Rare earth element (REE)-dependent growth of *Pseudomonas putida* KT2440 depends on the ABC-transporter PedA1A2BC and is influenced by iron availability

Matthias Wehrmann\textsuperscript{a}, Charlotte Berthelot\textsuperscript{b,c}, Patrick Billard\textsuperscript{b,c#}, Janosch Klebensberger\textsuperscript{a#}

\textsuperscript{a}University of Stuttgart, Institute of Biochemistry and Technical Biochemistry, Department of Technical Biochemistry, Stuttgart, Germany

\textsuperscript{b}Université de Lorraine, LIEC UMR7360, Faculté des Sciences et Technologies, Vandoeuvre-lès-Nancy, France

\textsuperscript{c}CNRS, LIEC UMR7360, Faculté des Sciences et Technologies, Vandoeuvre-lès-Nancy, France

Running title: Rare earth element uptake and interference in *Pseudomonas putida* KT2440

#Address correspondence to Janosch Klebensberger (janosch.klebensberger@itb.uni-stuttgart.de) and Patrick Billard (patrick.billard@univ-lorraine.fr)

Keywords: Lanthanides, rare earth elements, ABC-transporter, *Pseudomonas putida*, PQQ, PedE, PedH, mismetallation, dehydrogenases
Abstract

In the soil-dwelling organism *Pseudomonas putida* KT2440, the rare earth element (REE)-utilizing and pyrroloquinoline quinone (PQQ)-dependent ethanol dehydrogenase PedH is part of a periplasmic oxidation system that is vital for growth on various alcoholic volatiles. Expression of PedH and its Ca$^{2+}$-dependent counterpart PedE is inversely regulated in response to lanthanide (Ln$^{3+}$) bioavailability, a mechanism termed the REE-switch. In the present study, we demonstrate that copper, zinc, and in particular, iron availability influences this regulation in a pyoverdine-independent manner by increasing the minimal Ln$^{3+}$ concentration required for the REE-switch to occur by several orders of magnitude. A combined genetic- and physiological approach reveals that an ABC-type transporter system encoded by the gene cluster *pedA1A2BC* is essential for efficient growth with low (nanomolar) Ln$^{3+}$ concentrations. In the absence of *pedA1A2BC*, a ~100-fold higher La$^{3+}$ concentration is needed for PedH-dependent growth but not for the ability to repress growth based on PedE activity. From these results, we conclude that cytoplasmic uptake of lanthanides through PedA1A2BC is essential to facilitate REE-dependent growth under environmental conditions with poor REE bioavailability. Our data further suggest that the La$^{3+}$/Fe$^{3+}$ ratio impacts the REE-switch through the mismetallation of putative La$^{3+}$-binding proteins, such as the sensor histidine kinase PedS2, in the presence of high iron concentrations.

As such, this study provides an example for the complexity of bacteria-metal interactions and highlights the importance of medium compositions when studying physiological traits *in vitro* in particular in regards to REE-dependent phenomena.
**Introduction**

Metal ions are essential for all living organisms as they play important roles in stabilizing macromolecular cellular structures, by catalyzing biochemical reactions or acting as cofactors for enzymes (Gray, 2003; Merchant and Helmann, 2012). They can, however, also be toxic to cells at elevated levels through the generation of reactive oxygen species or by aspecific interactions such as mismetallation (Cornelis et al., 2011; Dixon and Stockwell, 2014; Foster et al., 2014). Bacteria have hence developed a sophisticated toolset to maintain cellular metal homeostasis (Andrews et al., 2003; Chandrangsu et al., 2017; Schalk and Cunrath, 2016; Semrau et al., 2018). Common mechanisms include release of metal-specific scavenger molecules, the activation of high-affinity transport systems, the production of metal storage proteins, and the expression of specific efflux pumps.

As it is the case for all strictly aerobic bacteria, the soil-dwelling organism *Pseudomonas putida* KT2440 has a high demand for iron but faces the challenge that the bioavailability of this metal is very poor under most toxic environmental conditions due to the fast oxidation of Fe$^{2+}$- and the low solubility of Fe$^{3+}$-species (Andrews et al., 2003). One strategy of many bacteria to overcome this challenge is to excrete self-made peptide-based siderophores (such as pyoverdines) into the environment that bind Fe$^{3+}$ with high affinity, and thereby increase its bioavailability (Baune et al., 2017; Cornelis and Andrews, 2010; Salah El Din et al., 1997). A second adaptation of *P. putida* cells to iron-limitation is a change in the proteomic inventory in order to limit the use of Fe-containing enzymes, exemplified by the switch from the Fe-dependent superoxide dismutase (SOD) to a Mn-dependent isoenzyme or by re-routing of entire metabolic pathways (Kim et al., 1999; Sasnow et al., 2016). In contrast, when Fe bioavailability is high, the production of the bacterioferritins Bfrα and Bfrβ is increased to enable intracellular storage and thereby improve cellular fitness under potential future conditions of iron starvation (Chen et al., 2010). The
regulatory mechanisms for metal homeostasis of *P. putida* cells in response to other essential metal ions such as Co, Cu, Mg, Mo, Ni, and Zn are less well explored. Genes encoding for transport systems associated with the uptake and efflux of these metals can, however, be found in its genome (Belda et al., 2016; Nelson et al., 2002), and some of these have been studied in more detail (Miller et al., 2009; Ray et al., 2013).

We have recently reported that *P. putida* KT2440 is capable of using rare earth elements (REEs) of the lanthanide series (Ln$^{3+}$) when growing on several alcoholic substrates (Wehrmann et al., 2017, 2019). Under these conditions, the cells use the pyrroloquinoline quinone (PQQ)-dependent ethanol dehydrogenase (EDH) PedH, to catalyze their initial oxidation within the periplasm. Like many other organism, *P. putida* harbors an additional, Ln$^{3+}$-independent functional homologue of PedH termed PedE that depends on a Ca$^{2+}$ ion as metal cofactor (Takeda et al., 2013; Wehrmann et al., 2017). Depending on the availability of REEs in the environment, *P. putida* tightly regulates PedE and PedH production (Wehrmann et al. 2017, 2018). In the absence of Ln$^{3+}$, growth is solely dependent on PedE whereas PedH transcription is repressed. The situation immediately changes in the presence of small amounts of Ln$^{3+}$ (low nM range) leading to a strong induction of the Ln$^{3+}$-dependent enzyme PedH and repression of its Ca$^{2+}$-dependent counterpart PedE. For *P. putida* KT2440 the PedS2/PedR2 two component system (TCS) is a central component of this inverse regulation (Wehrmann et al., 2018). Notably, the REE-switch in *P. putida* was also found to be influenced by environmental conditions, as the critical La$^{3+}$ concentrations required to support PedH-dependent growth differs dramatically depending on the medium used, ranging from 5 nM up to 10 µM (Wehrmann et al., 2017).

Lanthanides are only poorly available in natural environments (often picomolar concentrations) due to the formation of low soluble hydroxide and/or phosphate (Firsching and Brune, 1991; Meloche and Vrátný, 1959). The presence of active uptake systems to facilitate REE-dependent
growth in bacteria has thus been favored by many researchers (Aide and Aide, 2012; Cotruvo et al., 2018; Gu et al., 2016; Gu and Semrau, 2017; Markert, 1987; Picone and Op den Camp, 2019; Tyler, 2004). A transcriptomic study of *M. trichosporium* OB3b cells observed that multiple genes encoding for different active transport systems were among the most regulated in the presence of cerium (Gu and Semrau, 2017). In addition to these findings, it has been found that a specific TonB-dependent receptor protein as well as a TonB-like transporter protein are highly conserved in bacteria that carry genes encoding for Ln$^{3+}$-dependent MDHs (Keltjens et al., 2014; Wu et al., 2015). Only very recently, different studies indeed identified both an ABC-transporter and TonB-dependent receptor proteins that are needed for REE-dependent growth of methanotrophic and methylotrophs, strongly suggesting the existence of an uptake system that specifically transports a Ln$^{3+}$-chelator complex in these organisms (Groom et al., 2019; Ochsner et al., 2019; Roszczenko-Jasińska et al., 2019).

