Introduction

To date, biotechnology mostly uses inducible promoters to regulate the expression of recombinant proteins. However, especially in the context of industrial applications their use is connected to some disadvantages. The frequently employed T7 RNA polymerase-dependent expression system, for example requires the addition of isopropyl-β-D-thiogalactopyranoside (IPTG), making a production process expensive (Nocadello and Swennen, 2012). The need for monitoring the optical density (OD_{578}) of the cell culture and determining the optimal induction point makes an automatization of protein expression more difficult when using inducible promoters (Briand et al., 2016). Additionally, induction usually takes place in the exponential growth phase of the bacterial culture. In this phase, overexpression of proteins increases the metabolic burden of each cell and proteins which are detrimental for bacterial growth cannot be produced in some cases (Jaishankar and Srivastava, 2017). These disadvantages could be circumvented by using auto-inducing expression strategies for recombinant protein production.

The bacterial cell-to-cell communication mechanism of Quorum Sensing (QS), which is based on the production, release and detection of hormone-like signal molecules (auto-inducers), offers the possibility to link population-wide gene expression to a particular bacterial cell density (Waters and Bassler, 2005). In the last years, QS-based auto-inducible promoter systems were established for recombinant gene expression in both Gram-negative and Gram-positive bacteria (Anderson et al., 2006; Tsao et al., 2010; Nocadello and Swennen, 2012; Guan et al., 2015). In Pseudomonas putida, a Gram-negative soil bacterium with increasing importance for biotechnological applications, the QS-dependent RhlRI-promoter from Pseudomonas aeruginosa was used to express genes for rhamnolipid production (Cha et al., 2008; Cao et al., 2012). However, no cell density-dependent promoter system for recombinant protein expression in P. putida relying on a native QS-system has been available so far.

Previously, Espinosa-Urgel and Ramos (2004) identified the so far only known QS-system of P. putida KT2440, RoxS/RoxR, a two-component system formed by a sensor histidine kinase (RoxS) and a response regulator (RoxR). It was shown that RoxS/RoxR regulates a large
number of genes, responsible, for example for cytochrome oxidase activity and redox signalling, or, like the gene ddcA, involved in seed colonization. Expression of ddcA was directly dependent on cell density. A gene encoding for a yet unknown cytochrome c-type protein, PP_3332, was also shown to be controlled by the RoxS/RoxR system (Espinosa-Urgel and Ramos, 2004; Fernández-Piñar et al., 2008). For induction of expression, RoxS is believed to interact with tetradecanoic acid and fatty acids of similar chain lengths and subsequently phosphorylates RoxR (Fernández-Piñar et al., 2012). Phosphorylated RoxR regulates the expression of cell density-dependent genes by binding to a putative RoxR recognition element (RoxR RE) (Fernández-Piñar et al., 2008).

To enable this QS-system for recombinant protein production in P. putida, we utilized the regions upstream of the RoxS/RoxR-regulated genes ddcA and PP_3332 that are expected to contain all necessary functionalities for protein expression under control of RoxS/RoxR and inserted these into plasmids to mediate the auto-induced and cell density-dependent protein expression. To detect and quantify protein expression, we used the two fluorescent proteins superfolder green fluorescent protein (sfGFP) and mCherry. While sfGFP was expressed intracellularly, mCherry was expressed in the form of the autotransporter-fusion protein MATE-mCherry in order to evaluate the RoxS/RoxR system in the context of a more complex secretion pathway. Proteins expressed as MATE (maximized autotransporter mediated expression) fusion proteins are translocated to the cell surface via type Va secretion (Sichwert et al., 2015), which has recently also been demonstrated with P. putida as host (Tozakidis et al., 2016; Schulte et al., 2017).

We could show that both regions not only function as promoters for recombinant protein expression in P. putida KT2440, but also that the expression is auto-induced and cell density-dependent. The promoter strength could be enhanced by mutating the Pribnow box to the consensus sequence of the major α-factor of the σ70 family. This study complements the P. putida toolbox by a valuable promoter system with the perspective for industrial protein production.

