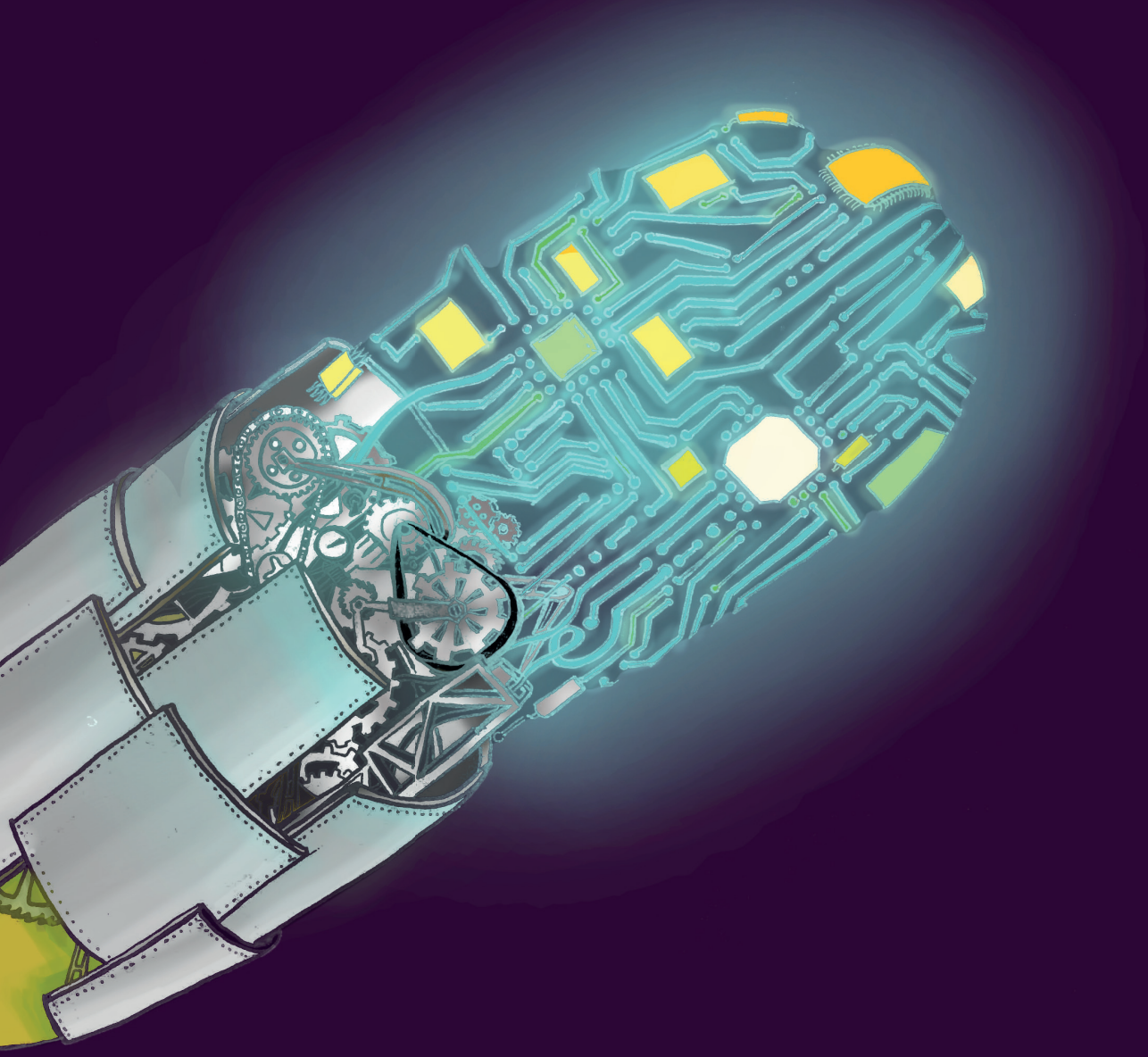

Microbial Lifestyle Engineering

Linde F.C. Kampers

MICROBIAL LIFESTYLE ENGINEERING

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This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

MICROBIAL LIFESTYLE ENGINEERING

Linde F. C. Kampers

Thesis

submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A. P. J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on **Thursday 4 June, 2020**
at **11:00 a.m.** in **the Aula**.

Linde F. C. Kampers
MICROBIAL LIFESTYLE ENGINEERING,
230 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2020)
With references, with summary in English and Dutch

ISBN: 978-94-6395-324-5
DOI: 10.18174/516082

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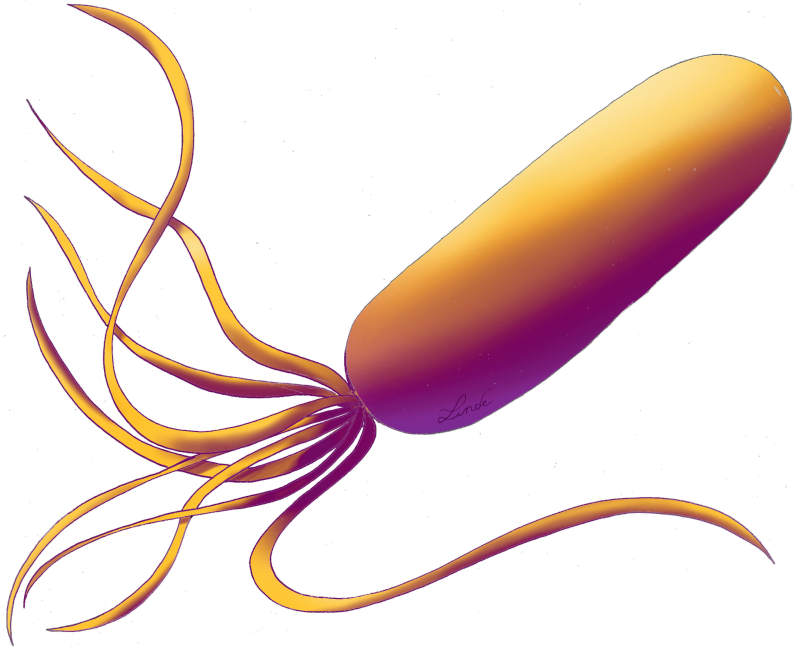
1 Introduction: An Ordered Approach to Feasible Bio-Based Sustainability



Linde F. C. Kampers

Introduction: Microbes for Feasible Sustainability

On the 29^{th} of July 2019, it was Earth Overshoot Day. On this symbolic day, the natural sources the Earth can renew in one year are depleted. Ever since the first Earth Overshoot Day on December 29^{th} 1970, this day has steadily crept forward every year [1, 2]. With an ever-growing world population causing food shortages and climate change [3] (Figure 1.1), it becomes increasingly clear that this is not a sustainable situation.

Microbes are pivotal agents of ecosystem and planetary functioning. They regulate and mediate biochemical cycles and recycle biological waste materials, constitute key producers and sinks of greenhouse gases and play essential roles in the quality and productivity maintenance of soil, sea, lakes and rivers. This exceptional diversity of applications that can be addressed by microbial technology can contribute substantially on many levels towards sustainability [4, 5]. Microorganisms can be used for many biotechnological tasks, including bio-fuel production [6–8], commodity chemical synthesis [9–11], and production of industrial and biopharmaceutical proteins [12–14]. As our knowledge about microorganisms grows, so does the horizon of bio-products. Nowadays worldwide, a large variety of differ-

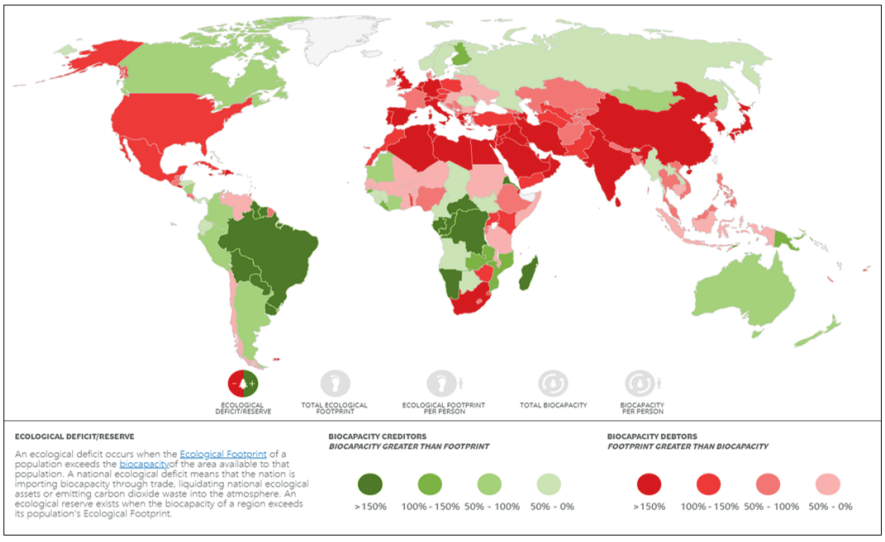


Figure 1.1: Ecological Footprint Atlas, [1]. Copyright 2019 Global Footprint Network. National Footprint Accounts, 2019 Edition.

ent microorganisms is used for industrial purpose, including bacteria, yeasts, fungi and mammalian cell-lines [15–26]. Production with microorganisms can offer high yields and pure products. Next to this, biological catalysis offers several advantages over chemical synthesis, as it offers the opportunity for cheap and stereo-selective production of chemical compounds [27]. Each organism has its own advantages and disadvantages. For instance, in many cases yeasts are considered superior to bacteria such as *Escherichia coli* for industrial production due to their ability to glycosylate, acetylate, fold, and perform many post-translational modifications required for production of functional eukaryotic proteins. Bacteria, on the other hand, can synthesize proteins with a higher yield than yeast [28, 29]. This has created an industrial landscape where for the production of one or few products a specific micro-organism is selected based on its unique characteristics. To intercept the disadvantages of the selected micro-organism and make most use of its specific advantages, the entire industrial set-up is adjusted accordingly. However, the development of synthetic biology as a tool to overcome specific characteristic disadvantages may become an industrial game-changer.

Microbial Lifestyle Selection or Design?

Each living organism has a specific habitable environment. The possibility of a microbe to survive in an environment depends on a scale on many different factors, including pH, temperature, aerobicity, salinity, and source availability. A classical way to visualize and to do research on the different habitats of microbial communities is using a Winogradsky column. A Winogradsky column is a model microbial ecosystem, prepared by filling a clear cylinder with for instance pond sediment with some additional supplements and incubated with light. Over time, environmental gradients develop, creating niches within the column. Esteban *et al.* studied the developed enrichment cultures from each niche from a Winogradsky column incubated for 60 days using a 16S RNA gene survey, and characterized the microbial community dynamics (Figure 1.2) [30].

Microbes have an unique ability to adapt to varying environments, also described as the strain robustness. Each ecological niche represent some specific characteris-

tics of its inhabitants. When applied in industry, these characteristics mirror the advantages and limitations of each species. Historically, microbes used in industry have simple nutrient requirements, can be easily cultivated in a laboratory and generally grow fast, resulting in over a hundred generations over few weeks. Many bacteria, such as *Escherichia coli* or *Pseudomonas putida*, have a generation time in the laboratory of 20 minutes, which results in over 500 generations per week. This allows for relatively fast adaptation of the entire community to new environmental conditions. Industrial microorganisms are in addition preferably safe, mesophilic, robust to fluctuations in pH, temperature and oxygen, and secrete their product for easy isolation. However, not one microbe has all these distinct characteristics, let alone is able to reliably produce a specific product in high yields.

Traditional industrial microbiology was merged with molecular biology to yield recombinant metabolic processes for the sustainable production of primary and secondary metabolites, proteins, bio-pharmaceuticals, and enzymes on an industrial scale. Examples of this are listed extensively in the review written by Adrio and Demain [16]. These include, but are not limited to: **amino acids** produced commer-

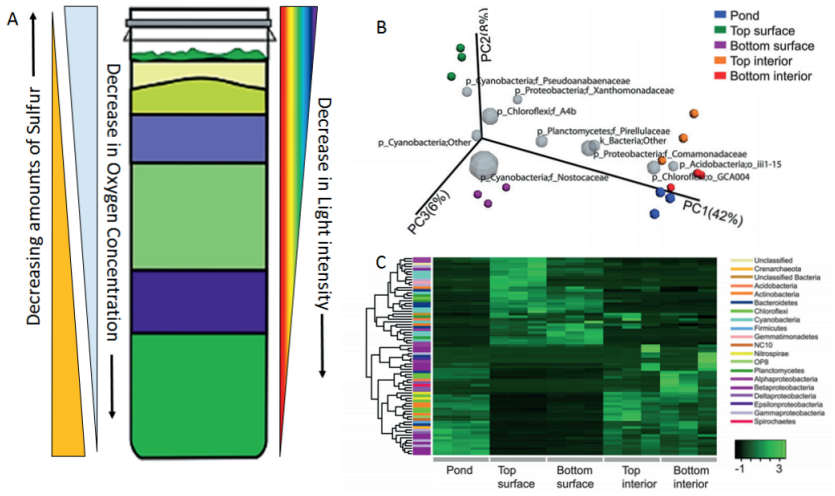


Figure 1.2: Winogradsky culture enrichment. A) a schematic visualisation of a Winogradsky column. B) A Biplot of the most abundant family-level taxa in the surface community of a Winogradsky column after 60 days of incubation. C) A heat-map of the normalized relative abundances showing Z-scores within the surface, interior and pond communities. Each row represents a unique genus and the coloured bar represents the phylum to which it belongs [30].

cially by *Corynebacterium*, *Brevibacterium* and *Serratia* strains, **vitamins** like biotin produced by *E. coli*, or riboflavin and vitamin C made with *Corynebacterium*, **organic acids** like acetic acid made with *E. coli*, *Corynebacterium* or *Acetobacter*, **lactic acid** for which a genetically engineered transgenic wine yeast is used, next to *Lactobacillus*, *Bacillus*, *Escherichia* or *Rhizopus*, **ethanol** using *Escherichia*, *Zymomonas* and *Saccharomyces*, **antibiotics** by *Acremonium*, *Penicillium* or *Streptomyces*, and a large variety of **other compounds** including nucleosides with *Bacillus*, carotenoids with *Escherichia* and solvents with *Clostridium* (Table 1.1) [16].

For over a hundred years, evolutionary methods have been applied to adapt species. A good example to adapt species to new specific conditions is through adaptive laboratory evolution (ALE) experiments. ALE is a frequently used and well-known method in biological studies to harbour adaptive changes in the form of mutations to accumulate in microbial populations during long term selection under specific environmental conditions. With ALE experiments, microorganisms are cultivated under strictly defined environmental conditions for a prolonged period of time, ranging from days to weeks to even years, and are often followed by long characterization studies and intensive analysis to define which random mutation caused the desired phenotype. It was first applied by William Dallinger [15, 104]. *Escherichia coli* and *Saccharomyces cerevisiae* are the most prominent organisms applied in ALE experiments [105–107], pushing for higher yield of products, the use of a different carbon source, or increased stress resistance to for example toxic substances, higher temperatures, varying pH or higher salinity. ALE allows for the cultivation and phenotype selection of strains adapted to the conditions of choice. This top-down approach takes a lot of time and resources, but do not require any background knowledge [108].

Within the fast-developing field of **Systems and Synthetic Biotechnology**, microorganisms can be changed in a directed way. This field has opened up many novel techniques to eliminate any disadvantages of industrially interesting bacterial strains all together through a bottom-up approach. With an ever-growing toolkit including model-driven circuit design, directed genome re-factoring, and metabolic engineering, microorganisms can be completely redirected into the production of novel natural or synthetic products, or even adapt a new lifestyle [16, 109–111].

