The PedS2/PedR2 Two-Component System Is Crucial for the Rare Earth Element Switch in *Pseudomonas putida* KT2440

Matthias Wehrmann, Charlotte Berthelot, Patrick Billard, Janosch Klebensberger

*University of Stuttgart, Institute of Biochemistry and Technical Biochemistry, Stuttgart, Germany*
*Université de Lorraine, LIEC UMR7360, Faculté des Sciences et Technologies, Vandoeuvre-lès-Nancy, France*
*CNRS, LIEC UMR7360, Faculté des Sciences et Technologies, Vandoeuvre-lès-Nancy, France*

**ABSTRACT** In *Pseudomonas putida* KT2440, two pyrroloquinoline quinone-dependent ethanol dehydrogenases (PQQ-EDHs) are responsible for the periplasmic oxidation of a broad variety of volatile organic compounds (VOCs). Depending on the availability of rare earth elements (REEs) of the lanthanide series (Ln³⁺), we have recently reported that the transcription of the genes encoding the Ca²⁺-utilizing enzyme PedE and the Ln³⁺-utilizing enzyme PedH are inversely regulated. With adaptive evolution experiments, site-specific mutations, transcriptional reporter fusions, and complementation approaches, we now demonstrate that the PedS2/PedR2 (PP_2671/PP_2672) two-component system (TCS) plays a central role in the observed REE-mediated switch of PQQ-EDHs in *P. putida*. We provide evidence that in the absence of lanthanum (La³⁺), the sensor histidine kinase PedS2 phosphorylates its cognate LuxR-type response regulator PedR2, which in turn not only activates pedE gene transcription but is also involved in repression of pedH. Our data further suggest that the presence of La³⁺ lowers kinase activity of PedS2, either by the direct binding of the metal ions to the periplasmic region of PedS2 or by an uncharacterized indirect interaction, leading to reduced levels of phosphorylated PedR2. Consequently, the decreasing pedE expression and concomitant alleviation of pedH repression causes—in conjunction with the transcriptional activation of the pedH gene by a yet unknown regulatory module—the Ln³⁺-dependent transition from PedE- to PedH-catalyzed oxidation of alcoholic VOCs.

**IMPORTANCE** The function of lanthanides for methanotrophic and methylotrophic bacteria is gaining increasing attention, while knowledge about the role of rare earth elements (REEs) in nonmethylotrophic bacteria is still limited. The present study investigates the recently described differential expression of the two PQQ-EDHs of *P. putida* in response to lanthanides. We demonstrate that a specific TCS is crucial for their inverse regulation and provide evidence for a dual regulatory function of the LuxR-type response regulator involved. Thus, our study represents the first detailed characterization of the molecular mechanism underlying the REE switch of PQQ-EDHs in a nonmethylotrophic bacterium and stimulates subsequent investigations for the identification of additional genes or phenotypic traits that might be coregulated during REE-dependent niche adaptation.

**KEYWORDS** lanthanides, LuxR-type regulator, PQQ, PedR2, PedS2, *Pseudomonas putida*, dehydrogenases, histidine kinase, periplasm, rare earth element switch, signal transduction, two-component regulatory systems
of two pyrroloquinoline quinone (PQQ)-dependent ethanol dehydrogenases (PQQ-EDHs)—namely, PedE and PedH—to carry out the initial oxidation steps in the periplasm of the cell (7, 8). In a recent study, we found that these two type I quinoproteins (9, 10) exhibit a similar substrate scope but require different metal cofactors (8). In contrast to PedE, which uses Ca\(^{2+}\) ions, PedH was characterized as a rare earth element (REE)-dependent enzyme that relies on the presence of lanthanides (Ln\(^{3+}\)) for catalytic activity. Notably, due to their low solubility in most natural environments, REEs have long been considered to have no biological function (11). However, the discovery of the widespread occurrence of the REE-dependent XoxF type of PQQ-dependent methanol dehydrogenases (PQQ-MDHs), together with the more recent description of Ln\(^{3+}\)-dependent PQQ-EDHs, has highlighted the important role of REEs for many bacterial species in various environmental compartments (8, 12–20).