Results and discussion

Construction of plasmids for QS-dependent recombinant gene expression

To generate cell density-dependent promoters for recombinant sfGFP expression in P. putida, a 132 bp part of the upstream genetic region of ddcA (PP_4615) was amplified from the chromosomal DNA of P. putida KT2440 (Fig. 1A, top panel). This region, termed P_{Rox132}, includes the RoxR RE, −10 (Pribnow box) and −35 regions and the ribosome binding site (RBS) of ddcA. Second, the 306 bp long complete integenic region of genes PP_3331 (uncharacterized protein) and PP_3332 (putative cytochrome c-type protein), termed P_{Rox306}, was amplified (Fig. 1A, bottom panel). To increase the promoter strength of P_{Rox306}, the Pribnow box was identified as TAGACT using the online software BPROM (Solovyev and Salamov, 2011), and two positions were mutated to obtain the consensus sequence of the primary α-factor of the σ70 family, TATAAT (Fig. 1B). This essential α-factor is dominantly present in bacteria (Paget and Helmann, 2003). The mutated P_{Rox306} was termed P_{Rox3061}. They were inserted into plasmid DNA upstream of the start codon of superfolder green fluorescent protein (sfGFP), resulting in plasmids pP_{Rox132-sfGFP}, pP_{Rox306-sfGFP} and pP_{Rox3061-sfGFP}.

The start of the transcripts produced under control of these promoters were determined by the SMART 5’ RACE technique (Sherwood et al., 2009). As depicted in Fig. 1B, the transcript started for P_{Rox132} with the sequence GGC in correct distance to the −10 region, and for P_{Rox306} as well as for P_{Rox3061} with the sequence TCC in correct distance to the −10 region. However, for P_{Rox3061}, additional transcriptional start sites besides GGC were identified. All of them were located upstream of the −35 region of P_{Rox132}. This could have been due to the upstream located replication gene of the plasmid. It cannot be excluded that this gene has been read through without termination and thereby overlapped with the gene transcripts controlled by P_{Rox132}.

Expression of sfGFP

To test the functionality of P_{Rox132}, P_{Rox306} and P_{Rox3061} as promoters, P. putida KT2440 cells were transformed with the abovementioned plasmids. Cell growth based on optical density at 578 nm (OD_{578}) as well as fluorescence intensity (FI) were monitored throughout cultivation of 100 ml cultures (Fig. 2A–C). As control, P. putida without plasmid was cultivated likewise. For comparison with established promoters, P. putida pPGAP-sfGFP and P. putida pP_{BAD-sfGFP} were also cultivated. In pPGAP-sfGFP, sfGFP is constitutively expressed under control of P_{GAP}, the promoter of glyceraldehyde-3-phosphate dehydrogenase from Zymomonas mobilis (Conway et al., 1987), which has been shown to be functional in P. putida in experiments before (data not shown). In pP_{BAD-sfGFP}, expression of sfGFP is controlled by the widely applied arabinose-inducible promoter (Guzman et al., 1995). Arabinose was added to this culture upon reaching an OD_{578} of 0.5. In addition to lysogeny broth medium (LB, Fig. 2A–B), P_{Rox3061} was also tested in minimal MOPS medium (Fig. 2C).

All strains with P_{Rox} promoters showed an increase in FI/OD_{578} during growth and hence expressed sfGFP. Therefore, both the P_{Rox132} and P_{Rox306} regions...
appeared to contain a functional promoter, and PRox306 was not corrupted when mutated to PRox3061. A truncated version of PRox306, starting at the RoxR RE and devoid of the region upstream, was also tested, but did not lead to expression of sfGFP (data not shown). This indicates that PRox306 contains elements important for RoxS/RoxR signalling that are not identified so far.

