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Cell density-dependent auto-inducible promoters for expression of recombinant proteins in *Pseudomonas putida*

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Summary

Inducible promoters such as P_{lac} are of limited usability for industrial protein production with *Pseudomonas putida*. We therefore utilized cell density-dependent auto-inducible promoters for recombinant gene expression in *P. putida* KT2440 based on the RoxS/RoxR Quorum Sensing (QS) system of the bacterium. To this end, genetic regions upstream of the RoxS/RoxR-regulated genes *ddcA* (P_{Rox132}) and PP_3332 (P_{Rox306}) were inserted into plasmids that mediated the expression of superfolder green fluorescent protein (sfGFP) and surface displayed mCherry, confirming their promoter functionalities. Mutation of the Pribnow box of P_{Rox306} to the σ^{70} consensus sequence ($P_{Rox3061}$) resulted in a more than threefold increase of sfGFP production. All three promoters caused cell density-dependent expression, starting transcription at optical densities (OD_{578}) of approximately 1.0 (P_{Rox132}, P_{Rox306}) or 0.7 ($P_{Rox3061}$) as determined by RT-qPCR. The QS dependency of P_{Rox306} was further shown by cultivating *P. putida* in media that had already been used for cultivation and thus contained bacterial signal molecules. The longer *P. putida* had grown in these media before, the earlier protein expression in freshly inoculated *P. putida* appeared with P_{Rox306}. This confirmed previous findings that a bacterial compound accumulates within the culture and induces protein expression.

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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-1-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 (Sichwart *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 σ-factor of the σ^{70}-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 intergenic 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 σ-factor of the σ^{70} family, TATAAT (Fig. 1B). This essential σ-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_{Rox132}, 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* pP_{GAP}-sfGFP and *P. putida* pP_{BAD}-sfGFP were also cultivated. In pP_{GAP}-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