With the present study, we show that a homologous ABC-transporter system, encoded by the gene cluster *pedA1A2BC*, is also essential for lanthanide-dependent growth in the non-methylotrophic organism *Pseudomonas putida* KT2440 under low (nanomolar) concentrations of REEs. Notably, no homolog of the TonB-dependent receptor proteins found in methanotrophic or methylotrophic strains could be identified within the genome of *P. putida* KT2440 indicating either a lack or substantial differences in the chemical nature of such a Ln$^{3+}$-specific chelator system. Finally, we show that the siderophore pyoverdine plays no essential role for growth under low REE concentrations but provide compelling evidence that in addition to Cu$^{2+}$ and Zn$^{2+}$ the Fe$^{2+/3+}$ to Ln$^{3+}$ ratio can significantly alter the REE-switch most likely through mismetallation.
Materials and methods

Bacterial strains, plasmids and culture conditions

The *E. coli* and *P. putida* KT2440 strains and the plasmids used in this study are described in Table 1. Maintenance of strains was routinely performed on solidified LB medium (Maniatis et al., 1982). If not stated otherwise, strains were grown in liquid LB medium (Maniatis et al., 1982) or a modified M9 salt medium (Wehrmann et al., 2017) supplemented with 5 mM 2-phenylethanol, 5 mM 2-phenylacetaldehyde, 5 mM phenylacetic acid, or 25 mM succinate as carbon and energy source at 28°C to 30°C and shaking. 40 µg mL⁻¹ kanamycin or 15 µg mL⁻¹ gentamycin for *E. coli* and 40 µg mL⁻¹ kanamycin, 20 µg mL⁻¹ 5-fluorouracil, or 30 µg mL⁻¹ gentamycin for *P. putida* strains was added to the medium for maintenance and selection, if indicated.

Liquid medium growth experiments

Liquid growth experiments were performed in biological triplicates by monitoring the optical density at 600 nm (OD₆₀₀) during growth in modified M9 medium supplemented with the corresponding carbon and energy sources (see above). For all experiments, washed cells from overnight cultures grown with succinate at 30°C and 180 rpm shaking were utilized to inoculate fresh medium with an OD₆₀₀ of 0.01 to 0.05. Depending on the culture vessel, the incubation was carried out in 1 ml medium per well for 96-well 2 ml deep-well plates (Carl Roth) at 350 rpm shaking and 30°C, or 200 µL medium per well for 96-well microtiter plates (Sarstedt) at 180 rpm shaking and 28°C. If needed, different concentrations of LaCl₃ (Sigma-Aldrich) were added to the medium.

Construction of plasmids
The 600 bp regions upstream and downstream of gene \textit{pvdD}, gene cluster \textit{pedA1A2BC} and genes tatC1 and tatC2 were amplified from genomic DNA of \textit{P. putida} KT2440 using primers pairs MWH56/MWH57 and MWH58/MWH59, MWH94/MWH95 and MWH96/MWH97, PBtatC1.1/PBtatC1.2 and PBtatC1.3/PBtaC1.4, and PBtatC2.1/PBtatC2.2 and PBtatC2.3/PBtatC2.4 to construct the deletion plasmids pMW50, pMW57, pJOE-tatC1 and pJOE-tatC2 (\textbf{Table 2}). The BamHI digested pJOE6261.2 as well as the two up- and downstream fragments were therefore joined together using one-step isothermal assembly (Gibson, 2011) and subsequently transformed into \textit{E. coli} BL21(DE3) or TOP10 cells. Sanger sequencing confirmed the correctness of the plasmids.

For measuring promoter activity of \textit{pedE} and \textit{pedH} in vivo, plasmids pTn7-M-pedH-lux and pTn7-M-pedE-lux were constructed. The DNA regions encompassing the promoters from \textit{pedE} and \textit{pedH} genes were amplified by PCR using the primer pairs p2674-FSac/p2674-RPst and p2679-FSac/p2679-RPst (Wehrmann et al., 2017). The PCR products were digested with SacI and PstI and inserted upstream the \textit{luxCDABE} operon hosted by plasmid pSEVA226. The cargo module bearing the \textit{pedE-lux} or \textit{pedH-lux} fusion was then passed from the resulting pSEVA226-based constructs to pTn7-M as PacI/SpeI fragments.

\textbf{Strain constructions}

For the deletion of chromosomal genes a previously described method for markerless gene deletions in \textit{P. putida} KT2440 was used (Graf and Altenbuchner, 2011). In short, after transformation of the integration vectors carrying the up- and downstream region of the target gene, clones that were kanamycin (Kan) resistant and 5-fluorouracil (5-FU) sensitive were selected and one clone was incubated in liquid LB medium for 24 h at 30°C and 180 rpm
shaking. Upon selection for 5-FU resistance and Kan sensitivity on minimal medium agarose plates, clones that carried the desired gene deletion were identified by colony PCR.

Integration of the pTn7-M based pedH-lux and pedE-lux fusions into the chromosome of P. putida KT2440 was performed by tetraparental mating using PIR2/pTn7-M-pedH-lux or PIR2/pTn7-M-pedE-lux as the donor, E. coli CC118 λpir/pTNS1 and E. coli HB101/pRK600 as helper strains and appropriate KT2440 strain as the recipient (Zobel et al., 2015). Briefly, cultures of the four strains grown under selective conditions were mixed, spotted on LB agar and incubated overnight at 28°C. Transconjugants were selected on cetrimide agar (Sigma-Aldrich) containing gentamicin. Correct chromosomal integration of mini-Tn7 was checked by colony PCR using Pput-glmSDN and PTn7R primers as described elsewhere (Choi et al., 2005).

**Promoter activity assays**

P. putida harboring a Tn7-based pedH-lux or pedE-lux fusion were grown overnight in M9 medium with 25 mM succinate, washed three times in M9 medium with no added carbon source, and suspended to an OD_{600} of 0.1 in the same medium with 1 mM 2-phenylethanol. For luminescence measurements, 198 µl of cell suspension was added to 2 µl of a 100-fold-concentrated metal salt solution in white 96-well plates with a clear bottom (µClear; Greiner Bio-One). Microtiter plates were incubated in a FLX-Xenius plate reader (SAFAS, Monaco) at 30°C with orbital shaking (600 rpm, amplitude 3 mm) and light emission and OD_{600} were recorded after the indicated time periods. Promoter activity was expressed as relative light units (RLU) normalized to the corresponding OD_{600}. Experiments were performed in triplicates, and data are presented as the mean value with error bars representing the standard deviation.
Results

*Pseudomonas putida* KT2440 makes use of a periplasmic oxidation system to grow on a variety of alcoholic substrates. Crucial to this system are two PQQ-dependent ethanol dehydrogenases (PQQ-EDHs), which share a similar substrate scope but differ in their metal cofactor dependency (Wehrmann *et al.*, 2017). PedE makes use of a Ca$^{2+}$-ion whereas PedH relies on the bioavailability of different rare earth elements (REE). During our studies, we found that the critical REE concentration that supports growth based on PedH activity differs dramatically depending on the minimal medium used. In a modified M9 medium, concentrations of about 10 µM of La$^{3+}$ were necessary to observe PedH-dependent growth with 2-phenylethanol while only about 20-100 nM La$^{3+}$ were required in MP medium (Wehrmann *et al.*, 2017). One major difference between the two minimal media lies in their trace element composition and the respective metal ion concentrations (Table 3). The concentrations of copper, iron, manganese, and zinc are between 2x and 7x higher in the modified M9 medium compared to MP medium, and other trace elements such as boron, cobalt, nickel, or tungsten are only present in one out of the two media. To study the impact of the trace element solution (TES) on growth in the presence of La$^{3+}$, we used the ΔpedE strain growing on 2-phenylethanol in M9 minimal medium in the presence and absence of TES.