While in the absence of Ln\(^{3+}\), the oxidation of methanol in methylotrophs is driven by Ca\(^{2+}\)-dependent PQQ-MDHs (MxaF type), the presence of small amounts of REE ions is usually sufficient to trigger a transcriptional switch to the XoxF type of PQQ-MDHs. This inverse regulation, called the REE switch, has been reported for many methanotrophic and/or methylotrophic organisms (13, 16, 21–24). From the growing number of studies, it has become apparent that the molecular mechanism underlying this switch for PQQ-MDHs is complex and can substantially differ among species. For example, the inverse regulation in the nonmethanotrophic methylotroph Methylobacterium extorquens strain AM1 is controlled by two different two-component systems (TCSs) (MxcQE and MxbDM) and the orphan response regulator MxaB (25–27). In this organism, it has been found that the transcriptional activation of both enzymes, the Ca\(^{2+}\)-dependent MxaF and the two Ln\(^{3+}\)-dependent XoxF1 and XoxF2, is entirely lost in a ΔxoxF1 ΔxoxF2 double mutant (28). As a consequence, a complex regulation in which the different binding affinities of the apo form (no Ln\(^{3+}\) bound to the enzyme) and holo form (Ln\(^{3+}\) bound to the enzyme) of the XoxF proteins to the periplasmic domain of the sensor histidine kinase MxcQ is essential to regulate the switch was postulated (23).

The type I methanotroph Methylomonas buryatense strain 5GB1C is lacking homologues of the aforementioned TCS systems MxcQE and MxbDM (13). In this organism, the REE switch is regulated predominantly by the sensor histidine kinase MxaY (29). Chu and coworkers (29) found that the activation of MxaY in the absence of lanthanum activates transcription of the Ca\(^{2+}\)-dependent enzyme MxaF by a so-far-unknown response regulator. In addition, the deletion of MxaY was found to almost entirely eliminate the Ln\(^{3+}\)-mediated transcriptional activation of the Ln\(^{3+}\)-dependent enzyme XoxF in a partially MxaB-dependent manner that also results in a severe growth defect, both in the presence and absence of Ln\(^{3+}\). As an additional layer of complexity, recent studies found that the presence of other metal ions such as copper, which is needed as a cofactor for the membrane-bound or particulate methane monooxygenase (pMMO) in methanotrophs, can significantly impact the REE-mediated switch (21, 22, 30).

In contrast to the increasing knowledge about the regulation of PQQ-MDHs in methylotrophs, the molecular basis underlying such a regulatory switch for PQQ-EDHs in nonmethylotrophic organisms is not established. In the present study, we identify the TCS encoded by PP_2671/PP_2672 (hereinafter referred to as PedS2/PedR2 according to the genetic nomenclature from Arias et al. [31]), consisting of the sensor histidine kinase PedS2 and its cognate LuxR-type transcriptional response regulator PedR2, as an essential regulatory module for the REE-mediated switch of PQQ-EDHs in P. putida KT2440. We provide evidence that the activity of PedS2 in the absence of lanthanides leads to phosphorylation of PedR2 (PedR2\(^{P}\)), which serves a dual regulatory function. On the one hand, PedR2\(^{P}\) acts as a strong transcriptional activator of the pedE gene, which is essential to allow growth with 2-phenylethanol in the absence of Ln\(^{3+}\). At the same time, PedR2\(^{P}\) also functions as a repressor of pedH. From our data, we conclude that the presence of Ln\(^{3+}\) ions triggers a reduction in PedS2 activity, either by a direct binding of the metal to the periplasmic region of PedS2 or by an uncharacterized
indirect interaction. This reduction of PedS2 activity, together with a proposed phosphatase activity of PedS2 under this condition, causes the accumulation of non-phosphorylated PedR2, which over time results in the loss of the regulatory activity of the protein, and facilitates—in concert with the positive-feedback function of PedH in the presence of Ln3+/H11001 ions—the switch between PedE- and PedH-dependent growth.

RESULTS

Identification of the sensor histidine kinase PedS2 as a lanthanide-responsive sensor. As a consequence of the inverse regulation of pedE and pedH, a pedH deletion strain does not grow within 48 h with 2-phenylethanol as the sole carbon source in the presence of a critical concentration of La3+/H11001 in the culture medium (8). To test whether strains can evolve to overcome the repression of pedE in the presence of Ln3+/H11001, an adaptive evolution experiment was performed (see Fig. 1 for a general scheme).

