J Ind Microbiol Biotechnol (2015) 42:851–866
DOI 10.1007/s10295-015-1615-8

BIOCATALYSIS

Making variability less variable: matching expression system and host for oxygenase-based biotransformations

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Received: 9 February 2015 / Accepted: 26 March 2015 / Published online: 16 April 2015

Abstract Variability in whole-cell biocatalyst performance represents a critical aspect for stable and productive bioprocessing. In order to investigate whether and how oxygenase-catalyzed reactions are affected by such variability issues in solvent-tolerant *Pseudomonas*, different inducers, expression systems, and host strains were tested for the reproducibility of xylene and styrene monooxygenase catalyzed hydroxylation and epoxidation reactions, respectively. Significantly higher activity variations were found for biocatalysts based on solvent-tolerant *Pseudomonas putida* DOT-TIE and S12 compared with solvent-sensitive *P. putida* KT2440, *Escherichia coli* JM101, and solvent-tolerant *Pseudomonas taiwanensis* VLB120. Specific styrene epoxidation rates corresponded to cellular styrene monooxygenase contents. Detected variations in activity strictly depended on the type of regulatory system employed, being high with the *alk*- and low with the *lac*-system. These results show that the occurrence of clonal variability in recombinant gene expression in *Pseudomonas* depends on the combination of regulatory system and host strain, does not correlate with a general phenotype such as solvent tolerance, and must be evaluated case by case.

Keywords *Pseudomonas putida* · Monooxygenase · Clonal variability · Solvent tolerance · *alk*-regulatory system

Introduction

Biotechnological processes are becoming more and more important in the chemical industry. The global industrial biotechnology market was valued at US $14.9 billion in 2013 and is expected to increase by 12.3 % per year from 2014 to 2020 [19]. The immense diversity of biocatalysts and respective substrates and products has led to widespread applications in industry across many sectors. For energy-dependent biocatalysis such as redox biocatalysis, reproducibility and biocatalyst stability typically are major challenges [52], which often are thwarted by unnatural conditions for the microbial biocatalyst, e.g., by toxic/inhibitory substrate and product concentrations. *Pseudomonas putida*, a microbial platform host with promising physiological attributes such as solvent tolerance [46], a broad enzymatic inventory [60], and high metabolic capacity for biocatalyst (re)production and redox cofactor regeneration [8], is an extraordinary candidate to overcome toxicity- and capacity-related process limitations. These promising properties of *Pseudomonas* strains enable the use of toxic solvents in whole-cell biotransformations [25], including oxygenase-based reactions [34], for which the dependence on cofactors, the formation of reactive oxygen species [36], and inherent enzyme instability ex vivo hamper the use of isolated enzymes [61]. Examples include

Electronic supplementary material The online version of this article (doi:10.1007/s10295-015-1615-8) contains supplementary material, which is available to authorized users.

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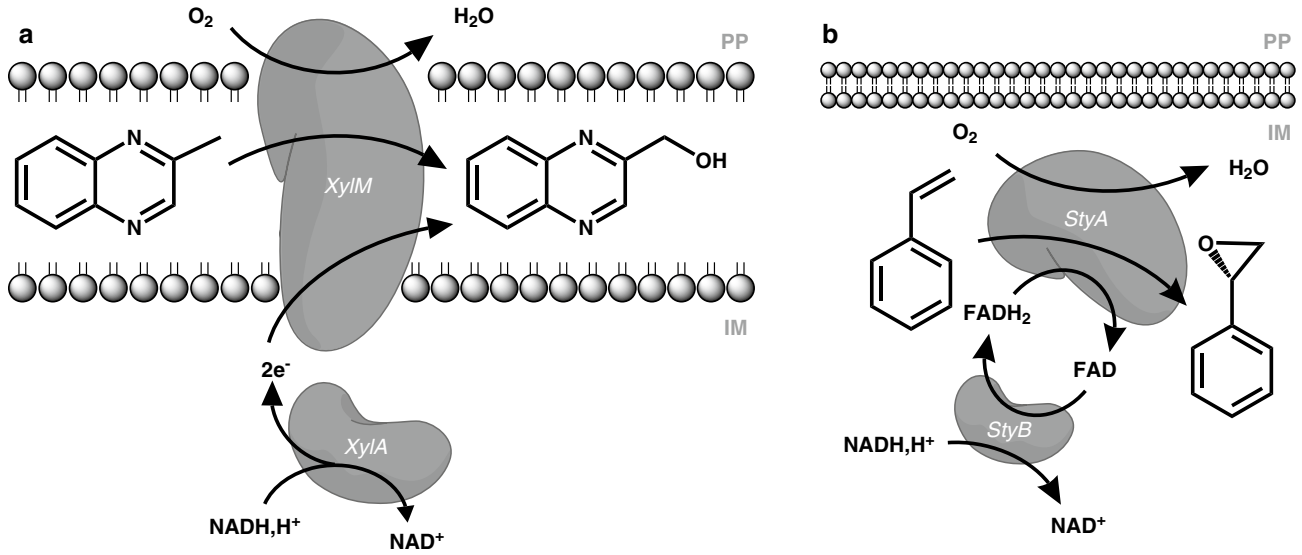

Fig. 1 Monooxygenases applied in activity assays. **a** The membrane-bound xylene monooxygenase (XMO), converting 2-methylquinoxaline to quinoxaline-2-yl-methanol, consists of two subunits, the hydroxylase component XylM and the NADH reductase component XylA responsible for electron transfer [59]. **b** Epoxidation of styrene to (*S*)-styrene oxide with the soluble styrene monooxygenase (SMO) consisting of the oxygenase StyA and the NADH-flavin reductase StyB [41]

P. putida DOT-T1E [46] and *P. putida* S12 [24] which, in contrast to solvent-sensitive *P. putida* KT2440 [38] or *Escherichia coli* strains [34], are highly tolerant toward toxic organic solvents with a log $P_{O/W}$ of 2–4, e.g., toluene (2.69) or styrene (2.95). When exposed to such solvents, tolerant strains differ from sensitive strains in their capability to adapt and grow, which involves the activation of protection mechanisms. Thereby, *cis–trans* isomerization of unsaturated fatty acids, as short-term response, decreases the permeability and fluidity of membranes [30], whereas, as long-term response, solvent efflux pumps actively extrude organic solvents [32] to avoid solvent accumulation in the cytoplasmic membrane.

Variations in cell morphology, stability of biocatalytic performance, and cell fitness of such solvent-tolerant strains are poorly understood so far. Phenotypic heterogeneities in isogenic microbial populations [4] often result from cellular stress responses [1], can be induced by recombinant gene expression [27] or the presence and accumulation of toxic compounds [2], and even have been observed in terms of variable gene expression patterns in glucose-limited chemostat reactors [40]. It was reported that, during well-mixed, high-cell-density fed-batch processes with *E. coli*, up to 20 % of the cells became depolarized and permeabilized indicating the development of high and low productive phenotypes [26]. If cells develop such phenotypic variations in a bioprocess, the whole process is hampered in terms of reproducibility, stability, and productivity.

The stochasticity in gene expression or gene expression "noise" in genetically identical cells exposed to the same environmental conditions has recently been investigated in detail [18, 31, 47]. Although heterogeneity in gene expression levels might represent a microbial strategy to prepare for diverse environmental conditions, which enhances robustness and may be stress induced [6], the development of subpopulations can heavily affect reproducibility and efficiency of biotechnological processes [1, 27].

The aim of this study was to investigate the stability and reproducibility of whole-cell monooxygenase activities in *Pseudomonas* strains differing with respect to phenotypic characteristics such as solvent tolerance. For this purpose, plasmids carrying genes encoding for membrane-bound xylene monooxygenase (XMO) or soluble styrene monooxygenase (SMO) were used in different *Pseudomonas* host strains. Whole-cell activities were investigated for the selective XMO- and SMO-catalyzed functionalizations of 2-methylquinoxaline to quinoxaline-2-yl-methanol and styrene to (*S*)-styrene oxide, respectively (Fig. 1). *E. coli* JM101 served as a control host strain, which has been used earlier for the production of 3,4-dimethylbenzaldehyde from pseudocumene using XMO [9, 11] and (*S*)-styrene oxide from styrene using SMO [35, 42]. The high variability in activity observed for some of the *Pseudomonas* host strains was then further investigated by testing different expression systems (*alk*/*lac*), inducers, and substrates.

Materials and methods

Bacterial strains, plasmids, media, and chemicals

Microbial strains and plasmids used in this work are listed in Table 1. Cells were grown in lysogeny broth medium [50]

Table 1 Bacterial strains and plasmids used in this study

Strain	Characteristics	References
E. coli		
DH5α	*sup*E44Δ*lac*U169(Φ80*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	[22]
DH10B	F^- mcrA Δ(*mrr hsd*RMS-*mcr*BC) Φ80d*lacZ*ΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*Δ139 Δ(*ara,leu*)7697 *gal*U *gal*K λ^- *rps*L *end*A1 *nup*G	[16]
JM101	*supE thi-1* Δ(*lac-proAB*) F′[*traD36 proAB*$^+$*lacI*^{q}*lacZ*ΔM15] (solvent sensitive)	[37]
P. putida		
DOT-T1E	Rif^r derivate of *P. putida* DOT-T1 (solvent tolerant)	[46]
DOT-T1E-PS34	Rif^{r}Km^{r}Sm^{r}Tel^r*ttgB*::9*phoA*-Km *ttgD*::*kilABttgH*::ΩSm (solvent sensitive)	[48]
DOT-T1Ecti0	Rif^{r}Km^{r}ctiT1::*9phoA*-Km (solvent sensitive)	[30]
KT2440	mt-2 derivative cured of the TOL plasmid pWW0 (solvent sensitive)	[5]
S12	(solvent tolerant)	[23]
P. taiwanensis VLB120	Orgin of *styA* and *styB* (solvent tolerant)	[33]

