

RESEARCH ARTICLE

Exploring engineered vesiculation by *Pseudomonas putida* KT2440 for natural product biosynthesis

Nora Lisa Bitzenhofer¹  | Carolin Höfel¹  | Stephan Thies¹  |
 Andrea Jeanette Weiler¹ | Christian Eberlein²  | Hermann J. Heipieper²  |
 Renu Batra-Safferling³  | Pia Sundermeyer^{4,5} | Thomas Heidler^{4,5}  |
 Carsten Sachse^{4,5,6}  | Tobias Busche^{7,8} | Jörn Kalinowski⁷ |
 Thomke Belthle^{9,10}  | Thomas Drepper¹  | Karl-Erich Jaeger^{1,11}  |
 Anita Loeschcke¹ 

¹Institute of Molecular Enzyme Technology (IMET), Heinrich Heine University Düsseldorf, Düsseldorf, Germany

²Department of Environmental Biotechnology, Helmholtz Centre for Environmental Research (UFZ), Leipzig, Germany

³Institute of Biological Information Processing – Structural Biochemistry (IBI-7: Structural Biochemistry), Forschungszentrum Jülich, Jülich, Germany

⁴Ernst-Ruska Centre for Microscopy and Spectroscopy with Electrons (ER-C-3/Structural Biology), Forschungszentrum Jülich, Jülich, Germany

⁵Institute for Biological Information Processing 6 (IBI-6/ Structural Cellular Biology), Forschungszentrum Jülich, Jülich, Germany

⁶Department of Biology, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

⁷Center for Biotechnology (CeBiTec), Bielefeld University, Bielefeld, Germany

⁸Bielefeld University, Medical School East Westphalia-Lippe, Bielefeld University, Bielefeld, Germany

⁹DWI—Leibniz-Institute for Interactive Materials, Aachen, Germany

¹⁰Functional and Interactive Polymers, Institute of Technical and Macromolecular Chemistry, RWTH Aachen University, Aachen, Germany

¹¹Institute of Bio- and Geosciences IBG-1: Biotechnology, Forschungszentrum Jülich, Jülich, Germany

Correspondence

Anita Loeschcke, Institute of Molecular Enzyme Technology, HHU Düsseldorf, Wilhelm-Johnen-Straße, 52428 Jülich, Germany.
 Email: a.loeschcke@fz-juelich.de

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Abstract

Pseudomonas species have become promising cell factories for the production of natural products due to their inherent robustness. Although these bacteria have naturally evolved strategies to cope with different kinds of stress, many biotechnological applications benefit from engineering of optimised chassis strains with specially adapted tolerance traits. Here, we explored the formation of outer membrane vesicles (OMV) of *Pseudomonas putida* KT2440. We found OMV production to correlate with the recombinant production of a natural compound with versatile beneficial properties, the tripyrrole prodigiosin. Further, several *P. putida* genes were identified, whose up- or down-regulated expression allowed controlling OMV formation. Finally, genetically triggering vesiculation in production strains of the different alkaloids prodigiosin, violacein, and phenazine-1-carboxylic acid, as well as the carotenoid zeaxanthin, resulted in up to three-fold increased product yields. Consequently, our findings suggest that the construction of robust strains by genetic manipulation of OMV formation might be developed into a useful tool which may contribute to improving limited biotechnological applications.

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INTRODUCTION

Secondary metabolites of microorganisms exhibit a variety of biological activities which make them applicable as pharmaceuticals, ingredients in cosmetics, and food additives. One approach to accessing these natural products is the biosynthesis in heterologous strains, which are engineered as whole-cell biocatalysts (Lin & Tao, 2017). In the last decades, *Pseudomonas* species have become promising bacterial cell factories for such bioproduction processes. One prominent representative of this group is *Pseudomonas putida*, mainly due to its inherent robustness as reviewed in detail in the last years (Bitzenhofer et al., 2021; Loeschcke & Thies, 2015, 2020; Nikel & de Lorenzo, 2018; Weimer et al., 2020).

The compounds which have been produced in *P. putida* include toxic aromatic acids like *p*-coumarate or cinnamate (Calero et al., 2018; Molina-Santiago et al., 2016; Schwanemann et al., 2020), antimicrobial compounds like violaceins and phenazines (Askitosari et al., 2019; Domröse et al., 2017; Zhang et al., 2017), or prodiginines and glidobactins (Cook et al., 2021; Domröse et al., 2015; Loeschcke & Thies, 2020), as well as different terpenoids including zeaxanthin, β -carotene, and lycopene (Beuttler et al., 2011; Hernandez-Arranz et al., 2019; Sánchez-Pascuala et al., 2019).

Despite these success stories, the high-level production of natural compounds is still challenging, amongst other reasons, due to chemical stress caused by high product and substrate concentrations, respectively, which can damage biomolecules or membranes, ultimately compromising the bioprocess (Nicolaou et al., 2010). It is thus intriguing to understand how bacteria have naturally evolved different strategies to respond and adapt to chemical stress. In *Pseudomonads*, an active extrusion of a chemical stressor via efflux transporters to avoid its intracellular accumulation or damage recovery/prevention mechanisms (e.g., the use of chaperones or redox enzymes) are prominent strategies to deal with chemical stress (Bitzenhofer et al., 2021; Blanco et al., 2016; Bösl et al., 2006; Hartl et al., 2011; Henderson et al., 2021; Nicolaou et al., 2010; Roca et al., 2008). Further, a *Pseudomonas*-characteristic and quite unique stress response is the conversion of *cis*-unsaturated fatty acids (FA) of the inner membrane (IM) to their *trans*-configuration by the *cis-trans*-isomerase (Cti) (Bitzenhofer et al., 2021; Heipieper et al., 2003; Tan et al., 2016). The Cti exerts an immediate response: The enzyme is described to be constitutively present in the periplasm and as soon as the membrane is sufficiently perturbed, it can access the *cis*-unsaturated FA in the membrane phospholipid bilayer and isomerises them to the corresponding *trans*-configuration (Mauger et al., 2021). Additionally, *P. putida* can also respond

by vesiculation to chemical stresses, i.e., releasing outer membrane vesicles (OMVs) into the extracellular space. This can be caused by structural changes in the cell envelope (Juodeikis & Carding, 2022). The two processes, *cis-trans*-isomerisation and OMV release, can be employed synchronously in response to the same stresses, but not obligatory so (Eberlein et al., 2018). The OMVs are mainly composed of phospholipids, lipopolysaccharides (LPS), and proteins (Avila-Calderón et al., 2021). Their release can lead to a more hydrophobic bacterial cell surface and thus enhance biofilm formation, which in turn increases bacterial resistance to chemical stressors (Atashgahi et al., 2018; Baumgarten et al., 2012). In addition, vesiculation can help to bring and/or keep the stressors out of the cell (Eberlein et al., 2019; Mozaheb & Mingeot-Leclercq, 2020) and OMVs may serve as an extracellular reservoir for chemical compounds, effectively reducing the concentration in or surrounding the cell (Domröse et al., 2015; Schwechheimer & Kuehn, 2015). An association of OMV release with natural bacterial export of secondary metabolites has been proposed (Batista et al., 2020; Choi et al., 2020; Mashburn & Whiteley, 2005; Tan et al., 2020). Recently, the engineering of such membrane structures was shown to enhance the recombinant production of hydrophobic metabolites in *Escherichia coli* (Yang et al., 2021).

To alleviate the difficulties of high-level microbial production, the construction of robust cell factories with specifically adapted tolerance features seems to be crucial. Here, we assess OMV formation as a biotechnologically exploitable tolerance trait. We present genetic engineering strategies to increase vesiculation and the production of different secondary metabolites in *Pseudomonas putida* KT2440.

EXPERIMENTAL PROCEDURE

Cultivation of *P. putida*

Pseudomonas putida wild-type KT2440 (Nelson et al., 2002) and the derived strains *P. putida* pig21, vio12, and PCA1 (Domröse et al., 2017), *P. putida* pig-r11, -43, and -r44 (Domröse et al., 2019), as well as *P. putida* crtΔX, which was constructed as previously described (Loeschcke et al., 2013; see Table S4), were cultivated under continuous shaking (130 rpm) at 30°C in 10 mL LB (lysogeny broth) medium (10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, 10 g L⁻¹ sodium chloride; Carl Roth®). Antibiotics were added to the culture medium when appropriate to the following final concentrations: 25 μg mL⁻¹ kanamycin, 25 μg mL⁻¹ irgasan, 25 μg mL⁻¹ gentamicin, and 50 μg mL⁻¹ tetracycline. For chemical induction of OMV formation, *P. putida* KT2440 was exposed to 1 mM 1-octanol (Acros organics, part of Thermo Fisher Scientific), 50 μM PQS in DMSO

(Biomol GmbH), or 0.6 mM prodigiosin (in DMSO, final concentration 3%; for extraction and purification see [Figure S15](#) and [M5](#) in [Appendix S1](#)) after reaching the logarithmic growth phase. To induce gene expression or to manipulate gene expression, respectively, 10 mM L-arabinose was added.