When ΔpedH cultures were incubated with 2-phenylethanol in the presence of 10 μM LaCl3 for longer than 5 days, growth was observed, indicating the occurrence of adapted strains (data not shown). When independent clones were isolated from such cultures and passaged several times on LB agar medium, their growth phenotype with 2-phenylethanol was much faster (<2 days) than that observed for

FIG 1 (A to D) Schemes of selection (A), clonal isolation (B), characterization and single nucleotide polymorphism (SNP) identification (C and D) in the two-component sensor histidine kinase PedS2 of the ΔpedH R73C, ΔpedH R111W, ΔpedH S178P spontaneous mutants. (A) Cells of the ΔpedH strain were incubated in M9 medium supplemented with 5 mM 2-phenylethanol and 10 μM LaCl3 in plastic Erlenmeyer flasks (n = 3) at 30°C with shaking at 180 rpm. (B) After growth was observed (>5 days), dilutions from each culture were plated onto LB agar plates and incubated at 30°C. Individual clones were further streaked on LB agar twice prior to further characterization. (C) Clones were characterized for their growth behavior in M9 medium with 5 mM 2-phenylethanol in the presence of 10 μM LaCl3. Subsequently, one clone from each culture exhibiting faster growth than the parental ΔpedH strain was used for PCR amplification of the pedS2 gene and multiple-sequence alignment analysis with the native sequence of the gene from the Pseudomonas Genome Database (52). (D and E) Visualization of domain composition of PedS2 of P. putida (D) and MxaY of Methylomicrobium buryatense SGB1C (E) using the prediction from the Simple Modular Architecture Research Tool (53). (F) Amino acid sequence alignment of the PedS2 and MxaY proteins generated with Clustal Omega (50).
their ΔpedH parental strain and very similar to the growth phenotype of the wild-type strain KT2440. Similar spontaneous suppressor mutants have been reported in methylotrophic organisms such as *Methylobacterium extorquens* strain AM1, *Methylomicrobium buryatense* strain 5GB1C, and *Methylobacterium aquaticum* strain 22A during growth with methanol (13, 16, 29). In *M. buryatense*, whole-genome sequencing revealed that the suppressor mutant strain was characterized by a mutation in the membrane-bound two-component sensor histidine kinase MxaY (29). In *Pseudomonas putida* KT2440, the gene \textit{PP}_{2671} (hereinafter referred to as \textit{pedS2}), located in close genomic proximity to \textit{pedE} (\textit{PP}_{2674}), encodes a membrane-bound histidine kinase sharing 25% amino acid sequence identity with MxaY (Fig. 1D to F). To test the hypothesis that mutations in \textit{pedS2} are responsible for the emergence of suppressor phenotypes in the ΔpedH mutant strain during growth in the presence of La³⁺, the ΔpedH and ΔpedH\_PedS2S178P strains were grown at 30°C and 350 rpm shaking with M9 medium in 96-well plates supplemented with 5 mM 2-phenylethanol in the presence of 10 μM La³⁺ (blue symbols) or in the absence of La³⁺ (green symbols). The gray areas in panels 2 to 4 show the time point by which the parental ΔpedH strain (circles) reached an OD₆₀₀ of >0.4 (dotted line). (B) Activities of the \textit{pedE} promoter in ΔpedH, ΔpedH\_PedS2S178P, ΔpedH \_PedS2, and ΔpedH ΔpedR2 strains in the presence (blue bars) of 1 μM La³⁺ or absence of La³⁺ (green bars) or measured in M9 medium supplemented with 1 mM 2-phenylethanol. Promoter activities are presented in relative light units (RLU × 10⁴) normalized to OD₆₀₀. All data represent the means for biological triplicates, and error bars correspond to the respective standard deviations.