Table 1.1: Industrial Microbial Applications. Adapted from Adrio and Demain, 2010 [16]

Product	Organism	Species	Source
Primary metabolites			
Amino acids	Bacteria	*Brevibacterium*, *Corynebacterium*, *Escherichia*, *Serratia*	[31–35] [36–39]
Vitamins	Bacteria	*Bacillus*, *Corynebacterium*, *Escherichia*, *Gluconobacter*, *Pantoea*, *Serratia*	[40–43] [44, 45]
Organic acids	Bacteria	*Acetobacter*, *Actinobacillus*, *Anaerobiospirillum*, *Clostridium*, *Corynebacterium*, *Escherichia*, *Lactobacillus*, *Mannheimia*, *Pichia*, *Rhizopus*, *Yarrowia*	[11, 46–49] [50–54]
	Fungi	*Candida*, *Kluyveromyces*, *Rhizopus*, *Saccharomyces*, Wine yeast	[55–59]
Alcohols	Bacteria	*Clostridium*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Zymomonas*	[46, 60–63] [64]
	Fungi	*Saccharomyces*	[65]
Nucleosides	Bacteria	*Bacillus*	[66]
Carotenoids	Bacteria	*Escherichia*	[67]
Glucosamine	Bacteria	*Escherichia*	[68]
Solvents	Bacteria	*Clostridium*, *Escherichia*	[69, 70]
Secondary metabolites			
Antibiotics	Bacteria	*Amycolatopsis*, *Escherichia*, *Saccharopolyspora*, *Streptomyces*	[71–75] [76–80] [81, 82]
	Fungi	*Acremonium*, *Aspergillus*, *Penicillium*,	[83, 84]
Anti-tumour agents	Bacteria	*Sorangium*, *Streptomyces*	[85]
Cholesterol-lowering agents	Fungi	*Aspergillus*	[86]
Antihelminthics	Bacteria	*Streptomyces*	[87]
Bio-pharmaceuticals			
Recombinant proteins	Bacteria	*Escherichia*, *Ralstonia*, *Staphylococcus*	[88]
	Fungi	*Hansenula*, *Pichia*, *Saccharomyces*	[28, 89]
	Mammalian cells	Chinese hamster ovary (CHO) cells, mouse myeloma (NSO) cells, baby hamster kidney (BHK) cells, green monkey kidney cells, human embryonic kidney (HEK) cells	[28, 29, 90–92]
	Insect cells	Lepidopteran cells (Spodoptera)	[28, 93, 94]
Enzymes			
	Bacteria	*Bacillus*, *Escherichia*	[95, 96]
	Fungi	*Aspergillus*, *Chrysosporium*	[97]
Miscellaneous			
Rapamycin	Bacteria	*Streptomyces*	[98]
Artemisin	Bacteria	*Escherichia*	[99]
	Fungi	*Saccharomyces*	[100, 101]
Spinosad	Bacteria	*Saccharopolyspora*	[102]
Xanthan gum	Bacteria	*Xanthomonas*	[103]

This has indeed led to the development and cultivation of a multitude of industrially competitive engineered strains [16].

Microbial lifestyle engineering

Due to their genetic accessibility, ease to culture in a lab-environment, and overall mesophilic robustness, it is obvious that a few microorganisms are widely applied in industry. However, that does not make these organisms perfect production platforms. The microbes are also not easily interchangeable: if one microbe is selected for a production process, all equipment has to be adapted for the specific needs and characteristics it has. This research will thus focus on the adaptation of the lifestyle of often-used microbes to develop new characteristics, and thus transform the microbe into the ultimate industrial workhorse.

Building an industrial workhorse

Thus far, there are only relatively few microbial organisms with a high degree of genetic accessibility. A well-known and largely applied example is *Escherichia coli*: easy to isolate and to maintain in a lab-environment, established as a great production strain of recombinant proteins, and the availability of an extensive engineering toolkit.

However, *E. coli* does have substantial downsides as opposed to other production hosts. Solubility is an issue as some proteins are packed into inclusion bodies. The bacteria have a limited growth temperature and pH range, which limits the products that can be made and drives production costs up. In 2000, Riehle and colleagues described the use of ALE to adapt six lines of *E. coli* for 2000 generations to the stressful high temperature of 41.5°C [105]. They postulated that with enough time and evolutionary pressure the short generation time and large population sizes of bacteria would ensure enough genomic mutations to adapt to higher temperatures. Still, this is slow-going, labour-intensive work with currently unknown limitations.

One of the up-and-coming candidates to challenge the established industrial work-horses is *Pseudomonas putida*. The gram-negative, rod-shaped *P. putida* KT2440, a derivative of toluene-degrading wild-type *P. putida* strain mt-2, which

was isolated from contaminated soil [112]. *P. putida* is in general renowned for its metabolic versatility, large reducing power, and tolerance to toxins and solvents. In particular, *P. putida* KT2440 is HV1-certified, genetically accessible, and has been successfully engineered to produce various compounds of industrial interest. These characteristics give *P. putida* KT2440 its high potential to be applied as the ultimate industrial platform. *P. putida* is rapidly making its name as a recognized synthetic biology and industrial workhorse.

Yet, also *P. putida* has substantial disadvantages for industrial applications, including its strict aerobic character and its relatively limited temperature growth range, which ranges from 18°C to 41°C. The constant requirement for homogeneously dissolved O_2 in large-scale cultivation results in increased costs due to aeration and stirring, and excludes the use of O_2-sensitive enzymes and pathway intermediates, as well as the production of O_2-sensitive products. Several closely related species in the *Pseudomonas* genus are facultative aerobes, suggesting that a limited number of genetic modifications may be required to redesign *P. putida* to adopt a micro-aerobic or even facultative anaerobic lifestyle. The latter has been experimentally attempted several times by engineering either anaerobic fermentation [113, 114] or anaerobic respiration [115–117].

The Design-Build-Test-Learn Cycle

Earlier attempts to resolve characteristic industrial disadvantages of bacteria like *E. coli* or *P. putida* have shown that it is a long, slow and complicated process. However, most attempts described have not made use of the current wealth of genomic, proteomic and metabolomic data that is nowadays available. Technical problems can be tackled in and ordered approach, using a Design-Build-Test-Learn cycle (DBTL-Cycle) (Figure 1.3). The DBTL-Cycle describes the process of using the plethora of information to tackle synthetic biology problems in a bottom-up approach [118]. In the design phase, *in silico* methods such as genome-scale metabolic models, domainome analysis, and transcriptomics are employed to design an genomically modified strain of interest. This goal can be to direct metabolic pathways towards the production of new products, recode the genome to implement safety-by-design, or to engineer the lifestyle of the micro-organism to be better suited for industrial applications. During the build phase, the design is made by engineering the genome *in vitro*. During the test-phase, specifically designed control experiments are performed to determine the effectiveness of the design. All the information that is gained from these experiments is fed back into the database used for the design, either to improve it in case the results where not as expected, or to fuel new research projects.

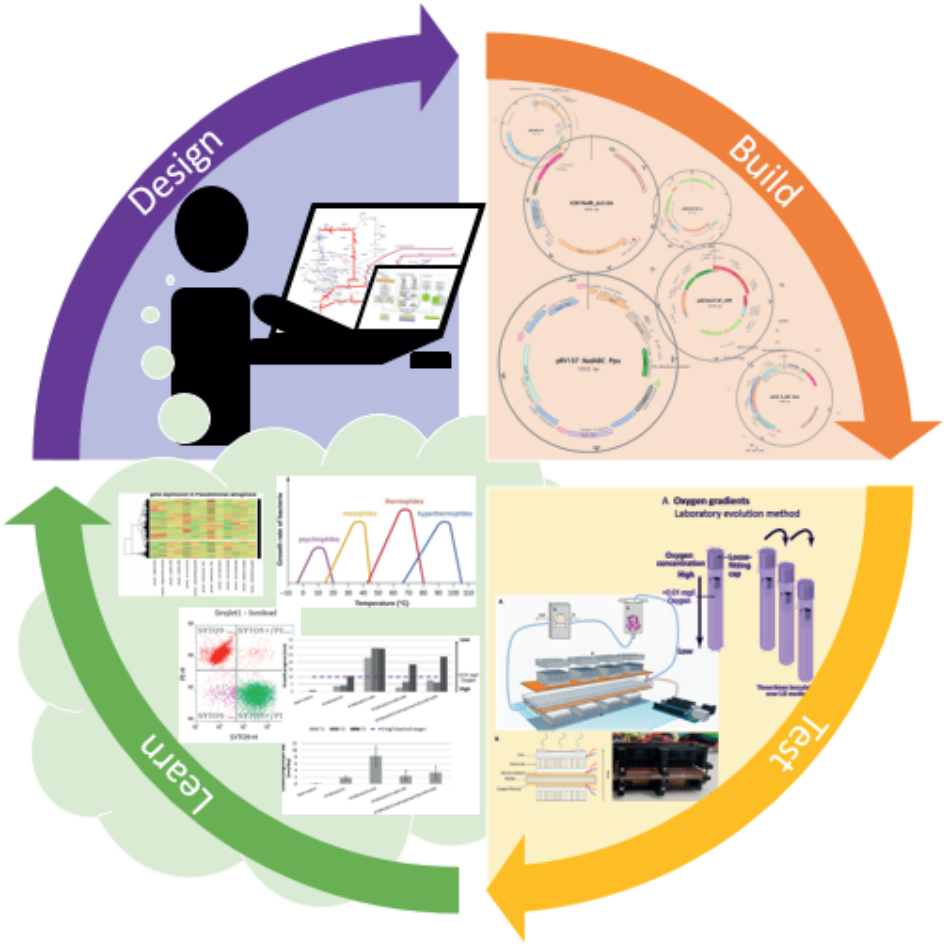


Figure 1.3: The Design-Build-Test-Learn Cycle.

In summary, microbes are of growing importance in industry. However, not all strains can equally well withstand the changes in environment that occur within large bioreactors, lacking the robustness to function across a wide range of conditions with little change in phenotype or fitness. New developments in biotechnology and synthetic biology have made it possible to improve microbes for industrial use, making their application towards sustainable industry more feasible. Classical methods such as strain evolution can be sped up by fusing them with an ordered approach, enabled by the wealth of genomic data available.

Thesis Aim and Outline

The aim of this thesis is to increase the environmental robustness of the industrially applied *Pseudomonas putida* KT2440 by applying an ordered approach and lifestyle engineering towards more affordable sustainable industry. The DBTL-cycle will be employed towards an ordered approach to tackle the main disadvantages for industrial applications: the oxygen requirements and its temperature tolerance.

In order to gain insight into the microbial living environment, information on the habitual restrictions in pH range and optimum, temperature range and optimum and oxygen requirements of 2502 bacterial strains was obtained via the Gold database [119]. Using MATLAB 2018b (The MathWorks, Natick, MA, USA), this data was translated into an overview of bacterial living environments per oxygen requirement (Supplementary data 1 and 2, Figure 1.4).

Looking at bacterial habitable environments based on temperature and pH per oxygen requirement group, it becomes apparent that temperature and oxygen requirements are often linked. The habitable pH range amongst bacteria does not vary much and does not seem influenced by the other factors. Among the 57 obligate anaerobic species and the 478 anaerobic, significantly more are thermophilic. Similar but less extreme thermophilic outliers are seen in the facultative aerobic species. The relative increase in anaerobic species at higher temperatures makes sense, since heat can limit oxygen availability in liquids. Overall, the aerobic, microaerobic and obligate aerobic species are more clustered.

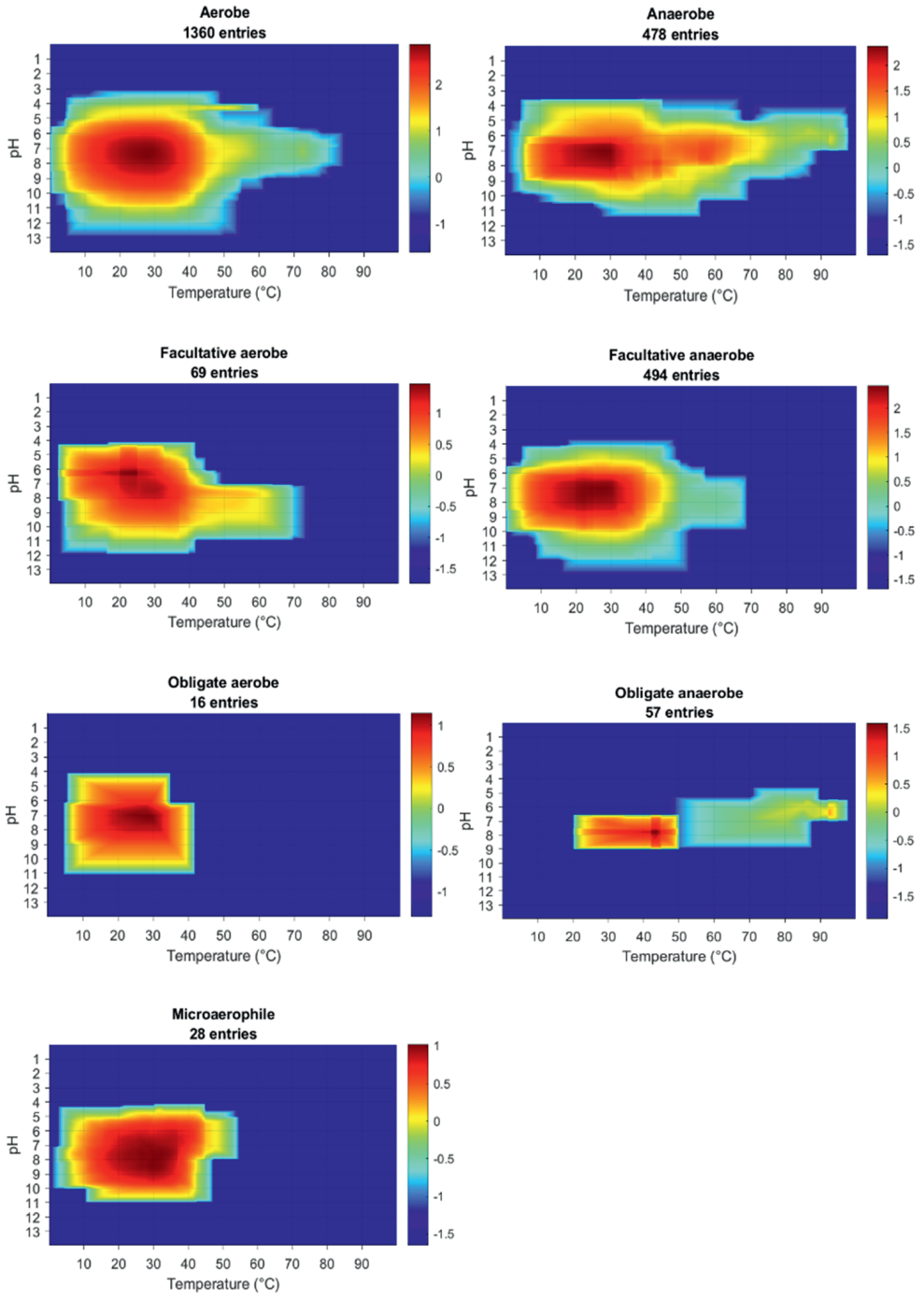


Figure 1.4: Bacterial living environments based on their oxygen requirements, pH range and optimum and temperature range and optimum. Colour intensity indicates bacterial density on a log scale. Per plot, the number of entries is indicated, representing the number of bacterial species listed under these oxygen requirements were used. Acknowledgements for figure: K.J.A. Martens and J.J. Koehorst.

In this thesis, a structured approach was applied by first determining which microbial improvements industry is looking for (**Chapter 2**). To this purpose, a series of interviews was conducted with companies from two different continents. Besides pinpointing the fields of interest from an industrial perspective, the interviews also clarified the limitations of the actual implementation of novel or (synthetically) adapted strains developed. Since strain safety was determined highly important, the claimed GRAS safety level of *P. putida* KT2440 was critically reviewed (**Chapter 3**).

A main interest in industry is increasing strain robustness. A major obstacle for the breakthrough of *P. putida* KT2440 as biotechnological host is its obligate aerobic metabolism. The *in silico*-directed strain improvement was initiated by the adaptation of strict aerobic *P. putida* KT2440 to micro-oxic and anoxic conditions. Adaptation to micro-oxic levels was done by first creating an *in silico*-guided design for a recombinant *P. putida* KT2440 strain capable of anaerobic fermentation. The bottlenecks uncovered were resolved by insertion of three genes via a plasmid, and the recombinant strains were monitored through an adaptive laboratory evolution method with oxygen gradients set up specifically for this purpose (**Chapter 4**). Performance of the resulting adapted strains were tested under anoxic conditions, but showed no improvement compared to a negative control strain. A more elaborate *in silico* analysis was then performed, combining protein domain analysis, transcriptomic analysis and genome-scale metabolic models to design a recombinant *P. putida* KT2440 strain capable of anaerobic respiration (**Chapter 5**).