Plasmid and strain construction

Oligonucleotides, plasmids, and strains, which were generated based on *P. putida* KT2440, are summarised in [Tables S3](#) and [S4](#).

All recombinant DNA techniques were essentially performed as described by Sambrook et al. (1989), using *Escherichia coli* strains DH5 α (Hanahan, 1983) and StellarTM cells (Takara Bio, Cat# 636763; see [Table S4](#)). Assembly procedures for vectors facilitating overexpression or repression of candidate genes are detailed in [M1](#) in [Appendix S1](#).

Plasmids were introduced into *P. putida* KT2440 by electroporation (Tu et al., 2016). Briefly, 1 mL of overnight cultures of *P. putida* strains was harvested by centrifugation (2 min, 11,000g) and washed with 1 mL H₂O (MilliQ®) twice. The cells were resuspended in 80 μ L H₂O (MilliQ®) and supplemented with 50 ng plasmid DNA. Electroporation was performed in a MicroPulser (25 μ F, 200 Ω , 4.5–5 ms, 20 kV cm⁻¹; Bio-Budget Technologies GmbH). Cells were incubated in 0.7 mL LB medium under continuous shaking (300 rpm) for 2 h before plating on LB agar plates with an appropriate antibiotic.

Determination of *cis-trans*-isomerase (Cti) activity

Fatty acid extraction and methylation were adapted from previous studies (Bligh & Dyer, 1959; Morrison & Smith, 1964). Briefly, cell samples corresponding to an optical density (OD_{700nm}) of 1 in 1 mL were harvested from *P. putida* cultures by centrifugation. Fatty acid methyl esters (FAME) were prepared by incubating the samples in 1 mL methanol and 1.75 mL chloroform under continuous shaking (1000 rpm) for 3 min. Then, 0.5 mL dH₂O was added, and the suspension was thoroughly mixed for 30 s. Centrifugation (10 min, 1000g) facilitated clear phase separation. Finally, the chloroform phase was transferred to a new glass vial (CS), and the solvent was removed by evaporation (Concentrator 5301; Eppendorf). For the methylation of fatty acids (FA), samples were incubated in BF₃-methanol (Merck) for 15 min at 95°C (Morrison & Smith, 1964). Lastly, FAME were extracted with hexane and stored at 4°C. FAME analysis was performed using gas chromatography with a flame ionisation detector (GC-FID, 6890N Network GC System, 7683B Series

Injector; Agilent Technologies). The instrument used a CP-Sil 88 column (CP7488; Varian) in stationary phase and helium as carrier gas. The temperature program was 40°C, 2 min isothermal, followed by a gradient-increase up to 220°C (8°C min⁻¹), and 10 min at 220°C. The FAME peak areas were used to determine their relative amounts. The FA was identified by co-injection of authentic reference compounds obtained from Supelco. *Trans/cis* ratio was calculated taking the sum of the FAME of *cis*-palmitoleic acid (C16:1 Δ 9*cis*) and *cis*-vacenic acid (C18:1 Δ 11*cis*) as divisor and the sum of their corresponding *trans* configuration as dividend (Heipieper et al., 1992).

Isolation of OMVs

Procedures were based on already published protocols by Heipieper and coworkers (Eberlein et al., 2019). After 7 h of cultivation (24 h for some experiments), *P. putida* cells were pelleted by centrifugation (at 5000g and 4°C for 15 min) and to ensure that all cells were removed, the supernatant was filtered through a membrane with 0.45 μ m pore size (Sarstedt). As we did not see any cells in either the TEM or the (MA)DLS analyses (see below), we consider the 0.45 μ m-filter to be appropriate. For final isolation, OMVs were sedimented by ultracentrifugation (at 100,000g and 4°C for 3 h; rotor 50.2 Ti; Beckman Coulter). The obtained vesicle pellet was re-suspended in 100 μ L 10 mM HEPES buffer (pH 6.8) and used for characterisation. For TEM analysis, OMVs obtained from one isolation batch were further purified by ultracentrifugation using a density gradient (2%, 15%, 40%, and 50% glucose) at 100,000g and 4°C for 18 h (rotor SW32 Ti; Beckman Coulter). OMV fractions were collected at sugar concentrations of approximately 10% and 30%, respectively (determined with a refractometer (OPTEC, Optimal Technology)). Vesicle bands at higher sugar concentrations (glucose concentration of about 40%–50%) were not collected due to accumulation of other impurities. Here, a larger batch of 50 mL LB medium was used, and the further procedure was applied unchanged.

OMV characterisation

Bradford assay

For an approximation of relative OMV quantity, the amount of proteins within the OMV fraction was determined by the Bradford assay as described before (Eberlein et al., 2019). For this purpose, 75 μ L of a 10-fold (or 30-fold) OMV dilution (in 10 mM HEPES buffer, pH 6.8) were mixed with 75 μ L Bradford reagent (20 mg Coomassie Brilliant Blue G-250, 10 mL ethanol, 20 mL phosphoric acid, ad 200 mL) in a microtiter plate



(MTP), and the absorption was measured at 595 nm using a plate reader (Tecan) after 5 min of incubation. Bovine serum albumin (BSA) solutions were used for calibration (0 to 0.25 mg mL⁻¹). Notably, other large cellular surface structures like flagellar components that co-sediment with OMVs would influence the Bradford results (Bauman & Kuehn, 2006). For prodigiosin-containing OMVs, the Bradford readout was corrected to take the absorption of the red pigment into account (see Figure S1 and M2 in Appendix S1).

Nile red assay

The lipophilic fluorescent dye Nile red was used as an indicator preferentially staining membranes to analyse OMV fractions. In an MTP, 2 µL of a 2% solution of Nile red (in DMSO; Sigma-Aldrich) was mixed with a 10-fold diluted OMV sample in a total volume of 150 µL. Fluorescence was measured using a plate reader (Tecan) with an excitation wavelength λ_{ex} = 543 nm, an emission wavelength λ_{em} of 598 nm, a bandwidth of 5 nm, and a gain of 150.

Biofilm formation assay

Quantification of biofilm formation as an indirect measurement of surface hydrophobicity was conducted according to O'Toole (2011). Cells were cultivated in an MTP in presence of the stressor 1-octanol (1 mM) or inducer for manipulation of gene expression (10 mM L-arabinose) at 30°C for 24 h. The supernatant was used to measure the optical density (OD_{580 nm}) of the planktonic cells. For quantification, the wells were washed, dried, and supplemented with 150 µL of a 0.1% crystal violet (CV) solution. The MTP was incubated at room temperature for 10 min, washed and dried again. To solubilise the CV, 150 µL 40% acetic acid was used and the absorption of the samples was measured at 550 nm in a fresh MTP (diluted with H₂O) using a plate reader (Tecan).

To correct for differences in growth behaviour, the data of all direct or indirect OMV assays were normalised to the respective cell density at 580 nm (or 700 nm in case of prodigiosin-containing samples).

DLS analysis

The average size of isolated OMVs was analysed by dynamic light scattering (DLS) using a SpectroSize 300 (Xtal Concepts) with a 660 nm laser at a fixed angle. Isolated OMVs were diluted to a corresponding optical density (OD_{580 nm}) of 0.2. DLS does not allow straightforward determination of the exact amount of OMVs in a solution. However, the intensity of the scattered light

depends on the concentration, diameter, and refractive index of the scatterer in relation to the refractive index of the solution (Makra et al., 2015). As the OMV samples were isolated at the same cultivation time, processed identically and the OMVs had a similar size distribution, the measured intensity of the signal (i.e. count rate in kHz) can be indicative of the vesicle concentration. To test this correlation, the signal intensity of a vesicle sample diluted to different concentrations was determined by DLS and the dilutions were plotted against the signal intensity, which showed a linear correlation (see Figure S7). Hence, DLS data were evaluated as size distribution (diameter in nm) and signal strength (of the count rate in kHz).

For MADLS, a Zetasizer Ultra (Malvern Panalytical GmbH, Herrenberg, Germany) was used, operating at three different scattering angles (θ = 15°, 90°, 175°) and a temperature of 25°C. The isolated vesicle fractions (after 7 or 24 h) were diluted 10-fold in HEPES buffer (10 mM, pH 6.8) for measurements in triplicates and analysed using a DTS0012 cuvette. The software ZSplorer was used to analyse the results.

TEM analysis

For imaging, isolated OMV samples (3 µL) were added to a glow-discharged Formvar–carbon film, hexagonal, 300-mesh copper grid, washed two times with H₂O (MilliQ®; 15 s) and stained with 2% uranyl acetate solution for 30 s. Surplus uranyl acetate was removed with a filter paper. Prepared grids were analysed using a TALOS L120C G2 transmission electron microscope (ThermoFisher Scientific) operated at 120 keV. Images were taken using the TEM Imaging & Analysis software (TIA; Thermo Fisher Scientific) on a 4 k × 4 k Ceta M16 CMOS camera.

Transcriptome analysis

For transcriptome analysis, cells were cultivated as described above, the cell pellet was harvested after 7 h, adjusted to an optical density (OD_{700 nm}) of 1 and flash frozen.