While a critical La$^{3+}$ concentration of 10 µM or higher was needed in the presence of TES to support PedH-dependent growth, this concentration dropped to as little as 10 nM La$^{3+}$ in the absence of TES (Figure 1A). Similarly, inhibition of PedE-dependent growth by La$^{3+}$ in strain ΔpedH differed dramatically depending on the presence of TES (Figure 1B). In the presence of TES, the addition of ≥ 100 µM of La$^{3+}$ was required for growth inhibition in the ΔpedH strain within 48 h of incubation, whereas a minimum of only ≥ 1 µM La$^{3+}$ was required in the absence of TES. From these experiments, we conclude that also a non-complemented minimal medium...
contains low, but sufficient, amounts of essential trace elements to allow growth even in the absence of TES. To find out whether the trace element mixture or a single trace element was causing the observed differences, we analyzed the growth of strain ΔpedE in more detail (Figure 2A). For concentrations of H$_3$BO$_3$, NaMoO$_4$, NiSO$_4$, and MnCl$_2$ similar to those found in complemented M9 medium, PedH-dependent growth with 2-phenylethanol in the presence of 10 nM La$^{3+}$ was observed. In contrast, upon the individual supplementation with 4 µM CuSO$_4$, 36 µM FeSO$_4$, or 7 µM ZnSO$_4$ PedH-dependent growth could not be observed with 10 nM La$^{3+}$, as it was the case upon the supplementation with TES. Since citrate is used as a metal chelator in TES, we further tested the impact of citrate on growth inhibition of CuSO$_4$, FeSO$_4$, and ZnSO$_4$ when used as additional supplement (Figure 2B). The addition of 50 µM of Na$_3$-citrate restored growth of the ΔpedE strain in the presence of 4 µM CuSO$_4$ and 7 µM ZnSO$_4$, even though cell growth was still impaired for Zn-containing medium. However, in cultures containing 36 µM FeSO$_4$ the addition of citrate had no effect, strongly indicating that FeSO$_4$ is predominantly responsible for the inhibition of PedH-dependent growth of the ΔpedE strain under low La$^{3+}$ concentrations in a TES complemented M9 medium.

To acquire iron under restricted conditions, P. putida KT2440 can excrete two variants of the siderophore pyoverdine (Salah El Din et al., 1997). Beside their great specificity towards Fe$^{3+}$, different pyoverdines can also chelate other ions including Al$^{3+}$, Cu$^{2+}$, Eu$^{3+}$, or Tb$^{3+}$, although with lower affinity (Braud et al., 2009a, 2009b). To test whether pyoverdine production in response to low iron conditions facilitates growth under low La$^{3+}$ conditions, the mutant strain ΔpedE ΔpvdD was constructed. This strain is no longer able to produce the two pyoverdines due to the loss of the non-ribosomal peptide synthetase pvdD (PP_4219; formerly known as ppsD) (Matilla et al., 2007), which was confirmed upon growth on agar plates (Figure 3C). In experiments with varying FeSO$_4$ supplementation, we found that PedH-dependent growth of
strain $\Delta pedE$ was only observed for FeSO$_4$ concentrations $\leq$ 10 µM under low (10 nM) La$^{3+}$ conditions (Figure 3A). With $\geq$ 20 µM FeSO$_4$ in the medium, no growth was observed. Strain $\Delta pedE\Delta pvdD$ exhibited the same FeSO$_4$-dependent growth phenotype as the parental strain under low La$^{3+}$ concentrations. Under high (10 µM) La$^{3+}$ conditions, strain $\Delta pedE$ exhibited PedH-dependent growth under any tested FeSO$_4$ concentration (Figure 3B). Notably, strain $\Delta pedE\Delta pvdD$ showed nearly the same growth pattern as $\Delta pedE$ under high La$^{3+}$ concentrations, with the exception of the condition where no FeSO$_4$ was added to the medium. Under this condition, no growth was observed.

From these data, it can be speculated that beside the PedH-dependent growth also the inhibition of PedE-dependent growth is dependent on the FeSO$_4$ to La$^{3+}$ ratio. In the presence of 10 nM La$^{3+}$, $pedE$ promoter activity was comparably high and increased with increasing FeSO$_4$ concentrations. In addition, strain $\Delta pedH$ grew readily on 2-phenylethanol under all these conditions even with no FeSO$_4$ supplementation (Figure 4A). When 10 µM La$^{3+}$ was available, no growth of the $\Delta pedH$ mutant was observed in presence of $\leq$ 20 µM FeSO$_4$ and the $pedE$ promoter activities were low (Figure 4B). However, when 40 µM FeSO$_4$ were present in the medium, representing a 4-fold excess compared to La$^{3+}$, PedE-dependent growth and an increased $pedE$ promoter activity was detected.

Due to the very low concentrations of REEs (nM range) required for REE-dependent growth it is commonly speculated that specific REE uptake systems must exist. From our previous results, we can conclude that pyoverdine is not such a system. A search of the genomic context of the $ped$ gene cluster identified a putative ABC transporter system located nearby the two PQQ-EDHs encoding genes $pedE$ and $pedH$ (Figure 5A). The ABC-transporter is predicted to be encoded as a single transcript by the online tool “Operon-mapper” (Taboada et al., 2018). It consists of four genes encoding a putative permease ($pedC$ [PP_2667]), an ATP-binding protein ($pedB$
[PP_2668]), a YVTN beta-propeller repeat protein of unknown function (pedA2 [PP_2669]) and
a periplasmic substrate-binding protein (pedA1 [PP_5538]). While efflux systems are usually
composed of the transmembrane domains and nucleotide binding domains, ABC-dependent
import system additionally require a substrate binding protein for functional transport (Biemans-
Oldehinkel et al., 2006). As the gene pedA1 is predicted to be such a substrate binding protein, it
is very likely that this transporter represents an import system.

ABC-dependent importers can be specific for carbon substrates or metal ions. Growth
experiments with ΔpedE, ΔpedH, ΔpedE ΔpedA1A2BC, and ΔpedH ΔpedA1A2BC demonstrated
that independent of La³⁺ (100 µM) availability, all strains were capable of growing with the
oxidized degradation intermediates of 2-phenylethanol, namely 2-phenylacetaldehyde and
phenylacetic acid, to a similar OD₆₀₀ within 48 h of incubation (Figure 5B), indicating that the
transport system is not involved in carbon substrate uptake.

When subsequently different La³⁺ concentrations were tested in a similar setup, we found that
PedH-dependent growth on 2-phenylethanol of strain ΔpedE ΔpedA1A2BC was inhibited for the
first 48 h of incubation under all concentrations tested, irrespectively of the presence or absence
of TES (Figure 6A). This was in contrast to the ΔpedE deletion strain, which grew in the
presence of ≥ 10 nM La³⁺ or ≥ 10 µM La³⁺ depending on TES availability (Figure 1A; Figure
6A pale symbols and lines). Upon an increased incubation time of 120 h, however, strain ΔpedE
ΔpedA1A2BC eventually did grow with 1 and 10 µM La³⁺ or 100 µM La³⁺ depending on TES
addition (Figure 6B). Notably, beside the substantial difference in lag-phase, also the critical
REE concentration for PedH-dependent growth of ΔpedE ΔpedA1A2BC was increased by 100-
fold compared to the ΔpedE strain under all conditions tested. Assuming that PedA1A2BC is
specific for REE uptake, growth with the Ca²⁺-dependent enzyme PedE should not be influenced
by a loss of the transporter function. When we tested the ΔpedH and ΔpedH ΔpedA1A2BC strain,
we indeed could not find any difference in growth as both strains exhibited a similar inhibition pattern for concentrations $\geq 1 \mu M$ $La^{3+}$ or $\geq 100 \mu M$ $La^{3+}$ depending on the absence or presence of TES in the medium (Figure 6C).

ABC-transporter systems, or the transported compounds, can be involved in transcriptional regulation of specific target genes (Biemans-Oldehinkel et al., 2006). Thus, the impaired growth under low $La^{3+}$ concentrations of the $\Delta pedE \Delta pedA1A2BC$ strain might be caused by the lack of transcriptional activation of the $pedH$ gene. To test this hypothesis, strain $\Delta pedE \Delta pedH \Delta pedA1A2BC$ was complemented with a $pedH$ gene independent of its natural promoter. Phenotypic analysis of this strain with 2-phenylethanol in the presence of TES and varying $La^{3+}$ concentrations revealed no difference in the growth pattern when compared to strain $\Delta pedE \Delta pedA1A2BC$ (Figure 6D). This indicated that the impaired growth phenotype of the ABC-transporter mutant is not due to a lack of transcriptional activation of $pedH$. To further validate this conclusion, $pedH$ promoter activities were measured during incubation with 2-phenylethanol in strain $\Delta pedA1A2BC$ and its parental strain in the absence and presence of 10 $\mu M$ $La^{3+}$ (Figure 7A). Both strains showed a similar and more than 20 fold increased $pedH$ promoter activity in response to $La^{3+}$ supplementation (26-fold for $KT2440^{*}:\text{Tn7-}pedH\text{-lux}$ and 23-fold in $\Delta pedA1A2BC:\text{Tn7-}pedH\text{-lux}$).