In subsequent growth experiments with 2-phenylethanol in the absence of La³⁺, the ΔpedH and ΔpedH\_PedS2S178P mutants showed similar growth behavior with a lag phase of <32 h (Fig. 2A1 and A2). In the presence of 10 μM La³⁺, however, the ΔpedH strain showed no growth within 72 h, whereas the ΔpedH\_PedS2S178P strain reached its maximum optical densities again after about 32 h of incubation, verifying that the
observed mutation in the histidine kinase pedS2 gene was sufficient to cause the suppressor phenotype. We speculated that the LuxR-type response regulator exaE (PP_2672; hereinafter referred to as pedR2), which is located adjacent to pedS2 within the genome of P. putida KT2440, represents the target of PedS2 activity. This assumption is based on the fact that PedR2 represents a homologue (65% amino acid sequence identity) of EraR (ExaA; PA1980), which forms a two-component system (TCS) with the cytosolic histidine kinase EraS (ExaD; PA1979) that activates expression of the pedE homologue exaA in Pseudomonas aeruginosa (34, 35). To test this hypothesis, pedS2 as well as its potential cognate response regulator-encoding gene pedR2, were deleted in a ΔpedH background. In addition, strains suitable for probing promoter activity of pedE in ΔpedH, ΔpedH_pedS2S178P, ΔpedH ΔpedS2, and ΔpedH ΔpedR2 mutant strains were constructed and subsequently analyzed during growth with 2-phenylethanol in the presence and absence of La3+ (Fig. 2B).

The PedS2/PedR2 TCS regulates pedE transcription in response to lanthanide availability. In accordance with the observed growth patterns, pedE promoter activities in the ΔpedH_pedS2S178P mutant were almost identical in both the presence and absence of La3+ (ratio of pedE promoter activity in cells grown without La3+ to promoter activity in cells grown with 1 μM La3+, 0.89 ± 0.02) but increased more than 2-fold (24-fold ± 1-fold and 27-fold ± 1-fold, respectively) compared to the pedE promoter activities determined for cells of the ΔpedH strain grown in the absence of La3+ (Fig. 2B). The ΔpedH ΔpedS2 double mutant also showed a La3+-independent growth phenotype similar to that of the ΔpedH_pedS2S178P strain but with a clear delay (<48 h versus <32 h) (Fig. 2A3). Promoter activities for pedE were almost identical in this strain in the presence and absence of La3+ (ratio of pedE promoter activity in cells grown without La3+ to promoter activity in cells grown with 1 μM La3+, 1.07 ± 0.09) (Fig. 2B).

In contrast, incubations of 72 h (Fig. 2A4) or even prolonged incubations for more than 7 days (data not shown) did not result in detectable growth of the ΔpedH ΔpedR2 double mutant with 2-phenylethanol, both in the presence and absence of La3+. Correspondingly, pedE promoter activities in this strain were low compared to those observed for cells of the ΔpedH strain in the presence of La3+ but in a range similar to those observed for the ΔpedH ΔpedS2 strain in the presence and absence of La3+. These data demonstrate that the PedS2/PedR2 system is the predominant element in the La3+-dependent regulation of pedE and that PedR2 is essential for PedE-dependent growth. However, as PedE-dependent growth can still be observed in the absence of PedS2 after prolonged incubations and in a PedR2-dependent manner (Fig. 2A3 and A4), we assume that at least one additional lanthanide-independent kinase must be able to phosphorylate PedR2, leading to transcriptional activation of pedE and functional production of the calcium-dependent enzyme under these conditions.

The PedS2/PedR2 TCS regulates the partial repression of pedH in the absence of lanthanides. On the basis of the critical role of PedS2/PedR2 in the regulation of pedE and the fact that LuxR-type regulators have been demonstrated to be capable of acting as both transcriptional activators and repressors (36, 37), we speculated that this TCS could also be involved in the regulation of pedH. To test this hypothesis, ΔpedE_pedS2S178P, ΔpedE ΔpedS2, and ΔpedE ΔpedR2 mutant strains were generated, and a transcriptional reporter suitable for probing pedH promoter activities was integrated into the genome of each of these strains.