Total RNA was isolated from three biological replicates using Quick-RNA Miniprep Plus kit (Zymo Research). The samples were treated with DNase (Zymo Research), and RNA was again purified with an RNA Clean&Concentrator-5 kit (Zymo Research). Ribosomal rRNA was removed with a riboPOOL for bacteria (siTOOLs Biotech GmbH). The purity of RNA and removal of rRNA was tested with an Agilent RNA Pico 6000 kit and an Agilent 2100 Bioanalyzer (Agilent Technologies). TruSeq Stranded mRNA Sample Preparation guide (Illumina) was then used to construct the cDNA library. The constructed cDNA library was

then sequenced with Illumina NextSeq500 high output mode paired-end using a read length of 75 bases.

Transcriptomics sequencing raw data files are available at the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession no. E-MTAB-12470.

The paired-end cDNA reads were mapped to the *P. putida* KT2440 genome sequence (accession number AE015451.2; Belda et al., 2016; Nelson et al., 2002) using bowtie2 v2.2.7 (Langmead & Salzberg, 2012) with default settings for paired-end read mapping. All mapped sequence data were converted from SAM to BAM format with SAMtools v1.3 (Li et al., 2009) and imported to the software ReadXplorer v2.2 (Hilker et al., 2016). Differential gene expression analysis of three biological replicates was performed using DESeq2 (Love et al., 2014) with ReadXplorer v2.2 (Hilker et al., 2016).

For significance of differentially transcribed genes, we used an adjusted *p*-value cutoff of ≤ 0.01 and a signal intensity ratio (*M*-value) cutoff of ≥ 2 or ≤ -2 .

Natural compound production in *P. putida* KT2440

Pseudomonas putida KT2440-derived previously generated strains, which carried the different biosynthetic gene clusters in the genome under a constitutive promoter (see Table S4), were transformed with plasmids for manipulation of the expression of candidate genes by electroporation. The cells were cultivated in 1 mL LB medium and incubated overnight in FlowerPlates® (Beckman Coulter GmbH (formerly m2p-labs GmbH)) at 30°C and shaking at 1200 rpm in a ThermoMixer® C (Eppendorf AG). For *P. putida* PCA1, Round Well Plates (Beckman Coulter GmbH (formerly m2p-labs GmbH)) were used instead of FlowerPlates® to lower oxygen transfer rates (culture volume: 750 µL). Main cultures were inoculated to an optical density (OD_{700nm}) of 0.05 in LB medium already containing 10 mM L-arabinose as inducer for gene overexpression or for the implementation of the CRISPRi system. The cells were incubated under above-described conditions for 48 h.

Extraction of natural products

Samples from cultivation of the producers were fractionated into cell pellet and supernatant by centrifugation (at 15,000 *g* and 4°C for 5 min). The pellets were extracted with 1 mL ethanol (p.a.; for prodigiosin: acidified ethanol (4% 1 M HCl) was used). The extracts were cleared by centrifugation (15,000 *g*, 10 min). Additionally, compounds were extracted from the supernatant by two-phase-extraction with ethyl acetate (2 × 500 µL; for PCA, the supernatant was acidified with 100 µL 6 M HCl) and evaporated afterwards (Concentrator 5301;

Eppendorf). After evaporation, products (except of PCA samples) were dissolved in 150 µL ethanol (p.a.; for prodigiosin, acidified ethanol was used). As PCA mainly accumulates in the supernatant, extracts from the supernatant were dissolved in 1 mL ethanol (p.a.) after evaporation.

Determination of product titres and analytics

Prodigiosin, (deoxy)violacein, and zeaxanthin were quantified spectrophotometrically based on their molar extinction coefficients (prodigiosin: $\epsilon_{535nm} = 139,800 M^{-1} cm^{-1}$; (deoxy)violacein: $\epsilon_{575nm} = 25,400 M^{-1} cm^{-1}$; zeaxanthin: $\epsilon_{450nm} = 144,500 M^{-1} cm^{-1}$ (Domröse et al., 2015; Loeschcke et al., 2013; Rodrigues et al., 2012)) in acidified (prodigiosin) or pure (zeaxanthin, (deoxy)violacein) ethanol, respectively, using a plate reader (Tecan, Maennedorf, Switzerland).

Phenazine-1-carboxylic acid (PCA) was quantified via HPLC-PDA analysis using an LC-10Ai series (Shimadzu GmbH), equipped with an SPD-M10Avp photodiode array detector (PDA). The column oven temperature and the flow rate were set to 30°C and 1 mL min⁻¹. As mobile phase water (A) and acetonitrile (B), both supplemented with 0.1% formic acid, were used. A C30-reverse-phase HPLC column (250 × 4.6 mm, 5 mm particle size, YMC-Europe GmbH) was applied and 10 µL of extracted samples were injected onto the column. Analyses started at 5% B for 2.5 min, before a gradient was used for 16.5 min to reach 98% B. This ratio was maintained for 2 min and then decreased again to 5% B within 1 min. For re-equilibration, the last condition was again maintained for 5 min (Domröse et al., 2017). Chromatograms were recorded at 366 nm. To identify and quantify the PCA signal, a reference (Apollo Scientific) was used for calibration (0–400 mg L⁻¹). PCA signals were observed at a retention time of 18.1 min.

RESULTS AND DISCUSSION

OMV release is a natural response of *P. putida* KT2440 to chemical stress

To identify a tolerance trait that might provide a useful starting point for the engineering of robust production chassis strains, the native stress response of *P. putida* KT2440 were examined first. We chose to focus on the characteristic membrane adaptation responses of *Pseudomonas* species to environmental stress (i.e., Cti activity and vesiculation), especially triggered by hydrophobic compounds. The established method to assess the activity of Cti relies on the determination of the ratio of *trans*- to *cis*-unsaturated membrane FAs

via GC–MS using common protocols for extraction and derivatisation (Heipieper et al., 1995). One common method to indirectly detect OMV formation is the quantification of proteins in the vesicle fraction which can be obtained by ultracentrifugation of culture supernatants (Eberlein et al., 2019). Positive controls for both assays were derived from previous studies: *P. putida* KT2440 wild type was treated with 1-octanol, which is known to trigger both Cti activity and vesiculation (Baumgarten et al., 2012; Eberlein et al., 2018, 2019; Heipieper et al., 1995), and with the *Pseudomonas* quinolone signal (PQS), a quorum sensing-associated compound that is known to induce vesiculation (Mashburn-Warren et al., 2009). In addition, we analysed the effect of the hydrophobic antimicrobial tripyrrole prodigiosin on the cell envelope stress response since *P. putida* KT2440 has been shown to be especially suitable for the production of this compound (Domröse et al., 2015, 2019). For this, we tested the effects of both, the external addition of prodigiosin to the wild type bacteria and the intrinsic prodigiosin production by *P. putida* pig21, which was previously established by chromosomal integration of

the prodigiosin biosynthetic *pig* gene cluster (Domröse et al., 2017).

Cell samples were subjected to analyses regarding changes in the *trans/cis* ratio of unsaturated membrane FA. In addition, vesicle fractions were isolated from the supernatant (see Figure 1A) and their protein content was determined as an estimation of produced vesicles. The untreated wild type was used as reference for the evaluation of all other samples (see Figure 1B,C).

As expected for the positive control, 1-octanol triggered both Cti activity and vesiculation. Interestingly, PQS only caused vesicle formation but no change in the *trans/cis* ratio of FA, while prodigiosin treatment caused the opposite response. External addition of prodigiosin resulted in a significant increase in Cti activity but did not induce hypervesiculation. The production strain *P. putida* pig21, like the PQS-treated wild type, showed again no Cti response but strong vesiculation. Interestingly, the isolated vesicle fraction of this strain exhibited the characteristic bright red colour of prodigiosin, suggesting that the compound had accumulated in these structures (see Figure 1A).