In LB medium, P BAD produced the highest sfGFP levels with final FI/OD578 values of over 400 after reaching the stationary phase. P Rox3061 induced expression with similar strength as P GAP (FI/OD578 = 143 vs. 132), which is approximately five times as high as with the unmutated P Rox306 (FI/OD578 = 29). However, P Rox3061 produced only roughly a third the amount of sfGFP compared to P BAD. P Rox306 exerted a much smaller final sfGFP yield (FI/OD578 = 8). While the strain with P GAP controlled expression already started to show fluorescence from the beginning of cultivation, the strains with PRox promoters did not show considerable fluorescence up to an OD578 of approximately 1.7, giving a first

Fig. 1. A. Genetic origins of PRox promoters. Top panel: P Rox132 is part of the upstream genetic region of ddcA (light grey). It starts with the RoxR recognition element (Rox RE), the regulatory regions (−35 and −10) and a ribosome binding site (RBS), which is located in the 5′ untranslated region (white). Bottom panel: PRox306 consists of the whole intergenic region between P. putida KT2440 genes PP_3332 and PP_3331. B. DNA sequences of P Rox132, P Rox306 and P Rox3061. The −35 and −10 regions predicted by BPROM (Solovyev and Salamov, 2011) are depicted in bold. Experimentally determined transcriptional start sites are marked with + 1. In P Rox3061, the −10 region was mutated to the consensus sequence of the major σ-factor of the σ70 family, TATAAT. The initiation codon of the regulated gene is underlined. The sequence of the Rox RE is shaded, the putative RBS sequence depicted in italics.
indication for its cell density dependency. In minimal medium, the protein production by PRox3061 was lower than in LB medium (FI/OD₅₇₈ = 42 vs. 143), whereas induction of protein expression was induced at a similar OD₅₇₈.

The accumulated amount of sfGFP within the cells was determined by a semi-quantitative Western blot. To this end, cells of each strain were harvested after reaching stationary phase, lysed, and their proteins were separated by SDS-PAGE. After blotting, sfGFP was labelled with specific antibodies and secondary HRP-conjugated antibodies and the intensities of the detected signals were analysed with ImageJ (Rueden et al., 2017) (Fig. 2D). The amount of sfGFP within the bacterial cells was consistent with the fluorescence intensities observed in the cultivation experiments.

To exclude an effect of different translational efficiencies on the amount of sfGFP produced under control of PRox3061, the Ribosome Binding Site (RBS) Calculator (Salis, 2011) was used. For PRox3061, translation efficiency of the RBS was given as nearly threefold higher than for PRox306 (307 vs. 140 arbitrary units). This means that the observed higher sfGFP yield with PRox306 was not an effect of the different RBS, but rather of different promoter strengths.

Surface display of mCherry

The applicability of the RoxS/RoxR QS-system was also tested for expression of a larger protein that is subjected to secretion, in the present case by the autotransporter

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Fig. 2. Expression of sfGFP by *P. putida* (A) under control of PRox132, PRox306 and PRox3061 and (B) under control of the constitutive promoter PGAP and the inducible promoter PBAD. C. Comparison of sfGFP expression under control of PRox3061 in LB and MOPS medium. D. Quantitative comparison of sfGFP accumulation within the cells with different promoters. *P. putida* cells containing plasmids for expression of sfGFP under control of the different promoters were cultivated in 500 ml shake flasks in LB or MOPS medium at 30 °C, and OD₅₇₈ and fluorescence intensity (FI, excitation: 485 nm, emission: 510 nm) were monitored. After reaching stationary phase, the cells were lysed, their proteins separated by SDS-PAGE and sfGFP detected by means of anti-sfGFP and secondary HRP-conjugated antibodies. The detected band intensities were quantified and provided relative to the band intensity of PRox3061. The data are derived from one representative experiment, with the mean of three technical replicates shown. The error bars represent the standard deviation.

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(type Va) pathway. To this end, plasmids were constructed in which the DNA sequence for sfGFP from the abovementioned plasmids was replaced by the sequence for MATE-mCherry (Fig. 3A). This protein was described in detail before (Sichwart et al., 2015). In brief, MATE proteins are translocated into the periplasm of the host and subsequently incorporated into the outer membrane to display the so-called passenger domain, in the case as presented here mCherry, on the cell surface. Surface display and functionality of MATE proteins with P. putida as a host have been shown before (Tozakidis et al., 2016; Schulte et al., 2017). The plasmids pPRox132-MATE-mCherry, pPRox306-MATE-mCherry and pPRox3061-MATE-mCherry were inserted into P. putida, and FI was monitored throughout cultivation in a microtiter plate (MTP) reader (Fig. 3B–C). An increasing FI/OD578 could be observed during growth of all strains except the control strain. Interestingly, in contrast to sfGFP expression, the promoter strength of PRox132 and PRox306 seemed to be similar, as can be seen by the final FI/OD578 values of approximately 1500 in both strains after 24 h. This is contradictory to the previous results, in which PRox306 mediated a much stronger sfGFP expression than PRox132. At this point, there is no verifiable explanation for this observation. It can only be speculated that the complex secretion mechanism of MATE-mCherry distorts the expression strength in a way that the amount of fluorescent mCherry on the cell