In contrast to PedE, the signal peptide of PedH contains two adjacent arginine residues, which is an indication that it might be transported to the periplasm in a folded state via the Tat (twin-arginine translocation) protein translocation system (Berks, 2015). Therefore, one could argue that the transport of lanthanides into the cytoplasm might be beneficial as the incorporation into the active site of PedH could be more efficient during protein folding compared to the complementation of the apoenzyme in the periplasm. An initial analysis of the PedH signal peptide using different online software tools (TatP, PRED-TAT, SignalP 5.0, TatFind) could
neither confirm nor refute this hypothesis (Almagro Armenteros et al., 2019; Bagos et al., 2010; Bendtsen et al., 2005; Rose et al., 2002). Therefore, we generated strains ΔpedE ΔtatC1 and ΔpedE ΔtatC2 in which the two individual TatC proteins (TatC1 [PP_1039] and TatC2 [PP_5018]) encoded in the genome of KT2440 are deleted. These strains should be restricted in the translocation of folded proteins into the periplasm, and if PedH would represent a Tat substrate, impaired growth on 2-phenylethanol in the presence of La\(^{3+}\) should be observable. However, neither tatC1 nor tatC2 mutation affected La\(^{3+}\)-dependent growth on 2-phenylethanol (Figure 7B). Additionally, various attempts to generate the double tatC1/C2 mutant strain were unsuccessful.
In the present study, we reveal that iron availability severely affects the REE-switch in *Pseudomonas putida* KT2440. This is evidenced by the reduction of the critical concentration of La$^{3+}$ that is required both to promote PedH-dependent growth and for the repression of growth based on PedE activity. By using a ΔpvdD deletion strain, we demonstrate that the production of the iron chelating siderophore pyoverdine is not required for PedH-dependent growth under low La$^{3+}$ conditions. Our data suggest that the observed effects during high Fe$^{2+/3+}$/La$^{3+}$ ratios are caused by mismetallation. In this scenario, the La$^{3+}$-binding sites of proteins could be occupied by Fe$^{2+/3+}$ ions that are in excess in the medium, and can also be present in the same 3+ oxidation state (Foster et al., 2014; Tottey et al., 2008; Tripathi and Srivastava, 2006; Webb, 1970).

Transcriptional data show that pedE repression can be influenced by iron in a concentration dependent manner. Further, the impact of iron is not identical for PedE and PedH-dependent growth (100 fold vs. 1000 fold). Since PedE regulation is solely dependent on PedS2 (Wehrmann et al., 2017; 2018), these data are thus supportive of such a mismetallation hypothesis, assuming that the sensor histidine kinase PedS2 and PedH have different binding affinities to La$^{3+}$ and/or Fe$^{2+/3+}$.

The same hypothesis might similarly explain why under high La$^{3+}$ concentrations in the absence of Fe$^{3+}$ supplementation, a pyoverdine-deficient strain is strongly impaired in growth. In this scenario the Fe$^{2+/3+}$ binding sites of pyoverdine-independent Fe transporters, such as the ferrichrome, ferrioxamine and ferric citrate uptake systems, might be occupied by La$^{3+}$ and prevent binding of Fe$^{2+/3+}$ ions (Cornelis, 2010; Jurkevitch et al., 1992). Consequently, a pyoverdine deficient strain would be unable to take up enough of this essential element that is, most likely, present at trace levels in the medium even without additional supplementation.
It is further interesting to point out that also micromolar $\text{Cu}^{2+}$ and $\text{Zn}^{2+}$ inhibited growth in presence of $\text{La}^{3+}$ in the nanomolar range, although these metals do not exist in the same $3+$ oxidation state under natural conditions. They are, however, the divalent transition metals that form the most stable complexes irrespective of the nature of the ligand, and as such also competitively bind non-cognate metal binding sites with high strength (Foster et al., 2014; Irving and Williams, 1953). Notably, $\text{Cu}^{2+}$ has also been reported to interfere with REE-dependent regulation of PQQ-dependent methanol dehydrogenases in $\text{M. trichosporium OB3b}$ (Gu et al., 2016; Gu and Semrau, 2017), and it is tempting to speculate that mismetallation might be involved in this process, too.

We provide compelling evidence that the predicted ABC-transporter $\text{PedA1A2BC}$ is essential for $\text{PedH}$-dependent growth under low concentrations of $\text{La}^{3+}$. Based on the $\text{PedE}$-dependent growth phenotype, we can further show that $\text{PedA1A2BC}$ is not involved in transcriptional repression of $\text{pedE}$ under low $\text{La}^{3+}$-conditions. The fact that a $\Delta\text{pedE} \Delta\text{pedA1A2BC}$ mutant strain can only grow with a 100-fold higher concentration of $\text{La}^{3+}$ compared to the $\Delta\text{pedE}$ single mutant strongly indicates that $\text{PedA1A2BC}$ functions as a $\text{La}^{3+}$-specific importer into the cytoplasm. In very recent studies it was demonstrated that in several $\text{Methylobacterium extorquens}$ strains a similar ABC-transporter system is required for $\text{Ln}^{3+}$-dependent growth (Ochsner et al., 2019; Roszczenko-Jasińska et al., 2019). A BLAST analysis revealed that these ABC transporters show high similarities to all four genes of the $\text{pedA1A2BC}$ operon (>43% sequence identity for $\text{pedA2}$ and >50% for $\text{pedA1}$, $\text{pedB}$ and $\text{pedC}$) and that all bacterial strains that have been reported to produce $\text{Ln}^{3+}$-dependent PQQ-ADHs thus far, carry homologues of this transporter system in their genome. Using a protein-based fluorescent sensor with picomolar affinity for REEs, Mattocks et al. were able to demonstrate that $\text{M. extorquens}$ indeed selectively takes up light REEs into its cytoplasm (Mattocks et al., 2019) and it was later shown that cytoplasmic REE-
uptake depends on the presence of the previously identified ABC-transporter system (Roszczenko-Jasińska et al., 2019).

Since the PedH enzyme, like all currently known Ln$^{3+}$-dependent enzymes, resides in the periplasm and since the purified apoenzyme of PedH can be converted into the catalytically active holoenzyme by Ln$^{3+}$ supplementation \textit{in vitro}, the question arises what the potential advantage of the postulated cytoplasmic Ln$^{3+}$ uptake for \textit{P. putida} would be. From our point of view, two different reasons can be imagined, namely that \textit{i}) the REE-dependent PedH protein is folded within the cytoplasm and the incorporation of the La$^{3+}$-cofactor is only possible or more efficient during the folding process; or \textit{ii}) La$^{3+}$ binds to a cytoplasmic protein that either represents a so-far uncharacterized transcriptional regulator or another REE-dependent enzyme.

It has been demonstrated that the location of protein folding can regulate metal binding (Tottey et al., 2008). As such, Ln$^{3+}$ insertion during folding in the cytoplasm, where metal concentrations are tightly regulated, could provide a means of preventing the Ln$^{3+}$ binding site of PedH from mismettallation with potentially competitive binders such as Cu$^{2+}$, Zn$^{2+}$, or Fe$^{2+/3+}$ in the periplasm. However, we could not find evidence that PedH is a Tat substrate and consequently transported into the periplasm as a folded protein (Berks, 2015), as the \textit{tatC1} or \textit{tatC2} mutants both still showed PedH-dependent growth. However, it cannot be excluded that the two Tat systems are functionally redundant since attempts to generate a \textit{tatC1/C2} double mutant strain proved unsuccessful.

We can further conclude that the putative La$^{3+}$ transport into the cytoplasm is not required to activate \textit{pedH} transcription. It is however possible that additional genes/proteins required for PedH-dependent growth rely on, or are regulated by, the cytoplasmic presence of REEs. In this context it is interesting to note that in a recent proteomic approach, we found that besides PedE and PedH, additional proteins of unknown function show differential abundance in response to
La$^{3+}$ availability (Wehrmann et al., 2019). It will hence be interesting to find out in future studies whether any of these proteins is required for PedH function.

In *M. extorquens* PA1 and *M. extorquens* AM1, almost identical TonB-dependent receptor proteins (>99 % sequence identity) were found to be crucial for REE-dependent growth suggesting a specific Ln$^{3+}$-binding chelator system in these organisms (Ochsner et al., 2019; Roszczenko-Jasińska et al., 2019). Interestingly, also in *Methylotuvimicrobium buryatense* 5GB1C a TonB-dependent receptor was identified that is crucial for the REE-switch to occur (Groom et al., 2019). Interestingly, the latter receptor only shows < 20% sequence identity to those of the *M. extorquens* strains and could thus not have been identified by homology searches.

