Adaptive laboratory evolution of *Pseudomonas putida* and *Corynebacterium glutamicum* to enhance anthranilate tolerance

Jannis Kuepper[1], Maike Otto[1,2], Jasmin Dickler[1], Swantje Behnken[3], Jorgen Magnus[3], Gernot Jäger[4], Lars M. Blank[1] and Nick Wierckx[2*]

[1] Institute of Applied Microbiology, RWTH Aachen University, 52074 Aachen, Germany

[2] Institute of Bio- and Geosciences (IBG-1: Biotechnology), Forschungszentrum Jülich GmbH, 52425 Jülich, Germany

[3] Bayer AG, 51373 Leverkusen, Germany

[4] Covestro Deutschland AG, 51365 Leverkusen, Germany

* Correspondence: Prof. Dr. Nick Wierckx; n.wierckx@fz-juelich.de

Keywords: *Pseudomonas putida, Corynebacterium glutamicum*, Adaptive laboratory evolution, anthranilic acid, industrial biotechnology

Abstract

Microbial bioproduction of the aromatic acid anthranilate (oAB, *ortho*-aminobenzoate) has the potential to replace its current, environmentally demanding production process. The host organism employed for such a process needs to fulfill certain demands to achieve industrially relevant product titers. As anthranilate is toxic for microorganisms, the use of especially robust production hosts can overcome issues due to product inhibition. The microorganisms *Corynebacterium glutamicum* and *Pseudomonas putida* are known for high tolerance towards a variety of chemicals and could serve as promising platform strains. In this study, the resistance of both wild-type strains towards anthranilate was assessed. To further enhance their native tolerance, adaptive laboratory evolution (ALE) was applied. Sequential batch fermentation processes were developed, adapted to the cultivation demands for *C. glutamicum* and *P. putida*, to enable long-term cultivation in the presence of anthranilate. Isolation and analysis of single mutants revealed phenotypes with improved growth behavior in the presence of anthranilate for both strains. The characterization and improvement of both potential hosts delivers an important basis for further process optimization and will aid to establish an industrially competitive method for microbial synthesis of anthranilate.

1 Introduction

Growing awareness of climate change and pollution has led to a thriving development of alternative, "green" production routes of diverse platform chemicals[1,2]. In recent years, several bio-based synthesis routes of aromatic compounds have been established, e.g. for phenol[3], cinnamic and p-hydroxycinnamic acid[4,5], styrene[6] or vanillin[7]. The aromatic amino acid anthranilate (*o*AB, *ortho*-aminobenzoate) is used as precursor for several plastic products, pharmaceutical compounds and in the food industry[8–10]. It can be converted to aniline, a primary precursor for methylene diphenyl di-isocyanate which is used to produce polyurethane foams[11,12]. Several microbial proof-of-concept production routes of anthranilate have been developed so far, utilizing a diverse range of production hosts including *Escherichia coli*[13], *Bacillus subtilis*[14], *Corynebacterium glutamicum*[15], and *Pseudomonas putida*[16]. Anthranilate inhibits specific enzymes and disturbs cellular processes like biofilm formation, as observed for a variety of microorganisms[17]. Besides this specific effect, the addition of high concentrations of anthranilate at neutral pH also causes osmotic stress[18]. Product toxicity is often a critical point for microbial production of aromatic compounds[19,20]. There are different possibilities to overcome this issue like *in situ* removal of toxic products from the medium by extraction or absorption[21,22]. Such methods, however, are usually more effective at higher product concentrations. For this reason, the utilization of stress-tolerant microorganisms enables a greater degree of freedom in process development, leading to better performance of anthranilate biocatalysis[16].

Corynebacterium glutamicum, a Gram-positive bacterium belonging to the order of *Actinomycetes*, is well-known as workhorse for the large-scale production of amino acids[23–26]. The application spectrum of the organism is constantly being broadened due to its versatile metabolism and stable growth behavior[27]. Examples of this include the production of the positional isomer of anthranilate, *para*-aminobenzoate (PABA)[28] and the synthesis of complex plant polyphenols[29]. The cytoplasmic membrane in Gram-positive bacteria is surrounded by a thick peptidoglycan cell wall that is especially stress-resistant. This robust physiological barrier renders the organism impenetrable to many chemicals and antibiotics[30]. *C. glutamicum* can also respond to external influences by inducing tolerance responses within the cells[31]. These include for example the production of non-enzymatic antioxidants[32] or the conversion of toxic agents into less harmful compounds[33]. Another promising candidate for anthranilate production is the Gram-negative bacterium *Pseudomonas putida.* The stress tolerance of this organism has been extensively studied in the past[34–36]. The cell envelope of Gram-negative bacteria is less physically resistant to external factors. However, *P. putida* is able to adapt the composition of its inner and outer membranes to lower the permeability for substrates. This can be achieved by shifting the ratio of saturated to unsaturated fatty acids to enhance membrane stability.[37] Furthermore, the organism is able to increase its substrate uptake rate and re-route its metabolism for production of reducing equivalents to cope with energy-consuming tolerance mechanisms[38–40]. In addition, some strains of *P. putida* express solvent pumps that can actively extrude toxic compounds from the inner membrane[41]. This enables them to withstand even highly toxic compounds such as toluene[38] or styrene[42]. It is unlikely that the solvent pumps grant a higher tolerance towards charged molecules like anthranilate, but other transporters are known to confer higher resistance to similar molecules like *p*-hydroxybenzoate or *p*-coumaric

acid[43,44]. With these traits, *C. glutamicum* and *P. putida* can be considered as interesting hosts for anthranilate production, as both organisms employ different tolerance mechanisms, particularly regarding their cell wall structure.

Similar to other industrial biotechnology processes and their respective products, anthranilate titers should ideally exceed 100 g L^{-1}, but this number greatly depends on the overall process, including the fermentation mode (batch, fed-batch, continuous) and the chosen downstream process.[45] While the native abilities of both *P. putida* and *C. glutamicum* set a strong starting point as production hosts for anthranilate, these capacities can be further exploited to gain more tolerant and thus more productive strains by applying adaptive laboratory evolution (ALE). ALE is a common strategy to obtain phenotypes with a better adaptation to certain stress conditions caused by naturally occurring mutations[46,47]. This technique has previously been successfully applied to *P. putida* to increase tolerance to organic solvents[48] and ionic liquids[49], the tolerance and metabolism of coumarate and ferulate[50], and the metabolism of ethylene glycol[51], 1,4-butanediol[52], and xylose[53]. With *C. glutamicum*, ALE has been used to enhance tolerance to lignocellulose-derived inhibitors[54], high temperature and solvents[55], and methanol[56,57], as well as for other objectives such as productivity, growth and substrate utilization[58].

In this study, we report an adaptation of strains of *C. glutamicum* and *P. putida* to gradually increasing concentrations of anthranilate by applying ALE. An assessment of anthranilate tolerance of the wild-type strains of both organisms set the starting point for ALE using sequential shake flask cultivations and sequential batch fermentations. Single colony isolation and evaluation resulted in the selection of improved strains of *P. putida* and *C. glutamicum* with enhanced tolerance towards anthranilate. An application of these improved strains for microbial anthranilate production could enhance production efficiencies and lay the basis for a less environmentally demanding production of this versatile aromatic acid.

2 Materials and methods

2.1 Bacterial strains and media

Wild-type strains of *P. putida* KT2440 (ATCC 47054) and *C. glutamicum* (ATCC 13032) were used in this study. The cultivation in shake flasks took place at 30°C and 250 rpm. *P. putida* KT2440 was cultivated in mineral salts medium (MSM) according to Wierckx *et al.*[59] at pH 7.0 and was composed of glucose (20 mM), K_2HPO_4 (3.88 g L^{-1}), $NaH_2PO_4.2H_2O$ (2.12 g L^{-1}), $(NH_4)_2SO_4$ (2 g L^{-1}), EDTA (0.01 g L^{-1}), $MgCl_2.6H_2O$ (0.1 g L^{-1}), $ZnSO_4.7H_2O$ (0.002 g L^{-1}), $CaCl_2.2H_2O$ (0.001 g L^{-1}), $FeSO_4.7H_2O$ (0.005 g L^{-1}), $Na_2MoO_4.2H_2O$ (0.0002 g L^{-1}), $CuSO_4.5H_2O$ (0.0002 g L^{-1}), $CoCl_2.6H_2O$ (0.0004 g L^{-1}) and $MnCl_2.2H_2O$ (0.001 g L^{-1}). Main cultures of *P. putida* KT2440 were inoculated from an overnight mineral medium pre-culture. Strains of *C. glutamicum* were cultivated in CGXII medium (pH 7.0) containing glucose (22 mM), MOPS (42 g L^{-1}, urea (5 g L^{-1}), $(NH_4)_2SO_4$ (20 g L^{-1}), $MgSO_4.7H_2O$ (0.25 g L^{-1}), KH_2PO_4 (1 g L^{-1}), K_2HPO_4 (1g L^{-1}), $CaCl_2$ (0.01 g L^{-1}), $FeSO_4.7H_2O$ (0.01 g L^{-1}), $NiCl_2.6H_2O$ (0.02 mg L^{-1}), $CuSO_4.5H_2O$ (0.0002 g L^{-1}), $ZnSO_4.7H_2O$ (0.001 g L^{-1}), $MnSO_4.H_2O$ (0.01 g L^{-1}), biotin solution (0.0002 g L^{-1}) and protocatechuate (0.003 g L^{-1}). Main cultures were inoculated from overnight CGXII medium plus 10% brain heart infusion (BHI) pre-cultures

which were inoculated from BHI pre-cultures. For tolerance experiments, the medium was supplemented with 2.5 to 15 g L^{-1} anthranilate (200 g L^{-1} stock solution pH 7). The strains were stored as cryo cultures containing 25 % glycerol at - 80°C.