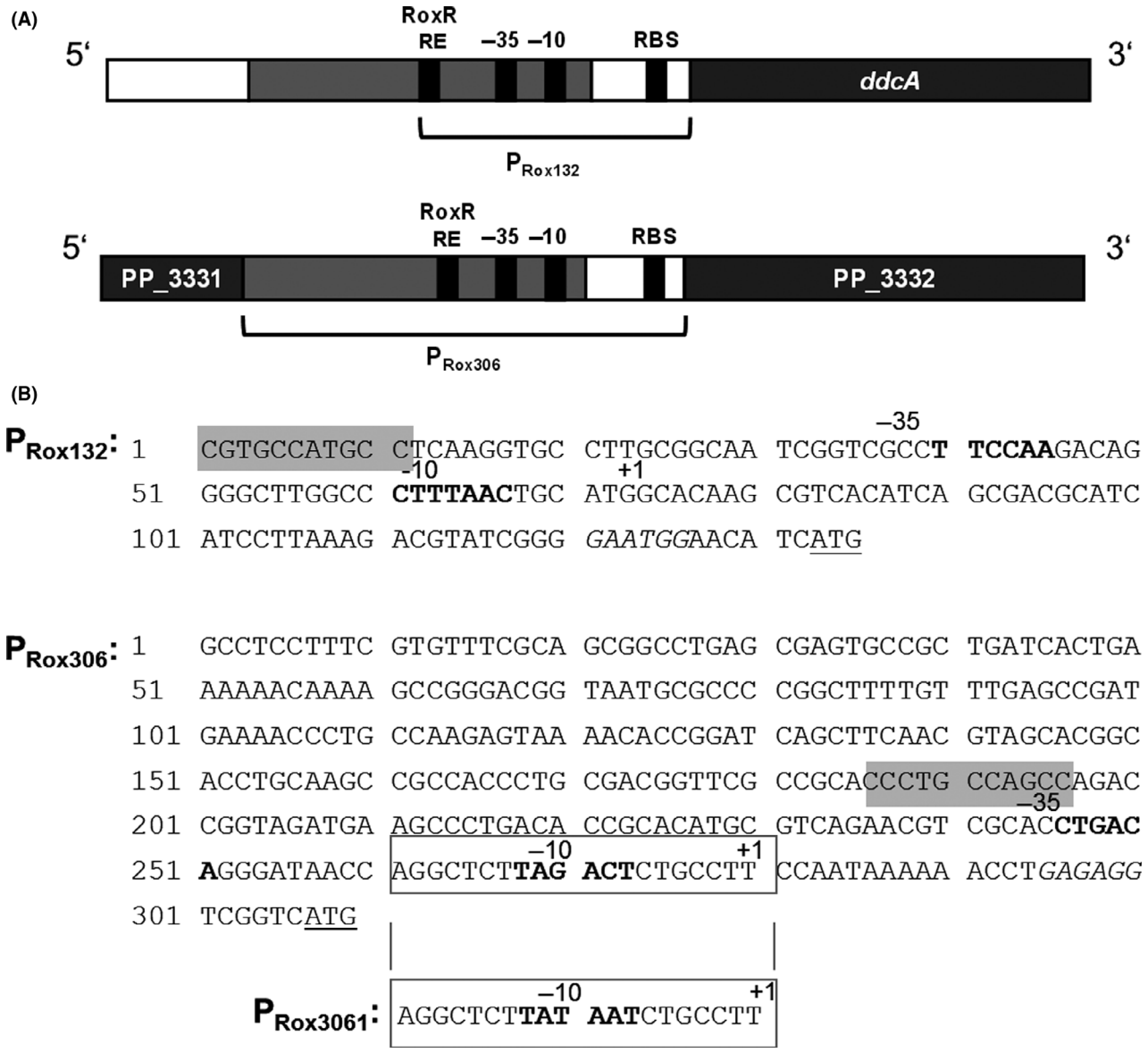

Fig. 1. A. Genetic origins of P_{Rox} promoters. Top panel: P_{Rox132} is part of the upstream genetic region of *ddcA* (light grey). It starts with the RoxR recognition element (Rox RE), the regulatory regions (−35 and −10) and a ribosome binding site (RBS), which is located in the 5′ untranslated region (white). Bottom panel: P_{Rox306} 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 σ^{70} 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.

appeared to contain a functional promoter, and P_{Rox306} was not corrupted when mutated to $P_{Rox3061}$. A truncated version of P_{Rox306}, 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 P_{Rox306} 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/OD_{578} values of over 400 after reaching the stationary phase. $P_{Rox3061}$ induced expression with similar strength as P_{GAP} (FI/OD_{578} = 143 vs. 132), which is approximately five times as high as with the unmutated P_{Rox306} (FI/OD_{578} = 29). However, $P_{Rox3061}$ produced only roughly a third the amount of sfGFP compared to P_{BAD}. P_{Rox132} exerted a much smaller final sfGFP yield (FI/OD_{578} = 8). While the strain with P_{GAP} controlled expression already started to show fluorescence from the beginning of cultivation, the strains with P_{Rox} promoters did not show considerable fluorescence up to an OD_{578} of approximately 1.7, giving a first