Another way to increase strain robustness is tackling their thermo-tolerance: the temperature range at which an organism can grow. A strong connection between NAD^+ availability and thermo-tolerance was discovered. Replacement of one single gene for that of a thermophilic heterolog in mesophilic prokaryotes *P. putida* and *E. coli* showed improved thermo-tolerance. Insertion of the aspartate NAD^+ biogeneration pathway in eukaryotic yeast *S. cerevisiae* resulted in a similar effect (**Chapter 6**). This discovery was patented (**Appendix chapter 6**) To determine the effect of this on an industrial scale, a down-scaled millifluidics system was developed to mimic temperature fluctuations that occur in large scale bioreactors (**Chapter 7**).

Finally, in **Chapter 8** the different applications of microbial lifestyle engineering for industrial applications are discussed, and a future outlook is presented.

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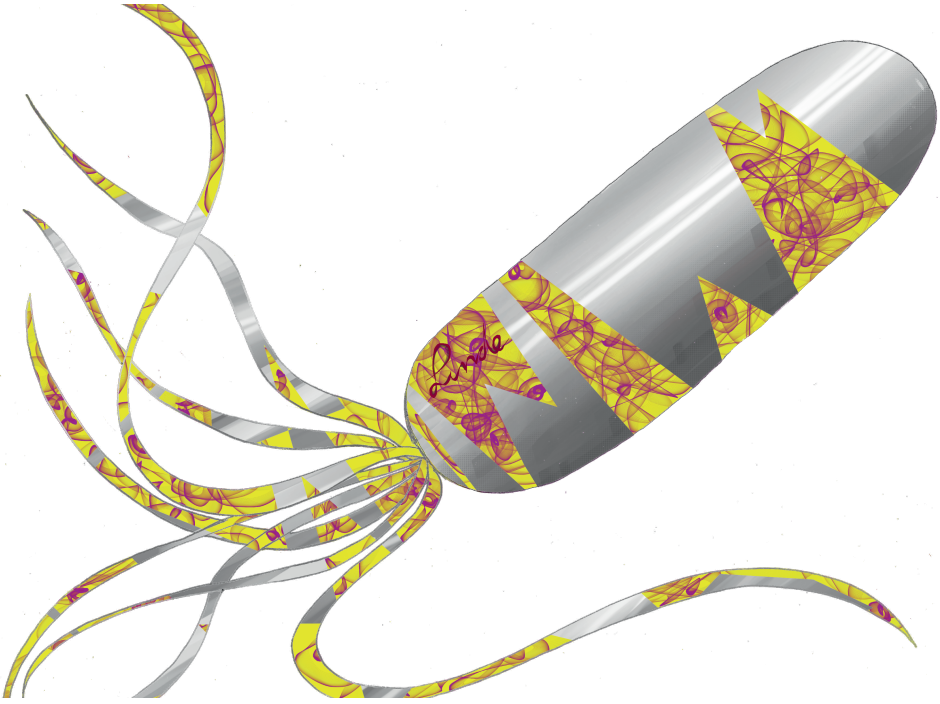
2

Exploring through the Valley of Death: Perceptions of Industry and Academia on Production Platform and Opportunities



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Abstract

Rational lifestyle engineering using computational methods and synthetic biology has made it possible to genetically improve industrial performance of microbial cell factories for the production of a range of biobased chemicals. However, only an estimated one in 5,000 to 10,000 innovations make it through the Valley of Death to market implementation.

Because of a perceived lack of systemic exploration, a qualitative and exploratory study was performed to gain in-depth insights into the views of industry and academia by conducting 12 interviews with 8 industrial participants and 4 academic participants. The characteristics that any cell factory must have were schematically listed, and commonly recognised opportunities were identified.

Industry is generally restricted in their research choices and flexibility by a series of technical, sector dependent and social factors, which often do not have to be taken into consideration in academia. Although it was found that academia must leave room to perform fundamental, curiosity driven research for innovative solutions that cannot be implemented in industry directly, if an academic research project is aimed for industrial applications, the end-user must be held in mind constantly to bridge the Valley of Death.

Introduction

The demand for industrial production by microorganisms is ever increasing. In 1990, the global market value of industrial enzymes was close to a billion USD, crossed the two billion USD mark in 2005 [1], was valued at over four and a half billion USD in 2016 and is expected to reach over six billion USD in 2022 [2]. Nowadays, bacteria, yeasts, fungi, and micro-algae are used in the industrial production processes of food, enzymes, vitamins, pharmaceuticals, biofuels, bioplastics, bio-insecticides, nanocomposites for electronic devices and a large variety of chemicals and enzymes with industrial value. The reasons to widely apply a diverse set of microorganisms are manifold; they represent a broad biochemical diversity, increase the feasibility of large scale production, can produce sustainable products, reduce processing time, require low energy input, increase cost effectiveness and can be selected for non-toxicity [3–12]. However, as heavily as the current biotechnology industry depends on production by microbes, production methods are not by far as stable as desired due to the biological variability of microorganisms [13]. Many external factors affect the production capacity of microorganisms, including temperature, pH, oxygen, ion and carbon source availability, cell density, and biofilm formation [14–23]. All factors combined, this microbial instability results in a high variability in yield.

For industrial production purposes it would be ideal to select only one microorganism as the general production platform. By focusing research on a single species the microbial toolkit would increase to improve stability in production and production costs would decrease as more universal bioreactor set-ups could be used. To be considered perfect, such an industrial microbial workhorse would have to check many boxes: it must be safe to work with (preferably generally regarded as safe, or GRAS), be genomically accessible, metabolically flexible, resistant to external industrial stress factors, grow on cheap medium, and be able to produce à la carte a high variety of products with high and stable yields. The field of systems and synthetic biology offers potential solutions for all of these requirements. By restructuring the genome and thus the metabolic pathways of microorganisms, microbes found in nature can be theoretically perfected to produce any à la carte product of

interest in a safe way. Where random evolution and selection are common methods to increase productivity, yield or performance of a species, *in silico* systems and *in vivo* synthetic biology is offering all tools to not only adapt microorganisms to gain novel characteristics in a directed way, but to create them.

Examples of research conducted in academia to improve microbial workhorses include the process of making the industrially applied *Escherichia coli* more resilient to temperature fluctuations [24]. Sandberg and colleagues exposed *E. coli* K-12 MG1655, which has an optimal growth temperature at 37°C, to adaptive laboratory evolution to improve strain performance at 42°C. Another example is the lifestyle adaptation of the industrially applied *Pseudomonas putida*, which was enriched with three heterologous genes and exposed to micro-oxic conditions through oxygen gradients [19]. So far, neither of these examples are actually applied in industry.

This large gap for academic research to advance from the technological demonstration to actual commercialization in industry is generally referred to as the "Valley of Death" [26]. In life sciences, including pharmaceuticals, biotechnology and therapeutic medical device development, it was estimated that only one in 5,000 to 10,000 innovations survive the long route from development to product commercialization [27–29]. There exists a large gap between results from academic research and industrial product development. The different levels of product development are known as **t**echnology **r**eadiness **l**evels, or TRLs (Table 2.1). This nine level system was instituted by NASA in the 1980s [25]. Academic research generally only covers TRL 1 to 3. At TRL 4 to 7, the discovery is considered too applied for scientific funding, while the private sector considers it too high risk to fund for market implementation. Industry generally covers TRL 8 and 9.

Table 2.1: Technology Readiness Levels (TRLs) as instated by NASA [25].

TRL	**Definition**
1	Basic principles observed and reported
2	Technology concept and/or application formulated
3	Analytical and experimental critical function and/or characteristic proof-of-concept
4	Component validation in laboratory environment
5	Component validation in relevant environment
6	System/subsystem model or prototype demonstration in relevant environment
7	System prototype demonstration in relevant environment
8	Actual system completed and qualified through test and demonstration
9	Actual system proven through successful operation

Reasons for new technology to not survive the Valley of Death include cumber-

some contracting or procurement of technology requirements, lack of exposure, lack of entrepreneurial management, lack of adequate funding for further development, and the lack of a strong link between technology development efforts and industrial deployment [27–29]. In an effort to decrease the amount of research that does not bridge the Valley of Death, funding committees now demand a direct collaboration between academia with industrial partners. Still, the Valley of Death remains as large as ever.

We approached multiple industrial and academic experts to discover their perceptions on how to improve chances of research surviving the Valley of Death. We aim to elucidate I) what factors influence the choice for an industrial micro-organism and II) opportunities between academia and industry.

Methods

Due to a perceived lack of systemic exploration of the factors that influence if novel research makes it through the Valley of Death to market implementation, a qualitative and exploratory study was performed to gain a comprehensive insight into the perception of industry and academia by conducting interviews.

Recruitment and Procedure

Recruitment took place by gathering a convenience sample based on our direct network and visited conferences. A list was devised of possible participants, from which 15 companies were selected based on their location, Europe or America, and field of research, pharmaceuticals, food, industrial chemicals, or production organism development. Some companies selected cover a combination of these fields. A variety of large and small and new and established companies were selected carefully from each continent, to determine the effect of a different regulation in industrial applications of bacteria and GMOs in particular, and over different companies from different sectors. Chief executive officers or chief technical officers were mainly approached to ensure the most overhead view on proceedings and the clearest perspective on the general aim, flexibility and workings of the company. In three cases, we were redirected to someone else within the company. Of the fifteen companies

approached, nine agreed to participate, five did not answer, and one declined participation citing confidentiality over proprietary information. Of the nine companies that agreed, one decided to withdraw from participating as they could not obtain consent from their legal department after receiving the transcripts.

Of the convenience sample, 4 European universities were selected based on their diverting expertise, similarly to companies. All universities approached agreed to participate. Three of the academic participants had present or past experience in working at a company. All participants were engaged in collaborations with multiple industrial sectors. The distribution of companies over continent and field are shown in Table 2.2.

Table 2.2: Participants in the interviews: the field occupied by participating companies and academics, and location.

		Pharmaceuticals	Food	Chemicals	Development of production platforms	Combination	Total
Industry	Europe	1	1	1	1	1	5
	USA	1			1	1	3
Academia	Europe			1	1	2	4

Potential participants were approached up to three times via email to make an appointment. If possible, interviews were conducted in person, otherwise face-to-face through Skype, WebEx or Bluejeans, according to the participants preference. During one interview, the camera of the participant failed and the interview was done by voice only. Both through e-mail and at the start of each interview the aim and methodology of the research was explained to the participants. Before the start of each interview, participants were presented with written information and contact details, and were asked to sign a declaration of consent. Interviews were conducted over a period of 8 months, from April to November 2019. After 8 interviews, the content of answers started to show overlap with previous interviews indicating data saturation.

Interviews

Based on literature and experience [19, 30–34], a topic list was constructed to guide the interviews:

1. **Choice of production platform** was addressed by questions such as which production organism is used now, what influences the choice for a production organism, including limitations or possible restrictions, company and set-up flexibility and what a production organism would need for the participating companies to switch.

2. **Opportunities between industry and academia** was addressed by questions including common ground, differences, challenges, opportunities, interesting fields of research, impact of science in industry, and collaboration. This topic included the presentation of a simplified overview of industrial production from the Design-Build-Test-Learn cycle [35] via laboratory scale to industrial production scale, where participants could indicate the main bottlenecks which indirectly represent the main short-term research opportunities for academia (Figure2.1). The presented figure was used to stimulate in-depth discussion on challenges and opportunities.

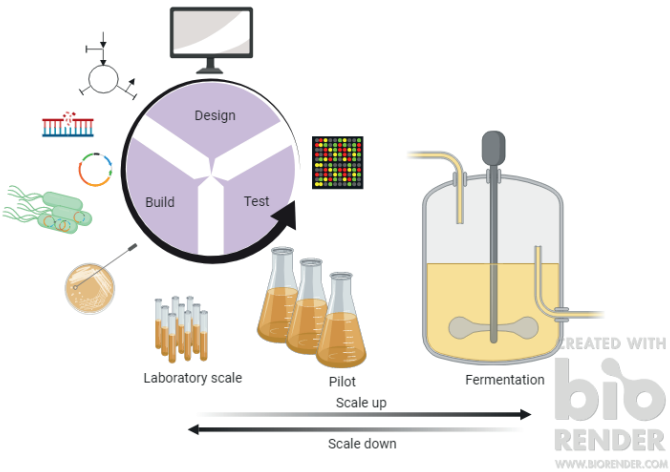

Figure 2.1: Overview of industrial development and production process. The schematic covers the main steps between the DBT-cycle and the industrial production scale.

Prior to data collection, the interview questions were discussed with an expert working in academia and working closely with industry, from which adjustments were made. Adjustments were focused on making the questions more open-ended and general to prevent bias and allow for a more in-depth discussion by leaving room for follow up questions (form S2). The use of appreciative inquiry in the questions [36] encouraged and inspired the participants to answer according to their own perspectives, ideas and experiences, as opposed to following a strict interview structure. In practice, this means that questions are asked with a positive spin, to focus more on solutions rather than on problems. A pilot interview with an expert working both in industry and academia was then held, indicating that the qualitative face-to-face interviews offered the expected in-depth information and clarity for both the participant and the interviewer, as it allowed for elaboration on specific topics, insight into technical differences or company aims. Special care was taken during the interviews to make sure there is a consensus of participants on definitions and terms used, by asking follow-up questions. Each interview was conducted by two members of the research team. The 13 interviews lasted on average 41 minutes, ranging from 26 to 54 minutes. With the participants' verbal and written consent, all interviews were audio-taped and transcribed (intelligent verbatim style). The data was pseudonymized. When necessary, audio files were used to confirm transcripts and listen to excerpts within their original context. Transcripts and specific quotes used from each transcript were shared with the participants to consent upon prior to analysis. All participants responded, and minor editorial changes were made.

Results

Data was processed using the six steps for qualitative data analysis [37]. All analysis was done by the two interviewers, LK and EAG. In the first step, transcripts were made and carefully read. In the second step, codes were formulated manually using the research questions. Thirdly, the transcripts were coded using the formulated top-down codes, and where necessary deriving new codes from the data. The processing of the transcripts was conducted via inductive category formation using QCAmap [38], according to a thematic content analysis [37, 39]. The interview-

ers discussed their respective individual top-down and bottom-up codes to define a final set of codes. In the fourth step, all codes were clustered into the research questions: A) Choice of Production Platform, and B) Opportunities between Industry and Academia. In step five and six, recurring themes found to affect multiple codes were derived from the data and defined carefully. The nine themes found affect either one or both research questions, and relate to the aims of industry and academia. To support the themes found, direct quotes are provided in the result section, selected to represent participants' opinions, views and experiences. An overview of the clusters, codes and derived themes can be found in Table 2.3.

Table 2.3: Themes, research questions and codes of transcriptions. Top-down codes were formed before coding, bottom-up codes were derived during transcription.

Themes	Top-down codes	Bottom-up codes
Research Question 1: Choice of production platform		
• I) Characteristics of microbial production platform • II) Industrial process and set-up flexibility • III) Regulations • IV) Public perception	• What production platform is used • Characteristics of used production platform • Flexibility on industrial set-up	• Limitations on choice of production platform • External influences on choice of production platform
Research Question 2: Opportunities between industry and academia		
• V) Genome engineering • VI) Scale-up • VII) Increase strain robustness	• Opportunities and developments • Distance between industry and academia • Impact of industry on academia	• Personal or company aim • Influence of industrial experiences
The aims of industry and academia		
• VIII) Curiosity-driven versus solution-based research • IX) The rise of small companies	• What production platform is used • Characteristics of used production platform • Flexibility on industrial set-up • Opportunities and developments • Distance between industry and academia • Impact of industry on academia	• Limitations on choice of production platform • External influences on choice of production platform • Personal or company aim • Influence of industrial experiences • Communication

(A) Choice of production platform

Four main themes affect the choice for a certain production organism: I) Characteristics of microbial production platform, II) Industrial process and set-up flexibility, III) Regulations, and IV) Public perception (Table 2.4).

Table 2.4: Factors affecting the choice of production platform with representational quotes from participants from industry (I) or academia (A), indicated by their numerical identifier.