2.2 Culture conditions

To investigate the tolerance of *C. glutamicum* towards anthranilate, batch fermentations were performed in controlled bioreactors. Cultivation took place in CGXII fermenter medium composed as described above without the addition of MOPS, Urea, biotin solution and protocatechuate. The bioreactors were inoculated with a starting OD_{600} of 0 .3 from overnight pre-cultures (first pre-culture in BHI, second pre-culture in CGXII + 10 % BHI). The reactors were operated containing 400 mL CGXII-fermenter medium at 30 °C, 500 to 1200 rpm (dO_2 regulated agitation cascade with a low limit of 35 % dO_2), 1 vvm aeration with compressed air, pH regulation to pH = 7 with 2 N KOH (no pH control above pH 7.0). The fermenters were supplemented with different anthranilate concentrations at different time points during the early exponential growth phase of the culture. Cultivation of *P. putida* KT2440 to assess its tolerance towards anthranilate took place in controlled bioreactors (New Brunswick, Eppendorf, Germany) in mineral medium with 20 mM glucose, a 2-fold PO_4-buffer concentration and a 2-fold $(NH_4)_2SO_4$. The bioreactors were inoculated with a starting OD_{600} of 0.2 from overnight pre-cultures. The reactors were operated with 400 mL mineral medium at 30 °C, 500 to 1200 rpm (dO_2 regulated agitation cascade with a low limit of 35 % dO_2), 1 vvm aeration with compressed air, pH regulation to pH = 7 with 2 N KOH (no pH control above pH 7.0). Anthranilate was added to the medium at the beginning of the fermentation.

P. putida KT2440 and *C. glutamicum* wild-type strains were sequentially inoculated in mineral salts medium (*P. putida*) or CGXII medium (*C. glutamicum*) with 5 g L^{-1} anthranilate in shake flasks and cultivated at 30 °C and 250 rpm. Every morning fresh sequential batch shake flasks were inoculated with a starting OD_{600} of about 0.2 and every evening with a starting OD_{600} of about 0.025. Evolved strains were tested for improved anthranilate tolerance as described above.

To further enhance the tolerance of *P. putida* towards anthranilate, a sequential batch fermentation was developed. Bioreactors were inoculated with a mineral medium overnight pre-culture of *P. putida* with a starting OD_{600} of 0.2. The fermentation medium was 400 mL mineral medium with 50 mM glucose, a 2-fold PO_4-buffer and $(NH_4)_2SO_4$ concentration, and 5 g L^{-1} anthranilate. The *P. putida* fermentations were performed at 30 °C, with 500 to 1200 rpm agitation (dO_2 regulated agitation cascade with a low limit of 35 % dO_2), with 1 vvm aeration of compressed air, the pH was regulated to pH 7 with 2 N KOH (no pH control above pH 7). An automatic refill of the fermenter was triggered when the following conditions were met: 1) fermentation medium reached pH 8.0, 2) the process time of one batch reached at least 20 hours and 3) the base pump was operated at a minimum of 8 AU. Base consumption is expressed in arbitrary units (AU) because calibration of pumps and sensors became unreliable during the very long term cultivation. The refill procedure was composed of 5 steps: 1) The broth was pumped out of the fermenter, leaving approximately 20 mL; 2) 40 mL of fresh medium were pumped into the fermenter; 3) the fermenter was pumped out again, 20 mL of fermentation broth remained in the system; 4) the fermenter was refilled with 380 mL of fresh

medium; 5) the desired concentration of anthranilate was added from a 200 g L^{-1} stock (pH 7) to the medium in less than 5 minutes immediately after the refill. The set point of the anthranilate concentration was manually increased (starting from 5g L^{-1}) at different time points to a final concentration of 15 g L^{-1} in steps of 0.25 to 1 g L^{-1}. During the refill process the pH control and the dO_2 cascade to control the stirrer were disabled. The last sample was analyzed after about 4200 hours.

A similar strategy was applied for *C. glutamicum* to increase its tolerance towards anthranilate. Bioreactors were inoculated with pre-cultures of *C. glutamicum* (first pre-culture in BHI, second pre-culture in CGXII + 10 % BHI) to a starting OD_{600} of about 0.2. The fermentation medium was 400 ml CGXII fermenter medium including 3 mg L^{-1} protocatechuate, 100 mM glucose and 100 µL L^{-1} antifoam. The fermentations were performed at 30 °C, with 500 to 1200 rpm agitation (dO_2 regulated agitation cascade with a low limit of 35 % dO_2), with 1 vvm aeration of compressed air, the pH was regulated to pH 7 with 2 M KOH (no pH control above pH 7). The addition of anthranilate was realized by gradually pumping the desired volume of a 200 g L^{-1} anthranilate stock (pH 7 set with KOH) into the fermenter from hour 4 to hour 7. To automatically control the trigger for a refill of the sequential batch fermenters with fresh medium the following constrains were set: 1) the process time of one batch had to reach at least 30 hours, 3) the base pump had to pump at least 50 AU and 3) the dO_2 had to reach at least 80 %. If these constrains were fulfilled the batch time was set to zero and the refill was triggered. The refill procedure is composed of the 4 steps described above for *P. putida*. Since anthranilate addition took place after the initial growth phase for *C. glutamicum*, step 5 was changed: At hour 4 the anthranilate pump was switched on for three hours. The final anthranilate concentration was set by varying the pump speed. The initial anthranilate concentration was set to 15g L^{-1}. The last samples were taken from the reactors after about 1400 hours.

2.3 Evolved single mutant isolation

To isolate single clones with the highest anthranilate tolerance from the populations evolved during the sequential batch fermentation, cryo cultures were re-inoculated in shake flasks and several dilutions were plated out on CGXII (*C. glutamicum*) or mineral medium (*P. putida*) agar plates supplemented with 0, 10, 15, 20, 25, and 30 g L^{-1} anthranilate. The plates were incubated at 30 °C.

2.4 Analytical methods

Bacterial growth was monitored as optical density at a wavelength of 600 nm (OD_{600}) with a spectrophotometer (Ultrospec 10 cell density meter, GE Healthcare) using cuvettes with 1 cm path length. Growth rates during the exponential growth phase were evaluated with exponential regression in Microsoft Excel 2016 using a minimum set of four data points.

For HPLC analysis 500 µL of medium was removed from the culture and centrifuged at 13,000 rpm for 2 minutes. The supernatant was transferred to HPLC glass vials for analysis.

Reverse phase HPLC was performed to monitor anthranilate and glycosyl-anthranilate concentrations in the cultivation medium. The analysis was carried out using a Beckmann System Gold 125 166 HPLC with an autosampler, UV/vis detector and a C18 reverse phase

column (LiChroCART 250-4 C18, 250 x 4 mm, Merck). Samples (5 µL) were injected at a flow rate of 1.2 ml min^{-1} and a column temperature of 30°C. The column was eluted with double-distilled water plus 0.1 % TFA and methanol. The eluent began as a mixture of 90 % water/TFA and 10 % methanol for 2 minutes before a linear gradient was applied for 10 minutes to reach 100 % methanol. This eluent was held for 2 minutes until a second linear gradient was applied over the course of 2 minutes to reach a mixture of 90 % water/TFA and 10 % methanol. The eluent was monitored at 257 nm. Anthranilate and glycosyl-anthranilate eluted at minute 4.5 and 4.7, respectively. Due to a lack of standards, all glycosyl-anthranilate values given are determined mathematically by subtraction.

The analysis of C6 sugars (glucose, gluconate and ketogluconate) was carried out in a Beckmann System Gold 125 166 HPLC with an autosampler using a polystyrol-divinylbenzol copolymer (PS-DVB) column (organic acid resin, 300 x 8.0 mm, CS-Chromatographie). Samples (5 µL) were injected at a flow rate of 0.8 ml/min and a column temperature of 75°C. The column was eluted with double-distilled water containing 5 mM H_2SO_4 for 11 minutes. The detection was accomplished with an UV/VIS detector at 210 nm and a refractory index (RI) detector (RI-101, Shodex).

3 RESULTS

3.1 Evaluation of tolerance of *P. putida* and *C. glutamicum* towards anthranilate

For the initial assessment of anthranilate tolerance, *P. putida* and *C. glutamicum* were cultivated in shake flasks in, respectively mineral salts medium (MSM) and CGXII-medium supplemented with different anthranilate concentrations prior to inoculation. At a concentration of 5 g L^{-1} anthranilate, a 63 % reduced growth rate and a 48 % reduced final OD_{600} were observed for *P. putida*, while growth was completely inhibited in the presence of 10 g L^{-1} anthranilate and above (Figure 1 A). The fitness of *C. glutamicum* was also significantly reduced with increasing amounts of anthranilate reflected by lower growth rates and a prolonged lag phase (Figure 1 B). However, the cultures containing 2.5 and 5 g L^{-1} anthranilate reached a final OD_{600} comparable to the control after 24 hours of cultivation. At 10 g L^{-1} anthranilate the final OD_{600} was reduced by 67% after 23h, however a prolonged cultivation could have resulted in a higher biomass concentration. The two organisms responded markedly different to anthranilate stress. *P. putida* grew slower and had a lower biomass yield, while *C. glutamicum* had a long lag phase but was not affected in its biomass yield. The long lag phases of *C. glutamicum* were circumvented by supplementation with anthranilate in the early exponential growth phase (Figure 1 C). In these cultures, the growth rates decreased with increasing anthranilate concentrations, but all cultures reached comparable final optical densities. At 10 g L^{-1} anthranilate the growth rate was reduced by 60 % while the final OD_{600} was reduced by only 19 % compared to the control. Applying a similar strategy to *P. putida* had no effect on anthranilate tolerance compared to *ab inito* addition.

https://doi.org/10.1099/mic.0.000982

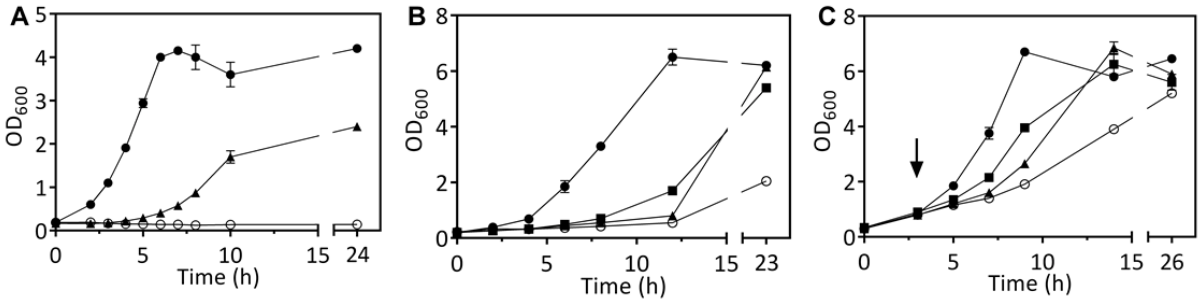

Figure 1. Growth of *P. putida* and *C. glutamicum* in the presence of anthranilate in shake flasks. (A) Growth curves of *P. putida* KT2440 in the presence of 0 (closed circle), 5 (triangle), and 10 (open circle) g L^{-1} anthranilate in MSM with 20 mM glucose. (B) Growth curves of *C. glutamicum* in the presence of 0 (closed circle), 2.5 (square), 5 (triangle) and 10 (open circle) g L^{-1} anthranilate in CGXII medium with 22 mM glucose. (C) Growth curves of *C. glutamicum* in CGXII medium with 22 mM glucose following an anthranilate pulse of 0, 2.5, 5 and 10 g L^{-1} anthranilate indicated by a black arrow. Error bars represent the standard error (n=2).