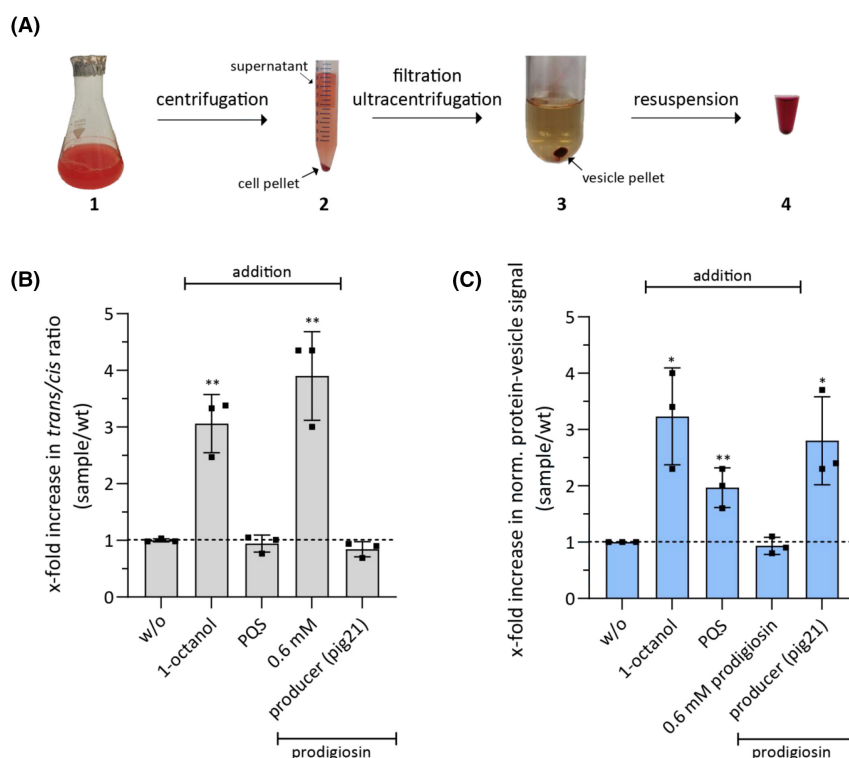


FIGURE 1 Cell envelope stress response of *Pseudomonas putida* after treatment with different stressors. (A) Procedure of OMV isolation using the prodigiosin producing strain *P. putida* pig21 as an example. Starting from a production culture (step 1), cells were harvested (step 2) and the supernatant was filtered (0.45 μ m pore size). Vesicle isolation was performed by ultracentrifugation (step 3). Subsequently, the obtained vesicle pellet was resuspended in buffer (step 4). (B) Increase in *trans/cis* ratio of membrane FA (indicative of Cti activity) of different *P. putida* samples. (C) Increase in protein-vesicle signal calculated from Bradford assay of different *P. putida* samples (correction for prodigiosin-containing samples had to be applied (see Figure S1)). The samples are taken after 7 h of cultivation (logarithmic phase) and the data are normalised to the cell density of the cell culture. Data are shown as x-fold increase of values of treated *P. putida* KT2440 wild type cells (1 mM 1-octanol, 50 μ M PQS or 0.6 mM prodigiosin) or the prodigiosin producer strain *P. putida* pig21 compared with the wild type sample without any treatment (w/o; indicated by the dashed line). The data are mean values of independent triplicate measurements with their respective standard deviation. Significant differences in comparison to the untreated wild type (w/o) are indicated by asterisks (determined by *T*-test; * $p \leq 0.05$, ** $p \leq 0.01$).

Hence, we find that the tested compounds, which share a relatively high value for the partition coefficient logP (1-octanol=3.0; PQS=4.74; prodigiosin=4.07 (calculated using XLOGP3 software (Cheng et al., 2007))) cause a membrane stress response. Interestingly, the quality of membrane adaptation clearly depends on the type of chemical stress. Further, the membrane adaptations appear to be influenced by the compound location and concentration dynamics ("shock treatment" of prodigiosin applied externally as a single full dose application versus steady increase of internally produced compound over the time of cultivation). External "shock treatment" might perturb the membrane to cause fatty acid conversion by Cti, while the production might be accompanied by the general vesiculation stress response which prevents Cti-critical concentrations at the IM. However, the validation of such hypotheses will require further analyses since any potential dynamic responses were not resolved in the end point measurements of the present study. Importantly, our data indicate that the meaningfulness of results obtained from examining cell responses after external application of a chemical stressor as a proxy for a production scenario can be limited and it is useful to analyse production strains themselves.

The observation of enhanced vesiculation in a *P. putida* production strain prompted us to investigate the OMV formation in this context in more detail. The quantification of OMVs by Bradford-based protein quantification as applied here is widely established (Eberlein et al., 2019; Rodriguez & Kuehn, 2020; Roier et al., 2016). However, potential changes in the protein content of OMVs in response to different conditions may influence the results (Bitto et al., 2021). Hence, the vesicle fraction of strain pig21 was additionally subjected to particle analysis by multi angle dynamic light scattering (MADLS), which validated enhanced vesicle formation in comparison to the wild type (see Table S1). We further characterised the OMVs produced by *P. putida* pig21 in comparison to the OMV-triggering controls by fixed angle dynamic light scattering (DLS) and transmission electron microscopy (TEM; see Figure 2). The OMV diameters determined by DLS ranged between 135 and 170 nm (comparable to the MADLS results), which is in the same range as previously described for *P. putida* OMVs (Baumgarten et al., 2012; Roier et al., 2016). Further, qualitative TEM analyses revealed that both OMVs from PQS-treated cells and *P. putida* pig21 producer cells apparently formed clustered agglomerates.

The formation of OMVs thus appears to be a native response of *P. putida* KT2440 to the production of the bioactive compound prodigiosin. We next evaluated whether vesiculation and compound production are quantitatively correlated. To this end, three previously established *P. putida* strains, pig-r43, pig-r11, and pig-r44, which produce prodigiosin at different levels

(Domröse et al., 2019), were subjected to OMV characterisation. Here, low, moderate, and strong OMV formation were found to correlate with the low, moderate, and high-level prodigiosin titres of the respective strains (see Figure 3A).

Additionally, the effect of OMV release triggered by chemical treatment with PQS and 1-octanol on the prodigiosin production levels of *P. putida* pig21 was investigated. Here, we also observed a correlation between OMV formation and compound production (see Figure 3B). An effect of the chemical stress on the *pig* gene transcription levels was excluded via RT-qPCR (see Figure S3). Hence, we deduced that the process of vesicle release itself may positively influence prodigiosin production titres and, therefore, both processes appear to influence each other: An increase in the amount of intracellularly produced prodigiosin increases the formation of OMVs and vice versa.

By measuring product concentration in the different fractions (pellet and OMV fraction; Figure 3), we demonstrate prodigiosin accumulation not only in the pellet but also in the vesicles. This confirms our initial visual observation (see Figure 1A). It is also consistent with the compound's hydrophobicity, based on which it was expected that prodigiosin would interact with the OMVs rather than accumulate in the aqueous medium.

Vesiculation can be triggered by genetic manipulation of *P. putida*

As a next step, we aimed to identify candidate genes associated with vesiculation that might allow targeted genetic manipulation of the process.

We first approached this question in an attempt to uncover the specific adaptations in vesiculating strains – based on the above-described observations. To this end, we analysed transcriptomic data of the prodigiosin producing strain *P. putida* pig21 in comparison to the wild type strain and the wild type triggered to form OMVs by addition of PQS or 1-octanol. Interestingly, both prodigiosin production and 1-octanol addition caused higher changes in the overall number of up- or down-regulated genes than PQS addition (see Table 1, see also Figure S4). This observation, along with the finding that PQS treatment did not increase Cti activity, is consistent with the fact that PQS cannot readily cross the outer membrane (OM) (Florez et al., 2017). Among the sample sets of strain pig21 and 1-octanol-treated wild type cells, we identified six common genes, which were highly up- or down-regulated compared to the untreated wild type (PP_2805 (*ethA*), PP_2807, PP_3494, PP_3533, PP_0710, and PP_4524; see Figure 4), which were thus selected as candidate genes presumably associated with OMV formation.

Interestingly, we detected the Cti transcript in all of the samples in comparable copies. It was thus not