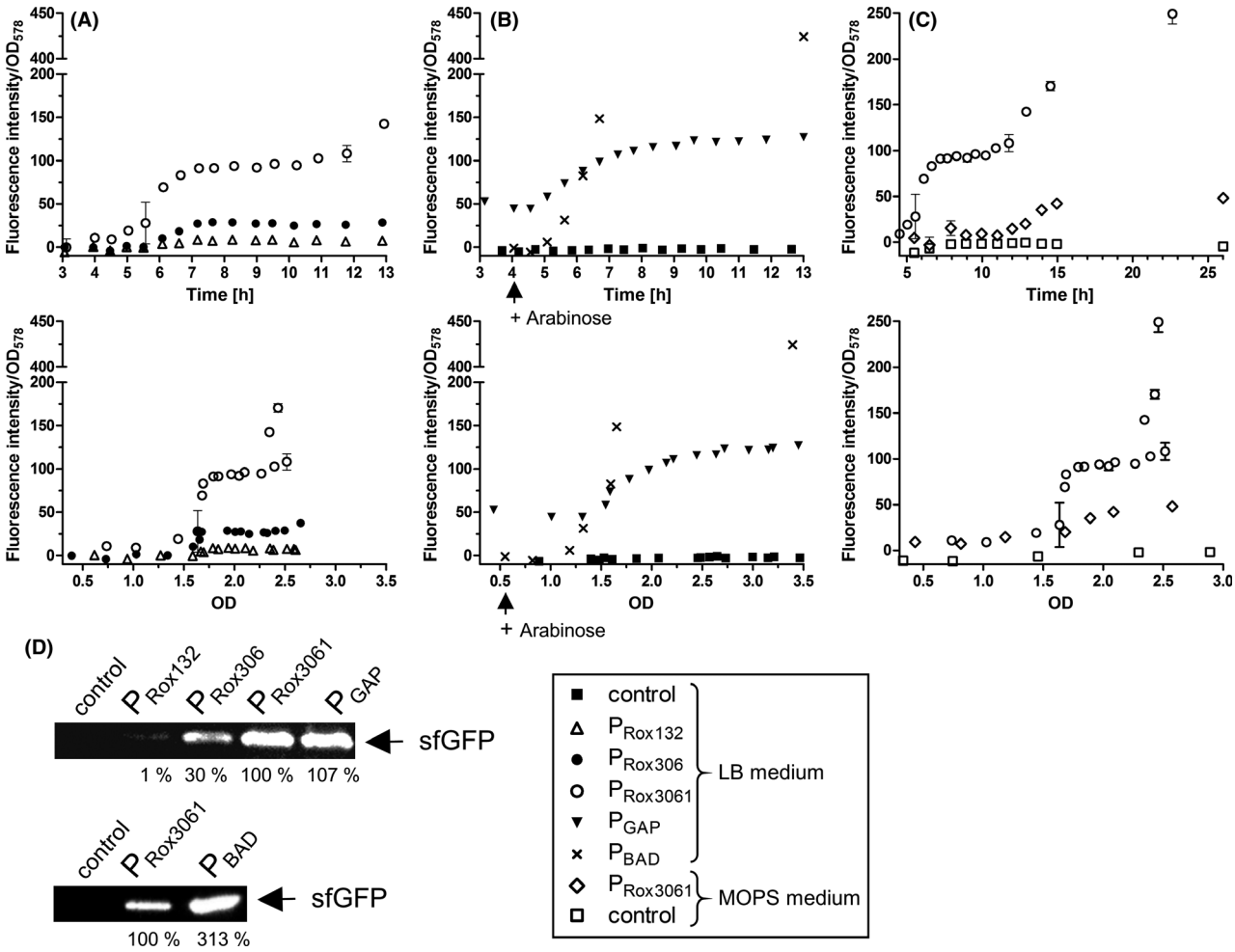

Fig. 2. Expression of sfGFP by *P. putida* (A) under control of P_{Rox132}, P_{Rox306} and $P_{Rox3061}$ and (B) under control of the constitutive promoter P_{GAP} and the inducible promoter P_{BAD}. C. Comparison of sfGFP expression under control of $P_{Rox3061}$ in LB and MOPS medium.D. Quantitative 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 OD_{578} 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 $P_{Rox3061}$. The data are derived from one representative experiment, with the mean of three technical replicates shown. The error bars represent the standard deviation.

indication for its cell density dependency. In minimal medium, the protein production by $P_{Rox3061}$ was lower than in LB medium (FI/OD_{578} = 42 vs. 143), whereas induction of protein expression was induced at a similar OD_{578}.