The tolerance of *C. glutamicum* towards anthranilate was further investigated in a batch fermentation in controlled bioreactors (Figure 2). The fermenters were supplemented with three manual injections of 5 g L^{-1} anthranilate at hour 4, 6 and 8 which resulted in a final concentration of 15 g L^{-1} anthranilate in the reactor, after which the reactors were operated for another 27 hours. This gradual addition of 15 g L^{-1} anthranilate posed significant stress to the cells, resulting in a reduced growth rate of 0.05 h^{-1}. However, as in shake flasks, the anthranilate exposure had no effect on the final OD_{600}. HPLC analysis of samples taken during the fermentation revealed the formation of a derivative product of anthranilate (Figure 2B) which was identified as glycosyl-anthranilate. This derivative is formed rapidly upon addition of anthranilate, then gradually decreases during cultivation as glucose is consumed. Its formation of glycosyl-anthranilate is catalyzed by cations in the cultivation medium and specially the high ammonium concentration of the CGXII-medium was identified as main catalytic agent[60–62]. To investigate the effects of different cations and anthranilate concentrations, shake flask experiments were performed where the ammonium as well as urea, MOPS, KH_2PO_4, K_2HPO_4, $MgSO_4$, and $CaCl_2$ were added to the media at 1-, 4-, and 8-fold dilution (corresponding to 20, 5, and 2.5 g L^{-1} $(NH_4)_2SO_4$). The growth of *C. glutamicum* in the presence of different concentrations of anthranilate was investigated under these medium conditions (Figure 2C). Without anthranilate stress, the decreased ammonium concentrations led to a lower growth rate. In the presence of anthranilate, the reverse effect was observed; the growth rate increased with decreasing ammonium concentrations.

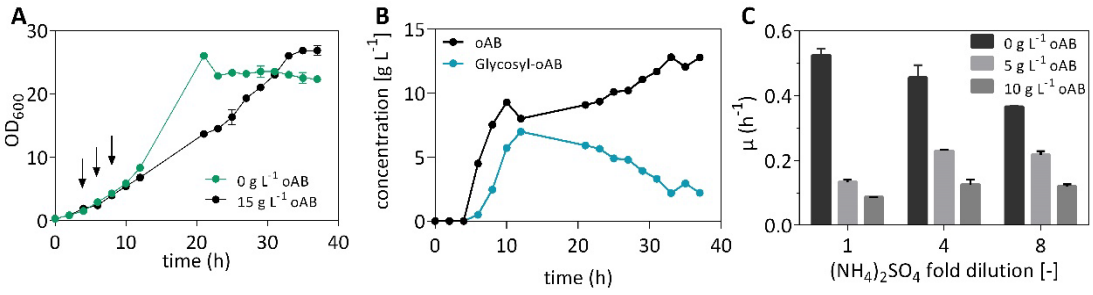

Figure 2. (A) Growth behavior of *C. glutamicum* in CGXII medium with 100 mM glucose at 0 g L^{-1} anthranilate and 15 g L^{-1} anthranilate during a batch fermentation. The final concentration of 15 g L^{-1} anthranilate was added gradually in 3 pulses of 5 g L^{-1}, indicated by the black arrows. Data points are the average of duplicate experiments. (B) Formation of glycosyl-anthranilate during the batch-fermentation shown in A. (C) Dependence of the growth rate of *C. glutamicum* on the concentration of $(NH_4)_2SO$, urea, MOPS, KH_2PO_4, K_2HPO_4, $MgSO_4$ and $CaCl_2$.

These components of the CGXII medium were added at 1x, 4x and 8x dilution in shake flasks experiments with 22 mM glucose. Anthranilate was added at concentrations of 0, 5, and 10 g L^{-1}. Error bars represent the standard deviation (n=3).

3.2 Adaptive laboratory evolution to increase anthranilate tolerance

3.2.1 ALE in shake flasks

Different strategies were applied to increase the tolerance of *P. putida* and *C. glutamicum* towards anthranilate. In a first approach, sequential batch shake flask experiments in the presence of increasing anthranilate concentrations were performed. With this approach, no improvement of tolerance was observed for *C. glutamicum*. In contrast, a gradual increase in growth rate and final OD_{600} were observed over time for *P. putida*, when the culture was sequentially inoculated in MSM in shake flasks with 5 g L^{-1} anthranilate (data no shown). A total of 16 sequential inoculations were performed to obtain the evolved strain *P. putida* oAB16. To analyze the improvement gained through the evolution in shake flasks a comparative experiment was performed with *P. putida* oAB16 and the wild-type. Both strains were cultivated in shake flasks in MSM containing anthranilate at varying concentrations (Figure 3). An increase in the growth rate of 196 % and a 132 % higher final OD_{600} was observed in the presence of 5 g L^{-1} anthranilate for *P. putida* oAB16 in comparison to the wild-type. While no growth was observed in the wild-type strain in the presence of 7.5 g L^{-1} anthranilate, the evolved strain could grow under these conditions with a growth rate of 0.2 h^{-1} and reached a final OD_{600} of 1.5. The improvement of tolerance towards anthranilate is stable after passing the strain through LB medium and cryo-conservation and it has no effect on the growth behavior under the control conditions without anthranilate.

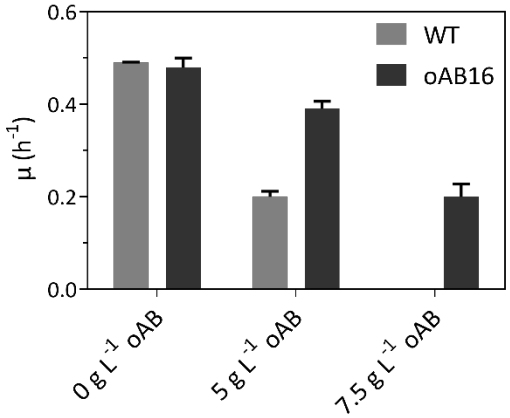

Figure 3. Growth rates of *P. putida* KT2440 and *P. putida* oAB16 in shake flasks in MSM with 20 mM of glucose and 0, 5 and 7.5 g L^{-1} anthranilate. Error bars represent the standard deviation (n=3).

3.2.2 ALE in controlled bioreactors

To further increase the tolerance towards anthranilate in a more controlled and less labor-intensive way, automated sequential batch fermentation strategies were developed. For *P. putida*, two bioreactors (Eppendorf, Germany) were inoculated with overnight pre-culture of the evolved strain oAB16. The initial fermentation was performed in MSM with 20 mM glucose plus 5 g L^{-1} anthranilate and operated as described in the materials and methods section. A

 https://doi.org/10.1099/mic.0.000982

typical batch fermentation profile of *P. putida* under anthranilate stress is shown in Figure 4, separated into the different phases of substrate consumption and conversion.