Experiments with 2-phenylethanol revealed that ΔpedE, ΔpedE ΔpedS2, and ΔpedE ΔpedR2 mutant strains showed La3+-dependent growth after a lag phase of <24 h and reached the maximum optical density at 600 nm (OD600) after ≈32 h (Fig. 3A1, A3, and A4). In contrast, the ΔpedE_pedS2S178P strain exhibited an extended lag phase (>24 h) and consistently reached the maximum OD600 only after prolonged incubations (≈32 h [Fig. 3A2]). In accordance with these growth results, the pedH promoter activities of ΔpedE, ΔpedE ΔpedS2, and ΔpedE ΔpedR2 strains were in a similar range, whereas pedH promoter activities in the ΔpedE_pedS2S178P strain were 45-fold ± 2-fold and 30-fold ± 2-fold lower than the promoter activities of the ΔpedE strain in the absence or presence
Assuming that the S178P mutation in PedS2 results in a sensor kinase activity that mimics that of the wild-type protein in the absence of lanthanides, it is very likely that PedS2 is responsible for the repression of pedH under the La3+/H11001-free conditions leading to the observed delay in growth. To find out whether this regulatory effect on pedH proceeds via the response regulator PedR2, as is the case for pedE, or is caused by an unknown additional target of PedS2, the ΔpedE PedS2S178P ΔpedR2 triple mutant strain was generated and characterized for its growth phenotype (Fig. 4A). In this experiment, the ΔpedE and ΔpedE PedS2S178P strains both grew with 2-phenylethanol but with clear differences in the corresponding lag phases and maximum growth rates (0.087 ± 0.003 h⁻¹ versus 0.057 ± 0.001 h⁻¹), confirming the previous results from growth in 96-well plates (Fig. 3A). In contrast, the additional deletion of the response regulator PedR2 eliminated the growth defect caused by the PedS2S178P allele, leading to a growth behavior of the ΔpedE PedS2S178P ΔpedR2 strain (Fig. 4A), which was indistinguishable (maximum growth rate, 0.089 ± 0.003 h⁻¹) from that of the ΔpedE strain.

The conserved phosphorylation site D53 in PedR2 is essential for the REE-mediated switch. In order to study the essentiality of the phosphorylation site at position D53 of PedR2 (38, 39), we used inducible low-copy-number constructs for the production of the wild-type PedR2 protein (pJEM[PedR2]) and a mutated variant, in which the conserved aspartate in the CheY-like receiver domain was replaced by an alanine (pJEM[PedR2D53A]). After transformation of these plasmids into the ΔpedH ΔpedR2 and ΔpedE PedS2S178P ΔpedR2 mutant strains, their growth with 2-phenylethanol in the presence and absence of La3⁺ was monitored. When the plasmid-borne wild-type regulator PedR2 was induced in cells of the ΔpedH ΔpedR2 strain, growth was observed after a lag phase of <<24 h (maximum growth rate, 0.032 ± 0.005 h⁻¹), whereas the PedR2D53A variant was unable to restore PedE-dependent growth in the same strain (Fig. 4B). Intriguingly, the reverse result was obtained in the ΔpedE PedS2S178P ΔpedR2 strain. Here, the PedR2D53A variant allowed PedH-dependent...
growth (maximum growth rate, 0.026 ± 0.002 h⁻¹), whereas the wild-type regulator PedR2 did not lead to significant growth within 120 h of incubation (Fig. 4C).

**DISCUSSION**

We recently demonstrated that in *P. putida* KT2440, the production of the two PQQ-EDHs PedE and PedH is both tightly and inversely regulated depending on lanthanide availability, representing the first reported REE switch for PQQ-EDHs in a nonmethylotrophic organism (8). In this study, we were able to show that Ln³⁺-dependent transcriptional activation of *pedH* is mostly, but not entirely, dependent on the presence of the PedH protein itself by a so-far unknown mechanism and transcriptional regulator (Fig. 5A). Notably, the Ln³⁺-dependent transcriptional repression of *pedE* remained elusive. In the current study, we present a detailed characterization of the mechanism underlying PedE and PedH regulation, in which the PedS2/PedR2 TCS acts as an essential signaling module for the REE-mediated switch between the two quinoproteins.

Similar to the recently characterized spontaneous mutant of *M. buryatense* (29), we found that a single nonsynonymous mutation within the periplasmic region of the sensor histidine kinase PedS2 (PedS2S178P), which differs from the LapD/MoxY domain found in MxaY of *M. buryatense* (Fig. 1), is sufficient to terminate the Ln³⁺-mediated repression of *pedE*. Notably, various mutations at different sites of the protein can cause the observed suppressor phenotype. This might explain the repeated and fast occurrence of these suppressor mutants in our experiments and would support a similar notion in *M. buryatense* (13, 29).

