

The Gram-negative soil bacterium *Pseudomonas putida* KT2440 emerges as biotechnological workhorse. However, it needs to be genetically manipulated to meet the challenges associated with industrial production processes. Accepting this as a challenge this thesis describes the development of a genetic tool that integrates into the genome of *P. putida* KT2440 resulting in a stable and reliable phenotype.

Another challenge of microbial production processes is the demand for reducing equivalents of numerous cellular natural and imposed non-natural reactions.

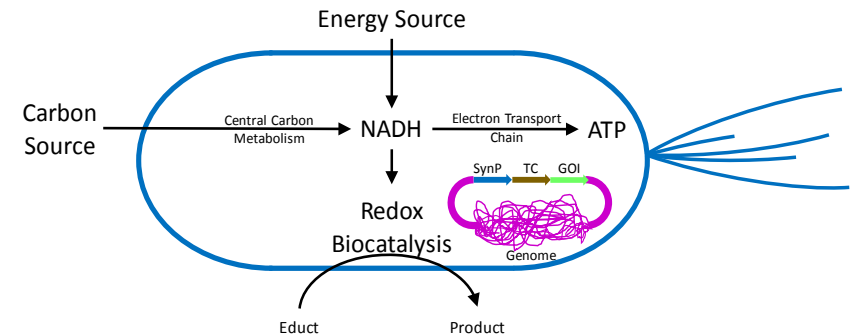
Hence, this thesis investigates in detail the redox metabolism of *P. putida* KT2440 and how to circumvent the shortage of reducing equivalents. The results reveal how *P. putida* KT2440 handles excess NADH while growing on glucose and being co-fed with formate without excreting low reduced compounds. Based on these results an assay was developed allowing the screening of redox enhancing substrates.

Sebastian Zobel

Engineering redox metabolism of *Pseudomonas putida* KT2440

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“Engineering redox metabolism of *Pseudomonas putida* KT2440”

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Für Paula

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Table of Contents

	Title	Page
	Acknowledgements	I
	Danksagung	III
	Funding	V
	List of Figures	IX
	List of Tables	X
	Summary	XII
	Zusammenfassung	XII
Chapter 1	General Introduction	1
Chapter 2	A Tn7-based device for calibrated heterologous gene expression in <i>Pseudomonas putida</i>	15
Chapter 3	Different approaches of increasing the glucose uptake rate in <i>Pseudomonas putida</i> KT2440	31
Chapter 4	Metabolic response of <i>Pseudomonas putida</i> to increased NADH regeneration rates	49
Chapter 5	A method to estimate NADH regeneration rates	63
Chapter 6	General Discussion	69
	Appendix	75
	List of Abbreviations	78
	References	79
	Curriculum vitae	99

List of Figures

Figure 1: Simplified central carbon metabolism of <i>P. putida</i>	5
Figure 2: Strategies to increase the NADH regeneration rate	6
Figure 3: Simplified electron transport chain of <i>P. putida</i> KT2440	7
Figure 4: Simplified glucose uptake system	9
Figure 5: Simplified co-feed strategies	11
Figure 6: Structural organization of mini-Tn7 vectors	21
Figure 7: GFP quantification in Tn7-BG monocopy systems	22
Figure 8: Screening for active promoters in <i>E. coli</i> PIR2	23
Figure 9: Single cell analysis of <i>P. putida</i> KT-BGXX strains	24
Figure 10: Development of fluorescence and biomass during growth of <i>P. putida</i> KT2440	24
Figure 11: Comparison of activities of individual synthetic promoters	26
Figure 12: Sequence logo of the synthetic promoters	28
Figure 13: Synthetic promoter activities in <i>P. putida</i> KT2440	38
Figure 14: Integration of the <i>atpAGD</i> operon into the pSEVA247Y backbone	39
Figure 15: Growth rate profile of <i>P. putida</i> KT2440 harboring pSEVA247atpAGDYXX constructs	40
Figure 16 Fluorescence microscopy of wild type <i>P. putida</i> KT2440	42
Figure 17: Activity with pSEVA247lacZYXX constructs in <i>P. putida</i> KT2440 and <i>E. coli</i> DH5 α	42
Figure 18: Impact of pSEVA231atpAGDXX constructs on the growth of <i>P. putida</i> KT2440 and <i>E. coli</i> JM101	43
Figure 19: Activity of pSEVA231lacZXX constructs in <i>P. putida</i> KT2440 and <i>E. coli</i> JM101	44
Figure 20: SDS PAGE of <i>E. coli</i> JM101 and <i>P. putida</i> KT2440 harboring different SynP- <i>atpAGD</i> constructs	45
Figure 21: Co-feed of formate and glucose in shake flask experiments	55
Figure 22: Flux distribution of <i>P. putida</i> KT2440 with glucose and formic acid	58
Figure 23: NAD(P)H regeneration rate determined by flux analysis for the different cultivation conditions	59
Figure 24: Assay validation	65
Figure 25: Addition of formate increases resazurin reduction rate	66
Figure 26: Resazurin reduction rates with formate as additional NADH source	66
Figure 27: Simplified illustration of biotechnological processes compared to growth	70

List of Tables

Table 1: NAD(P)H balance during complete oxidation of glucose to CO ₂ and biomass	5
Table 2: Strains and plasmids used in this study.....	18
Table 3: Oligonucleotides and sequences synthesized in this work	18
Table 4: Quantitative data of the calibrated synthetic promoter library in <i>P. putida</i> KT2440.....	25
Table 5: Sequence of integrated synthetic promoters	27
Table 6: Strains and plasmids used in this study.....	33
Table 7: Primers used in this study	35
Table 8: Fluorescence microscopy settings.....	37
Table 9: Physiology of positive clones driving <i>atpAGD</i> expression.....	41
Table 10: Sequence of synthetic promoters chosen for <i>atpAGD</i> overexpression with plasmid pSEVA247atpAGDYXX.....	41
Table 11: Sequence of synthetic promoters chosen for <i>atpAGD</i> overexpression with plasmid pSEVA231atpAGDXX.....	43
Table 12: Physiology growth experiment S12 and S12.49 in minimal medium	46
Table 13: Physiology of <i>P. putida</i> KT2440 growing at different pH values	46
Table 14: Strains used in this study.....	51
Table 15: ¹³ C fraction of proteinogenic amino acids.....	56
Table 16: Physiology of cultures grown in bioreactors	57
Table 17: Calculation of the NAD(P)H yield on glucose	59
Table 18: Comparison of <i>E. coli</i> , <i>S. cerevisiae</i> , and <i>P. putida</i> based on biomass yields on glucose...	71
Table 19: Physiology of <i>P. putida</i> KT2440 strains harboring pSEVA231atpAGDXX constructs	75
Table 20: Physiology of <i>E. coli</i> JM101 strains harboring pSEVA231atpAGDXX constructs	75
Table 21: Sequence of synthetic promoters chosen for <i>atpAGD</i> overexpression.....	76
Table 22: Experimentally determined constraints for the calculation of intracellular fluxes	76
Table 23: Stoichiometric model of <i>P. putida</i> KT2440 with estimated net fluxes	77

Summary

Pseudomonas putida is a remarkable soil microorganism and emerges as biotechnological platform host, which despite its remarkable natural abilities needs to be optimized to meet the challenges associated with industrial processes, e.g., homogenic behavior of a producing culture and an efficient supply of reducing equivalents. The overall goal of this thesis was twofold, i) the development of a genetic tool, which minimizes expression variability leading to a consistent phenotype of *P. putida* and ii) increasing the NADH regeneration rate leading to hyper producing strain suited for redox biocatalysis.

The heterologous expression device was designed in a way that it integrates into a single site of the chromosome circumventing copy number variations of classical plasmid based devices. It is composed of a synthetic promoter, a translational coupler and a gene of interest. The synthetic promoter is hence the only sequence determining the strength of gene expression. The reliability of this device was demonstrated by fluorescence measurements with genomically integrated constructs exhibiting a stable expression during the main stage of growth. By this means a calibrated synthetic promoter library was developed exhibiting a 30-fold range of activities. The modular structure of the heterologous expression device allows simple exchange of the gene of interest and hence a community tool is presented.

The aim of increasing the NADH regeneration rate was pursued by two different approaches. Since the central carbon metabolism is the main source of NADH, the first approach aimed at increasing the glucose uptake rate, which in *E. coli* was shown to be controlled by the demand for ATP. However, this strategy adapted to *P. putida* KT2440 did not lead to an increased glucose uptake rate, since it is likely equipped with mechanisms sensing and thereby preventing wastage of ATP. With the second approach we took advantage of *P. putida* KT2440 being equipped with an NAD⁺-dependent formate dehydrogenase converting formate to CO₂ and NADH. Co-feed experiments of glucose and formate indicated that formate can be used as an electron source for NADH as an increased biomass yield on glucose was observed. Thus, a co-feed of formate constitutes a promising approach of increasing the NADH regeneration rate likely supporting redox biocatalysis with *P. putida* KT2440 under process relevant conditions. In addition, based on these results we developed an easy and fast whole cell assay for the estimation of NADH regeneration rates and the screening of redox enhancing substrates.

All together this thesis contributes to the progress of implementing *P. putida* KT2440 as versatile and industrially relevant workhorse with the successful development of a reliable expression tool and the simple formate co-feeding strategy of increasing the regeneration rate of the main redox cofactor NADH.

Zusammenfassung

Das bekannte Bodenbakterium *Pseudomonas putida* gewinnt größer werdendes Interesse für eine Anwendung in der industriellen Biotechnologie. Trotz seiner bemerkenswerten natürlichen Fähigkeiten muss dieser Organismus für eine industrielle Anwendung optimiert werden, um Anforderungen industrieller Prozesse, wie bspw. das Aufrechterhalten einer homogenen katalytischen Aktivität und einer effizienten Versorgung mit Reduktionsäquivalenten, zu genügen. Aufgrund dieser Hürden bestand das Ziel dieser Arbeit i) sowohl in der Entwicklung eines genetischen Werkzeugs, das die Expressionsvariabilität minimiert als auch ii) in der Steigerung der NADH Regenerationsrate mit dem Ziel eines überproduzierenden Stammes, der für den Einsatz in der Redox Biokatalyse geeignet ist.

Um das erste Ziel zu erreichen, wurde ein genetisches Element entwickelt, das in eine definierte und nur einmal existierende Stelle im Genom integriert und so Schwankungen der Kopieanzahl klassischer Vektorsysteme umgeht. Das Hauptelement dieses Werkzeugs besteht aus einem synthetischen Promoter, der über ein Verbindungsstück an das zu exprimierende Gen gekoppelt ist. Hierbei bestimmt nur der synthetische Promoter die Expressionsstärke. Die Zuverlässigkeit dieses Werkzeuges wurde anhand von Fluoreszenzmessungen mit genomisch integrierten Konstrukten mit sich im Wachstum befindenden *P. putida* KT2440 Kulturen nachgewiesen. So konnte eine kalibrierte Bibliothek synthetischer Promotoren entwickelt werden, deren Aktivitäten sich um das bis zu 30-fache unterscheiden. Das modulare Baukastensystem ermöglicht einfaches Austauschen des zu exprimierenden Gens. Das Werkzeug ist für einen breiten mikrobiologischen Einsatz konzipiert.

Das zweite Ziel wurde auf unterschiedliche Weise verfolgt. Da im zentralen Kohlenstoffwechselweg das meiste NADH regeneriert wird, wurde versucht, über eine erhöhte Glukoseaufnahme eine erhöhte NADH Regenerationsrate zu erzielen. In *E. coli* wurde gezeigt, dass der Bedarf an ATP die Glukoseaufnahme kontrolliert. Daher wurde eine ATP verschwendende Reaktion in *P. putida* integriert, die, wahrscheinlich aufgrund von Regulationsmechanismen, nicht zu einer erhöhten Glukoseaufnahme führte. Das Ziel einer erhöhten NADH Regenerationsrate wurde dennoch über einen zweiten Ansatz erreicht. Hierbei wurde gezeigt, dass Formiat von *P. putida* KT2440 über eine NAD⁺-abhängige Formiatdehydrogenase zu CO₂ und NADH oxidiert wird. In einer Co-Verstoffwechslung mit Glukose konnte so zusätzliches NADH bereitgestellt werden, das zu einer erhöhten Biomasseausbeute auf Glukose führte. Dieser vielversprechende Ansatz könnte die Redox Biokatalyse mit *P. putida* unter prozessrelevanten Bedingungen unterstützen. Basierend auf diesen Ergebnissen wurde ein einfach durchzuführender Ganzzell-Assay entwickelt, der das Screening von Cosubstraten ermöglicht, die eine erhöhte NADH Regenerationsrate verursachen.

Durch die Entwicklung eines zuverlässigen Expressionswerkzeugs und die gut beschriebene Nutzung von Formiat als Donor von Elektronen zur Erhöhung der NADH Regenerationsrate tragen die Ergebnisse dieser Arbeit zur Entwicklung von *P. putida* KT2440 als Plattformwirt der industriellen Biotechnologie bei.

Chapter 1

General Introduction

The flexibility of life is remarkable. Every imaginable biological niche has been conquered by incredibly different organisms. However, when speaking of life society often only thinks of the visible life, meaning plants and animals. But the invisible life often remains unnoticed. As last member of the food chain, the activity of soil organisms and their role as distributors and destruent of nutrients are essential for the functioning of ecosystems¹, especially those microorganisms living in the soil and in the narrow zone of plant roots, the so-called rhizosphere². The enormous diversity of soil microorganisms evolved under the selection pressure of a broad variety of biotic (e.g., sort of carbon source, carbon and nitrogen availability, competition) and abiotic factors (e.g., temperature, pH, salinity, UV-light intensity). Thus, soil microorganisms developed numerous adaptation strategies³ to react to frequently changing environmental conditions. One of these strategies is the response to dry/wet cycles with *de novo* synthesis of organic solutes or the uptake of extracellular ions to provide turgor pressure^{4,5}. Metal ion contamination is compensated by a shift in the use of the carbon source requiring less resources during catabolism^{6,7} to support the higher energy demands caused by an increased oxidative stress^{8,9}. Their innate capability to use different carbon sources for the generation of biomass and energy originates from the degradation of plant material mainly by white rot basidiomycetes¹⁰, which are able to attack the highly resistant lignocellulosic biomass.

1.1 Fungi and bacteria are the key to carbon recycling

Plant material mainly consists of cellulose (~40%), hemicellulose (~30%), and lignin (~30%). Cellulose is a linear biopolymer comprised of only D-glucose subunits while hemicellulose is a more crosslinked polymer composed of different pentoses, hexoses, and pectin¹¹. These degradable biopolymers are surrounded and protected by lignin, an often called “recalcitrant” complex aromatic hydrophobic biopolymer comprised of methylated phenylpropanoid monomers, which confers a high tolerance towards biotic and abiotic stresses making it almost completely resistant to microbial degradation¹². Nevertheless, the biological degradation of lignocellulosic biomass is essential for the recycling of carbon. Although certain bacteria are capable of lignin degradation¹³, the degradation process of plant material is mainly initiated by white-rot and brown-rot basidiomycetes releasing enzymes such as laccases, lignin peroxidases, manganese peroxidases, and versatile peroxidases with the help of mediators capable of attacking the structure of lignin^{10,14,15}. Hence, low molecular weight aromatic compounds originating from lignin and polysaccharides from cellulose and hemicellulose are accessible for microbial mineralization^{13,16}. As a consequence, soil microorganisms developed many degradation pathways for using a broad range of different carbon sources for the generation of energy and biomass.

1.2 Soil microorganisms – experts in utilizing a broad variety of carbon sources

Soil microorganisms are highly versatile. They are equipped with different catabolic pathways like the Entner-Doudoroff pathway^{17,18} which is fueled by sugars, or with the β -oxidation pathway which catabolizes fatty acids^{19,20}. But the most versatile metabolic pathway of soil microorganisms is the β -ketoadipate pathway²¹. This pathway is the major route for the degradation of lignin derived

aromatic hydrocarbons like *p*-hydroxybenzoate, vanillate, ferulate and *p*-coumarate naturally occurring in the soil²². All these and other soil aromatic compounds like anthranilate and tryptophan are converted either to protocatechuate, catechol or gentisate prior to entering the aromatic ring cleavage within the *ortho*-, *meta*- or *para*-cleavage pathway with β -ketoadipate as common metabolite²¹. β -ketoadipate itself leads to the intermediates succinyl-CoA and acetyl-CoA fueling the TCA cycle. Hence, soil microorganisms being endowed with all these pathways are able to catabolize a myriad of different carbon sources.

Consequently, carbon catabolite repression is a common phenomenon of soil microorganisms, which is described as the regulatory system of microorganisms to use a preferred carbon source among others for the generation of biomass and energy^{23,24}. Singh et al.²⁵ described this phenomenon in *Bacillus subtilis*, which prefers many carbon sources over xylose with glucose as the most preferred substrate. Previously, carbon catabolite repression was described as the preference of glucose over other carbon sources which was first described in *Escherichia coli* preferring glucose over lactose and the soil microorganism *Streptomyces coelicolor* A3(2) which prefers glucose over maltose²⁶. But glucose, although being the most abundant sugar monomer, is not the preferred growth substrate of all microorganisms probably reflecting the abundance of the carbon source in the environmental niche. Other explanations could be the avoidance for the same carbon source in the highly competitive soil environment, e.g. *Clostridium thermocellum* prefers cellulobiose over glucose²⁷ and species of the genus *Pseudomonas* prefer succinate and amino acids^{28,29}. Vinuselvi et al.³⁰ described carbon catabolite repression as a “boon” to microorganisms since this regulatory mechanism constitutes an essential fitness advantage due to optimized uptake and assimilation of nutrients in constantly changing environmental conditions.

However, there is also evidence that certain microorganisms are able to simultaneously metabolize more than one substrate. For example, *Lactobacillus brevis*³¹ and *C. thermohydrosulfuricum*³² have shown to grow on glucose/xylose mixtures and *Pseudomonas* strain B1 grew on benzene/toluene mixtures³³. Carbon catabolite repression was not observed in these systems likely due to substrate similarity. Enzymes responsible for one or the other substrate were constitutively expressed even when the organism grew on one substrate alone, which means that the breakdown pathways are connected in a particular way. A simultaneous metabolization of two growth substrates whose breakdown relies on two different pathways like glucose and benzoate is not reported in literature as such a combination likely leads to carbon catabolite repression. Nevertheless, the ability of a simultaneous metabolization of a growth and a non-growth substrate seems to be a relative common phenomenon among soil microorganisms. This phenomenon was defined by Dalton and Stirling³⁴ as “the transformation of a non-growth substrate in the obligate presence of a growth substitute or another transformable compound”. This ability is widely exploited in bioremediation processes such as the biotransformation of trichloroethylene³⁵ and pharmaceuticals and personal care products³⁶ which accumulate in the environment.

In conclusion, soil microorganisms are endowed with a considerable genetic inventory which becomes increasingly interesting for industrial purposes like bioremediation of toxic organic compounds and industrial biocatalysis of fine chemicals. A versatile representative of the soil microbial consortium is *P. putida* which is the main actor of this thesis and shall be presented briefly.

1.3 *Pseudomonas putida* – a multifaceted soil microorganism

P. putida belongs to the γ -proteobacteria, it is Gram-negative, polar flagellated and possesses a chemo-organotrophic aerobic respiratory metabolism³⁷. It metabolizes sugars through the Entner-Doudoroff pathway³⁸. By this means it is able to tolerate high levels of oxidative stress⁹. Furthermore, *P. putida* is capable of using a broad variety of organic compounds as sole carbon source³⁹ which are processed through the β -ketoadipate pathway. It is able to adapt to various environmental conditions partly due to its branched, adaptable and very effective respiratory chain⁴⁰. Its remarkable solvent-tolerance⁴¹ is of great importance for the production of aromatic compounds like phenol⁴² and *p*-coumerate⁴³, and for its application in two-phase systems^{44,45}. One representative of *P. putida*, *P. putida* KT2440, is generally regarded as safe⁴⁶ which is a prerequisite for industrial applications. The genome of *P. putida* KT2440 was published already a decade ago⁴⁷ followed by a genome scale model^{48,49} which makes *P. putida* KT2440 amenable to detailed genetic engineering. Recently, it was shown that *P. putida* is able to boost its redox metabolism^{45,50}, which was defined by Blank et al.⁵¹ as the sum of all modules in which redox equivalents are produced or consumed. This is of special interest for redox biocatalytic reactions.

Unfortunately, most of the increased redox cofactor regeneration rate was not channeled to the biocatalytic redox reaction rather than to processes supporting cellular homeostasis resulting from the exposure to organic solvents⁴⁵. Hence, a redox biocatalytic reaction demands redox equivalents not only directly by transferring electrons enzymatically to the products. Its impact goes far beyond the enzymatic reaction as various factors associated with the production system are dependent on energy, e.g., expression of heterologous enzymes concomitant with proliferation in the presence of antibiotics, solvent extrusion in two-phase systems⁴¹, and uncoupling reactions resulting in reactive oxygen species⁵².

In this respect, it seems not to be sufficient to expect the cell to adapt to all these energy demanding processes by the driven by demand concept⁴⁵. It is rather important to support the cell to increase the regeneration rate of the redox cofactors which may lead to an increased activity of *P. putida* as a whole cell biocatalyst.

1.4 Strategies supplying increased redox equivalent demands

The term redox equivalent is commonly used to describe a redox cofactor. Next to the most abundant cofactors NADH and NADPH belonging to the class of dihydropyridines, there are redox cofactors belonging to the group of flavins (e.g., FAD/FADH₂) and quinones (PQQ/PQQH₂)^{51,53}, which only play a minor role considering their frequency of use.

Assuming that glucose is the sole carbon and energy source of metabolically active *P. putida*, NADH and NADPH are regenerated during the breakdown of glucose through the Entner-Doudoroff pathway down to pyruvate which is further metabolized in the TCA cycle (Figure 1, Table 1). Overall, ten NAD(P)H molecules can theoretically be regenerated during complete oxidation of glucose to CO₂ with metabolically active but non-growing cells. In this balance the pentose phosphate pathway was not included as it only plays a minor role of the total carbon flux in *P. putida*⁴⁵.

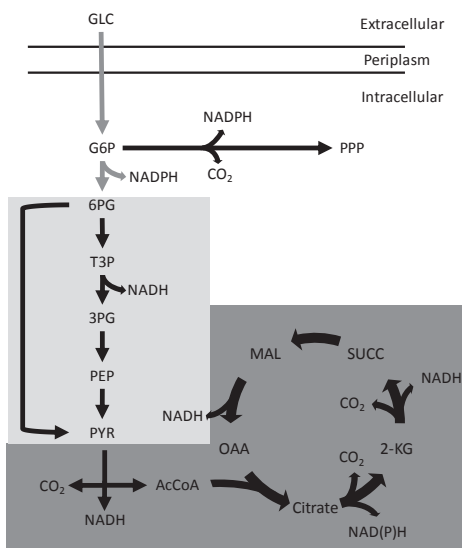


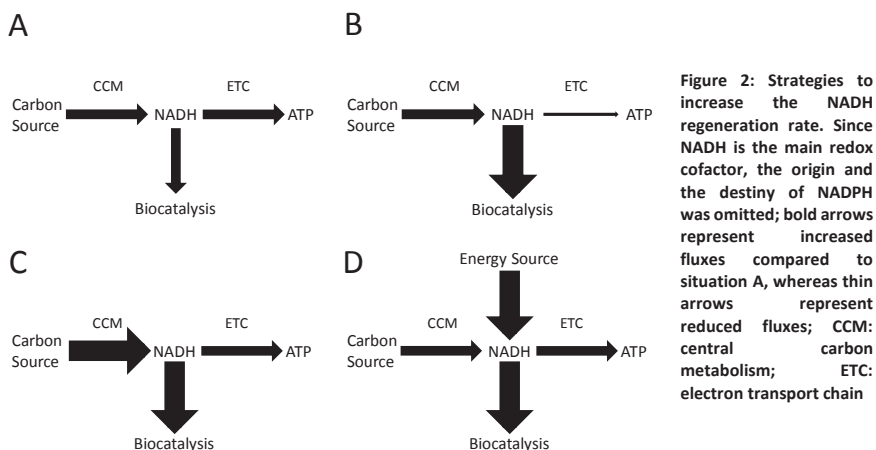
Figure 1: Simplified central carbon metabolism of *P. putida* adapted from Blank et al.⁴⁵, consisting of uptake and initial conversion of GLC to 6PG (grey arrows), the pentose phosphate pathway (PPP) (grey arrows), the Entner-Doudoroff (ED) pathway (light grey background) and the TCA cycle (dark grey background); reactions in which the redox cofactors are regenerated are indicated; Abbreviations: GLC – glucose, G6P – glucose-6-phosphate, T3P – triose-3-phosphate combining glyceraldehyde-3-phosphat, 1,3-bisphosphoglycerate, 3-phosphoglycerate, and 2-phosphoglycerate, PEP – phosphoenolpyruvate, PYR – pyruvate, AcCoA – acetyl-CoA, 2-KG – α -ketoglutarate, SUCC – succinate, MAL – malate

Table 1: NAD(P)H balance during complete oxidation of glucose to CO₂ and biomass

	Section ^a	NADH ^b	NADPH ^c
	ED pathway	1	1
	TCA cycle	8	

- a: In this calculation the pentose phosphate pathway was not included as it only plays a minor role in *P. putida*⁴⁵
b: The calculation of 8 NADH in the TCA cycle is the sum of both pyruvate molecules; additionally, it was assumed that the isocitrate dehydrogenase is NAD⁺-dependent
c: The regeneration of one NADPH molecule within the peripheral pathway (Figure 1) was considered to be part of the ED pathway

Whereas NADH mainly serves as electron donor for the electron transport chain, the destiny of NADPH is to serve as electron donor in anabolic reactions. However, *P. putida* is able to circumvent the shortage of one or the other reduced cofactor through the action of transhydrogenases⁵¹. Considering an oxygenase-based heterologous redox biocatalytic reaction, this reaction constitutes a demanding competitor to anabolism and the electron transport chain, as most oxygenases are NAD(P)H-dependent^{51,54}. Consequently, the cell faces an increased redox cofactor demand and a trade-off between cellular viability and biocatalytic activity.



But knowing the origin and the fate of NADH, one can imagine different strategies of how to increase the regeneration rate of NADH or decrease its consumption in order to satisfy both needs (Figure 2). Figure 2A illustrates an unmanipulated system in which NADH is regenerated during catabolism of glucose and is required for the generation of ATP and the imposed biocatalytic reaction. Figure 2B demonstrates a reduced flux towards the electron transport chain leaving more NADH available for biocatalysis. In Figure 2C an increased flux through the central carbon metabolism is depicted, which concomitantly results in an increased regeneration rate of NADH making it faster available for biocatalysis, and Figure 2D illustrates an artificial NADH regeneration system through the addition of an external energy source which increases the NADH regeneration rate. These three possibilities shall be presented and evaluated in terms of feasibility.

1.4.1 Reduction of the electron flow towards the electron transport chain

The electron transport chain is located within the cytoplasmic membrane of bacteria and translocates protons generating a proton gradient or a proton motive force which is the driving force for numerous processes such as the synthesis of ATP⁵⁵ or the motion of flagella^{56,57}. The organization of the electron transport chain differs among the bacteria⁵⁸. Therefore, only the electron transport chain of *P. putida* KT2440⁴⁰ which is comparable to that of *P. aeruginosa*^{59,60} is introduced.