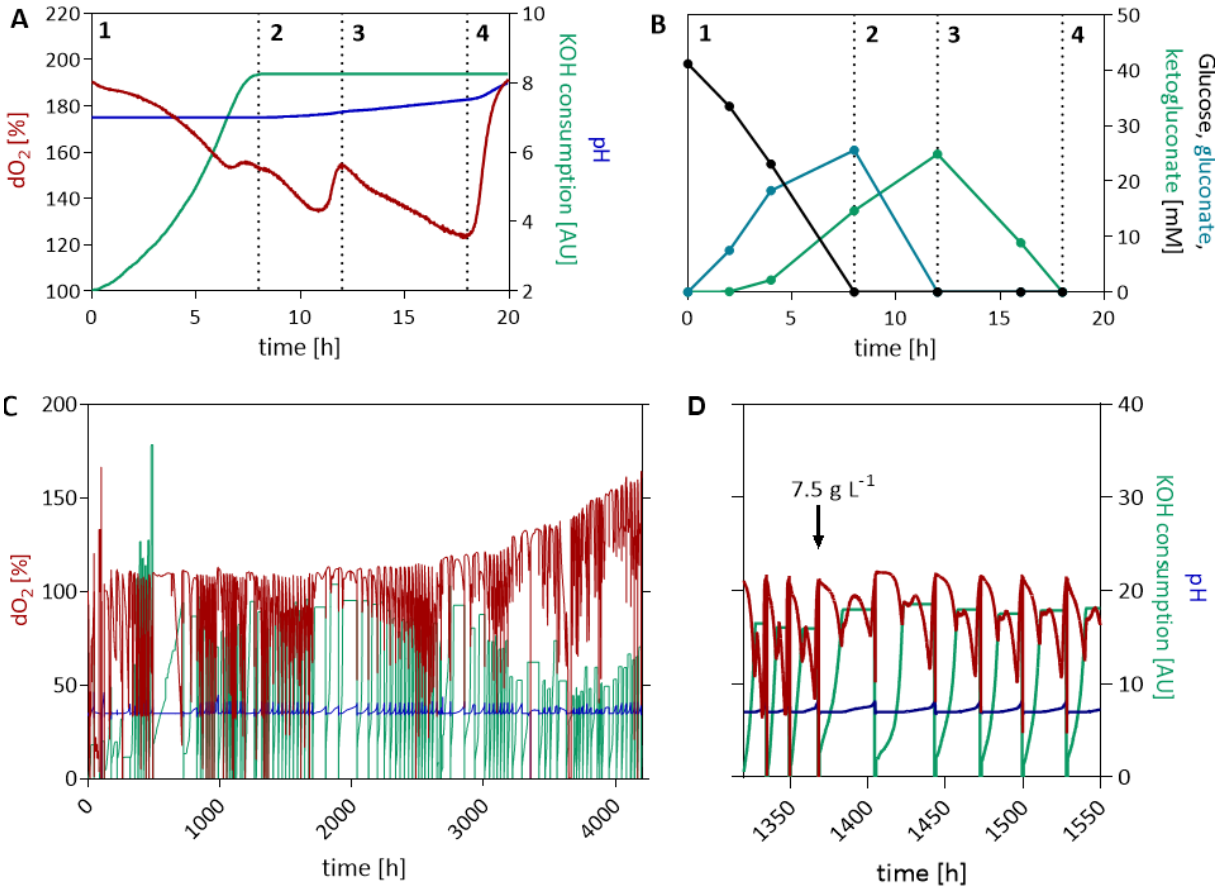

Figure 4. Sequential batch fermentation of *P. putida* in the presence of anthranilate. (A) A typical batch fermentation profile of *P. putida* on MSM with 20 mM glucose as sole carbon source under anthranilate stress. The black lines indicate the different phases of consumption and conversion of glucose, gluconate and 2-ketogluconate. red: dO_2 [%], blue: pH, green: KOH consumption [AU]). Dissolved oxygen values of >100% are due to signal drift of the Clark electrode over the long incubation time. (B) Formation and consumption of C6-substrates. 1: Beginning of the batch, the cells grow primarily on glucose, 2: All glucose is consumed or converted, the cells grow primarily on gluconate, 3: All gluconate is consumed or converted, the cells grow primarily on 2-ketogluconate, 4: End of the batch, all glucose, gluconate and 2-ketogluconate are consumed. (C) During 112 batches within 4200 hours of sequential batch fermentation the initial concentration of 5 g L^{-1} was gradually increased to 15 g L^{1} in steps of 0.25 - 1 g L^{-1} anthranilate. An exemplary series of batches after an increase of the anthranilate concentration to 7.5 g L^{-1} is shown on the right (D).

The trigger for initiating a new batch of the sequential batch fermentation strategy was based on the pH shift in the cultivation broth of *P. putida* when growing on glucose. During growth of *P. putida* in a batch fermentation, glucose is converted into gluconate by the glucose dehydrogenase and further into 2-ketogluconate by the gluconate dehydrogenase[63,64]. Both glucose oxidation products are secreted from the periplasmic space leading to an acidification of the medium. Consequently, the fermentation medium was titrated with 2 M KOH. When the glucose is fully consumed, *P. putida* takes up gluconate and later 2-ketogluconate to form more biomass. By the consumption of these organic acids the pH of the fermentation medium increases, which was not compensated by the addition of acid. The consequent rise in pH marked the trigger of a new batch as described in the materials and methods section, removing

most of the culture broth and adding fresh medium. The initial anthranilate concentration of 5 g L^{-1} was manually increased at indicated time points. During the adaptation to increasing amounts of anthranilate in the sequential batches of the fermentation the strain *P. putida* oAB16 was eventually able to tolerate concentrations of 15 g L^{-1} anthranilate after about 4200 hours (112 batches, Figure 4 C). It was further observed that the longer the cells were grown in the sequential batch fermentation in the presence of a certain concentration of anthranilate, the shorter the process time was for one batch and the higher the final OD_{600} was at the end of a batch (Figure 4D). For some batches, the concentrations of glucose, gluconate and 2-ketogluconate and the presence of anthranilate and glycosyl-anthranilate were monitored by HPLC. This confirmed that glucose, gluconate and 2-ketogluconate were completely consumed by the end of the batch (Figure 4 B). A decrease of the anthranilate concentration, either through catabolism or glycosylation was not observed.

A different strategy was developed to increase the tolerance of *C. glutamicum* towards anthranilate. Three bioreactors were inoculated with overnight cultures of the wild-type strain. The initial fermentation was performed in CGXII-medium with anthranilate added gradually during the growth phase. A typical batch fermentation profile under anthranilate stress is shown in Figure 5.

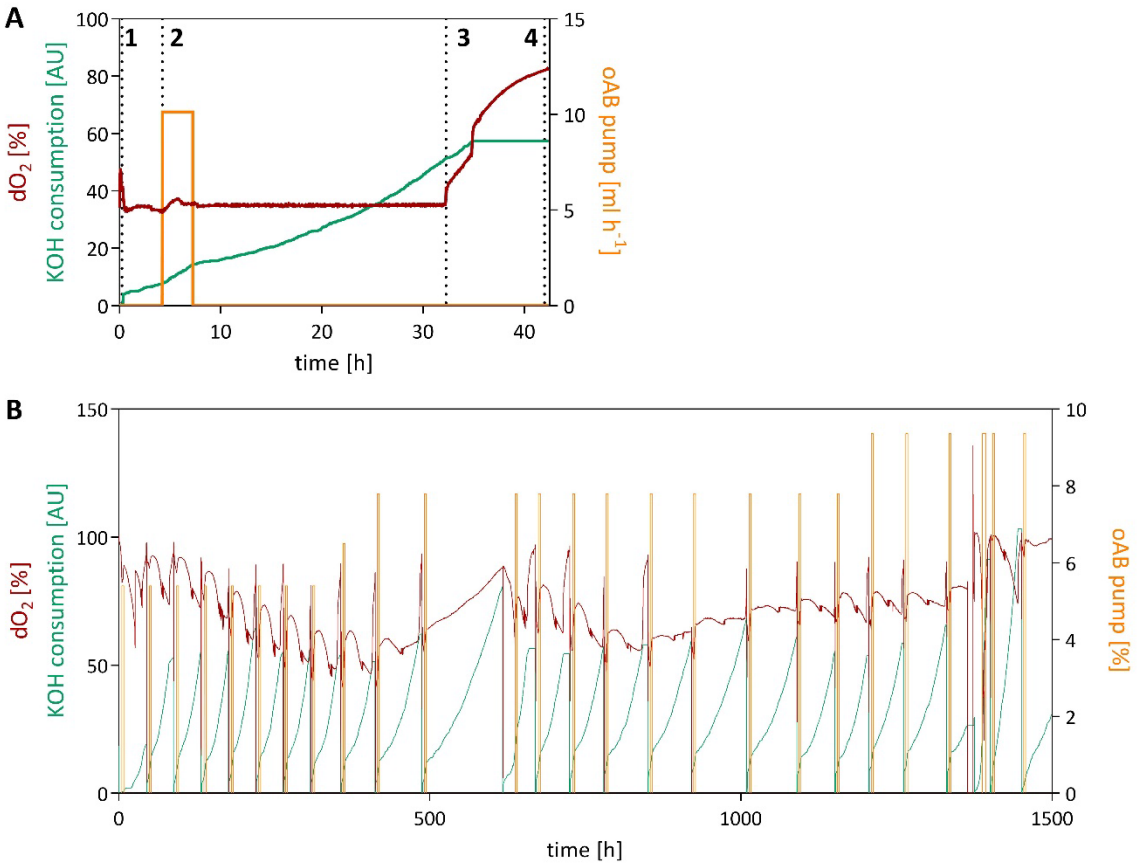

Figure 5. Sequential batch fermentation of *C. glutamicum* in the presence of anthranilate. (A) A typical batch fermentation profile of *C. glutamicum* with glucose as sole carbon source under anthranilate stress. (red: dO_2[%], green: KOH consumption [AU], orange: anthranilate pump [%]). The black lines indicate the different phases of one batch. 1: Beginning of batch, 2: Start of anthranilate addition, 3: Glucose is limited, beginning of starvation. 4: End of batch, glucose is consumed. (B) During 24 batches within 1400 hours of sequential batch fermentation the initial concentration of 15 g L^{-1} was gradually increased to 25.9 g L^{-1}.

The pH was maintained at pH 7 with 2 N KOH since *C. glutamicum* acidifies the medium during growth. At the end of a batch fermentation an increase in dO_2 can be observed as a consequence of starvation, which was set as a trigger to start the next batch. The addition of anthranilate was realized by gradually pumping the required volume of a 200 g L^{-1} anthranilate stock into the fermenter from hours 4 to 7. Fermenters were sampled at different time points for analysis and cryo conservation. With this strategy, 26 sequential batches over a process time of about 1,400 hours were performed per reactor (Figure 5). The anthranilate concentration was increased from initially 15 g L^{-1} to a maximum of 25.9 g L^{-1} anthranilate (Figure 5B). The concentration of glucose, anthranilate, and glycosyl-anthranilate was monitored by HPLC as described above. The same phenomenon described in Figure 2 was also observed during the sequential batch fermentations. During the anthranilate addition phase when the glucose concentration was high, about half of the anthranilate was spontaneously converted into glycosyl-anthranilate. As glucose was consumed, the concentration of glycosyl-anthranilate decreased to zero resulting in the anthranilate concentration that was initially added to the culture.

3.2.3 Single mutant selection

Cryo conservation samples were taken from the SBF at different time points during ALE. . These samples were diluted and inoculated on agar plates with anthranilate to evaluate the tolerance enhancement of evolved populations. Figure 6 shows an example from one sequential batch fermentation of *C. glutamicum*. Samples from ALE later time points showed a clear increase of colony forming units (CFU) on CGXII plates with high anthranilate concentrations.

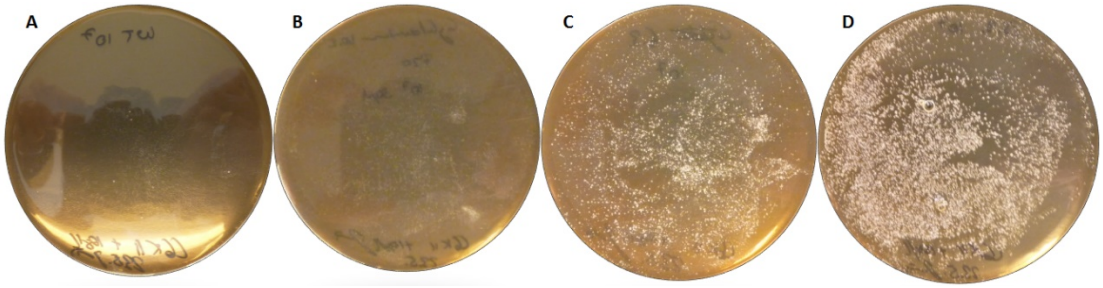

Figure 6. Agar plate evaluation of *C. glutamicum* cultures from one sequential batch fermentation on CGXII medium plates with 22 mM glucose supplemented with 10 g L^{-1} anthranilate. (A) *C. glutamicum* wild-type (B) SBF1 hour 336 (C) SBF1 hour 936 (D) SBF1 hour 1392 after 24h of incubation at 30°C.