In *P. putida* KT2440, no close homolog to any of the aforementioned TonB-dependent receptors could be identified (< 30% sequence identity). *P. putida* preferentially resides in the rhizosphere whereas *M. buryatense* and *M. extorquens* PA1 were isolated from the sediment of a soda lake (pH 9 – 9.5) and the phyllosphere of *Arabidopsis thaliana*, respectively (Kaluzhnaya et al., 2001; Knief et al., 2010). One could therefore speculate that a specific Ln$^{3+}$-chelator system is perhaps not relevant in the rhizosphere due to the large reservoir of REEs within the soil and the usually acidic environment near the plant roots caused by the secretion of organic acids for phosphate solubilization (Raghothama and Karthikeyan, 2005; Ramos et al., 2016). The lack of a homologous TonB-dependent receptor could also be explained by structural differences in the REE-specific chelator system that might be employed by *P. putida* compared to that of the methylotrophic bacteria. Lastly, it is also possible that in *P. putida* the REE uptake across the outer membrane proceeds via the same chelator systems that are used for pyoverdin independent Fe-acquisition. This could further provide another explanation for the impact of the Fe$^{2+/3+}$ to La$^{3+}$ ratio on the REE-switch.
Overall, the present study expands the crucial role of a conserved ABC-transporter system, which was very recently identified as Ln$^{3+}$-specific inner membrane transport system in methano- and methylotrophs, to non-methylotrophic organisms. It further provides new insight into the complexity of bacterial-metal interactions and demonstrates that Cu, Zn, and in particular Fe ions can strongly interfere with the REE-switch in *P. putida* most likely through mismetallation. The body of knowledge how REEs impact protein function, gene regulation, and consequently physiology of different microorganisms is rapidly increasing. As such, it will be very interesting to see when some of the most interesting questions, such as the cytoplasmic function of REEs or the nature and potential structural diversity of specific REE-chelator systems, will be resolved by future studies.
Funding Information

The work of Matthias Wehrmann and Janosch Klebensberger was supported by an individual research grant from the Deutsche Forschungsgemeinschaft (DFG, KL 2340/2-1). The work of Charlotte Berthelot and Patrick Billard was supported by the French National Research Agency through the National Program “Investissements d’Avenir” with the reference ANR-10-LABX-21-01/LABEX RESSOURCES21.
Acknowledgements

The authors further declare no conflict of interest. Janosch Klebensberger and Matthias Wehrmann would like to thank Prof. Bernhard Hauer for his continuous support.
References

Aide, M. T., and Aide, C. (2012). Rare earth elements: Their importance in understanding soil genesis. *ISRN Soil Sci.* 2012, 1–11. doi:10.5402/2012/783876.

Almagro Armenteros, J. J., Tsirigos, K. D., Sønderby, C. K., Petersen, T. N., Winther, O., Brunak, S., et al. (2019). SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nat. Biotechnol.* 37, 420–423. doi:10.1038/s41587-019-0036-z.

Andrews, S. C., Robinson, A. K., and Rodríguez-Quíñones, F. (2003). Bacterial iron homeostasis. *FEMS Microbiol. Rev.* 27, 215–237. doi:10.1016/S0168-6445(03)00055-X.

Arias, S., Olivera, E. R., Arcos, M., Naharro, G., and Luengo, J. M. (2008). Genetic analyses and molecular characterization of the pathways involved in the conversion of 2-phenylethylamine and 2-phenylethanol into phenylacetic acid in *Pseudomonas putida U.* *Environ. Microbiol.* 10, 413–432. doi:10.1111/j.1462-2920.2007.01464.x.

Bagos, P. G., Nikolaou, E. P., Liakopoulos, T. D., and Tsirigos, K. D. (2010). Combined prediction of Tat and Sec signal peptides with hidden Markov models. *Bioinformatics* 26, 2811–2817. doi:10.1093/bioinformatics/btq530.

Baune, M., Qi, Y., Scholz, K., Volmer, D. A., and Hayen, H. (2017). Structural characterization of pyoverdines produced by *Pseudomonas putida* KT2440 and *Pseudomonas taiwanensis* VLB120. *BioMetals* 30, 589–597. doi:10.1007/s10534-017-0029-7.

Belda, E., van Heck, R. G. A., José Lopez-Sanchez, M., Cruveiller, S., Barbe, V., Fraser, C., et al. (2016). The revisited genome of *Pseudomonas putida* KT2440 enlightens its value as a robust metabolic chassis. *Environ. Microbiol.* 18, 3403–3424. doi:10.1111/1462-2920.13230.

Bendtsen, J. D., Nielsen, H., Widdick, D., Palmer, T., and Brunak, S. (2005). Prediction of twin-arginine signal peptides. *BMC Bioinformatics* 6, 167. doi:10.1186/1471-2105-6-167.
Berks, B. C. (2015). The Twin-Arginine Protein Translocation Pathway. *Annu. Rev. Biochem.* 84, 843–864. doi:10.1146/annurev-biochem-060614-034251.

Biemans-Oldehinkel, E., Doeven, M. K., and Poolman, B. (2006). ABC transporter architecture and regulatory roles of accessory domains. *FEBS Lett.* 580, 1023–1035. doi:10.1016/j.febslet.2005.11.079.

Boyer, H. W., and Roulland-Dussoix, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J. Mol. Biol.* 41, 459–72. doi:10.1038/s41598-018-31191-1.

Braud, A., Hannauer, M., Mislin, G. L. A., and Schalk, I. J. (2009a). The *Pseudomonas aeruginosa* pyochelin-iron uptake pathway and its metal specificity. *J. Bacteriol.* 191, 3517–25. doi:10.1128/JB.00010-09.

Braud, A., Hoegy, F., Jezequel, K., Lebeau, T., and Schalk, I. J. (2009b). New insights into the metal specificity of the *Pseudomonas aeruginosa* pyoverdine-iron uptake pathway. *Environ. Microbiol.* 11, 1079–1091. doi:10.1111/j.1462-2920.2008.01838.x.

Chandrangsu, P., Rensing, C., and Helmann, J. D. (2017). Metal homeostasis and resistance in bacteria. *Nat. Rev. Microbiol.* 15, 338–350. doi:10.1038/nrmicro.2017.15.

Chen, S., Bleam, W. F., and Hickey, W. J. (2010). Molecular analysis of two bacterioferritin genes, *bfralpha* and *bfrbeta*, in the model rhizobacterium *Pseudomonas putida* KT2440. *Appl. Environ. Microbiol.* 76, 5335–43. doi:10.1128/AEM.00215-10.

Choi, K.-H., Gaynor, J. B., White, K. G., Lopez, C., Bosio, C. M., Karkhoff-Schweizer, R. R., et al. (2005). A Tn7-based broad-range bacterial cloning and expression system. *Nat. Methods* 2, 443–448. doi:10.1038/nmeth765.

Cornelis, P. (2010). Iron uptake and metabolism in pseudomonads. *Appl. Microbiol. Biotechnol.* 86, 1637–1645. doi:10.1007/s00253-010-2550-2.
Cornelis, P., and Andrews, S. C. (2010). Iron uptake and homeostasis in microorganisms. *Caister Acad. Press*, 300.

Cornelis, P., Wei, Q., Andrews, S. C., and Vinckx, T. (2011). Iron homeostasis and management of oxidative stress response in bacteria. *Metallomics* 3, 540. doi:10.1039/c1mt00022e.

Cotruvo, J. A., Featherston, E. R., Mattocks, J. A., Ho, J. V, and Laremore, T. N. (2018). Lanmodulin: A highly selective lanthanide-binding protein from a lanthanide-utilizing bacterium. *J. Am. Chem. Soc.* 140, 15056–15061. doi:10.1021/jacs.8b09842.

Dixon, S. J., and Stockwell, B. R. (2014). The role of iron and reactive oxygen species in cell death. *Nat. Chem. Biol.* 10, 9–17. doi:10.1038/nchembio.1416.

Firsching, F. H., and Brune, S. N. (1991). Solubility products of the trivalent rare-earth phosphates. *J. Chem. Eng. Data* 36, 93–95. doi:10.1021/je00001a028.

Foster, A. W., Osman, D., and Robinson, N. J. (2014). Metal preferences and metallation. *J. Biol. Chem.* 289, 28095–103. doi:10.1074/jbc.R114.588145.

Gibson, D. G. (2011). Enzymatic assembly of overlapping DNA fragments. *Methods Enzymol.* 498, 349–61. doi:10.1016/B978-0-12-385120-8.00015-2.