The proton motive force is generated by the oxidation of NADH molecules, normally with the NADH dehydrogenase NDH-I, transferring their electrons to ubiquinone (UQ) which serves as an electron shuttle inside the cytoplasmic membrane (Figure 3). Furthermore, *P. putida* KT2440 is endowed with a second NADH dehydrogenase, NDH-II (PP_0626)⁶¹. Up to now it is not reported whether NDH-II of *P. putida* KT2440 also translocates protons. Therefore, it is assumed that NDH-II of *P. putida* KT2440 functions comparably to the NDH-II of *E. coli* which does not translocate protons⁶² and transfers

electrons from NADH to ubiquinone⁶³. The reduced ubiquinone (UQH₂) transfers the received electrons either to the terminal oxidases *bd* or *bo₃* or to the *bc₁* complex, which is connected to another three terminal oxidases *cbb₃-1*, *cbb₃-2* and *aa₃* via cytochrome c. It is important to note that all terminal oxidases translocate different numbers of protons across the membrane per oxygen atom consumed (H⁺/O ratio) ranging from approximately 3 to 6 determined for *P. aeruginosa*⁶⁰. Due to these variable H⁺/O ratios, different ATP yields can be obtained for each oxidized NADH molecule since all five different terminal oxidases are expressed under different nutrient and oxygen conditions⁶⁴.

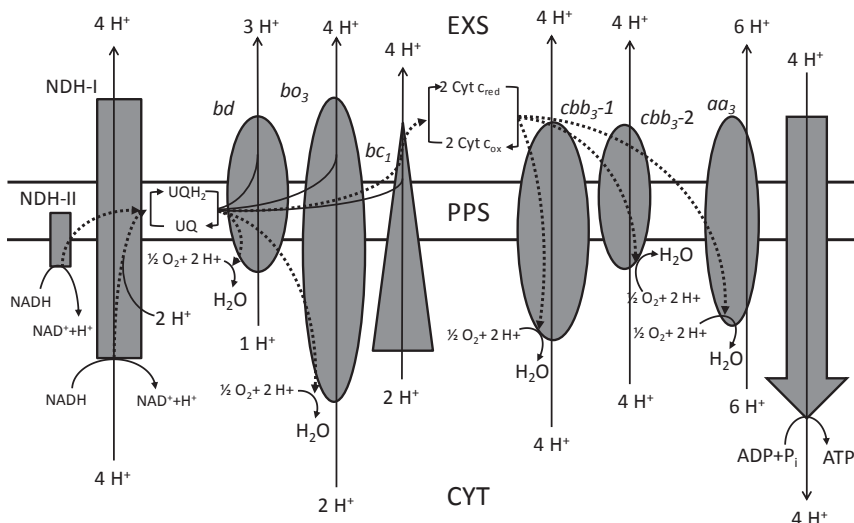


Figure 3: Simplified electron transport chain of *P. putida* KT2440 adapted from Blank et al.⁵¹; rectangular proteins indicate NADH dehydrogenases NDH I and NDH II; ellipsoid proteins indicate terminal oxidases with given H⁺/O ratios on top, the triangular and the arrow proteins indicate *bc₁* complex and ATP synthase, respectively; dotted arrows indicate the flux of two electrons, solid lines to and from ubiquinone (UQ, UQH₂) indicate flux of two protons, solid horizontal lines indicate the cytoplasmic membrane; Cyt c – cytochrome c, CYT - cytoplasm, PPS - periplasmic space, EXS – extracellular space

These ATP yields can range theoretically from 0.75 ATP/NADH (scenario 1) to 3.5 ATP/NADH (scenario 2), provided that the H⁺/O ratios of the terminal oxidases remain constant. The ATP yields of scenario 1 can be obtained if only NDH-II is active, which transfers electrons from NADH to UQ which in turn transfers the received electrons to the solely expressed *bd* oxidase translocating only 3 H⁺. Assuming that 4 protons are needed to regenerate one molecule of ATP⁵⁵ this would result in 0.75 ATP/NADH. Scenario 2 can be explained when *P. putida* KT2440 expresses the NADH dehydrogenase NDH-I and only terminal oxidase *aa₃* which means that the electrons have to pass the *bc₁* complex which additionally translocates protons. Overall this would result in 14 H⁺ yielding 3.5 ATP per NADH.

As the electron transport chain constitutes the main competitor for NADH inside the cell, a very efficient electron transport chain would be desirable in combination with an NADH-dependent biocatalytic reaction. The above illustrated scenario 2 describes such an efficient electron transport chain requiring less NADH for the regeneration of ATP which means that more NADH would be available for the biocatalytic reaction. Unfortunately, a suppressor mutant of *P. aeruginosa* expressing solely the *aa₃* oxidase was only able to grow slowly. Furthermore, NDH-II seems only to be active under NADH rich conditions due to the higher K_m values observed in *E. coli* presented by Ebert et al.⁵⁰. Thus, NDH-II needs to be deactivated prior to the establishment of NADH rich conditions in order to prevent NADH loss.

Overall, manipulation of the electron transport chain as means to increase the NADH availability for an imposed redox biocatalytic reaction seems to be very challenging and can only be achieved accompanied by a reduction of the cellular performance. However, there are other strategies likely leading to an increased NADH regeneration rate.

1.4.2 Increase of the carbon uptake rate

Most of the NADH is regenerated during catabolism of glucose. Hence, an increased flux through the central carbon metabolism results in an increased NADH regeneration rate (Figure 2C). A closer look into the uptake and the catabolism of glucose reveals how *P. putida* KT2440 might be domesticated for an increased glucose uptake.

P. putida KT2440 is a Gram-negative bacterium. Gram-negative bacteria are protected by two membranes, the outer membrane and the cytoplasmic membrane (Figure 4) surrounding the periplasmic space. The periplasmic space embeds a thin peptidoglycan layer. Peptidoglycan is a biopolymer consisting mainly of the two glucose derivatives, *N*-acetylglucosamine and *N*-acetylmuramic acid, which serve as structural components providing strength towards osmotic pressure of the cytoplasm. The cytoplasmic membrane is composed of phospholipids with integrated lipoproteins, while the composition of the outer membrane is mostly changed to polysaccharides connected to lipids. Both the outer and the cytoplasmic membranes are hydrophobic and can only be passed by certain molecules which enter the periplasmic space mostly via specific porins. The path of the molecules inside the periplasmic space into the cytoplasm leads through specific transporters.

Before glucose can be catabolized within the Entner-Doudoroff pathway of *P. putida* KT2440, it enters the periplasmic space via an OprB porin⁶⁵ first described in *P. aeruginosa*⁶⁶ where it is likely received by a periplasmic glucose-binding protein. This glucose-binding protein was observed in *P. aeruginosa*, *P. putida* CSV86⁶⁷ and many other Gram-negative bacteria⁶⁸ leading to the assumption that it is also present in *P. putida* KT2440. This protein delivers the glucose molecule to the ABC transporter harboring two ATP binding domains. ABC is short for ATP-binding cassette meaning that the hydrolysis of two ATP is required for the import of glucose⁶⁸ into the cytoplasm constituting a severe investment in the uptake. Subsequent to the transfer into the cytoplasm,

glucose is converted to 6-phosphogluconate, which fuels the Entner-Doudoroff pathway. However, this expensive path of glucose through the OprB porin and the ABC transporter is called the “high-affinity” pathway with a K_m value of $8\ \mu\text{M}$ ⁶⁶. *P. putida* KT2440 is able to utilize two ATP molecules for the import of glucose likely due to the low cellular maintenance demand reflecting the amount of ATP which is required for cellular maintenance processes⁵⁰. This in turn points back to the above described efficient electron transport chain, which requires low amounts of NADH for the generation of ATP. Nevertheless, *P. putida* KT2440 is endowed with a second, the “low-affinity” pathway with a K_m value of $1\text{--}2\ \text{mM}$ ⁶⁶ yielding either one or two intermediates prior to the uptake.

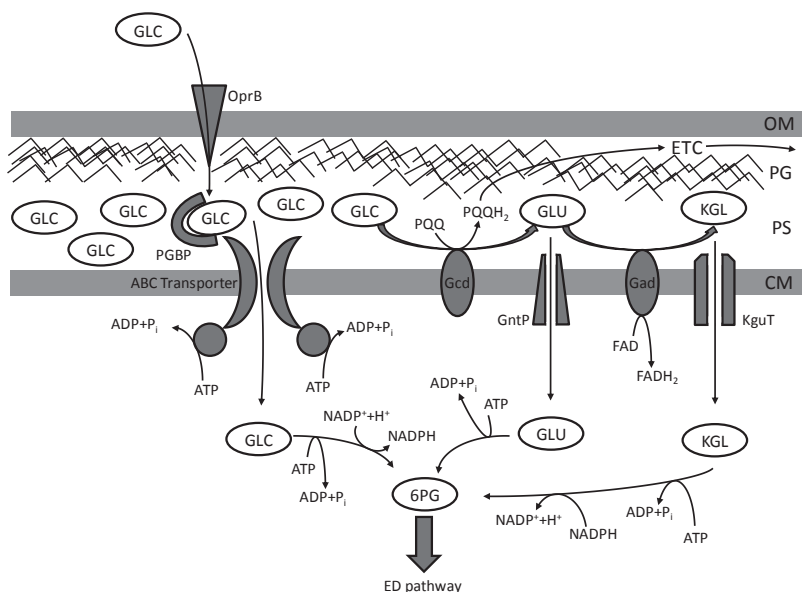


Figure 4: Simplified glucose uptake system adapted from Del Castillo et al.⁶⁵, GLC – glucose, OprB – OprB porin, PGBP – periplasmic glucose binding protein, PQQ(H₂) – pyrroloquinoline quinone, GLU – gluconate, KGL – 2-ketogluconate, Gcd – glucose dehydrogenase, Gad – gluconate dehydrogenase, GntP – gluconate transporter, FAD(H₂) – flavin adenine dinucleotide, KguT – 2-ketogluconate transporter, 6PG – 6-phosphogluconate; OM – outer membrane, ETC – electron transport chain, PG – peptidoglycan layer, PS – periplasmic space, CM – cytoplasmic membrane

As illustrated in Figure 4, glucose gets converted to gluconate and further to 2-ketogluconate via the cytoplasmic membrane-bound glucose dehydrogenase (Gcd) and the gluconate dehydrogenase (Gad)⁶⁵ before both intermediates are converted to the central metabolite 6-phosphogluconate. The conversion of glucose to gluconate yields another cofactor, pyrroloquinoline quinone (PQQ(H₂)) which acts as electron donor in the electron transport chain⁵¹. Since the transporters of gluconate and 2-ketogluconate are annotated as proteins belonging to the H⁺ symporter and the major facilitator superfamily⁶¹, their uptake is not connected to the investment of ATP. Notwithstanding,

the ATP-dependent uptake of glucose seems to be the major path of glucose into the cytoplasm. The “lower affinity” pathway yet is essential to this bacterium since mutants deficient in conversion or uptake enzymes of the “lower affinity” pathway exhibited deficient growth⁶⁵.

Now knowing the catabolic route of glucose, one can imagine that overexpression of essential glycolytic enzymes may lead to an increased glucose uptake rate. However, Sudarsan et al.⁶⁹ grew *P. putida* KT2440 successively in a continuous culture on benzoate, glucose, fructose and back to benzoate and demonstrated that although severe intracellular flux changes were observed, these changes were not reflected in the gene expression levels. This led the authors to the assumption that all proteins of central carbon metabolism may be subject to metabolic and/or translational modifications rather than to transcriptional regulation. Hence, overexpression of central enzymes like the 2-keto-3-deoxy-6-phosphogluconate aldolase responsible for the conversion of 6-phosphogluconate to pyruvate and glyceraldehyde-3-phosphate (Figure 1) may not lead to an increased uptake of glucose into the cell.

Interestingly, Wylie and Worobec⁷⁰ observed that overexpression of the OprB porin in *P. aeruginosa* led to an increased glucose uptake rate above wild-type levels. However, they also observed that at glucose concentrations of 100 μ M *P. aeruginosa* only expressing the high affinity pathway took up glucose at the same speed as a non-expressing OprB strain. *P. putida* KT2440 is also endowed with the OprB porin. Hence, this strategy may not lead to the desired effect since biocatalytic processes are applied with glucose concentrations in the range of $g\ l^{-1}$.

If glucose molecules diffuse unspecifically through the outer membrane at high concentrations and OprB deficient *P. aeruginosa* strains exhibit the same uptake rates as OprB overexpressing strains, the inner membrane may constitute the limiting hurdle. Therefore, overexpression of the ABC transporter in combination with the periplasmic glucose binding protein in *P. putida* KT2440 may lead to an increased glucose uptake rate. A similar strategy was already followed by Ruyter et al.⁷¹ in *E. coli*. *E. coli* imports glucose with the phosphoenolpyruvate:carbo-hydrate phosphotransferase (PTS) system, which depends on the phosphorylation of glucose by phosphoenolpyruvate as an ATP equivalent. However, overexpression of the glucose transporter did not result in an increased glucose uptake rate. Maybe this phenomenon is unspecific and not only limited to the PTS system in *E. coli* meaning that overexpression of the ABC transporter in *P. putida* KT2440 might not lead to an increased glucose uptake. Moreover, the ABC transporter without the periplasmic glucose binding protein is translated from a four gene operon⁶⁵. If overexpressed, this four gene operon would already result in an increased metabolic burden.

The key to an increased glucose uptake rate was presented by Koebman et al.⁷² showing that the demand of ATP controls the uptake of glucose. This strategy applied to *P. putida* KT2440 seems to be of high potential although one has to be aware of the reduction of the cellular performance.

However, there are less invasive strategies of increasing the NADH regeneration rate such as the supply of an additional energy donor next to glucose as the carbon and energy source (Figure 2D).

1.4.3 Addition of an external energy source

Figure 5 illustrates two possible co-feed strategies one can imagine to support the elevated NADH demand during a biocatalytic reaction. Figure 5A describes an unusual system in which an unspecific enzyme catalyzes the product formation as well as the regeneration of NADH. Such a system was reported by Itoh et al.⁷³ in which recombinant *E. coli* cells expressed a phenylacetaldehyde reductase from *Corynebacterium* strain ST-10 which is responsible for the conversion of ethyl 4-chloro-3-oxobutanoate (substrate 1) to ethyl (*R*)-4-chloro-3-hydroxy butanoate (product 1) in the presence of 2-propanol (substrate 2) being oxidized to acetone (product 2). Unfortunately, such a one enzyme system can be impaired by different pH optima for each of the reactions. Thus, a two-enzyme system (Figure 5B) might be more suitable for a supported NADH regeneration.

Kizaki et al.⁷⁴ applied such a two enzyme regeneration system in *E. coli* expressing a carbonyl reductase from *Candida magnoliae* for the transformation of ethyl 4-chloro-3-oxobutanoate to ethyl (*S*)-4-chloro-3-hydroxybutanoate (Enzyme 1) in combination with the glucose dehydrogenase from *B. megaterium* responsible for the NADH regeneration (Enzyme 2).

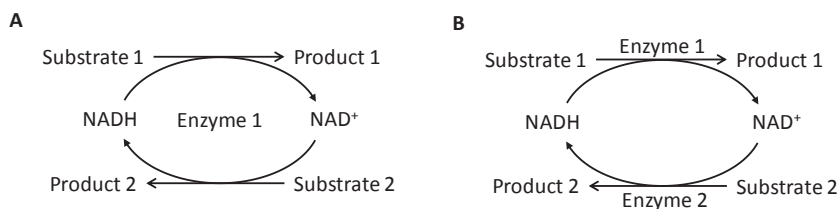


Figure 5: Simplified co-feed strategies illustrating how NADH as main electron donor in biocatalytic process can be efficiently regenerated, adapted from Hummel and Gröger⁷⁵; A) The regeneration of NADH is carried out by the same enzyme catalyzing the desired reaction, B) The oxidation of a second substrate, which electrons are transferred to NADH, is dependent on a second enzyme

However, such a regeneration system may be inappropriate for *P. putida* KT2440 as glucose should be applied as the primary carbon and energy source within this thesis, and the conversion of glucose to gluconate within the periplasmic space yielding PQQH₂ as cofactor is of great importance to this organism. Heterologous expression of an NADH regenerating glucose dehydrogenase might constitute a severe competitor to the natural glucose dehydrogenase which then might end up in a reduced cellular performance.

The most promising two enzyme strategy, which is often applied, is a co-feed of formate which is oxidized to CO₂ by an NAD⁺-dependent formate dehydrogenase (FDH)⁷⁶. This FDH often originates from the yeast *C. boidinii* which is known to be NAD⁺-dependent. However, *P. putida* KT2440 is endowed with an NAD⁺-dependent FDH as part of an highly efficient formaldehyde detoxification machinery⁷⁷. Thus, a co-feed of formate to *P. putida* KT2440 grown on glucose seems to be a promising strategy of a supported NADH regeneration.

1.5 Scope of this thesis

This PhD thesis was part of the European project “*Pseudomonas* 2.0: industrial biocatalysis using living cells” within the “European Research Area - Industrial Biotechnology”. The aim of this project was to implement *Pseudomonas* species as a novel biocatalyst into the European bioindustry since *Pseudomonas* are not yet well established besides *Bacillus*, *C. glutamicum* and *E. coli* due to low biocatalytic activities under industrially relevant conditions. The epoxidation of styrene to enantiomerically pure (S)-styrene oxide can be considered as industrially relevant since (S)-styrene oxide is of high potential as building block for biologically active compounds⁷⁸. This model reaction combines various challenges like solvent tolerance, reliable constitutive heterologous gene expression of the styrene monooxygenase from *P. taiwanensis* VLB120 and efficient supply with reducing equivalents in the form of NADH. In view of this background the goal of this PhD thesis was to develop a reliable expression device to reduce expression variability and to increase the NADH regeneration rate of the model organism *P. putida* KT2440.

This chapter is meant as an introduction into the remarkable capabilities of *P. putida* KT2440, which qualifies this soil microorganism for an application within the industrial biotechnology. The focus is on the central carbon metabolism comprised of the peripheral conversion of glucose to the central intermediate 6-phosphogluconate which is further metabolized through the Entner-Doudoroff pathway and the TCA cycle yielding theoretically ten NADH molecules per molecule glucose. Most of the NADH is needed to fuel the electron transport chain leaving only a small fraction of NADH for a biocatalytic reaction. Thus, different strategies with the aim of increasing the NADH regeneration rate were assessed.

Although *P. putida* KT2440 as the most prominent non-pathogenic representative of the *Pseudomonas* family is equipped with all the above mentioned remarkable capabilities, these capabilities are only the entrance ticket to an application in industrial biocatalysis. Hence, this organism has to undergo a certain optimization to meet special requirements such as a defined and reliable gene expression. Chapter 2 describes the development of a calibrated promoter library as a reliable tool for metabolic engineering of not only *Pseudomonas* species. This library was developed in cooperation with a project partner and is made available to the public through the SEVA database^{79,80}.

As demonstrated by Sudarsan et al.⁶⁹ the central carbon metabolism is rigid as most of the proteins of the central carbon metabolism may be subject to metabolic and/or translational modifications. Hence, a manipulation of the central carbon metabolism may not lead to the desired effect of an increased glucose uptake rate. Therefore, the goal of increasing the NADH regeneration rate through an increased glucose uptake rate beyond the driven by demand concept⁴⁵ was approached with a fine-tuned overexpression of an ATP-wasting enzyme through the use of the synthetic promoter library technique⁸¹ (Chapter 3). Although highly active synthetic promoters were obtained, overexpression of the ATPase was not observed. It is likely that *P. putida* KT2440 possess

sophisticated mechanisms to prevent ATP wastage. Thus, a simpler and less invasive strategy was pursued.

In Chapter 4 a co-feed of the low reduced additional energy source formate was evaluated as alternative strategy to increase the NADH regeneration rate. Therefore, we co-fed *P. putida* KT2440 grown on glucose with formate, which is mostly converted by its inherent NAD⁺-dependent FDH and investigated its effect on the physiology of growing *P. putida* KT2440 cultures and on the carbon fluxes inside the cell with ¹³C carbon tracer experiments. The additional energy input resulted mainly in a severely increased NADH regeneration rate and biomass yield on glucose, a decreased carbon flux through the TCA cycle, and an impressive increase in the uptake of oxygen likely due to an altered electron transport chain.

Next to formate there are other promising co-substrates which might serve as an additional energy source. Thus, we developed an assay for the detection of substrates which support the regeneration of NADH as the most available techniques are cumbersome (Chapter 5). The assay was developed based on the observations made in Chapter 4 and constitutes a promising tool for the screening of redox enhancing substrates.

Finally, Chapter 6 discusses the different approaches and evaluates their feasibility for an industrial application.

Chapter 2

A Tn7-based device for calibrated heterologous gene expression in *Pseudomonas putida*

The soil bacterium *P. putida* is increasingly attracting a considerable interest as a platform for advanced metabolic engineering through synthetic biology approaches. However, genomic context, gene copy number and transcription/translation interplay often enter a considerable uncertainty to the design of reliable genetic constructs. In this work we have established a standardized heterologous expression device, in which the only variable is promoter strength, the remaining parameters of the flow having stable default values. To this end, we tailored a mini-Tn7 delivery transposon vector that inserts the constructs in a single genomic point of *P. putida*'s chromosome. This was then merged with a promoter insertion site, an unvarying translational coupler and a downstream location for placing the gene(s) of interest under fixed assembly rules. This arrangement was exploited to benchmark a collection of synthetic promoters with low transcriptional noise in this bacterial host. Growth experiments and flow cytometry with single-copy promoter-GFP constructs revealed a robust, constitutive behavior of these promoters, whose strengths and properties could be faithfully compared. We argue this standardized expression device to significantly extend the repertoire of tools available for reliable metabolic engineering and other genetic enhancements of *P. putida*.

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Author Contributions:

Sebastian Zobel and Ilaria Benedetti contributed equally to this work. Victor de Lorenzo, Nick Wierckx, and Lars M. Blank conceived and designed the project. Ilaria Benedetti constructed and benchmarked the Tn7-based vectors and performed flow cytometry experiments. Sebastian Zobel and Lara Eisenbach constructed and screened the synthetic promoter library and quantitatively characterized selected promoters. All authors designed experiments and analyzed results. Sebastian Zobel, Ilaria Benedetti, and Nick Wierckx wrote the chapter with the help of Victor de Lorenzo and Lars M. Blank

2.1 Introduction

Non-pathogenic *Pseudomonads* are highly promising hosts for biotechnology applications^{42,82}. They are equipped with a remarkable set of genes that endows them with a highly versatile metabolism. On the one hand, this enables the catabolism of a wide variety of substrates including, besides glycerol⁸³ and in some cases xylose⁸⁴, also a wide range of aromatics³⁹. On the other hand, it enables the production of many industrially relevant chemicals from these substrates, including several aromatics^{42,45,85}, furandicarboxylic acid⁸⁶ and polyhydroxyalkanoates⁸⁷ (see Tiso et al.⁸² for an overview). *Pseudomonads* are also well-known for their ability to withstand stressful conditions in biotransformations, including exposure to organic solvents^{88,89} and oxidative stress⁹. Moreover, they are promising candidates for electron-demanding biotransformations due to their capability of regenerating the cofactor NADH at a high rate^{45,50}.

Nevertheless, there are challenges associated with these biotechnological applications, which require significant metabolic engineering efforts to overcome hurdles of efficiency, stability and eventually economy. *P. putida* has thus been the subject of genetic engineering for over three decades, accompanied by a continuous development of genetic tools⁹⁰ and accelerated by the publication of its complete genome sequence at an early stage⁴⁷. This has been followed recently by the complete genome sequences of other *Pseudomonads* often used for biocatalysis^{91,92}. Now, *P. putida* is rapidly becoming a synthetic biology workhorse^{93–95}. For this, standardized and well-characterized tools are a prerequisite, especially including means to express genes in a defined and reliable manner.

One of these tools are promoter libraries, either hybrid, aiming at the variation of the promoter strength by modification of up-⁹⁶ and downstream elements⁹⁷, or synthetic, as first described in *L. lactis* and *E. coli* in 1998⁸¹. Synthetic promoter libraries enable the efficient fine-tuning of gene expression by using a degenerate promoter-primer containing basic promoter consensus elements⁹⁸. Examples of applications of synthetic promoters are diverse, including modulation of enzyme activities in metabolic pathways⁹⁹, protein production¹⁰⁰ and even the heterologous expression of an ATPase, a highly toxic ATP-wasting enzyme⁷². Since its first description, several different prokaryotes have undergone optimization with this synthetic promoter technology, such as *C. glutamicum*¹⁰¹ and *S. lividans*¹⁰².

However, quantitative experiments with plasmid-based expression systems are problematic due to plasmid loss¹⁰³ and copy number variations¹⁰⁴. These drawbacks can be avoided by introducing the expression cassette into a neutral site of the chromosome¹⁰⁵. This has given rise over the years to e.g. a large number of Tn7-based transposon vectors^{106–108}. However, this is not sufficient, as translation dramatically varies depending on the sequence of the non-coding 5'-region of the gene of interest (GOI)^{22,109}. This can be circumvented with translational couplers (bicistronic design), which add a downstream region to the promoter that encodes a short peptide and reduces GOI-specific effects on translation¹¹⁰. The bicistronic design contains two Shine-Dalgarno sequences (SD).

The first (SD1) is followed by a short coding cistron (Figure 6C). The second (SD2) is encoded entirely within the coding sequence of the leading peptide and it is translationally coupled to the GOI. This design limits interaction of mRNA secondary structures across 5' untranslated regions¹¹¹ and fixes translation efficiency, thereby eliminating this uncertainty of the gene expression flow.

In this work we have developed a standardized heterologous expression device in which the only variable is the strength of the transcriptional signal that originates the whole gene expression flow but eliminates uncertainties stemming from copy numbers and transcription/translation interference. As shown below, this device has been instrumental to benchmark a collection of calibrated synthetic promoters with different expression levels and kinetic/stochastic properties. These promoters, which deliver a stable and constitutive expression of downstream genes, are made available to the community through the Standard European Vector Architecture Database (SEVA)^{79,80}.

2.2 Material and Methods

2.2.1 DNA Techniques

Mini-Tn7 vectors were designed as DNA segments flanked by the restriction sites *AscI*/*SwaI* and composed of terminal sequences of Tn7 (Tn7L and Tn7R), a resistance to gentamycin (Gm^R), two terminators T0 and T1, and a multi-cloning site (MCS) compatible with pSEVA architecture^{79,80}; this sequence was previously assembled in synthetic pMK (Table 2) using *XhoI* and *AscI* cloning sites. Subsequently the Tn7 fragment was digested with *AscI*/*SwaI* and ligated into R6K vector¹¹² pS211, obtaining pTn7-M. In order to obtain a device compatible with the MCS of mini-Tn7s the translational coupler BCD2¹¹⁰ was edited by including a *BclI* and an *EcoRI* site as part of the sequence coupled to the reporter gene (Figure 6C). As reporter the *msf-gfp* gene was included, encoding the monomeric and super-folder GFP (MsfGFP¹¹³), downstream to BCD2 and comprehended between the two *EcoRI* sites. The entire fragment BCD2-*msfgfp* (BG) was cloned as *SacI*/*BamHI* into pTn7-M, resulting in pBG. The constitutive promoter *P_{em7}*¹¹⁴ was digested from pSEVA2513⁸⁰ and cloned as *PacI*/*AvrII* into pBG, obtaining pBG13 (Table 2). To obtain the BCD2-less vector pM13, we passed the *msfgfp* gene borne by pSEVA237M to pTn7-M as a *HindIII*/*SpeI* segment and the resulting plasmid then inserted with the *P_{em7}* promoter as a *AvrII*/*PacI* fragment. Synthetic promoters were obtained by PCR with phusion high fidelity polymerase (New England Biolabs, Ipswich, MA, United States) using primers SZ1 and SZ2 (Table 3) with template pBG (Table 2). The vector and the PCR product containing the synthetic promoter library were digested with *PacI* and *NcoI*. The restriction site *NcoI* is located within the *msfgfp*. After ligation, the vector (pBGXX) was transformed via electroporation into *E. coli* PIR2 cells (Life Technologies, Carlsbad, Ca, USA) according to the suppliers' instructions. Greenish growing colonies, which indicated active synthetic promoters, were selected for further analysis. Plasmids carrying active synthetic promoters were isolated with the QIAGEN plasmid mini kit and were submitted to sequencing using primer SZ3.