Plasmid	Characteristics	References
pCom8	Expression vector for *E. coli* and *Pseudomonas*, pRO1600 and pMB1*ori*, *alk*-regulatory system (*alkS*, P_{alkB}), Gm^r	[57]
pCom10	pCom8, Km^r	[57]
pSPZ10	pBR322 derived, pMB1 *ori*, *alk*-reg. sys. (*alkS*, P_{alkB}), *styA* and *styB*, Km^r	[44]
pSPZ3	pBR322 derived, pMB1 *ori*, *alk*-reg. sys. (*alkS*, P_{alkB}), x*ylM* and *xylA*, Km^r	[43]
pTEZ225	pCom8, *xylM* and *xylA*, Gm^r	This study
pTEZ240	pCom8, *styA* and *styB*, Gm^r	This study
pTEZ220	pCom10, *xylM* and *xylA*, Km^r	This study
pStyAB	pCom10, *styA* and *styB*, Km^r	This study
pStyAB-lac	pCom10 derived, contains *lac*-regulatory system (*lac*I, P_{lacUV5}), *styA* and *styB*, Km^r	This study
pLac_alaD_ω-TA	placI-derived vector (Merck), *lac*I, P_{lacUV5}, carries *alaD* and *cv2025* ω-TA, Cam^r	unpublished

Gm^r = gentamycin resistance, Km^r = kanamycin resistance, Rif^r = rifampin resistance, Tel^r = tellurite resistance, Tet^r = tetracycline resistance, Cam^r = chloramphenicol resistance

or M9* medium [43] adjusted with 10 M NaOH to a pH of 7.4, either with 0.5 % (w/v) glucose or citrate as sole carbon source for *E. coli* or *Pseudomonas*, respectively. Citrate was used to avoid catabolite repression of the P_{alkB} promoter in *Pseudomonas* strains [58]. Thiamine (10 mg L^{-1}), kanamycin (50 µg mL^{-1}), and gentamycin (50 µg mL^{-1}) were added when appropriate. Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) or Carl Roth (Karlsruhe, Germany) in the highest purity available and used without further purifications. Quinoxaline-2-yl-methanol, quinoxaline-2-carbaldehyde, and quinoxaline-2-carboxylic acid were obtained as a gift from Georgios Ionidis (formerly ETH Zurich, Institute of Biotechnology, Zurich, Switzerland).

Molecular biology methods

Electrocompetent *Pseudomonas* cells were prepared according to the method of Choi et al. [14] and plasmids were introduced by electroporation (2500 V, Equibio EasyjecT Prima, Kent, UK). Transformation of *E. coli* was performed as described earlier [50].

DNA manipulation methods and agarose gel electrophoresis were performed as described by Sambrook and Russel [50]. Enzymes (Phusion High-Fidelity Polymerase, T4-ligase, restriction enzymes) and buffers were purchased from Thermo Scientific Molecular Biology (St. Leon-Rot, Germany) and oligonucleotides from Sigma-Aldrich. Plasmids and DNA fragments were isolated using the peqGOLD plasmid Miniprep Kit I from peqLab (Erlangen, Germany) and purified via NucleoSpin Gel and PCR Clean-up from Macherey–Nagel (Düren, Germany) according to supplier protocols.

For the construction of plasmids (pTEZ225, pTEZ220, pTEZ240), the monooxygenases genes *styAB* and *xylMA* were isolated from the plasmids pSPZ10 and pSPZ3, respectively. The *xylMA* genes were excised as an *Asp718*I/*Bam*HI fragment which was inserted into the pCom8 vector digested with the same enzymes yielding plasmid pTEZ225 (10,055 bp). pTEZ220 (10,084 bp) was constructed in analogy to pTEZ225 with pCom10 (Km^r) instead of pCom8 (Gm^r) as backbone. The *styAB* genes were excised as a *Hin*dIII/*Kpn*I fragment which was inserted into pCom8 digested with same enzymes yielding pTEZ240 (9720 bp).

Table 2 Oligonucleotide primers used in this study

Primer	Sequence (5′ → 3′)[a]	Characteristics
F1	CGGCCATATGAAAAAGCGTATCGG	pStyABFw (*Nde*I)
B1	CGGCCATATGTCAATTCAGTGGCAACG	pStyABBw (*Nde*I)
F2	GCGTCTAGAAATTCTCATGTTAGTCATGCC	LacI_LacUV5_Fw_*Xba*I
B2	GCGAGTACTGGTTCCTAGATCCTGTGTGA	LacI_LacUV5_Bw_*Sca*I
F3	GCGAGTACTGGAGAATTCCATATGAAAAAGC	pCOM_AMP_Fw_*Sac*I
B3	GCGTCTAGAAAATAATCGGCATTAAGTGA	pCOM_AMP_Bw_*Xba*I

[a] Restriction sites are underlined

For the construction of pStyAB, the *styAB* genes were amplified via PCR from pTEZ240 using primers F1 and B1 (Table 2). The 1832 bp PCR product was purified via agarose gel electrophoresis, digested with *Nde*I, and ligated into pCom10.

The plasmid pStyAB-lac was constructed via PCR using the primers F3 and B3 (Table 2) and the template pStyAB. The resulting 6418 bp fragment, containing the *styAB* genes and the plasmid backbone, was purified via agarose gel electrophoresis, digested with *Xba*I and *Sac*I, and ligated with purified and identically digested PCR fragments of pLac_alaD_ω-TA generated with F2 and B2 as primers (1655 bp) (Table 2) to insert the *lac*-regulatory system.

Growth conditions and clonal heterogeneity experiments

Cultivations were carried out in screw-capped baffled Erlenmeyer flasks at 30 °C and 200 rpm in a Multitron shaker (Infors, Bottmingen, Switzerland) and inoculated with single colonies from LB agar plates, which had been incubated for 12–16 h. To determine variabilities, 8 clones (colonies) were picked, grown for 6–8 h in liquid LB precultures, and transferred as a 1 % (v/v) inoculum into an overnight M9* preculture, by which the main M9* culture was inoculated to a starting cell concentration of 0.02–0.03 g cell dry weight (CDW) per liter. Recombinant gene expression was induced after 3.5 h of cultivation with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) or 0.025 % (v/v) (0.22 mM) dicyclopropyl ketone (DCPK) and 0.1 % (v/v) (0.61 mM) *n*-octane for P_{lac}- or P_{alkB}-based expression vectors, respectively. After induction, incubation was continued for 4 h, before cells were harvested by centrifugation and resuspended to 0.5 g_{CDW} L^{-1} in 50 mM KPi buffer (pH 7.4) supplemented with 0.5 % (w/v) citrate or glucose.

To perform activity assays, cell suspension aliquots of 1 mL were distributed into 10-mL Teflon-sealed Pyrex tubes and incubated for 5 min at 30 °C in a rotary shaker (Aquatron, Infors) at 300 rpm. After this equilibration time, the biotransformation was started by the addition of the substrate to a concentration of 1.5 mM for styrene and 8 mM for 2-methylquinoxaline. Incubation was continued for 10 min (or 5 min when 1 mM toluene was used as substrate). The styrene epoxidation assays were stopped by addition of 1 mL ice-cold diethyl ether containing 0.2 mM *n*-decane as internal standard. After 1 min extraction by mixing and phase separation via centrifugation, the organic phase was transferred into a GC vial for analysis (see below). Reactions performed with 2-methylquinoxaline or toluene as substrates were stopped by acidification with 0.8 % (v/v) perchloric acid or by the addition of 0.5 volumes of acetonitrile. After centrifugation, the cell-free supernatant was analyzed via reversed-phase high-performance liquid chromatography (HPLC, see below).

Analytical procedures

Biomass concentrations were monitored by measuring the optical density at a wavelength of 450 nm (OD_{450}) using a Libra S11 spectrophotometer (Biochrom, Cambridge, UK). Gravimetrical determination of the CDW:OD_{450} correlation factor was done as described [50]. One OD_{450} unit corresponds to a biomass concentration of 0.186 g_{CDW} L^{-1} for *P. taiwanensis* VLB120 [21], 0.166 g_{CDW} L^{-1} for *E. coli* JM101, 0.211 g_{CDW} L^{-1} for *P. putida* DOT-T1E and S12, and 0.235 g_{CDW} L^{-1} for *P. putida* KT2440.

Styrene, (*S*)-styrene oxide, and 2-phenylethanol quantification were performed by GC analysis as described before [35]. Analysis of 2-methylquinoxaline samples was performed with a LaChrom Elite HPLC system (Hitachi High Technologies America, Pleasanton, CA) equipped with a Hypersil ODS C18 125-4.0 (5 µm) column from Agilent Technologies (Palo Alto, CA). The mobile phase consisted of acetonitrile and water containing 0.1 % (v/v) perchloric acid operating a gradient as follows: 3 min isocratic with 5 % acetonitrile followed by an increase to 25 % of acetonitrile over 7 min and up to 100 % acetonitrile within 6 min. The flow rate was set to 1 mL min^{-1} and the temperature to 25 °C. Detection occurred at a wavelength of 236 nm. Toluene samples were separated with an HPLC instrument from Merck Hitachi (interface D-7000, UV-detector

L-7400, pump L-7100, autosampler L-7200) equipped with a Nucleosil C18 column (pore size, 100 Å; particle size, 5 µm; inner diameter, 4 mm; length, 2 cm) from Macherey–Nagel (Oensingen, Switzerland). The mobile phase consisted of acetonitrile and H_2O containing 0.1 % (v/v) perchloric acid. The elution profile was 35–43 % acetonitrile over 5 min, followed by 43–80 % over 5 min and then 80 % acetonitrile isocratic for 2 min. The flow rate was set to 1.0 mL min^{-1} and the detection occurred at 210 nm.

Protein separation for StyA detection was performed as described earlier [50], via sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Results

Solvent-tolerant *P. putida* shows a high variability in recombinant XMO activity

To investigate clonal heterogeneity of oxygenase catalysis in recombinant *Pseudomonas*, XMO-based specific whole-cell activities and their variability were tested in *Pseudomonas* strains with different phenotypes, especially regarding solvent tolerance, and compared among each other and with *E. coli* JM101. The *Pseudomonas* strains tested included the solvent-tolerant *P. putida* strains DOT-T1E and S12 and solvent-sensitive *P. putida* KT2440, which, as *E. coli*, is highly susceptible toward low $\log P_{O/W}$ solvents.