![Diagram of MATE-mCherry fusion protein](attachment:image.png)

**Fig. 3.** A. Scheme of the unprocessed MATE-mCherry fusion protein. After cleavage of the signal peptide (SP), the protein consists of a 6xHis epitope, the passenger mCherry, OmpT and fXa cleavage sites, a PEYFK epitope and the EhaA linker and β-barrel (Sichwart et al., 2015). B,C. Expression of MATE-mCherry under control of PRox132, PRox306 and PRox3061. P. putida without plasmid and with plasmid pPRox132-MATE-mCherry (B), pPRox306-MATE-mCherry and pPRox3061-MATE-mCherry (C) were cultivated in LB medium in a 24-well MTP at 30°C. OD578 and fluorescence intensity (FI, excitation: 580 nm, emission: 620 nm) were monitored. These data are mean values of biological triplicates. Error bars are not visible due to small standard deviations that are covered by the icons.

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surface does not reflect the strength of the promoters. In contrast, the mCherry yield was approximately twofold higher with $P_{\text{Rox3061}}$ than with $P_{\text{Rox306}}$ after 24 h, as similarly observed in the sfGFP-experiments.

To verify surface display of mCherry, *P. putida* cells expressing MATE-mCherry and sfGFP under control of $P_{\text{Rox132}}$ were analysed via fluorescence microscopy (Fig. 4). While sfGFP-fluorescence was located within the cytoplasm as expected (Fig. 4B), mCherry-fluorescence was localized at the cell membrane of *P. putida* cells (Fig. 4D). Cells expressing MATE-mCherry were slightly smaller (1.4–1.9 μm in length) (Fig. 4C) than cells expressing sfGFP (Fig. 4A, 2.5–2.8 μm in length). It is likely that the expression of membrane-anchored MATE-mCherry caused morphological changes in *P. putida* KT2440 as described previously, for example for *Escherichia coli* as host (Wagner et al., 2007; Gubelini et al., 2011).

**Cell density dependency of $P_{\text{Rox132}}$ and $P_{\text{Rox306}}$ on transcriptional level**

In order to investigate if protein expression with $P_{\text{Rox}}$ promoters is indeed depending on the cell density of *P. putida*, transcription levels of MATE-mCherry as a function of OD$_{578}$ were determined (Fig. 5). To this end, *P. putida* strains expressing MATE-mCherry under control of $P_{\text{Rox132}}$, $P_{\text{Rox306}}$ and $P_{\text{Rox3061}}$ as described above were cultivated, samples were taken at different time points and the OD$_{578}$ was determined. MATE-mCherry mRNA transcripts of these samples were quantified by means of reverse transcription quantitative PCR (RT-qPCR) taking transcription of *rpoD* as reference. The gene *rpoD*, encoding for a transcription factor of the σ$^{70}$-factor family, has been shown before to be transcribed in equal amounts at all bacterial growth phases (Savli et al., 2003; Wang and Nomura, 2010). *P. putida*
expressing MATE-mCherry under control of the constitutive promoter PBG35 (Zobel et al., 2015) was used as control. PBG35 was used here instead of PGAP because cells transformed with pPGAP-MATE-mCherry were not viable, perhaps because protein expression was too high. While PBG35 showed gene expression levels independent of the growth phase of the bacteria (Fig. 5A), PRox promoters showed a sharp increase in relative gene expression at a particular cell density. In case of PRox132, gene expression was upregulated at an OD578 of 1.0 5-fold in comparison with the initial gene expression level (Fig. 5B). In case of PRox306, gene expression was upregulated threefold at the same OD578 of approximately 1 (Fig. 5C). With PRox3061, a sevenfold increase of MATE-mCherry mRNA transcripts was observed at an OD578 of 0.7, further increasing up to a factor of 10 at an OD578 of 1.0 (Fig. 5D). These experiments confirmed that PRox promoters are auto-induced and dependent on the cell density of P. putida KT2440.