Chapter 2

Table 2: Strains and plasmids used in this study		
Strains	Description	Reference
<i>E. coli</i>		
DH5αλpir	λpir phage lysogen of DH5α	De Lorenzo Lab collection
HB101	<i>F mcrB mrr hsdS20(r_S m_S) recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20(Sm^R) glnV44 λ</i>	Boyer and Roulland-Dussoix ¹¹⁵
CC118λpir	<i>Δ(ara-leu) araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE(Am) recA1</i> , lysogenized with λpir phage	Herrero et al. ¹¹⁶
PIR2	<i>F Δlac169 rpoS(Am) robA1 creC510 hsdR514 endA reacA1 uidA(ΔMluI)::pir</i>	Life Technologies
<i>P. putida</i>		
KT2440	Wild-type strain derived of <i>P. putida</i> mt-2 cured of the pWW0 plasmid	Bagdasarian et al. ¹¹⁷
KT-13	<i>Gm^R</i> , <i>P. putida</i> KT2440 with genomic insertion of pM13	This work
KT-BG	<i>Gm^R</i> , <i>P. putida</i> KT2440 with genomic insertion of pBG	This work
KT-BG13	<i>Gm^R</i> , <i>P. putida</i> KT2440 with genomic insertion of pBG13	This work
KT-BGXX ^a	<i>Gm^R</i> , <i>P. putida</i> KT2440 with genomic insertion of pBGXX	This work
Plasmids		
pRK600	<i>Cm^R</i> , <i>ori ColE1</i> , <i>tra+mob+</i> of RK2	Keen et al. ¹¹⁸
pTnS-1	<i>Ap^R</i> , <i>ori R6K</i> , <i>TnSABC+D</i> operon	Choi et al. ¹¹⁹
pS211	<i>Kan^R</i> , <i>ori R6K</i> , standard multiple cloning site	De Lorenzo Lab collection
pSEVA2513	<i>Kan^R</i> , <i>ori ColE1</i> , <i>P_{em7}</i>	Martínez-García et al. ⁸⁰
pSEVA237M	<i>Kan^R</i> , <i>ori pBBR1</i> , <i>msfgfp</i>	Martínez-García et al. ⁸⁰
pMK	<i>Kan^R Gm^R</i> , <i>ori ColE1</i> , miniTn7 cassette	Gene Art
pTn7-M	<i>Kan^R Gm^R</i> , <i>ori R6K</i> , <i>Tn7L</i> and <i>Tn7R</i> extremes, standard multiple cloning site	This work
pM13	<i>Kan^R Gm^R</i> , <i>ori R6K</i> , <i>Tn7L</i> and <i>Tn7R</i> extremes, <i>P_{em7}-msfgfp</i> fusion	This work
pBG	<i>Kan^R Gm^R</i> , <i>ori R6K</i> , <i>Tn7L</i> and <i>Tn7R</i> extremes, <i>BCD2-msfgfp</i> fusion	This work
pBG13	<i>Kan^R Gm^R</i> , <i>ori R6K</i> , pBG–derived, <i>P_{em7}</i>	This work
pBGXX ^a	<i>Kan^R Gm^R</i> , <i>ori R6K</i> , pBG–derived	This work
a: the tag XX represents numbered variants of synthetic promoters		

Table 3: Oligonucleotides and sequences synthesized in this work		
Name	Sequence 5'-3' ^a	Reference
SZ1	GACTTAATTAANNNNNNTTGACANNNNNNNNNNNNNNTATAATN NNNNNACCTAGGCCCAAGTTCACTTA	This work
SZ2	ACACCATAGGTCAGGGTAGTC	This work
SZ3	GCTGCGTTCGGTCAAGGTTTC	This work
BCD2 standardized linker	CCTAGGGCCCCAAGTTCACCTAAAAAGGAGATCAACAATGAAGCAATTTT CGTACTGAAACATCTTAATCATGCTAAGGAGGTTTCTAATGATCATGGG AATTCAT	This work
5-Pput-glmS UP	AGTCAGAGTTACGGAATTGTAGG	Schweizer ¹²⁰
3-Tn7L	ATTAGCTTACGACGCTACACCC	Schweizer ¹²⁰
a: the restriction sites <i>PacI</i> (TTAATTA) and <i>AvrII</i> (CCTAGG) are underlined		

2.2.2 Genome integration

To integrate the BGXX constructs into the *P. putida* KT2440 genome, *E. coli* PIR2 pBGXX (donor strain), *E. coli* DH5 α - λ pir pTnS-1 (strain leading transposase), *E. coli* pRK600 (helper strain) and *P. putida* KT2440 (recipient strain) were streaked one above the other on a lysogeny broth (LB) plate without antibiotics. This plate was incubated overnight at 30 °C. The next day cell material was taken from the bacterial lawn and streaked on cetrимide agar containing 30 mg l⁻¹ gentamycin (Gm) and incubated overnight at 30 °C. The next day one colony was streaked on LB-Gm³⁰ and again incubated overnight at 30 °C. To verify correct insertion of the transposon into the *att* site some clones resistant to Gm were selected and checked via colony PCR. One colony was picked, resuspended in 50 μ l of 60% alkaline PEG 200 (addition of 2 M KOH until pH=13.3-13.5) and incubated for 15 min at room temperature. 1 μ l of this suspension was used for the PCR (primers 5-Pput-glmSUP and 3-Tn7L, Table 3) and the products of amplification showed a size of 400 bp¹²⁰.

2.2.3 Bacterial strains, plasmids, and cultivation conditions

Strains and plasmids used are presented in Table 2. For cloning and screening purposes, *E. coli* cells were cultivated in liquid LB with 5 g l⁻¹ NaCl¹²¹. For solid cultivation 1.5% (w/v) agar-agar was added to the LB medium. Screening for active promoters in *E. coli* was done in 10 ml liquid LB. Fluorescence was measured after overnight growth. Cultures for flow cytometry experiments with *P. putida* KT2440 were carried out at 30 °C in M9 minimal medium supplemented with 2 mM MgSO₄ and 20 mM of glucose as the sole carbon source¹²². For quantitative assessment of synthetic promoters a minimal medium¹²³ containing 3.88 g l⁻¹ K₂HPO₄ and 1.63 g l⁻¹ NaH₂PO₄ with 20 mM of glucose as sole carbon source was used for liquid cultivation. For *E. coli* minimal medium cultures 10 mg l⁻¹ of thiamine was added. The volume to liquid ratio during liquid cultivation in Erlenmeyer flasks was always 1:10. For precultures 100 ml Erlenmeyer flasks were used, for main cultures 500 ml flasks. *P. putida* KT2440 was cultivated at 30 °C without any antibiotics in liquid minimal medium due to stable genomic integration. *E. coli* was cultivated at 37 °C. Cultivation was done in a Minitrone shaker (INFORS, Bottmingen, Switzerland) with orbital shaking (amplitude 50 mm) at 200 rpm shaking speed. When required, gentamycin (Gm 10 mg l⁻¹), kanamycin (Kan 50 mg l⁻¹), ampicillin (Ap 150 mg l⁻¹) and chloramphenicol (Cm 30 mg l⁻¹) were added to growth media, unless stated otherwise. The relationship between optical density at a wavelength of $\lambda = 600$ nm (OD₆₀₀) and fluorescence during growth with *P. putida* KT2440 was evaluated with linear regression in Microsoft Excel 2010 using a minimum set of 5 data points.

2.2.4 Fluorescence measurements

Fluorescence of samples from shake-flask cultures in liquid media was measured in 96 well plates (Greiner Bio-One, Solingen, Germany) in a synergyMX well-plate reader (Biotek, Bad Friedrichshall, Germany). Each sample was measured in triplicates with a volume of 200 μ l. The excitation wavelength was set to $\lambda=395$ nm. Fluorescence emission was measured at $\lambda=509$ nm at a read height of 8 mm. For *P. putida* KT2440 cells the gain was set to 75. For *E. coli* cells growing in LB medium the gain was reduced to 50 to compensate for autofluorescence of the LB medium¹²⁴. In order to prevent influence of different cell numbers on the fluorescence measurement after overnight growth, the

OD₆₀₀ was set to 1 for every construct to be integrated into the genome. This procedure was also followed for the comparison between *E. coli* PIR2 and *P. putida* KT2440 in minimal medium. Promoter activities were estimated based on the linear trend of a fluorescence over OD₆₀₀ plot, similar to Leveau and Lindow¹²⁵.

Single-cell fluorescence was analyzed with a MACSQuant VYB (Miltenyi) flow cytometer. MsfGFP was excited at $\lambda = 488$ nm, and the fluorescence signal was recovered with a 525(40) BP filter. Overnight *P. putida* KT2440 cultures were diluted 1:100 in fresh media containing 20 mM succinate or glucose as carbon sources and incubated for 4 – 5 h at 30 °C. After this pre-incubation, cells were analyzed at mid-exponential phase (OD₆₀₀ = 0.4), and for every aliquot 30,000 events were analyzed. The data processing was performed using FlowJo software (9.6.2 version), and data in Figure 7 were analyzed by Microsoft Excel 2010.

2.3 Results and discussion

2.3.1 Development of a novel promoter probe vector with minimal transcriptional noise

We chose the mini-Tn7 transposon as system to chromosomally integrate the constructs because it is targeted at high frequency into the *att*Tn7 site and it integrates as single copy located downstream of the *glmS* gene^{107,108}. Moreover, it integrates unidirectional and the integrated constructs are considered innocuous¹⁰⁸. Mini-Tn7-based vectors have proven useful for various genetic applications including gene expression analysis, functional characterization of genes, and single copy gene complementation^{119,120}. The scarcity of formatted tools makes their application difficult; we therefore designed standardized and minimized Tn7 vectors, which reflect SEVA architecture⁸⁰. As a backbone vector, we used a R6K suicide pS211 and cloned a mini-Tn7 device at the *AscI*/*SwaI* site (Figure 6); this module has two Tn7 extremes, two terminators T1 and T0, a Gm^R marker and the SEVA variant multi-cloning site (Figure 6B). The monocopy system optimized for *P. putida* KT2440 was obtained by implementing an additional part that consists of a translational coupler and a reporter protein, in this case MsfGFP¹¹³. We safely assumed that the basic mechanism of translation initiation in *P. putida* is the same as in *E. coli* and thus we chose a translational coupler that was described as highly efficient in *E. coli*. From the collection described in Mutalik et al.¹¹⁰, the unit BCD2 was selected and edited to obtain a standardized fragment compatible with our Tn7 format (Figure 6C). The resulting BG segment (=BCD2-*msfGFP*) contains an *AvrII*/*PacI* site for cloning promoters, two ribosome binding sites RBS (BCD2 linker) included as *AvrII*/*BclI* sites, and a translationally coupled *msfGFP*¹¹³ placed as an *EcoRI*/*EcoRI* fragment. The construct composed of the elements synthetic promoter-BCD2-*msfGFP* will be referred to as pBGXX, in which XX represents the number of individual synthetic promoters (Table 2). The synthetic promoters were designed by a strategy presented by Hammer et al.⁹⁸. The -35 and -10 consensus sequences of prokaryotic sigma-70 promoters were kept constant, whereas the spacer surrounding these sequences was randomized (Table 3). As a positive control the synthetic constitutive sigma-70 promoter P_{em7} promoter was used¹¹⁴ (see sequence in Figure 6D).

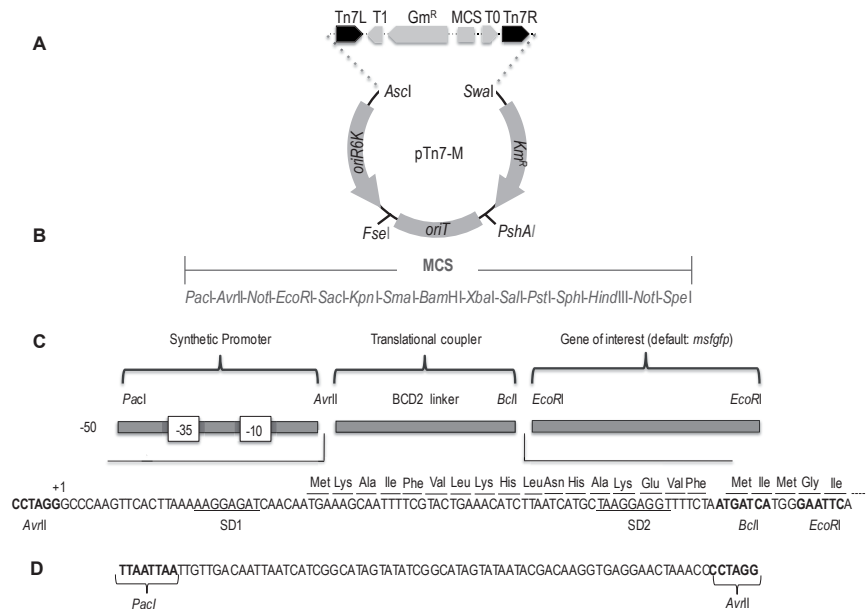


Figure 6: Structural organization of mini-Tn7 vectors. A) Functional elements of the plasmid include [i] a backbone vector bearing a Kan^R marker, origin of replication *oriR6K*¹¹² and the origin of transfer *oriT* and [ii] a Tn7 module cloned between *Ascl* and *SwaI* sites that bears a Gm^R marker, two terminators T1 and T0, a SEVA version of MCS, and two Tn7 sites recognized by a transposase (which is provided in trans). B) List of restriction sites found at the MCS. C) Structure of standardized module cloned into pTn7 vectors. A module carrying the synthetic promoter, BCD2 translational coupler¹¹⁰ and *msfgfp*¹¹³ (pBGXX), was designed to be compatible with standardized mini-Tn7s; the synthetic promoter is always included between *PacI*/*AvrII* sites; the linker is placed between *AvrII* and *BclI* and is translationally coupled to the *msfgfp* gene. The latter is inserted between two *EcoRI* sites to facilitate its replacement with other reporters or other genes of interest. The sequence of leader peptide is indicated between SD1 and SD2. D) complete sequence of *P_{em7}* promoter used as positive control.

2.3.2 Genome integrated controls exhibit increased expression with reduced transcription variability

In order to verify the functionality of the newly designed promoter probe vector, positive and negative control constructs were integrated into the genome of *P. putida* KT2440 and checked for GFP signal by flow cytometry (Figure 7). The GFP signal of the integrated promoterless negative control BG was comparable to the wild type *P. putida* KT2440 background control, suggesting a negligible background activity. To assess the performance of the new construct, we evaluated the effect of BCD2 linker on GFP production, for which we compared the activity of *P_{em7}*, alone or with the linker BCD2. After transposon insertion of the constructs, *P. putida* KT2440 cells were grown on succinate and analyzed by flow cytometry; as shown in Figure 7, GFP activity was improved by 60% when the translational coupler was present, demonstrating the functionality of standardized BCD2 in other Gram-negative bacteria than *E. coli*. Interestingly, besides the primary objective of

transcriptional noise reduction¹¹⁰, in this case BCD2 also enhanced GFP expression of KT-BG13 in comparison with the construct without BCD2 (KT-13). This increase may be explained by slight differences in the sequence behind the SD and the start of *msfgfp* of the two constructs. For individual analysis of vector and promoter, sequences were submitted to GenBank.

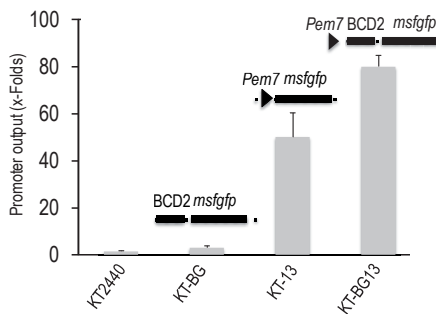


Figure 7: GFP quantification in Tn7-BG monocopy systems. Modules bearing promoter *P_{em7}*, translational coupler BCD2 and *msfgfp* were inserted into the *P. putida* KT2440 genome on a Tn7 mini-transposon. The composition of the Tn7 cargos is shown above the bars. Cells were grown overnight in M9 medium and diluted to an OD₆₀₀ of 0.4. The GFP signal was quantified by flow cytometry. Promoter intensity was normalized to the mean GFP fluorescence of the *P. putida* KT2440 background control. *P. putida* KT2440 with the insertion *P_{em7}-msfgfp* (KT-13) was used in this case as positive control. Error bars indicate SEM of at least three replicates.

2.3.3 Plasmid based screening of a synthetic promoter library in *E. coli*

Promoter probe plasmids have been extensively used to compare promoter activities in *E. coli* and *Pseudomonas* strains, principally *P. aeruginosa* and *P. putida*^{126–128}. These analyses showed that consensus sequence, which includes -10 and -35 regions, was highly similar to that of sigma-70 promoters in *E. coli*^{129,130}. Moreover, some experiments described how promoters bearing consensus sequences -10 and -35 (and placed in broad host range plasmids) were active similarly in *E. coli* and *Pseudomonas*¹²⁹. Sigma factors involved in transcription are also very similar in these strains, and transcriptional mechanisms respond identically to changes in promoter sequences recognized by sigma-70^{130,131}. Comparison between both *P. aeruginosa* *rpoD* and *E. coli* sigma-70 indicated that some regions involved in the interactions with -10 and -35 were virtually identical. The rule of thumb is that intrinsic promoter strength is kept in either host within the same activity range - changes are more dramatic only in regulated promoters, which is not the case in this work. On these basis, we proceeded to analyze synthetic promoters in *E. coli* cells, and further we compared the activity of these promoters in *P. putida* KT2440 strain.

An initial plasmid-based selection of synthetic promoters was performed in *E. coli* PIR2 cells since it decreased the screening effort drastically. Transformation of pBGXXs bearing synthetic promoters into *E. coli* PIR2 yielded greenish glowing colonies on LB-Kan⁵⁰ plates. Overnight liquid cultures revealed a gradual distribution of fluorescence, indicating the presence of promoters with different activities (Figure 8). The negative control construct BG shows a level of fluorescence that is comparable to the background signal from the LB medium, indicating that there is no transcriptional background activity originating from upstream elements. After compensation for this background, the fluorescence levels of this library range from 79±2 (BG28) to 23,203±833 (BG51) relative fluorescence units (RFUs), indicating a dynamic range of approximately 3 orders of magnitude.

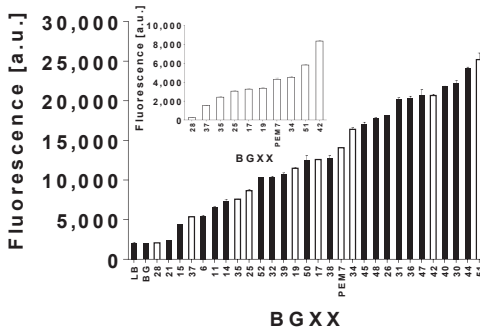


Figure 8: Screening for active promoters in *E. coli* PIR2. LB indicates the growth medium background; BG indicates the empty vector negative control without promoter; *P_{em7}* is a positive control promoter; error bars indicate SEM of at least three replicates. The promoters marked in white were selected for the genome integration. Inset: Reevaluation of selected synthetic promoters in *E. coli* PIR2. Fluorescence was measured after overnight growth in LB-Kan⁵⁰ media; the presented data are compensated for the background of the negative control BG. Samples were diluted to an OD₆₀₀ of 1.0 for fluorescence measurement using a plate reader. Error bars indicate SEM of at least three replicates.

The distribution of promoter activities in Figure 8 shows a relatively linear increase up to the maximum with the positive control *P_{em7}* being a medium strength promoter within this library. This continuous increase indicates that the upper limit of promoter strength may not have been reached. Indeed, this is a relatively common phenomenon, which can also be observed for other synthetic promoter libraries^{26,52}. This is understandable considering the degeneracy of the synthetic promoter oligonucleotide, which in this case contains $4^{28} = 7.2 \cdot 10^{16}$ possible combinations, which are further expanded by primer errors such as indels or SNP's in the -10 and -35 region^{23,52}. Out of these, only a handful of synthetic promoters were evaluated, typically showing a distribution of activities like in Figure 8. From the library presented in Figure 8 we selected nine promoters of different strength to be integrated into the genome of *P. putida* KT2440. The selected synthetic promoters were re-analyzed in *E. coli* PIR2 following overnight growth and dilution to an OD₆₀₀ of 1.0 in order to exclude errors from the medium-throughput screening and to avoid the influence of different cell concentrations on the GFP fluorescence level (highlighted in the inset).

2.3.4 Assessment of a genome integrated and calibrated synthetic promoter library in *P. putida* KT2440

In order to quantitatively assess the strength of the synthetic promoters, a stable and single copy expression is essential. Therefore, we integrated the mini-Tn7 transposon constructs with the selected synthetic promoters, along with the promoterless negative control BG and the *P_{em7}* positive control pBG13, into the *attTn7* site of the genome of *P. putida* KT2440. Prior to quantitatively assessing the integrated synthetic promoters in growth experiments, cultures containing specific promoters were examined for population homogeneity by flow cytometry. We analyzed promoter activity in cells growing in the exponential phase (OD₆₀₀ = 0.4) in minimal media with glucose as typical glycolytic carbon source. Flow cytometry revealed single peaks for all constructs. Thus, regarding GFP distribution in single cells, we noted a typical unimodal behavior¹³³ (Figure 9) in which bacterial population is homogeneously distributed with a stable single copy expression during exponential growth phase¹³⁴.

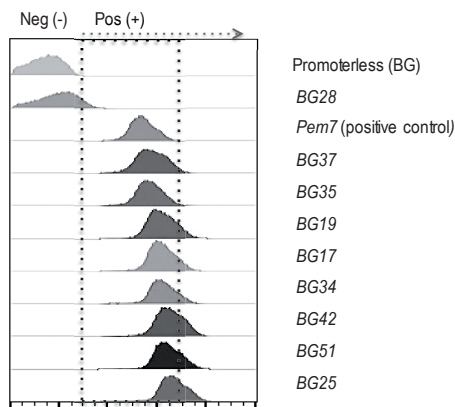


Figure 9: Single cell analysis of *P. putida* KT-BGXX strains. Cells grown overnight were diluted 1:100 in M9 medium supplemented with glucose as carbon source. At exponential phase ($OD_{600}=0.4$), samples were analyzed by flow cytometry. For each assay, 30,000 cells were recovered and *P. putida* KT-BG was used as negative control strain.

This last feature also gives evidence that all synthetic promoters assayed have a constitutive nature. With small variations, such constitutive activity was kept regardless of the growth substrate added to the medium, although in some cases there was a dependence on the growth phase (data not shown). To get a detailed and quantitative insight into the activity of the synthetic promoters during different growth stages, OD_{600} and fluorescence measurement were assessed over time in shake-flask cultivations in minimal medium with glucose as sole carbon source. Although absolute fluorescence levels differ for each construct, a similar trend is observed for all constructs, as exemplarily shown in Figure 10 for BG17.

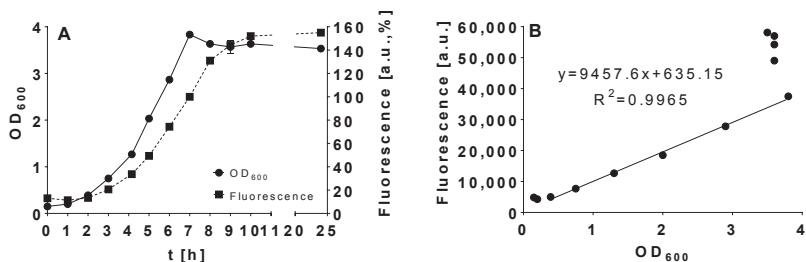


Figure 10: Development of fluorescence and biomass during growth of *P. putida* KT2440 in shake flasks carrying BG17 in minimal medium containing 20 mM of glucose. A) The relative fluorescence was measured in a well-plate reader and was set to 100% at the onset of the stationary phase (t=7); Error bars indicate SEM (n=3), but these are mostly so small that they are covered by the symbols; B) Linear relationship between fluorescence and biomass during the exponential phase of growth.

During the exponential phase, fluorescence develops parallel to growth and a linear relationship can be observed between fluorescence and growth over a broad period of time that corresponds to the main growth phase (see Figure 10B). This indicates that the promoter is equally active throughout growth. The same behavior was observed for every other promoter analyzed, with the lowest linear

correlation coefficient value (R^2) between fluorescence and growth of 0.986 for BG19 (Table 4). The quantitative physiology data are summed up in Table 4. The given slopes of the linear functions are compensated for the background of the negative control BG. A similar phenotype of synthetic promoters during growth was also observed by Rud et al.¹⁰⁰ working with *L. plantarum* and by Hartner et al.¹³⁵ working with *Pichia pastoris*.

Table 4: Quantitative data of the calibrated synthetic promoter library in *P. putida* KT2440

Promoter number/ SEVA name ^a	Growth Rate μ^b	Slope of linear function ^b	R^2 values of linear fit ^c	Activity related to BG42 [%]
BG	0.72±0.02	0±47	$R^2_1=0.974$ $R^2_2=0.986$	0±5
BG28 / 14a	0.60±0.02	433±6	$R^2_1=0.999$ $R^2_2=0.999$	3±1
BG35 / 14b	0.66±0.00	3226±122	$R^2_1=0.989$ $R^2_2=0.992$	25±4
BG37 / 14c	0.61±0.01	4091±204	$R^2_1=0.999$ $R^2_2=0.996$	32±5
BG34	0.63±0.01	5517±296	$R^2_1=0.992$ $R^2_2=0.996$	43±5
BG19	0.66±0.01	5583±242	$R^2_1=0.986$ $R^2_2=0.997$	44±4
Pem7 / 13	0.64±0.03	5723±574	$R^2_1=0.984$ $R^2_2=0.996$	45±10
BG51 / 14d	0.63±0.00	5726±397	$R^2_1=0.998$ $R^2_2=0.998$	45±7
BG17 / 14e	0.61±0.00	8216±384	$R^2_1=0.997$ $R^2_2=0.998$	64±5
BG25 / 14f	0.58±0.01	11646±344	$R^2_1=0.987$ $R^2_2=0.998$	91±3
BG42 / 14g	0.55±0.00	12787±820	$R^2_1=0.995$ $R^2_2=0.996$	100±6

a: Selected promoters were re-named as SEVA cargo sorted by their relative activity.

b: Growth rates and slopes are given as mean values from at least two replicates with SEM; values given for the slope of the linear function are compensated for the reference BG.

c: A minimum set of 5 data points (fluorescence over OD plot) showing the best R^2 values were taken into account for the calculation of the slope, R^2_1 and R^2_2 refer to individual replicates

However, fluorescence increased by 42-67% even after the onset of the stationary phase (e.g. 55% increase for BG17 at t=7-24 h (Figure 10A). In part, this is maybe due to the maturation time of the GFP. However MsfGFP matures in minutes¹¹³, whereas the fluorescence increases for several hours after depletion of the carbon source. In addition, this phenomenon was also observed by Jensen and Hammer⁸¹ using β -galactosidase as a reporter gene, which does not require the longer maturation times typical for GFP. It is more likely that the orthogonal synthetic promoters simply remain active in the stationary phase, and MsfGFP protein synthesis continues from amino acids turned over from degradation of the total cell protein pool¹³⁶. Indeed, protein production continues under starvation from the proteins produced during exponential growth^{137,138}. Therefore, quantitative analyses of synthetic constitutive promoters would be best during the exponential phase to minimize over-

estimation of activities. Upon inoculation, a slight initial decrease of fluorescence is observed, likely due to a dilution through cell division. Apparently, this diluting effect is higher than the production of new MsfGFP in the initial stage of growth. Only when the culture reaches the early exponential phase ($t=2h$, Figure 10A) does the effect of newly expressed MsfGFP become obvious.