For each strain, eight different colonies of freshly transformed cells were picked separately and cultivated in parallel, induced, and tested for the hydroxylation of 2-methylquinoxaline to quinoxaline-2-yl-methanol as described in "Materials and methods". Relative activity variations from clone to clone (i.e., colony to colony picked) are represented by the coefficient of variation c_v (Table 3, Fig. S1), the ratio of the standard deviation among the activities for the eight clones to the mean activity. The experimental error σ_{exp} of a reproducibility study of one recombinant strain is calculated via the standard deviation of all measured activities (each clone analyzed in duplicates) normalizing the mean activities of each clone to 100 %. Based on the assumption that, for a homogenous population, the experimental error and the biological variation should not exceed 10 % each, strains were classified to exhibit low variabilities, if the overall variation does not exceed 20 %. To ensure the overall reproducibility of the cultivation and assay strategy, the experimental setup was standardized in a way that each clone was treated equally and separately from the beginning on (inoculation of LB agar plate), which resulted in similar numbers (45–50) of cell divisions at the start of the resting cell activity assay.

To avoid insufficient supply of the inducer, induction was routinely carried out with 0.025 % (v/v) DCPK. This concentration is sufficient for full induction [10], although it provoked a slight reduction in growth rates for all tested *Pseudomonas* strains (data not shown). The most significant variation was observed with *P. putida* DOT-T1E and *P. putida* S12 showing an up to 13-fold higher variation compared with *E. coli* JM101 and solvent-sensitive *P. putida* KT2440 (Table 3, Fig. S1). The lowest activity variation was found for *E. coli* JM101 (pSPZ3) with a coefficient of variation of 6 % being in the same range as the experimental error. The finding that the two solvent-tolerant strains showed high variability, whereas solvent-sensitive *P. putida* KT2440 and *E. coli* JM101 showed low variability suggests that high variability may be inherent to solvent-tolerant strains. The high activity variations of the two recombinant *P. putida* DOT-T1E strains indicate that the heterogeneity is not related to the type of selection marker used; the plasmids used only differed with respect to the antibiotic resistance (kanamycin for pTEZ220 and gentamycin for pTEZ225).

As control experiment for activity assay validation, the oxidation of the substrate toluene (Table 3, Fig. S1) was investigated with the same eight transformants of *E. coli* JM101 (pSPZ3), *E. coli* JM101 (pTEZ225), and *P. putida* DOT-T1E (pTEZ225) used for the conversion of 2-methylquinoxaline. A similar variability was found for DOT-T1E recombinants, whereas reproducible activities of *E. coli* JM101 (pSPZ3) and *E. coli* JM101 (pTEZ225) were in accordance with reported activities [10].

Average specific 2-methylquinoxaline and toluene hydroxylation rates were higher for *E. coli* JM101 (pTEZ225) (53.2 ± 10.5 U g_{CDW}^{-1}/121.4 ± 26.5 U g_{CDW}^{-1}) than for *E. coli* JM101 (pSPZ3) (39.5 ± 4.0 U g_{CDW}^{-1}/103.3 ± 13.5 U g_{CDW}^{-1}). This might be due to the higher plasmid copy number (PCN) for pTEZ225 compared with pSPZ3 in *E. coli*. In contrast to plasmid pSPZ3, plasmid pTEZ225 lacks the *rop* gene, whose product is involved in the repression of plasmid DNA replication. Furthermore, pTEZ225 harbors two origins of replication, pRO1600 and pMB1, whereas only the pMB1 origin is present on pSPZ3.

To exclude the transformation procedure as cause for variability of whole-cell activities, a single colony of a *P. putida* DOT-T1E (pTEZ225) transformant was spread on solid LB medium and 8 descending colonies were tested for XMO-catalyzed 2-methylquinoxaline hydroxylation in independent whole-cell activity assays. The tested clones originating from the same transformant cell again showed a high variation in activity. These results show that, during cultivation on agar plates (25–30 generations) and/or in liquid cultures (~20 generations), genetic

Table 3 Summary of catalytic performance in repeated whole-cell activity assays for all tested biocatalyst

Biocatalyst	Solvent tolerance	Specific activity			Coeff. of variation c_v (%)	Exp. error (%)	n (–)	Antibiotic resistance
		Min (U g_{CDW}^{-1})	Max (U g_{CDW}^{-1})	Average (U g_{CDW}^{-1})				
Xylene monooxygenase								
P. putida DOT-T1E pTEZ225	+	0.4 ± 0.0	45.1 ± 1.7	23.1 ± 18.5	80	7.2	8	Gm^r
P. putida DOT-T1E pTEZ225[a]	+	1.9 ± 0.3	122.9 ± 16.3	63.4 ± 48.2	76	12.0	8	Gm^r
P. putida DOT-T1E pTEZ220	+	2.4 ± 0.0	32.8 ± 1.8	17.4 ± 10.0	58	6.1	8	Km^r
P. putida S12 pTEZ225	+	3.9 ± 0.1	22.5 ± 0.5	12.2 ± 7.3	58	2.6	8	Gm^r
P. putida KT2440 pTEZ225	–	27.9 ± 4.4	45.6 ± 2.4	35.3 ± 5.9	17	7.1	8	Gm^r
E. coli JM101 pTEZ225	–	38.4 ± 1.0	70.5 ± 0.7	53.2 ± 10.5	20	5.2	8	Gm^r
E. coli JM101 pTEZ225[a]	–	94.0 ± 21.6	157.5 ± 6.9	121.4 ± 26.5	22	8.9	8	Gm^r
E. coli JM101 pSPZ3	–	35.7 ± 1.1	41.9 ± 1.5	39.5 ± 4.0	6	4.9	8	Km^r
E. coli JM101 pSPZ3[a]	–	86.3 ± 7.1	127.9 ± 0.4	103.3 ± 13.5	13	12.1	8	Km^r
P. putida DOT-T1E pTEZ225[b]	+	5.6 ± 0.1	22.0 ± 0.6	17.2 ± 6.6	38	2.0	8	Gm^r
Styrene monooxygenase								
P. putida DOT-T1E pTEZ240	+	3.9 ± 0.8	80.2 ± 6.5	52.7 ± 23.3	43	9.0	8	Gm^r
P. putida DOT-T1Ecti0 pTEZ240	–	10.6 ± 0.9	47.5 ± 3.8	25.7 ± 12.3	48	11.5	8	Gm^r
P. putida DOT-T1E-PS34 pTEZ240	–	2.9 ± 0.1	80.0 ± 1.7	37.2 ± 30.4	82	7.7	7	Gm^r
P. putida KT2440 pTEZ240	–	6.7 ± 0.4	13.0 ± 2.0	8.8 ± 2.0	23	15.3	8	Gm^r
E. coli JM101 pStyAB	–	57.5 ± 1.3	71.2 ± 5.5	61.2 ± 4.4	7	5.3	8	Km^r
P. putida DOT-T1E pStyAB	+	0.8 ± 0.7	68.5 ± 6.9	27.2 ± 20.5	75	9.1	32	Km^r
P. putida KT2440 pStyAB	–	14.8 ± 1.2	22.4 ± 1.6	18.6 ± 2.1	12	4.8	16	Km^r
P. taiwanensis VLB120 pStyAB	+	30.4 ± 2.2	54.5 ± 4.8	38.9 ± 7.7	20	7.5	16	Km^r
P. putida DOT-T1E pTEZ240[c]	+	29.8 ± 1.4	104.3 ± 1.8	57.2 ± 28.9	51	7.7	8	Gm^r
E. coli JM101 pTEZ240[d]	–	49.5 ± 2.9	73.8 ± 1.0	61.0 ± 9.2	15	2.8	7	Gm^r
P. putida DOT-T1E pStyAB-lac	+	87.0 ± 10.1	146.2 ± 20.2	107.6 ± 14.8	14	8.5	16	Km^r
P. putida DOT-T1E pStyAB-lac + DCPK	+	106.7 ± 28.5	150.4 ± 8.5	130.7 ± 13.3	10	7.7	8	Km^r
P. putida KT2440 pStyAB-lac	–	21.2 ± 0.1	28.7 ± 0.2	24.0 ± 2.9	12	7.1	8	Km^r

n: number of colonies picked and tested for variability in specific activity after cultivation in LB and M9* liquid media including induction

[a] Results based on activity assays using toluene instead of 2-methylquinoxaline as substrate

[b] Results based on one mother colony which was spread to 8 descending colonies

[c] Induction agent is *n*-octane instead of DCPK

[d] Results based on *E. coli* JM101 colonies harboring pTEZ240 plasmids which were previously extracted from different induced cultures of *P. putida* DOT-T1E-PS34 showing high variability

or physiological alterations must have taken place, which influenced XMO overproduction or activity of the recombinants (Table 3).

Variations in activity appear independently of the enzyme system used and of specific solvent tolerance mechanisms

The variability of whole-cell activities using the membrane-bound XMO opened the question, if this behavior is enzyme specific or connected to its membrane-bound nature. Thus, the SMO from *P. taiwanensis* VLB120 was tested as an alternative monooxygenase, which is soluble and hence physiologically different from membrane-bound XMO. The variability of SMO-catalyzed styrene epoxidation was investigated in different *Pseudomonas* strains and in *E. coli* JM101 by means of whole-cell activity assays (Table 3, Fig. S2). *P. putida* S12 was not tested as host strain for SMO catalysis, as this strain harbors a SMO and is able to degrade and grow on styrene [23].

Again, high specific activity variations occurred in *P. putida* DOT-T1E whereas solvent-sensitive *P. putida* KT2440 showed a lower variability. Thus, the type of enzyme, substrate, and/or product can be excluded as factors contributing to the observed variability. The

lower coefficient of variation obtained with *P. putida* KT2440 compared with *P. putida* DOT-T1E supported the possibility of a correlation between solvent tolerance of the host strain and the variability of recombinant monooxygenase activity, e.g., via altering the intracellular inducer or substrate concentrations or via competition for energy.

Thus, solvent-sensitive deletion mutants of *P. putida* DOT-T1E were tested as hosts for the SMO-catalyzed styrene epoxidation. The variabilities in SMO activity of both *P. putida* DOT-TlEcti0 (pTEZ240), which lacks the *cis–trans* isomerase, and *P. putida* DOT-T1E-PS34 (pTEZ240), which is deficient in all three major solvent efflux pumps, were at least as high as the variability observed with the parental strain. The most prominent solvent tolerance mechanisms apparently did not interact with recombinant oxygenase catalysis. This, however, does not exclude a connectivity with solvent tolerance regulation and respective population heterogeneity, which might result from the addition of the inducer DCPK ($\log P_{O/W} = 0.58$) being an organic solvent itself.