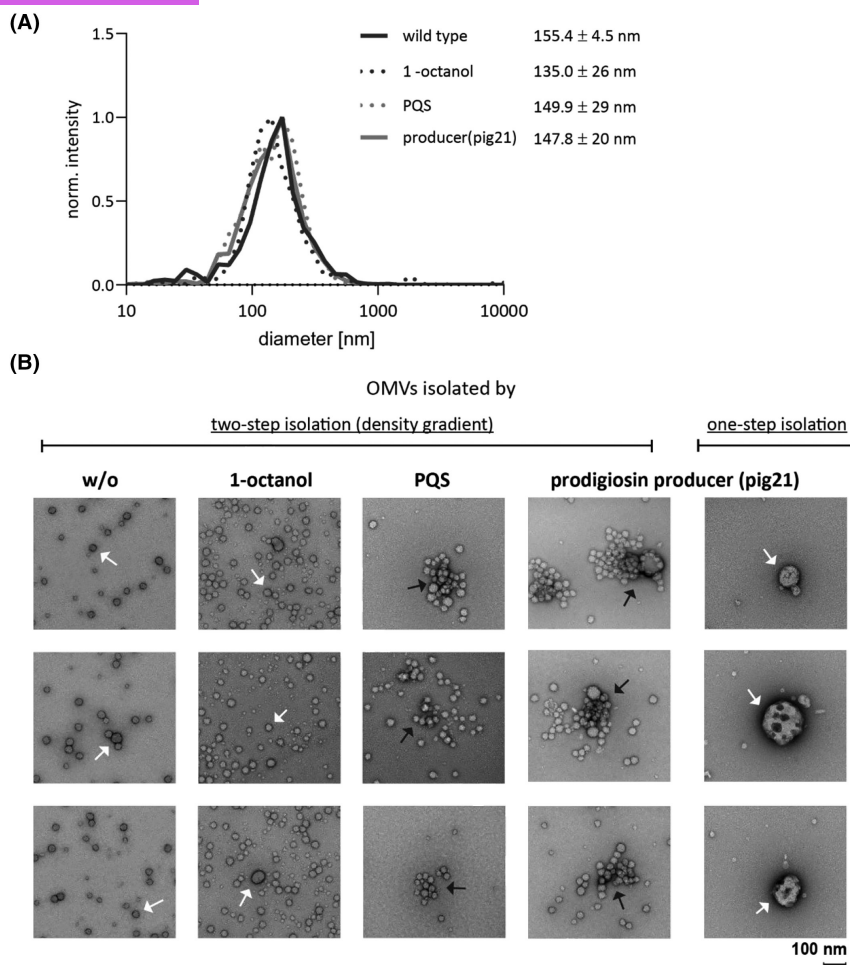


FIGURE 2 *Pseudomonas putida* wild type and prodigiosin producer strain OMVs. (A) Diameter of isolated OMVs of differently treated *P. putida* KT2440 wild type samples (1 mM 1-octanol or 50 μ M PQS; w/o: without addition of a stressor) and the prodigiosin producer strain *P. putida* pig21 determined by DLS measurements (diameter plot: intensity is normalised to maximum). Here, OMVs were isolated by a single ultracentrifugation step (one-step isolation). (B) TEM analysis of negatively stained OMVs of the above measured samples. Left side: for higher background purity, the OMV samples were additionally purified using a glucose density gradient (2%–50%) after the first ultracentrifugation step (two-step isolation). Here, only smaller types of vesicles were collected (20–90 nm). Right side: vesicles from the producer *P. putida* pig21 are shown after one-step isolation (identical to DLS sample preparation). Vesicles showed diameters of about $\sim 143.9 \pm 45$ nm, comparable to the results from DLS measurements (see A and Table S1). White arrows indicate vesicles, black arrows clustered agglomerates. For every OMV sample, TEM images of three different grid squares are shown (see also Figure S2). Magnification: 92,000 $\times$. Scale: 100 nm.

regulated, although 1-octanol treatment was shown to enhance the *trans/cis* ratio of unsaturated membrane FA. This is in accordance with previous descriptions of the enzyme being constitutively expressed and constantly present in the periplasm, from where it can slide into a perturbed membrane to reduce fluidity and increase cell stability (Eberlein et al., 2018; Mauger et al., 2021).

As a second approach, additional candidate genes were identified based on existing knowledge. The release of OMVs can be caused by (i) a reduction of local connections between the OM and the peptidoglycan layer (PG), (ii) an increase in local OM curvature, or (iii) an increase in periplasmic pressure (Juodeikis & Carding, 2022). We looked for different genetic targets whose native function is connected to one of the potential

OMV generation mechanisms and whose manipulation can thus affect vesiculation. Several genes have been identified as putative candidates involved in OMV formation in Gram-negative bacteria (see Table S2; Avila-Calderón et al., 2021; Juodeikis & Carding, 2022; Kulp & Kuehn, 2010; Schwechheimer & Kuehn, 2015). While *P. putida* features similar genes or respective proteins, which could be identified by sequence homology searches (see M6 in Appendix S1), their role in OMV formation has never been described or examined. Based on the results of the literature- and sequence-based searches as well as transcriptome analysis, we selected a set of 21 candidate genes potentially associated with vesiculation, which encode inner and outer membrane proteins (IMPs and OMPs), regulatory elements, periplasmic proteins, PG-degrading enzymes,

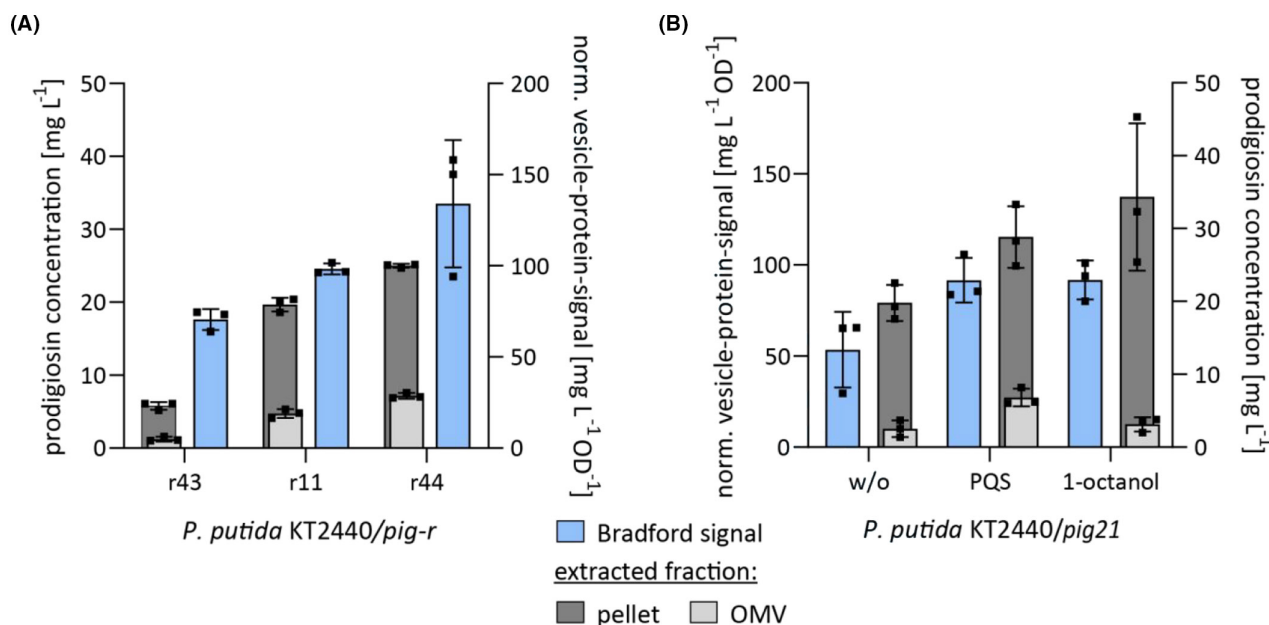


FIGURE 3 Correlation between prodigiosin production and OMV formation in *P. putida* producer strains. (A) Investigation of the effect of prodigiosin production (grey) on vesicle formation (blue) using a low (*pig-r43*), a moderate (*pig-r11*), and a high (*pig-r44*) prodigiosin producer strain. (B) Investigation of the effect of OMV formation (blue) on the prodigiosin titres (grey) of the producer strain *P. putida* *pig21*. For this, vesiculation is triggered by addition of different vesiculation-inducing stressors (1 mM 1-octanol or 50 µM PQS; w/o: without addition of a stressor). OMV amount was analysed by Bradford assay (correction for prodigiosin containing samples had to be applied (see Figure S1)). The prodigiosin concentration was determined in both fractions, the pellet fraction (dark grey) and the isolated OMV fraction (light grey). All samples were taken after 24 h of cultivation, and the data are normalised to the cell density of the cell culture. The data are mean values of independent triplicate measurements with their respective standard deviation. Bars are sorted in ascending order.

TABLE 1 Overall number of up- or down-regulated genes (identified by transcriptome analysis).

Number of regulated genes ^a	<i>Pseudomonas Putida</i> KT2440		
	Strain <i>pig21</i>	Wild type + 1-octanol	Wild type + PQS
Up	53	29	2
Down	74	7	–

^aFor significance of differentially transcribed genes as compared to the untreated wild type, we used an adjusted *p*-value cutoff of ≤0.01 and a signal intensity ratio (*M*-value) cutoff of ≥2 or ≤-2.

or enzymes involved in iron homeostasis (see Table 2). For each gene, a manipulation strategy to either up- or down-regulate the expression was chosen, depending on the previous findings.

Thus far, knockout strains have been generated and described in the literature for many of the chosen targets. To conveniently test a range of candidate genes in the present study, we chose to implement repression of the target gene expression (i.e., respective protein production) by CRISPR interference (CRISPRi; Batianis et al., 2020; Qi et al., 2013; Tan et al., 2018). With this method, the repression is 'portable on a plasmid' and can be easily implemented and tested in several strain backgrounds, as has been done before to manipulate

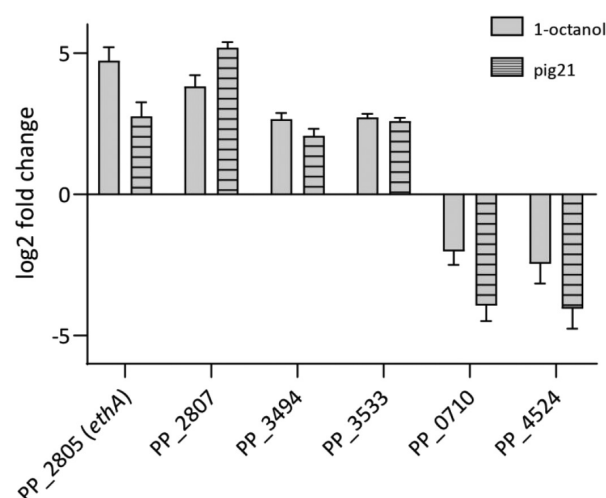


FIGURE 4 Transcriptome analysis of differently stressed *Pseudomonas putida* cells. Highly up- or down-regulated genes of 1-octanol treated *P. putida* KT2440 and *P. putida* *pig21* cells as compared to the untreated wild type. Filled bars show *M*-values for 1-octanol-treated cells, hatched bars the prodigiosin producer. The data are mean values of independent triplicate measurements with their respective standard deviation.