ALE cryo cultures of *P. putida* (at 4200 h) and *C. glutamicum* (at 1400 h) were plated on agar plates containing up to 20 and 30 g L^{-1} anthranilate and single colonies were picked. To select the best performing single clones, growth of these single colonies was monitored in liquid cultures in a growth profiler® (Enzyscreen, The Netherlands). Twelve evolved mutants of *P. putida* from each reactor were cultivated in 96-well plates in MSM containing 20 mM of glucose. No growth defects were observed compared to the wild-type in the control condition (0 g L^{-1} anthranilate). In the presence of 2 g L^{-1} anthranilate, the evolved strains of *P. putida* showed varying fitness levels indicated by both growth rate and final optical density on glucose. While some clones show improved growth rates and reach higher biomass, some also grew significantly worse under anthranilate stress compared to the wild-type. Interestingly, there is a clear difference in performance under anthranilate stress of the populations obtained from the

two parallel sequential batch fermentations. Colonies from fermenter 1 showed impaired growth, while colonies from fermenter 2 showed improved performance under anthranilate stress (Figure 7A). For *C. glutamicum*, eight isolated mutants from each of the three reactors were cultivated in 96-well plates in the growth profiler in CGXII-medium supplemented with 10 g L^{-1} anthranilate prior to inoculation. No growth defects were observed compared to the wild-type in the control condition (0 g L^{-1} anthranilate) (Data not shown). In the presence of anthranilate, the evolved strains of *C. glutamicum* overall showed enhanced tolerance compared to the wild-type. Figure 7 shows the growth rate and OD_{600} on glucose of isolated strains in the presence of anthranilate compared to the wild-type. While most strains showed an improved growth rate in the presence of anthranilate, the biomass generation of most strains remained below that of the wild-type. Colonies isolated from the three fermenters showed similar behavior when exposed to anthranilate (Figure 7B).

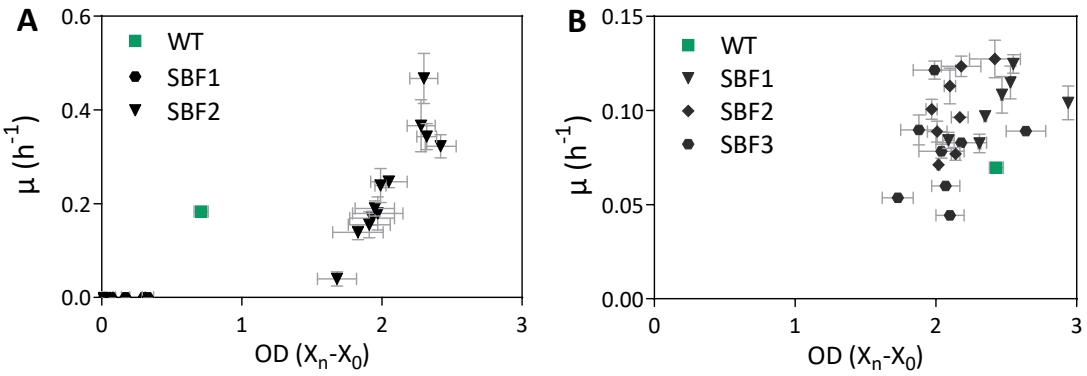

Figure 7. (A) Growth rate (μ) and biomass formation of isolated evolved colonies of *P. putida* from fermenter 1 (SBF1) and fermenter 2 (SBF2) compared to the un-evolved wild-type in MSM containing 20 mM glucose and 2 g L^{-1} anthranilate. (B) Growth rate (μ) and OD of isolated colonies of *C. glutamicum* from fermenter 1 (SBF1), fermenter 2 (SBF2) and 3 (SBF3) compared to the un-evolved wild-type in CGXII-medium containing 22 mM glucose 10 g L^{-1} anthranilate. Error bars represent the standard deviation (n=4).

3.2.4 Evaluation of selected ALE strains

The two most promising strains of *P. putida* were re-named oAB128.1 and oAB128.2 and cultivated in controlled bioreactors in the presence of 10 g L^{-1} anthranilate (Figure 8). In contrast to the wild-type, both evolved strains can grow in the presence of 10 g L^{-1} anthranilate. Under these conditions, the best performing evolved *P. putida* strain oAB128.1 has 33 % of the growth rate and 68 % of the final OD_{600} compared to the wild-type growing without anthranilate stress. The SBF of *P. putida* was continued after the last sampling at 4200 h as a backup measure. One batch of this SBF from hour 4630 to 4660 (the anthranilate concentration in the reactor was at 15 g L^{-1} at this point) was analyzed in parallel in order to assess the effect of adaptation versus evolution. The growth rate of this strain at 15 g L^{-1} was comparable to strain *P. putida* oAB128.1 at 10 g L^{-1}, but the culture had a shorter lag phase compared to *P. putida* oAB128.1. Cryo

 https://doi.org/10.1099/mic.0.000982

conservation and passing of the strains on rich medium had no effect on the phenotype of improved tolerance.

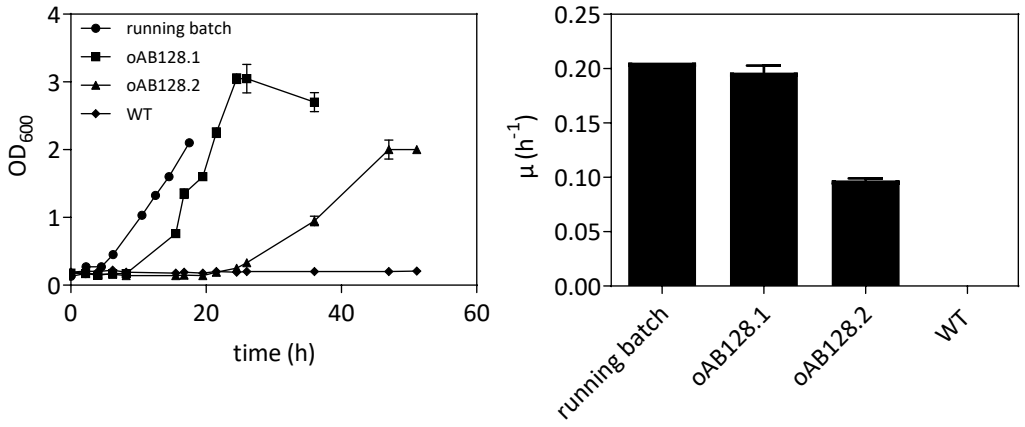

Figure 8. Growth behavior and growth rates in controlled bioreactors of *P. putida KT2440* (WT), oAB128.1 and oAB128.2 in the presence of 10 g L^{-1} anthranilate and of the running batch fermentation in the presence of 15 g L^{-1} anthranilate. Error bars represent the standard error (n=2).

Two of the best performing evolved strains of *C. glutamicum*, now named oAB26.1 and oAB26.2, were subsequently evaluated in batch fermentations in controlled bioreactors. The fermenters were supplemented with three manual anthranilate injections at hour 3, 4 and 5, which resulted in final concentrations of 15 or 25 g L^{-1} anthranilate in the reactor (Figure 9). In comparison to the wild-type, both evolved strains grew to a higher final OD_{600} at both concentrations of anthranilate. While growth of Evo26.1 do not significantly differ from the wild type, the best performing evolved strain *C. glutamicum* oAB26.2 has a 2.2-fold increased growth rate and 1.4-fold higher final OD_{600} compared to the wild-type at 25 g L^{-1} anthranilate.

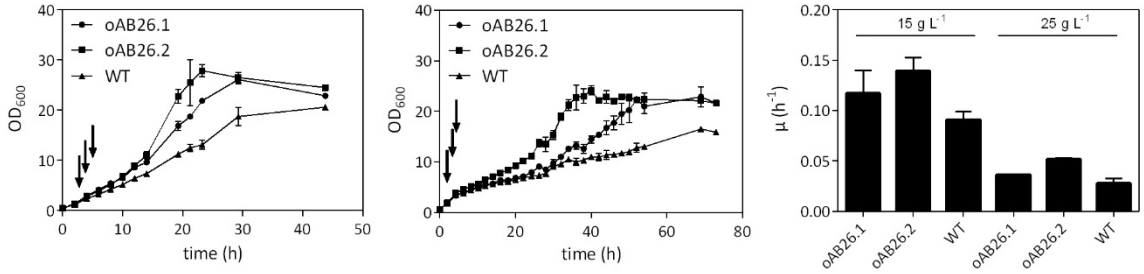

Figure 9. Growth behavior of *C. glutamicum* oAB26.1 and oAB26.2 in controlled bioreactors in CGXII medium in the presence of 15 (A) and 25 (B) g L^{-1} anthranilate. The arrows mark the time point of pulsed anthranilate addition. Error bars represent the standard error (n=2).

Table 1 shows a comparison of the evolved strains of *P. putida* KT2440 and *C. glutamicum* ATCC 13032 compared to the respective wild-types at different concentrations of anthranilate. The tolerance towards anthranilate was improved for both organisms by applying evolutionary engineering techniques. In direct comparison between the two organisms, the wild-type of *P. putida* KT2440 may have slightly higher growth rates, but an overall higher sensitivity towards

anthranilate than *C. glutamicum*. Growth of wild-type *P. putida* KT2440 is completely inhibited in the presence of 10 g L^{-1} anthranilate, whereas the evolved strains can grow at this concentration. The wild-type of *C. glutamicum* can already grow in the presence of 25 g L^{-1} anthranilate, however with only 10 % of the reference growth rate (0 g L^{-1} anthranilate) and lower biomass formation. Evolution yielded a strain that reached 22 % of the growth rate and virtually the same final OD_{600} at 25 g L^{-1} anthranilate compared to the wild-type without anthranilate stress.

Table 1. Comparison of the growth rate and the final OD_{600} of *P. putida* KT2440 and *C. glutamicum* wild-type and evolved strains from sequential batch fermentations at different anthranilate concentrations. (-) indicates no growth at the indicated concentration.