To exclude effects related to the inducer used for recombinant gene expression, DCPK was replaced by less toxic *n*-octane ($\log P_{O/W} = 5.15$) for the induction of SMO synthesis in *P. putida* DOT-T1E (pTEZ240). *P. putida* DOT-TIE is unable to grow on and metabolize *n*-octane, whereas the reduction of DCPK by host-specific dehydrogenases cannot be completely excluded. The nearly identical catalytic performance in both cases (DCPK: 52.7 U g_{CDW}^{-1}, $c_v = 43$ %; *n*-octane: 57.2 U g_{CDW}^{-1}, $c_v = 51$ %) indicates that the variability in SMO activity is independent of the agent used for induction (Table 3, Fig. S2).

Variability of SMO activity in recombinant *Pseudomonas* correlates with SMO expression levels and is strain specific

For a more detailed statistical and mechanistic characterization of the observed variability of in vivo SMO activities, expression analyses were performed and a higher number of colonies was tested. To further elucidate whether this high variability is a common feature of solvent-tolerant *Pseudomonas* strains, solvent-tolerant *P. taiwanensis* VLB120 was included as host strain in these studies. Since *P. taiwanensis* VLB120 is the source of the SMO used in this study, possible interferences of the plasmid-based recombinant gene expression with the native styrene degradation operon were investigated. In a control experiment, *P. taiwanensis* VLB120 incubated with 0.025 % (v/v) DCPK did not show any styrene conversion (SMO activity) in resting cell activity assays (data not shown). Thus, interferences originating from the native *sty* operon can be excluded for the conditions applied.

SMO overproduction in different strains using plasmid pStyAB (differing from pTEZ240 only in its kanamycin instead of gentamycin resistance marker) revealed that specific SMO activities correlate with intracellular levels of the StyA component of SMO as visualized via SDS-PAGE analysis (Fig. 2). StyB was not visible on the gel, due to its low abundance and intrinsic stability, as it has been reported for the wild-type strain and recombinant *E. coli* [29, 41]. Specific styrene epoxidation activities were comparable to those obtained with plasmid pTEZ240, again indicating that the change of the selection marker does not remarkably influence whole-cell activities.

Significant variations in activity and StyA contents from clone to clone only occurred with *P. putida* DOT-T1E (pStyAB) indicating that host-specific differences in gene expression, plasmid proliferation, or enzyme stability, rather than in cofactor or substrate availability, caused the difference in activity reproducibility. The fact that solvent-tolerant *P. taiwanensis* VLB120 (pStyAB) showed a low variability in activity contradicts the suggested correlation of variability and solvent tolerance.

A detailed characterization of the variability in *P. putida* DOT-T1E (pStyAB) on the basis of 32 independently assayed clones revealed an apparent random distribution of activities between 0.8 and 68.5 U g_{CDW}^{-1} (Fig. 3). This heterogeneous behavior in SMO activity of independent cultures is a result of strong variations in the intracellular SMO protein concentrations with a calculated coefficient of variation of 75 %. A statistical test according to Shapiro and Wilk [53] was employed to evaluate, whether the activities determined for *P. putida* DOT-T1E (pStyAB) and *P. putida* KT2440 (pStyAB) are normally distributed. The calculated *p* value of 0.758 for recombinant *P. putida* KT2440 was well above the significance level of 0.05 which indicates a normal Gaussian distribution of respective activities (Fig. 3). In contrast, the *p* value of 0.056 obtained for *P. putida* DOT-T1E (pStyAB) provides only poor evidence for a normal distribution, but, considering the high activity range between 0 and 68.5 U g_{CDW}^{-1}, suggests a random distribution of measured activities.

The variations in specific whole-cell activities apparently were related to variable oxygenase expression levels, which may be due to mutations on the plasmid sequence or unstable plasmid segregation. To investigate these possibilities, plasmids were isolated from different cultures of induced *P. putida* DOT-T1E-PS34 (pTEZ240) showing highly variable activities and used to transform *E. coli* JM101 cells. The constant size and recovery of the plasmid isolated (results not shown) and the regained reproducibility of activity when introduced in *E. coli* JM101 as host strain (Table 3) indicated that plasmid-related alterations did not occur during cultivation and induction and, hence, were not the cause for the observed variabilities.

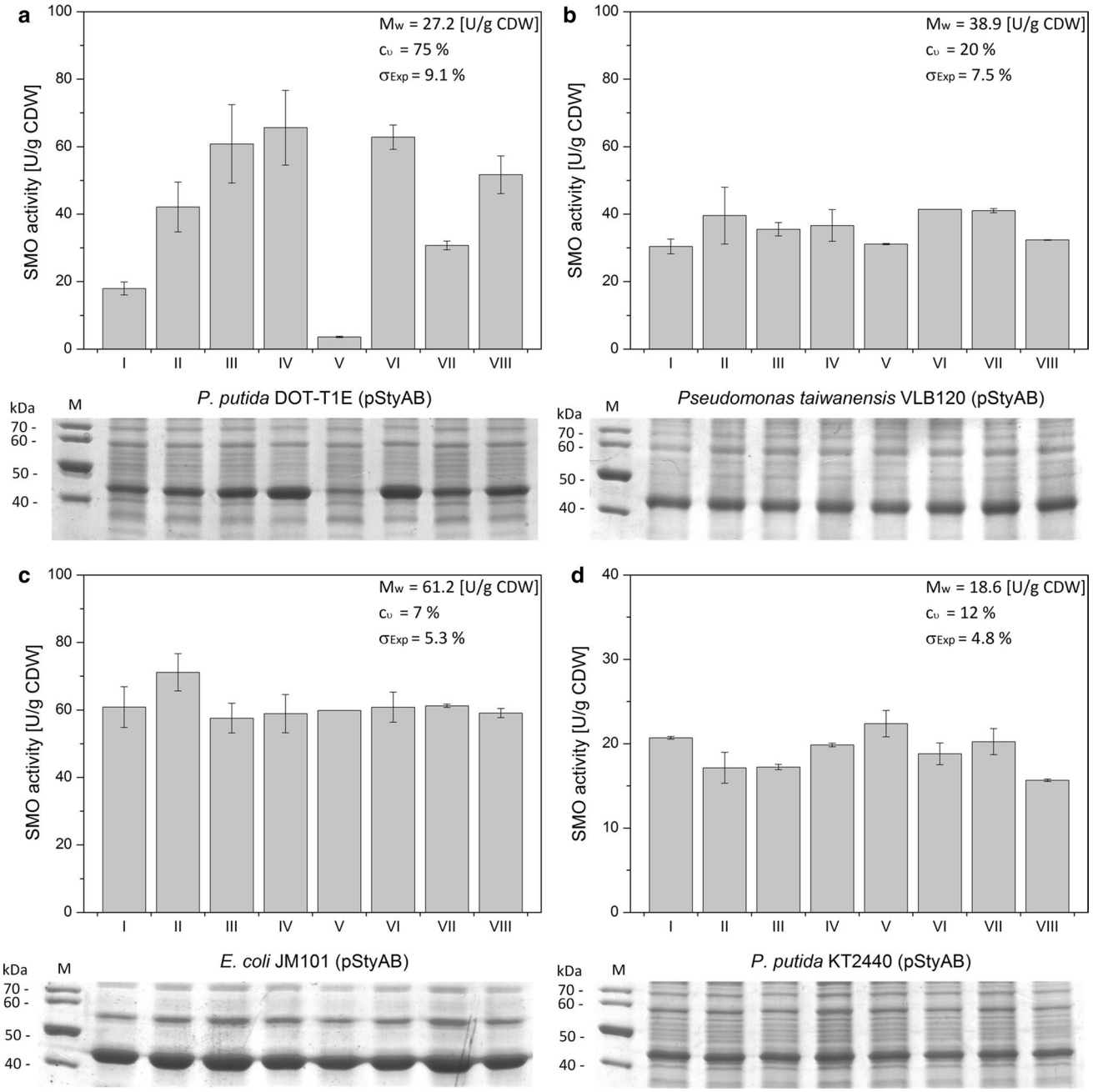

Fig. 2 Variability in SMO activity and expression levels using recombinant strains harboring the plasmid pStyAB. Specific styrene epoxidation activities were determined with resting cells of solvent-tolerant *P. putida* DOT-T1E (**a**) and *P. taiwanensis* VLB120 (**b**) and of solvent-sensitive *E. coli* JM101 (**c**) and *P. putida* KT2440 (**d**). Exemplary results of resting cell activities are displayed for 8 independent clones 4 h after induction with 0.025 % (v/v) DCPK. SDS-PAGE analysis of each induced clone used for activity assays (**a**–**d**) revealed the corresponding StyA content (at 46.4 kDa)

Exchange of the *alk*-regulatory system with the *lac*-regulatory system results in a decreased activity variation

To investigate if the *styAB* expression variability in *P. putida* DOT-T1E is due to an inhomogeneous transcriptional regulation, and thus, besides the strain, depends on the type of regulatory system employed, the *alk*-regulatory system was substituted by the *lac*-regulatory system for *styAB* expression. In contrast to the *alk*-regulatory system, which is subject to catabolite repression by glucose but not by organic acids such as citrate and succinate [49], the *lac*-regulatory system, due to its orthologous nature, is not subject to catabolite repression in *Pseudomonas*