Factor	Representative Quote	Source
I. Characteristics of microbial production platform		
Titre-rate-yield	"The micro-organism itself is not so relevant, the whole picture should be okay. Generally speaking, you can say that for any fermentation process you need to have a good titre, a good rate and a good yield, so the famous try-numbers, titre-rate-yield."	I.2
Toolbox availability	"I think trying to predict and overcome toxicity problems of given molecules to given organisms is very difficult. Trying to adapt one organism to secrete something where another does it more naturally is very difficult. I don't see why you would do it, I think it is a better strategy to develop the broadest range of tools for different organisms including extremophiles."	I.5
	"Genetic accessibility, wealth of available data, free available data"	A.6
Genomic Accessibility	"If a host is genetically accessible we can start to metabolically engineer it from day one, that is preferred to do six or nine months of method development to even get DNA inside the cell."	I.12
	"[The main advantage] is that we don't have to worry about the genetic tools"	A.11
Product of interest	"The higher the cell complexity is, the more advanced the product should be. This statement somehow mimics necessary R&D expenses that need to be covered by cost calculations."	A.9
	"These microbes have not been evolved to make a 100 grams per liter of compounds A or B, so we typically start with a selection where we screen different hosts for tolerance towards the products."	I.12
Expertise available	"Everybody needs to be trained on the new host, and please don't forget that as soon as you have transferred something to a production line, there will be lots of questions coming up in the aftermath. For instance, downstream processing matters need to be checked and must fulfill given constraints, too. That requires experts who care for those questions."	I.9
Production of unwanted side-products	"We produce flavour and fragrance ingredients (...) they should smell and taste in the right way afterwards, after purification, so what we have to avoid is off odours."	I.3
Fundamental interests	"There is this fundamental interest in understanding how new insights, new activities in new networks emerged under natural conditions because if we are able to domesticate the corresponding mechanisms then we should be able to reproduce them in the laboratory and make them work much faster and much more efficiently. The basic questions that we are interested in are not limited to [*micro – organism*], it just happens that [*micro – organism*] is a very good experimental system to address them."	A.10

Factor	Representative Quote	Source
II. Industrial process and set-up flexibility		
Investment requirements	"It depends on the numbers, and on the cost, but changes in bioreactor setup of course mean that you have to either build or find another bioreactor."	I.3
Choice of industrial setup	"[The producer] might want a semi-continuous process. The cell may have high productivity but it dies after a week; they will swap that out to something that has a lower productivity but can last for three weeks in a continuous or semi-continuous culture. It's all about space-time yield for low value products."	I.5
Downstream processing	"There is also the downstream processing part. In essence, you need to separate your product equally simple from the broth as you can do with established hosts."	I.9
Complete industrial process	"If your fermentation is going too fast, faster than the downstream can handle, you're creating a bottleneck."	I.2
Stage of development	"[Producers] will like small changes where they don't have to invest in new capital (...) but if it's a process that's not been implemented at pilot scale then they're more likely to make a radical change as long as it's consistent with their engineers' understanding of what the eventual process will look like."	I.5
III. Regulations		
GMO regulations	"If you're heading for products of food, feed, flavour or fragrance markets, you may face severe opposition regarding the use of recombinant strains, per se."	I.9
Safety regulations	"All proteins that are produced for food and feed, they have to apply to EFSA or FDA regulations, and the more similar you can do a process, the more easy it is to get permission."	I.4
IV. Public Perception		
Social acceptability	"Sometimes the social acceptability of it as well. I mean you might find that *Klebsiella* or *Salmonella* would be good hosts to make something and you could use perfectly viable lab strains. But they might prefer *Lactobacillus* because it's a food grade organism and public perception ultimately might be very different."	I.5
Halal or Kosher	"It's not just GMO regulations, it's not just FDA and what have you, it's also these kinds of considerations [like halal or kosher] that must be taken into account. So you really have to think through all the consequences if you want to introduce a new microbe in your process and make other products."	I.2

The factors that influence the choice of production platform in industry and academia can thus be divided into technical factors, like the characteristics of the production platform and industrial process and set-up flexibility, sector-specific factors like regulations, and social factors like public perception. In industry there are many technical, sector-based and social restrictions to choosing a production host. As they must keep the end-user in mind throughout the entire development and production process, products must be made as cost-effective, fast, green and safe as possible.

For academia, mostly technical factors play a role when deciding to work with a specific micro-organism. These boil down to toolbox availability, genomic acces-

sibility, product of interest and fundamental interests. Only the latter is specific to academia, the rest is shared with industry. This means that academics are more free to work with any micro-organism, even with microbes that cannot be applied (yet) in industrial processes.

(B) Opportunities between Academia and Industry

To determine what the main future opportunities are, participants were asked for future research of interest, bottlenecks and what scientific progress they expected to have most impact (Figure 2.2).

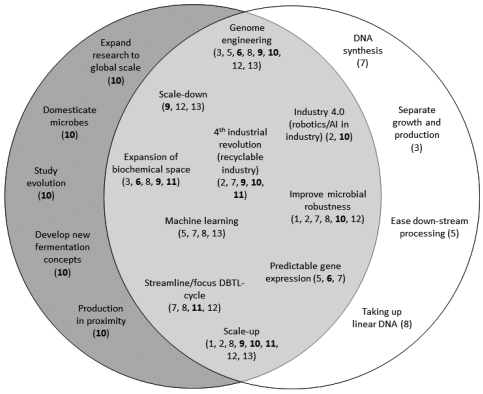

Figure 2.2: Venn diagram of research areas of interest per field. A schematic overview of the research areas indicated as interesting by academia (left) and industry (right). Overlapping research of interest is in the middle. Per subject, the identifier of each participant is indicated. Identifiers of academic collaborators are printed in bold.

From this overview, three main technical themes could be deduced: V) Genome engineering, VI) Scale-up, and VII) Improve robustness. These will be discussed in more detail.

V) Genome engineering

When presented with Figure 2.1, most industrial participants identified the whole or part of the DBTL-cycle as the main opportunity for improvement.

> "You test [a new design], you find something out and then you have to design, you get a lot of data, you get some results, you don't understand them, and you have to analyse. The learn part is the bottleneck because all the other things are automatable or scalable to some extent, so the learning bit is not." *Interviewee 3*

For industry, the possibility to affordably construct an entire genome combined with the rise of novel tools such as CRISPR-Cas for genome engineering substantially ease this bottleneck.

> "On the build side [of the DBTL-cycle] there's a lot of change. Synthetic DNA is becoming cheaper and cheaper by the day, sequencing is becoming cheaper by the day, CRISPR is like 6 years old and [recently] there was a new version released that really put the whole town plus all of the genetic engineering upside down. [...] It's just changing the timelines and the ability so much." *Interviewee 12*

For academia, such tools pose promising ways to improve upon fundamental research, which could eventually be required for understanding strain design.

> "I would hope that it will accelerate the way at which we can generate industrially meaningful strains, and it will expand the extent of manipulation that we can achieve. So rather than looking at one gene or one pathway, we should hopefully be able to look at one entire section of metabolism in one go, and this would hopefully have a very good influence on the strain design that we want to achieve." *Interviewee 6*

VI) Scale-up

To reliably scale-up results obtained in perfect lab-conditions to industrial scale is challenging at best. Scaling-up was recognised as the second main bottleneck. Even in academia, the importance of reliable scale-up to bridge the Valley of Death was recognised:

> "You can develop the most sophisticated CRISPR system, the most sophisticated recombineering, the most sophisticated reactions, you can do wonderful things in the lab: if you cannot scale them up, then industry will not be interested, period." *Interviewee 10*

From an industrial point of view, there are few different reasons why scale-up can be a bottleneck, besides technical difficulties:

> "The availability of equipment is actually the whole bottleneck. There is not a lot of pilot facilities in the US, so either you build your own or you go to Europe for piloting. That's non-trivial. I think there's a lot of syn-bio companies that are mostly comprised of genetic engineers or metabolic engineers that have never seen a large-scale fermentor and a large scale production process including purification. So if you start a scale-up trajectory without people that know what they're doing then it's going to be a bottleneck. But if you have a team, [...] then you should be able to do that in a pretty smooth trajectory. There's always going to be surprises and hiccups. What I've also seen is that lots of syn-bio companies have very aggressive timelines, probably due to their investors pushing really hard, and then they are going to take short-cuts and that's going to blow up in your face. There is not a technical bottleneck, but it's more of a managerial bottleneck at that point." *Interviewee 12*

VII) Improve strain robustness

When asked directly, all participants indicated that the robustness of their used or preferred production platform could be improved. Reasons for improving robust-

ness circle back to scale-up without fail, since industrially used production organisms are grown in conditions that are strictly controlled.

> "Robustness in an industrial sense is that it is operationally robust: meaning it is not very sensitive to infections, it is not very sensitive to minor variations that you always have in the conditions of the process, e.g. due to scale. So there should be some tolerance in the microbes to slight variations in the process conditions, which of course we try to control that as much as possible." *Interviewee 2*

In the few cases that robustness was not recognised as an issue, it was solely because the production platform of interest already performed optimally at large scale:

> "We experience our micro-organism is very robust under the conditions that are relevant to us, that is industrial fermentors, so we don't see a need for further improvement there." *Interviewee 3*

Robustness is thus only deemed important to aid production platforms to perform reliably on industrial scale. Even in academia, this link was made.

> "Fluctuations on large scale are much more prominent then on small scale, and constant performance through larger fluctuations in large scale most probably could be improved." *Interviewee 6*

Overall, the long- and short-term interests of academia and industry align very well. However, there were some differences in interest. In industry, interests were often very practical, all leading to an increase in titre-rate-yield. Subjects included the use of a different C-source, separated growth- and production cycles, and ease down-stream processing. Amongst academia, topics of interest that did not align with those in industry were less aimed at application and practicality, but of a more fundamental or futuristic nature. These included for example the development of new fermentation concepts, expanding of research to global scale and studying evolution.

The aims of industry and academia

Two themes where found vital in this difference in aim, and as such influence both research questions: VIII) Curiosity-driven versus solution-based research, and IX) the rise of small companies.

VIII) Curiosity-driven versus solution-based research

The precarious balance between curiosity-driven research and solution-based research, the latter mainly with industrial application in mind or on stakeholders' demand, can be defined most clearly by looking at the collaborations between academia and industry. All participants, both from industry and academia, indicated current involvement in projects of collaboration. However, these collaborations are not necessarily initiated by industry nor based on industrial interests. By academics, it was more described as follows:

> "It's rarely the case that somebody [from industry] comes to me and says "*This is something that you should work on*", but of course you talk to people at conferences, at seminars, and you try to understand what the problems are, and you try to find out if you can solve some of these problems with the tools that you have available. But it's a bit of a balance, I try to make sure that a substantial part of our activities does not have a direct line. In a way, it's curiosity driven." *Interviewee 6*

Collaborations between academia and industry are not based on a demand-supply model, but rather on academics trying to fill existing or predicted gaps in industry. All academic partners did indicate a strong influence on their work from industry, even if not direct. However, a way to deal with this potential struggle was explained:

> "We are driven by curiosity and obviously we have to frame our curiosity and our personal interest in the interest of our sponsors, and in the interest of society, and this is perfectly fine. Just remember that the big artist in the Renaissance were sponsored by the popes, by the emperors and so, and [the artists] had to produce things that [the sponsors] liked.

> I think this is not completely shocking that we are constrained by the ideas of our sponsors. I think that our challenge [...] has a point even of enjoyment. It is how you connect your own personal interest and our own curiosity with these big demands that your sponsors have for your research." *Interviewee 10*

Industry thus does not approach academia with questions, but rather shops around at conferences for interesting opportunities they can easily adapt and apply. This indicates that they rely heavily on research performed in academia to make large strides in innovation.

Industry indicates this when finding new investment opportunities:

> "The biggest opportunities lie in the combination for a company like us, or a hospital research group for example, that have developed something novel, something proprietary, but [they, academia] have no understanding of the hurdles they'll face or the means to demonstrate its viability to be a competitive product in the market. Because that's what's going to be necessary to attract investment. To give it a chance of survival." *Interviewee 5*

The clear distinction between technical, sector-driven and social factors influencing main research choices and definition of opportunities can be derived from the difference in the main aim of each field. Where industry looks for applications and adaptable solutions to day-to-day problems, academics aim to discover answers to fundamental questions without being limited by end-user demands. Communication to align these different aims towards one goal is vital:

> "First of all, there has to be good communication, because academia works in a completely different way to industry. Industry is all about timelines, deadlines, deliverables, project management, progress towards measurable output. Academia is about testing, discovery, interesting products, interesting processes, new methods, means of discovering where science can go." *Interviewee 5*

IX) The rise of small companies

Small companies that specialise in commercialising academic findings are called start-ups. Start-ups do not only focus on technical factors, but work on the sector-based and social factors that might influence the successful industrial introduction of their product as well.

Some see start-ups as a natural way to close the gap between academia and industry, by presenting interesting research ready for commercialisation on a silver platter:

> "It's those partnerships in between, it's the networking and the identification of viable product opportunities that are emerging from either academic groups, hospital groups, virtual companies and then the partnerships that takes that feasibility demonstration onto viability. That's where the opportunity is. Bridging that gap from feasibility to viability. Because that's what derisks it, and brings in funding to take it forward. I think otherwise so many things can sit in academia, very interesting, very exciting, and probably more so in Europe than in the United States, and they just don't see the light of day because they haven't taken the next step and that's where certainly a company like ours, but other small companies can come in and make that difference." *Interviewee 5*

Others indicate that the rise of start-ups widens the gap between academia and industry. However, they do understand why companies prefer dealing with companies:

> "What we observe is that all these new developments, new technologies and everything, is mostly the business of small companies. The big companies just become very conservative. [...] Big companies prefer to deal with smaller companies than dealing directly with scientists, and the reason [...] is that scientists have too big a mouth. That means that the moment you have discovered something interesting you immediately start spreading the word, and this is something that the companies don't like at all." *Interviewee 10*

Discussion

The importance of technical, sector-based and social factors

The importance of three factors, technical, sector-based and social, can be found when considering currently applied industrial microorganisms. Current host systems widely applied include bacteria, yeasts, filamentous fungi and unicellular algae. Industrially applied strains all come with their own strengths and drawbacks [5, 40, 41]. Technical factors for this are the ability to produce complex chemicals, growth speed, the ability to perform post-translational modifications and the use of cheap medium components. Sector-based factors include the used micro-organism to be a natural producer of the product of interest, the fermentation and production process to fit within the complete industrial process, or familiarity with working with a specific strain from out the company history. Social factors for the wide application of these production hosts include public familiarity and the GRAS status of a species.

Academic participants only cited technical reasons for the selection of a microorganism such as ease to work with, genomic accessibility and wealth of publicly available data. In industry, sector-based factors such as product of interest, and social influences derived from demands of the end-user or regulations applied by the government also strongly influence choices made. Still, the main research opportunities and future prospects found are of a technical nature. Genome engineering opportunities, scale-up, and increased strain robustness all focus on improving the production process from a technical point of view. Mismatches leading to an innovation not making it through the Valley of Death thus may occur when the social and sector-based aspects are not kept in mind.

Changing the used production organisms in an established industrial process is considered a rigorous industrial shift, depending heavily on research. Adapting an existing production process is expensive, requires specific scientific knowledge and takes time to develop and test. When changing production platform affects the industrial setup, the gain in yield and characteristics has to be phenomenal to make up for the money of the investment. Additionally, microbes with possibly more interesting traits to better produce specific substances are being discovered every

day. It is thus not surprising that there is a lot of research focused not on finding the perfect production host for specific purposes, but on the *de novo* design and construct of one. This is either done by making a minimal cell, only including traits specifically selected as required for survival, or by adapting an existing production microbial platform.

Computational methods are paving the way towards big data processing towards one goal of interest, in any available species. However, implementation and testing of many different designs through the DBTL cycle and predictability of gene expression were identified as the bottlenecks towards progress, by both industrial and academic participants. This offers a great research opportunity for academia.