Effect of conditioned medium on MATE-mCherry expression

To further substantiate the cell density-dependent regulation of the PRox promoters, P. putida pPRox306-MATE-mCherry was cultivated in the presence of conditioned medium (CM), that is medium that was used for P. putida cultivation before, subsequently harvested and released from the bacteria. CM should therefore contain all signal molecules (in addition to waste and other metabolites) secreted by P. putida. CM has been used to assess cell density-dependence of ddcA expression before (Espinosa-Urgel and Ramos, 2004). In CM, the concentration of auto-inducers is supposed to be the higher the longer P. putida has been cultivated therein. 24 h CM (final OD578 of the culture: 5.0) should have a higher auto-inducer concentration than 12 h CM (final OD578 = 3.0) and 8 h CM (final OD578 = 2.3) due to the increasing cell number and the longer time the cells produced auto-inducers. The higher the concentration of auto-inducers, the earlier the induction of QS-regulated promoters should consequently take place as the induction threshold is reached earlier due to the auto-inducer concentration provided by CM. These considerations were confirmed by cultivation experiments with CM (Fig. 6). When cultivated in LB medium supplied with 20% (v/v) 24 h CM, P. putida KT2440 pPRox306-MATE-mCherry showed increasing fluorescence after 7 h of cultivation. In contrast, when cultivated in LB medium without CM (fresh medium), the cells started to show fluorescence after 9 h (Fig. 6A). Cultures in LB medium supplemented with 20% (v/v) 12 h CM started to increase FI after 7.5 h (Fig. 6B). Hence, the induction of PRox306

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was shown to be cell density-dependent. Signalling molecules, enriched in CM during cultivation of *P. putida* KT2440 cells, are assumed to be responsible for the earlier increase in FI/OD<sub>578</sub> of *P. putida* KT2440 pPRox306-MATE-mCherry cultivated in presence of different CM. Fernández-Pinario et al. (2012) identified tetradecanoic acid and fatty acids of similar chain lengths to be the auto-inducers of the RoxS/RoxR QS-system. Whether these molecules were present in CM as applied here was not investigated. Nevertheless, further studies would be very interesting as the production of the relevant signal molecules could be modulated and adapted to individual needs.

Conclusions

The RoxS/RoxR QS-system discovered by Espinosa-Urgel and Ramos (2004) could successfully be applied to provide new auto-inducible, cell density-dependent promoters for the recombinant protein production in *P. putida* KT2440 in this study. As the underlying RoxS/RoxR QS signalling is native to *P. putida*, it is sufficient to incorporate the corresponding promoter sequences into a plasmid of choice to mediate protein expression. The mutated version of the P<sub>Rox306</sub> promoter, P<sub>Rox3061</sub>, leads to stronger expression. Still, this promoter does not reach the overexpression of the strong PBAD promoter. For applications that demand a maximal output of protein, further development will be necessary to increase the promoter strength. The P<sub>Rox</sub> promoters are not restricted to induce expression of cytosolic proteins, but can be used for surface display of recombinant proteins as well, making them interesting tools for the generation of whole-cell biocatalysts. They will contribute to expand the application of *P. putida* KT2440 in industrial biotechnology.

**Fig. 6.** Influence of conditioned medium (CM) on expression of MATE-mCherry. *P. putida* pPRox306-MATE-mCherry was cultivated in LB medium to an OD<sub>578</sub> = 0.8. Five millilitres of the cell suspension were harvested and suspended in mixtures of 4 ml fresh LB and 1 ml of different CM. The cells were then cultivated in a 24-well MTP for another 12 h while monitoring OD<sub>578</sub> and fluorescence intensity (FI). (A) 24 h CM, (B) 12 h CM, (C) 8 h CM, (D) 4 h CM. These data are mean values of biological triplicates. Error bars are not visible due to small standard deviations that are covered by the icons.