Graf, N., and Altenbuchner, J. (2011). Development of a method for markerless gene deletion in *Pseudomonas putida*. *Appl. Environ. Microbiol.* 77, 5549–5552. doi:10.1128/AEM.05055-11.

Gray, H. B. (2003). Biological inorganic chemistry at the beginning of the 21st century. *Proc. Natl. Acad. Sci.* 100, 3563–3568. doi:10.1073/pnas.0730378100.

Groom, J., Ford, S. M., Pesesky, M. W., and Lidstrom, M. E. (2019). A mutagenic screen identifies a TonB-dependent receptor required for the lanthanide metal switch in the Type I methanotroph *Methylovorivibrio bryatense 5GB1C*. *J. Bacteriol.* doi:10.1128/JB.00120-19.
Gu, W., Farhan Ul Haque, M., DiSpirito, A. A., and Semrau, J. D. (2016). Uptake and effect of rare earth elements on gene expression in *Methylosinus trichosporium* OB3b. *FEMS Microbiol. Lett.* 363, fnw129. doi:10.1093/femsle/fnw129.

Gu, W., and Semrau, J. D. (2017). Copper and cerium-regulated gene expression in *Methylosinus trichosporium* OB3b. *Appl. Microbiol. Biotechnol.* 101, 8499–8516. doi:10.1007/s00253-017-8572-2.

Herrero, M., de Lorenzo, V., and Timmis, K. N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.* 172, 6557–6567. doi:10.1128/jb.172.11.6557-6567.1990.

Irving, H., and Williams, R. J. P. (1953). 637. The stability of transition-metal complexes. *J. Chem. Soc.*, 3192. doi:10.1039/jr9530003192.

Jurkevitch, E., Hadar, Y., Chen, Y., Libman, J., and Shanzer, A. (1992). Iron uptake and molecular recognition in *Pseudomonas putida*: receptor mapping with ferrichrome and its biomimetic analogs. *J. Bacteriol.* 174, 78–83. doi:10.1099/13500872-140-7-1697.

Kaluzhnaya, M., Khmelenina, V., Eshinimaev, B., Suzina, N., Nikitin, D., Solonin, A., et al. (2001). Taxonomic characterization of new alkaliphilic and alcalitolerant methanotrophs from Soda Lakes of the southeastern Transbaikal region and description of *Methylociromium buryatense* sp.nov. *Syst. Appl. Microbiol.* 24, 166–176. doi:10.1078/0723-2020-00028.

Keen, N. T., Tamaki, S., Kobayashi, D., and Trollinger, D. (1988). Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. *Gene* 70, 191–197. doi:10.1016/0378-1119(88)90117-5.

Keltjens, J. T., Pol, A., Reimann, J., and Op den Camp, H. J. M. (2014). PQQ-dependent
methanol dehydrogenases: rare-earth elements make a difference. *Appl. Microbiol. Biotechnol.* 98, 6163–83. doi:10.1007/s00253-014-5766-8.

Kim, Y. C., Miller, C. D., and Anderson, A. J. (1999). Transcriptional regulation by iron of genes encoding iron- and manganese-superoxide dismutases from *Pseudomonas putida*. *Gene* 239, 129–135. doi:10.1016/S0378-1119(99)00369-8.

Knief, C., Frances, L., and Vorholt, J. A. (2010). Competitiveness of diverse *Methylobacterium* strains in the phyllosphere of *Arabidopsis thaliana* and identification of representative models, including *M. extorquens* PA1. *Microb. Ecol.* 60, 440–52. doi:10.1007/s00248-010-9725-3.

Maniatis, T., Fritsch, E., Sambrook, J., and Laboratory, C. S. H. (1982). *Molecular Cloning*: A *Laboratory Manual*. Cold Spring Harbor, N.Y. Cold Spring Harbor Laboratory.

Markert, B. (1987). The pattern of distribution of lanthanide elements in soils and plants. *Phytochemistry* 26, 3167–3170. doi:10.1016/S0031-9422(00)82463-2.

Matilla, M. A., Ramos, J. L., Duque, E., de Dios Alché, J., Espinosa-Urgel, M., and Ramos-González, M. I. (2007). Temperature and pyoverdine-mediated iron acquisition control surface motility of *Pseudomonas putida*. *Environ. Microbiol.* 9, 1842–1850. doi:10.1111/j.1462-2920.2007.01286.x.

Mattocks, J. A., Ho, J. V., and Cotruvo, J. A. (2019). A selective, protein-based fluorescent sensor with picomolar affinity for rare earth elements. *J. Am. Chem. Soc.* 141, 2857–2861. doi:10.1021/jacs.8b12155.

Meloche, C. C., and Vrátný, F. (1959). Solubility product relations in the rare earth hydrous hydroxides. *Anal. Chim. Acta* 20, 415–418. doi:10.1016/0003-2670(59)80090-8.

Merchant, S. S., and Helmann, J. D. (2012). Elemental economy: microbial strategies for optimizing growth in the face of nutrient limitation. *Adv. Microb. Physiol.* 60, 91–210.
Miller, C. D., Pettee, B., Zhang, C., Pabst, M., McLean, J. E., and Anderson, A. J. (2009). Copper and cadmium: responses in *Pseudomonas putida* KT2440. *Lett. Appl. Microbiol.* 49, 775–783. doi:10.1111/j.1472-765X.2009.02741.x.

Mückschel, B., Simon, O., Klebensberger, J., Graf, N., Rosche, B., Altenbuchner, J., et al. (2012). Ethylene glycol metabolism by *Pseudomonas putida*. *Appl. Environ. Microbiol.* 78, 8531–9. doi:10.1128/AEM.02062-12.

Nelson, K. E., Weinel, C., Paulsen, I. T., Dodson, R. J., Hilbert, H., Martins dos Santos, V. A. P., et al. (2002). Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.* 4, 799–808. doi:10.1046/j.1462-2920.2002.00366.x.

Ochsner, A. M., Hemmerle, L., Vonderach, T., Nüssli, R., Bortfeld-Miller, M., Hattendorf, B., et al. (2019). Use of rare-earth elements in the phyllosphere colonizer *Methylobacterium extorquens* PA1. *Mol. Microbiol.*, 0–2. doi:10.1111/mmi.14208.

Picone, N., and Op den Camp, H. J. M. (2019). Role of rare earth elements in methanol oxidation. *Curr. Opin. Chem. Biol.* 49, 39–44. doi:10.1016/j.cbpa.2018.09.019.

Raghothama, K. G., and Karthikeyan, A. S. (2005). Phosphate acquisition. *Plant Soil* 274, 37–49. doi:10.1007/s11104-004-2005-6.

Ramos, S. J., Dinali, G. S., Oliveira, C., Martins, G. C., Moreira, C. G., Siqueira, J. O., et al. (2016). Rare earth elements in the soil environment. *Curr. Pollut. Reports* 2, 28–50. doi:10.1007/s40726-016-0026-4.

Ray, P., Girard, V., Gault, M., Job, C., Bonneu, M., Mandrand-Berthelot, M.-A., et al. (2013). *Pseudomonas putida* KT2440 response to nickel or cobalt induced stress by quantitative proteomics. *Metallomics* 5, 68–79. doi:10.1039/C2MT20147J.
Rose, R. W., Brüser, T., Kissinger, J. C., and Pohlschröder, M. (2002). Adaptation of protein secretion to extremely high-salt conditions by extensive use of the twin-arginine translocation pathway. *Mol. Microbiol.* 45, 943–950. doi:10.1046/j.1365-2958.2002.03090.x.

Roszczenko-Jasińska, P., Vu, H. N., Subuyuj, G. A., Crisostomo, R. V., Cai, J., Raghuraman, C., et al. (2019). Lanthanide transport, storage, and beyond: genes and processes contributing to XoxF function in *Methylorubrum extorquens* AM1. bioRxiv, 647677. doi:10.1101/647677.

Salah El Din, A. L. M., Kyšík, P., Stephan, D., and Abdallah, M. A. (1997). Bacterial iron transport: Structure elucidation by FAB-MS and by 2D NMR (1H, 13C, 15N) of pyoverdin G4R, a peptidic siderophore produced by a nitrogen-fixing strain of *Pseudomonas putida*. *Tetrahedron* 53, 12539–12552. doi:10.1016/S0040-4020(97)00773-4.