Due to the genomic insertion of the constructs there was no need for continuous antibiotic selection, resulting in generally high growth rates in these cultures. However, a difference between the negative control BG and the promoter library is apparent, but the difference is not significant in most cases (Welch-test, $p>0.05$). This indicates a low level of stress, which only becomes significant with two promoters (BG25: $p=0.028$ and BG28: $p=0.038$). Surprisingly, one of these two promoters is the weakest promoter tested (BG28). Moreover, there is no apparent relation between promoter strength and growth rate reduction.

When comparing the activities of the promoter library in *E. coli* PIR2 and *P. putida* KT2440, the former host enables overall higher fluorescence signals (Figure 11). This can be attributed to the fact that in *E. coli*, the promoter probe construct lies on a multi-copy plasmid, whereas in *P. putida* a single copy of the construct is integrated into the genome. The copy number of R6K-based plasmids in *E. coli* PIR2 is approximately $15 \cdot 10^{139}$, while the fluorescence levels in *E. coli* are only 5 ± 1 -fold higher than in *P. putida*.

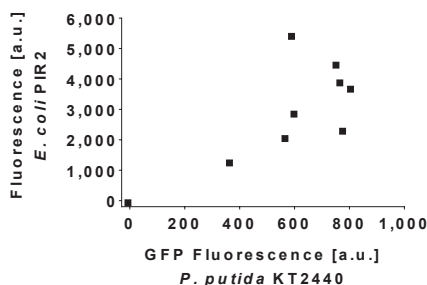


Figure 11: Comparison of activities of individual synthetic promoters in *E. coli* PIR2 and *P. putida* KT2440. Both strains were cultivated overnight in the same minimal medium; for *E. coli* 50 mg l⁻¹ Kan was added; Samples were diluted to an OD₆₀₀ of 1.0 for fluorescence measurement using a plate reader. The gain factor for the measurement of fluorescence was set to 50 and signals were compensated for the background signal by subtraction of the BG negative controls in the respective organism.

This would indicate that, relatively speaking, the expression of the single-copy *P. putida* constructs is more efficient, meaning that *E. coli* exhibited less fluorescence per copy of construct since the fluorescence levels were not 15-fold higher as one could expect from the 15-fold higher copy number. This is due to several reasons: high-level gene expression from a multi-copy plasmid in dividing cells imposes a high metabolic burden, which is further exacerbated by antibiotic selection, especially using kanamycin, which specifically inhibits protein synthesis. This was circumvented with the genome-integrated constructs. Possible explanations for the observed lower fluorescence per copy number in *E. coli* could be plasmid copy number variations or subpopulation plasmid loss^{31,60}.

In sum, although the promoters tested in this work keep some general trends in *E. coli* and *P. putida*, the specific parameters that rule their output do change in either host (Figure 11). This is not

altogether unexpected⁸¹ and should be taken into account when expression devices or genetic circuits are passed from one species to another¹⁰⁰.

2.3.5 Sequence analysis of synthetic promoters

The strength of a promoter depends on its sequence⁸¹, but also on its up-⁹⁶ and downstream⁹⁷ elements as well as a combination thereof¹⁴¹. Since in this study up- and downstream elements as well as the -35 and -10 motifs of sigma-70 promoters were kept constant, only the sequence of the spacers indicated in Table 5 (see Design) can contribute to the strength of the calibrated synthetic promoters.

Table 5: Sequence of integrated synthetic promoters upstream of the BG construct sorted by their relative activity (see Table 4)

Name	Sequence ^a	GC % ^b
Design ^c	<u>TTAATTAA</u> NNNNN TTGACA NNNNNNNNNNNNNNNN TATAAT NNNNNNA <u>CCTAGG</u>	
BG28	<u>TTAATTAA</u> CTAGG TTGACA -----TGGAT TATAAT GTATGTAC <u>CCTAGG</u>	
BG28s ^d	ATGTCAGACGCTC <u>TTAATTAA</u> CTAGG TTGACA TGGAT TATAAT GTATGTAC <u>CCTAGG</u>	43
BG35	<u>TTAATTAA</u> TTTAT TTGACA TGCGTGATGTTTAGAAT TATAAT TTGGGGAC <u>CCTAGG</u>	36
BG37	<u>TTAATTAA</u> GTGAAT TTGACA TGTCAAATTTTATGTTG TATAAT AATACTAC <u>CCTAGG</u>	25
BG34	<u>TTAATTAA</u> ATAAT TTGACA TCGAAATCGAACACATG TATAAT CGCTTAAC <u>CCTAGG</u>	36
BG19	<u>TTAATTAA</u> CCAAT TTGACA ATCAACAGGAAACACTT TATAAT ACGAGAAC <u>CCTAGG</u>	39
BG51	<u>TTAATTAA</u> TCTACT TTGACA TCCGACATTCGCGACTG TATAAT AAGTTGAC <u>CCTAGG</u>	50
BG17	<u>TTAATTAA</u> CGGAG TTGACA CACTCGAAAAGCCGAGT TATAAT CGATGAC <u>CCTAGG</u>	57
BG25	<u>TTAATTAA</u> AGCCCC TTGACA TGACATGGTTTGGAGG TATAAT GTGGCGAC <u>CCTAGG</u>	64
BG42	<u>TTAATTAA</u> AGCCCAT TTGACA AGGCTCTCGCGGCCAGG TATAAT TGCACGAC <u>CCTAGG</u>	75

- a: The restriction sites for *PacI* (TTAATTAA) and *AvrII* (CCTAGG) are underlined; the conserved -35 (**TTGACA**) and -10 (**TATAAT**) regions of sigma-70 promoters are shown in bold; synthetic promoters are sorted by activity as indicated in Table 4.
- b: GC content [%] indicated for the spacer region (see “N” in the Design).
- c: Part of the oligonucleotide design which represents the synthetic promoters (see primer S21 in Table 3).
- d: BG28s illustrates the shifted sequence due to the 13 bp deletion within the center spacer region

The sequence logo of the synthetic promoters reveals that certain positions might have an impact on the strength of the synthetic promoters, especially positions -37, -29, -25 and -13. These positions are dominated by only two of the four nucleotides (Figure 12). For all other variable positions at least three of the four nucleotides are occurring nearly at the same frequency. Hence, it is not really possible to conclude whether certain positions within the spacers significantly influence the strength of these synthetic promoters. Except for BG28, all promoters have a complete sigma-70 sequence that matches the initial oligonucleotide design, i.e. no deletions in the whole sequence or mutations in the -10 and -35 consensus sequences. Due to a 13 bp deletion in the sequence of BG28, the center spacer was shortened from 17 bps to 4 bps so that the original -35 consensus sequence TTGACA is now shifted to TTAATT. Likely this accounts for the relatively weak activity of this promoter (Table 4) since the varied -35 consensus sequence is more different from the conserved consensus TTGACC¹³¹.

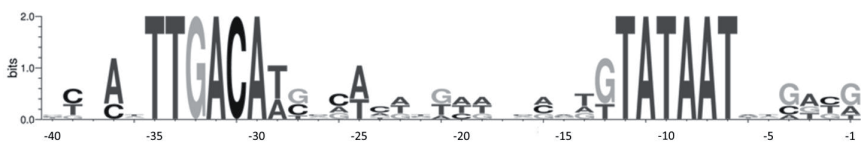


Figure 12: Sequence logo of the synthetic promoters; The sequence logo¹⁴² was constructed using WebLogo3¹⁴³. The synthetic promoter in construct BG28 was omitted due to the shift in the center spacer (see Table 5)

A comparable phenomenon can be observed in a study by Mutalik et al.¹¹⁰, who varied bases within the -35 or -10 consensus sequences, resulting in a relative over-representation of weak promoters. Contrary to the *E. coli* pre-screening, the three synthetic promoters 34, 19, and 51 have a very similar activity (Table 4). The sequences of promoters 34 and 19 have some similarity, i.e. ten matching bases out of a total of 28 randomized bases and a similar CA-rich stretch in the center region. However, BG51 has no clear similarity to the other two. Generally speaking, the composition of the spacer sequences indicates a possible relationship between the GC content and the promoter activity (Table 5). Except for BG28 and 35, the promoter activity increases with the GC content of the spacer sequences. A similar relationship was already observed by Seghezii et al.¹⁰², who stated that G-rich promoters are stronger than G-poor promoters. However, it should be noted that the dataset inspected here is too small to raise general sequence-activity correlations¹³².

2.4 Conclusion

A new and reliable genetic device was constructed in which promoters can easily be exchanged as the only variable for fixing a pre-determined expression level of given genes of interest. The expression system is inserted into the genome in an innocuous and specific site that occurs in a wide range of Gram-negative bacteria^{35,64}. Seven synthetic promoters were selected and re-named as SEVA cargo number 14, followed by an alphabetical letter to reflect their expression level from lowest (a) to highest (g) (see Table 4). These promoters make up a calibrated promoter library, which covers a range of activity of almost three orders of magnitude. A spread of activities with steps of 7-27% will enable fine-tuning of gene expression while circumventing the laborious expression analysis usually needed when using synthetic promoter libraries. This calibrated promoter library significantly expands the range of dependable constitutive expression levels available for applications ranging from metabolic engineering to microbial physiology, thereby contributing to the continuous development of *P. putida* as a biotechnological workhorse.

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Chapter 3

Different approaches of increasing the glucose uptake rate in *Pseudomonas putida* KT2440

NADH constitutes the main electron acceptor and donor in cofactor dependent cellular reactions. An increase in the demand of NADH through the imposition of an heterologous NADH-dependent reaction might cause an insufficient supply of this cofactor. Hence, increasing the regeneration rate of NADH of the microbial host is a promising approach of circumventing NADH shortage. Since NADH is mainly regenerated in reactions of the central carbon metabolism, increasing the metabolic rate of the microbial host may lead to an increased NADH regeneration rate. We tried to increase the metabolic rate of the soil microorganism *P. putida* KT2440 through overexpression of an ATP wasting enzyme, which was shown to drastically increase the glucose uptake rate in *E. coli*. However, *P. putida* KT2440 was able to circumvent the intended ATP wastage with the aim of increasing the NADH regeneration rate through an increased glucose uptake rate likely through sophisticated regulatory mechanisms.

Author Contributions:

Sebastian Zobel has conceived and designed the project, performed the experiments, analyzed the results, and has written the chapter with the help of Nick Wierckx. Nick Wierckx and Lars M. Blank have conceived and supervised the study. Zahra Shukoor has characterized the synthetic promoter library illustrated in Figure 13.

3.1 Introduction

Whole cell biocatalytic reactions require vital and metabolically active cells, especially when non-natural electron demanding reactions are imposed on the organism. Often these kinds of reactions are NADH-dependent and constitute an invasive intervention for the cells since NADH as a major electron carrier has to supply the electron transport chain sufficiently in order to sustain the proton motive force and maintain the cell's viability. Since nature promotes growth, the imposed reaction can be limited by the supply of NADH¹⁴⁵. Hence, on the one hand, an increased regeneration rate of NADH would support the organism with sufficient NADH to keep its viability and on the other hand the NADH demanding reaction might not be limited by the supply of electrons. One possibility of how the NADH regeneration rate could be increased will be described in Chapter 4. This chapter pursues an alternative approach.

The main source of NADH is the central carbon metabolism composed of a breakdown of a carbon source like glucose down to pyruvate, which is further metabolized in the oxidative TCA cycle⁶⁹. Thus, one option with the aim of increasing the NADH regeneration rate would be an increased flux through the central carbon metabolism, which requires an increased uptake rate of the carbon source.

Schaaff et al.¹⁴⁶ addressed the enzymes acting in the breakdown of glucose. Overexpression of the key glycolytic enzymes in the yeast *Saccharomyces cerevisiae* did not lead to an increased glucose uptake rate. Next to glucose catabolizing enzymes, overexpression of glucose transporters would be another possibility to manipulate the uptake rate. But Ruyter et al.⁷¹ have shown that an increased expression level of glucose uptake enzymes above wild type levels in *E. coli* did not result in an increased glucose uptake rate either. Consequently, Koebmann et al.⁷² concluded that other factors determine the rate at which glucose is taken up and oxidized and unveiled that in *E. coli* a low ATP level is the major determinant. They accomplished lower ATP levels through overexpression of the F_1 subunit of the (F_1F_0) H^+ -ATP Synthase (EC 3.6.1.34) which hydrolyses ATP if it is independently expressed from the membrane bound F_0 subunit. Zhu et al.¹⁴⁷ were able to establish lower ATP levels in *E. coli* with a different but similar approach by knocking out the genes responsible for the F_0 subunit and the ArcA regulator system resulting in comparable glucose uptake rates, whereas Liu et al.¹⁴⁸ demonstrated that the glucose uptake rate of eukaryotic hosts can also be increased with different approaches of lowering the ATP level by manipulating the electron transport chain in the yeast *Torulopsis glabrata*.

Next to *E. coli* and *S. cerevisiae*, *P. putida* is emerging as an industrially relevant workhorse^{93,149} and has proven to be able to increase its glucose uptake and NADH regeneration rate under solvent stress^{45,50}. However, these high rates were only observed under severe energy demanding conditions. In this study we tried to increase the glucose uptake rate of *P. putida* KT2440 with an approach motivated by Koebmann et al.⁷² through the overexpression of its *atpAGD* operon, representing the genes for the F_1 subunit of the ATP synthase. We expected *P. putida* KT2440 to stay viable and metabolically active under these harsh conditions due to its remarkable ability of generating energy at a high rate and thus being encouraged to increase the flux of carbon through

its central carbon metabolism. However, *P. putida* KT2440 could not be animated to boost the rate of its central carbon metabolism with this approach most likely due to promoter silencing mechanisms or other abilities to prevent the expression of adverse genes. Even two different alternatives did not result in an increased glucose uptake rate. Finally, other approaches which might result in an elevated glucose uptake rate are discussed.

3.2 Material and Methods

3.2.1 Bacterial strains, plasmids and cultivation conditions

The strains and plasmids used in this study are presented in Table 6. For solid cultivation LB-medium¹²¹ with 5 g l⁻¹ NaCl was used. The LB-medium was solidified with the addition of 1.5% (w/v) agar-agar. For liquid cultivation different media were used. *P. putida* KT2440 harboring pSEVA247 constructs, *P. putida* S12 and S12.49 were cultivated in a minimal medium according to Hartmans et al.¹²³.

Table 6: Strains and plasmids used in this study

Strain	Genotype / relevant characteristics	Source
<i>E. coli</i>		
DH5α	<i>F endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG</i>	Hanahan ¹⁵⁰
JM101	<i>Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(r^k m^k), λ-supE thi Δ(lac-proAB) F' [traD36 proAB⁺ lacI^q lacZ ΔM15]</i>	Kuhn et al. ¹⁵¹
K12	<i>F, lambda^d, rph-1</i>	Blattner et al. ¹⁵²
<i>P. putida</i>		
KT2440	Derived from <i>P. putida</i> mt-2, cured of the pWW0 plasmid	Bagdasarian et al. ¹¹⁷
S12	Wild-type strain	Hartmans et al. ¹⁵³
S12.49	<i>P. putida</i> S12 evolutionary engineered in the presence of benzene	Volkers et al. ¹⁵⁴
Plasmids		
pTn1	<i>Gm^R, Amp^R, pUC ori, nag repressor and nag promoter</i> (salicylate inducible), <i>Tn-1</i> terminator	Wierckx et al. ⁴²
pJS-LacZ	<i>Gm^R, Amp^R, pUC ori, nag repressor and nag promoter</i> (salicylate inducible), synthetic promoter fused to the <i>lacZ</i> operon, <i>Tn-1</i> terminator	This study
pSEVA247Y	<i>Kan^R, ori pRO1600/ColE1, yfp operon, t0 and t1 terminator</i>	Silva-Rocha et. al. ⁷⁹
pSEVA247atpAGDXX ^a	<i>Kan^R, ori pRO1600/ColE1, synthetic promoter, atpAGD operon, yfp operon,</i>	This study
pSEVA247lacZYXX ^a	<i>Kan^R, ori pRO1600/ColE1, synthetic promoter, lacZ operon, yfp operon</i>	This study
pSEVA231	<i>Kan^R, ori pBBR1, t0 and t1 terminator</i>	Silva-Rocha et al. ⁷⁹
pSEVA231atpAGDXX ^a	<i>Kan^R, ori pBBR1, synthetic promoter, atpAGD operon, t0 and t1 terminator</i>	This study
pSEVA231lacZXX ^a	<i>Kan^R, ori pBBR1, synthetic promoter, lacZ, t0 and t1 terminator</i>	This study

a: XX represents variants of synthetic promoters

E. coli JM101 and *P. putida* KT2440 harboring pSEVA231 constructs were cultivated in M9* minimal medium with 10 mg l⁻¹ of thiamine described by Panke et al. in 1999¹⁵⁵. Both minimal media contained 20 mM of glucose. *E. coli* strains were always cultivated at 37 °C and *P. putida* strains at 30 °C. Precultures were inoculated the day before the experiment with cell material taken from the

bacterial lawn of the solid cultivation in 10 ml of liquid medium in a 100 ml Erlenmeyer flask. This 1:10 ratio of liquid medium to total volume of the flask was also applied in the main culture with 50 ml of liquid medium in a 500 ml non-baffled Erlenmeyer flask. All strains were cultivated at 200 rpm shaking speed with an amplitude of 50 mm in a Minित्रon shaker (INFORS, Bottmingen, Switzerland).

For growth experiments in the growth profiler 1152 (Enzysscreen, Haarlem, Netherlands) precultures were set up in 5 ml liquid medium in tubes the day before the experiment. The 96-square half deep well microplates were filled with 185 μ l of minimal medium and were inoculated with 15 μ l of the preculture. The shaking speed was set to 225 rpm with a temperature of 30 °C.

When required 50 mg l⁻¹ of Kan was added to solid or liquid medium. In case of Gm a concentration of 30 mg l⁻¹ was chosen for solid medium and 10 mg l⁻¹ for liquid medium.

3.2.2 Analytics and calculation of extracellular rates

The OD of the liquid culture was monitored at a wavelength of λ = 600 nm. Extracellular metabolites in the supernatant were quantified using the Ultimate 3000 HPLC system from Thermo Fisher Scientific (Waltham, MA, USA). The supernatant was obtained following centrifugation of the samples for 3 min at 17,000 x g out of which 5 μ l of the supernatant were used for the quantification of the analytes. Extracellular metabolites were eluted at 60 °C on a 300 x 8 mm organic acid resin column and a 40 x 8 mm organic acid resin precolumn (both CS Chromatographie, Langerwehe, Germany) with 5 mM H₂SO₄ as mobile phase at a flow rate of 0.7 ml min⁻¹.

The extracellular rates (growth rate, carbon uptake as the sum of glucose, gluconate and 2-ketogluconate, and acetate production rate) were determined using the software SigmaPlot (V 12.5.0.38) according to Heyland et al.¹⁵⁶.

3.2.3 DNA Techniques

Genomic DNA of *E. coli* K12 and *P. putida* KT2440 were isolated using the High Pure PCR Template Preparation Kit from Roche (Basel, Switzerland). The genomic DNA of *E. coli* K12 was used for the amplification of the *lacZ* operon fused to a synthetic promoter library using primers pSynPro_ *lacZ* and pLacZrev (Table 7) with the Phusion High-Fidelity DNA Polymerase from New England Biolabs (Ipswich, MA, United States). The resulting PCR fragment and the empty vector pTn1 (Table 6) were digested with *Bam*HI and *Xba*I. Additionally, the vector was dephosphorylated with an alkaline phosphatase. After ligation with a T4 DNA ligase, the product was transformed via a standard heat shock protocol into chemically competent *P. putida* KT2440. *P. putida* KT2440 was made chemically competent with a standard protocol using 0.1 M CaCl₂ and 0.1 M CaCl₂ with 15% (v/v) glycerol.

The genomic DNA of *P. putida* KT2440 was used for the amplification of the *atpAGD* operon using primers SZ9 and SZ10 with the Phusion High-Fidelity DNA Polymerase. The synthetic promoter sequence within primer SZ9 was designed according to Hammer et al.⁹⁸. The PCR product and the empty vector pSEVA247Y and pSEVA231 were digested with *Xba*I and *Hind*III before ligation. In addition, the vectors were dephosphorylated with an alkaline phosphatase. The ligation product was transformed into *P. putida* KT2440 and *E. coli* JM101 either according to Choi et al.¹⁵⁷ or into

prepared electrocompetent cells which were made competent as follows:

A 10 ml LB NaCl-free liquid preculture was inoculated and grown overnight. The next day four 500 ml Erlenmeyer flasks with 50 ml NaCl-free LB medium were inoculated to an OD₆₀₀ of 0.05 and were cultivated until the OD₆₀₀ reached 0.6 to 0.8. Subsequently, the flasks were kept on ice for 5 min before the culture was harvested and centrifuged for 10 min at 3,000 x g at 4 °C. All steps following the harvesting of the cells were kept on ice. The supernatant was discarded. Two pellets were resuspended with an inoculation loop in 30 ml each of 10% glycerol in ddH₂O and combined and resuspended with one of the remaining pellets.

Table 7: Primers used in this study

Primer	Sequence (5'→3') ^a	Description
pSynPro_lacZ	GCGGATCCNNNNNTTGACANNNNNNNNNNNNNNNT ATAATNNNNNNNGGTACCACTCGAGTGGAGAACGCTAT GACCATGATTACGGATTAC	Degenerated oligo with variable spacers attached to lacZ ,
pLacZrev	GCTCTAGATTATTTTGGACACCAGACCAACTG	reverse primer for SPL with lacZ
SZ2	CTTCGGGCTCGTATGTTGTG	Sequencing pJS-LacZ
SZ9	GCTCTAGANNNNNTTGACANNNNNNNNNNNNNNNT ATAATNNNNNNNGAAGGGGCGAGCAGAGCAATG	Degenerated oligo with variable spacers attached to atpA ,
SZ10	GAAAGCTTCGCTCAACCTGATAGCGCC	Reverse primer SPL pSEVA247Y,
SZ11	CAGGAAGCGAGTAATCAG	Colony PCR and sequencing pSEVA247atpAGDYXX
SZ12	GAACTGTGGCCGTTTAC	Colony PCR and sequencing pSEVA247atpAGDYXX
SZ15	GTTCGCAAGCTGGAAGAAAC	Sequencing pSEVA247atpAGDYXX
SZ16	CTCTGATCGCCTCTTCAAC	Sequencing pSEVA247atpAGDYXX
SZ17	TCAAGCATCACTGGGACTAC	Sequencing pSEVA247atpAGDYXX
SZ26	GGCCATCGTCCACATATC	Control forward and sequencing pSEVA231atpAGD
SZ27	GGCTACAGCTCGTTTAC	Control reverse pSEVA231atpAGDXX
SZ33	CCCTCTAGATGCTCTTGACACTCTGACAGAACGTAGGTATAA TTAGATGGAAGGGGCGAGCAGAGCAATGACCATGATTACG GATTCACT	Synpro21 of pSEVA247atpAGDY21 connected to lacZ ,
SZ34	CCCTCTAGAGGGAATTGACAACTGTTTTGGGAGATCTATAA TCGCGAAGAAGGGGCGAGCAGAGCAATGACCATGATTACG GATTCACT	Synpro24 of pSEVA247atpAGDY24 connected to lacZ ,
SZ35	CCTCTAGACCGCCTTGACATGGGTGGTATCTGGTCCTATAA TCGCTGGGAAGGGGCGAGCAGAGCAATGACCATGATTACG GATTCACT	Synpro124 of pSEVA247atpAGDY124 connected to lacZ ,
SZ36	GCAGGCTTTATTTTGGACACCAGACCA	Reverse primer lacZ,
SZ43	CCCTCTAGAGTTTTTGGACAGCGGAAAGGCACGGCTATAA TATATTGGAAGGGGCGAGCAGAGCAATGACCATGATTACGG ATTCACT	Synpro20 of pSEVA231atpAGD20 connected to lacZ ,
SZ44	CCCTCTAGATGCGGTTGACAGGACGGGAAGCTACGTTATA ATGAGGACGAAGGGGCGAGCAGAGCAATGACCATGATTAC GGATTCACT	Synpro38 of pSEVA231atpAGD38 connected to lacZ ,

a: The restriction sites of *Bam*HI (GGATCC), *Kpn*I (GGTACC), *Xho*I (CTCGAG), *Xba*I (TCTAGA), and *Hind*III (AAGCTT) are underlined; nucleotides in italics represent the sequence of specific synthetic promoters; nucleotides in bold represent the annealing part of the primer

The two tubes were centrifuged again. The supernatant was discarded and one pellet was resuspended in 20 ml of 10% glycerol and pooled together with the other pellet followed by a third centrifugation step. The supernatant was discarded again and the pellet was resuspended in 200 μ l of 10% glycerol. This cell suspension was subdivided into aliquots of 50 μ l which were stored at -80 °C. For transformation aliquots were thawed on ice and mixed gently with 1-10 ng of the ligation product followed by electroporation in a cuvette with a 0.2 cm gap with a pulse of 2.5 kV, 200 or 400 Ω and 25 μ F. Immediately after the pulse, 1 ml of LB-medium was added and the transformed cells were incubated for 1 h before 1/10 and 9/10 were spread on selective plates.

Clones resistant to Kan were selected and checked for successful integration of the construct after inoculation of 5 ml LB-Kan⁵⁰ medium and overnight growth followed by a plasmid clean up with the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Another method used was colony PCR for which colonies were resuspended in 50 μ l of 60% alkaline PEG 200 (addition of 2 M KOH until pH=13.3-13.5) and incubated for 15 min at room temperature. 1 μ l of this suspension was used for the PCR. In both cases the GoTaq MasterMix (Promega, Madison, WI, United States) was applied for validating the successful integration. In case of the synthetic promoter library based on pSEVA247Y, primers SZ11 and SZ12 were used. When pSEVA231 was chosen as backbone for the synthetic promoter library, primers SZ26 and SZ27 were used.

For the sequencing of the synthetic promoters and the *atpAGD* operon in pSEVA247atpAGDYXX constructs, samples were submitted to MWG eurofins with primers SZ11, SZ15, SZ16, SZ17 and SZ18. In case of pSEVA231atpAGDXX constructs, the samples were only mixed with primer SZ26 to unveil the sequence of the synthetic promoters.

In order to test the activities of certain promoters, new primers were designed and used for the amplification of the *lacZ* gene with genomic DNA of *E. coli* K12. When synthetic promoters of pSEVA247atpAGDYXX constructs were investigated, primers SZ33, SZ34 or SZ35 with SZ36 were used. In case of pSEVA231atpAGDXX constructs, primers SZ43 and SZ44 with SZ36 were applied. In all cases the constructs and the empty vectors were digested with *Xba*I and *Hind*III and ligated and transformed in the manner described above. When *lacZ* constructs were transformed, selective LB-Kan⁵⁰ plates were supplemented with 0.2 mM IPTG (Isopropyl β -D-1-thiogalactopyranoside) and 0.1 g l⁻¹ X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside).

3.2.4 Fluorescence microscopy

For fluorescence microscopy of living cells, samples were taken from a standard main culture grown to mid exponential phase in Hartmans' minimal medium¹²³. The samples were diluted 1:10 in minimal medium without glucose. The cells were fixed on 50 μ l of minimal medium without glucose and 1% (w/v) agarose between the microscope slide and the cover slip. The minimal medium containing agarose cooled down between two slides before use.

The pictures were taken with AF 6000LX system from Leica (Wetzlar, Germany) with an HC PL APO63x/1.40 OIL objective and an EL 6000 (LQ-HXP 120 LEJ) illuminant. The settings for the two channels are given in Table 8.

Table 8: Fluorescence microscopy settings

	Channel 1	Channel 2
Filter	brightfield	YFP
Gain	1.3	10
Exposure time [milliseconds]	14.61	1000.531

3.2.5 Enzymatic assays

For measurement of the β -galactosidase activity samples were taken from a liquid culture grown to mid-exponential phase at $OD_{600} = 1.0$. In a total volume of 1 ml, sample and buffer (pH = 7.0) from Hartmans minimal medium¹²³ were mixed in different ratios. To this solution 2 drops of chloroform and 1 drop of 1 g l⁻¹ SDS solution were added followed by vortexing the mixture for 10 s and an incubation step for 5 min at 37 °C. The β -galactosidase activity was measured according to the instructions by Miller¹⁵⁸.