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Experimental procedures

Bacterial strains and culture conditions

Cloning was carried out in Escherichia coli DH5α (DSM No.: 6897). The cells were cultivated either in lysogeny broth (LB) medium (10 g l⁻¹ tryptone/peptone, 5 g l⁻¹ yeast extract and 10 g l⁻¹ NaCl) at 37°C and 200 rpm or on LB agar plates at 37°C, both containing 50 μg ml⁻¹ kanamycin. P. putida KT2440 (DSM No.: 6125) was cultivated either in LB medium, MOPS medium (LaBauve and Wargo,) or on LB agar plates, with 50 μg ml⁻¹ kanamycin when necessary, at 30°C. Main cultures were inoculated to OD₅₇₈ of 0.05 from an overnight culture and cultivated either in shaking flasks at 30°C and 200 rpm or in 24-well microtiter plates (MTP) in an MTP reader at 30°C with constant shaking (Infinite M200 Pro, Tecan, Männedorf, Switzerland).

To demonstrate cell density-dependence of PₚRox306, P. putida KT2440 pPₚRox306-MATE-mCherry was cultivated in LB medium with 20% (v/v) of conditioned medium (CM). CM was prepared by cultivating P. putida KT2440 cells in LB medium for 4, 8, 12 and 24 h respectively. The cells were then separated by centrifugation, and the supernatant was filtered by a filter with a pore size of 0.22 μm.

Construction of expression plasmids

DNA sequences of PₚRox132 and PₚRox306 were amplified directly from the chromosomal DNA of P. putida KT2440 via PCR with specific primers. PₚGAP was amplified from the chromosomal DNA of Z. mobilis (DSM No.: 3580). Promoter DNA fragments were inserted into pBBR1MCS-2 plasmids (Kovach et al., 1995) containing sfGFP sequence (Wu et al., 2009) via In-Fusion Cloning as described by the manufacturer, resulting in pPₚRox132-sfGFP, pPₚRox306-sfGFP and pPₚGAP-sfGFP. pPₚRox306-sfGFP was generated by mutating the PₚRox306 DNA sequence of pPₚRox306-sfGFP via an In-Fusion Cloning strategy described elsewhere (Raman and Martin, 2014). To generate pPₚRox132-MATE-mCherry, pPₚRox306-MATE-mCherry and pPₚRox306-MATE-mCherry, promoter sequences were amplified via PCR from the sfGFP-plasmids and inserted into a pBBR1MCS-2 plasmid encoding for a MATE-mCherry fusion protein (Tozakidis et al., 2014; Sichwert et al., 2015). pPₚBG35-MATE-mCherry was prepared analogously. The pPₚBG35-promoter was synthesized commercially according to the DNA sequence described elsewhere (Zobel et al., 2015). All cloning primers are listed in Table S1. The sequences of all constructed plasmids were verified by Sanger sequencing.

Analysis of fluorescent proteins

Expression of MATE-mCherry was analysed with the MTP reader. Each cavity of a 24-well plate was loaded with 1.1 ml of main culture, and cells were cultivated as described before. For measuring sfGFP produced by cultures cultivated in flasks, 200 μl of the culture was transferred to a 96-well plate, and the OD₅₇₈ as well as the fluorescence intensity were measured at given time-points. sfGFP: Excitation 485 nm, emission 510 nm; MATE-mCherry: Excitation 580 nm, emission 620 nm. The OD₅₇₈ was determined directly in the MTP and converted by a conversion formula to correct for the non-linearity of the OD₅₇₈ at higher cell densities (Meyers et al., 2018).

Fluorescence microscopy

Pseudomonas putida KT2440 pPₚRox306-sfGFP and pPₚRox132-MATE-mCherry were cultivated at 30°C, 200 rpm in LB medium for 8 and 24 h respectively. 1 ml of cell culture was harvested (11 000 g, 1 min) each. The cells were washed three times with 1 ml PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) and suspended in 1 ml PBS. 10 μl of each cell suspension was mixed with 10 μl DABCO/Mowiol on a microscope slide and covered with a coverslip.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis

