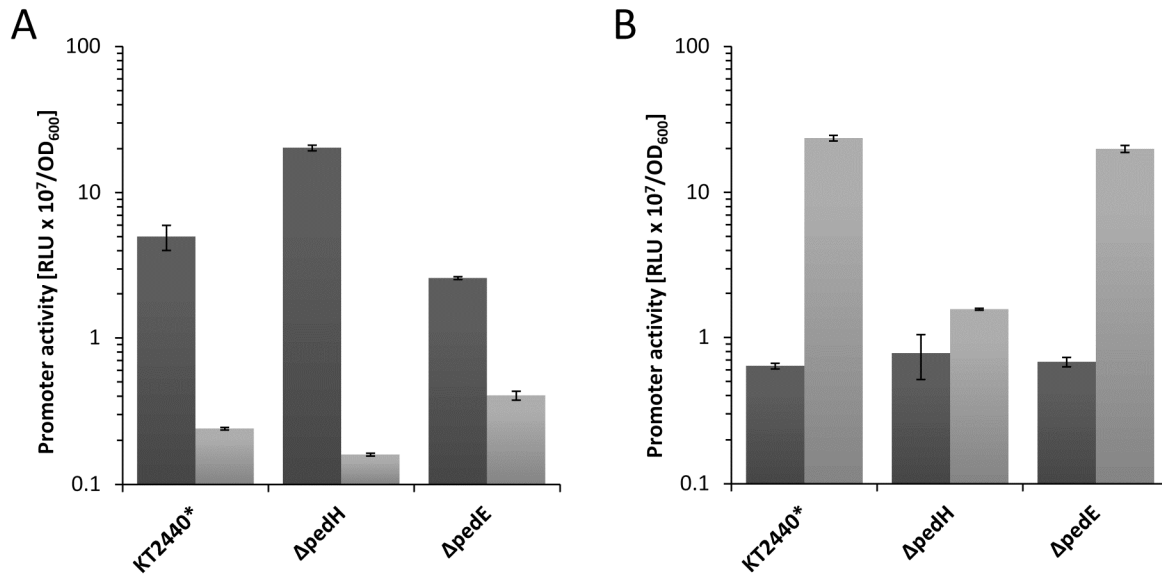
714



715

716 **FIG 6: A)** Activities of the *pedE* (dark grey bars) and *pedH* (light grey bars) promoters in strain
717 KT2440* during incubation in liquid MP medium (A) supplemented with 1 mM of 2-
718 phenylethanol in the presence of varying concentrations of La³⁺. Promoter activities are
719 presented as relative light units (RLU × 10⁷) normalized to OD₆₀₀. **B)** Growth of KT2440* (black
720 circles), $\Delta upp \Delta pedE$ (black diamonds), $\Delta upp \Delta pedH$ (black squares) and $\Delta upp \Delta pedE \Delta pedH$ (black
721 triangles) in liquid MP medium with 5 mM of 2-phenylethanol in the presence of different La³⁺
722 concentrations. Growth was determined as the optical density at 600 nm after incubation at
723 30°C for 24 h. Data are presented as the mean values from biological triplicates and error bars
724 represent the corresponding standard deviation.

725



726

727 **FIG 7:** Activities of the *pedE* (A) and *pedH* (B) promoters in strains KT2440* (Δupp strain used as
728 the parental strain for knockout mutants), $\Delta pedE$, $\Delta pedH$ and $\Delta pedE\Delta pedH$ in liquid MP medium
729 with 1 mM of 2-phenylethanol in the absence (dark grey bars) or presence of 10 nM La³⁺ (light
730 grey bars). Promoter activities are presented as relative light units (RLU $\times 10^7$) normalized to
731 OD₆₀₀. Data are presented as the mean values from biological triplicates and error bars
732 represent the corresponding standard deviation. The promoter activities for each strain in the
733 presence and absence of lanthanides were statistically analyzed (two-tailed *t*-test; $\alpha = 0.05$; $N =$
734 3; GraphPad Prism, version 7.03) and found to be significantly different ($P < 0.01$).

735 **TABLES**

736 **Table 1:** Specific activities of PedE and PedH with various alcohols and aldehydes. Data are
737 presented as the mean value of three independent measurements with the corresponding
738 standard deviation. 10 mM of substrate was used in H₂O if not indicated otherwise.

Substrate	Specific activity [U mg ⁻¹]	
	PedE	PedH
Methanol	0.61 ± 0.10	0.80 ± 0.05
Ethanol	6.7 ± 0.9	11.0 ± 0.3
Ethanolamine	0.55 ± 0.09	1.6 ± 0.2
1-Butanol	5.8 ± 0.1	11.5 ± 0.7
2-Butanol	4.4 ± 0.7	7.6 ± 0.4
2,3-Butanediol	0.39 ± 0.03	0.78 ± 0.04
1-Hexanol	5.2 ± 0.1	10.4 ± 1.1
1-Octanol ^a	3.5 ± 0.2	4.7 ± 0.7
2-Phenylethanol	6.7 ± 1.1	10.2 ± 1.4
Acetaldehyde	4.7 ± 0.5	6.7 ± 0.4
Butyraldehyde	6.1 ± 0.4	10.3 ± 0.6
Hexanal ^a	3.8 ± 0.1	6.2 ± 0.3
Octanal ^a	1.9 ± 0.2	3.6 ± 0.6
Cinnamyl alcohol ^a	2.4 ± 0.1	3.9 ± 0.1
Cinnamaldehyde ^b	n. d. ^c	n. d. ^c
Farnesol ^b	2.0 ± 0.3	3.8 ± 0.5

739 ^a 10 mM substrate in DMSO.

740 ^b 500 μM substrate in DMSO.

741 ^c Activity below detection limit.