3.2.6 SDS PAGE

For the SDS PAGE a whole main culture of the respective strain was harvested and centrifuged for 10 min at 3,000 x g and 4 °C. The pellet was resuspended in 3 ml of the buffer of the growth medium, mixed with one scoop of glass beads with a diameter of 0.75 - 1 mm. This mixture was vortexed three times for 30 s followed by three sonication steps for 45 s interrupted by a 30 s pause. The mixture was always kept on ice. Subsequently, the protein concentration was measured according to Bradford¹⁵⁹.

For the SDS Page 10 µg of protein were loaded on the Any kD Mini-PROTEAN TGX Gel in a Mini-PROTEAN® Tetra Cell (both Bio-Rad, Munich, Germany) and the SDS Page was carried out according to the instructions from Bio-Rad.

3.3 Results and discussion

An increased NADH regeneration rate might enhance the biocatalytic performance of *P. putida* KT2440 domesticated for the production of high value compounds. Since NADH is mostly regenerated within the central carbon metabolism, an increased flux through the central carbon metabolism would also result in an increased NADH regeneration rate. In this study we tried to increase the glucose uptake rate in *P. putida* KT2440 by increasing the ATP demand via overexpression of an ATP wasting enzyme.

3.3.1 Background expression and read through are too toxic for successful subcloning in *E. coli* DH5α

The initial strategy was to insert the *atpAGD* construct on a plasmid with an inducible promoter. *E. coli* DH5α was chosen as standard host for subcloning purposes. But repeated cloning procedures did not lead to living cells. Other cloning strategies like blunt end ligation instead of sticky end ligation, different vectors or cloning strategies like PLICing¹⁶⁰ also failed. Due to all these different strategies, which did not result in living colonies, we came to the conclusion that despite the absence of inducers and transcriptional terminators it is very likely that a toxic background expression and

read through occurred¹⁶¹. Due to the high copy number of the chosen vector in *E. coli*, even low background expression would be multiplied, leading to lethal cellular concentrations of the ATPase. Hence, the overexpression of an ATPase needs very weak promoters which can be found within a synthetic promoter library. Therefore, a synthetic promoter library was designed out of which those promoters with a defined activity and strength can be chosen to drive the overexpression of the *atpAGD* operon based on the strategy presented by Koebmann et al.⁷². Moreover, no subcloning in *E. coli* is required and the constructs can be directly investigated in the desired host. For initial screening purposes and determination of synthetic promoter activities, *lacZ* of *E. coli* K12 was chosen as reporter gene.

3.3.2 A slight imbalance of the F₁ to the F₀ subunit of the ATP synthase impedes growth of *P. putida* KT2440

A synthetic promoter library was created by PCR with primers pSynPro_*lacZ* and pLacZrev (Table 7) leading to 72 blue and 36 white colonies, which were picked from an X-gal plate and assessed for their promoter strength by a Miller assay¹⁵⁸. With the new designed synthetic promoter library a broad activity of synthetic promoters was found ranging from 123±4 Miller Units (MU) to 8,747±739 MU (Figure 13). Koebmann et al.⁷² presented in their study promoters which were even less active than the weakest promoter found here, which nevertheless had a huge impact on the *E. coli* physiology.

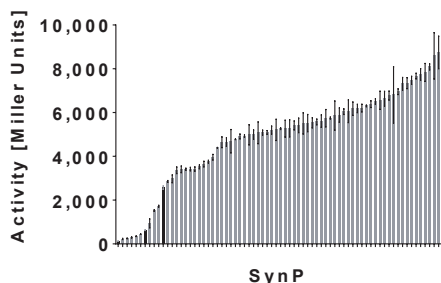


Figure 13: Synthetic promoter activities in *P. putida* KT2440; 72 promoters were tested for activity; bars indicate the mean of at least two replicates with SEM, black bars indicate synthetic promoters chosen for cloning of the *atpAGD* operon

However, *P. putida* KT2440 is able to regenerate NADH at a high rate to support an elevated NADH demand⁵⁰ and it is endowed with a very flexible electron transport chain⁴⁰, which can be compared to the electron transport chain of *P. aeruginosa*⁵⁹, and can be adapted to different ATP needs. Therefore, we thought that *P. putida* KT2440 would be able to stand an elevated ATP demand compared to that observed in *E. coli* by Koebmann and coworkers. Hence, out of this library two weak synthetic promoters SynP2 (123±2 MU) and SynP37 (630±22 MU) and the medium strong synthetic promoter SynP30 (2,569±103 MU) were chosen for the overexpression of the *atpAGD* operon (marked in black in Figure 13). However, even the weakest promoter (SynP2) seemed to be too strong in order to overexpress the ATPase genes in *P. putida* KT2440. Maybe even in *P. putida* KT2440 a slight imbalance of the F₁ to the F₀ subunit of the ATP synthase was already too

toxic since only clones with the *lacZ* gene were found originating from the uncut template.

Thus, a new strategy was developed since designing a synthetic promoter library independent from the GOI was not rewarding. Therefore, a new synthetic promoter library directly linked to the GOI was designed. By this means only those transformed cells survive, which express the ATPase at a viable range.

3.3.3 Despite active promoters no phenotype in *P. putida* KT2440

For this purpose the plasmid pSEVA247Y⁷⁹ was chosen as backbone. It already includes *yfp* as reporter gene which translates into the yellow fluorescent protein (YFP). YFP is an ideal reporter gene since it is easy to detect with living cells without the need for laborious enzymatic assays. The *atpAGD* operon was amplified with primers SZ9 and SZ10 (Table 7), ligated into the vector and transformed directly to *P. putida* KT2440. With this strategy many positive clones carrying *atpAGD* were detected (Figure 14).

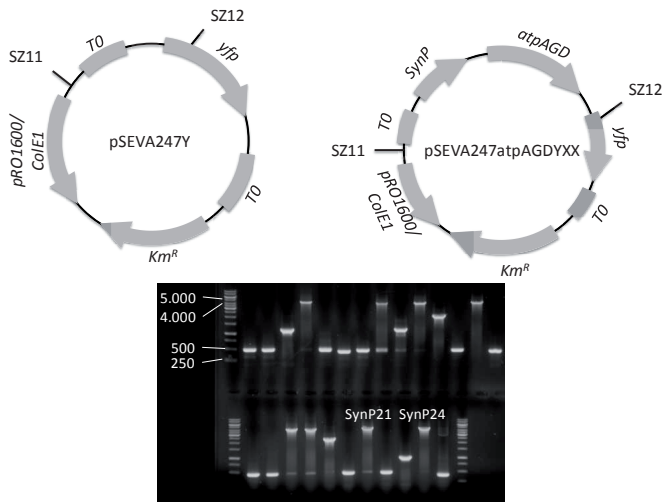


Figure 14: Integration of the *atpAGD* operon into the pSEVA247Y backbone; pSEVA247Y backbone with annealing sites of primers SZ11 and SZ12 resulting in an 0.4 kb fragment; pSEVA247atpAGDYXX with annealing sites of primers SZ11 and SZ12 resulting in a 4.3 kb fragment; gel picture verifying successful PCR; clones carrying SynP21 and SynP24 are indicated exemplary.

E. coli cells exhibiting an elevated ATP demand drastically changed the ratio of glucose being converted either to energy or biomass⁷². Hence, a reduced growth rate might indicate active *atpAGD* expression since cells would be challenged with a higher energy demand which would not allow the cells to grow at the same rate. Therefore, all positive clones were compared in a 96 well format under the same conditions in a growth profiler (Figure 15). Surprisingly, most of the positive clones even grew faster than wild type *P. putida* KT2440 and those positives growing more slowly than the wildtype were not impacted significantly (according to an unpaired Welch t-test, $P < 0.05$). Even with

a new batch of successfully transformed cells the expected phenotype was not observed (data not shown). This is maybe due to several reasons.

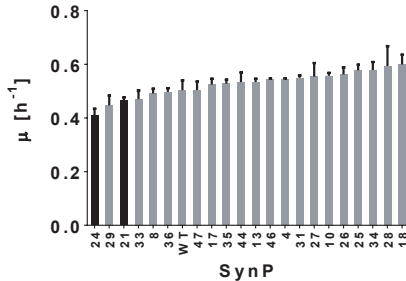


Figure 15: Growth rate profile of *P. putida* KT2440 harboring pSEVA247atpAGDYXX constructs; columns represent the mean with SEM of three replicates; WT: wild type of *P. putida* KT2440 without the empty vector control; black bars indicate SynPs which underwent further investigation.

Having a detailed look at the results of the PCR verifying the presence of the *atpAGD* construct in the original clone (Figure 14), it appeared as if the culture consists of two subpopulations since next to the band of 4.3 kb, a shorter band of 0.4 kb appeared (see SynP21 and SynP24). This indicates the presence of the empty vector in the same culture originating from low concentrations of the uncut empty vector and a potential double transformation event into a single cell^{162,163}. The empty vector pSEVA247Y (4.3 kb) is nearly half in size compared to the pSEVA247atpAGDYXX construct (8.2 kb) leading to a shortened replication time for the empty vector. Consequently, the *atpAGD* construct may have been diluted within the culture resulting sooner or later in outgrowth of the subpopulation carrying the *atpAGD* construct by those cells harboring the empty vector. Hence, we made sure that only pure cultures were investigated in more detail, which were obtained by repeated re-streaking and resuspension of single colonies followed by overnight growth on selective solid medium.

To exclude technical effects arising from the high-throughput cultivation platform, those clones exhibiting the lowest growth rates in the growth profiler were re-evaluated under standard conditions in shake flasks (Table 9). Under these standard growth conditions the differences in growth previously observed during cultivation in the growth profiler were no longer apparent when comparing *P. putida* KT2440 harboring the empty vector control with *P. putida* KT2440 harboring pSEVA247atpAGDYXX. *P. putida* KT2440 driving *atpAGD* expression from SynPs24 and 124 performed even better than the control in all categories under investigation.

Thus, growth conditions cannot explain why the expected phenotype was not observed. Additionally, *E. coli* JM101 was transformed with pSEVA247atpAGDY21, 24 and 124 in order to evaluate the effect on a different host. Maybe *E. coli* JM101 is more prone to a massive ATP wastage like *E. coli* BOE270⁷². However, it was not possible to cultivate the transformed *E. coli* JM101 strains in liquid media although some colonies were detected following transformation. This indicates the presence of active promoters, likely leading to *atpAGD* expression levels that are too toxic for *E. coli* JM101 to tolerate.

Different approaches of increasing the glucose uptake rate in *P. putida* KT2440

Table 9: Physiology of positive clones driving *atpAGD* expression from SynP21, 24, and 124 in duplicates in shake flasks^a

SynP ^b	μ		CUR		$Y_{x/s}$	
	[h ⁻¹]	[%]	[mmol g _{CDW} ⁻¹ h ⁻¹]	[%]	[g _{CDW} g _s ⁻¹]	[%]
Control	0.33±0.01	100±3	8.4±0.0	100±1	0.50±0.02	100±4
21	0.32±0.01	95±2	8.8±0.2	106±2	0.50±0.00	101±1
24	0.39±0.01	117±2	8.5±0.1	102±1	0.59±0.00	119±1
Control	0.49±0.01	100±1	4.0±0.1	100±2	0.35±0.01	100±3
124	0.54±0.02	110±3	4.5±0.0	112±1	0.43±0.01	124±3

a: the values for the control strain harboring the empty vector pSEVA247Y were set to 100%, values represent the mean of two replicates with SEM; μ - growth rate, CUR - carbon uptake rate as the sum of glucose, gluconate and 2-ketogluconate, $Y_{x/s}$ - biomass yield on glucose

b: Growth experiments were carried out in indicated sets, strains harboring different pSEVA247atpAGDYXX constructs in duplicates with *P. putida* KT2440 carrying the empty vector pSEVA247Y as a control

Another reason why the pSEVA247atpAGDYXX constructs did not impact the growth of *P. putida* KT2440 could be a reduced activity of the synthetic promoters which arose from possible secondary mRNA structures¹⁰⁹ or SNP's and indels within the promoter sequence⁸¹. Regarding the first synthetic promoter library described in paragraph 3.3.2 with pTn1 as template vector, deletions within the sequence of SynP2, SynP37 and SynP30 led to a shifted -35 region which were responsible for their rather low activity (see Table 21 in the Appendix) as already observed in Chapter 2. But in this case, the sequence of all three SynPs under investigation perfectly matched the initial primer design (Table 10). Moreover, the whole *atpAGD* operon of SynP21 and SynP24 was sequenced, which perfectly matched the sequence of the wild type gene. Hence, mutations within the sequence of the promoter or the *atpAGD* operon could be excluded as reason for the absence of a phenotype.

Table 10: Sequence of synthetic promoters chosen for *atpAGD* overexpression with plasmid pSEVA247atpAGDYXX

Name	Sequence ^a
Design ^b	<u>TCTAG</u> ANNNNN TTGACA NNNNNNNNNNNNNNNNNNNNNNNNNNNN TATAAT NNNNNNNGAAGGG
SynP21	<u>TCTAGATGCTCT</u> TTGACA CTCTGACAGACGTAGG TATAAT TAGATGGAAGGG
SynP24	<u>TCTAGAGGGGAAT</u> TTGACA AACTGTTTGGGAGATCT TATAAT CGCGAAGAAGGG
SynP124	<u>TCTAGACCGCC</u> TTGACA TGGGTGGTATCTGGTCCT TATAAT CGCTGGGAAGGG

a: The restriction sites for *Xba*I (TCTAGA) is underlined; the conserved -35 (**TTGACA**) and -10 (**TATAAT**) regions of sigma⁷⁰-promoters are shown in bold

b: Part of the oligonucleotide design which represents the synthetic promoters (see primer S29 in Table 7)

Nevertheless, it was still possible that the promoters were not active. With *yfp* as the reporter gene, fluorescence microscopy served as a means of detecting active promoters (Figure 16). Wild type *P. putida* KT2440 was not fluorescent at all (Figure 16A) whereas cells harboring the promoterless empty vector control pSEVA247Y exhibited a homogenous fluorescence (Figure 16B). Surprisingly, once *P. putida* KT2440 carried the plasmid with the SynP-*atpAGD* insert, some cells exhibited fluorescence and some did not (Figure 16C) indicating that the expression of the *yfp* gene from the SynPs, which lies behind the *atpAGD* operon, is lower than the background expression of cells harboring the promoterless empty vector control pSEVA247Y (Figure 16B). Thus, in order to rule out polar effects of the *atpAGD* operon we replaced this operon with *lacZ* from *E. coli* K12 as an alternative reporter gene and tested SynP21, SynP24 and SynP124 for their activity (Figure 17).

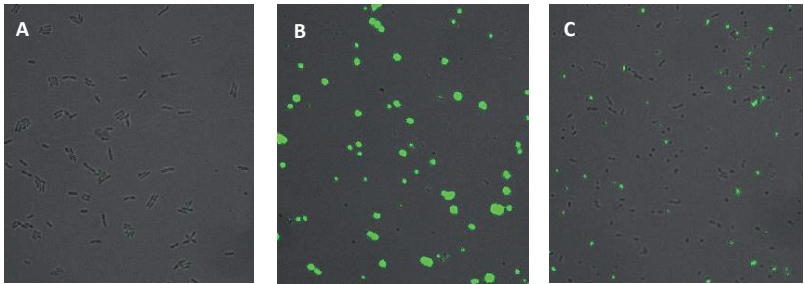


Figure 16 Fluorescence microscopy of wild type *P. putida* KT2440 (a) : Fluorescence microscopy of *P. putida* KT2440 harboring the empty vector control pSEVA247Y (b), and pSEVA247atpAGDY21 (c); although the yellow fluorescent protein was used as a reporter, we chose to illustrate fluorescent cells in green due to higher contrast

Astonishingly, all three SynPs tested exhibited unexpectedly high activities in *P. putida* KT2440. The pSEVA247lacZYXX constructs were also transformed into *E. coli* DH5 α for testing purposes in an *E. coli* host. The activities were even higher in *E. coli* DH5 α explaining why *E. coli* JM101 did not grow in liquid media since 44 MU reduced the growth of *E. coli* BOE270 already by 34%⁷².

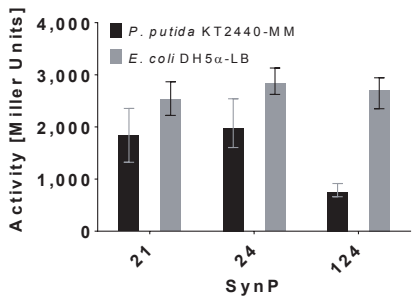


Figure 17: Activity with pSEVA247lacZYXX constructs in *P. putida* KT2440 and *E. coli* DH5 α ; the activities in *P. putida* KT2440 were tested in minimal medium (MM); for screening purposes the SynPs in *E. coli* were tested in LB; bars represent the mean of three replicates with min to max values

So why is there no phenotype in *P. putida* KT2440 although the tested SynPs exhibited high activities and sequencing of the *atpAGD* operon perfectly matched the annotated gene? Maybe pSEVA247Y as backbone is too unstable due to two origins of replications (pRO1600/ColE1) so that after a couple of generations the culture loses the plasmid. That is why pSEVA231 was chosen as an alternative since it is equipped with only one origin of replication, pBBR1, known to be a broad host range vector resulting in comparable plasmid copy numbers at least in *Bordetella bronchiseptica* and *E. coli*¹⁶⁴. Unfortunately, this new strategy of overexpressing the F₁-ATPase in *P. putida* KT2440 and *E. coli* JM101 is coupled to the design of a third synthetic promoter library within this study.

3.3.4 Sophisticated regulatory mechanisms prevent ATP wastage in *P. putida* KT2440

Although the expression system was designed with the *atpAGD* operon of *P. putida* KT2440, it should affect the metabolism of *E. coli* JM101 comparably to *P. putida* KT2440 since the translated protein

dephosphorylates ATP and produces heat, which should be independent from the host. Hence, it was possible to search for plasmids with SynP-*atpAGD* constructs in both hosts simultaneously. An exchange of constructs between both hosts revealed how growth of both hosts was impacted by the same construct (Figure 18, Table 19 and Table 20 in the Appendix).

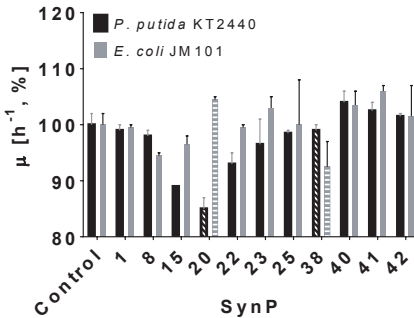


Figure 18: Impact of pSEVA231atpAGDXX constructs on the growth of *P. putida* KT2440 and *E. coli* JM101; bars represent the mean of three replicates with min to max values

There are differences in the carbon uptake rate and the biomass yield on glucose but they are rather small compared to those reported by Koebmann et al.⁷². Nevertheless, it is interesting to see that in some *E. coli* JM101 strains the acetate production rate increased with specific SynP-*atpAGD* constructs which was accompanied by a reduced biomass yield on glucose. However, the aim of drastically increasing the carbon uptake rate was not achieved in either host with the possible exception of SynP40 in *P. putida* KT2440. The carbon uptake rate in this strain was increased by 15% compared to the empty vector control. But SynPs 20 and 38 were chosen based on their effect on the growth rate for further evaluation, since they impacted growth the most at least in one of the two hosts (SynP20 in *P. putida* KT2440 and SynP38 in *E. coli* JM101, marked in Figure 18).

The sequence of both SynPs matched the initial primer design (Table 11) so that SNP's or indels were excluded as reason for inactive promoters in both hosts comparable to the previous investigated synthetic promoter library in 3.3.3.

Table 11: Sequence of synthetic promoters chosen for *atpAGD* overexpression with plasmid pSEVA231atpAGDXX

Name	Sequence ^a
Design ^b	<u>TCTAG</u> ANNNNN TTGACA NNNNNNNNNNNNNNNNNNNN TATAAT NNNNNNNGAAGGG
SynP20	<u>TCTAGA</u> AGTTT TTGACA AGCGCGAAAGGCACGGC TATAAT TATATTGGAAGGG
SynP38	<u>TCTAGAT</u> GCGGT TTGACA GGACGGGGAAGCTACGT TATAAT GAGGACGAAGGG

a: The restriction sites for XbaI (TCTAGA) is underlined; the conserved -35 (**TTGACA**) and -10 (**TATAAT**) regions of σ^{70} -promoters are shown in bold

b: Part of the oligonucleotide design which represents the synthetic promoters (see primer S29 in Table 7)

But again a perfect sequence is only an indication for an active promoter. Thus, the *atpAGD* operon was replaced with the *lacZ* operon of *E. coli* K12 for quantification of SynP activities. Indeed, they were much more active than the SynPs of the previous synthetic promoter library (Figure 19).

Moreover, SynPs were much more active in *P. putida* KT2440 than in *E. coli* JM101. However, in either host no phenotype was observed when the tested SynPs were placed upstream of the *atpAGD* operon, which in case of *E. coli* is especially interesting since the SynPs are 10- (SynP38: 505±28 MU) to 20-fold (SynP20: 808±62) more active than the highest reported activity by Koebmann and coworkers⁷².

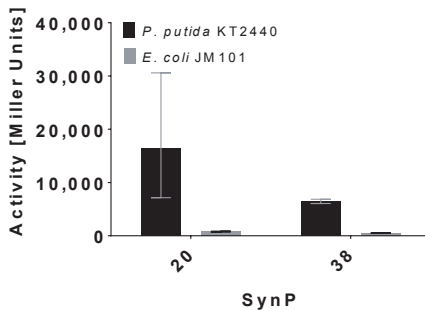


Figure 19: Activity of pSEVA231lacXXX constructs in *P. putida* KT2440 and *E. coli* JM101; bars represent the mean of three replicates with min to max values

Two different synthetic promoter libraries with different vectors were investigated but the aim of increasing the carbon uptake rate in *P. putida* KT2440 was not achieved neither with a synthetic promoter library based on the pSEVA247Y nor based on the pSEVA231 vector system, although the SynPs exhibited a high activity and the F₁-ATPase should be active since its *atpAGD* genes perfectly matched the annotated genes at least in the case of the pSEVA247Y system. Thus, it is likely that the ATPase was just not translated from mRNA or both hosts possess mechanisms to prevent gene expression from adverse genes. Hence, two active SynPs were investigated for protein expression with SDS PAGE analysis. Since the SynPs express the *lacZ* gene, a comparison of a SynP expressing *lacZ* and the *atpAGD* operon should reveal a difference in the expression of the two genes.

In case of SynPs expressing the *atpAGD* genes, three bands with a size of 55 kDa (subunit α), 49 kDa (subunit β) and 32 kDa (subunit γ) were expected. For the β -galactosidase strong bands at a size of 116 kDa were expected. The SDS PAGE of *E. coli* JM101 did not unveil clear differences in protein expression and an overexpression of either *atpAGD* or *lacZ* could not be detected since all four mutants exhibited a comparable expression pattern to the wild type control (Figure 20A).

The SDS PAGE of *P. putida* KT2440 revealed that only in the case of *lacZ* the host expressed the genes downstream of the SynP (Figure 20B). No bands could be detected when the SynPs expressed the *atpAGD* genes but as soon as they were replaced with *lacZ*, a (strong) band could be detected between 100 and 130 kDa. This is an indication that *P. putida* KT2440 is somehow able to shut down adverse genes, maybe through promoter silencing mechanisms. This would also explain why cells harboring the pSEVA247atpAGDY construct in Figure 16 did not fluoresce, apparently the presence of the *atpAGD* operon suppresses expression of the downstream *yfp*.

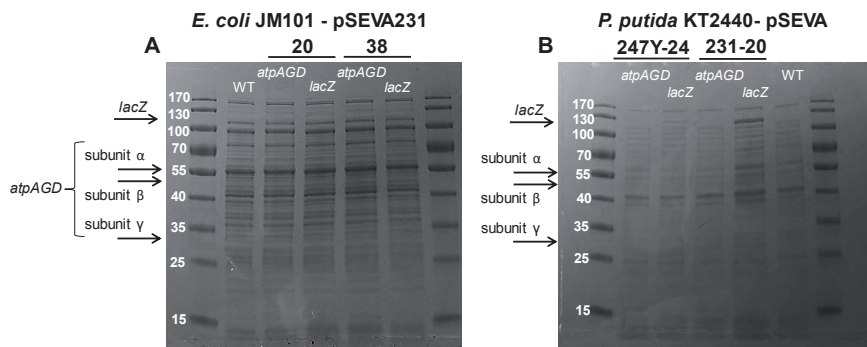


Figure 20: SDS PAGE of *E. coli* JM101 (A) and *P. putida* KT2440 (B) harboring different SynP-*atpAGD* constructs; since pSEVA247atpAGDYXX construct did not lead to growing cells in *E. coli* JM101, SDS PAGE analysis was performed with pSEVA231atpAGDXX constructs; pSEVA247Y constructs driving expression from SynP24 and pSEVA231 construct driving expression from SynP20 were chosen based on their highest activity among the SynPs tested for the SDS PAGE analysis in *P. putida* KT2440; wild type (WT) *P. putida* KT2440 and *E. coli* JM101 were chosen as controls

It might also be that the synthetic promoters express the *atpAGD* genes but its mRNA is extraordinarily unstable when being expressed independently from the genes expressing the F_0 subunit of the ATP synthase so that it gets degraded immediately. Another reason for the absence of the protein may be that *P. putida* senses the imbalance of the F_1 subunit to the F_0 subunit of the ATP-Synthase and strongly regulates the balance of both subunits by degrading the excess of the F_1 subunit or increasing the expression of the F_0 subunit. All these reasons make sense considering the soil as natural habitat of *P. putida* where the competition for resources is more challenging than for example in the natural habitat of *E. coli*. Thus, the sensing and shut down of adverse genes, responsible for wastage of carbon, let *P. putida* grow efficiently in its natural environment.

Overall, increasing the carbon uptake rate via overexpression of an ATP wasting enzyme as means to increase the NADH regeneration rate remains unsolved in *P. putida* KT2440. Therefore, other alternatives were evaluated with the aim of increasing the carbon uptake rate in order to boost the redox metabolism. Two alternatives are presented in the following.

3.3.5 Alternative strategies aiming at an increased glucose uptake rate in *P. putida* KT2440

Microbial growth in the presence of organic solvents requires huge amounts of energy⁸⁹ which may cause an increased carbon uptake rate in order to compete with the elevated energy demand. This fact could be turned into an advantage with an evolutionary engineering approach by growing microorganisms permanently in the presence of an organic solvent. By this means the microorganisms would get used to the higher energy demand and might steadily increase their need for carbon even when the stress causing solvent is not present. Volkers et al.¹⁵⁴ have shown that *P. putida* S12 can be adapted to increasing benzene concentrations with an evolutionary engineering approach. We investigated whether the adapted strain *P. putida* S12.49 got used to the higher energy demand and still took up more carbon although benzene was not present (Table 12).

Unfortunately, the adapted strain *P. putida* S12.49 performed below the wild type control in growth rate, carbon uptake rate and biomass yield on glucose suggesting that this approach did not lead to an increased metabolic rate. However, these results do not suffice for a general rejection of the strategy since only one evolutionary engineering approach was evaluated. Maybe this strategy succeeds with a different strain and/or a different solvent.