Besides the essentiality for *pedE* regulation, our experimental data further provide strong evidence that PedS2 is also involved in the repression of *pedH* in the absence of lanthanides, but not in its Ln³⁺-dependent activation. This is based on the observation that the ΔpedE ΔpedS2 deletion strain did not show any differences in growth and *pedH* activation, while the ΔpedE PedS2S178P suppressor mutant displayed decreased *pedH* promoter activity and a strongly increased lag phase in growth experiments in the presence of La³⁺. The complementation assay with the inducible PedR2 variants further demonstrates that the *pedS2*-dependent regulation of *pedE* and *pedH* is mediated by the LuxR-type response regulator PedR2 for both genes. From these data, we conclude that in the absence of La³⁺ ions, the PedS2 sensor histidine kinase is active and triggers phosphorylation of PedR2 at the conserved position D53 (PedR2⁵³⁵³) (38, 39). The phosphorylated state of PedS2 subsequently has a dual regulatory function, namely, the
activation of pedE and concomitant repression of pedH transcription (Fig. 5A). In this context, it is interesting to note that expression of the exaA gene in Azospirillum brasilense Sp7, which encodes a pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase, is dependent on /H9268 and its interaction with a LuxR-type response regulator that shares 45% sequence identity with PedR2 (40). Whether the transcriptional activation of pedE is dependent on a similar interaction of PedR2 with a specific sigma factor is currently unknown.

To our surprise, a strain lacking PedS2 (ΔpedH ΔpedS2 strain) was still able to grow on 2-phenylethanol in the absence of Ln3/H11001, even though it exhibited an increased lag phase and low pedE promoter activities (Fig. 2A3). As our study provides strong evidence that phosphorylation of PedR2 is essential for transcriptional activation of pedE, this observation indicates that an additional so-far unidentified kinase beside PedS2 is capable of phosphorylating PedR2 to facilitate growth of this strain under these conditions (indicated in Fig. 5 as a gray membrane-bound protein). Given that such an additional kinase exists, it is even more surprising that a pedH single mutant, in contrast to the aforementioned ΔpedH ΔpedS2 double mutant, is able to grow in the presence of REEs only when PedS2 is mutated (e.g., PedS2S178P [Fig. 2A1]). This suggests that the activity of the additional kinase toward PedR2 in the presence of La3+ is repressed as long as PedS2 is functional. As an intrinsic phosphatase activity has been found for many bacterial sensory histidine kinases (33, 41, 42), we hence propose that PedS2 also exhibits phosphatase activity on PedR2 in the presence of La3+, thereby ensuring specificity of the signal transduction pathway and eliminating interference from other nonspecific kinases. In our working hypothesis, the presence of lanthanides in the medium leads to the repression of PedS2 kinase activity, most likely by direct binding of the metal ions to its periplasmic domain. The reduced kinase activity and postulated phosphatase activity of PedS2 in the presence of Ln3+ consequently leads to the accumulation of unphosphorylated PedR2, which finally results in the loss of its regulatory functions. In addition, the transcription of pedH is activated via a yet unknown pathway, in which a functional PedH protein is an essential component, most likely by acting as a lanthanide sensor (8).
In conclusion, it appears that the REE-mediated switches in *Methylobacterium extorquens* AM1 and *Methylomicrobiun buryatense* during growth with methane are predominantly dependent on only one lanthanide-responsive pathway, which either proceeds via the XoxF1 and XoxF2 proteins or via the MxaY protein (23, 29). Our results establish that in *P. putida* KT2440, a combination of at least two independent pathways are important to orchestrate the inverse regulation of pedE and pedH in response to lanthanides efficiently.