Pseudomonas putida KT2440 cells were cultivated in 200 ml LB medium. At different time points during cultivation, the OD₅₇₈ was determined and the total RNA of 2.5·10⁸ cells was purified (peqGOLD Bacterial RNA Kit and peqGOLD DNase I Digest Kit; PEQLAB Biotechnologie, Erlangen, Germany) according to the manufacturer's protocol. DNase I digestion was performed for 30 min at room temperature. 1000 ng of total RNA was reversely transcribed (qScript™ XLT cDNA SuperMix; Quanta bio, Beverly, MA, USA) according to the manufacturer’s description. 50 ng of complementary DNA was amplified with specific primers (Table S2). The qPCR was performed using GreenMasterMix (2×) High ROX (Genaxxon bioscience, Ulm, Germany) and theRotor-Gene Q 2 Plex HRM qPCR cycler (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The expression level of MATE-mCherry was analysed relative to the expression level of P. putida reference gene rpoD (Savli et al., 2003; Wang and Nomura, 2010) according to a mathematical model of Pfaffl (2001).

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Determination of +1 transcription site

To identify the transcriptional start site, the technique ‘Switching Mechanism At 5’ end of RNA Transcript Rapid Amplification of cDNA Ends’ (SMART™ RACE cDNA Amplification kit; Takara, Saint-Germain-en-Laye, France) was used according to the manufacturer’s description. RNA was isolated as stated above from cells expressing MATE-mCherry. First-strand cDNA synthesis was performed following the instructions for the 5’RACE cDNA Amplification with random primers. The RACE-PCR was performed using a gene-specific primer (5’-GATTACGCAAGCTTCAACTGGCCGCTAC CGTCGCGCCAC-3’) and the provided universal primer mix that anneals to the SMART sequence at the 5’ end of the cDNA. The RACE product was cloned into the provided pRACE vector. The resulting plasmids were sequenced by Sanger sequencing.

Semi-quantitative Western Blot analysis

Pseudomonas putida KT2440 cells were cultivated in 20 ml LB medium. Afterwards, cells were harvested by centrifugation (3500 g, 5 min; 4°C) and the sediment was suspended in buffer (composed of 5 μl of 1 μg ml⁻¹ aprotinin, 50 μl of 1 mM phenylmethanesulfonyl fluoride and 500 μl of 1 mg ml⁻¹ DNase I solution in 5 ml ddH₂O). The O₅₇₈₅ was adjusted to 5. Sodium dodecyl sulphate (SDS) sample buffer (100 mM Tris/HCl, 200 mM dithiothreitol, 4 % (w/v) SDS, 0.2 % (w/v) bromophenol blue, 20 % (v/v) glycerol pH 6.8) was added to the cell suspension in a volume ratio of 1:1 and heated for 15 min at 95°C. Samples were simultaneously separated by SDS-PAGE in two gels. One of the resulting gels was stained in a Commassie Brilliant Blue solution. The other gel was used for Western blot analysis. Proteins were transferred to a polyvinylidene fluoride membrane by electroblootting (mini-trans blot; Bio-Rad, München, Germany). The membrane was blocked with phosphate-buffered saline with 0.1 % Tween 20 (PBS-T) and 3 % bovine serum albumin (BSA) for 3 h at room temperature (RT), followed by incubation with the primary antibody (1:500 in PBS-T containing 0.01 % sodium azide and 5 % BSA, goat-anti GFP; SIGGEN Antibodies, Carcavelos, Portugal) over night at 4°C. The membrane was then washed three times with PBS-T and incubated with a secondary horseradish peroxidase (HRP) conjugated antibody solution (1:5000 in PBS-T containing 3 % BSA, donkey anti-rabbit; Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h at RT. After washing the membrane for three times with PBS-T, it was coated with an Immuno Cruz Western Blot Luminol Reagent (Santa Cruz Biotechnology), and the resulting chemiluminescence was detected with a chemiluminescent reader (ChemoCam ECL imager; Intas, Göttingen, Germany).

Chemiluminescence intensity of each band was determined using the program ImageJ (Rueden et al., 2017) and normalized to the overall band intensity of the respective sample in the Coomassie stained gel, which was also determined using ImageJ.

Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Oligonucleotides used for gene cloning in this study.

**Table S2.** Oligonucleotides used for qRT-PCR in this study.