External influences on the connection between academia and industry

The main differences arise from the difference in perspective between academia and industry. Where academia focuses on solving one problem at a time, industry always keeps the bigger picture in mind. At the same time, academia is encouraged to perform curiosity driven research, answering fundamental questions. Where industry works on solving the problems of today, academia focuses on the problems of tomorrow. In spite of this difference in aim, both parties need each other for funding, direction, creativity, innovation and exploring boundaries. Both parties also agree that communication could be improved to improve cooperation and increase opportunities for process or product development and introduction to the market.

These differences found between the general aims, way of thinking and factors included in decision-making between academia and industry can be largely brought back to the different technology readiness levels (TRLs) they operate on [25]. As earlier indicated, academia works at TRL 1-3 while industry focuses on TRL 8-9. The enormous gap left from TRL 4-7 is the most high-risk phase of new product development.

To close this gap, a solution may have naturally emerged. In between established industry and academia, a third party can be distinguished: small start-up companies. Rather than direct adaptation of academic research in industry, new companies emerge straight out of academia, which are acquired by large companies when proving particularly promising. Closely linked to both industry and academia, the rules

in start-up companies appear to be different. In short, they are set up specifically to bridge TRL 4-7. Small companies are more willing to take risks, and easier change their production platform. The continuous process of using novel advanced technologies to form a firm has moved to the heart of innovation strategy [42]. Start-up initiatives focusing on the technical, sector-based and social factors by presenting the best, most lucrative results achieved in academia on a silver platter, attracting their own initial funding and stirring commercial interest. This trend is currently seen throughout many different sectors, with the well-known example of the once-startup multinational IT company Facebook acquiring multiple startups [43].

Government funded science indicated a first academic revolution. Entrepreneurial science, where collaborations were formed with industry, indicated the second academic revolution [44]. Nowadays, government funded research often even requires collaborations with industrial partners, as seen in European projects such as EmPowerPutida, P4SB or IBISBA [45, 46]. The swift rise of start-ups has chimed in a third academic revolution, where patenting novel discoveries will grow in importance. This has impacted the role of academia. All academic and some industrial participants have indicated a growing distance between academia and industry, impacting the communication and collaborations. Some have indicated this growing distance to be worrisome; start-up companies present commercialized techniques that can be quickly integrated in any company, masking the years of university driven research and development that occurred before forming the start-up.

However, the natural rise of start-ups should be seen as an opportunity. Start-ups can ease the transition of academic research to actual industrial application, closing the Valley of Death by forming an independent bridge between the academics and industry. It also opens up room for academics to focus on what they do best: education and research. It was earlier recognised [47] that having opportunities in academia to translate their research effectively into concrete products benefits academic institutions, faculty members, industry, and society [44]. A case could be made for collaborations to include some attention for the possibility of development of start-ups, which might lead to more attention to promising results and how to grow them into market applications from an early point on.

Strengths and limitations

As after approximately 8 interviews data saturation occurred, where no new information was introduced, it indicates a good reflection of the community. The large spread of different companies in different biotechnology sectors, of different sizes and spread over different locations around the globe have served to generalise the community adequately so the universal bottlenecks, challenges and opportunities could be easily recognised. However, another interesting approach to this research could be in the subtle differences from companies in different continents. A larger sample would be more reflective of the continental community. Companies and research facilities from other continents could add to this. Additionally, only European academic instances were included in the research.

Conclusions

Within biotechnology, production platform development is a main branch of research. Existing production platforms are improved, or new ones are developed. From an academic point of view, accessibility and ease to work with are the main reasons to choose for one micro-organism. For industry, technical factors such as the characteristics, industrial process and set-up flexibility, sector-based factors such as regulations and social factors such as public perception are important influences. To ensure research innovation makes it through the Valley of Death to market commercialisation, these factors must be taken into account early on.

Universally recognised research topics of interests are genome engineering, scale-up, and improved strain robustness. The main cause for lack of market introduction are the vastly different technology readiness levels that academia and industry operate on. Industry always focuses on solution-based research to improve the titre-rate-yield, while academia focuses on curiosity-driven, fundamental research, steps removed from industrial application. Neither party wants this to change, while both indicate a need for improved communication so that no opportunities are lost. Start-ups can serve as the bridge over the Valley of Death, connecting feasibility to viability, by acting as a communication channel between academia where the research originates, and industry to help set-up and fund along the way.

Competing interests

The authors declare that they have no competing interests. All data generated or analysed during this study are included in this published article and its supplementary information files. VAPMdS gratefully acknowledges financial support from the Wageningen University IP/OP project, and the European Horizon 2020 project EmPowerPutida (Project reference 635536). The funders had no role in study design, data collection and analysis, or preparation of the manuscript.

Author's contributions

Conceived the *in silico* study: VAPMdS/LFCK Literature research: LFCK Interview setup: LFCK/EAG/AW Interviews: LFCK/EAG Data Analysis: LFCK-/EAG/AW Work Supervision: AW/PJS/VAPMdS Wrote Manuscript: LFCK Proofread Manuscript: AW/PJS/VAPMdS Arranged Funding: VAPMdS

Acknowledgements

We thank all our participants for taking part in our research. We thank dr. R. A. Weusthuis for his advice on the interview.

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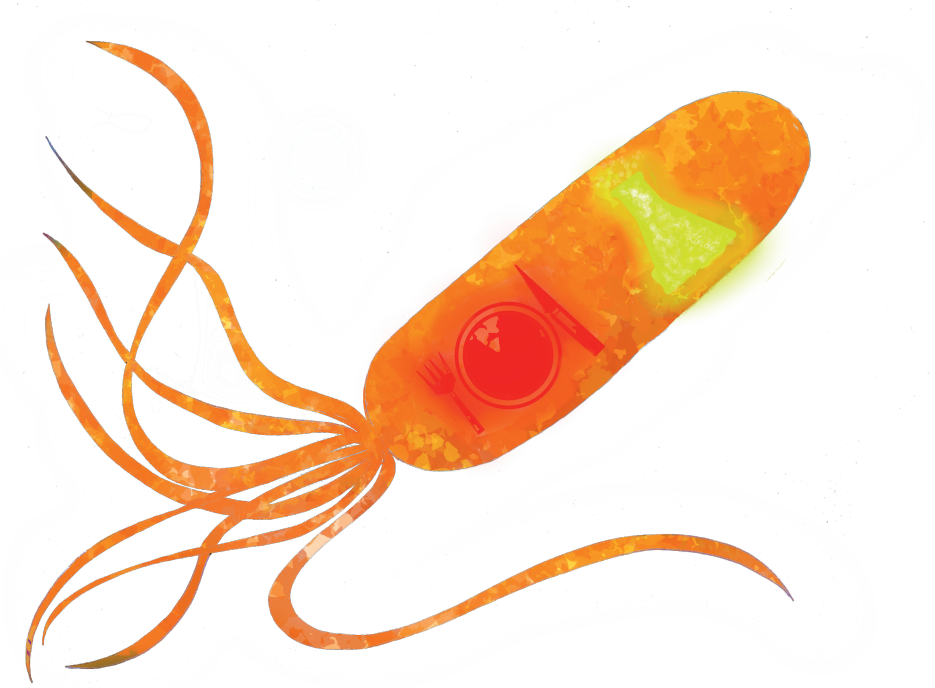
3

Pseudomonas putida KT2440 is HV1 certified, not GRAS



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Published in **Microbial Biotechnology**

Abstract

Pseudomonas putida is rapidly becoming a workhorse for industrial production due to its metabolic versatility, genetic accessibility and stress-resistance properties. Often, the *P. putida* strain KT2440 is described as Generally Regarded as Safe, or GRAS. This description is incorrect. *Pseudomonas putida* KT2440 is HV1 certified, not GRAS.

After the discovery and full sequencing of the bacterium thus far known as *Pseudomonas putida* KT2440 [1–3], this strain has rapidly become a workhorse for industrial biotechnology owing to its metabolic versatility, genetic accessibility and stress-resistance properties [4–6]. This specific set of characteristics is made more accessible by the recent expansion of its metabolic toolkit, including specific inducible promoters [7] and introduction of the CRISPR-system [8–10]. Its many benefits as a species have launched *P. putida* KT2440 as a model organism for industrial production of biobased chemicals such as citrulline [11], muconate [12], precursors of biobased plastics [13], precursor of biodegradable plastics [5, 14] and waste biodegradation purposes [15, 16].

Often *P. putida* KT2440, and sometimes the *P. putida* species, is described as Generally Regarded as Safe, or GRAS, indicating it is safe to use as a food additive [17] (e.g. [4, 6–10, 15, 16, 18–53]).

When a reference is given by the authors for this GRAS claim, the following is used: the US Food and Drug Regulation Administration (FDA) report, specifically the FDA vol. 47, no. 77, Appendix E, page 17197, Certified host-vector systems from April 21st, 1982 (see supplementary file) [54]. However, *P. putida* KT2440 is herein not certified as GRAS, but as Host-Vector (HV) system safety level 1: "HV1 – *Pseudomonas putida* strain KT2440 with plasmid vectors pKT262, pKT263 and pKT264". The HV1 certification indicates that *P. putida* KT2440 is determined to be safe to work with at a P1 (or ML1) facility, as elaborately described by the FDA on page 17181 of the report [54]. Similarly to *E. coli* K12, no additional safety measures are required to work with this strain.

The GRAS status determined by the FDA is only awarded to chemicals or specific strains of microorganisms that are proven to be safe for ingestion [17]. This is not proven for *P. putida* KT2440, contrary to food-related microorganisms such as *Lactobacillus helveticus* R0052, *Lactobacillus casei* subsp. *paracasei* Lpc-37, *Bifidobacterium bifidum* R0071, or *Streptococcus salivarius* K12, which are examples of strains that carry the GRAS status. Intense literature research suggests the GRAS reference occurs due to incorrect transitive referencing. A full GRAS certificate list can be found at the FDA website: `https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices`.

However, this does not mean that *P. putida* KT2440 is unsafe to work with. The safety of the strain is proven by the overall absence of virulence factors [19, 55] and the intensive research over the past 40 years by a large community without any fortuitous cases of infection reported. This correct certification does therefore not change the many useful applications of this strain. Furthermore, the widespread use of KT2440, the massive evidence of safe performance and the development of more refined methods for risk assessment than those available in the early 80s ask for a revised classification of the strains at stake under the current FDA and EFSA regulations.

As a note, the phylogenetic classification of *P. putida* KT2440 within the *P. putida* group has been questioned for long [56], and recent work has provided a new classification [57] (Moore et al., in preparation). However, for the purpose of safety certification, the specific bacterium formerly known as *P. putida* KT2440 remains classified as HV1, regardless of its precise phylogenetic status.

We urge other scientists to properly describe the safety status of all used strains, since we also discovered the same is true for for example *Corynebacterium glutamicum*. Similarly to *P. putida* KT2440, *C. glutamicum* is sometimes reported to have GRAS status (e. g. [58–61]), but when checked using the website stated above, only cultured corn starch fermented by *C. glutamicum* is GRAS, not the organism itself.

Acknowledgements

We are grateful to Prof. Victor de Lorenzo for fruitful discussions and advice. This work was funded by the H2020 project Empowerputida, grant agreement nr. No 635536

Author's contributions

Conceived subject: LFCK, Literature search and analysis: LFCK/RJMV, Wrote Manuscript: LFCK, Work supervision: RJMV/VAPMdS, Arranged Funding: VAPMdS. The authors declare that they have no competing interests.

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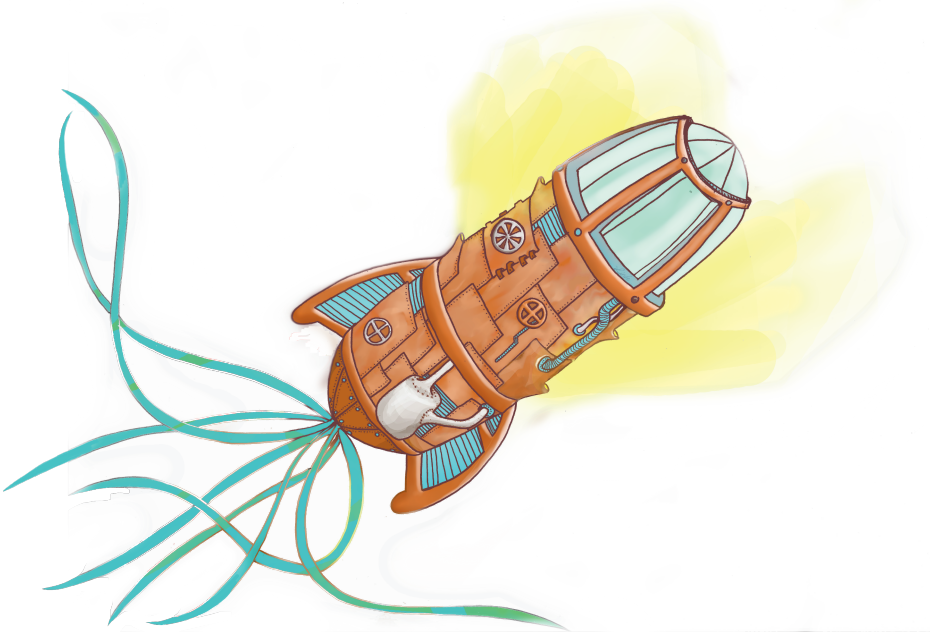
4

In silico-guided engineering of *Pseudomonas putida* towards growth under micro-oxic conditions



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Published in **Microbial Cell Factories**

Abstract

Pseudomonas putida is a metabolically versatile, genetically accessible, and stress-robust species with outstanding potential to be used as a workhorse for industrial applications. While industry recognises the importance of robustness under micro-oxic conditions for a stable production process, the obligate aerobic nature of *P. putida*, attributed to its inability to produce sufficient ATP and maintain its redox balance without molecular oxygen, severely limits its use for biotechnology applications. Here, a combination of genome-scale metabolic modelling and comparative genomics is used to pinpoint essential O_2-dependent processes. These explain the inability of the strain to grow under anoxic conditions: a deficient ATP generation and an inability to synthesize essential metabolites. Based on this, several *P. putida* recombinant strains were constructed harbouring acetate kinase from *Escherichia coli* for ATP production, and a class I dihydroorotate dehydrogenase and a class III anaerobic ribonucleotide triphosphate reductase from *Lactobacillus lactis* for the synthesis of essential metabolites. Initial computational designs were fine-tuned by means of adaptive laboratory evolution. We demonstrated the value of combining *in silico* approaches, experimental validation and adaptive laboratory evolution for microbial design by making the strictly aerobic *Pseudomonas putida* able to grow under micro-oxic conditions.

Introduction

The *Pseudomonas* genus is known for its metabolic versatility, high supply of reducing power, and tolerance to toxins and solvents [1–3]. In particular, *Pseudomonas putida* KT2440 is HV1 certified (safe to use in an ML1 or P1 environment), genetically accessible [4–9], and has been successfully engineered to produce various compounds of industrial interest [10, 11]. Therefore, *P. putida* KT2440 is a recognized synthetic biology- and industrial workhorse [4, 10–13]. Genome-scale, constraint-based models of metabolism (GSMs) have been developed to analyse its bio-degradative and biotechnological capacities [4–9]. GSMs provide a comprehensive list of all genome-encoded reactions of an organism and can be used to make predictions of growth in different media and/or environmental conditions [14].

Several species in the *Pseudomonas* genus are facultative anaerobes (e.g *P. aeruginosa*, *P. fluorescens*, *P. denitrificans*), suggesting that the strictly aerobic *P. putida* could be designed towards a micro-aerobic or even facultative anaerobic lifestyle with a limited number of genetic modifications. The latter has been experimentally attempted several times by engineering either anaerobic fermentation or anaerobic respiration.

The first attempt to create a *P. putida* strain capable of anaerobic fermentation was by Sohn *et al.* [6]. They created a GSM of *P. putida* KT2440 from which they concluded that *P. putida* KT2440 cannot grow anaerobically due to insufficient ATP generation. Anaerobic ATP generation was then enhanced through the expression of acetate kinase via a plasmid, which resulted in 10 times more surviving *P. putida* KT2440 cells in minimal medium with glucose as only carbon source after 8 days of exposure to anoxic conditions.