Table 12: Physiology growth experiment S12 and S12.49 in minimal medium^a

Strain	μ		GUR		$Y_{X/S}^b$	
	[h ⁻¹]	[%]	[mmol g _{CDW} ⁻¹ h ⁻¹]	[%]	[g _{CDW} g _S ⁻¹]	[%]
S12	0.65±0.01	100±2	4.4±0.1	100±1	0.83±0.03	100±4
S12.49	0.56±0.02	85±4	3.9±0.6	89±13	0.81±0.15	98±18

- a: the values for the control strain *P. putida* S12 were set to 100%, values represent the mean of two replicates with SEM; μ - growth rate, GUR - glucose uptake rate since *P. putida* S12 does not accumulate gluconate and 2-ketogluconate, $Y_{X/S}$ - biomass yield on glucose
- b: OD to CDW factor of 0.505 for *P. putida* KT2440 was chosen likely leading to overestimation of the biomass yield on glucose

The second alternative uses an artificial ATP sink by lowering the pH value of the surrounding medium. Strains growing in the presence of an increased proton concentration have to actively pump out protons at the expense of ATP. Therefore, *P. putida* KT2440 was challenged with pH values below pH = 7.0. But this strategy with *P. putida* KT2440 was not rewarding either (Table 13).

Table 13: Physiology of *P. putida* KT2440 growing at different pH values^a

pH	μ		CUR		$Y_{X/S}$	
	[h ⁻¹]	[%]	[mmol g _{CDW} ⁻¹ h ⁻¹]	[%]	[g _{CDW} g _S ⁻¹]	[%]
pH = 7.0	0.56±0.02	100±3	6.0±0.2	100±4	0.52±0.04	100±7
pH = 6.5	0.46±0.00	82±0	4.6±0.1	77±1	0.56±0.00	107±1

- a: the values for the control at pH = 7.0 were set to 100%, values represent the mean of two replicates with SEM; μ - growth rate, CUR – carbon uptake rate as the sum of glucose, gluconate and 2-ketogluconate, $Y_{X/S}$ - biomass yield on glucose

A further lowering of the pH value to pH = 6.0 even led to lysis of the cells. In this specific experiment the pH value was varied by increasing the amount of NaH₂PO₄ as acidic buffer component of the mineral medium according to the Henderson Hasselbach equation leading to a concentration of NaH₂PO₄ of 113 mM (pH = 6.5) and 357 mM (pH = 6.0). This corresponds to an 8- and 26-fold increase of Na⁺ and PO₄³⁻ ions compared to pH = 7.0. As a consequence, it is likely that *P. putida* KT2440 did not grow in these experiments due to drastically increased sodium and phosphate ion concentrations rather than due to the lowered pH value.

3.4 Summary and Outlook

Increasing the glucose uptake rate as means to increase the NADH regeneration rate is not trivial with *P. putida* KT2440. Several alternatives were investigated with the aim of increasing the glucose uptake rate through overexpression of an ATP wasting enzyme. In case of an inducible expression system it is highly likely that a low background expression was already too toxic for the cells to grow.

When the inducible expression system was replaced by a constitutive system, *P. putida* KT2440 was not able to grow either even when the ATPase was expressed from weak promoters. Upon direct transformation of the ATPase under the control of another constitutive expression system, *P. putida* KT2440 was somehow able to shut down very active synthetic promoters to prevent massive ATP wastage. Also two other strategies which did not require overexpression of the toxic *atpAGD* operon were not rewarding so that the aim of this study remains unsolved. Additionally, as a control *E. coli* JM101 was also challenged with active promoters expressing the *atpAGD* operon from *P. putida* KT2440. Although the SynPs were 10- to 20-fold more active than the highest reported synthetic promoter activities by Koebmann et al.⁷², the expected phenotype was not observed suggesting that *E. coli* JM101 is capable of tolerating higher stress levels than *E. coli* BOE270. In order to compare *P. putida* KT2440 with *E. coli* BOE270, the constructs designed by Koebmann and coworkers would have to be tested in *P. putida* KT2440. However, our results suggest that the *atpAGD* operon likely suppresses downstream reporter genes. Thus, it is likely that the extremely low activity of their described synthetic promoters is not indicative of the actual promoter activity. It might be that their promoters are comparably active to those described in *E. coli* JM101 in this study (Figure 18), if they only express the *lacZ* reporter gene. Hence, increasing the glucose uptake rate with the aim of increasing the NADH regeneration rate may be accomplished with less invasive strategies out of which some shall be discussed in the following:

A transcriptome analysis of cells growing in the presence of organic solvents or other agents like 2,4-dinitrophenol which cause an increased glucose uptake rate might be a promising alternative^{45,50}. By this means those overexpressed genes compared to an unstressed control could be identified which might cause the increased carbon uptake. By overexpressing these gene(s) with promoters with a defined activity a stress situation could be simulated without the addition of the stress causing agent to the medium.

Despite the results presented by Schaaff et al.¹⁴⁶ that overexpression of key glycolytic enzymes did not result in an increased glucose uptake rate, Papagianni et al.¹⁶⁵ tried to overexpress the phosphofructokinase (Pfk) as bottleneck enzyme of the Embden-Meyerhof-Parnas pathway in *L. lactis*. Indeed, they observed that *L. lactis* doubled its glucose uptake rate when Pfk was overexpressed. Alternatively, the results presented by Ruyter et al.⁷¹ that *E. coli* did not increase the uptake rate of glucose when the key enzyme of its PTS uptake system was overexpressed may constitute a promising alternative when being adapted to the uptake system of *P. putida*. *P. putida* takes up glucose with an ABC (ATP binding cassette) transporter, which requires two molecules of ATP for every glucose molecule compared to only one molecule of ATP with the PTS system in *E. coli*. Therefore, overexpression of the ABC transport system in combination with the periplasmic glucose binding protein (Figure 4) in *P. putida* and deletion of alternative pathways of glucose uptake may be sufficient to create an ATP sink resulting in an increased glucose uptake.

Furthermore, *P. putida* KT2440 is able to generate inorganic polyphosphates¹⁶⁶ which enables this microorganism to tolerate higher stress levels than its competitors in its natural environment. Overexpression of the polyphosphate generating enzyme polyphosphate kinase might also constitute an ATP sink.

A simpler method would be to lower the dissolved oxygen (DO) in the surrounding medium. Less oxygen in the medium means less oxygen available for the electron transport chain resulting in a lower amount of ATP produced, which in turn means less NADH consumed. The yeast *T. glabrata* was challenged with low oxygen levels together with an elevated oxidoreduction potential resulting in a 1.5-fold increase of the glucose uptake rate compared to an unstressed control at saturating DO concentrations¹⁶⁷. The reason for this elevated glucose uptake rate is the need for ATP which could only be satisfied by substrate-level phosphorylation. However, since the electron transport chain regenerates NAD⁺, which accepts the electrons from glucose, one has to provide an alternative NADH sink like an intended NADH-dependent biocatalytic reaction which does not depend on the supply of oxygen. This constitutes a promising and less invasive strategy, which can be adapted to *P. putida*. Since *P. putida* converts glucose to pyruvate using the Entner-Doudoroff pathway which lacks the key glycolytic enzyme Pfk and thus only produces one ATP during glucose catabolism, this approach might even result in a more increased glucose uptake rate compared to those values reported by Qin et al.¹⁶⁷ If the Entner-Doudoroff pathway is not able to support the ATP demand through substrate-level phosphorylation a heterologous expression of a Pfk in *P. putida* KT2440 may be sufficient to promote an increase in glucose uptake under low DO concentrations although it was shown that *P. putida* KT2440 did not rearrange the carbon flow through its central carbon metabolism with a heterologous Pfk expression⁹.

In all, it seems that different bacterial genera cope very differently with energy demanding stresses and that the control of metabolic rate is not easily overridden. Overall, our attempts to engineer an artificial ATP demand in *P. putida* KT2440 were not successful due to its flexibility both in its energy metabolism, but also in its genetic mechanisms. Likely this has an evolutionary background of its natural soil habitat protecting the cell from invasion of foreign DNA.

3.5 Acknowledgements

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Chapter 4

Metabolic response of *Pseudomonas putida* to increased NADH regeneration rates

The efficient use of carbon and energy sources by *E. coli* or *S. cerevisiae* can be hampered by overflow metabolism leading to a loss of carbon through the extrusion of byproducts such as ethanol or acetate for maintenance of the redox balance or energy homeostasis. In contrast, *P. putida* efficiently utilizes many different carbon sources without the overflow of byproducts even under stressful conditions. This implies a high degree of flexibility to cope with conditions that require a significantly altered distribution of carbon to either biomass or energy in the form of NADH. Co-feeding of the reduced C1 compound formate to *E. coli* heterologously expressing the NAD⁺-dependent formate dehydrogenase of the yeast *C. boidinii* boosted various NADH-demanding applications. By contrast, *P. putida* KT2440 as emerging biotechnological workhorse is inherently equipped with an NAD⁺-dependent formate dehydrogenase. This study provides a detailed insight into the use of formate as NADH electron donor together with glucose as carbon source by *P. putida* KT2440. Our results show that the addition of formate leads to an increased NADH regeneration rate resulting in a beneficial effect on the biomass yield on glucose due to a significant flux rerouting from catabolism to anabolism.

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Author contributions:

Sebastian Zobel and Jannis Küpper have conceived the project, performed the experiments, and analyzed the results. Birgitta E. Ebert has performed the flux analysis. Sebastian Zobel, Nick Wierckx and Birgitta E. Ebert have written the chapter. Nick Wierckx and Lars M. Blank have conceived and supervised the study.

4.1 Introduction

The term “redox biocatalysis” can be defined as the biocatalytic production of chemicals, requiring the supply of reducing equivalents⁵¹. In enzymatic biocatalysis these reducing equivalents are redox cofactors such as NADH and NADPH, which have to be added externally when isolated enzymes are applied. Since the requirement of stoichiometric amounts of NADH and NADPH can significantly influence the overall costs of such a process, redox biocatalysis with isolated enzymes is only profitable with an efficient artificial cofactor regeneration system^{168,169}. Hence, the use of whole cells is advantageous since the cells synthesize their own cofactors, which are constantly regenerated by their metabolism. However, the pool of redox cofactors, mainly consisting of NADH and NADPH, is tightly regulated between the reduced and oxidized form and even between the pools of both molecules in many organisms through transhydrogenases^{50,51}. To maintain redox homeostasis, the cell has to adjust the metabolism in case of redox biocatalysis to counteract the increased redox cofactor consumption. Indeed, high NADH regeneration rates do not automatically translate into corresponding biocatalytic activity, e.g., due to an increased maintenance demand⁵². This is especially relevant under biological stress, where an increased redox cofactor regeneration rate may additionally stabilize the viability of the cell being confronted with a redox biocatalytic reaction.

Since NADH is regenerated during catabolism of a carbon source such as glucose, one could argue that by increasing the flux through the central carbon metabolism the NADH regeneration rate would be increased. However, recent studies have shown that increasing the uptake rate of a sugar is not that trivial^{71,72,146}. Another biological alternative could be the use of higher reduced substrates than glucose such as sorbitol¹⁷⁰. This strategy may require further genetic engineering if the bacterial host chosen for the redox biocatalytic reaction is not able to catabolize this alternative carbon source. Moreover, sorbitol is more expensive than glucose, imposing additional economic restraints, which consequently limits its applicability to high value products.

The most promising and most applied strategy to increase the NADH regeneration rate is a co-feed of an additional substrate, which serves as means to regenerate NADH. Here different approaches are reported. Zhang et al.¹⁷¹ improved xylitol production with a glucose dehydrogenase cofactor regeneration system and Ouyang et al.¹⁷² used isopropanol as electron donor to regenerate NADH and reached higher titers of (*R*)-[3,5-*Bis*(trifluoromethyl) phenyl] ethanol. The most commonly applied strategy to increase the NADH regeneration rate is a co-feed of formate, which is irreversibly oxidized to innocuous CO₂ by an NAD⁺-dependent FDH (EC 1.2.1.2, FDH) from the yeast *C. boidinii*. This approach was successfully applied with numerous hosts in diverse processes^{76,173–176}. However, the use of heterologous genes may increase the metabolic burden especially when these genes are expressed from plasmids requiring antibiotic selection. Hence, a host inherently equipped with an active NAD⁺-dependent FDH might be more suitable for an NADH demanding redox biocatalytic reaction when co-fed with formate.

P. putida is a potential candidate for this kind of supported redox biocatalysis as it is capable of using C1 compounds as electron donors¹⁷⁷. Moreover, it is naturally capable of regenerating NADH at a

high rate⁵⁰ and it is endowed with active FDHs⁷⁷ out of which at least one is expected to be NAD⁺-dependent (PP_2183-2186)⁶¹.

In this study, we investigated the use of formate by *P. putida* KT2440 in co-feed experiments, in which formate is mainly oxidized to CO₂ by an NAD⁺-dependent FDH. By this means, the NADH regeneration rate was drastically increased leading to a significant flux rerouting within the metabolism in the absence of an NADH consuming redox biocatalytic reaction.

4.2 Material and Methods

4.2.1 Bacterial strains and cultivation conditions

Bacterial strains used are presented in Table 14. For solid media LB¹²¹ containing 5 g l⁻¹ NaCl with 1.5% (w/v) agar-agar was used. In shake flask experiments a minimal medium¹²³ with 20 mM glucose was used with a volume to liquid ratio of 1:10. The medium was adapted to achieve a higher buffer capacity through a 4-fold increase of the phosphate buffer (final concentrations: 15.52 g l⁻¹ K₂HPO₄ and 6.52 g l⁻¹ NaH₂PO₄, pH = 7.0).

Table 14: Strains used in this study

Strains	Description	Source
<i>P. putida</i> KT2440	Wildtype <i>P. putida</i> KT2440	Bagdasarian et al. ¹¹⁷
ΔPP_0489	Mutant of <i>P. putida</i> KT2440 with a kanamycin marker integrated into the alpha subunit of the FDH (PP_0489-0492)	Bio-Ilberis R&D, Granada, Spain
ΔPP_2158	Mutant of <i>P. putida</i> KT2440 with a kanamycin marker integrated into the alpha subunit of the FDH (PP_2183-2186)	Consejo Superior de Investigaciones Científicas, EEZ, Department of Environmental Protection, Granada, Spain

Precultures were incubated in 100 ml Erlenmeyer flasks, main cultures in 500 ml Erlenmeyer flasks. The medium was supplemented with formate as sodium formate out of a 2 M stock solution (pH = 7.0). Liquid cultures were incubated at 30 °C, 200 rpm shaking speed with an amplitude of 50 mm in a Minitron shaker (INFORS, Bottmingen, Swiss). Bioreactor experiments were carried out in the Bioflow 115 systems from Eppendorf (Hamburg, Germany) with a working volume of 0.4 l. The stirring speed was set to 700 rpm, with 0.5 l min⁻¹ head space aeration with air. The temperature was controlled at 30 °C. The pH was regulated to 7.0 by the addition of 4 M H₂SO₄ and 4 M NaOH. In the fermenter experiments the standard buffer concentration was 3.88 g l⁻¹ K₂HPO₄ and 1.63 g l⁻¹ NaH₂PO₄ and formic acid was added instead of sodium formate. Continuous cultivations were performed at a dilution rate of D = 0.2 h⁻¹. Physiological data were determined during steady state after at least five volume changes.

4.2.2 Analytical methods

Bacterial growth was monitored as optical density at a wavelength of λ = 600 nm (OD₆₀₀) with an Ultrospec 10 Cell Density Meter (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The conversion factor of OD₆₀₀ to cell dry weight (CDW) was determined to 0.505. Samples taken from

liquid cultivation were centrifuged for 3 min at 17,000 g for the determination of extracellular metabolites. The supernatant was analyzed using an Ultimate 3000 HPLC system from Thermo Fisher Scientific (Waltham, MA, USA). Analytes were eluted at 60 °C using a 300 x 8 mm organic acid resin column together with a 40 x 8 mm organic acid resin precolumn (both CS Chromatographie, Langerwehe, Germany) with 5 mM H₂SO₄ as mobile phase at a flow rate of 0.7 ml min⁻¹ and detected with a UV detector, which was part of the HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) at λ = 210 nm and a Shodex-101 RI detector at 35 °C (Showa Denko, Tokyo, Japan).

¹³C labeling of proteinogenic amino acids was determined using a Trace GC Ultra Gas chromatography and mass spectrometry (GC-MS). Analytes were separated on a TG5 SilMs 30 m x 0.25 mm x 0.25 μ m column with helium as carrier gas. Both GC-MS and column were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Samples taken during steady state of the chemostat and during exponential growth in shake flask and bioreactor batch fermentation experiments were processed according to Schmitz et al.¹⁷⁸.

Off-gas during bioreactor experiments was monitored using BlueInOne Ferm (BlueSens, Herten, Germany) off-gas sensors.

4.2.3 Calculation of extracellular fluxes

Growth rate and carbon uptake were calculated according to Heyland et al.¹⁵⁶ with SigmaPlot (V 12.5.0.38) for all experiments. For shake flask experiments the formate uptake rate was calculated with Microsoft Excel 2010. For this, the formic acid concentration was plotted against the cell dry weight in the same time frame as for the growth and carbon uptake rate. The data were fitted with a linear regression and the slope was multiplied with the growth rate determined with SigmaPlot.

4.2.4 Enzyme Assays

For the *in vitro* quantification of the FDH activities, a liquid culture (20 mM glucose, 60 mM formate) cultivated to mid-exponential phase (OD₆₀₀ = 1.0 - 1.5) was harvested and centrifuged for 10 min at 6,500 g and 4 °C. The pellet was washed once with minimal medium without a carbon source and centrifuged for 10 min at 6,500 g and 4 °C. Cells were lysed using the BugBuster Protein Extraction Reagent from Merck Millipore (Darmstadt, Germany). The overall protein concentration was determined according to Bradford¹⁵⁹. The protein extraction supernatant was kept on ice until it was used.

For the NAD⁺-dependent soluble FDH assays¹⁷⁹ minimal medium without a carbon source and the protein extraction supernatant were first added to a cuvette in different ratios in 800 μ l and heated to 30 °C. After 5 min, 100 μ l of 20 mM NAD⁺ were added for the quantification of the background activity. Subsequently, 100 μ l of 500 mM sodium formate (pH = 7.0) were added. Absorption was monitored at a wavelength of 340 nm. The activity is presented in units (U) referring to 1 μ mol of NADH formed or formate consumed per minute.

To investigate the NAD⁺-independent membrane bound FDH¹⁸⁰ the protein extraction pellet was resuspended in 700 μ l BugBuster Protein Extraction Reagent and diluted 1:10 with minimal medium without a carbon source. This solution was mixed with minimal medium without a carbon source in

different ratios in 760 μl in a cuvette heated to 30 °C. After 5 min, 100 μl of 20 mM phenaziniummethylsulfat (PMS) were added. Another 5 min later, 40 μl of 50 mM dichlorophenolindophenol (DCPIP) were added to measure the background activity. NAD⁺-independent FDH activity was measured after 100 μl of 500 mM sodium formate were added. Activity was monitored at a wavelength of $\lambda = 600\text{ nm}$. Both assays were performed in a Cary 60 UV-Vis spectrophotometer from Agilent Technologies (Santa Clara, CA, United States).

4.2.5 Labeling experiments

In every labeling experiment a ratio of 20:80 of U-¹³C labeled glucose (Euriso-top, Saint-Aubin Cedex, France) and naturally labeled glucose at a total concentration of 20 mM was used. The only exception was the labeling experiment in shake flasks with 30 mM of sodium formate to test incorporation of formate into the biomass, where 100% of U-¹³C glucose was used. The preculture was cultivated overnight on naturally labeled glucose. The main culture was inoculated with 3% of the estimated biomass concentration of the preculture at the harvesting point. During mid-exponential growth and after five generations of growth on the labeled substrate, samples of 3 mg CDW were taken for mass distribution measurements of proteinogenic amino acids.

All cultivations for flux analysis were performed in bioreactors as described above using 20 mM glucose and 120 mM formic acid (where indicated). For the continuous cultivation the shake flask preculture and the batch cultivation in the bioreactor (before switching to continuous cultivation conditions) were performed using naturally labeled glucose. When a metabolic steady state was reached (after five volume changes) the carbon source was changed from naturally labeled glucose to labeled glucose in the aforementioned ratio. Samples for the mass distribution measurements of proteinogenic amino acids were taken after five volume changes when the isotopic steady state on labeled glucose was reached. The formate co-feed was started by adding 120 mM of formic acid to the medium reservoir. After another five volume changes the steady state on glucose and formate was reached.

4.2.6 Flux analysis

Flux calculations were performed with OpenFLUX using standard settings and applying the gradient based search algorithm for sensitivity analysis¹⁸¹. The stoichiometric model (Table 23 in the Appendix) was based on a previously published model of the central carbon metabolism of *P. putida*⁵⁰. A partially cyclically operating Entner-Doudoroff pathway has recently been reported for *P. putida*¹⁸². However, as the applied ¹³C labeling strategy and mass distribution measurements of proteinogenic amino acids did not allow to resolve such a cyclic operation, we neglected this mode of operation and only allowed positive flux values for the phosphoglucose isomerase catalyzed conversion of glucose-6-phosphate to fructose-6-phosphate. The initial model contained a phosphoenolpyruvate carboxykinase reaction, which decarboxylates oxaloacetate to phosphoenolpyruvate. The gene encoding this enzyme, however, has been annotated as a pseudogene due to an authentic frameshift^{47,61,182}. Indeed, in all initial simulations the optimal phosphoenolpyruvate carboxykinase flux was zero, supporting this annotation. However, the fluxes were not resolved well and the simulations could not exclude the formation of a metabolic cycle

(phosphoenolpyruvate > pyruvate > oxaloacetate > phosphoenolpyruvate). As the net reaction of this cycle is the hydrolysis of one ATP and its suppression does not alter the flux distribution, we excluded the phosphoenolpyruvate carboxykinase reaction in the final simulations.

To compute the intracellular fluxes the model was constrained with the experimentally determined carbon uptake rate (i.e., the sum of glucose, gluconate, and 2-ketogluconate), formate uptake rate, growth rate (see Table 22 in the Appendix), and mass isotopomer distributions of proteinogenic amino acids (average data of independent bioreactor fermentations were used). Mass distribution vectors were corrected for unlabeled biomass introduced with the inoculum and natural abundance of heavy isotopes using iMS2Flux¹⁸³.

4.3 Results

The balancing of redox cofactors has an impact on the cellular performance of a biocatalyst like *S. cerevisiae*, which transfers electrons to acetaldehyde resulting in ethanol during the Crabtree effect induced by excess glucose leading to carbon and energy loss. By contrast, *P. putida* KT2440 is known to direct carbon sources only to biomass, CO₂ and energy without the formation of byproducts⁴⁵. Here we investigated the effect of excess reducing equivalents on the central carbon metabolism of *P. putida* KT2440 through the addition of formic acid.

4.3.1 Formate is co-metabolized with glucose by *P. putida* KT2440

A cell has to co-metabolize a suitable additional energy source with the main carbon source in order to benefit from the additional energy input and to boost redox cofactor regeneration rates. Therefore, the ability of *P. putida* to co-metabolize glucose and formate was investigated by cultivations on mixtures of these two compounds in shake-flasks. A typical experiment of a co-feed of glucose and formate with *P. putida* KT2440 is shown in Figure 21A. Indeed, glucose and formate were consumed simultaneously by *P. putida* KT2440 during growth.

A combination of 60 mM of formate with 20 mM glucose was considered to be optimal for shake-flask cultures in the mineral medium used. Other formate concentrations investigated were 30, 80, and 100 mM. With 30 mM formate the improvement in growth rate was comparable to 60 mM formate. With 80 and 100 mM formate, growth rates decreased due to a pH increase during the batch cultivation, since formate was added as sodium salt. Moreover, the formate uptake rate doubled with increased formate concentrations from 30 mM to 60 mM. However, an increase from 30 to 80 mM (increase factor of 2.7) or from 30 to 100 mM (increase factor of 3.3) did not result in an corresponding formate uptake rate increase, i.e. the formate uptake rate increased by 2.5 in the case of 80 mM formate and 3.1 in the case of 100 mM formate (Figure 21B). Thus, 60 mM of formate was chosen as standard condition in further shake flask experiments.

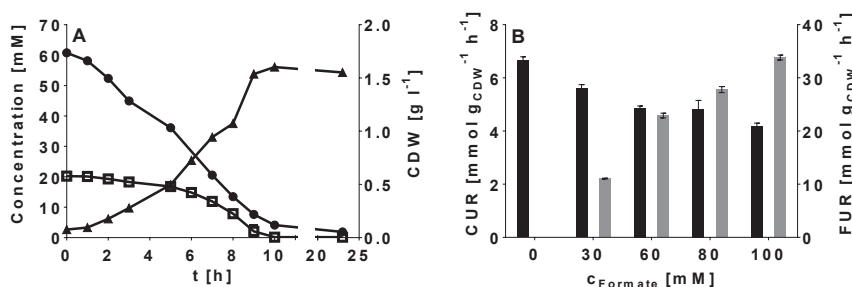


Figure 21: Co-feed of formate and glucose in shake flask experiments; data represent the mean of two independent growth experiments in shake flasks; error bars indicate the SEM, A) Concentration profile of C6 components (as the sum of glucose, gluconate, and 2-ketogluconate, $\square$), formate ($\bullet$), and biomass (CDW, $\blacktriangle$) for the experiment with 60 mM initial formate concentration; B) Uptake rates of C6 components (CUR, black bars) and formate (FUR, grey bars) of cultures with 20 mM glucose supplemented with different initial formate concentrations.

4.3.2 *P. putida* KT2440 is flexible in the use of formate

For NADH-limited redox biocatalysis a transfer of the electrons from formate to NAD⁺ would be preferable since an increased NADH regeneration rate may directly lead to an increased redox biocatalytic performance. *P. putida* KT2440 is equipped with at least two FDHs⁷⁷. The genes *fmdE-fmdH* (PP_2183-2186) likely encode a soluble NAD⁺-dependent FDH since the FmdG protein contains a predicted NAD⁺ binding domain (determined by SMART domain search¹⁸⁴). However, the cofactor-dependency, if any, of the second FDH *fmdA-fmdD* (PP_0489-0492) in *P. putida* KT2440 is not clarified. These genes encode elements typical for membrane-bound FDHs and include binding sites for iron-sulfur clusters and molybdenum-cofactors. The encoded proteins have high sequence identity (78-83%) to the nitrate-inducible FDH of *P. aeruginosa*^{185,186}. This enzyme complex transfers its electrons directly to nitrate reductase under anaerobic nitrate respiration. Since *P. putida* KT2440 is not able to respire nitrate, we hypothesize an alternative electron acceptor in this species. Possibly, electrons are transferred to the membrane-soluble quinone pool, as it is the case with the *E. coli* enzyme¹⁸⁷. Indeed, *in vivo* mediation by a quinone cannot be excluded for the *P. aeruginosa* enzyme either¹⁸⁸. However, the *fmdA* gene contains a rare opal (TGA) codon encoding a selenocysteine^{61,189}. The latter is highly conserved⁶¹, but likely often mis-annotated as a stop codon in databases. This selenocysteine is crucial for the activity of the enzyme, and relies on the addition of selenium to the medium. By omission of this element, possible unwanted side-activity of this NAD⁺-independent enzyme is likely suppressed, although some residual activity may be present¹⁸⁹. In order to ascertain which FDH is active under standard shake flask conditions, growth experiments with single FDH knock-out mutants were performed. These experiments support the observation by Roca et al.⁷⁷ that formate disappears from the fermentation broths no matter which FDH was knocked out. Surprisingly, the formate uptake rate nearly stayed the same in both mutants (Δ PP_0489: 12.3 ± 1.1 mmol g_{CDW}⁻¹ h⁻¹; Δ PP_2185: 11.7 ± 0.01 mmol g_{CDW}⁻¹ h⁻¹) compared to the wildtype (12.3 ± 0.8 mmol g_{CDW}⁻¹ h⁻¹).