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 P_{Rox132} and P_{Rox306}, the Ribosome Binding Site (RBS) Calculator (Salis, 2011) was used. For P_{Rox132}, translation efficiency of the RBS was given as nearly threefold higher than for P_{Rox306} (307 vs. 140 arbitrary units). This means that the observed higher sfGFP yield with P_{Rox306} 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

(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 pP_{Rox132}-MATE-mCherry, pP_{Rox306}-MATE-mCherry and $pP_{Rox3061}$-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/OD_{578} could be observed during growth of all strains except the control strain. Interestingly, in contrast to sfGFP expression, the promoter strength of P_{Rox132} and P_{Rox306} seemed to be similar, as can be seen by the final FI/OD_{578} values of approximately 1500 in both strains after 24 h. This is contradictory to the previous results, in which P_{Rox306} mediated a much stronger sfGFP expression than P_{Rox132}. 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

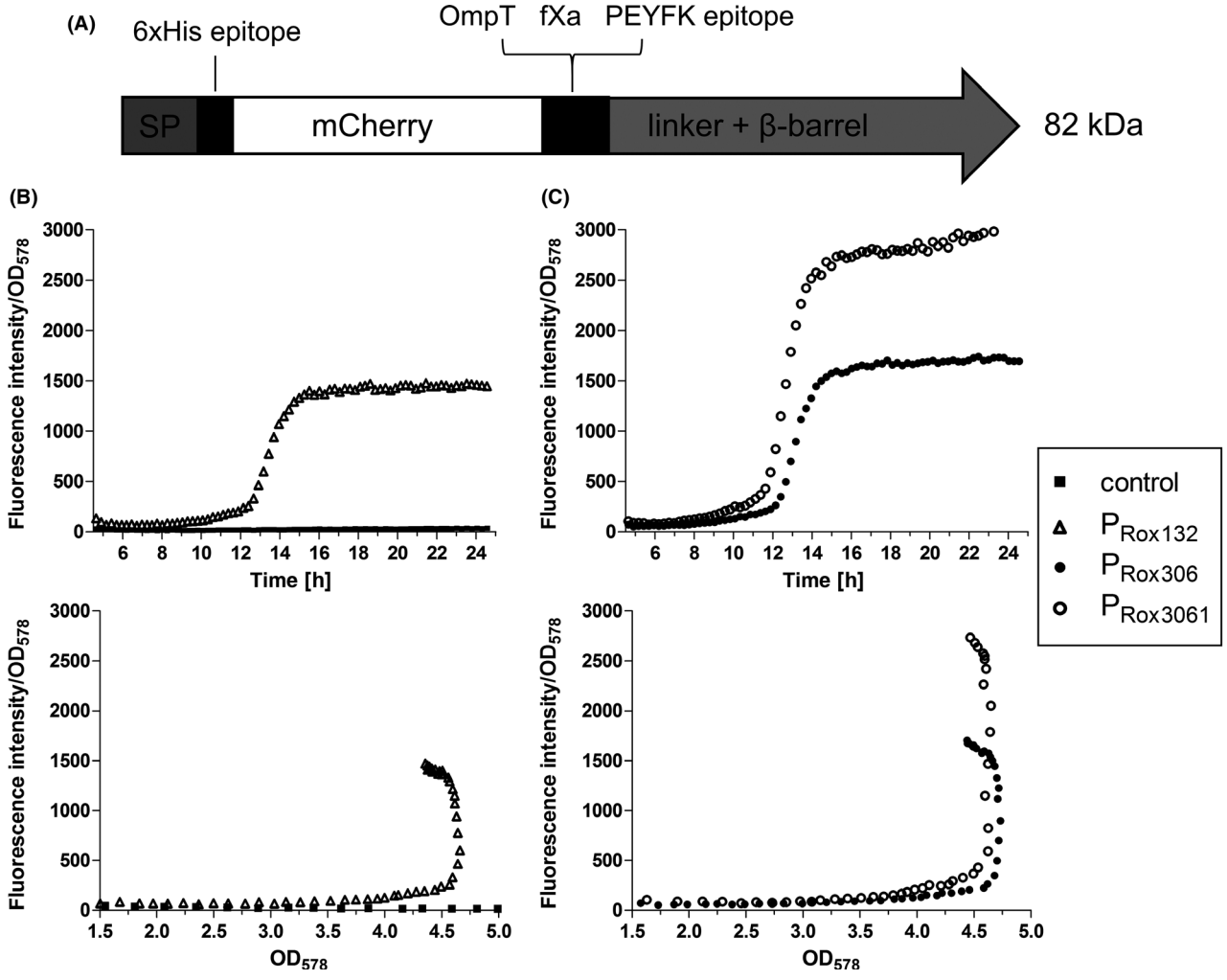

Fig. 3. A. Scheme of the unprocessed MATE-mCherry fusion protein. After cleavage of the signal peptide (SP), the protein consists of a 6xHis epitope, the passenger mCherry, OmpT and fXa cleavage sites, a PEYFK epitope and the EhaA linker and β-barrel (Sichwart *et al*., 2015). B,C. Expression of MATE-mCherry under control of P_{Rox132}, P_{Rox306} and $P_{Rox3061}$. *P. putida* without plasmid and with plasmid pP_{Rox132}-MATEmCherry (B), pP_{Rox306}-MATE-mCherry and $pP_{Rox3061}$-MATE-mCherry (C) were cultivated in LB medium in a 24-well MTP at 30°C. OD_{578} 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.

surface does not reflect the strength of the promoters. In contrast, the mCherry yield was approximately twofold higher with $P_{Rox3061}$ than with P_{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_{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; Gubellini *et al*., 2011).

Cell density dependency of P_{Rox132} and P_{Rox306} on transcriptional level

In order to investigate if protein expression with P_{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_{Rox132}, P_{Rox306} and $P_{Rox3061}$ 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*