Strain	**anthranilate [g L^{-1}]**	**μ [h^{-1}]**	**max OD_{600}**	**$\mu_{(Evo)}/\mu_{(ref.)}$ [%]**	**$OD_{600\ (Evo)}$ / $OD_{600\ (ref.)}$ [%]**
P. putida WT	0	0.49 ±0.01	4.2 ±0.1	Ref. condition	Ref. condition
P. putida WT	5	0.21 ±0.05	2.4 ±0.1	41.8 ±7.2	55.3 ±2.6
P. putida WT	10	-	-	-	-
P. putida oAB128.1	10	0.19 ±0.01	3.1 ±0.1	38.9 ±1.5	72.6 ±1.7
P. putida oAB128.2	10	0.10 ±0.00	2.1 ±0.1	19.9 ±0.7	48.8 ±1.7
C. glutamicum WT	0	0.24 ±0.02	27.6 ±0.02	Ref. condition	Ref. condition
C. glutamicum WT	15	0.11 ±0.00	20.6 ±0.5	48.4 ±3.2	74.7 ±1.9
C. glutamicum WT	25	0.03 ±0.00	16.4 ±0.2	10.2 ±1.0	59.3 ±0.6
C. glutamicum oAB26.1	15	0.13 ±0.00	26.1 ±0.8	55.8 ±1.1	94.6 ±3.1
C. glutamicum oAB26.2	15	0.16 ±0.02	27.5 ±0.4	68.1 ±6.2	99.6 ±1.3
C. glutamicum oAB26.1	25	0.04 ±0.00	23.1 ±1.5	15.4 ±1.3	83.8 ±5.8
C. glutamicum oAB26.2	25	0.05 ±0.00	24.6 ±1.7	22.1 ±0.7	89.2 ±0.6

4 Discussion

In this study, *C. glutamicum* and *P. putida* displayed different reactions to anthranilate stress. The initial tolerance of wild-type strain of *P. putida* was significantly lower than that of *C. glutamicum*. A recently published study showed a similar trend for toxicity tests with PABA, with *C. glutamicum* growing in the presence of up to 55 g L^{-1} PABA, while growth of the strain *P. putida* S12 was inhibited at concentrations above 13.7 g L^{-1} [28]. However, the impact of ALE on *C. glutamicum* was less significant. The fact that *C. glutamicum* is only affected in growth rate, and not biomass yield, suggests a more passive tolerance towards anthranilate that does not require energy. Its robust cell envelope has shown to be impenetrable to a variety of compounds including antibiotics and drugs.[65–67] This passive defense of the cell wall apparently provides excellent protection against anthranilate, but the cells do suffer from lower growth rates, likely due to plasmolysis from osmotic pressure[18,68] and enzyme inhibition by

anthranilate. In contrast, with *P. putida* anthranilate stress rather results in decreased biomass yield. This indicates a higher maintenance demand, suggesting an active, energy consuming mechanism of resistance towards anthranilate. Strains of *P. putida* can adapt their inner and outer membrane by changing the lipid composition through increased lipid turnover and altered expression of membrane proteins.[69] The induction of chaperones such as GroESL can help to alleviate protein misfolding caused by plasmolysis. Also and the active export of anthranilate may be a possibility[35,44,70]. The extra energy required to induce and maintain these mechanisms is reflected by reduced biomass formation.

ALE was instrumental in increasing anthranilate tolerance for both organisms. *C. glutamicum* shows a higher tolerance in terms of the maximal concentration of anthranilate, but has a much lower innate growth rate than *P. putida*. The adapted mutants *C. glutamicum* oAB26.1 and oAB26.2 grew with rates between 0.13-0.15 h^{-1} in the presence of 15 g L^{-1} anthranilate, while *P. putida* oAB128.1 reaches a growth rate of 0.2 h^{-1} in the presence of 10 g L^{-1}, while growing at a similar rate in the running batch with 15 g L^{-1} anthranilate. The latter suggests the possibility of further improvement by pre-incubating the cells with the stressor. This so-called adaptation is a common strategy for realizing the full potential of solvent-tolerant Pseudomonads.[71] If this can be realized, the growth rate of *P. putida* is likely higher than that of *C. glutamicum* under similar stress conditions. However, *C. glutamicum* was cultivated for a total of 26 batch fermentations (~169 generations). In contrast, *P. putida* was pre-cultivated in shake flasks and in an SBF during 112 batch fermentations (~528 generations), giving more time for beneficial mutations to occur. For comparison, ALE experiments for thermotolerance indicate a difference in the number of total generations required for the development of stable mutations in different species. While mutations due to ALE towards thermotolerance occurred after 100-200 generations in *E. coli*[72], around 370 generations were required to obtain comparable phenotypes of *C. glutamicum*[73]. As both experiments were performed in different laboratories, these numbers only give a rough estimate for the effects of ALE with different organisms. It does however compare to the fact that in our study, short-term ALE in shake flasks did not show tolerance improvements for *C. glutamicum*, in contrast to *P. putida*.

Growth of *C. glutamicum* was significantly enhanced when supplied with anthranilate during the early exponential growth phase. In a biotechnological context, a similar gradual increase of anthranilate during growth can be expected. This later addition likely reduces the overall stress level compared to *ab inito* addition of a similar anthranilate concentration. This makes direct comparison of the tolerance levels of the two organisms difficult. This is further exacerbated by the glycosylation of anthranilate. Throughout the fermentation, the consumption of glucose shifted the equilibrium of the glycosylation in the reverse direction, causing anthranilate concentrations to increase back to the initially added level at the end of the culture. Since wildtype *P. putida* oxidizes glucose to gluconate and 2-ketogluconate, it causes the glucose concentration to decrease much more rapidly than *C. glutamicum*. The cultivation media for ALE and tolerance assessments could be unified for both bacteria to allow a more direct comparison. However, this would mean that each organism is evaluated in sub-optimal conditions, making it less relevant for an eventual biotechnological application.

Dilution of CGXII medium components had an interesting effect on the growth of *C. glutamicum*. Without anthranilate stress, the diluted medium led to a lower growth rate. With

anthranilate stress, the reverse effect was observed and growth rates increased with decreasing ammonium concentrations. Assuming that glycosyl-anthranilate is less toxic than anthranilate, as was shown for PABA[28], this indicates that glycosyl-anthranilate is still formed in spite of the lower concentration of ammonium as the catalyst for glycosylation. The observed effect likely is a combination of anthranilate and ammonium toxicity, as it leads to an increased osmotic pressure in the medium[18]. However, as this effect is rather small, evolution experiments were performed in medium with high ammonium concentrations. This is closer to the envisioned industrial process which demands high ammonium concentrations, given that anthranilate is an amine.

Growth profiler analysis of the evolved single mutants of *P. putida* showed differences in performance under anthranilate stress between the single mutants obtained from the two parallel SBFs. In these screening conditions, clones from fermenter 1 showed significantly impaired growth under anthranilate stress compared to the wild-type. In contrast, single mutants of *C. glutamicum* evolved in three parallel fermentations overall show comparable phenotypes towards anthranilate stress. Evolution inherently leads to a mixed population of individual cells with varying degrees of fitness. While parallelism between replicate cultures is a common observation in ALE, the divergence in evolutionary pathways can drive the evolution of multiple traits and lead to differently evolved populations[74,75]. This can possibly further lead to the emergence of specialized subpopulations, resulting in symbiotic growth behavior to counteract the applied stress condition during ALE. In this case, the isolation of single colonies will lead to a loss of growth advantage in the presence of anthranilate for the individual mutants, as observed for colonies from fermenter 1 of *P. putida*. However, no growth defect was observed on agar plates with anthranilate, rather suggesting a difference in tolerance in sessile versus planktonic growth. Possibly, stable biofilm or microcolony formation[76,77] in the *P. putida* SFB1 was disrupted by the isolation procedure, leading to the reduced growth in the subsequent liquid culture.

5 Conclusions

Using adaptive laboratory evolution, strains of *C. glutamicum* and *P. putida* with enhance anthranilate tolerance were obtained. The foundation of the evolution during ALE is genetic mutation. To gain deeper insight into the genetic basis of the improved tolerance of both organisms, a systems analysis is required. Sequencing of the genome of evolved strains should give insights into mutations that occurred during ALE and set a starting point for characterization and reverse engineering of anthranilate tolerance mechanisms. In addition, transcriptome or proteome analysis can unravel changes in the expression of tolerance mechanisms and the underlying regulatory cascades. Ultimately, the implementation of production pathways for anthranilate into the evolved strains will be required to ascertain whether the higher stress-tolerance towards externally added anthranilate will also benefit the microbial production of this compounds. There is a difference between externally imposed anthranilate and the intracellular production of the same molecule. In the latter case, an impenetrable barrier will likely hinder anthranilate production, especially in the absence of secretion mechanisms.

6 Conflict of Interest

The authors declare that the research was conducted as part of a contract research between Bayer AG and RWTH Aachen University.

7 Author Contributions

NW, LMB, SB and GJ conceived the study. NW supervised the study with the help of GJ, SB, JM und LMB. NW, JK, SB and GJ designed the experiments. JK, SB und JD performed the experiments. All authors analyzed and interpreted the data. MO wrote the manuscript with the help of NW and JK. All authors have read and approved the manuscript.

8 Funding

NW and MO were supported by the German Research Foundation through the Emmy Noether project WI 4255/1-1.

9 References

(1) Ghobadi, M., and Jafari, H. R. (2015) Environmental impact assessment of petrochemical industry using fuzzy rapid impact assessment matrix. *J. Pet. Environ. Biotechnol. 06*, 127.

(2) Cho, C., Choi, S. Y., Luo, Z. W., and Lee, S. Y. (2015) Recent advances in microbial production of fuels and chemicals using tools and strategies of systems metabolic engineering. *Biotechnol. Adv. 33*, 1455–1466.

(3) Wynands, B., Lenzen, C., Otto, M., Koch, F., Blank, L. M., and Wierckx, N. (2018) Metabolic engineering of *Pseudomonas taiwanensis* VLB120 with minimal genomic modifications for high-yield phenol production. *Metab. Eng. 47*, 121–133.