4.3.3 The metabolic fate of formate in *P. putida*

In vitro enzyme assays elucidated how *P. putida* KT2440 distributes formate between the FDHs under the investigated conditions. Most of the formate was converted by the NAD⁺-dependent FDH. An activity of approx. 247±20 U g_{Protein}⁻¹ was determined which is nearly sevenfold higher than the PMS-DCPIP-dependent FDH activity of 37±7 U g_{Protein}⁻¹. No activity was observed when NAD⁺ was replaced with NADP⁺ as cofactor in the enzymatic assays. This confirms that the NAD⁺-independent FDH was not very active under the investigated conditions, which is likely due to the omission of selenium from the medium. With this distribution of activity between the two different FDHs a co-feed of formate is highly promising for NADH demanding biotransformations.

These results indicate that under the investigated conditions *P. putida* KT2440 metabolizes formate mostly through an NAD⁺-dependent FDH. However, certain Pseudomonads, such as species AM1, are known to incorporate C1 compounds into the biomass¹⁹⁰. Therefore, we investigated whether *P. putida* KT2440 is equipped with known pathways incorporating formate into the biomass, which would impair the NADH yield from formate.

In nature there are three known pathways of C1 compound assimilation, the ribulose bisphosphate (RuBP) pathway, the ribulose monophosphate (RuMP) pathway, and the serine pathway. The RuBP pathway is a CO₂ assimilation pathway, which is unique to autotrophic organisms and is thus not present in heterotrophic organisms like *P. putida* KT2440. A C1 compound enters the RuMP pathway via formaldehyde¹⁷⁷, which is more strongly reduced than formate. Hence, *P. putida* KT2440 would have to reduce formate first before incorporating it as formaldehyde into the biomass via fusion to ribulose 5-phosphate through a hexulose phosphate synthase. This would result in glucose-6-phosphate, which is central to the primary metabolism. Assimilation via the serine pathway, which is mainly present in methylotrophic microorganisms¹⁹¹, would attach formate as C1 compound to the C2 carbon backbone of glycine, resulting in serine^{190,192}.

Table 15: ¹³C fraction of proteinogenic amino acids derived from cultures with 20 mM 100 % ¹³C-labeled glucose co-fed with 30 mM unlabeled formate^a.

Amino Acid ^b	¹³ C Fraction [%]
<u>Aspartate</u>	<u>91±2.8</u>
<u>Isoleucine</u>	<u>92±0.3</u>
<u>Lysine</u>	<u>90±0.4</u>
<u>Methionine</u>	<u>90±1</u>
<u>Threonine</u>	<u>89±1.1</u>
<u>Glutamate/Glutamine</u>	<u>94±3.6</u>
Serine	99±0.6
Alanine	100±0.5
Glycine	99±0.6
Leucine	99±0.6
Tyrosine	97±2.8
Valine	100±0.7
Phenylalanine	100±0.5

a: out of 15 detectable amino acids¹⁹⁴ proline and histidine were left out due to low resolved detection resulting in too high measurement errors
b: Underlined amino acids are derived from TCA cycle intermediates

In order to ascertain whether formate is incorporated into the biomass through any of these pathways, *P. putida* KT2440 was cultured in mineral medium with 100% ^{13}C -labeled glucose with the addition of unlabeled formate. If the unlabeled formate is incorporated into the biomass, the labeling of the proteinogenic amino acids is diluted. The results of the labeling experiments are summarized in Table 15 and indicate that only those amino acids originating from intermediates of the TCA cycle (see underlined values¹⁹³) show a dilution of the ^{13}C fraction by $9\pm 1\%$. This labeling pattern is consistent with the direct oxidation of formate to CO_2 . Most likely, the unlabeled carbon is only incorporated as CO_2 , derived either from the air or from the oxidation of unlabeled formate, through anaplerotic reactions such as phosphoenolpyruvate or pyruvate carboxylase. Hence, formate can be considered as an energy source, providing reduction equivalents, primarily NADH, by its oxidation to CO_2 , which is stripped from the medium.

4.3.4 Addition of formate increases the biomass yield on glucose

To further investigate how a culture of *P. putida* KT2440 benefits from a co-feed of formate and glucose, pH-controlled bioreactor experiments were performed containing 120 mM of formate. With formate as additional energy source, the biomass yield on glucose increased considerably by $25\pm 11\%$ in batch experiments and by $24\pm 6\%$ in continuous cultures when formate was added, while the growth rate remained unaffected (Table 16). The carbon uptake rate (sum of glucose, gluconate and 2-ketogluconate) decreased by approximately $19\pm 5\%$ when formate was added in both cultivation conditions. An increased CO_2 evolution rate (CER) was expected in the cultures with a formate co-feed. In the case of the batch cultivation the CER increased 5.8 ± 1.6 -fold compared to the culture without formate, while in the C-limited chemostat culture the rate only increased 2.3 ± 0.4 -fold upon the addition of formate. The addition of formate also affected the O_2 uptake rate (OUR), which increased approximately 3.4 ± 1.0 -fold in batch and 1.4 ± 0.3 -fold in chemostat cultivations.

Table 16: Physiology of cultures grown in bioreactors^a

	Batch	Batch + FA	Chem. ^b	Chem. + FA
μ [h^{-1}]* ^b	0.61 ± 0.01	0.61 ± 0.02	0.2 ± 0.01	0.2 ± 0.01
$Y_{X/S}$ [$\text{g}_{\text{CDW}} \text{g}_{\text{glucose}}^{-1}$]	0.56 ± 0.05	0.70 ± 0.00	0.55 ± 0.02	0.69 ± 0.02
CUR [$\text{mmol g}_{\text{CDW}}^{-1} \text{h}^{-1}$]	6.0 ± 0.5	4.9 ± 0.0	2.0 ± 0.1	1.6 ± 0.1
FUR [$\text{mmol g}_{\text{CDW}}^{-1} \text{h}^{-1}$]	-	64.1 ± 9.2	-	7.6 ± 0.9
CER [$\text{mmol g}_{\text{CDW}}^{-1} \text{h}^{-1}$]	11.8 ± 2.7	68.7 ± 9.3	4.1 ± 0.4	9.4 ± 1.2
OUR [$\text{mmol g}_{\text{CDW}}^{-1} \text{h}^{-1}$]	10.6 ± 2.9	35.4 ± 4.8	3.7 ± 0.4	5.2 ± 0.8

a: data represent the mean of at least two independent bioreactor experiments with SEM for measured values (asterisk) and half of error range for estimated values; 20 mM of glucose was used and formate (FA) was added where indicated at a concentration of 120 mM; μ : growth rate, $Y_{X/S}$: biomass yield on glucose; CUR: uptake rate of C6 components (as the sum of glucose, gluconate and 2-ketogluconate); FUR: formate uptake rate; OUR: O_2 uptake rate; CER: CO_2 evolution rate

b: The SEM of the growth rate for the chemostat experiments was assumed to be 0.01 as it is depending on the feeding pump rates, which exhibit low deviations.

4.3.5 Flux Analysis reveals a decreased flux of carbon through the TCA cycle upon formate addition

¹³C-based metabolic flux analysis allows the determination of intracellular reaction rates and was applied here to elucidate the impact of a formate co-feed on the activity of central carbon metabolism pathways. The addition of formate resulted in a significant flux rerouting from catabolism to anabolism as indicated by the decreased flux through the TCA cycle (Figure 22). The pentose phosphate pathway only played a minor role, independent of the investigated cultivation conditions supporting the observations of previous investigations^{45,182,195}. The optimal values for the glyoxylate shunt were zero, but an active glyoxylate shunt cannot be completely ruled out. Interestingly, the pool of oxaloacetate is almost completely replenished from pyruvate rather than from malate in all conditions investigated since malate was mostly converted to pyruvate by the malic enzyme.

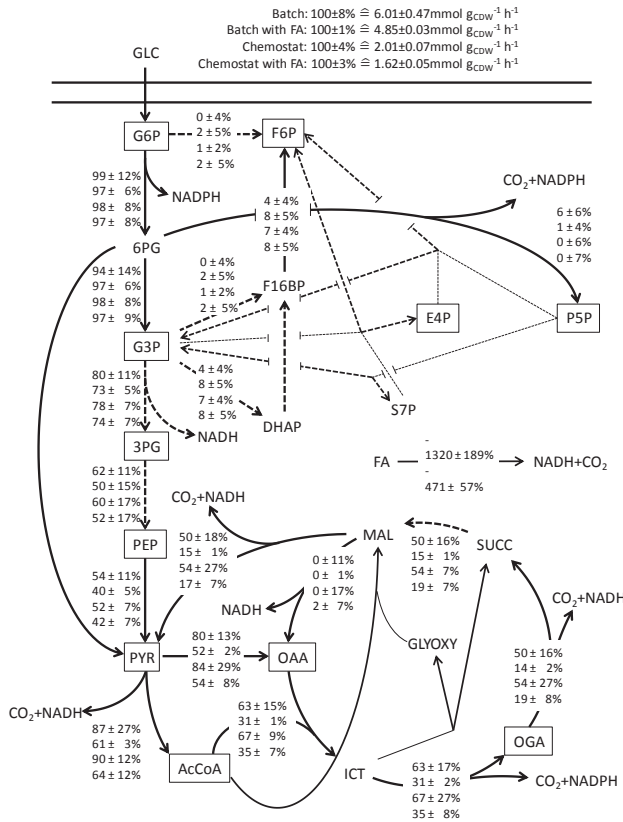


Figure 22: Flux distribution of *P. putida* KT2440 with 20 mM glucose and 120 mM formic acid (FA, where indicated) adapted from Blank et al.⁴⁵; dashed lines indicate reversible reactions; the illustrated direction indicates the estimated net flux (forward minus reverse reaction) the glucose uptake rate was determined as the sum of glucose, gluconate and 2-ketogluconate which was set to 100%, all other values are related to this value; boxed metabolites indicate biomass precursors; given values are connected to bold arrows; for thin arrows the reader is referred to Table 23 in the Appendix; values represent from top to bottom batch, batch with FA, chemostat, and chemostat with FA

The effect of formate oxidation on the NAD(P)H regeneration rate is illustrated in Figure 23. The addition of 120 mM formate resulted in a total NAD(P)H regeneration rate increase from 28 ± 7 to approximately 80 ± 10 mmol $\text{g}_{\text{CDW}}^{-1} \text{ h}^{-1}$ during unlimited growth comparable to NAD(P)H regenerations rates observed for *P. putida* DOT-T1E in the presence of octanol¹⁴⁵. During C-limited growth the total NAD(P)H regeneration rate increased from 9.5 ± 2.7 to 13.2 ± 2.0 mmol $\text{g}_{\text{CDW}}^{-1} \text{ h}^{-1}$. Assuming that all formate is converted to CO_2 by the NAD^+ -dependent FDH, NADH from formate accounts for approximately $80 \pm 15\%$ of the total NAD(P)H regeneration rate during batch cultivation and for $58 \pm 11\%$ percent during limited growth. In both cases the regeneration rate of NADH from glucose oxidation decreased by approximately $58 \pm 15\%$ when *P. putida* KT2440 was supplied with formate (Table 17). NAD(P)H available for miscellaneous processes such as a potential redox biocatalytic reaction increased by 3.9 ± 1.6 fold during unlimited growth and by 1.6 ± 0.7 fold during limited growth (Figure 23).

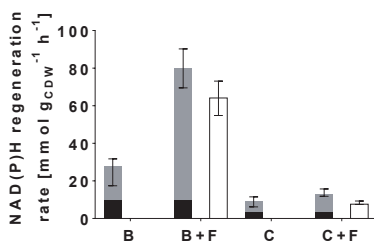


Figure 23: NAD(P)H regeneration rate determined by flux analysis for the different cultivation conditions; it was assumed that the formate uptake rate presented in Table 17 corresponds equally to an NADH regeneration rate; the bar height represents the total NAD(P)H regeneration rate, black bars represent NAD(P)H used for the formation of biomass, while grey bars represent the NAD(P)H available for miscellaneous processes, white bars indicate the proportion of total NADH originating from formate; B: Batch; B+F: Batch + Formate; C: Chemostat; C+F: Chemostat + Formate

Table 17: Calculation of the NAD(P)H yield on glucose

	Batch	Batch + FA	Chem.	Chem. + FA
CUR [mmol _{glucose} $\text{g}_{\text{CDW}}^{-1} \text{ h}^{-1}$] ^a	6.01 ± 0.47	4.85 ± 0.03	2.01 ± 0.07	1.62 ± 0.05
NAD(P)H [mmol _{NADH} $\text{g}_{\text{CDW}}^{-1} \text{ h}^{-1}$] ^b	27.8 ± 7.2	15.9 ± 1.2	9.5 ± 2.7	5.6 ± 1.0
NADH yield [mmol _{NADH} mmol _{glucose} ⁻¹]	4.6 ± 1.2	3.3 ± 0.2	4.7 ± 1.3	3.5 ± 0.6

a: CUR: carbon uptake rate as indicated in Table 16

b: sum of all NAD(P)H regenerating reactions only from glucose (see Table 23 in the Appendix)

4.4 Discussion

This study revealed that a co-feed of formate increases the regeneration rate of redox cofactors in *P. putida* KT2440, most likely leading mainly to the intended NADH. This increased redox cofactor regeneration rate resulted in a significant flux rerouting towards anabolism and an increased biomass yield on glucose.

This strategy of increasing the NADH regeneration rate through the addition of formate was also reported for the industrially well-established strains *S. cerevisiae*¹⁷⁴ and *E. coli*⁷⁶. However, *E. coli* is equipped with three FDHs, none of which is NAD^+ -dependent¹⁹⁶ and therefore needs to be genetically manipulated. The *in vitro* determined *P. putida* KT2440 FDH activity of approx. $136 \text{ U g}_{\text{CDW}}^{-1}$ (assuming that 55% of the CDW is composed of proteins) is lower than the heterologous

FDH activity in *E. coli* of approx. 250 U g_{CDW}⁻¹ expressed from a multi-copy plasmid⁷⁶. However, the use of *P. putida*'s native NAD⁺-dependent FDH has certain advantages, such as the omission of plasmid-encoded heterologous FDH constructs, which need to be maintained by antibiotic selection. In contrast, *S. cerevisiae* is naturally endowed with two isoenzymes of the NAD⁺-dependent FDH¹⁹⁷ and the reported activities in carbon limited cultures are comparable to the FDH activity determined here in *P. putida* KT2440.

The enzyme activity determined in the *in vitro* assay (247 U g_{Protein}⁻¹) is comparable to the one derived from the estimates of the continuous cultivation (127 U g_{CDW}⁻¹ $\hat{=}$ 231 U g_{Protein}⁻¹). However, during unlimited growth the FDH activity was increased to approximately 1,000 U g_{CDW}⁻¹ (1,800 U g_{Protein}⁻¹). Assuming that the specific activity of the FDH of *P. putida* KT2440 is comparable to that of *Pseudomonas* sp. 101¹⁹⁸ of 2,430 U g_{Protein}⁻¹, this would mean that *P. putida* is able to increase the amount of the FDH from approximately 10% during limited growth to 74% during unlimited growth of the total protein content. This highlights the importance of the FDH as part of a formaldehyde detoxification machinery⁷⁷.

However, it might be that not all of the formate is channeled through the NAD⁺-dependent FDH in *P. putida* KT2440 as indicated by growth experiments with knock-out mutants. Both annotated FDHs oxidize formate to CO₂ with either the regeneration of NADH (PP_2183-2186) or possibly a quinol, which can feed electrons into the electron transport chain (PP_0489-0492). As indicated above, most of the formate is likely converted by the NAD⁺-dependent FDH in the exponential phase of growth, as demonstrated by *in vitro* enzyme assays. However, since *P. putida* KT2440 is endowed with more than the two annotated FDHs⁷⁷ a transfer of the electrons from formate to NAD⁺ with growing cells can only be ensured, if the genes encoding the potential NAD⁺-independent FDH(s) and the unspecific dehydrogenases are identified and disrupted.

Considering the metabolic network of *P. putida* KT2440, ten molecules of NAD(P)H can theoretically be regenerated per molecule of glucose being completely oxidized with non-growing but metabolically active cells. This study demonstrates that this maximal theoretical yield can only be achieved by approximately 50% with growing cells independent of carbon-excess or -limited conditions (Table 17). As a result of the formate co-feeding the cells even decreased the NADH yield per consumed molecule of glucose to one third of the maximal yield demonstrating that the cell changed the distribution of carbon to the benefit of biomass synthesis.

The central aim of this study was to investigate the fundamental effects of an additional energy source on the metabolism of *P. putida* KT2440. While co-catabolism of formate increased the biomass yield on glucose indicating energy-limited growth during glucose as sole carbon and energy source, the growth rate remained unaffected under excess energy conditions demonstrating that the growth rate is limited by the capacity of the biomass synthesis machinery. Apparently, 0.7 g_{CDW} g_S⁻¹ was the maximum achievable biomass yield on glucose with formate as additional energy source since this threshold value was reached in both cultivation conditions. *P. putida* throttled the glucose uptake rate when formate was co-fed to the culture demonstrating that the carbon uptake of this organism is driven by demand.

Conversely, the uptake of formate was more subject to a passive diffusion mechanism rather than driven by demand since the formate uptake rate and thus the NADH regeneration rate highly exceeded cellular demands demonstrated by a maximum achievable biomass yield accompanied by a severely increased OUR. The surplus NADH regeneration rate from the FDH activity must be balanced in order to maintain cellular homeostasis. Since we did not detect any reduced extracellular metabolites, the electron transport chain remains as the only fate of NADH with oxygen as the final electron acceptor. The surplus energy is likely 'burned' with a less efficient branch of the electron transport chain^{40,59}, which was included in our model containing alternative reactions differing in the number of protons transferred over the membrane during oxidation of ubiquinol. Although these flux estimates could only be poorly resolved, they are supported by an experimentally determined oxygen uptake rate that showed a similar increase (data not shown). However, even the less efficient electron transport chain resulted in an increased ATP regeneration, which was likely balanced in futile reactions. Note that *P. putida* is able to accumulate polyhydroxyalkanoates¹⁹⁹ under carbon excess conditions which was not included in our model, which may explain the unclosed electron balance. Additionally, evaporation of formate and hence an overestimated formate oxidation rate may be another explanation for the unclosed electron balance.

These observations have clear implications for possible applications of a formate co-feed in redox biocatalysis. Our results indicate that unlimited growth conditions might be favorable due to the severely increased NADH regeneration rate available for miscellaneous processes compared to a carbon-limited condition. This rate would be further supported if the cells operated an efficient electron transport chain during redox biocatalysis. However, redox biocatalysis can be related to many energy dependent processes such as protein overexpression and uncoupling reactions⁴⁵ so that the surplus NADH from formate co-feeding might not be fully exploited for the intended redox biocatalytic reaction.

Altogether, a co-feed of formate in addition to a carbon source seems to be a promising strategy to supply the cell with an additional energy source, e.g., to support a redox biocatalytic reaction with *P. putida* KT2440 as a host. Therefore, *P. putida* KT2440, as emerging biotechnological workhorse, may constitute an alternative in terms of NADH supplied redox biocatalytic reactions to long-established chassis such as *S. cerevisiae* and *E. coli*, especially in systems that are dependent on reducing equivalents, such as monooxygenase-based systems.

4.5 Acknowledgements

We thank Estrella Duque (Consejo Superior de Investigaciones Científicas, EEZ, Department of Environmental Protection, Granada, Spain) for providing the NAD⁺-dependent FDH knock-out mutant. We acknowledge financial support from the Federal Ministry of Education and Research (BMBF), Germany through the ERA-IB project "Pseudomonas 2.0". N. Wierckx was supported by the German Research Foundation through the Emmy Noether project WI 4255/1-1.

Chapter 5

A method to estimate NADH regeneration rates

We introduce a rapid whole cell assay for the estimation of NADH regeneration rates based on the fluorescent dye resazurin. A co-feed of formate and glucose, known to increase the intrinsic NADH regeneration rate of *P. putida* KT2440, was chosen as model system for the validation of this assay.

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Author contributions:

Sebastian Zobel and Jannis Küpper have conceived the project, performed the experiments, and analyzed the results. Sebastian Zobel has written the chapter with the help of Nick Wierckx. Nick Wierckx and Lars M. Blank have conceived and supervised the study.

5.1 Introduction

NADH is the main energy carrier for hundreds of reactions within cellular metabolism resulting in a tightly regulated ratio of its oxidized form NAD^+ to its reduced form NADH in order to maintain cellular homeostasis²⁰⁰. Thus, challenging a microbe with an NADH-dependent redox sink, such as a biocatalytic reaction, influences this ratio and therefore might have an impact on the cellular performance due to an imbalance of the NAD^+/NADH ratio. Consequently, the microbe must increase the regeneration rate of NADH to supply efficiently the increased NADH demand. Indeed, whole-cell redox biocatalysis can be limited by an insufficient regeneration rate of NADH^{52,145}.

Many studies focus on the determination of the intracellular NAD(H) concentration, which is difficult due to the instability of the analyte (reviewed in Blank et al.⁵¹). For an estimation of the NADH regeneration rate, metabolic flux analysis is a reliable method^{50,52}. However, this method is quite laborious for medium- and high-throughput analysis.

Therefore, we developed a rapid assay to estimate NADH regeneration rates based on the fluorescent dye resazurin. Resazurin is blue and non-fluorescent, it is non-toxic, water soluble, and cell-permeable and is only reduced to the pink fluorescent resorufin by living cells²⁰¹. Thus, it is easy to detect and an ideal indicator for cell viability and has been applied as biosensor for efflux pumps²⁰², beer-spoilage²⁰³, and organic pollutants²⁰⁴. Since resazurin is reduced to resorufin by NADH-dependent dehydrogenases²⁰⁵ the resazurin reduction rate can be determined in a simple whole cell assay.

P. putida was chosen as an appropriate candidate for the development of this assay as it was shown that it can adapt its redox metabolism to various environmental conditions like during biocatalysis in the presence of organic solvents⁴⁵ or during growth in the presence of an uncoupler⁵⁰. *P. putida* is able to increase its redox metabolism when being co-fed with glucose and formate, compared to glucose alone (Chapter 4). This co-feeding regime was chosen to test whether the resazurin-based assay is suitable for a relative estimation of NADH regeneration rates.

5.2 Materials and Methods

We cultured *P. putida* KT2440 to late exponential phase (30 °C, 250 rpm) in minimal medium¹²³ with 20 mM glucose in a Minitron shaker (INFORS, Bottmingen, Switzerland) and in the same medium supplemented with formate where indicated. Before the resazurin reduction was measured the cells were harvested by centrifugation for 3 min at 7.600 x g, washed twice in 36 mM phosphate buffer (pH = 7.0) and resuspended in distilled water without carbon source to a final cell dry weight (CDW) of $2.6 \pm 0.0 \text{ g}_{\text{CDW}} \text{ l}^{-1}$ ($\text{OD}_{600}=5.1 \pm 0.0$). The concentrated cells and all other assay components were kept on ice until they were used. For the assay 50 μl of concentrated cells, 20 μl of fivefold concentrated phosphate buffer, 10 μl of 200 mM glucose, 10 μl of 300 or 600 mM sodium formate, and 10 μl of 70 nM resazurin were added in the above mentioned order. If the resazurin reduction rate was determined with glucose as the only electron donor for NADH regeneration in the assay,

the amount of fivefold concentrated phosphate buffer was increased to 30 μl in order to maintain a total volume of 100 μl in each well. The analysis was started subsequent to the addition of resazurin. The development of the fluorescent resorufin was measured in well plates (Greiner 96 Flat Bottom Black Polystyrene, Greiner Bio-One, Kremsmünster, Austria) with an Infinite 200 PRO microplate reader (Tecan, Maennedorf, Switzerland) at 30 °C with an excitation wavelength of $\lambda = 544$ nm and an emission wavelength of $\lambda = 590$ nm²⁰⁴ in a kinetic whole cell assay (3 mm shaking amplitude, Z-position 20 mm) reflected by the increase in relative fluorescent units (RFU).

5.3 Results and discussion

Controls revealed that medium components did not influence the reduction of resazurin, which only occurred in the presence of cells (Figure 24). Even in the absence of glucose the cells reduced resazurin to resorufin for more than 40 min at a nearly constant rate reflected by the linear RFU increase. This background activity likely arises from the metabolism of intracellular carbon storage polymers such as polyhydroxyalkanoate to maintain cell viability¹⁹⁹. The addition of 1 mM glucose did not result in an increased resazurin reduction rate. This concentration is below the K_m of the low-affinity glucose uptake system of *Pseudomonads*²⁰⁶, possibly leading to a low glucose uptake rate that is indistinguishable from the background reduction rate, and the induction of the high-affinity uptake system is delayed under these conditions. In contrast, 10 mM glucose significantly boosted the redox metabolism of *P. putida* KT2440. A further increase to 20 mM resulted in nearly the same rate, which is in line with a saturation of the low-affinity uptake system^{70,207}. Therefore, 20 mM glucose was chosen as standard condition in the assay to ensure a sufficiently long analysis time and comparability to previous experiments (Chapter 4). Further analysis with different cell concentrations applied in the assay revealed that a CDW of approximately 1.3 g_{CDW} l⁻¹ (OD₆₀₀ = 2.5) was preferable resulting in short analysis times while preventing oxygen limitation in the 96-well plate.

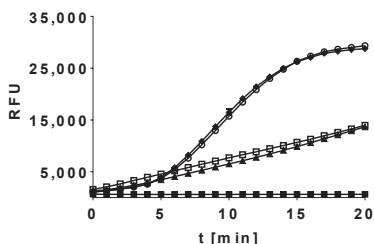


Figure 24: Assay validation. The reduction of resazurin was carried out in a total of 100 μl ; in every assay a 1.5-fold medium buffer concentration and 7 nM of resazurin were added: supernatant + 20 mM glucose (■), cells without carbon source (□), cells + 1 mM glucose (▲), cells + 10 mM glucose (◆), cells + 20 mM glucose (○); the cell concentration was 2.5 g_{CDW} l⁻¹; symbols represent the mean of three replicates with SEM.

After assay validation, we investigated whether a co-feed of formate, known to increase the NADH regeneration rate (Chapter 4), increases the resazurin reduction rate. Hence, cells incubated solely with glucose were compared to those being co-fed with 30 mM formate in the main culture (Figure 25). Indeed, those cells that were grown in the presence of formate and were supplied with 30 mM

formate in the assay exhibited an increased resazurin reduction rate, which can be attributed to an increased NADH regeneration.

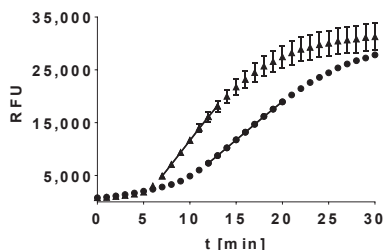


Figure 25: Addition of formate increases resazurin reduction rate; the slope (solid line) illustrates the range for which resazurin reduction rates were determined; cells ($1.28 \text{ g}_{\text{CDW}} \text{ l}^{-1}$) with 20 mM glucose (●), cells ($1.29 \text{ g}_{\text{CDW}} \text{ l}^{-1}$) with 20 mM glucose and 30 mM formate (▲); data represent the mean of three replicates with SEM

The rate was determined similar to Tizzard et al.²⁰⁴ in $\text{RFU min}^{-1} \text{ mg}_{\text{CDW}}^{-1}$ as the slope of the linear range (RFU min^{-1} , a minimum time period of 5 min with the highest correlation coefficient R^2) correlated to the biomass (CDW) used in a well. Figure 26 illustrates cells solely grown on glucose being co-fed with formate only in the assay. Surprisingly, these cells exhibited an increased resazurin reduction rate upon formate addition in the assay comparable to those being exposed to 60 mM formate during cultivation (data not shown). This supports the observation by Roca et al.⁷⁷ that the FDH is constitutively expressed by *P. putida* KT2440 as part of an urgently needed detoxification machinery allowing growth in its natural soil habitat.