Several recent studies suggest that in methano- and methylotrophic bacteria, the REE switch might affect more genes than only the genes needed for the periplasmic oxidation system itself (16, 22, 43). Reports on physiological consequences are, however, inconsistent as some studies found no effects (13, 16, 23, 44), whereas other studies reported a stimulating effect on biofilm formation, growth rates, and overall yields in the presence of REE (43, 45). We think it is not unlikely that additional REE-mediated regulatory effects also exist in *P. putida* KT2440 in a context-dependent manner. Thus, one of our current foci is to investigate the global regulatory impact and physiological consequences of the presence and absence of lanthanides under various environmental conditions.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and culture conditions.** A detailed description of the bacterial strains, plasmids, and primers used in this study can be found in Tables 1 and 2. If not stated otherwise,
TABLE 2 Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5′ → 3′)</th>
<th>Annealing temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWH85</td>
<td>GAAATACGAGAAAGTACGAAATG</td>
<td>60</td>
</tr>
<tr>
<td>MWH86</td>
<td>TGTCAGCCTGTGGGTCCTGTGGGTC</td>
<td>60</td>
</tr>
<tr>
<td>MWH90</td>
<td>GCCATACGACCTGTTAAGGCTGCTG</td>
<td>60</td>
</tr>
<tr>
<td>MWH91</td>
<td>CGATACGACCTGTTAAGGCTGCTG</td>
<td>60</td>
</tr>
<tr>
<td>MWH92</td>
<td>GAAACCGGACCTGGTCTGCTGCTG</td>
<td>60</td>
</tr>
<tr>
<td>MWH93</td>
<td>GCACTGCGAAGTAACTGCGGAGCAGG</td>
<td>60</td>
</tr>
<tr>
<td>MWH98</td>
<td>GCCATACGACCTGTTAAGGCTGCTG</td>
<td>60</td>
</tr>
<tr>
<td>MWH99</td>
<td>GTCCTACGACCTGTTAAGGCTGCTG</td>
<td>60</td>
</tr>
<tr>
<td>MWH100</td>
<td>GCTCGGGGAGGACCTGCTGCTG</td>
<td>60</td>
</tr>
<tr>
<td>MWH101</td>
<td>GCACTGCGAAGTAACTGCGGAGCAGG</td>
<td>60</td>
</tr>
<tr>
<td>MWH106</td>
<td>GCCATACGACCTGTTAAGGCTGCTG</td>
<td>60</td>
</tr>
<tr>
<td>MWH107</td>
<td>GCACTGCGAAGTAACTGCGGAGCAGG</td>
<td>60</td>
</tr>
<tr>
<td>MWH108</td>
<td>GCACTGCGAAGTAACTGCGGAGCAGG</td>
<td>60</td>
</tr>
<tr>
<td>MWH109</td>
<td>GCACTGCGAAGTAACTGCGGAGCAGG</td>
<td>60</td>
</tr>
</tbody>
</table>

Escherichia coli and Pseudomonas putida KT2440 strains were maintained on solidified LB medium. Routinely, strains were cultured in liquid LB medium (46) or a modified M9 salt medium (8) supplemented with 25 mM succinate or 5 mM 2-phenylethanol as a source of carbon and energy at 30°C and shaking, if not stated otherwise. For maintenance and selection, 40 μg ml⁻¹ kanamycin or 15 μg ml⁻¹ gentamicin for E. coli or 40 μg ml⁻¹ kanamycin, 20 μg ml⁻¹ 5-fluorouracil, or 15 μg ml⁻¹ gentamicin for P. putida strains was added to the medium, if indicated.

Liquid medium growth experiments. All liquid growth experiments were carried out using modified M9 medium with 25 mM succinate or 5 mM 2-phenylethanol as the sole source of carbon and energy (see above) in 125-ml or 250-ml polycarbonate Erlenmeyer flasks (Corning) or in 96-well 2-ml deep-well plates (Carl Roth) as described previously (8). Briefly, washed cells from overnight cultures grown with succinate at 30°C and 180 rpm shaking were used to inoculate fresh medium with an optical density at 600 nm (OD 600) of 0.01 and incubated at 30°C and 180 rpm (growth experiments in polycarbonate Erlenmeyer flasks) or 350 rpm (growth experiments in 96-well plates) at 30°C. Maximum growth rates were calculated from three time points during the exponential phase of growth.