Sasnow, S. S., Wei, H., and Aristilde, L. (2016). Bypasses in intracellular glucose metabolism in iron-limited *Pseudomonas putida*. *Microbiologyopen* 5, 3–20. doi:10.1002/mbo3.287.

Schalk, I. J., and Cunrath, O. (2016). An overview of the biological metal uptake pathways in *Pseudomonas aeruginosa*. *Environ. Microbiol.* 18, 3227–3246. doi:10.1111/1462-2920.13525.

Semrau, J. D., DiSpirito, A. A., Gu, W., and Yoon, S. (2018). Metals and methanotrophy. *Appl. Environ. Microbiol.* 84, e02289-17. doi:10.1128/AEM.02289-17.

Silva-Rocha, R., Martínez-García, E., Calles, B., Chavarría, M., Arce-Rodríguez, A., de las Heras, A., et al. (2013). The Standard European Vector Architecture (SEVA): a coherent platform for the analysis and deployment of complex prokaryotic phenotypes. *Nucleic Acids Res.* 41, D666–D675. doi:10.1093/nar/gks1119.

Studier, F. W., and Moffatt, B. A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* 189, 113–130.
Taboada, B., Estrada, K., Ciria, R., and Merino, E. (2018). Operon-mapper: a web server for precise operon identification in bacterial and archaeal genomes. *Bioinformatics*, 1–3. doi:10.1093/bioinformatics/bty496.

Takeda, K., Matsumura, H., Ishida, T., Samejima, M., Igarashi, K., Nakamura, N., et al. (2013). The two-step electrochemical oxidation of alcohols using a novel recombinant PQQ alcohol dehydrogenase as a catalyst for a bioanode. *Bioelectrochemistry* 94, 75–78. doi:10.1016/j.bioelechem.2013.08.001.

Tottey, S., Waldron, K. J., Firbank, S. J., Reale, B., Bessant, C., Sato, K., et al. (2008). Protein-folding location can regulate manganese-binding versus copper- or zinc-binding. *Nature* 455, 1138–1142. doi:10.1038/nature07340.

Tripathi, V. N., and Srivastava, S. (2006). Ni2+-uptake in *Pseudomonas putida* strain S4: a possible role of Mg2+-uptake pump. *J. Biosci.* 31, 61–7. doi:10.1007/BF02705236.

Tyler, G. (2004). Rare earth elements in soil and plant systems - A review. *Plant Soil* 267, 191–206. doi:10.1007/s11104-005-4888-2.

Webb, M. (1970). The mechanism of acquired resistance to Co2+ and Ni2+ in Gram-positive and Gram-negative bacteria. *Biochim. Biophys. Acta - Gen. Subj.* 222, 440–446. doi:10.1016/0304-4165(70)90134-0.

Wehrmann, M., Berthelot, C., Billard, P., and Klebensberger, J. (2018). The PedS2/PedR2 two-component system is crucial for the rare earth element switch in *Pseudomonas putida* KT2440. *mSphere* 3, 1–12. doi:10.1128/mSphere.00376-18.

Wehrmann, M., Billard, P., Martin-Meriadec, A., Zegeye, A., and Klebensberger, J. (2017). Functional role of lanthanides in enzymatic activity and transcriptional regulation of pyrroloquinoline quinone-dependent alcohol dehydrogenases in *Pseudomonas putida*.
Wehrmann, M., Toussaint, M., Pfannstiel, J., Billard, P., and Klebensberger, J. (2019). The cellular response towards lanthanum is substrate specific and reveals a novel route for glycerol metabolism in *Pseudomonas putida* KT2440. *bioRxiv*, 567529.

doi:10.1101/567529.

Wu, M. L., Wessels, J. C. T., Pol, A., Op den Camp, H. J. M., Jetten, M. S. M., and van Niftrik, L. (2015). XoxF-type methanol dehydrogenase from the anaerobic methanotroph *Candidatus Methylomirabilis oxyfera*. *Appl. Environ. Microbiol.* 81, 1442–51.

doi:10.1128/AEM.03292-14.

Zobel, S., Benedetti, I., Eisenbach, L., de Lorenzo, V., Wierckx, N., and Blank, L. M. (2015). Tn7-Based Device for Calibrated Heterologous Gene Expression in *Pseudomonas putida*. *ACS Synth. Biol.* 4, 1341–1351. doi:10.1021/acssynbio.5b00058.
### Table 1: Strains and plasmids used in the study

| Strains                  | Relevant features                                                                 | Source or reference                  |
|--------------------------|----------------------------------------------------------------------------------|-------------------------------------|
| KT2440*                  | KT2440 with a markerless deletion of *upp*. Parent strain for deletion mutants.   | (Graf and Altenbuchner, 2011)       |
| Δ*pedE*                  | KT2440* with a markerless deletion of *pedE*                                      | (Mückschel et al., 2012)            |
| Δ*pedA1A2BC*             | KT2440* with a markerless deletion of *pedA1A2BC*                                | (PP_5538, PP_2669, PP_2668, PP_2667) |
| Δ*pedE ΔpedA1A2BC*       | Δ*pedE* with markerless deletion of gene cluster *pedA1A2BC*                     | This study                          |
| Δ*pedE ΔpvdD*            | Δ*pedE* with a markerless deletion of *pvdD* (PP_4219)                             | This study                          |
| Δ*pedH*                  | KT2440* with a markerless deletion of *pedH*                                      | (Mückschel et al., 2012)            |
| Δ*pedH ΔpedA1A2BC*       | Δ*pedH* with a markerless deletion of gene cluster *pedA1A2BC*                    | This study                          |
| Δ*pedE ΔpedH*            | KT2440* with a markerless deletion of *pedE* and *pedH*                           | (Mückschel et al., 2012)            |
| Δ*pedE ΔtatC1*           | Δ*pedE* with a markerless deletion of *tatC1* (PP_1039)                            | This study                          |
| Δ*pedE ΔtatC2*           | Δ*pedE* with a markerless deletion of *tatC2* (PP_5018)                            | This study                          |
| E. coli BL21 (DE3)       | *F*−*ompT gal dcm lon hsdS41(rB− mB−) Δ(DE3 lacI lacUV5-T7 gene 1 ind1 sam7 nin51)* | (Studier and Moffatt, 1986)         |
| E. coli TOP10            | *F*− mcrA Δ(mrr-hsdRMS-mcrBC) F80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA λ− | Invitrogen                          |
| E. coli HB101            | *F*− mcrB mrr hsdS20(rB− mB−) recA13 leuB6 araA14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20(SmR) gln V44 λ− | (Boyer and Roulland-Dussoix, 1969)   |
| E. coli PIR2             | *F* Δlac169 rpoS(Em) robA1 creC510 hsdR514 endA recA1(ΔMlui):pir                 | Invitrogen                          |
| E. coli CC118Δpir        | Δ(ara-leu) araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE(Em) recA1 λpir phage lysogen | (Herrero et al., 1990)              |
| KT2440*::Tn7M-pedH-lux   | KT2440* with insertion of Tn7-M-pedH-lux                                         | This study                          |
| ΔpedA1A2BC::Tn7M-pedH-lux| ΔpedA1A2BC with insertion of Tn7-M-pedH-lux                                       | This study                          |
| KT2440*::Tn7M-pedE-lux   | KT2440* with insertion of Tn7-M-pedE-lux                                         | This study                          |