In a later *in vivo* study, Nikel *et al.* [15] reasoned that in the absence of aerobic respiration there is not only a lack of ATP generation, but also an accumulation of NADH that cannot be re-oxidized to NAD^+ via the electron transfer chain. Therefore they expressed acetate kinase, pyruvate decarboxylase and alcohol dehydrogenase II to facilitate energy generation and redox rebalancing, respectively. This approach also yielded approximately 10 times more surviving *P. putida* KT2440 cells compared to the control strain after 7 days of exposure to anoxic conditions, with a

viability rate of 58±4% when both these enzymes were expressed versus 37±3% for sole expression of acetate kinase [6, 15].

In a different approach Steen *et al*. [16] introduced the nitrate or nitrite respiration machinery in *P. putida* KT2440. Nitrate and nitrite respiration are common anaerobic alternatives to O_2 respiration in other *Pseudomonas* species, but are completely absent in *P. putida* strains. Therefore, Steen *et al*. separately expressed the *nar* or *nir-nor* operons from *P. aeruginosa* ATCC 17933 in *P. putida* KT2440 to enable nitrate or nitrite respiration respectively. *P. putida* KT2440 expressing the *nar* operon yielded a 50-fold higher number of colony forming units (CFUs) compared to the control strain when incubated for 5 days under anoxic conditions in the presence of nitrate, and *P. putida* KT2440 expressing the *nir-nor* operon displayed an up to 80-fold higher number of CFUs in the presence of nitrite.

Another approach to attempt anaerobic respiration in *P. putida* was the use of phenazines to transfer electrons from the cell to an electrode, to re-establish functioning of the electron transfer chain under oxygen limiting conditions. Schmitz *et al*. [17] expressed seven core phenazine biosynthesis genes *phzA-G* and the two specific genes *phzM* and *phzS* required for pyocyanin synthesis from *P. aeruginosa* PAO1 into *P. putida* KT2440. They observed that phenazines facilitate electron discharge to the electrode, although the wild-type *P. putida* KT2440 unexpectedly also showed a limited ability to interact with the electrode. The production of phenazines resulted in sustained metabolism under oxygen-limited conditions for up to two weeks, with doubled biomass production in the presence of a poised electrode when compared to wild-type *P. putida* KT2440.

Similarly, Lai and colleagues [18] showed that adding the redox mediators thionine chloride, tris(2,2'-bipyridine)cobalt(II) diperchlorate, or potassium hexacyanoferrate(III) to the culture medium could enable wild-type *P. putida* F1 to discharge electrons to an electrode. This resulted in survival of *P. putida* F1 under anoxic conditions over 300 hours with a higher adenylate energy charge, indicating the cells could generate energy using the anode as terminal electron acceptor.

The attempts for anaerobic fermentation or anaerobic respiration yielded similar levels of survival under anoxic conditions. None of the studies reported growth of the strains under anoxic conditions [6, 15–18]. Overall, anaerobic fermentation

would be preferred over anaerobic respiration since this eliminates the need of supplementing the electron acceptor (oxygen or nitrate/nitrite) homogeneously through the bioreactor, and fermentation often results in products of industrial value such as lactate. Recombinant strain behaviour through fermentation under micro-oxic conditions was not explored in any of the aforementioned studies.

In this work, we explore the roles oxygen plays in *P. putida* KT2440 by focusing on growth under anoxic *in silico* and micro-oxic conditions *in silico* and *in vivo*. In our pursuit of these goals we took advantage of the available knowledge on *P. putida* KT2440 metabolism, as well as of the wealth of genomic data on *P. putida* KT2440 and other *Pseudomonas* species [14]. Specifically, we used a GSM to probe *P. putida* KT2440 metabolism, while comparative genomics was used to pinpoint the distinct genetic features of *Pseudomonas* species capable of growing under anoxic conditions through anaerobic fermentation. These *in silico* approaches identified several limitations of *P. putida* KT2440 to grow under micro-oxic or anoxic conditions. These limitations were systematically addressed in recombinant *P. putida* KT2440 strains, which were then exposed to micro-oxic conditions through an oxygen gradient set-up to evaluate their capabilities.

Materials and methods

Genome-scale, constraint-based metabolic models

In this study we used the *P. putida* KT2440 genome-scale metabolic models iJN746 [5] and iJP962 [7].

GSM simulations

iJN746 and iJP962 were analysed using Flux Balance Analysis (FBA) in the CobraPy toolbox [14, 19]. Growth predictions were performed by setting biomass production as maximization objective in FBA. The minimal glucose medium conditions were set by allowing 'unlimited' uptake (up to 1000 mmol gdw^{-1} h^{-1}, gdw: grams dry weight) of copper, cobalt, iron, protons, water, sodium, nickel, ammonia, phosphate, sulphate, and nitrate. In addition, the glucose uptake rate was set to be maximally 6.14 mmol gdw^{-1} h^{-1}, based on experimentally measured uptake rates [20]. The

in silico medium composition closely resembles the composition of DeBont minimal medium which was used for the *in vivo* experiments.

Rich medium conditions were set by allowing unlimited uptake (up to 1000 mmol gdw^{-1} h^{-1}) of all compounds (except oxygen) for which exchange reactions are present in the GSM. Under oxic conditions, the O_2 uptake rate was set to maximally 18.5 mmol $gdw^{-1}h^{-1}$. Reactions for previous designs or to complement newly identified reactions were added using the CobraPy 'addReaction' function.

Genome annotation

We selected six genomes of facultative anaerobic strains from the *Pseudomonas* genus (*P. aeruginosa* PAO1 and M18, *P. stutzeri* DSM10701 and A1501, *P. denitrificans* ATCC13867 and *P. fluorescens* F113) and six genomes of obligate aerobic *P. putida* strains (KT2440, F1, S16, W619, GB1, and BIRD1). All genomes were obtained from the EnsemblBacteria repository in March 2015 [21]. Genomes were annotated in SAPP [22, 23] using Prodigal for gene prediction (version 2.6) [24], 2010] and with InterProScan version 5.4-47.0 [25] for functional annotation, with the selected applications: TIGRFAM, ProDom, SMART, PROSITE Pattern, PfamA, PRINTS, SUPERFAMILY, Coils, Gene3d.

Bacterial strains and cultivation conditions

Bacterial strains used in this study can be found in [26]. *E. coli* CC118λpir was used for cloning procedures and plasmid maintenance, and was routinely cultivated at 37°C under oxic conditions in LB medium (containing 10 g/l tryptone, 10 g/l NaCl and 5 g/l yeast extract), optionally containing antibiotics for selection (50 μg/ml kanamycin as indicated). For solid medium, 15 g/l agar was added to the medium. *P. putida* KT2440 was routinely cultivated under oxic conditions at 30°C in LB medium. Growth and fluorescence experiments were performed in De Bont minimal medium (DBG) [27] (3.88 g/l K_2HPO_4, 1.63 g/l $NaH_2PO_4 \cdot 2H_2O$, 2.00 g/l $(NH_4)_2SO_4$, 0.1 g/l $MgCl_2 \cdot 6H_2O$, 10 mg/l EDTA, 2 mg/l $ZnSO_4 \cdot 7H_2O$, 1 mg/l $CaCl_2 \cdot 2H_2O$, 5 mg/l $FeSO_4 \cdot 7H_2O$, 0.2 mg/l $Na_2MoO_4 \cdot 2H_2O$, 0.2 mg/l $CuSO_4 \cdot 5H_2O$, 0.4 mg/l $CoCl_2 \cdot 6H_2O$, 1 mg/l $MnCl_2 \cdot 2H_2O$), with 4.5 g/l glucose as

the sole carbon source. The medium was supplemented with 50 μg/ml kanamycin as indicated. Pre-cultures were prepared by picking and transferring a single colony from solid LB medium with the appropriate antibiotic selection into culture flasks for oxic overnight cultivation at 200 rpm at indicated cultivation temperatures.

Construction of Plasmids

Plasmids used in this study are described in [26]. DNA segments were amplified from the indicated template by colony-PCR using Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturers' protocol (Primers are listed in [26]). Restriction enzymes were obtained from NEB (New England BioLabs®inc, Ipswich, MA USA). L-aspartate oxidase was isolated from both *E. coli* BW25113 or *P. putida* KT2440. Both genes were designed with NdeI and BamHI restriction sites on the 5'- and 3'-ends, respectively. DNA fragments were purified from agarose gel using the KG Gel Purification Kit (Machery-Nagel GmbH and Co. Düren, Germany) and ligated into the pSEVA638 backbone (Standardized SEVA plasmid system [28, 29]) using T4 DNA Ligase (Roche Applied Science, Indianapolis, IN USA). Acetate kinase (*ackA*) was isolated from *E. coli*, the two-subunit gene cluster class I dihydroorotate hydrogenase (*pyrK-pyrD B*) and the two-subunit gene cluster class III ribonucleotide triphosphate reductase (*nrdD-nrdG*) were ordered using the gene from *L. lactis* as a template but codon-optimized for *P. putida* KT2440. The genes were designed with PacI and SpeI restriction sites on the 5'- and 3'-ends, respectively. Similarly purified DNA segments were ligated into the pSEVA2213 backbone (Standardized SEVA plasmid system [28, 29]). Each construct additional carries the fluorescent protein EcFbFP [30] which does not depend on oxygen to check if the genes are transcribed and in roughly what amount the proteins are present. The empty plasmid control only carries *EcFbFP*.

Micro-oxic experiments

Oxygen gradients are based on Bailey and Scott's Diagnostic Microbiology [31]. Oxygen gradients were set up in sterile 10 ml glass test tubes capped with loosely fitting caps and filled halfway with LB medium. After autoclaving, the medium was supplemented with sterile components: antibiotics as indicated, 5 g/l L-cysteine and 0.5 g/l sodium thioglycollate to remove O_2 from the medium, 1 mg/l resazurin to indicate O_2 presence and 4 g/l agarose to stabilize the gradients. After heating the medium to just below boiling point for 10 min to expel O_2, the tubes were cooled to room temperature. 24 Hours after preparation, the tubes were inoculated drop-wise with 10μl of a pre-culture.

Strain performance in oxygen gradients was tested while oxygen gradient test tubes were slanted at a 45° angle or upright. Multiple agar or agarose concentrations and variants were tested [26]. Micro-oxic conditions within the gradient were verified with a micro-electrode (similar set-up as described in [32, 33]). The resazurin colour change from pink to colourless coincides with a dissolved oxygen concentration around the micro-electrode detection limit of 0.01 mg/l [26]. Growth of the strains through the oxygen gradient was monitored via a Mirazoom MZ-902 time-lapse camera (OOWL tech, Kowloon, Hong Kong) set-up in a well-lit non-shaking 30°C incubator. Time-lapse movies were analysed using Fiji 1.51p (ImageJ 1.51p, Rasband, National institutes of Health, Bethesda, MA USA) [26]. Growth in the oxygen gradients started from the surface of the medium (high in oxygen) downwards (gradually lower in oxygen), and was measured in mm. Sampling of bacteria was done by selecting the bacteria growing at the lowest oxygen concentration with a 3 ml syringe (Thermo Fisher Scientific, Waltham, MA USA) and 1.5" needle (BD Microlance, Switzerland).

Anoxic cultivation

Anoxic cultivation of *E. coli* JW2558 was performed in 50 ml glass 20 mm aluminium crimp cap vials with rubber stoppers (Glasgerätebau Ochs laborfachhandel e.K.) in 30 ml DBG medium supplemented with 0.75 g/l L-cysteine, 1 mg/l resazurin and with 50 μg/ml gentamycin as selection marker. Before inoculation, the vials were gas exchanged with CO_2/N_2. Inoculation was done with aerobically precultured bacterial sample, with a starting OD of ca. 0.03. Samples were taken using CO_2 flushed 1.5" needles (BD Microlance) and 3-5 ml syringes (ThermoFisher) avoiding O_2 exposure. Anoxic conditions were ensured as in samples taken the resazurin turned from colourless to bright pink within seconds. Growth rates were determined by OD_{600} measurements. Medium only served as a blank control.

Statistical analysis

All reported experiments were independently repeated three times, testing biological triplicates. Figures represent the mean values of corresponding biological triplicates and the standard deviation. The level of significance of the differences when comparing results was evaluated by means of analysis of variance (ANOVA), with α=0.05.

Results

Model evaluation confirms the strict aerobic nature of *P. putida* KT2440

Previous designs of anaerobically surviving *P. putida* strains were conceptually based on insufficient anaerobic ATP generation and redox balancing [6, 15–18]. Implementation of acetate kinase resulted in prolonged survival times under anoxic conditions [6, 15, 16]. Therefore, we first re-evaluated these designs in the context of the *P. putida* KT2440 models iJP962 [7] and iJN746 [5]. GSMs iJP962 and iJN746 describe the known metabolism of *P. putida* KT2440 as well as its requirements for survival and growth. When analysed with Flux Balance Analysis (FBA) [14], these GSMs can predict whether or not *P. putida* grows under various conditions [5, 7]. The analysis of the two GSMs led to similar results and hereafter we will specifically report those obtained with iJP962.

iJP962 Was first confirmed to correctly predict the obligate aerobic nature of wild-type *P. putida* KT2440. It predicted the maximal achievable growth rate under anoxic conditions in both rich and minimal medium to be zero, which is in line with the obligate aerobic nature of *P. putida*. iJP962 Was then used to contextualize the anaerobic *P. putida* designs referred to above. The GSM was expanded with reactions corresponding to the expressed heterologous genes from previous experimental designs [6, 15–17]. These expanded GSMs still predicted that anaerobic growth was not possible in neither minimal nor rich medium. These predictions are consistent with the experimental observations that none of the previous designs enabled *P. putida* KT2440 to grow anaerobically. In addition, these predictions imply that iJP962 captures previously undescribed limitations to anaerobic growth in *P. putida* KT2440.

Limited energy supply and inability to generate biomass inhibit *P. putida* to survive under anoxic conditions

The model allows identification of reactions which indirectly use oxygen, thereby resulting in limited survival under micro-oxic conditions. To identify additional limitations, we performed two independent *in silico* analyses: GSM simulations and

domainome, comparative genomic analysis. The GSM simulation approach focused on identifying essential O_2-dependent metabolic reactions in iJP962, whereas the comparative genomics approach focused on pinpointing the genetic differences between obligate aerobic *P. putida* strains and other facultative anaerobic *Pseudomonas* species (Figure 4.1). To identify reactions in the GSM that involve O_2 and are essential for growth, we first set the growth medium to a minimal glucose medium under oxic conditions. Then, we iteratively deleted each reaction that involves O_2 one at a time and predicted whether or not growth was possible. Growth was no longer possible upon the deletion of either (i) protoporphyrinogen oxidase, (ii) L-aspartate oxidase, or (iii) dihydroorotate dehydrogenase. These enzymes are required for the biosynthesis of heme, NAD^+/$NADP^+$, and pyrimidines, respectively (Figure 4.2).

Next, we evaluated whether the lack of anaerobic alternatives to these three reactions is the only limitation to *in silico* anaerobic growth. We expanded iJP962 with anaerobic alternatives for L-aspartate oxidase, dihydroorotate dehydrogenase and protoporphyrinogen oxidase and again simulated growth. iJP962 now predicted anaerobic growth of the modified *P. putida* on glucose minimal medium, suggesting that the lack of anaerobic alternatives to the aforementioned three reactions is the only limitation to anaerobic growth.

The predicted anaerobic growth rate with the three aforementioned metabolic alternatives was, however, very low (0.007 h^{-1}) compared to the aerobic growth rate using the unamended model (0.450 h^{-1}). Although low, this does suggest that according to the GSM the modified *P. putida* is capable of producing all biomass constituents necessary for growth. The comparatively low growth rate suggests a lack of resources or an inefficient conversion thereof.

The limiting factor was identified to be ATP formation. Under anoxic conditions, most ATP required for biomass formation is *in silico* generated by *oxygen-dependent* cytochrome reactions. Therefore, we further expanded iJP962 with reactions corresponding to the previous anaerobic *P. putida* designs that dealt with anaerobic ATP generation [6, 15–17]. The addition of acetate kinase to the model doubled the predicted growth rate to 0.014 h^{-1}, while addition of the nitrate respiration machinery increased the predicted growth rate to 0.171 h^{-1}. Based on the modelling results, we hypothesized that the anaerobic ATP generation is insufficient.