OMVs (Yu et al., 2023). We further regarded repression advantageous over complete deletion since it should also allow addressing rather essential genes.

TABLE 2 Candidate genes that may affect OMV formation by *Pseudomonas putida* KT2440 (sorted by localisation).

<i>Pseudomonas putida</i> protein [Genbank ID]	Locus tag (<i>Pseudomonas</i> genome DB; Winsor et al., 2016)	Product description (<i>Pseudomonas</i> genome DB; Winsor et al., 2016)	Identification strategy	Manipulation strategy (regulation)
Outer membrane (OM)				
OprI (Lpp) [NP_744471]	PP_2322	OM lipoprotein	Literature, sequence (BLASTp)	Down-regulation (CRISPRi, NT strand)
OprL (Pal) [NP_743383]	PP_1223	Peptidoglycan-associated lipoprotein	Literature, sequence (BLASTp)	Down-regulation (CRISPRi, T strand)
OmpA [NP_743248]	PP_1087	OmpA family OM protein	Literature, sequence (BLASTp)	Down-regulation (CRISPRi, NT strand)
MitA [NP_747074]	PP_4971	Outer membrane-bound lytic murein transglycosylase A	Literature, sequence (BLASTp)	Up-regulation (overexpression)
Periplasm (PP)				
Spr [NP_743826]	PP_1669	NLP/P60 family protein	Literature, sequence (BLASTp)	Up-regulation (overexpression)
MrcA [NP_747185]	PP_5084	Penicillin-insensitive transglycosylase/penicillin-sensitive transpeptidase	Literature, sequence (BLASTp)	Down-regulation (CRISPRi, NT)
PvdQ [NP_745045]	PP_2901	Acyl-homoserine lactone acylase	Literature, sequence (BLASTp)	Down-regulation (CRISPRi, NT strand)
Inner membrane (IM)				
MlaE [NP_743120]	PP_0959	Phospholipid ABC transporter permease	Literature, sequence (BLASTp)	Down-regulation (CRISPRi, NT strand)
PP_0287 (AsmA) [NP_742454]	PP_0287	Hypothetical protein	Literature, sequence (BLASTp)	Up-regulation (overexpression)
WbpL [NP_743959]	PP_1804	Glycosyl transferase	Literature, sequence (BLASTp)	Down-regulation (CRISPRi, NT strand)
ToIA [NP_743381]	PP_1221	Colicin S4/filamentous phage transport protein	Literature, sequence (BLASTp)	Down-regulation (CRISPRi, NT strand)
Cytoplasm (CP)				
RpoE [NP_743585]	PP_1427	RNA polymerase sigma E factor	Literature, sequence (BLASTp)	Up-regulation (overexpression)
HidE [NP_4934]	PP_4934	Bifunctional heptose 7-phosphate kinase/heptose 1-phosphate adenylyltransferase	Literature, sequence (BLASTp)	Down-regulation (CRISPRi, NT strand)
WaaC [NP_742509]	PP_0342	ADP-heptose-LPS heptosyltransferase	Literature, sequence (BLASTp)	Down-regulation (CRISPRi, NT strand)
EthA [NP_744949]	PP_2805	FAD-containing monooxygenase	Transcriptomics	Up-regulation (overexpression)
PP_2807 [NP_744951]	PP_2807	Hypothetical protein	Transcriptomics	Up-regulation (overexpression)
Unknown localisation				
PP_0710 [NP_742871]	PP_0710	Hypothetical protein	Transcriptomics	Down-regulation (CRISPRi, NT strand)
PP_3494 [NP_745631]	PP_3494	Hypothetical protein	Transcriptomics	Up-regulation (overexpression)



Therefore, we assembled a broad-host-range CRISPRi vector (pBTBX-2-CRISPRi, see Figure S5A) based on a catalytically inactive variant of the endonuclease Cas9 from *Streptococcus pyogenes* (SpCas9 D10A H840A). We first evaluated the effect of the newly constructed system on both prodigiosin and pyoverdine production. Here, we could verify the functionality of the CRISPRi system in dependence on the guidingRNA (gRNA) target DNA strand (template (T) or non-template (NT)), on the distance from the translational start codon, as well as on the concentration of the inducer L-arabinose (see Figure S5B). Due to the most effective down-regulation, gRNAs targeted to the NT DNA strand close to the translational start codon were selected for down-regulation of the expression of candidate genes. However, the efficiency of down-regulation was dependent on the addressed gene (see Figure S5B). Accepting the limitation that gene-to-gene differences in down-regulation efficiency may occur, we deemed the system applicable to conveniently screen the candidate genes, for which respective gRNA-encoding sequences were cloned.

Candidate genes, whose up-regulation appeared promising, were cloned in the vector pBTBX-2-mcs (Prior et al., 2010), which facilitates L-arabinose-dependent expression (see Figure S6; Hogenkamp et al., 2022). Using both strategies, plasmids for the manipulation of 18 targets could be cloned (for *tonB*, PP_3533, and PP_4524, which were also regarded as promising, cloning was not successful) and introduced into *P. putida* KT2440 to test whether a hypervesiculation phenotype could be implemented.

It is unknown which aspect of vesiculation in terms of quality and quantity might be relevant for supportive effects of natural compound production. Hence, we performed different assays: Bradford and Nile red assay were applied for protein- or lipid-quantification in isolated vesicle fractions as these are also the commonly addressed components for quantifying OMV yields (Klimentová & Stulík, 2015). Further, we analysed the particles via DLS to investigate vesicle sizes; moreover, as the DLS signal intensity may be correlated to the scatterer concentration (see Figure S7), the signal strength was evaluated as potential indicator of relative OMV concentrations. In addition, we measured biofilm formation using crystal violet (CV; Baumgarten et al., 2012; Kulp & Kuehn, 2010) as a measure for vesiculation-induced, increased cell surface hydrophobicity and aggregation (see Figure 5). For all assays, 1-octanol treatment was again used as a positive control. To exclude any effect by *dcas9* expression on the vesiculation response, a CRISPRi empty vector control (ev (Ci)) was used in all assays.

The different assays for analysis of vesiculation gave comparable results for down- or up-regulated expression of most target genes. Since OMV features may well change upon manipulations, we did not expect to

find the exact same outcome in all assays for all strains. Around a third of the manipulations had no or only a minor impact. Notably, in the cases where manipulation relied on CRISPRi, the screening approach cannot distinguish between a manipulation having no effect and a guide RNA failing to implement repression of target gene expression. Around two thirds of the manipulations gave moderately to significantly increased signals. Interestingly, manipulation of OMPs barely caused a change in vesiculation in samples taken from the logarithmic growth phase, however, a stronger effect became apparent after 24 h (see also Figure S8). We also observed differences in the vesiculation phenotype of bacteria examined during the logarithmic and stationary growth phases e.g., upon overexpression of sigma factor *rpoE*, which regulates cell envelope integrity (Rouvière et al., 1995; Schwechheimer et al., 2013). In contrast, the sizes of vesicles produced by differently engineered hypervesiculating strains did not vary significantly (see Figure 5D and Figure S9). In summary, we could identify a range of genes, whose manipulation led to the implementation of hypervesiculation phenotypes in *P. putida* KT2440.

For validation, we aimed to delete the two CRISPRi-downregulated genes, *pvdQ* and *hldE*. However, *hldE* could not be deleted despite several attempts. This underlines the usefulness of a CRISPRi-based down-regulation of genes of interest. The *pvdQ* mutant, which was obtained without difficulties, showed a similarly increased protein and lipid signal in the vesicle fraction as the respective CRISPRi strain (see Figure S10). A limited number of manipulations were chosen for an assessment of tolerance engineering in the generation of robust chassis for natural compound production. We selected the target genes PP_2087 (up), *ethA* (up), *hldE* (down), *mltA* (up), and *pvdQ* (down) because their manipulation led to effects across all assays, which was achieved by both, up- and downregulation approaches. Moreover, these included different types of proteins with different cellular localisations, which are associated with different vesiculation mechanisms.

PP_2087 encodes a protein with sequence-similarity to *E. coli* AsmA, which is predicted to be localised at the IM and to play a role in the assembly of OMPs, LPS biogenesis, OM fluidity, and the tolerance towards hydrophobic antibiotics (Deng & Misra, 1996; Levine, 2019; Misra & Miao, 1995; Xiong et al., 1996). EthA is a cytoplasmatic FAD-containing monooxygenase exhibiting the consensus motifs of Baeyer-Villiger monooxygenases, which was up-regulated in both, 1-octanol-treated and prodigiosin-producing cells and might naturally be involved in the degradation of alkanes and other xenobiotics (Blum et al., 2021; Minerdi et al., 2012; Rehdorf et al., 2007; Van Bogaert et al., 2011; Winsor et al., 2016). However, the association of this monooxygenase with cell envelope properties and vesiculation is unclear.