(4) Vargas-Tah, A., Martínez, L. M., Hernández-Chávez, G., Rocha, M., Martínez, A., Bolívar, F., and Gosset, G. (2015) Production of cinnamic and *p*-hydroxycinnamic acid from sugar mixtures with engineered *Escherichia coli*. *Microb. Cell Fact. 14*, 6.

(5) Nijkamp, K., Westerhof, R. G. M., Ballerstedt, H., De Bont, J. a M., and Wery, J. (2007) Optimization of the solvent-tolerant *Pseudomonas putida* S12 as host for the production of *p*-coumarate from glucose. *Appl. Microbiol. Biotechnol. 74*, 617–624.

(6) McKenna, R., and Nielsen, D. R. (2011) Styrene biosynthesis from glucose by engineered *E. coli*. *Metab. Eng. 13*, 544–54.

(7) Kunjapur, A. M., Tarasova, Y., and Prather, K. L. J. (2014) Synthesis and accumulation of aromatic aldehydes in an engineered strain of *Escherichia coli*. *J. Am. Chem. Soc. 136*, 11644–11654.

(8) Shafiq, M., Zia-Ur-Rehman, M., Khan, I. U., Arshad, M. N., and Khan, S. A. (2011) Synthesis of novel anti-bacterial 2,1-benzothiazine 2,2-dioxides derived from methyl anthranilate. *J. Chil. Chem. Soc. 56*, 527–531.

(9) Sun, X., Lin, Y., Huang, Q., Yuan, Q., and Yan, Y. (2013) A novel muconic acid biosynthesis approach by shunting tryptophan biosynthesis via anthranilate. *Appl. Environ. Microbiol. 79*, 4024–4030.

(10) Raffensperger SP, Park P, V. R. (1961) Stabilization of grape flavored soft drink mixes

containing methyl anthranilate. Patent US 3005715 A.

(11) Jaeger, G., Magnus, J., and Moussa, A. S. (2015) Production of aniline via anthranilate. Patent WO2015124686A1.

(12) McCullough, W. G., Piligian, J. T., and Daniel, I. J. (1957) Enzymatic decarboxylation of the aminobenzoates. *J. Am. Chem. Soc. 79*, 628–630.

(13) Balderas-Hernandez, V. E., Sabido-Ramos, A., Silva, P., Cabrera-Valladares, N., Hernandez-Chavez, G., Baez-Viveros, J. L., Martinez, A., Bolivar, F., and Gosset, G. (2009) Metabolic engineering for improving anthranilate synthesis from glucose in *Escherichia coli*. *Microb. Cell Fact. 8*, 19.

(14) Cooper, I. B., and Euler, K. (1995) Production of anthranilic acid by a strain of *Bacillus subtillis* resistant to sulfaguanidine and fluorotryptophan. *US 5422256 A* 2–6.

(15) Jaeger, G., Magnus, J., Moussa, A. S., Olf, G., Lolli, G., Behnken, S., Youn, J. W., Yalfani, M., and Sprenger, G. (2015) Recombinant strain producing *o*-aminobenzoate and fermentative production of aniline from renewable resources via 2-aminobenzoic acid. Patent WO/2015/124687

(16) Kuepper, J., Dickler, J., Biggel, M., Behnken, S., Jäger, G., Wierckx, N., and Blank, L. M. (2015) Metabolic engineering of *Pseudomonas putida* KT2440 to produce anthranilate from glucose. *Front. Microbiol. 6*, 1–9.

(17) Li, X.-H., Kim, S.-K., and Lee, J.-H. (2017) Anti-biofilm effects of anthranilate on a broad range of bacteria. *Sci. Rep. 7*, 8604.

(18) Csonka, L. N. (1989) Physiological and genetic responses of bacteria to osmotic stress. *Microbiol. Rev. 53*, 121–47.

(19) McKenna, R., Thompson, B., Pugh, S., and Nielsen, D. R. (2014) Rational and combinatorial approaches to engineering styrene production by *Saccharomyces cerevisiae*. *Microb. Cell Fact. 13*, 123.

(20) Schwanemann, T., Wynands, B., Otto, M., and Wierckx, N. (2020) *Pseudomonas* as versatile aromatics cell factory. *Biotechnol. J. in press*

(21) Domröse, A., Klein, A. S., Hage-Hülsmann, J., Thies, S., Svensson, V., Classen, T., Pietruszka, J., Jaeger, K. E., Drepper, T., and Loeschcke, A. (2015) Efficient recombinant production of prodigiosin in *Pseudomonas putida*. *Front. Microbiol. 6*, 1–10.

(22) McKenna, R., Moya, L., McDaniel, M., and Nielsen, D. R. (2015) Comparing *in situ* removal strategies for improving styrene bioproduction. *Bioprocess Biosyst. Eng. 38*, 165–174.

(23) Eggeling, L., and Bott, M. (2005) Handbook of *Corynebacterium glutamicum*. Taylor & Francis. ISBN 9780849318214

(24) Becker, J., Gießelmann, G., Hoffmann, S. L., and Wittmann, C. (2016) *Corynebacterium glutamicum* for sustainable bioproduction: From metabolic physiology to systems metabolic engineering. *Adv. Biochem. Eng. Biotechnol. 162*, 217–263.

(25) Becker, J., and Wittmann, C. (2012) Bio-based production of chemicals, materials and fuels – *Corynebacterium glutamicum* as versatile cell factory. *Curr. Opin. Biotechnol. 23*, 631–640.

(26) Baritugo, K.-A., Kim, H. T., David, Y., Choi, J., Hong, S. H., Jeong, K. J., Choi, J. H., Joo, J. C., and Park, S. J. (2018) Metabolic engineering of *Corynebacterium glutamicum* for fermentative

production of chemicals in biorefinery. *Appl. Microbiol. Biotechnol. 102*, 3915–3937.

(27) Kogure, T., and Inui, M. (2018) Recent advances in metabolic engineering of *Corynebacterium glutamicum* for bioproduction of value-added aromatic chemicals and natural products. *Appl. Microbiol. Biotechnol. 102*, 8685–8705.

(28) Kubota, T., Watanabe, A., Suda, M., Kogure, T., and Hiraga, K. (2016) Production of *para*-aminobenzoate by genetically engineered *Corynebacterium glutamicum* and non-biological formation of an N-glucosyl byproduct. *Metab. Eng. 38*, 322–330.

(29) Kallscheuer, N., Classen, T., Drepper, T., and Marienhagen, J. (2019) Production of plant metabolites with applications in the food industry using engineered microorganisms. *Curr. Opin. Biotechnol. 56*, 7–17.

(30) Jordan, S., Hutchings, M. I., and Mascher, T. (2008) Cell envelope stress response in Gram-positive bacteria. *FEMS Microbiol. Rev. 32*, 107–146.

(31) Dover, L. G., Cerdeño-Tárraga, A. M., Pallen, M. J., Parkhill, J., and Besra, G. S. (2004) Comparative cell wall core biosynthesis in the mycolated pathogens, *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*. *FEMS Microbiol. Rev. 28*, 225–250.

(32) Liu, Y. B., Long, M. X., Yin, Y. J., Si, M. R., Zhang, L., Lu, Z. Q., Wang, Y., and Shen, X. H. (2013) Physiological roles of mycothiol in detoxification and tolerance to multiple poisonous chemicals in *Corynebacterium glutamicum*. *Arch. Microbiol. 195*, 419–429.

(33) Lee, S. Y., Kim, B. N., Han, J. H., Chang, S. T., Choi, Y. W., Kim, Y. H., and Min, J. (2010) Treatment of phenol-contaminated soil by *Corynebacterium glutamicum* and toxicity removal evaluation. *J. Hazard. Mater. 182*, 937–940.

(34) Rojas, A., Duque, E., Schmid, A., Hurtado, A., Ramos, J. L., and Segura, A. (2004) Biotransformation in double-phase systems: Physiological responses of *Pseudomonas putida* DOT-T1E to a double phase made of aliphatic alcohols and biosynthesis of substituted catechols. *Appl. Environ. Microbiol. 70*, 3637–3643.

(35) Mukhopadhyay, A. (2015) Tolerance engineering in bacteria for the production of advanced biofuels and chemicals. *Trends Microbiol. 23*, 498–508.

(36) Verhoef, S., Wierckx, N., Westerhof, R. G. M., de Winde, J. H., and Ruijssenaars, H. J. (2009) Bioproduction of *p*-hydroxystyrene from glucose by the solvent-tolerant bacterium *Pseudomonas putida* S12 in a two-phase water-decanol fermentation. *Appl. Environ. Microbiol. 75*, 931–936.

(37) Isken, S., Derks, A., Wolffs, P. F. G., and De Bont, J. A. M. (1999) Effect of organic solvents on the yield of solvent-tolerant *Pseudomonas putida* S12. *Appl. Environ. Microbiol. 65*, 2631–2635.

(38) Ramos, J. L., Duque, E., Gallegos, M.-T., Godoy, P., Ramos-Gonzalez, M. I., Rojas, A., Teran, W., and Segura, A. (2002) Mechanisms of solvent tolerance in Gram-negative bacteria. *Annu. Rev. Microbiol. 56*, 743–768.

(39) Isken, S., and de Bont, J. A. M. (1998) Bacteria tolerant to organic solvents. *Extremophiles 2*, 229–238.

(40) Belda, E., van Heck, R. G. A., José Lopez-Sanchez, M., Cruveiller, S., Barbe, V., Fraser, C., Klenk, H. P., Petersen, J., Morgat, A., Nikel, P. I., Vallenet, D., Rouy, Z., Sekowska, A., Martins dos Santos, V. A. P., de Lorenzo, V., Danchin, A., and Médigue, C. (2016) The revisited genome of *Pseudomonas putida* KT2440 enlightens its value as a robust metabolic chassis. *Environ. Microbiol.*

18, 3403–3424.

(41) Kieboom, J., Dennis, J. J., Zylstra, G. J., and de Bont, J. A. (1998) Active efflux of organic solvents by *Pseudomonas putida* S12 is induced by solvents. *J. Bacteriol. 180*, 6769–6772.

(42) Velasco, a, Alonso, S., García, J. L., Perera, J., and Díaz, E. (1998) Genetic and functional analysis of the styrene catabolic cluster of *Pseudomonas* sp. strain Y2. *J. Bacteriol. 180*, 1063–71.