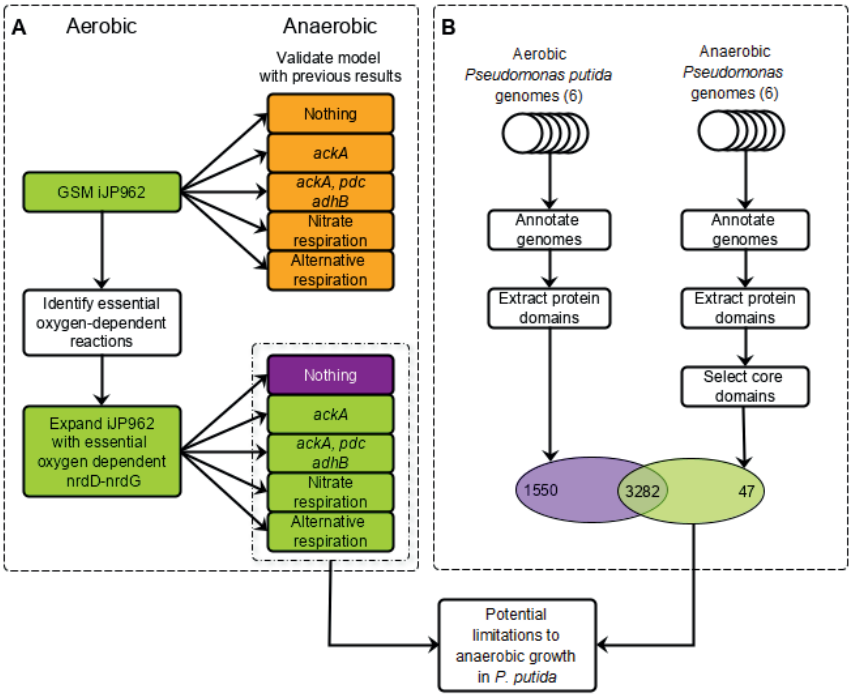

Figure 4.1: Overview of in silico approaches to identify limitations to anaerobic growth in P. putida. (A) Summary of genome-scale GSM predictions using iJP962 [7] given an aerobic environment (left) and expansions with indicated reaction sets given an anaerobic environment (right). The colours indicate no growth (orange), poor growth (purple) and growth (green) (B) Comparative genomics workflow. Genomes of the P. putida group and the anaerobic Pseudomonas group were systematically annotated using SAPP [22, 23], the protein domains were extracted, and the domains common to all anaerobic Pseudomonas species (the core domains) were selected. Predictions from both methods were combined to obtain a final design.

Although the GSM-based approach successfully identified several limitations to anaerobic growth in *P. putida*, this identification is restricted to metabolism as described in iJP962. Other cellular processes that may rely on O_2 are not described. Therefore, we also used comparative genomics to perform an analysis of protein domain content and pinpoint genetic differences between select groups of obligate aerobic *P. putida* strains and facultative anaerobic *Pseudomonads*. The *P. putida* group consisted of the following strains: KT2440, F1, S16, W619, GB1, and BIRD1. The facultative anaerobic *Pseudomonas* group consisted of *P. aeruginosa* PAO1 and M18, *P. stutzeri* DSM10701 and A1501, *P. denitrificans* ATCC13867 and *P. fluorescens* F113.

All genomes were *de novo* annotated to avoid artefacts from differences in the annotation procedures. Annotated protein domains were extracted from each genome to compare the presence of functional protein domains. The domains from the anaerobic *Pseudomonas* group were then further filtered to select only those domains that are shared by all members of the group: core domains. These core domains were compared to the domains present in the *P. putida* strains to identify domains common to all selected anaerobic *Pseudomonas* species and absent from all selected *P. putida* strains. This resulted in a shortlist of 47 anaerobic-only protein domains (Figure 4.1, [26]).

These domains thus identify the genetic makeup of the anaerobic lifestyle and can be divided in three main categories: i) Domains functional in ATP generation (16), ii) Domains of unknown function (13), and iii) Other domains (18).

Category i) comprises nitrate respiration, and acetate kinase (IPR000890). Furthermore, two domains that are related to ribonucleotide-triphosphate reductase (IPR012833, IPR012840) have been identified which are crucial for this work, as discussed further below. Category ii), domains of unknown function, must be excluded from the design as nothing can be said about them with the current knowledge.

For the majority of the remaining domains belonging to category iii) it is not clear how they would contribute to the desired anaerobic lifestyle. For example, several domains associated with siderophore transport (IPR003538) and pilus assembly (IPR008707, IPR013362, IPR013374, IPR025746) are found. While they have been related to virulence in *P. aeruginosa* [34, 35], which is part of its pathogenic lifestyle, they do not appear to be linked to anaerobicity. Other domains may be beneficial but not essential for anaerobic growth. The identified iron-sulfur clusters (IPR007202, IPR018298) for example were absent in the investigated aerobic species. Typically these clusters are oxygen sensitive, which may explain their absence. The iron sulfur clusters have a regulatory role [36, 37] and are implicated as intracellular sensors and in electron transport. Hence, these clusters do not appear essential for a conversion from an aerobic to an anaerobic lifestyle.

Design of anaerotolerant *P. putida*

Together, the *in silico* approaches provide a holistic view on the metabolic processes required to enable designing a *P. putida* strain capable of anaerobic growth by fermentation. The main bottlenecks found include L-aspartate oxidase (NadB), dihydroorotate dehydrogenase (PyrD), protoporphyrinogen oxidase (HemY), ribonucleotide-triphosphate reductase (RNRs), and anaerobic energy generation. We address these below.

L-aspartate oxidase (NadB) catalyses the conversion of L-aspartate to iminoaspartate, a precursor in $NAD(P)^+$ biosynthesis. In iJP962 this conversion requires O_2 as electron acceptor. However, in *E. coli* L-aspartate oxidase is known to use either O_2 or fumarate as electron acceptors [38]. The necessity of exchanging *nadB* in *P. putida* KT2440 must be checked experimentally. This was done by constructing a plasmid with either *nadB* from *P. putida* KT2440 or *nadB* from *E. coli* BW25113 and transforming it into *nadB* knock-out strain *E. coli* JW2558. The growth dynamics of the resulting recombinant *E. coli* strains were compared under anoxic conditions.

Dihydroorotate dehydrogenase (PyrD) catalyzes the production of orotate, which is required for pyrimidine biosynthesis, and ultimately for the synthesis of RNA and DNA. In iJP962 this enzyme interacts directly with O_2, but in reality *P. putida pyrD* encodes a membrane-bound class II dihydroorotate dehydrogenase [39], which interacts with quinones rather than directly with O_2 [40]. The reoxidation of the quinones requires the flow of electrons towards the terminal electron acceptor O_2. Thus, *P. putida pyrD* is indirectly dependent on O_2 via the electron transfer chain. To circumvent the need for the electron transfer chain, a biochemically characterised class I dihydroorotate dehydrogenase that uses fumarate, FAD^+, or NAD^+ [40] could be obtained from the non-pathogenic *Lactobacillus lactis* [41] and introduced in *P. putida* KT2440.

Protoporphyrinogen oxidase (HemY) converts protoporphyrinogen IX to protoporphyrin IX, which is further converted to heme. Heme is involved in many cellular processes–including respiration–and is essential for most organisms, excluding some specific species capable of fermentation under anoxic conditions [42, 43]. It is unclear whether heme is essential for an anaerobically fermenting *P. putida*

strain, but it is most likely required for anaerobic respiration. There are two alternative protoporphyrinogen oxidases in gram negative bacteria: HemG and HemJ. The protein HemG is quinone-dependent instead of O_2-dependent, but is not found in any *Pseudomonas* species. The protein HemJ is found in *Pseudomonads*, including *P. aeruginosa* [44] and *P. putida*, but it is unknown whether or not it relies on O_2 [42]. We have thus not included an alternative biosynthesis gene for the heme precursor protoporphyrin IX, as it is likely that protoporphyrin IX can be synthesized by the endogenous *P. putida* HemJ.

Ribonucleotide-triphosphate reductases (RNRs) are required for the biosynthesis of deoxynucleotides (dNTPs). There are three classes of RNRs: Class I is strictly aerobic, class II is O_2-independent, and class III is O_2 sensitive [45, 46]. *P. putida* only has a class I RNRs and is not able to produce DNA under anoxic conditions. In contrast, *P. aeruginosa* contains RNR of all three classes [46]. RNR knockout experiments in *P. aeruginosa* revealed that the class II enzyme contributes most to anaerobic dNTP production [46], and that the class II RNRs are reported to be essential for anaerobic growth in *P. aeruginosa* [47]. Therefore, a class II RNR seems the most promising candidate to enable anaerobic nucleotide production in *P. putida*.

The identified limitations and alternative options found using the *in silico* methods were combined into a consolidated design of a *P. putida* KT2440 strain capable of *in-silico* growth under anoxic conditions through anaerobic fermentation. The predicted lack of ATP was resolved by acetate production, based on the previously successfully expressed acetate kinase for fermentation [6, 15]. The newly proposed design includes (i) a quinone-independent gene cluster class I dihydroorotate dehydrogenase (*pyrK-pyrD B*) which was taken from the non-pathogenic *L. lactis* [48], (ii) the class III ribonucleotide-triphosphate reductase (*nrdD-nrdG*) which was taken from the non-pathogenic *L. lactis*, (iii) acetate kinase (*ackA*) which was taken from *E. coli*, and possibly (iv) L-aspartate oxidase (*nadB*) from *E. coli* (Figure 4.2).

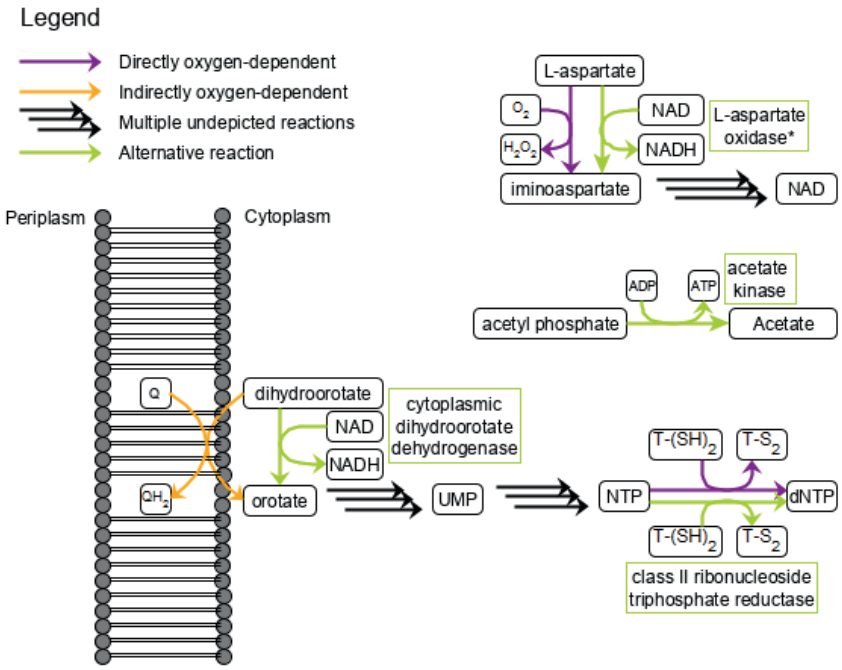

Figure 4.2: Final design of P. putida KT2440 capable of growth under anoxic conditions through anaerobic fermentation. QH2: quinol, Q: quinone; T-(SH)2: reduced thioredoxin, T-S2 oxidized thioredoxin NTP: nucleoside triphosphate, dNTP: deoxynucleoside triphosphate, UMP: uridine monophosphate.

The *P. putida* L-aspartate oxidase can function without oxygen as electron acceptor

To determine the need to replace the *nadB* gene to facilitate anaerobic survival of *P. putida*, plasmids carrying either *nadB* from *E. coli* (p638 *nadBEco*) or the *nadB* gene from *P. putida* (p638 *nadBPpu*) were inserted into the *E. coli nadB* knockout strain *JW2558*. The strains were cultured anaerobically, monitoring growth through sampling. Both strains grow similarly under anoxic conditions, proving that the endogenous L-aspartate oxidase of *P. putida* does not need oxygen as an electron acceptor and therefore does not have to be replaced (Figure 4.3).

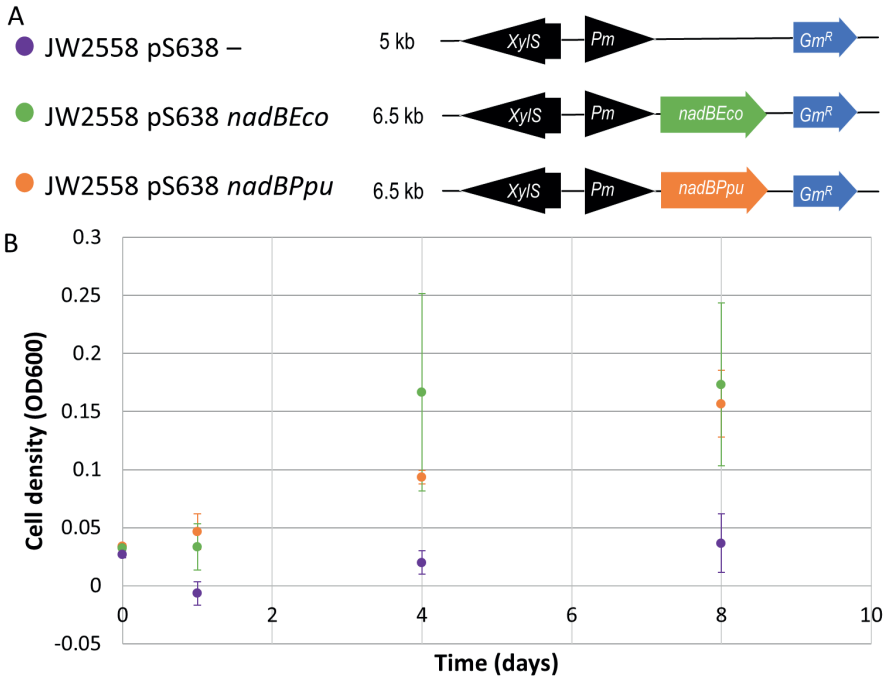

Figure 4.3: Testing of P. putida L-aspartate oxidase under anoxic conditions. A) Schematic overview of constructs for E. coli JW2558: p638 - (empty plasmid control) (purple), p638 nadBEco (green) and p638 nadBPpu (orange). B) Anaerobic growth analysis of all strains.

Expression of acetate kinase, dihydroorotate dehydrogenase and ribonucleotide triphosphate reductase in *P. putida* does not alter aerobic growth

To verify the *in silico* analysis, recombinant strains were constructed carrying *ackA*, *pyrK-pyrD B*, *nrdD-nrdG*, or all three (*ackA-(pyrK-pyrD B)–(nrdD-nrdG)*) (Figure 4.4A). The genes of interest were placed in the same SEVA pS2213 backbone, with an RK2 origin of replication and an *EcFbFP* gene included directly behind the gene of interest. In the empty plasmid, only the fluorescence gene is present.

A 64-hour growth experiment under oxic conditions in a platereader was performed, measuring the OD_{600} and fluorescence (Figure 4.4B-D). The similar growth curves of the different recombinant strains show no difference in plasmid burden, while plasmid sizes vary between 4 kb (empty plasmid) and 10 kb (pS2213 *ackA-(pyrK-pyrD B)-(nrdD-nrdG)*) (Figure 4.4B).