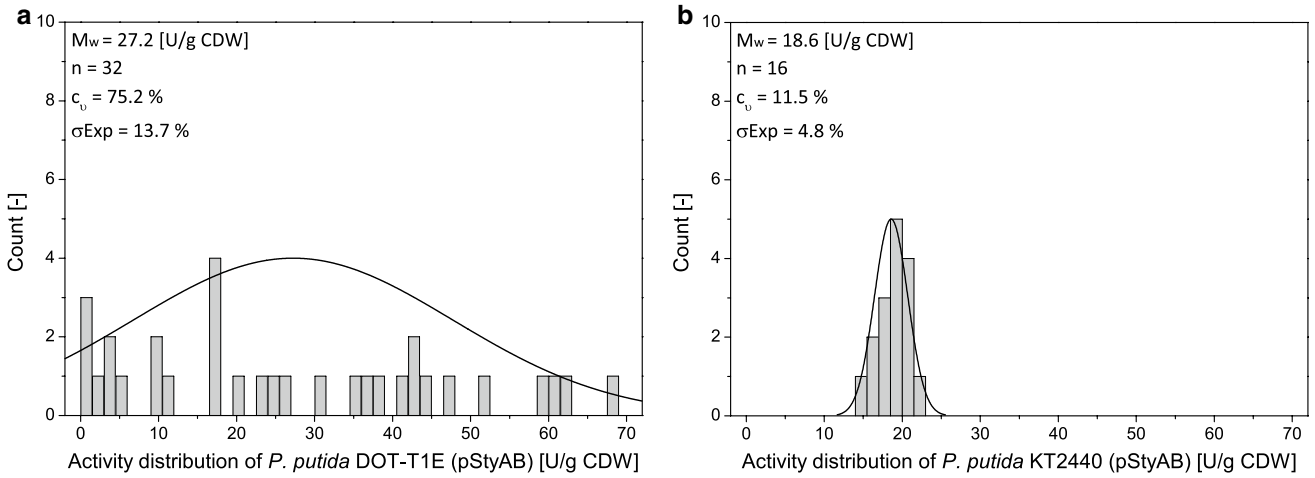

Fig. 3 Distribution of specific activities measured for recombinant *P. putida* DOT-T1E (panel **a**) and *P. putida* KT2440 (panel **b**) harboring the pStyAB plasmid. Specific styrene epoxidation activities were determined for 32 and 16 colonies, respectively. *Lines* represent the calculated Gaussian distribution of the measured specific activities

at all. The non-degradable, non-volatile, and non-toxic sugar derivative IPTG served as induction agent for the pStyAB-lac plasmid in which the *lac* operon is regulated via the LacI repressor and the P_{lacUV5} promotor. Upon induction, the LacI repressor dissociates from the operator region once IPTG has bound resulting in expression of the *styAB* genes (negative regulation). This is another difference to the *alk*-regulatory system (positive regulation) where, in the presence of the induction agent, the transcriptional regulator AlkS activates gene expression via the P_{alkB} promotor as well as its own formation. As origin of replication, ribosomal binding site, and transcriptional terminators were kept identical, emerging differences in gene expression can be attributed to the exchange of the regulatory system.

In accordance with the results obtained with the *alk*-regulatory system, epoxidation activities of *P. putida* KT2440 (pStyAB-lac) did not show heterogeneous behavior, but increased by ~30 % (Fig. 4). For *P. putida* DOT-T1E (pStyAB-lac), in sharp contrast to *P. putida* DOT-T1E (pStyAB), no significant activity variation occurred in reproducibility assays. Instead, a high reproducible average activity of 107 U g_{CDW}^{-1} was obtained, with a coefficient of variation of 14 %. These results were supported by SDS-PAGE analysis verifying that the low variation is based on homogenous intracellular SMO levels. As a control experiment to exclude an influence of the inducer DCPK as trigger for variability, 0.025 % (v/v) DCPK was added during induction of *styAB* expression in *P. putida* DOT-T1E (pStyAB-lac). Interestingly, even higher activities of 130.8 ± 13.1 U g_{CDW}^{-1} were obtained with a good reproducibility. The non-variable recombinant oxygenase activity and gene expression in *P. putida* DOT-T1E, when the plasmid with the *lac*-system was used, indicates that the variability emerging with the *alk*-based regulatory system is specific to this regulatory system, which behaves differently depending on the *Pseudomonas* host strain.

Discussion

Energy-dependent production of chemicals and proteins in recombinant bacteria is coupled to cell viability and often hampered by cellular stress responses. The negative influence of plasmid-based gene expression on growth efficiency and cell viability is well documented and is typically caused by utilization of cellular resources for plasmid replication [7], antibiotic resistance mechanisms [3], or transcription and translation for recombinant protein production [13]. In addition, inducer, enzyme, substrate, or product toxicity may on the one hand activate energy-dependent cell protection mechanisms, and on the other hand directly impair the cell metabolism and viability [25]. Based on differential regulatory predispositions of individual cells, leading to different phenotypes in cultures descending from respective clones (discussed below as extrinsic noise), all these effects may trigger or uncover cellular heterogeneity. The results presented here show that the clonal heterogeneity of oxygenase activity in recombinant *Pseudomonas* using the pCom-based system, on which recombinant gene expression is regulated via the AlkS/P_{alkB} system, was not related to plasmid replication, mutation, antibiotic resistance or solvent tolerance mechanisms, or type of enzyme, substrate/product, or inducer used. Clonal heterogeneity was, however, related to the

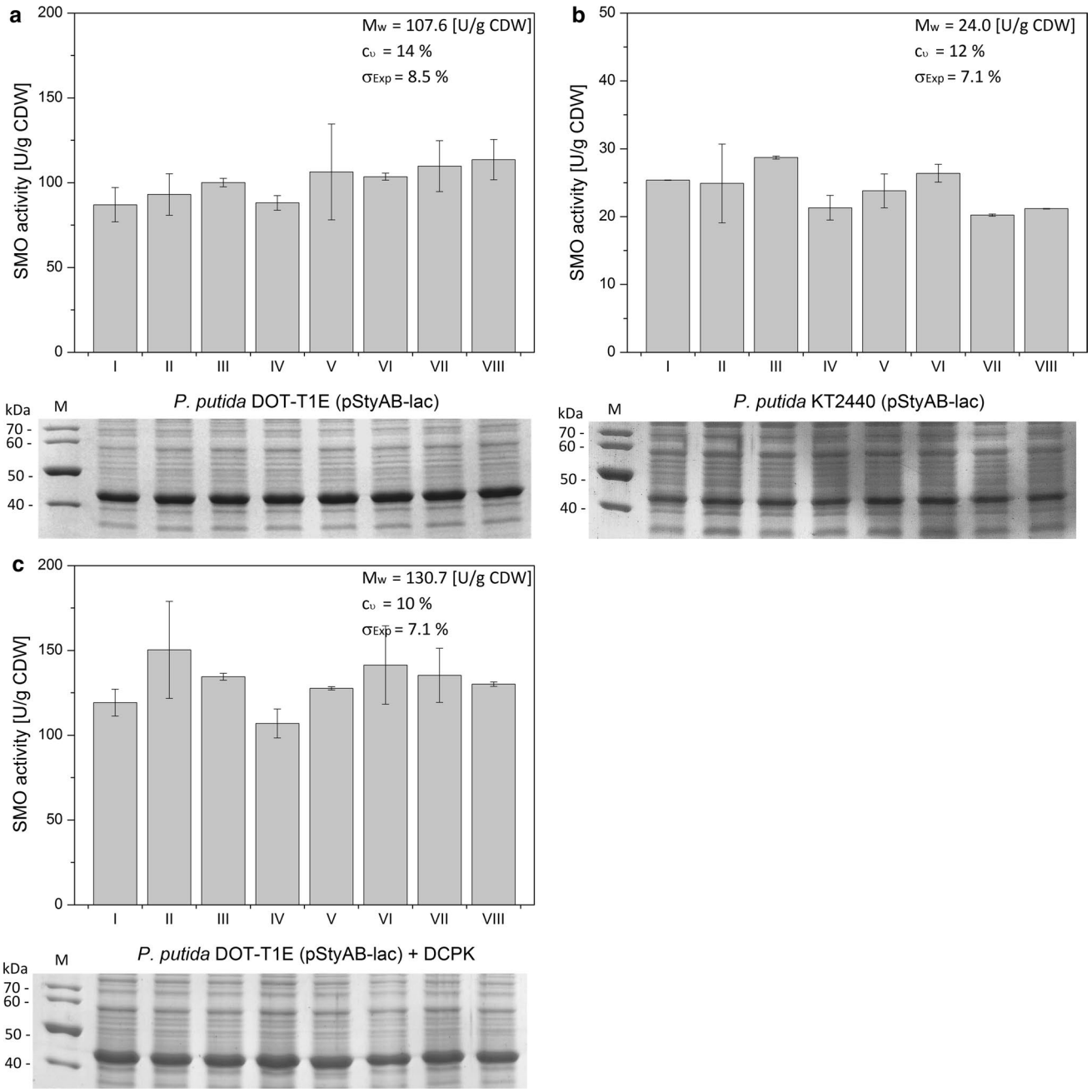

Fig. 4 Variability in SMO activity and expression levels in *P. putida* DOT-T1E (**a** + **c**) and *P. putida* KT2440 (**b**) harboring the plasmid pStyAB-lac. Specific styrene epoxidation activities were determined with resting cells originating from 8 different clones 4 h after induction with 1 mM IPTG and addition of 0.025 % (v/v) DCPK in a control experiment (**c**). SDS-PAGE analysis of each induced clone used for activity assays revealed the corresponding StyA content (at 46.4 kDa)

combination of strain and expression system used, which determines the recombinant gene expression efficiency via the susceptibility of regulation to extrinsic noise (the interaction of the regulatory network of the host strain and the regulatory system on the expression vector) or via intrinsic noise related to the regulatory system itself as discussed in the following.

Noise in gene expression based on the *alk*-regulatory system

Cell-to-cell variability in recombinant gene expression can be initiated by two different types of noise, intrinsic and extrinsic noise (Fig. 5) [18]. Extrinsic noise is based on cell-to-cell differences in regulatory network