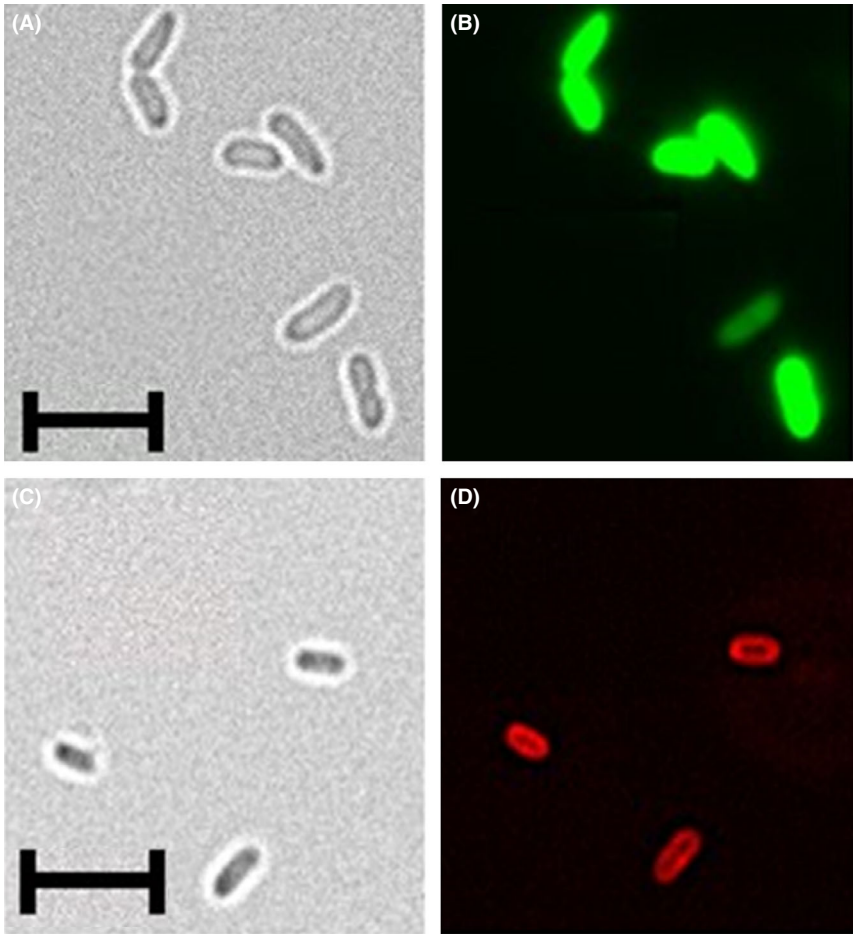

Fig. 4. Analysis of *P. putida* $pP_{Rox3061}$-sfGFP (A,B), and pP_{Rox132}-MATE-mCherry (C,D) *via* fluorescence microscopy. The strains were cultivated at 30°C, 200 rpm in LB medium for 8 h and 24 h respectively. 2.5×10^6 cells were washed three times with PBS and fixed on a microscope slide with DABCO/Mowiol. The samples were analysed with the 100× oil immersion lens of a BZ-9000 fluorescence microscope (Keyence, Neu-Isenburg, Germany).A,C. Brightfield pictures. (B) GFP-filter, excitation 472/30 nm, emmission 593/40 nm.D. TexasRed-filter, excitation 560/40 nm, emmission 630/75 nm. The length of the scales corresponds to 5 μm.

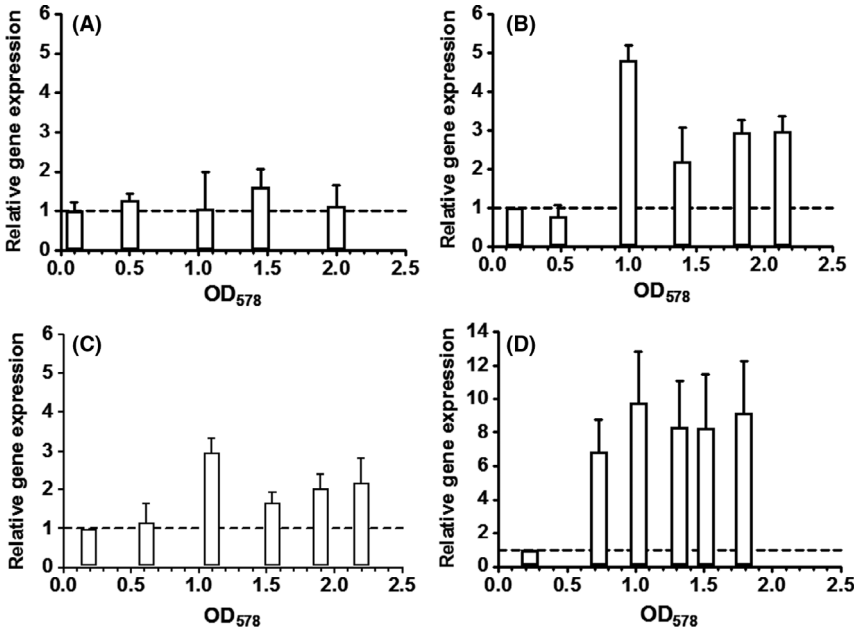

Fig. 5. RT-qPCR analysis of MATE-mCherry expression under control of P_{BG35} (A), P_{Rox132} (B), P_{Rox306} (C) and $P_{Rox3061}$ (D). *P. putida* cells containing plasmids for expression of MATE-mCherry under control of different promoters were cultivated in LB medium at 30°C, 200 rpm. At different time points, 2.5×10^8 cells were removed from the cultures and the total amount of RNA was isolated. 1000 ng of total RNA was reversely transcribed to cDNA. 50 ng cDNA was amplified and analysed with specific primers in a qPCR cycler. The gene expression of MATE-mCherry was determined relative to the gene expression of the reference gene *rpoD* (Fujita *et al*., 1995). The measured Cq values were analysed by applying a model of Pfaffl (2001). All RT-qPCR analyses were performed as biological triplicates, each of them conducted as technical triplicates. Error bars indicate the standard deviation.