Construction of plasmids. For construction of the deletion plasmids pMW55, pMW56, and pMW61, the 600-bp regions upstream and downstream of the pedH gene (PP_2671) or amino acid residue S178 in the pedS2 gene (PP_2672) were amplified from genomic DNA of P. putida KT2440 using primers MWH85 to MWH86, MWH90 to MWH93, MWH98 to MWH101, or MWH106 to MWH109 (Table 2). The two up- and downstream fragments and BamHI-digested pJOE6261.2 were then joined together using one-step isothermal assembly (47). Upon subsequent transformation of the constructs into E. coli BL21(DE3) cells, the correctness of the plasmids was confirmed by Sanger sequencing. Plasmids pEM-[PedR2] and pEM[PedR2D53A] encoding PedR2 or PedR2 with mutated amino acid residue 53 (D→A) under a rhamnose-inducible promoter were ordered from an external source (Eurofins).

Strain constructions and isolation of suppressor mutants. The pedS2 (PP_2671) and pedR2 (PP_2672) negative mutants as well as the PedS2178P allele were constructed using a recently described system for markerless gene deletion in P. putida KT2440 (48). Briefly, the integration vectors harboring the up- and downstream regions of the target genes (pMW55 and pMW61) or the up- and downstream regions of the region to be mutated, including the desired S178P mutation (pMW56) were transformed into P. putida KT2440* and kanamycin-resistant (Kanr) clones were selected on LB Kan agarose plates. After incubation at 30°C for 24 h in LB medium without selection markers, clones that were 5-FU resistant (5-FUr) and Kanr were tested for successful gene deletion using primer pair MWH90/MWH93 or MWH106/MWH109 for the pedS2 or pedR2 gene, respectively. The presence of the underlying PedS2178P mutation was verified by Sanger sequencing after the pedS2 gene of 5-FUr and Kanr clones was amplified using primer pair MWH85/MWH86. ΔPedH suppressor mutant strains were isolated from 25-ml liquid M9 cultures with 5 mM 2-phenylethanol as the sole source of carbon and energy supplemented with 10 μM La³⁺ upon 5-day incubation at 30°C and 180 rpm. Strains were passaged three times in 12 cm agar plates and reevaluated for growth in liquid M9 medium supplemented with 5 mM 2-phenylethanol and 10 μM La³⁺. From cultures that showed growth with a lag phase similar to that of the wild-type KT2440 strain, the pedS2 gene was amplified by PCR using primer pair M85/M86, and mutations were identified by Sanger sequencing.

To construct reporter strains for the analysis of pedE and pedH promoter activity in different genetic backgrounds, plasmids pUC18-mini-Tn7-pedE-lux-Gm and pUC18-mini-Tn7-pedH-lux-Gm (8) were co-electroporated with the helper plasmid pTN52 into selected mutant strains of P. putida KT2440 (Table 1). Proper chromosomal integration of the mini-Tn7 element in gentamicin-resistant transformants was verified by colony PCR using P pedE and P pedH primers as described previously (R49).

Reporter gene fusion assays. For quantitative measurement of pedE and pedH promoter activity, strains of P. putida harboring a Tn7-based pedE-lux or pedH-lux transcriptional reporter fusion were grown overnight in LB medium with gentamicin (15 μg·ml⁻¹), diluted to an OD 600 of 0.2 in fresh LB medium, and grown to an OD 600 of 0.6. The cells were then washed three times in M9 medium without a carbon source and finally adjusted to an OD 600 of 0.2 in M9 medium with 1 mM 2-phenylethanol. For luminescence measurements, 198 μl of a cell suspension was added to 2 μl of a 100 μM LaCl₃ solution.
in white 96-well plates with a clear bottom (μClear; Greiner Bio-One). Microtiter plates were placed in a humid box to prevent evaporation and incubated at 28°C with continuous agitation (180 rpm), and light emission and OD600 were recorded at regular intervals in an FLX-Xenius plate reader (SAFAS, Monaco) for 6 h. For both parameters, the background provided by the M9 medium was subtracted, and the luminescence was normalized to the corresponding OD600. Experiments were performed with biological triplicates, and data are presented as the mean values with error bars representing the corresponding standard deviations.

**Sequence identity determination.** Protein sequence identities were determined based on amino acid sequence alignments of the proteins of interest generated using the Clustal Omega multiple-sequence alignment tool (50).

**ACKNOWLEDGMENTS**

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 in part by Labex Resources 21 (ANR-10-LABX-21-01).

We thank Bernhard Hauer for his continuous support.

We declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**REFERENCES**