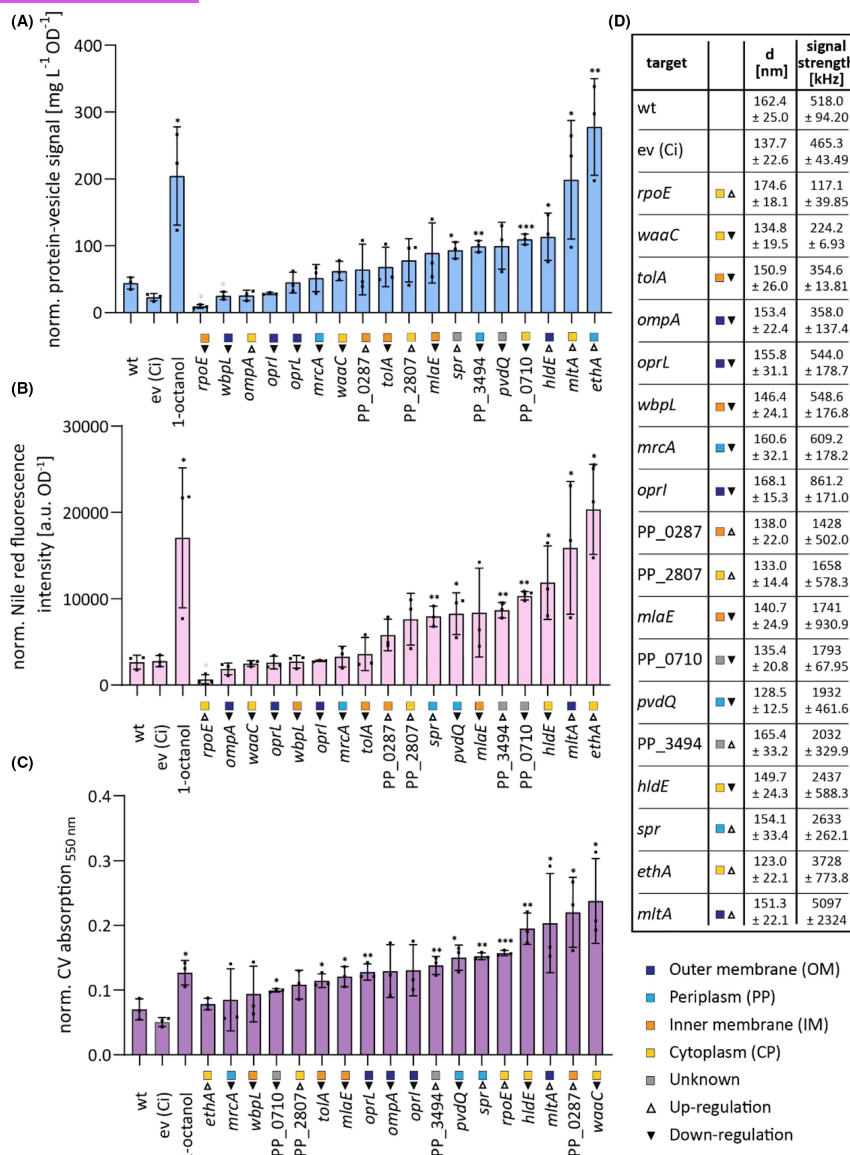


FIGURE 5 Characterisation of OMVs formed by engineered *Pseudomonas putida* strains. (A) Amount of protein in the OMV fraction isolated from engineered *P. putida* strains calculated by Bradford assay (based on $\lambda_{\max}$ = 595 nm) after growth for 7 h (logarithmic phase). The data are normalised to the cell density of the cell culture. (B) Amount of lipid in the OMV fraction formed by engineered *P. putida* strains calculated by Nile red assay (based on λ_{ex} = 543 nm; λ_{em} = 598 nm) after growth for 7 h (logarithmic phase). The data are normalised to the cell density of the cell culture. (C) Quantification of biofilm formation using crystal violet (CV) (based on $\lambda_{\max}$ = 550 nm). The data are normalised to the cell density of the planktonic cells in each sample (after growth for 24 h). (D) Characterisation of OMV particles formed by engineered *P. putida* strains by determination of diameters (d) and the signal strength of count rates with DLS (see also Figure S7 showing a correlation of the sample concentration with signal intensity). Bars and DLS data are sorted in ascending order. Putative cellular localisation of proteins is indicated by different colours (OM: dark blue; PP: light blue; IM: orange; CP: yellow; unknown localisation: grey). Implemented manipulation of gene expression is indicated by arrows (empty arrows: up-regulation; filled arrows: down-regulation). ev (Ci): CRISPRi empty vector control; The data are mean values of independent triplicate measurements with their respective standard deviation. Significant differences in comparison to the untreated wild type are indicated by asterisks (determined by *T*-test; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

HldE is a bifunctional enzyme involved in the biosynthesis of the LPS component L-glycero-D-mannoheptose (L,D-Hep) and localised in the cytoplasm, as reported for *E. coli* (Kneidinger et al., 2002; McArthur et al., 2005; Valvano et al., 2000). In a study with this organism, HldE, together with other enzymes from LPS biosynthesis, was identified as involved in the formation of OMVs (Yang et al., 2021). MltA is a conserved lytic transglycosylase which is presumably

anchored in the OM and cleaves linkages between N-acetylglucosamine and N-acetylmuramic acid in the PG layer, thus far described for *E. coli* and *P. aeruginosa* (Chen et al., 2022; Lommatzsch et al., 1997; Mueller & Levin, 2020). PvdQ is a periplasmic (de)acetylase conserved in fluorescent *Pseudomonads*. In *P. putida* KT2440, it is involved in pyoverdine biosynthesis, where it catalyses the deacylation of the ferripectin precursor in the periplasm as

a maturation step to release the peptide from the IM (Koch et al., 2010; Ringel & Brüser, 2018).

Engineered vesiculation can help to improve the production of diverse natural products

Our results suggested that hypervesiculation could support biosynthetic compound production by *P. putida* KT2440. To verify this hypothesis, we chose the tripyrrolic compound prodigiosin (1), the indolo-carbazoles violacein (2), and deoxyviolacein (3) that are co-produced by the respective biosynthetic pathway, phenazine-1-carboxylic acid (PCA) (4), and the carotenoid zeaxanthin (5). These are all relatively hydrophobic but chemically rather diverse bioactive compounds, for which previously characterised *P. putida* KT2440-derived production strains (and product analysis assays) were readily available. The expression vectors to up- or down-regulate the expression of the identified genes PP_0287, *ethA*, *hldE*, *mltA* and *pvdQ* were introduced in the four previously constructed strains *P. putida* pig21, vio12, PCA1, and crtΔX, which carried the respective biosynthetic gene clusters in the genome where they were constitutively expressed (Domröse et al., 2017; Loeschke et al., 2013). Compound production was assessed by spectrophotometry or HPLC-PDA analyses (see Figure 6).

Production titres of the natural compounds increased to different levels depending on the used engineered producer strain. While for violaceins and zeaxanthin, a smaller increase of titres was observed upon manipulation of some target genes, prodigiosin and PCA titres were more clearly enhanced. Interestingly, this overall trend is not correlated with the hydrophobicity of the compounds (logP values: prodigiosin=4.07; violacein=1.88; deoxyviolacein=2.24; PCA=1.79; zeaxanthin=10.91; calculated by XLOGP3 software (Cheng et al., 2007)).

Previous reports on membrane or vesicle association of some compounds might explain the overall trends in parts: It is known that the final reaction of prodigiosin biosynthesis occurs at the membrane, and the release of prodigiosin or related structures by vesicles or unknown mechanisms has been suggested (Domröse et al., 2015; Matsuyama et al., 1986; Schrempf & Merling, 2015; Tan et al., 2020). A transporter has not yet been described. Similarly, release of violacein via OMVs has been described (Batista et al., 2020; Choi et al., 2020). Phenazines also end up in the culture broth of producing bacteria via unknown mechanisms (Bator et al., 2020; Jin et al., 2015; Sakhtah et al., 2016; Schmitz et al., 2015; Sporer et al., 2018). To our knowledge, a membrane association is not known. In contrast, carotenoids like zeaxanthin are thought to be associated with whole cells and to span the lipid double layer (Gruszecki & Strzałka, 2005). In this context, we evaluated the distribution of compounds between