expressing MATE-mCherry under control of the constitutive promoter P_{BG35} (Zobel *et al*., 2015) was used as control. P_{BG35} was used here instead of P_{GAP} because cells transformed with pP_{GAP}-MATE-mCherry were not viable, perhaps because protein expression was too high. While P_{BG35} showed gene expression levels independent of the growth phase of the bacteria (Fig. 5A), P_{Rox} promoters showed a sharp increase in relative gene expression at a particular cell density. In case of P_{Rox132}, gene expression was upregulated at an OD_{578} of 1.0 5-fold in comparison with the initial gene expression level (Fig. 5B). In case of P_{Rox306}, gene expression was upregulated threefold at the same OD_{578} of approximately 1 (Fig. 5C). With $P_{Rox3061}$, a sevenfold increase of *MATE-mCherry* mRNA transcripts was observed at an OD_{578} of 0.7, further increasing up to a factor of 10 at an OD_{578} of 1.0 (Fig. 5D). These experiments confirmed that P_{Rox} 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 P_{Rox} promoters, *P. putida* pP_{Rox306}-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 OD_{578} of the culture: 5.0) should have a higher auto-inducer concentration than 12 h CM (final $OD_{578} = 3.0$) and 8 h CM (final $OD_{578} = 2.3$) 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 pP_{Rox306}-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 P_{Rox306}

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_{578} of *P. putida* KT2440 pP_{Rox306}-MATE-mCherry cultivated in presence of different CM. Fernández-Piñar *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_{Rox306} promoter, $P_{Rox3061}$, leads to stronger expression. Still, this promoter does not reach the overexpression of the strong P_{BAD} promoter. For applications that demand a maximal output of protein, further development will be necessary to increase the promoter strength. The P_{Rox} 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.