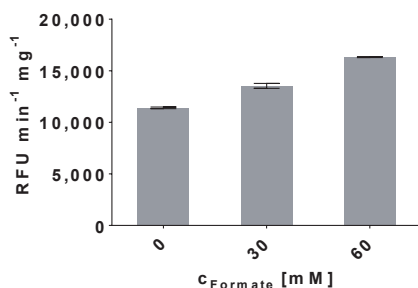


Figure 26: Resazurin reduction rates with formate as additional NADH source; bars represent main cultures grown solely on glucose; concentrations on the x-axis represent the applied concentration of formate in the assay

In conclusion, the development of a whole-cell assay for the rapid investigation of NADH regeneration rates was successful. However, the derivation of absolute NADH regeneration rates remains difficult since no 1:1 ratio of a regenerated NADH to a reduced resazurin molecule can be assumed due to cellular competition for this cofactor. More quantitative estimations would require at least the knowledge of the Michaelis Menten constants of the resazurin reducing enzymes. Nevertheless, this assay constitutes a good tool for screening of redox enhancing substrates, mutants or conditions, which may be useful for improving redox-cofactor dependent whole-cell biocatalysis.

5.4 Acknowledgements

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Chapter 6

General Discussion

6.1 The benefit of formate as additional energy source

The volumetric productivity which is the amount of product formed in a certain volume and time [$\text{g l}^{-1}\text{h}^{-1}$] is a major determinant of biotechnological processes, especially for continuous cultivations. An important variable of the volumetric productivity is the product yield which is defined as the product (p) formed per added substrate (s) [$\text{g}_p \text{g}_s^{-1}$] and which can dramatically change depending on the chosen host and the biotechnological process (Figure 27).

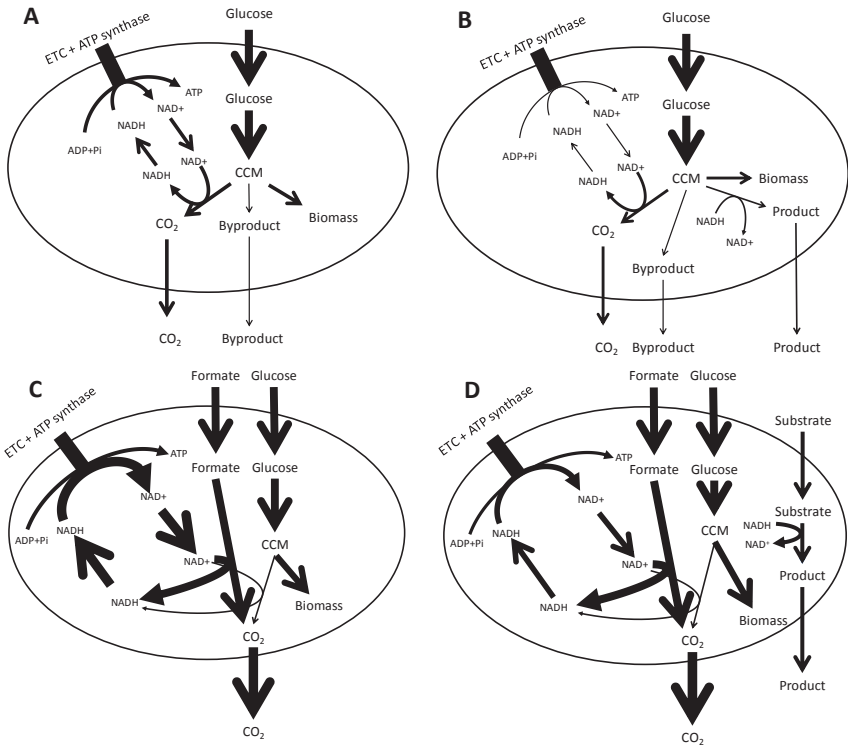
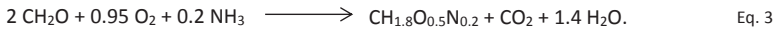
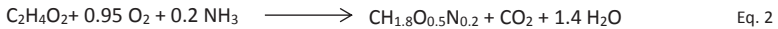
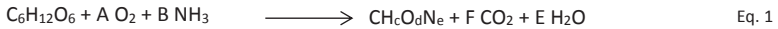


Figure 27: Simplified illustration of biotechnological processes compared to growth; A) microbial growth under standard conditions including the formation of byproducts which constitutes a drain of carbon; B) fermentation in which glucose serves as carbon source for biomass, a potential byproduct, the product, and energy; C) growth of *P. putida* KT2440 with formate as additional energy source; d) biotransformation of *P. putida* KT2440 with formate as additional energy source; arrow thickness indicates lower or higher fluxes

Under standard growth conditions glucose as carbon source, together with oxygen and ammonia as nitrogen source, is converted to energy (in the form of NADH and ATP) and biomass, which can be described with its elemental composition formula $\text{CH}_2\text{O}_d\text{N}_e$ (Eq. 1):



If glucose is described with a simplified formula of $\text{C}_2\text{H}_4\text{O}_2$ (Eq. 2)²⁰⁸ or as a one carbon atom molecule CH_2O ($M_{\text{Cmol, Glucose}}=30 \text{ g Cmol}^{-1}$, Eq. 3), it is obvious that glucose carbon is theoretically converted in a 1:1 ratio into biomass ($\text{CH}_{1.8}\text{O}_{0.5}\text{N}_{0.2}$, $M_{\text{Cmol, biomass}}=24.6 \text{ g Cmol}^{-1}$)²⁰⁹ and CO_2 , which is connected to the generation of energy mostly within the TCA cycle (Figure 27), meaning that a maximum theoretical yield of $0.82 \text{ Cmol}_{\text{biomass}} \text{ Cmol}_{\text{glucose}}^{-1}$ can be achieved. For hosts like *E. coli* and *S. cerevisiae* the formation of byproducts like acetate and ethanol occurs due to overflow metabolism in *E. coli*²¹⁰ and the Crabtree effect in *S. cerevisiae*²¹¹ (Figure 27A). The formation of byproducts is undesired as it decreases the biomass and product yield especially during a product forming fermentation (Figure 27B) and increases the costs of the overall process since byproducts have to be removed efficiently. A great advantage of *P. putida* is the absence of byproduct formation not only under standard growth conditions but also under energy demanding conditions⁴⁵. Thus, *P. putida* generally achieves higher biomass yields on glucose compared to *E. coli* and *S. cerevisiae* especially during unlimited growth (Table 18).

Table 18: Comparison between *E. coli*, *S. cerevisiae*, and *P. putida* based on observed biomass yields on glucose

	Aerobic cultivation condition		max. theor. Yield [%] ^a	Reference
	Growth rate [h^{-1}]			
<i>E. coli</i>				
K12 TG-1	limited	$D=0.044\text{-}0.415$	40-90	Kasyer et al. ²¹²
K12 MG1655	unlimited	$\mu_{\text{max}}=0.63$	53	Leighty and Antoniewicz (2012) ²¹³
JM101	unlimited	$\mu_{\text{max}}=0.69$	64	Martínez-Goméz et al. ²¹⁴
<i>S. cerevisiae</i>				
H1022	limited	$D=0.05\text{-}0.45$	22-74	Kaspar and Meyenburg ²¹⁵
CEN.PK 113-7D	unlimited	$\mu_{\text{max}}=0.07\text{-}0.44$	18-24	Blank and Sauer ²¹⁶
CEN.PK 113-7D	limited	$D=0.025\text{-}0.15$	58-74	Vos et al. ²¹⁷
CEN.PK 113-7D	limited	$D=0.1$	73	Luttik et al. ²¹⁸
<i>P. putida</i>				
KT2440	limited	$D=0.1\text{-}0.7$	67	Lieder et al. ²¹⁹
	limited	$D=0.2$	77	This study – Chapter 4
	unlimited	$\mu_{\text{max}}=0.73$	83	Ebert et al. ⁵⁰
	unlimited	$\mu_{\text{max}}=0.61$	85	This study – Chapter 4
	unlimited	$\mu_{\text{max}}=0.57$	85	Blank et al. ⁴⁵
Gpo12	unlimited	$\mu_{\text{max}}=0.48$	80	Blank et al. ⁴⁵
S12	unlimited	$\mu_{\text{max}}=0.52$	62	Blank et al. ⁴⁵
DOT-T1E	unlimited	$\mu_{\text{max}}=0.60$	77	Blank et al. ⁴⁵

a: $0.82 \text{ Cmol}_{\text{biomass}} \text{ Cmol}_{\text{glucose}}^{-1}$ was assumed to be the maximal theoretical biomass yield on glucose based on the average biomass formula $\text{CH}_{1.8}\text{O}_{0.5}\text{N}_{0.2}$

Hence, *P. putida* seems to be an optimal biocatalyst for a biotechnological application due to the absence of carbon loss. As demonstrated in Chapter 4, a co-feed of formate to glucose resulted in a shift in the ratio of energy to biomass which changed to the benefit of biomass (Figure 27C) resulting in approximately 104% ($0.7 \text{ g}_{\text{biomass}} \text{ g}_{\text{glucose}}^{-1} \cong 0.85 \text{ Cmol}_{\text{biomass}} \text{ Cmol}_{\text{glucose}}^{-1}$) of the maximum theoretical biomass yield. Therefore, the additional energy source may increase the volumetric

productivity as the cell concentration increases. Moreover, our results suggest that the increased cell population of *P. putida* KT2440 might be able to meet the energy challenges associated with a hypothetical biocatalytic reaction due to the severely increased NADH regeneration rate drastically exceeding cellular needs. The effect of formate as NADH electron donor might even be increased during a biotransformation process (Figure 27D) since glucose only serves as carbon and energy source and not as carbon source for the formation of the product.

However, for an application in an industrial scale different co-feeding and process strategies need to be evaluated in order to obtain the highest volumetric productivities. During the batch fermentation process described in Chapter 4 formate was completely removed out of the fermentation broth while during continuous cultivation approximately 8 mM of formate remained in the fermentation broth due to several reasons. It is likely that the transport of formate into the cytoplasm was limiting. Another reason may be that the FDH or the NADH dehydrogenase(s) of the electron transport chain already operated at their highest activities. It is likely that *P. putida* KT2440 benefits most from formate additionally to glucose during growth until a certain threshold concentration above which *P. putida* KT2440 has to compensate for the surplus NADH from formate through burning it at the electron transport chain. This threshold concentration would be the concentration to optimally support growth. Therefore, a lower concentration than 120 mM during an initial biomass forming phase likely results in a comparable increase in biomass yield prior to a subsequent biotransformation during which the formate concentration should be increased. If the reason for the remaining formate in the fermentation broth is the limiting enzyme activity of the NADH dehydrogenase of the electron transport chain, the NADH-dependent substrate converting enzyme should exhibit a lower K_m value in order to minimize loss of formate through the dissipation of energy at the electron transport chain.

6.2 Renewable sugars vs. lignocellulose

Although it is known that *P. putida* prefers succinate²⁸ or benzoate⁶⁹ over glucose, the application of glucose or other sugars like fructose and glycerol is common practice in industrial biotechnology. A shift to the preferred benzoate as carbon source would only be sustainable and imaginable if pyrolysis of the renewable lignocellulosic plant material became economic. However, pyrolysis of wood biomass results in a broad product spectrum of e.g. acids, esters, and alcohols derived from hemicellulose and cellulose and phenols, guaiacols, and syringols from lignin²²⁰. This mixture of different compounds needs a laborious treatment before specific compounds can be used in biotechnological applications. Thus, microbes being equipped with a considerable genetic inventory for industrial purposes need to be engineered in the efficient use of one of the above mentioned sugars as long as lignocellulosic derived carbon sources do not constitute an economic alternative.

6.3 The perfect biotechnological host

The overall aim of life is survival meaning reproduction and adaptation to changing environmental conditions through diversity which is not in line with the objectives of a biotechnological process

such as a homogenic culture with a limited growth connected to a high production rate²²¹. The regulatory network within a living cell is incredibly complex guaranteeing the survival in their natural, frequently changing environment. Most of this network does not constitute any benefit in a controlled technical environment and may even result in an unnecessary drain of resources. Hence, a microbial host needs to be engineered not only in the efficient use of a carbon source but also in terms of metabolic robustness, phenotypic stability and a stable gene expression which also should be addressed as a design objective of a perfectly domesticated cellular host²²¹. Martinez-Garcia et al²²² already demonstrated that by deleting multiple genes responsible for, e.g., a drain of energy resulted in a *P. putida* KT2440 strain which was superior to the wild type strain. The developed calibrated promoter library during this work can contribute to such an engineered host for these purposes since the synthetic promoters guarantee a stable expression during the main growth phase and contribute to a more homogenic behavior of the microbial culture.

Alternatively to the “top-down” engineering approach of existing organisms described above, there are advances in the “bottom-up” approach describing the construction of a synthetic organism with a minimal set of genes which is essential for the survival in a technical environment²²³. The biggest challenge in this approach is the identification of the universal minimal set of genes, since reduced essential genomes for several species were already unraveled ranging from 271 protein coding genes for *B. subtilis*²²⁴ to 878 for *S. cerevisiae*²²⁵ owing to their natural environment²²³. This “bottom-up” approach of designing the perfect microbial host for biotechnological applications may lead to a library of modular genes out of which one can choose those which perfectly fit for the desired application. Here, too, the presented strategy for a calibrated synthetic promoter library may contribute to such an approach of a synthetically constructed technical organism which will for the present remain a vision.

Appendix

Table 19: Physiology of different *P. putida* KT2440 strains harboring pSEVA231atpAGDXX constructs^a

SynP ^b	μ		CUR		$Y_{X/S}$	
	[h ⁻¹]	[%]	[mmol g _{CDW} ⁻¹ h ⁻¹]	[%]	[g _{CDW} g _S ⁻¹]	[%]
Control	0.47±0.01	100±2	5.2±0.1	100±2	0.50±0.02	100±4
α	0.44±0.01	94±2	5.0±0.2	96±4	0.49±0.01	98±2
1	0.46±0.00	99±1	4.7±0.0	90±0	0.55±0.01	110±1
8	0.46±0.00	98±1	5.0±0.3	96±6	0.51±0.03	102±5
Control	0.44±0.01	100±2	4.2±0.0	100±0	0.58±0.01	100±2
15	0.39±0.00	89±0	4.2±0.0	100±1	0.52±0.00	89±1
20	0.38±0.01	85±2	4.3±0.0	101±1	0.49±0.01	84±2
22	0.41±0.01	93±2	4.6±0.0	107±1	0.50±0.01	87±2
Control	0.43±0.00	100±1	4.5±0.1	100±1	0.53±0.01	100±2
23	0.41±0.02	96±4	4.8±0.8	107±17	0.49±0.06	92±11
25	0.42±0.00	98±1	4.5±0.0	101±0	0.52±0.00	98±1
38	0.42±0.00	99±1	4.6±0.0	104±0	0.51±0.00	95±1
Control	0.44±0.00	100±0	4.3±0.1	100±2	0.57±0.01	100±2
40	0.46±0.01	104±2	4.9±0.0	115±0	0.52±0.01	90±1
41	0.45±0.01	102±2	4.5±0.1	105±2	0.56±0.02	98±3
42	0.45±0.00	101±1	4.5±0.1	104±3	0.56±0.02	98±4

- a: the values for the control strain harboring the empty vector pSEVA231 were set to 100%, values represent the mean of two replicates with SEM; μ - growth rate, CUR - carbon uptake rate as the sum of glucose, gluconate and 2-ketogluconate, $Y_{X/S}$ - biomass yield on glucose
- b: Growth experiments were carried out in sets of three strains harboring different pSEVA231atpAGDXX constructs in duplicates with *P. putida* KT2440 carrying the empty vector pSEVA231 as a control

Table 20: Physiology of different *E. coli* JM101 strains harboring pSEVA231atpAGDXX constructs^a

SynP ^b	μ		CUR		$Y_{X/S}$		APR	
	[h ⁻¹]	[%]	[mmol g _{CDW} ⁻¹ h ⁻¹]	[%]	[g _{CDW} g _S ⁻¹]	[%]	[mmol g _{CDW} ⁻¹ h ⁻¹]	[%]
Control	0.63±0.01	100±2	7.4±0.0	100±0	0.48±0.01	100±2	4.0±0.1	100±3
α	0.61±0.01	97±1	7.0±0.3	96±4	0.48±0.02	102±5	4.0±0.1	100±2
1	0.63±0.00	99±1	7.0±0.2	95±3	0.50±0.02	104±4	4.1±0.1	103±3
8	0.59±0.00	94±0	7.0±0.0	95±0	0.47±0.00	100±0	4.3±0.0	107±1
Control	0.60±0.01	100±2	6.6±0.0	100±0	0.51±0.01	100±2	3.2±0.0	100±1
15	0.58±0.01	97±2	7.0±0.2	107±4	0.46±0.02	91±5	4.3±0.3	136±8
20	0.63±0.00	105±1	7.0±0.2	106±3	0.50±0.02	98±3	4.1±0.1	128±3
22	0.59±0.01	99±0	7.0±0.0	106±0	0.47±0.00	94±0	4.3±0.0	134±1
Control	0.58±0.01	100±1	8.3±0.0	100±0	0.39±0.00	100±0	3.8±0.1	100±3
23	0.60±0.01	103±2	9.0±0.5	109±7	0.37±0.03	96±8	5.0±0.5	132±14
25	0.58±0.05	100±8	8.0±0.7	97±8	0.41±0.01	105±17	4.0±0.1	105±2
38	0.54±0.03	93±4	6.7±0.0	81±0	0.45±0.02	114±16	3.7±0.2	97±6
Control	0.51±0.01	100±2	7.2±0.0	100±0	0.42±0.01	100±3	2.8±0.2	100±7
40	0.56±0.01	104±3	7.6±0.0	104±0	0.41±0.01	99±30	3.5±0.2	126±7
41	0.57±0.01	106±1	7.3±0.1	101±1	0.44±0.00	105±0	3.4±0.1	122±4
42	0.55±0.03	102±6	7.5±0.5	104±7	0.41±0.05	99±12	3.7±0.1	132±4

- a: the values for the control strain harboring the empty vector pSEVA231 were set to 100%, values represent the mean of two replicates with SEM; μ - growth rate, CUR - carbon uptake rate as the sum of glucose, gluconate and 2-ketogluconate, $Y_{X/S}$ - biomass yield on glucose, APR - acetate production rate
- b: Growth experiments were carried out in sets of three strains harboring different pSEVA231atpAGDXX constructs in duplicates with *E. coli* JM101 carrying the empty vector pSEVA231 as a control

Appendix

Table 21: Sequence of synthetic promoters chosen for *atpAGD* overexpression with plasmid pJS-LacZ

Name	Sequence ^a	Activity [MU]
Design ^b	GGATCC NNNNNTTGACANNNNNNNNNNNNNNNNTATAATNNNNNN GGTACC	
SynP2	GGATCC -----T- <u>A</u> -----TTTTTATAATCCGAGCG GGTACC	123±4
SynP2s ^c	TCCAACGCGTTGGGAGCTCTCC GGATCC TATTTTATAATCCGAGCG GGTACC	
SynP37	GGATCC AAATGGTTGACA----CTTATTTCTATGAGATAATTCAAAA GGTACA	630±17
SynP37s ^c	ATCC GGATCC AAATGGTTGACACTTATTTCTATGAGATAATTCAAAA GGTACA	
SynP30	GGATCC CAGGGCT---AA-----TGTATAATAGACGCG GGTACA	2,569±79
SynP30s ^c	GGGAGCTCTCCGGATCC GGATCC CAGGGCTAATGTATAATAGACGCG GGTACA	

a: The restriction sites for *Bam*HI (**GGATCC**) and *Kpn*I (**GGTACC**) are in bold; the conserved -35 (TTGACA) and -10 (TATAAT) regions of sigma⁷⁰-promoters are underlined

b: Part of the oligonucleotide design which represents the synthetic promoters (see primer pSynPro_lacZ in Table 7: Primers used in this study)

c: 2Bs, 37Bs and 30Bs illustrate the shifted sequence due to deletions within SynP sequence

Table 22: Experimentally determined constraints for the calculation of intracellular fluxes (see Table 23)

	Batch	Batch + FA	Chemostat	Chemostat + FA
μ [h ⁻¹]	0.61±0.01	0.61±0.02	0.2±0.01	0.2±0.01
CUR [mmol g _{CDW} ⁻¹ h ⁻¹] ^a	5.73±0.42	4.59±0.32	2.15±0.05	1.52±0.08
FUR [mmol g _{CDW} ⁻¹ h ⁻¹] ^b		60.2±1.5		8.0±0.1

a: CUR is the sum of glucose, gluconate and 2-ketogluconate

b: FUR is the formate uptake rate

Table 23: Stoichiometric model of *P. putida* KT2440 with estimated net fluxes calculated with OpenFLUX¹⁶⁰ for the different cultivation conditions described in Chapter 4^a

		Batch	Batch + FA	Chemostat	Chemostat + FA
	GLC_EX + ATP = G6P ^b	6.01±0.47	4.85±0.03	2.01±0.07	1.62±0.05
	G6P = 6PG + NADPH	5.97±0.71	4.72±0.28	1.97±0.15	1.57±0.13
	6PG = P5P + CO ₂ + NADPH	0.35±0.37	0.02±0.19	0.00±0.12	0.00±0.12
	G6P = F6P	0.01±0.24	0.10±0.25	0.03±0.05	0.04±0.08
	6PG = G3P + PYR	5.62±0.84	4.70±0.31	1.97±0.15	1.57±0.15
	F16BP = F6P	0.25±0.25	0.39±0.25	0.14±0.07	0.12±0.08
(r)	F16BP = DHAP + G3P	-0.25±0.25	-0.39±0.25	-0.14±0.08	-0.12±0.08
(r)	DHAP = G3P	-0.25±0.25	-0.39±0.25	-0.14±0.08	-0.12±0.08
(r)	P5P + P5P = S7P + G3P	0.00±0.12	-0.11±0.06	-0.04±0.04	-0.04±0.04
(r)	E4P + P5P = F6P + G3P	-0.22±0.12	-0.33±0.06	-0.11±0.04	-0.11±0.04
(r)	S7P + G3P = E4P + F6P	0.00±0.12	-0.11±0.06	-0.04±0.04	-0.04±0.04
(r)	G3P = 3PG + ATP + NADH	4.82±0.67	3.52±0.27	1.56±0.13	1.19±0.12
(r)	3PG = PEP	3.73±0.66	2.43±0.27	1.21±0.14	0.83±0.12
	PEP = PYR + ATP	3.25±0.67	1.94±0.27	1.05±0.14	0.67±0.12
	PYR = ACCOA + CO ₂ + NADH	5.20±1.65	2.96±0.15	1.81±0.25	1.04±0.20
	ACCOA + OAA = ICT	3.78±0.91	1.51±0.06	1.35±0.18	0.57±0.11
	ICT = OGA + CO ₂ + NADPH	3.78±1.04	1.50±0.10	1.35±0.55	0.57±0.12
	OGA = SUC + CO ₂ + ATP + NADH	2.97±0.98	0.69±0.10	1.08±0.55	0.31±0.12
(r)	SUC = MAL + QH ₂	2.97±0.96	0.70±0.06	1.08±0.14	0.31±0.11
	MAL = OAA + NADH	0.00±0.63	0.00±0.06	0.00±0.35	0.03±0.11
	MAL = PYR + CO ₂ + NADH	2.97±1.1	0.70±0.06	1.08±0.55	0.28±0.12
	OAA + ATP = PEP + CO ₂	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	PYR + CO ₂ + ATP = OAA	4.80±0.78	2.53±0.10	1.68±0.58	0.88±0.13
	ICT = GLYOXY + SUC	0.00±0.71	0.01±0.09	0.00±0.34	0.00±0.10
	ACCOA + GLYOXY = OAA	0.00±0.71	0.01±0.09	0.00±0.34	0.00±0.10
	CO ₂ =CO ₂ _EX	11.77±2.73	68.71±9.34	4.07±0.41	9.37±1.24
	O ₂ _EX=O ₂	10.55±2.91	35.44±4.75	3.67±0.39	5.16±0.77
	FA_EX= FA = CO ₂ +NADH	-	64.1±4.8	-	7.6±0.8
	NADPH=NADPH_B	9.71±0.00	9.79±0.00	3.19±0.00	3.19±0.00
	NADH_B=NADH	1.79±0.00	1.8±0.0	0.59±0.00	0.59±0.00
	NADH=NADPH	-0.38±2.12	3.54±0.45	-0.13±0.63	1.04±0.3

a: Values are given in mmol g_{CDW}⁻¹ h⁻¹, net fluxes means forward reaction minus reverse reaction of reversible reactions indicated with (r); “_EX” – extracellular; “_B” indicates fluxes towards biomass

b: This value illustrates the carbon uptake rate as the sum of glucose, gluconate and 2-ketogluconate;

List of Abbreviations

Abbreviations used in the text		Stoichiometric model	
Ap	Ampicillin	GLC	Glucose
ATP	Adenosine triphosphate	G6P	Glucose-6-phosphate
bps	Base pairs	6PG	6-phosphogluconate
CDW	Cell dry weight	P5P	Pentose-5-phosphate
Chem.	Chemostat	F6P	Fructose-6-phosphate
Cm	Chloramphenicol	G3P	Glyceraldehyde-3-phosphate
FA	Formic acid	F16BP	Fructose-1,6-bisphosphate
FDH	Formate dehydrogenase	DHAP	Dihydroxyacetone phosphate
FUR	Formate uptake rate	S7P	Seduheptulose-7-phosphate
Gm	Gentamicin	E4P	Erythrose-4-phosphate
GOI	Gene(s) of interest	3PG	3-phosphoglycerate
Indel	Combination of <u>in</u> sertion and <u>de</u> letion	PEP	Phosphoenolpyruvate
Kan	Kanamycin	PYR	Pyruvate
kb	Kilo base pairs	ACCOA	Acetyl-CoA
K_m	Michaelis Menten constant	OAA	Oxaloacetate
LB	Lysogeny broth	ICT	Isocitrate
MCS	Multiple cloning site	OGA	2-Oxoglutaric acid, α -ketoglutarate
NAD ⁺ /NADH	Nicotinamide adenine dinucleotide	SUC	Succinate
NADP ⁺ /NADPH	Nicotinamide adenine dinucleotide phosphate	MAL	Malate
OD ₆₀₀	Optical density measured at λ = 600 nm	QH2	Reduced quinone
PCR	Polymerase chain reaction	GLYOXY	Glyoxylate
PEG	Polyethylene glycol	FA	Formic acid
RFU	Relative fluorescence units		
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis		
SEM	Standard deviation from the mean		
SNP's	Single nucleotide polymorphism		
SynP	Synthetic promoter		
TCA cycle	Tricarboxylic acid cycle		
Organisms		Units	
<i>B. subtilis</i>	<i>Bacillus subtilis</i>	°C	degree Celsius
<i>C. boidinii</i>	<i>Candida boidinii</i>	Cmol	molar amount of carbon
<i>C. thermohydrosulfuricum</i>	<i>Clostridium thermohydrosulfuricum</i>	g	Gram
<i>E. coli</i>	<i>Escherichia coli</i>	g	gravitational force
<i>L. plantarum</i>	<i>Lactobacillus plantarum</i>	h	hour
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>	kDa	kilodalton
<i>P. pastoris</i>	<i>Pichia pastoris</i>	kV	kilovolt
<i>P. putida</i>	<i>Pseudomonas putida</i>	l	Liter
<i>P. taiwanensis</i>	<i>Pseudomonas taiwanensis</i>	M	molar
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>	mg	milligram
<i>S. coelicor</i>	<i>Streptomyces coelicolor</i>	min	Minutes
<i>S. lividans</i>	<i>Streptomyces lividans</i>	ml	milliliter
<i>T. glabrata</i>	<i>Torulopsis glabrata</i>	mM	millimolar
		mm	millimeter
		mmol	millimol
		MU	Miller units
		ng	nanogram
		nm	nanometer
		rpm	revolutions per minute
		s	seconds
		w/v	mass (weight) per volume
		λ	Wavelength
		μ F	microfarad
		μ l	microliter
		μ M	micromolar
		Ω	Ohm

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