### Plasmids

| Plasmids                  | Description                                                                 | Source or reference              |
|---------------------------|------------------------------------------------------------------------------|----------------------------------|
| pJOE6261.2                | Suicide vector for gene deletions                                            | (Graf and Altenbuchner, 2011)    |
| pMW10                     | pJeM1 based vector for rhamnose inducible expression of PedH with C-terminal 6x His-tag | (Wehrmann et al., 2017)          |
| Construct     | Description                                                                 | Source                        |
|---------------|------------------------------------------------------------------------------|-------------------------------|
| pMW50         | pJOE6261.2 based deletion vector for gene *pvdD* (PP_4219)                   | This study                    |
| pMW57         | pJOE6261.2 based deletion vector for gene cluster *pedA1A2BC*                | This study                    |
| pTn7-M        | Km<sup>R</sup> Gm<sup>R</sup>, ori R6K, Tn7L and Tn7R extremities, standard multiple cloning site, oriT RP4 | (Zobel et al., 2015)          |
| pRK600        | Cm<sup>R</sup>, ori ColE1, Tra' Mob<sup>+</sup> of RK2                       | (Keen et al., 1988)           |
| pTNS1         | Ap<sup>R</sup>, ori R6K, TnSABC+D operon                                     | (Choi et al., 2005)           |
| pSEVA226      | Km<sup>R</sup>, ori RK2, reporter vector harboring the luxCDABE operon       | (Silva-Rocha et al., 2013)    |
| pSEVA226-pedH | pSEVA226 with a *pedH-luxCDABE* fusion                                       | This study                    |
| pSEVA226-pedE | pSEVA226 with a *pedE-luxCDABE* fusion                                       | This study                    |
| pTn7-M-pedH-lux | pTn7-M with a *pedH-luxCDABE* fusion                                       | This study                    |
| pTn7-M-pedE-lux | pTn7-M with a *pedE-luxCDABE* fusion                                       | This study                    |
| Primer | Name | Sequence 5’ → 3’ | Annealing temperature |
|--------|------|------------------|----------------------|
| MWH56  | GCCGCTTTGGTCGCCGCAACCAGCGAGTTGCA | 60°C |
| MWH57  | CCCGAAGCTTTTACATCTCCTACCAGGCG | 60°C |
| MWH58  | ATGTTCAAGCTTTGGGGGCC | 60°C |
| MWH59  | GCAGGTCGACTCTAAGCTTACAGATGCTGCTGCAG | 60°C |
| MWH94  | GCCGCTTTGGTCCCGCAACAACGCCAGGCC | 60°C |
| MWH95  | GCCAGGTTAAACACACTCCACGGGCGATGG | 60°C |
| MWH96  | AGTGTGTAAACCTGGGCTGTGTAACCC | 60°C |
| MWH97  | GCAGGTCGACTCTAGAGCCAGGAGTTGCTATGC | 60°C |
| PBtatC1.1 | CGATGCGCCGCTTTGGTCCCGCCATCCGTCATGCCT | 66°C |
| PBtatC1.2 | CGATGCGCCGCTTTGGTCCCGCCATCCGTCATGCCT | 66°C |
| PBtatC1.3 | AAAATGCTTTCCCGCCGCTTTGCGGGCCGTG | 72°C |
| PBtatC1.4 | CCTGCAAGTGCTTGACTCTAGAGGGCCATGCGAGCTTG | 72°C |
| PBtatC2.1 | CGATGCGCCGCTTTGGTCCCGCCATCCGTCATGGAACAC | 72°C |
| PBtatC2.2 | AGCAACAGGTGGGCTGCGGGGTCTTGAAGAG | 72°C |
| PBtatC2.3 | CGCGAGCCACCTGGTCTTTGCTAAGG | 62°C |
| PBtatC2.4 | CCTGCAAGTGCTTGACTCTAGAGATCACCAGCTGTACC | 62°C |
| Trace Element          | M9 medium | MP medium |
|------------------------|-----------|-----------|
| Na$_3$-citrate         | 51 µM     | 45.6 µM   |
| H$_3$BO$_3$            | 5 µM      | -         |
| CoCl$_2$               | -         | 2 µM      |
| CuSO$_4$               | 4 µM      | 1 µM      |
| FeSO$_4$               | 36 µM     | 18 µM     |
| MnCl$_2$               | 5 µM      | 1 µM      |
| NaMoO$_4$/($\text{NH}_4$)$_6$Mo$_7$O$_{24}$ | 0.137 µM | 2 µM |
| NiCl$_2$               | 0.084 µM  | -         |
| Na$_2$WO$_4$           | -         | 0.33 µM   |
| ZnSO$_4$               | 7 µM      | 1.2 µM    |
Figures

**Figure 1:** Growth of strain ΔpedE (A, dots) and ΔpedH (B, squares) in 1 mL liquid M9 medium in 96-well deep-well plates with 5 mM 2-phenylethanol and various concentrations of La$^{3+}$ in the presence (orange) or absence (blue) of trace element solution (TES). OD$_{600}$ was determined upon 48 h of incubation at 30°C and 350 rpm. Data are presented as the mean values of biological triplicates and error bars represent the corresponding standard deviations.

**Figure 2:** A) Growth of ΔpedE in 1 mL liquid M9 medium in 96-well deep-well plates with 5 mM 2-phenylethanol and 10 nM La$^{3+}$ in the presence of trace element solution (TES) or individual components thereof. B) Growth of ΔpedE as described in A with and without the additional supplementation of 50 µM Na$_3$-citrate. OD$_{600}$ was determined upon 48 h of incubation at 30°C and 350 rpm. Data are presented as the mean values of biological triplicates and error bars represent the corresponding standard deviations. *Probes with 137 nM NaMoO$_4$ also contain 5 µM H$_3$BO$_3$ and 84 nM NiSO$_4$.

**Figure 3:** (A and B) Growth of ΔpedE (blue squares) and ΔpedE ΔpvdD (light blue diamonds) in 1 mL liquid M9 medium in 96-well deep-well plates without TES with 5 mM 2-phenylethanol and various concentrations of FeSO$_4$ in the presence of 10 nM La$^{3+}$ (A) or 10 µM La$^{3+}$ (B). OD$_{600}$ was determined upon 48 h of incubation at 30°C and 350 rpm. Data are presented as the mean values of biological triplicates and error bars represent the corresponding standard deviations. (C) Pyoverdine production by strains ΔpedE (left) and ΔpedE ΔpvdD (right) grown on cetrimide agar plates examined under blue light.
Figure 4: Activities of the pedE promoter (blue bars) in strain KT2440* during incubation in M9 medium with 2-phenylethanol, no TES and 10 nM (A) or 10 µM La$^{3+}$ (B) as well as different FeSO$_4$ concentrations. Promoter activities were determined upon 8 h of incubation at 600 rpm and 30°C. Growth of strain ΔpedH (orange dots) in M9 medium with 2-phenylethanol, no TES and 10 nM (A) or 10 µM (B) La$^{3+}$ as well as different FeSO$_4$ concentrations. Cells were incubated for 48 h in 96 deep-well plates at 30°C and 350 rpm prior to OD$_{600}$ measurements. Data are presented as the mean values of biological triplicates and error bars represent the corresponding standard deviations.

Figure 5: A) Genomic organization of the ped cluster in P. putida KT2440. Nomenclature in analogy to P. putida U as suggested by Arias et al. (Arias et al., 2008) B) Growth of ΔpedH, ΔpedH ΔpedA1A2BC, ΔpedE, and ΔpedE ΔpedA1A2BC strains in liquid M9 medium with TES and 5 mM phenylacetaldehyde (orange bars) or 5 mM phenylacetic acid (green bars) and either 0 µM La$^{3+}$(dark green and dark orange bars) or 100 µM La$^{3+}$(light green and light orange bars). OD$_{600}$ was determined upon 48 h of incubation at 30°C and 180 rpm. Data are presented as the mean values of biological triplicates and error bars represent the corresponding standard deviations.

Figure 6: Growth of strains ΔpedE ΔpedA1A2BC (A and B, triangles) and ΔpedH ΔpedA1A2BC (C, diamonds) in liquid M9 medium with 5 mM 2-phenylethanol with (orange) or without (blue) TES and various concentrations of La$^{3+}$. Pale circles and squares represent the growth of ΔpedE and ΔpedH parental strains. Growth of strains ΔpedE and ΔpedH in A) and C) represents the restated data from Figure 1 for better comparability. D) Growth of strain ΔpedE ΔpedH ΔpedA1A2BC harboring plasmid pMW10 (light orange triangles) in liquid M9 medium with 5
mM 2-phenylethanol, 20 µg/ml kanamycin, TES and various La$^{3+}$ concentrations. OD$_{600}$ was determined upon 48 h (A and C) or 120 h (B and D) of incubation at 30°C and 350 rpm. Data are presented as the mean values of biological triplicates, and error bars represent the corresponding standard deviations.

**Figure 7**: A) Promoter activities of the *pedH* promoter in the KT2440* and ΔpedA1A2BC background during incubation with 2-phenylethanol in presence (orange) or absence (blue) of 10 µM La$^{3+}$. Promoter activities were determined upon 3 h of incubation at 600 rpm and 30°C. B) Growth of strain ΔpedE ΔpedA1A2BC on 2-phenylethanol in presence (orange) or absence (blue) of 10 µM La$^{3+}$. OD$_{600}$ was determined upon 48 h of incubation at 30°C and 180 rpm. Experiments were conducted in presence of TES. Data are presented as the mean values of biological triplicates and error bars represent the corresponding standard deviations.