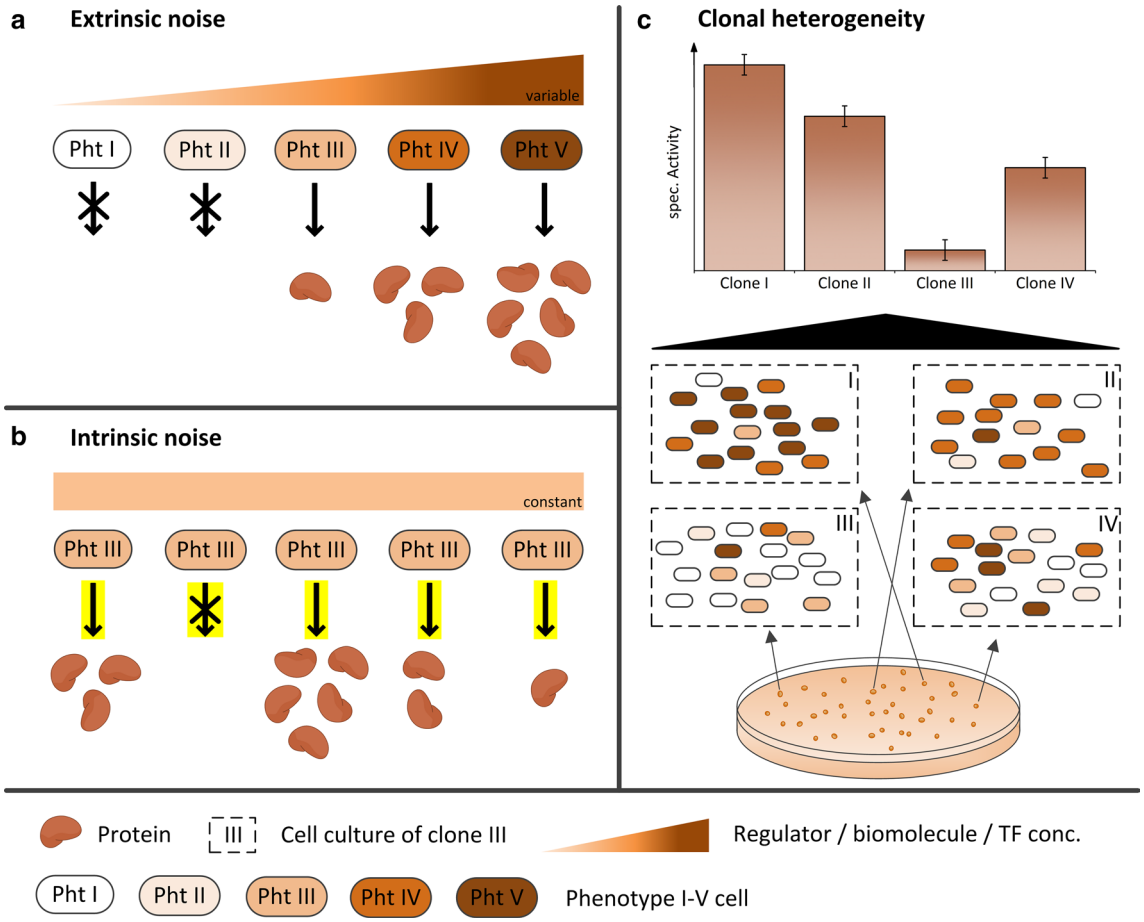

Fig. 5 Clonal heterogeneity in *Pseudomonas* and sources of noise during recombinant gene expression. **a** Extrinsic noise in isogenic cells is caused by fluctuations in concentrations of biomolecules [ribosomes, transcription factors (TF), DNA/RNA polymerases] involved in the basic regulatory machinery controlling gene expression. Differences in such concentrations in individual cells can be considered as differing predispositions and may, under certain conditions, result in phenotypes with differing gene expression patterns. This may include threshold concentrations, e.g., of TFs, below which the expression of certain genes is not induced at all. In the extreme case, this may result in a digital behavior (expression or no expression) with respect to the production of a recombinant protein. **b** Intrinsic noise is defined as the noise occurring, when the concentrations of the above-mentioned biomolecules are constant, i.e., do not differ in isogenic cells. Thus, intrinsic noise only depends on the specific expression system considered and not on the predisposition of the regulatory network. Such noise occurs when the rate of gene expression is controlled by random microscopic events (*yellow boxes*), i.e., coincidental contacts/collisions between the respective partners (inducer, promotor, regulator) present at low concentrations. The resulting output also differs from cell to cell, but is established and changes upon random events following a probabilistic pattern. Both, intrinsic and extrinsic noise act in parallel and are origins of cell-to-cell heterogeneity. **c** Possible scheme of cell-to-cell variability in clonal heterogeneity assays with *P. putida* DOT-T1E cells. The predisposition of specific regulatory molecules in one single clone defines the efficiency of recombinant gene expression and finally the specific activity of a descending culture. Overall, activity variations from clone to clone (culture to culture) are driven by the extent to which such extrinsic noise occurs (color figure online)

operation. Thereby, random fluctuations of non-discrete signals occur, i.e., uneven contents of biomolecules made by fluctuations during discrete gene expression and cell division events. These variations in defined biomolecule or regulator concentrations (ribosomes, RNA polymerases, DNA polymerases, transcription factors) are either too small, not having an effect on regulatory network operation, or are sensed by the regulatory network resulting in a cellular response such as the expression of specific genes [18, 45]. In contrast, intrinsic noise (Fig. 5) is inherent to the operation of a discrete regulatory system (e.g., the *alk*- or the *lac*-regulatory systems) and also occurs in cells, which are identical not only regarding their genetic inventory but also regarding the concentrations and states of their cellular components. In this case, the reaction rate, e.g., for recombinant gene expression, is driven by the frequency of the collision of the individual partners (regulator, promoter, inducer) involved [18, 56]. Thereby, variability emerges due to low concentrations of these partners leading to a high stochasticity of respective

interactions. As stated by Elowitz et al. [18], the accuracy of the respective gene regulation is limited by intrinsic noise, even if host-intrinsic regulatory protein concentrations do not vary. For the *alk*-regulatory system on the pCom plasmid, AlkS may be the major source of intrinsic noise as it positively regulates its own formation on the transcriptional level [12], and may be present at very low concentrations in non-induced cells. Such intrinsic noise leads to phenotypic cell-to-cell variability within a culture and is subject to short-term changes based on events occurring randomly. As it is not dependent on regulatory network predispositions, it does, from a probabilistic perspective, not become apparent looking at the average expression level in a whole culture and is not reflected in differences among cultures originating from different clones. Thus, the clonal variability observed in this study upon induction of cultures cannot be explained by intrinsic noise. On the other hand, extrinsic noise may contribute via unequal partitioning during cell division or variable predispositions of regulatory networks, e.g., variable predispositions for solvent stress, antibiotic resistance, or plasmid proliferation. For example, the abundance of a regulator enhancing gene expression under control of the *alk*-regulatory system might be bequeathed from the source clone to daughter cells. If, after transformation, regulator concentrations differ from clone to clone, the following generations would inherit a more or less efficient recombinant protein production (Fig. 5). A similar mechanism based on individual predispositions as cause for cell-to-cell variability in one culture was described by Siegele and Hu [54]. Variations in gene expression occurred due to an 'autocatalytic induction mechanism' based on the *araBAD*-regulatory system in *E. coli*. Thereby, the regulator protein AraC activates gene expression of the host-specific transporter systems for arabinose uptake upon binding of arabinose and stimulates induction of the P_{araBAD} promotor. Based on their predisposition, only those cells initiated gene expression upon induction by arabinose, in which the basic Ara transporter level exceeded a certain threshold. In this study, however, the uptake of the induction agents, *n*-octane and DCPK, is not coupled to an active transport system, but occurs via passive diffusion across the cell membranes. Thus, the intracellular abundance of the inducer is assured independently of the genetic background of the strains used and of predispositions regarding inducer uptake.

In this study, we clearly show that variability in recombinant gene expression in *P. putida* DOT-T1E and S12 is connected to the *alk*-regulatory system, which originates from another *Pseudomonas* strain, *P. putida* GPo12. The application of a regulation system not originating from *Pseudomonas*, the *lac*-regulation system, did not result in significant clonal heterogeneity for all *Pseudomonas* strains tested. With a variability in activity of only 14 %, *P. putida* DOT-T1E (pStyAB-lac) showed stable and reproducible SMO activities as high as 107.62 ± 14.8 U g_{CDW}^{-1}, which is the highest specific styrene oxide formation rate reported for *P. putida* DOT-T1E so far. These results indicate that stochastic predispositions of the regulatory network, owing to extrinsic noise in individual cultures of *P. putida* DOT-T1E(pStyAB) and S12 (pStyAB) leads to the high variability in gene expression. This can be attributed to the interaction of the regulatory network of the employed strain with the *alk*-regulatory system or with plasmid replication. The latter, however, would require an additional interference of plasmid replication with the *alk*-regulatory system, as the backbone (replication regulation) of the pStyAB-lac plasmid (showing low variability) is identical to pStyAB.

In contrast, solvent-tolerant *P. taiwanensis* VLB120 [33] did not show a high variability in *styAB* gene expression regulated via the *alk*-system. Although this strain has the tendency to develop two different morphotypes when growing in a biofilm, no differences in activity of these two types have been observed [20]. The apparent consistency of expression and activities indicates that possible stochastic variabilities occurring in the regulatory network of this strain do not affect AlkS/P_{alkB}-mediated expression. These observations point out the fact that microbial heterogeneity must be analyzed on a case-by-case basis and therefore should be incorporated in the initial screening phases for the selection of the best microbial host as proposed by Delvigne and Goffin [15]. However, such heterogeneity obviously does not only depend on the host strain, but on the combination of the host strain and the regulatory system used.

Possible implications of solvent tolerance regarding variability in gene expression

Variations obtained with the *alk*-regulatory system for recombinant gene expression and respective whole-cell oxygenase activities were significantly lower for solvent-sensitive *P. putida* KT2440 and *E. coli* JM101 as well as for solvent-tolerant *P. taiwanensis* VLB120 than for solvent-tolerant *P. putida* DOT-T1E and *P. putida* S12. The high activity variations (c_v = 82 and 48 %, respectively) obtained with solvent efflux pump and *cis–trans* isomerase knockout strains of *P. putida* DOT-T1E heterologously expressing the XMO genes under control of the *alk*-regulatory system indicate that respective tolerance mechanisms do not directly cause the heterogeneous behavior, e.g., via variable predispositions for the pumping of inducers, substrates, and products out of the cells. In addition, due to its low hydrophobicity ($\log P_{O/W}$ of 0.58), DCPK does not vigorously accumulate in the membrane and may not cause solvent adaptation as it has been predicted for 1-hexanol

and 1-butanol [25, 39]. This and the finding that induction of recombinant gene expression via DCPK and *n*-octane—solvents with differing polarities—both resulted in high activity variations further confirm that the major solvent tolerance mechanisms are not directly involved in the observed variability in gene expression.

Nevertheless, the addition of a solvent like DCPK does influence cell physiology and, in consequence, process performance [17]. Related stress responses, which may well involve the solvent tolerance regulatory network, and variability in their predisposition may cause the observed variability in biocatalytic performance via an interaction with the *alk*-regulatory system or plasmid proliferation. Such a scenario can be excluded as DCPK addition during SMO gene expression under control of the *lac*-regulatory system resulted in low variations regarding SMO expression levels and activities. Thus, the observed variability appears to be related to stress-independent, but host-specific interactions of the *alk*-regulatory system with the host regulatory network.