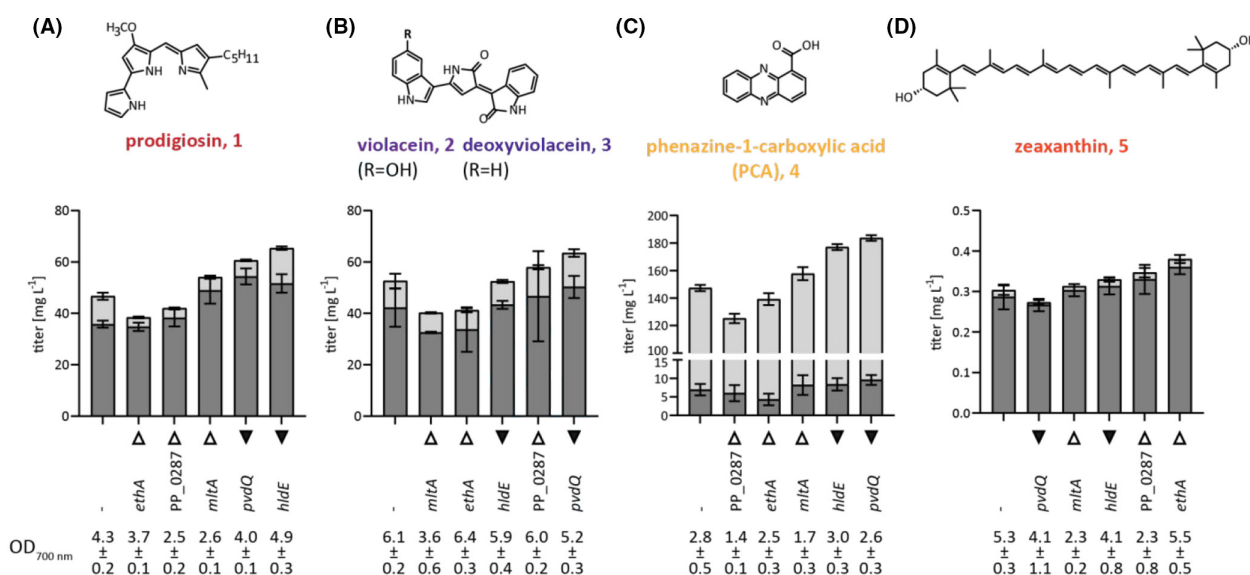


FIGURE 6 Recombinant natural compound production by *Pseudomonas putida* engineered for OMV release. Natural product titres were determined after extraction from both pellet fraction (dark grey) and supernatant (light grey) of the engineered *P. putida* strains (genetic targets addressed by up- (empty arrows) or down-regulation (filled arrows) are indicated) and compared to the respective producer strains (indicated as '-'). (A) Prodigiosin (1) produced by *P. putida* pig21. (B) Violacein (2)/deoxyviolacein (3) produced by *P. putida* vio12. (C) PCA (4) produced by *P. putida* PCA1. (D) Zeaxanthin (5) produced by *P. putida* crtΔX. Implemented manipulation of gene expression is indicated by arrows (empty arrows: up-regulation; filled arrows: down-regulation). Bars are sorted in ascending order. Cell densities reached at the end of the cultivation (after 48h) are given below graphs. The data are mean values of independent triplicate measurements with their respective standard deviation.

the supernatant and cell pellet fractions. Here, we observed that vesiculation did not exclusively support the accumulation of compounds in the OMV-containing supernatant fraction, but that the engineered strains showed an increase of compound levels also in the cell pellet fraction (see [Figure 6](#)). However, we cannot exclude that OMVs may also be present in the cell pellet fraction.

The specific genetic manipulations had rather differential effects: While expression of *ethA* led to enhanced titres only in the zeaxanthin producer, repression of *pvdQ* increased those of all other products. In the prodigiosin producer, *hldE* repression was most effective. An increase in vesiculation upon manipulation of these targets (*hldE*, *pvdQ*, and *ethA*) in the production strains was verified by MADLS analysis (see [Figure S11](#)). The effect was not very pronounced in all cases, which might be due to the fact that the production can already cause relatively strong vesiculation (as demonstrated for prodigiosin, see [Figure 1](#) and [Table S1](#)). OMV formation might be naturally limited by lipid supply and cellular integrity. Since excessive vesiculation must perturb cell envelope integrity, we tested cell viability by propidium iodide (PI) staining and found the engineered cells to be more damaged (see [Figure S12](#)). This indicates that vesiculation engineering may require careful fine-tuning for each case in order to achieve best results.

Notably, some of the engineered strains also did not reach the same cell densities as the parental production strains, which influenced the titres in the cultures (see [Figure 6](#)). Therefore, in terms of product yields [mg per gDCW], the manipulations had a higher (by up to 1.5- to 3-fold) and in parts a different effect (see [Figure S13](#)).

Our findings did not reveal a single outstanding genetic target which would allow improving production of all compounds. This cannot be expected as also suggested by findings reported for membrane engineering with *E. coli* (Yang et al., 2021). Differential effects may be caused, for example, by the temporal dynamics of vesiculation and biosyntheses. It was interesting to note that the ratio of violacein/deoxyviolacein was slightly changed by the manipulations (see [Figure S14](#)). Compared to the parental strain, cell pellet extracts of the manipulated strains showed a decrease of deoxyviolacein relative to violacein (except for *pvdQ* repression). Compared to these samples, supernatant extracts showed an increase in the relative deoxyviolacein levels (except for *ethA* expression). This might indicate that the more hydrophobic compound deoxyviolacein is more amenable to being excreted or to accumulate in vesicles.

Apart from optimising compound release by vesiculation, limitations in the biosynthetic pathways must also be considered. An example is zeaxanthin biosynthesis which is limited by the pool of available precursors (Hernandez-Arranz et al., 2019; Sánchez-Pascuala et al., 2019). Concerted efforts encompassing metabolic

engineering and a fine-tuned engineering of the host tolerance shall boost production yields and allow to implement the biosynthetic production of highly toxic compounds which would otherwise be inaccessible.

CONCLUSION

In summary, we show that OMV formation is a natural response of *P. putida* KT2440 not only to external chemical stressors but also to the production of small molecules. Moreover, we demonstrate that this phenotype can be genetically engineered, which can improve production yields. In the future, the engineering of OMV formation may help to create robust chassis strains and serve as a potential tool to improve hitherto limited yields of natural products for biotechnological applications.

AUTHOR CONTRIBUTIONS

Nora Lisa Bitzenhofer: Conceptualisation (equal); data curation (lead); formal analysis (lead); methodology (equal); validation (lead); visualisation (lead); writing – original draft (lead); writing – review and editing (equal).

Carolin Höfel: Investigation (supporting); writing – review and editing (equal).

Stephan Thies: Conceptualisation (supporting); funding acquisition (equal); validation (equal); writing – review and editing (equal).

Andrea Jeanette Weiler: Investigation (supporting); methodology (supporting); resources (supporting); writing – review and editing (equal).

Christian Eberlein: Data curation (equal); formal analysis (equal); investigation (equal); writing – review and editing (equal).

Hermann J. Heipieper: Data curation (equal); formal analysis (equal); resources (equal); writing – review and editing (equal).

Renu Batra-Safferling: Data curation (equal); formal analysis (equal); resources (equal); writing – review and editing (equal).

Pia Sundermeyer: Investigation (equal); methodology (equal); writing – review and editing (equal).

Thomas Heidler: Investigation (equal); methodology (equal); writing – review and editing (equal).

Carsten Sachse: Data curation (equal); formal analysis (equal); resources (equal); writing – review and editing (equal).

Tobias Busche: Data curation (equal); formal analysis (equal); methodology (equal); writing – review and editing (equal).

Jörn Kalinowski: Data curation (equal); formal analysis (equal); resources (equal); writing – review and editing (equal).

Thomke Belthle: Investigation (equal); methodology (equal); writing – review and editing (equal).

Thomas Drepper: Formal analysis (equal); supervision (equal); writing – review and editing (equal).

Karl-Erich Jaeger: Funding acquisition (equal); writing – original draft (equal); writing – review and editing (equal).

Anita Loeschcke: Conceptualisation (equal); data curation (equal); formal analysis (equal); funding acquisition (equal); supervision (lead); validation (equal); writing – original draft (equal); writing – review and editing (equal).



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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no competing interests associated with the manuscript.

DATA AVAILABILITY STATEMENT

Transcriptomic data are available at the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession no. E-MTAB-12470. All data are available upon request.

ORCID

Nora Lisa Bitzenhofer <https://orcid.org/0000-0002-2790-4929>

Carolin Höfel <https://orcid.org/0000-0002-4230-2942>

Stephan Thies <https://orcid.org/0000-0003-4240-9149>

Christian Eberlein <https://orcid.org/0000-0003-4521-8145>

Hermann J. Heipieper <https://orcid.org/0000-0002-3723-9600>

Renu Batra-Safferling <https://orcid.org/0000-0002-8597-4335>

Thomas Heidler <https://orcid.org/0000-0002-5997-3945>

Carsten Sachse <https://orcid.org/0000-0002-1168-5143>

Thomke Belthle <https://orcid.org/0000-0001-6802-4189>

Thomas Drepper <https://orcid.org/0000-0002-0096-8084>

Karl-Erich Jaeger <https://orcid.org/0000-0002-6036-0708>

Anita Loeschcke <https://orcid.org/0000-0001-5184-8499>

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SUPPORTING INFORMATION

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