(43) Verhoef, S., Ballerstedt, H., Volkers, R. J. M., de Winde, J. H., and Ruijssenaars, H. J. (2010) Comparative transcriptomics and proteomics of *p*-hydroxybenzoate producing *Pseudomonas putida* S12: novel responses and implications for strain improvement. *Appl. Microbiol. Biotechnol. 87*, 679–690.

(44) Calero, P., Jensen, S. I., Bojanovič, K., Lennen, R. M., Koza, A., and Nielsen, A. T. (2018) Genome-wide identification of tolerance mechanisms toward *p*-coumaric acid in *Pseudomonas putida*. *Biotechnol. Bioeng. 115*, 762–774.

(45) Yim, H., Haselbeck, R., Niu, W., Pujol-Baxley, C., Burgard, A., Boldt, J., Khandurina, J., Trawick, J. D., Osterhout, R. E., Stephen, R., Estadilla, J., Teisan, S., Schreyer, H. B., Andrae, S., Yang, T. H., Lee, S. Y., Burk, M. J., and Van Dien, S. (2011) Metabolic engineering of *Escherichia coli* for direct production of 1,4-butanediol. *Nat. Chem. Biol. 7*, 445–452.

(46) Dragosits, M., and Mattanovich, D. (2013) Adaptive laboratory evolution – principles and applications for biotechnology. *Microb. Cell Fact. 12*, 64.

(47) Maughan, H., and Nicholson, W. L. (2011) Increased fitness and alteration of metabolic pathways during *Bacillus subtilis* evolution in the laboratory. *Appl. Environ. Microbiol. 77*, 4105–4118.

(48) Kusumawardhani, H., Furtwängler, B., Blommestijn, M., Kaltenytė, A., Poel, J. van der, Kolk, J., Hosseini, R., and Winde, J. H. de. (2020) Adaptive laboratory evolution restores solvent tolerance in plasmid-cured *Pseudomonas putida* S12; a molecular analysis. *bioRxiv* 2020.08.01.232264.

(49) Lim, H. G., Fong, B., Alarcon, G., Magurudeniya, H. D., Eng, T., Szubin, R., Olson, C. A., Palsson, B. O., Gladden, J. M., Simmons, B. A., Mukhopadhyay, A., Singer, S. W., and Feist, A. M. (2020) Generation of ionic liquid tolerant *Pseudomonas putida* KT2440 strains via adaptive laboratory evolution . *Green Chem. 22*, 5677–5690.

(50) Mohamed, E. T., Werner, A. Z., Salvachúa, D., Singer, C., Szostkiewicz, K., Jiménez-Díaz, M., Eng, T., Radi, M. S., Simmons, B. A., Mukhopadhyay, A., Herrgård, M. J., Singer, S. W., Beckham, G. T., and Feist, A. M. (2020) Adaptive laboratory evolution of *Pseudomonas putida* KT2440 improves p-coumaric and ferulic acid catabolism and tolerance. *Metab. Eng. Commun. 11*, e00143.

(51) Li, W., Jayakody, L. N., Franden, M. A., Wehrmann, M., Daun, T., Hauer, B., Blank, L. M., Beckham, G. T., Klebensberger, J., and Wierckx, N. (2019) Laboratory evolution reveals the metabolic and regulatory basis of ethylene glycol metabolism by *Pseudomonas putida* KT2440. *Environ. Microbiol. 21*, 3669–3682.

(52) Elmore, J. R., Dexter, G. N., Salvachúa, D., O'Brien, M., Klingeman, D. M., Gorday, K., Michener, J. K., Peterson, D. J., Beckham, G. T., and Guss, A. M. (2020) Engineered *Pseudomonas putida* simultaneously catabolizes five major components of corn stover lignocellulose: Glucose, xylose, arabinose, p-coumaric acid, and acetic acid. *Metab. Eng. 62*, 62–71.

(53) Bator, I., Wittgens, A., Rosenau, F., Tiso, T., and Blank, L. M. (2020) Comparison of three xylose pathways in *Pseudomonas putida* KT2440 for the synthesis of valuable products. *Front.*

Bioeng. Biotechnol. 7, 480.

(54) Wang, X., Khushk, I., Xiao, Y., Gao, Q., and Bao, J. (2018) Tolerance improvement of *Corynebacterium glutamicum* on lignocellulose derived inhibitors by adaptive evolution. *Appl. Microbiol. Biotechnol. 102*, 377–388.

(55) Oide, S., Gunji, W., Moteki, Y., Yamamoto, S., Suda, M., Jojima, T., Yukawa, H., and Inui, M. (2015) Thermal and solvent stress cross-tolerance conferred to *Corynebacterium glutamicum* by adaptive laboratory evolution. *Appl. Environ. Microbiol. 81*, 2284–2298.

(56) Wang, Y., Fan, L., Tuyishime, P., Liu, J., Zhang, K., Gao, N., Zhang, Z., Ni, X., Feng, J., Yuan, Q., Ma, H., Zheng, P., Sun, J., and Ma, Y. (2020) Adaptive laboratory evolution enhances methanol tolerance and conversion in engineered *Corynebacterium glutamicum*. *Commun. Biol. 3*, 1–15.

(57) Leßmeier, L., and Wendisch, V. F. (2015) Identification of two mutations increasing the methanol tolerance of *Corynebacterium glutamicum*. *BMC Microbiol. 15*, 1–11.

(58) Stella, R. G., Wiechert, J., Noack, S., and Frunzke, J. (2019) Evolutionary engineering of *Corynebacterium glutamicum*. *Biotechnol. J. 14*, 1800444.

(59) Wierckx, N. J. P., Ballerstedt, H., Bont, J. A. M. De, and Wery, J. (2005) Engineering of solvent-tolerant *Pseudomonas putida* S12 for bioproduction of phenol from glucose. *Appl. Environ. Microbiol.*

(60) Sanders, P., and Parks, L. (1962) Some observations on the fluorescence of anthranilic acid. *Anal. Biochem. 3*, 354–356.

(61) Araki, K., Takakura, H., Miyajima, Y., Akashi, Y., Kawanishi, K., Kakita, S., and Kondo, Y. (1999) Accumulation of anthranilic acid and N-glucosylanthranilic acid by a *Corynebacterium glutamicum* mutant resistant to DL-serine hydroxamate. *J. Gen. Appl. Microbiol. 45*, 169–176.

(62) Berger, L., and Lee, J. (1946) Arylamine-N-glycosides. Part I. Arylamine-N-D-ribopyranosides and N-D-ribofuranosides. *J. Org. Chem. 11*, 75–83.

(63) Daddaoua, A., Krell, T., and Ramos, J. L. (2009) Regulation of glucose metabolism in *Pseudomonas*. The phosphorylative branch and Entner-Doudoroff enzymes are regulated by a repressor containing a sugar isomerase domain. *J. Biol. Chem. 284*, 21360–21368.

(64) Wierckx, N., Ruijssenaars, H. J., de Winde, J. H., Schmid, A., and Blank, L. M. (2009) Metabolic flux analysis of a phenol producing mutant of *Pseudomonas putida* S12: Verification and complementation of hypotheses derived from transcriptomics. *J. Biotechnol. 143*, 124–129.

(65) Eggeling, L., and Sahm, H. (2001) The cell wall barrier of *Corynebacterium glutamicum* and amino acid efflux. *J. Biosci. Bioeng. 92*, 201–213.

(66) Alderwick, L. J., Harrison, J., Lloyd, G. S., and Birch, H. L. (2015) The mycobacterial cell wall-Peptidoglycan and arabinogalactan. *Cold Spring Harb. Perspect. Med. 5*, a021113.

(67) Lee, J.-Y., Seo, J., Kim, E.-S., Lee, H.-S., and Kim, P. (2013) Adaptive evolution of *Corynebacterium glutamicum* resistant to oxidative stress and its global gene expression profiling. *Biotechnol. Lett. 35*, 709–717.

(68) Wood, J. M. (2015) Bacterial responses to osmotic challenges. *J. Gen. Physiol. 145*, 381–8.

(69) Kusumawardhani, H., Hosseini, R., and de Winde, J. H. (2018) Solvent tolerance in bacteria: Fulfilling the promise of the biotech era? *Trends Biotechnol. 36*, 1025–1039.

(70) Segura, A., Molina, L., Fillet, S., Krell, T., Bernal, P., Muñoz-Rojas, J., and Ramos, J. L. (2012) Solvent tolerance in Gram-negative bacteria. *Curr. Opin. Biotechnol. 23*, 415–421.

(71) Weber, F. J., Ooijkaas, L. P., Schemen, R. M., Hartmans, S., and de Bont, J. A. (1993) Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. *Appl. Environ. Microbiol. 59*.

(72) Bennett, A., Dao, K., and Lenski, R. (1990) Rapid evolution in response to high-temperature selection. *Nature 245*, 118–143.

(73) Oide, S., Gunji, W., Moteki, Y., Yamamoto, S., Suda, M., Jojima, T., Yukawa, H., and Inui, M. (2015) Thermal and solvent stress cross-tolerance conferred to *Corynebacterium glutamicum* by adaptive laboratory evolution. *Appl. Environ. Microbiol. 81*, 2284–2298.

(74) Pelosi, L., Kühn, L., Guetta, D., Garin, J., Geiselmann, J., Lenski, R. E., and Schneider, D. (2006) Parallel changes in global protein profiles during long-term experimental evolution in *Escherichia coli*. *Genetics 173*, 1851–1869.

(75) Tenaillon, O., Rodriguez-Verdugo, A., Gaut, R. L., McDonald, P., Bennett, A. F., Long, A. D., and Gaut, B. S. (2012) The molecular diversity of adaptive convergence. *Science (80-.). 335*, 457–461.

(76) Tolker-Nielsen, T., Brinch, U. C., Ragas, P. C., Andersen, J. B., Jacobsen, C. S., and Molin, S. (2000) Development and dynamics of *Pseudomonas* sp. biofilms. *J. Bacteriol. 182*, 6482–9.

(77) López-Sánchez, A., Leal-Morales, A., Jiménez-Díaz, L., Platero, A. I., Bardallo-Pérez, J., Díaz-Romero, A., Acemel, R. D., Illán, J. M., Jiménez-López, J., and Govantes, F. (2016) Biofilm formation-defective mutants in *Pseudomonas putida*. *FEMS Microbiol. Lett.* (Rumbaugh, K., Ed.) *363*, fnw127.