Possible effects of DCPK enabling increased activities independent of induction

Interestingly, the addition of 0.025 % (v/v) DCPK to *P. putida* DOT-T1E (pStyAB-lac) leads to an increase in mean activity of around 21 %, compared with the same recombinant strain in the absence of DCPK. Semi-quantitative analysis via SDS-PAGE (Fig. 4) did not show a significant difference in StyA levels, suggesting that this activity increase is not owing to different intracellular SMO concentrations. However, differences in active SMO concentrations cannot be excluded. An increased mass transfer of the substrate styrene across the membrane due to increased membrane fluidity caused by the addition of a solvent [55], in this case DCPK, may be another explanation for the higher epoxidation rates. Alternatively, energy metabolism may cause this behavior via an increased NADH availability. Previous studies on *P. putida* DOT-T1E and S12 revealed that, upon exposure to low $\log P_{O/W}$ solvents like octanol ($\log P_{O/W} = 2.9$) and toluene ($\log P_{O/W} = 2.7$), rates for central energy metabolism including NAD(P)H regeneration were significantly increased to cope with higher energy demands for solvent tolerance mechanisms [8]. The same phenomenon may be caused by the addition of low DCPK amounts, in this case fostering styrene epoxidation via increased NADH supply.

Plasmid copy number and variability

Recently, flow cytometry studies showed that recombinant *P. putida* KT2440 forms subpopulations regarding expression under control of the *alk*-regulatory system from the pCom10-based plasmid pA-EGFP_B [27]. The SMO used in the respective study is the same as the SMO used in this study and was tagged with a green fluorescence protein (eGFP). After induction, a productive, fluorescing (68 %) and a non-productive, non-fluorescing (32 %) subpopulation emerged. In our study using the pStyAB and pStyAB-lac plasmids with the same plasmid backbone and monooxygenase genes as present on pA-EGFP_B, *P. putida* KT2440 might also exhibit such a bimodal behavior with a rather robust ratio of the two subpopulations. The formation of a non-productive subpopulation would be a plausible explanation for the low activities of 18.6 ± 2.1 U g_{CDW}^{-1} and 24.0 ± 2.9 U g_{CDW}^{-1} obtained with *P. putida* KT2440 containing pStyAB and pStyAB-lac, respectively. PCN determination via digital droplet PCR showed that the subpopulation formation of the *P. putida* KT2440 strain occurs due to instable plasmid proliferation despite the presence of kanamycin as selection marker [28]. It has been reported that long exposure to kanamycin can trigger the formation of a bacterial population able to survive without harboring a resistance gene [51]. Thus, the question arises whether recombinant *P. putida* DOT-T1E and S12 also tend to the formation of subpopulations regarding PCN and if heterogeneous subpopulation ratios in individual cultures are the cause for the observed variability in recombinant gene expression and epoxidation activity. If so, plasmid replication would be affected by the *alk*-regulatory system as indicated above. The fact that agarose gel band intensities for plasmids isolated from different *P. putida* DOT-T1E-PS34 cultures did not differ (results not shown), points to a direct interference of the *alk*-regulatory system with the host regulatory network, rather than the involvement of plasmid replication. Further investigations on a possible involvement of plasmid replication in recombinant *P. putida* DOT-T1E are in progress.

Conclusion

In this study, we characterized recombinant monooxygenase catalysis in different *Pseudomonas* strains regarding specific resting cell activities, expression levels, and respective variability. A standardized experimental setup was developed to ensure similar generation numbers (45–50) for each tested clone at the start of the resting cell activity assay. These clonal heterogeneity assays revealed high variations in specific resting cell activities for recombinant *P. putida* DOT-T1E and S12 and low variations for *E. coli* JM101, *P. putida* KT2440, and *P. taiwanensis* VLB120 upon monooxygenase gene expression under the control of the *alk*-regulatory system. Such activity variations from colony to colony picked were found to correlate with the recombinant protein amount in the cells. A direct connectivity of this variability with the proposed major solvent

tolerance mechanisms (that is, *cis–trans* isomerization and active solvent efflux), mutations, antibiotic resistance, and type of enzyme, substrate/product, or inducer used could be excluded. By changing the regulatory system used for recombinant gene expression to the *lac*-system, which relieved any high variability, it was shown that the interplay between the *alk*-regulatory system and the host-specific regulatory network of the employed strain, an extrinsic type of noise, apparently plays an important role regarding the appearance of such variability. In addition, it can be stated that the development of gene expression variability is not limited to the induction period, but already starts during growth on the agar plate after transformation.

Thus, this study highlights that stability and reproducibility of recombinant plasmid-based gene expression strongly depend on the combination of host strain and expression system employed. The results point out that the general usage of the *alk*-regulatory system in combination with *Pseudomonas putida* as host strain has to be scrutinized. In general, we demonstrate that the evaluation of hosts, strains, and regulatory systems regarding reproducibility and variability is crucial for prospective industrial applications. The methods developed for the analysis of clonal heterogeneity will serve as stepping stone for such future investigations. Overall, future research should not only target cell-to-cell variability in a single culture/bioprocess, but also clonal variability, finally allowing the selection of the best performing microbial host and expression system.

Acknowledgments We are grateful to Georgios Ionidis (formerly ETH Zurich, Inst. of Biotechnology, Zurich, Switzerland) for providing the chemicals quinoxaline-2-yl-methanol, quinoxaline-2-carbaldehyde, and quinoxaline-2-carboxylic acid. Financial support of the Federal Ministry of Education and Research—BMBF (ERA-IB Project "Pseudomonas 2.0", EIB.10.0.41) is acknowledged.

References

1. Alonso S, Rendueles M, Diaz M (2012) Physiological heterogeneity of *Pseudomonas taetrolens* during lactobionic acid production. Appl Microbiol Biotechnol 96(6):1465–1477. doi:10.1007/s00253-012-4254-2
2. Amanullah A, Hewitt CJ, Nienow AW, Lee C, Chartrain M, Buckland BC, Drew SW, Woodley JM (2003) Measurement of strain-dependent toxicity in the indene bioconversion using multiparameter flow cytometry. Biotechnol Bioeng 81(4):405–420. doi:10.1002/Bit.10479
3. Andersson DI, Hughes D (2010) Antibiotic resistance and its cost: is it possible to reverse resistance? Nat Rev Microbiol 8(4):260–271. doi:10.1038/nrmicro2319
4. Avery SV (2006) Microbial cell individuality and the underlying sources of heterogeneity. Nat Rev Microbiol 4(8):577–587. doi:10.1038/nrmicro1460
5. Bagdasarian M, Lurz R, Ruckert B, Franklin FCH, Bagdasarian MM, Frey J, Timmis KN (1981) Specific-purpose plasmid cloning vectors II. Broad host range, high copy number, Rsf 1010-derived vectors, and a host-vector system for gene cloning in Pseudomonas. Gene 16(1–3):237–247. doi:10.1016/0378-1119(81)90080-9
6. Beaumont HJ, Gallie J, Kost C, Ferguson GC, Rainey PB (2009) Experimental evolution of bet hedging. Nature 462(7269):90–93. doi:10.1038/nature08504
7. Birnbaum S, Bailey JE (1991) Plasmid presence changes the relative levels of many host cell proteins and ribosome components in recombinant *Escherichia coli*. Biotechnol Bioeng 37(8):736–745. doi:10.1002/bit.260370808
8. Blank LM, Ionidis G, Ebert BE, Bühler B, Schmid A (2008) Metabolic response of *Pseudomonas putida* during redox biocatalysis in the presence of a second octanol phase. FEBS J 275(20):5173–5190. doi:10.1111/j.1742-4658.2008.06648.x
9. Bühler B, Bollhalder I, Hauer B, Witholt B, Schmid A (2003) Use of the two-liquid phase concept to exploit kinetically controlled multistep biocatalysis. Biotechnol Bioeng 81(6):683–694. doi:10.1002/Bit.10512
10. Bühler B, Schmid A, Hauer B, Witholt B (2000) Xylene monooxygenase catalyzes the multistep oxygenation of toluene and pseudocumene to corresponding alcohols, aldehydes, and acids in *Escherichia coli* JM101. J Biol Chem 275(14):10085–10092. doi:10.1074/jbc.275.14.10085
11. Bühler B, Witholt B, Hauer B, Schmid A (2002) Characterization and application of xylene monooxygenase for multistep biocatalysis. Appl Environ Microbiol 68(2):560–568. doi:10.1128/Aem.68.2.560-568.2002
12. Canosa I, Sanchez-Romero JM, Yuste L, Rojo F (2000) A positive feedback mechanism controls expression of AlkS, the transcriptional regulator of the *Pseudomonas oleovorans* alkane degradation pathway. Mol Microbiol 35(4):791–799
13. Carneiro S, Ferreira EC, Rocha I (2013) Metabolic responses to recombinant bioprocesses in *Escherichia coli*. J Biotechnol 164(3):396–408. doi:10.1016/j.jbiotec.2012.08.026
14. Choi KH, Kumar A, Schweizer HP (2006) A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J Microbiol Methods 64(3):391–397. doi:10.1016/j.mimet.2005.06.001
15. Delvigne F, Goffin P (2014) Microbial heterogeneity affects bioprocess robustness: dynamic single-cell analysis contributes to understanding of microbial populations. Biotechnol J 9(1):61–72. doi:10.1002/biot.201300119
16. Durfee T, Nelson R, Baldwin S, Plunkett G 3rd, Burland V, Mau B, Petrosino JF, Qin X, Muzny DM, Ayele M, Gibbs RA, Csorgo B, Posfai G, Weinstock GM, Blattner FR (2008) The complete genome sequence of *Escherichia coli* DH10B: insights into the biology of a laboratory workhorse. J Bacteriol 190(7):2597–2606. doi:10.1128/JB.01695-07
17. Ebert BE, Kurth F, Grund M, Blank LM, Schmid A (2011) Response of *Pseudomonas putida* KT2440 to increased NADH and ATP demand. Appl Environ Microbiol 77(18):6597–6605. doi:10.1128/AEM.05588-11
18. Elowitz MB, Levine AJ, Siggia ED, Swain PS (2002) Stochastic gene expression in a single cell. Science 297(5584):1183–1186. doi:10.1126/science.1070919
19. Grand View Research MRC (2014) Biotechnology market (Biopharmacy, Bioservices, Bioagriculture, Bioindustrial, Fermentation, DNA sequencing, Tissue Engineering, Regeneration) analysis and segment forecasts to 2020. http://www.grandviewresearch.com/industry-analysis/biotechnology-market
20. Gross R, Lang K, Bühler K, Schmid A (2010) Characterization of a biofilm membrane reactor and its prospects for fine chemical synthesis. Biotechnol Bioeng 105(4):705–717. doi:10.1002/Bit.22584
21. Halan B, Schmid A, Buehler K (2010) Maximizing the productivity of catalytic biofilms on solid supports in membrane aerated