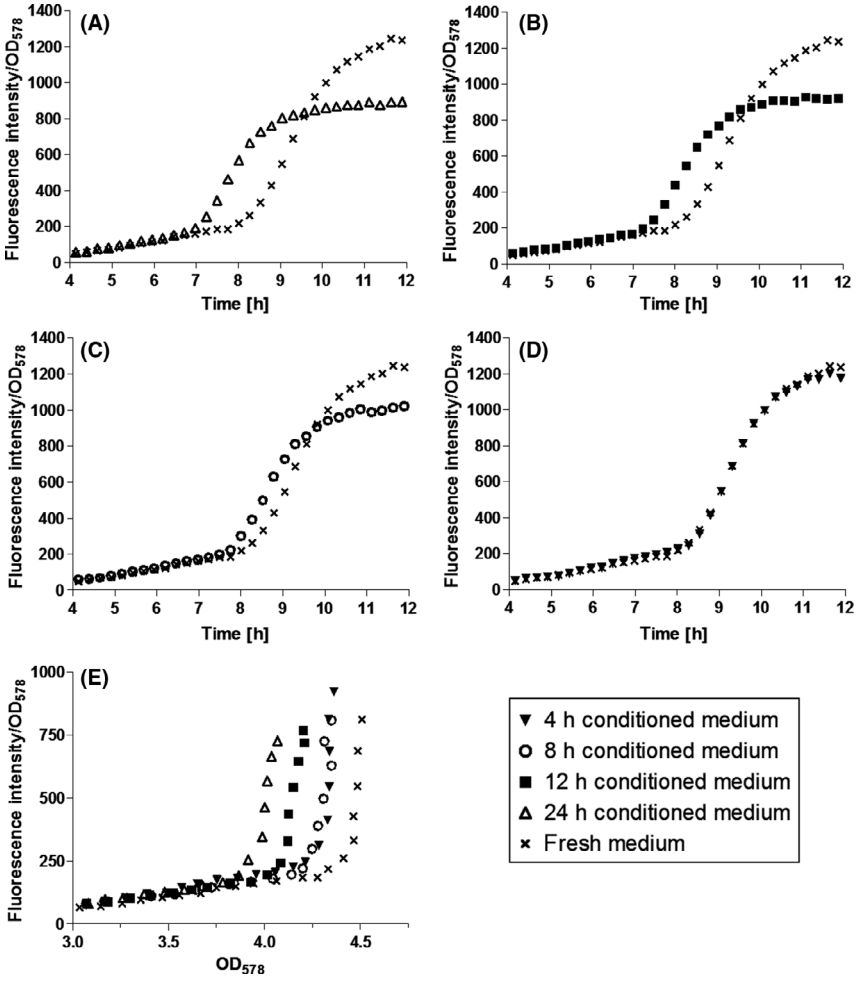

Fig. 6. Influence of conditioned medium (CM) on expression of MATE-mCherry. *P. putida* pP_{Rox306}-MATE-mCherry was cultivated in LB medium to an OD_{578} = 0.8. Five millilitres of the cell suspension were harvested and suspended in mixtures of 4 ml fresh LB and 1 ml of different CM. The cells were then cultivated in a 24-well MTP for another 12 h while monitoring OD_{578} 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.

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 OD_{578} 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 a MTP reader at 30°C with constant shaking (Infinite M200 Pro, Tecan, Männedorf, Switzerland). To demonstrate cell density-dependence of P_{Rox306}, *P. putida* KT2440 pP_{Rox306}-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 filtrated by a filter with a pore size of 0.22 μm.

Construction of expression plasmids

DNA sequences of P_{Rox132} and P_{Rox306} were amplified directly from the chromosomal DNA of *P. putida* KT2440 *via* PCR with specific primers. P_{GAP} 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 pP_{Rox132}-sfGFP, pP_{Rox306}-sfGFP and pP_{GAP}-sfGFP. $pP_{Rox3061}$-sfGFP was generated by mutating the P_{Rox306} DNA sequence of pP_{Rox306}-sfGFP *via* an In-Fusion Cloning strategy described elsewhere (Raman and Martin, 2014). To generate pP_{Rox132}-MATE-mCherry, pP_{Rox306}-MATE-mCherry and $pP_{Rox3061}$-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; Sichwart *et al*., 2015). pP_{BG35}-MATE-mCherry was prepared analogously. The P_{BG35}-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 OD_{578} as well as the fluorescence intensity were measured at given timepoints. sfGFP: Excitation 485 nm, emission 510 nm; MATE-mCherry: Excitation 580 nm, emission 620 nm. The OD_{578} was determined directly in the MTP and converted by a conversion formula to correct for the non-linearity of the OD_{578} at higher cell densities (Meyers *et al*., 2018).

Fluorescence microscopy

Pseudomonas putida KT2440 pP_{Rox306}-sfGFP and pP_{Rox132}-MATE-mCherry were cultivated at 30°C, 200 rpm in LB medium for 8 h 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 Na_2HPO_4, 2 mM KH_2PO_4, 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. The samples were analysed with the 100× oil immersion lens of a BZ-9000 fluorescence microscope (Keyence, Neu-Isenburg, Germany). sfGFP-fluorescence was excited at 472/30 nm and detected at 593/40 nm with the GFP-filter. mCherry-fluorescence was excited at 560/40 nm and detected at 630/75 nm with the TexasRed-filter.

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 OD_{578} was determined and the total RNA of $2.5{\cdot}10^8$ 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 the Rotor-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).

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′-GATTACGCCAAGCTTCAACTGGCCGCTAC 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 ddH_2O). The OD_{578} 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 electroblotting (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; SICGEN 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). Chemilumiscence 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.