reactors. Biotechnol Bioeng 106(4):516–527. doi:10.1002/bit.22732
22. Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166(4):557–580. doi:10.1016/S0022-2836(83)80284-8
23. Hartmans S, van der Werf MJ, de Bont JA (1990) Bacterial degradation of styrene involving a novel flavin adenine dinucleotide-dependent styrene monooxygenase. Appl Environ Microbiol 56(5):1347–1351
24. Heipieper HJ, de Bont JA (1994) Adaptation of *Pseudomonas putida* S12 to ethanol and toluene at the level of fatty acid composition of membranes. Appl Environ Microbiol 60(12):4440–4444
25. Heipieper HJ, Neumann G, Cornelissen S, Meinhardt F (2007) Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. Appl Microbiol Biotechnol 74(5):961–973. doi:10.1007/s00253-006-0833-4
26. Hewitt CJ, Caron GNV, Nienow AW, McFarlane CM (1999) The use of multi-parameter flow cytometry to compare the physiological response of *Escherichia coli* W3110 to glucose limitation during batch, fed-batch and continuous culture cultivations. J Biotechnol 75(2–3):251–264. doi:10.1016/S0168-1656(99)00168-6
27. Jahn M, Seifert J, von Bergen M, Schmid A, Bühler B, Müller S (2012) Subpopulation-proteomics in prokaryotic populations. Curr Opin Biotechnol 24(1):79–87. doi:10.1016/j.copbio.2012.10.017
28. Jahn M, Vorpahl C, Turkowsky D, Lindmeyer M, Bühler B, Harms H, Müller S (2014) Accurate determination of plasmid copy number of flow-sorted cells using droplet digital PCR. Anal Chem 86(12):5969–5976. doi:10.1021/ac501118v
29. Julsing MK, Kuhn D, Schmid A, Bühler B (2012) Resting cells of recombinant *E. coli* show high epoxidation yields on energy source and high sensitivity to product inhibition. Biotechnol Bioeng 109(5):1109–1119. doi:10.1002/bit.24404
30. Junker F, Ramos JL (1999) Involvement of the *cis/trans* isomerase Cti in solvent resistance of *Pseudomonas putida* DOT-T1E. J Bacteriol 181(18):5693–5700
31. Kaern M, Elston TC, Blake WJ, Collins JJ (2005) Stochasticity in gene expression: from theories to phenotypes. Nat Rev Genet 6(6):451–464. doi:10.1038/nrg1615
32. Kieboom J, Dennis JJ, Zylstra GJ, de Bont JA (1998) Active efflux of organic solvents by *Pseudomonas putida S12* is induced by solvents. J Bacteriol 180(24):6769–6772
33. Köhler KA, Rückert C, Schatschneider S, Vorhölter FJ, Szczepanowski R, Blank LM, Niehaus K, Goesmann A, Pühler A, Kalinowski J, Schmid A (2013) Complete genome sequence of *Pseudomonas* sp. strain VLB120 a solvent tolerant, styrene degrading bacterium, isolated from forest soil. J Biotechnol 168(4):729–730. doi:10.1016/j.jbiotec.2013.10.016
34. Kuhn D, Bühler B, Schmid A (2012) Production host selection for asymmetric styrene epoxidation: *Escherichia coli* vs. solvent-tolerant *Pseudomonas*. J Ind Microbiol Biotechnol 39(8):1125–1133. doi:10.1007/s10295-012-1126-9
35. Kuhn D, Kholiq MA, Heinzle E, Bühler B, Schmid A (2010) Intensification and economic and ecological assessment of a biocatalytic oxyfunctionalization process. Green Chem 12(5):815–827. doi:10.1039/b921896c
36. Leak DJ, Sheldon RA, Woodley JM, Adlercreutz P (2009) Biocatalysts for selective introduction of oxygen. Biocatal Biotransform 27(1):1–26. doi:10.1080/10242420802393519
37. Messing J (1979) A multipurpose cloning system based on the single-stranded DNA bacteriophage M13. Recomb DNA Tech Bull 3(2):43–49
38. Nelson KE, Weinel C, Paulsen IT, Dodson RJ, Hilbert H, Martins dos Santos VA, Fouts DE, Gill SR, Pop M, Holmes M, Brinkac L, Beanan M, DeBoy RT, Daugherty S, Kolonay J, Madupu R, Nelson W, White O, Peterson J, Khouri H, Hance I, Chris Lee P, Holtzapple E, Scanlan D, Tran K, Moazzez A, Utterback T, Rizzo M, Lee K, Kosack D, Moestl D, Wedler H, Lauber J, Stjepandic D, Hoheisel J, Straetz M, Heim S, Kiewitz C, Eisen JA, Timmis KN, Dusterhoft A, Tummler B, Fraser CM (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ Microbiol 4(12):799–808. doi:10.1046/j.1462-2920.2002.00366.x
39. Neumann G, Kabelitz N, Zehnsdorf A, Miltner A, Lippold H, Meyer D, Schmid A, Heipieper HJ (2005) Prediction of the adaptability of *Pseudomonas putida* DOT-T1E to a second phase of a solvent for economically sound two-phase biotransformations. Appl Environ Microbiol 71(11):6606–6612. doi:10.1128/Aem.71.11.6606-6612.2005
40. Nikolic N, Barner T, Ackermann M (2013) Analysis of fluorescent reporters indicates heterogeneity in glucose uptake and utilization in clonal bacterial populations. BMC Microbiol 13:258. doi:10.1186/1471-2180-13-258
41. Otto K, Hofstetter K, Rothlisberger M, Witholt B, Schmid A (2004) Biochemical characterization of StyAB from *Pseudomonas* sp. strain VLB120 as a two-component flavin-diffusible monooxygenase. J Bacteriol 186(16):5292–5302. doi:10.1128/JB.186.16.5292-5302.2004
42. Panke S, Held M, Wubbolts MG, Witholt B, Schmid A (2002) Pilot-scale production of (S)-styrene oxide from styrene by recombinant *Escherichia coli* synthesizing styrene monooxygenase. Biotechnol Bioeng 80(1):33–41. doi:10.1002/Bit.10346
43. Panke S, Meyer A, Huber CM, Witholt B, Wubbolts MG (1999) An alkane-responsive expression system for the production of fine chemicals. Appl Environ Microbiol 65(6):2324–2332
44. Panke S, Wubbolts MG, Schmid A, Witholt B (2000) Production of enantiopure styrene oxide by recombinant *Escherichia coli* synthesizing a two-component styrene monooxygenase. Biotechnol Bioeng 69(1):91–100. doi:10.1002/(SICI)1097-0290(20000705)69:1<91:AID-BIT11>3.0.CO;2-X
45. Pedraza JM, van Oudenaarden A (2005) Noise propagation in gene networks. Science 307(5717):1965–1969. doi:10.1126/science.1109090
46. Ramos JL, Duque E, Huertas MJ, Haidour A (1995) Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high-concentrations of aromatic-hydrocarbons. J Bacteriol 177(14):3911–3916
47. Rausenberger J, Fleck C, Timmer J, Kollmann M (2009) Signatures of gene expression noise in cellular systems. Prog Biophys Mol Biol 100(1–3):57–66. doi:10.1016/j.pbiomolbio.2009.06.003
48. Rojas A, Duque E, Mosqueda G, Golden G, Hurtado A, Ramos JL, Segura A (2001) Three efflux pumps are required to provide efficient tolerance to toluene in *Pseudomonas putida* DOT-T1E. J Bacteriol 183(13):3967–3973. doi:10.1128/JB.183.13.3967-3973.2001
49. Rojo F, Dinamarca MA (2004) Catabolite repression and physiological control. In: Ramos JL, Filloux A (eds) *Pseudomonas*, virulence and gene regulation, vol 2. Springer, New York, pp 365–387
50. Sambrook J, Russel DW (2001) Molecular cloning: a laboratory manual, Band 1. New York
51. Sanchez-Romero MA, Casadesus J (2014) Contribution of phenotypic heterogeneity to adaptive antibiotic resistance. Proc Natl Acad Sci USA 111(1):355–360. doi:10.1073/pnas.1316084111
52. Schrewe M, Julsing MK, Bühler B, Schmid A (2013) Whole-cell biocatalysis for selective and productive C-O functional group introduction and modification. Chem Soc Rev 42(15):6346–6377. doi:10.1039/c3cs60011d
53. Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). Biometrika 52(3–4):591–611. doi:10.2307/2333709

54. Siegele DA, Hu JC (1997) Gene expression from plasmids containing the *araBAD* promoter at subsaturating inducer concentrations represents mixed populations. Proc Natl Acad Sci USA 94(15):8168–8172
55. Sikkema J, de Bont JA, Poolman B (1995) Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59(2):201–222
56. Silva-Rocha R, de Lorenzo V (2010) Noise and robustness in prokaryotic regulatory networks. Annu Rev Microbiol 64:257–275. doi:10.1146/annurev.micro.091208.073229
57. Smits TH, Seeger MA, Witholt B, van Beilen JB (2001) New alkane-responsive expression vectors for *Escherichia coli* and *Pseudomonas*. Plasmid 46(1):16–24. doi:10.1006/plas.2001.1522
58. Staijen IE, Marcionelli R, Witholt B (1999) The $P_{alkBFGHJKL}$ promoter is under carbon catabolite repression control in *Pseudomonas oleovorans* but not in *Escherichia coli* alk^+ recombinants. J Bacteriol 181(5):1610–1616
59. Suzuki M, Hayakawa T, Shaw JP, Rekik M, Harayama S (1991) Primary structure of xylene monooxygenase: similarities to and differences from the alkane hydroxylation system. J Bacteriol 173(5):1690–1695
60. Timmis KN (2002) *Pseudomonas putida*: a cosmopolitan opportunist par excellence. Environ Microbiol 4(12):779–781. doi:10.1046/j.1462-2920.2002.00365.x
61. Woodley JM (2006) Microbial biocatalytic processes and their development. Adv Appl Microbiol 60:1–15. doi:10.1016/S0065-2164(06)60001-4