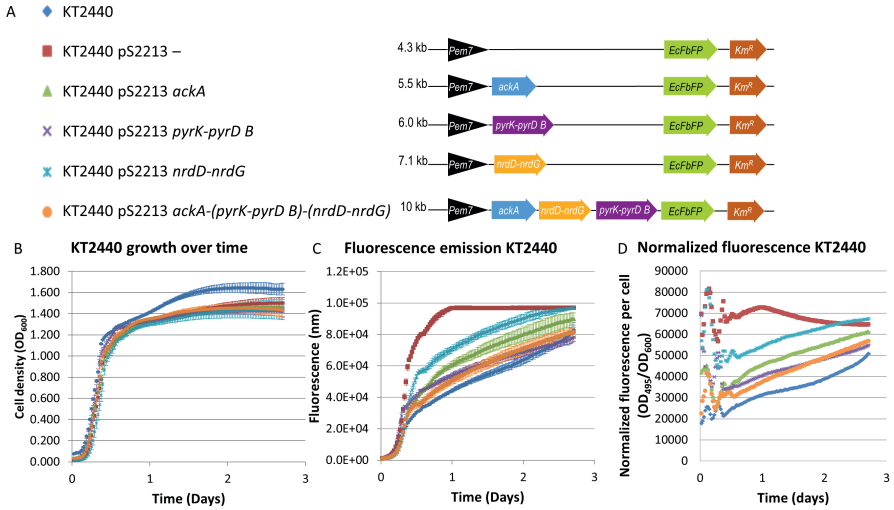

Figure 4.4: Building the design of P. putida KT2440 capable of growth under anoxic conditions and testing under oxic conditions. A) Schematic overview of constructs for P. putida KT2440: 2213F - (empty plasmid control), pS2213 ackA, pS2213 pyrK-pyrD B, pS2213 nrdD-nrdG and pS2213 ackA-(pyrK-pyrD B)-(nrdD-nrdG). The symbols in front of the strain names function as the legend for the graphs. B) Aerobic growth analysis of all strains. C) Aerobic fluorescence assay of all strains at excitation of 450 and emission of 495. D) Fluorescence relative to cell density. Measurements for B, C and D were taken every 30 min for 64 hours at 30°C. Cultures were shaken continuously.

All recombinant strains carrying plasmids emit significantly more fluorescence than the wild-type counterpart (Figure 4.4C, D). There are large differences in fluorescence between those strains. In 12 hours, the relative fluorescence of the strain carrying the empty plasmid was 2.3 to 1.4 times as high as the recombinant strains. At that point, the fluorescence intensity of strain KT2440 pS2213*nrdD-nrdG* exceeds all other strains by a factor 1.6 (Figure 4.4D). Remarkably, strain KT2440 pS2213 *ackA-(pyrK-pyrD B)-(nrdD-nrdG)* and strain KT2440 pS2213 *pyrK-pyrD B* emitted similar amounts of fluorescence. In spite of the size of the two-subunit gene cluster class III ribonucleotide triphosphate reductase, relatively more fluorescence was recorded in *P. putida* KT2440 pS2213 *nrdD-nrdG* than when solely acetate kinase is expressed. In this context, the differences in fluorescence between strains can be used as a rough indication of difference in gene transcription, as the genes were placed in the same backbone under de same promoter and with equal copy numbers.

These data show that under oxic conditions all plasmids pose an equal burden, but the level of gene transcription varies significantly between strains.

Additional ATP generation results in growth of *P. putida* KT2440 under micro-oxic conditions

The recombinant strains were exposed to micro-oxic conditions in an adaptive laboratory evolution set-up using an oxygen gradient first for two days, followed by two four-day cycles. Strain performance was continually monitored using a time-lapse camera, taking a picture every 20 seconds. The end of one round was marked by either stagnation of growth and visible cell-death, or the ability of the strains to grow under micro-oxic conditions, defined as below the micro-electrode detection limit. Recombinant strains were passed three times over oxygen gradients to adapt the strains to micro-oxic conditions (Figure 4.5A). Since KT2440 pS2213 *pyrK-pyrD B* underperformed in preliminary oxygen gradient experiments, this strain was excluded (data not shown). KT2440 pS2213 - (negative control), KT2440 pS2213 *ackA*, KT2440 pS2213 *nrdD-nrdG* and KT2440 pS2213 *ackA-(pyrK-pyrD B)-(nrdD-nrdG)* were monitored for three consecutive rounds of culturing in the oxygen gradients. Medium was used as a blank control.

Over multiple consecutive rounds, all recombinant strains except the empty plasmid control showed improved performance under micro-oxic conditions (Figure 4.5B). This is reflected by the difference in progress towards a dissolved oxygen concentration of <0.01 mg/l along the consecutive passages over the oxygen gradients. In contrast to the other transformant strains, no growth beyond detectable oxygen levels was achieved with the empty plasmid between passage 2 and 3. To ensure that there was no growth possible beyond the detection limit, we deliberately tested the empty plasmid in further cycles. The results show that the empty plasmid control only adapts to the limit of detectable oxygen levels over multiple passages, but does not surpass it (Figure S6).

After a period of four days the strains progressed to areas with <0.01 mg/l oxygen (Figure 4.5, Supplementary data). While in the set-up, time-wise, the first passage (T1, two days) is different from the following cycles (T2 and T3, four days), presently it is unclear what causes the sudden increase in performance from T2 to T3 observed in strains carrying either pS2213 -, pS2213 *nrdD-nrdG* or pS2213 *ackA-(pyrK-pyrD B)-(nrdD-nrdG)* (Figure 4.5B). When solely acetate kinase is expressed,

strain performance in micro-oxic conditions is directly improved. The differences between the strains with pS2213 *ackA* and pS2213 *ackA-(pyrK-pyrD B)-(nrdD-nrdG)* could either be explained by the additional metabolic load caused by gene expression under micro-oxic conditions (which in itself imposes a burden), or by the effect of acetate kinase held back by the additional expression of *pyrK-pyrD B* and *nrdD-nrdG*. When analysing the overall daily progress of the strains (Figure 4.5C) it again becomes apparent that overall the strain expressing acetate kinase performs significantly better. Extra energy generation is therewith proven to be a vital requisite for *P. putida* to accommodate micro-aerobic growth.

The results of the O_2-gradients show that after multiple rounds over the oxygen gradient expression of *nrdD-nrdG* alone in *P. putida* KT2440 result in growth under micro-oxic conditions (Figure 4.5).

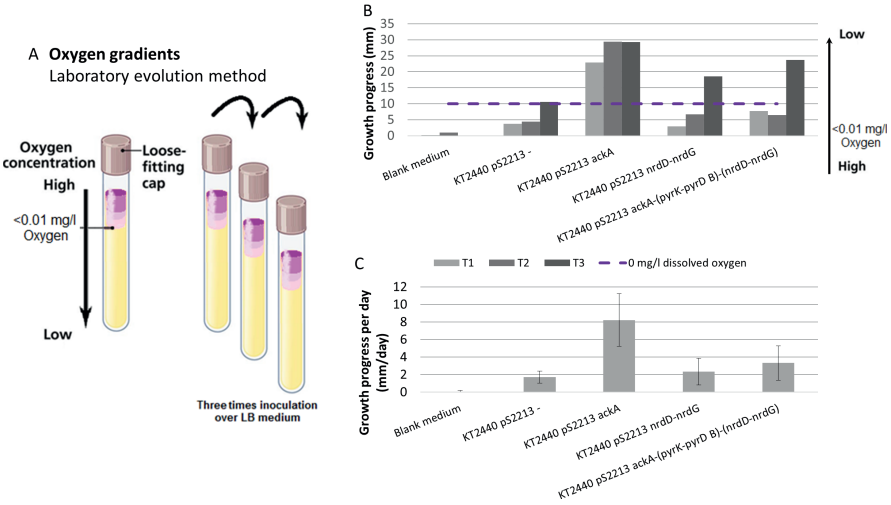

Figure 4.5: Testing of transformed strains under micro-oxic conditions. A) Screening assay using an adaptive laboratory evolution method with oxygen gradients. Recombinant strains were passed three times over oxygen gradients to adapt the strains to micro-oxic conditions in LB medium. B) Ingrowth in oxygen gradients over consecutive rounds. The first adaptation round was monitored over two days, followed by two four-day cycles. Strain performance of P. putida KT2440 pS2213 -, KT2440 pS2213 ackA, KT2440 pS2213 nrdD-nrdG and KT2440 pS2213 ackA-(pyrK-pyrD B)-(nrdD-nrdG) throughout the experiment was monitored continuously with a time-lapse camera set-up, and was depicted as ingrowth in mm from the surface of the growth medium down. Above the purple dashed line the oxygen concentration is <0.01 mg/L. Non-inoculated medium was taken as control. C) The average progression of the recombinant strains in the oxygen gradients over time, depicted in mm/day.

Discussion

We discuss below the work described along four major lines:

1) The integrated computational analysis herein described provided a coherent, systems-wide basis to understand the factors underlying the aerobic nature of *P. putida*. By enabling to explain previous unsuccessful experimental efforts aiming at making *P. putida* grow under anoxic conditions, this endeavour emphasised the value of applying an *a priori* systems, model-driven design perspective to metabolic engineering, as opposed to the common practice of designing experiments based on fractional knowledge of parts of the system. Also, the combination of GSM simulations and comparative genomics can be of widespread use as they are inherently complementary. GSMs, already available for many organisms [7, 9], describe an organism in high detail; whereas comparative genomics pinpoints the genetic basis underlying the differences between organisms.

2) According to the understanding of *P. putida* KT2440 metabolism as represented in iJP962, the adjustments we found through comparative genomics and protein domain content analysis should enable anaerobic growth. However, it must be noted that although this metabolic model offers a clear and direct insight into the known metabolism of *P. putida* KT2440, it is far from complete. Due to necessity, metabolic models represent a significantly simplified metabolism. Not enough experimental data is available to describe redox balances and regulatory pathways in a model in full detail, especially considering that much remains unknown about regulatory pathways and protein domain functions. The model contains all reactions known regardless if they are active or not, which means that pathways or reactions that are unlikely to be active under certain conditions are active in the model. The metabolic model therefore offers a good starting point, but should not be considered to provide a perfect design to the ambitious goals we set out. Hence, we rather see the model predictions regarding anaerobic growth as a guide towards achieving micro-oxic robustness.

3) When testing the fermentative design of *P. putida* KT2440 as detailed in Figure 4.5, we used adaptive laboratory evolution methods combined with insertion of the genes/gene clusters that enabled the strain to grow under micro-oxic conditions.

The oxygen gradient analysis proved a robust and reliable way to follow the progress of recombinant strains versus control strains under micro-oxic conditions within two to four days' time over multiple passages. Under oxic conditions, fluorescence levels (Figure 4.4) indicate that acetate kinase concentrations in *P. putida* KT2440 pS2213 *ackA* are higher than in KT2440 pS2213 *ackA-(pyrK-pyrD B)-(nrdD-nrdG)*. Under micro-oxic conditions KT2440 pS2213 *ackA* outperforms all other strains, including KT2440 pS2213 *ackA-(pyrK-pyrD B)-(nrdD-nrdG)*. This suggests that acetate kinase directly leads to improved performance under micro-oxic conditions: the more acetate kinase, the larger the ATP availability, the better the strain performance under micro-oxic conditions. However, the accumulation of acetate due to AckA activity could lower the pH to a growth-inhibiting environment. The production of acetate (yielding ATP) could also reduce carbon flow towards the product of interest, lowering the yield. Yet, acetate is just one way to increase the ATP pool. Other options include growing cells on more energy-ready carbon sources such as gluconic acid, knocking out energy demanding pathways [12], applying a different terminal electron acceptor by offering an anode in combination with phenazines or electron mediators in a bioelectrochemical system [17, 18] or switching to anaerobic respiration [16]. The use of genetic switches at the level of acetyl-CoA could also be used to alleviate the potential toxicity exerted by acetate [49]. The experimental set-up with overall improved strain performance in micro-oxic conditions over multiple oxygen gradient passages shows the value of an adaptation set-up in relation to GSM predictions. GSM predictions regarding growth rates and fluxes typically relate better to strains already having undergone adaptive laboratory evolution [50, 51]. Hence, improvement of strain performances under micro-oxic conditions is expected upon further adaptive laboratory evolution.

4) After multiple rounds over the oxygen gradients, dihydroorotate dehydrogenase and ribonucleotide triphosphate reductase had an added benefit in this assay. The ability of growing under micro-oxic conditions of KT2440 pS2213 *nrdD-nrdG* and KT2440 pS2213 *ackA-(pyrK-pyrD B)-(nrdD-nrdG)* after three passages over oxygen gradients, as opposed to *P. putida* KT2440 pS2213 –, indicate the added value of these genes. However, pyrK-pyrD B and nrdD-nrdG are expected to be only of value under anoxic conditions, since aerobic endogenous versions of these genes are

present in the KT2440 genome. We expect that a switch to anoxic conditions will result in the exclusive use of the anaerobic versions of the proteins.

These four items represent as well the core elements of the Design-Build-Test-Learn (DBTL) engineering cycle of Synthetic Biology [52]. This integrated, highly iterative workflow illustrates how such strategies can efficiently assist lifestyle engineering of industrial traits and provide the means to enable a new era in tailored, agile biomanufacturing.

Conclusions

The obligate aerobic metabolism of *P. putida* is a major obstacle for the breakthrough of this bacterium to be widely used as a biotechnological host. The integrated computational analysis herein described provided a coherent, solid basis to understand the factors underlying the aerobic nature of *P. putida*. The model-driven redesign of an obligate aerobe into a strain tolerant to low-oxygen concentrations constitutes an important fundamental step in the rational engineering of such biological systems.

The initial design addressed five metabolic bottlenecks: protoporphyrinogen oxidase (*hemY*), L-aspartate oxidase (*nadB*), dihydroorotate dehydrogenase (*pyrD*), ribonucleotide-triphosphate reductase (*RNRs*) and anaerobic energy conservation (through substrate-level phosphorylation by acetate kinase, *ackA*). This design could be brought back to the addition of five genes upon further evaluation. Introduction of *hemY* was determined not to be essential as the endogenous HemJ can possibly fulfil its role and *P. putida* NadB was experimentally proven to function under anoxic conditions.

Upon testing the final design under micro-oxic conditions, the increased availability of ATP through the synthesis of acetate in *P. putida* KT2440 pS2213 *acka* results in growth, unlike the wild-type strain. The importance of ATP availability under anoxic conditions was demonstrated earlier [6, 15]. Moreover, an increased ATP pool may also have beneficial effects on other processes, including thermo-tolerance [12, 53]. This work thus confirms that insufficient ATP generation is a main bottleneck for cell growth under oxygen-limiting conditions.

The final design included three enzymes. The two newly introduced gene sets,

pyrK-pyrD B encoding for class I dihydroorotate dehydrogenase and *nrdD-nrdG* encoding for class III ribonucleotide triphosphate reductase, recognized through the *in silico* approach, proved effective upon introduction after multiple rounds of adaptive laboratory evolution pressure. This not only highlights the value of the *in silico* approach, but also demonstrates the importance of design testing under micro-oxic conditions to allow for strain adaptation. Together, these enzymes make up the anaerobic version of the pathway for dNTP production, an essential pathway for strain performance under anoxic conditions.

Combined they achieve the highest impact, although sole introduction of *nrdD-nrdG* also enabled improved micro-oxic performance on its own. This suggests that *nrdD-nrdG* is the main bottleneck of the two metabolic activities. The impact so far does not compare to the implementation of *ackA*, since the aerobic homologs are still present and functional. We believe that these newly recognized genes will prove to be essential under fully anoxic conditions.

Altogether, this work provides insights into the non-linear process of transforming a strict aerobic species into a facultative anaerobic bacterium.

Competing interests

The authors declare that they have no competing interests. All data generated or analysed during this study are included in this published article and its supplementary information files. VAPMdS gratefully acknowledges financial support from the Wageningen University IP/OP project, the European Horizon 2020 projects EmPowerPutida (Project reference n°635536) and IBISBA (Project reference n°730976) and the TTW-NWO project Safechassis (Project reference n°15814). PIN gratefully acknowledges the Novo Nordisk Foundation for financial support. The funders had no role in study design, data collection and analysis, or preparation of the manuscript.

Author's contributions

Conceived the project: RvH/VAPMsD Conceived the *in silico* study: RvH/MSD/-VAPMdS Conceived and designed the experiments: LFCK/PJS Performed the GSM simulations: RvH/SD Performed the Comparative Genomics: SD/ES Plasmid design and construction: SD/PIN Growth and fluorescence experiments: LFCK Oxygen Gradients method development and experiments: LFCK/RJMV Data Analysis: LFCK/RvH/SD Work Supervision: RJMV/MSD/RvH/PJS/PIN Wrote Manuscript: RvH/LFCK/PJS/RJMV/VAPMdS Arranged Funding: VAPMdS

Acknowledgements

We thank dr. R. A. Weusthuis for fruitful discussions regarding the metabolic insights in this project, prof. dr. ir. A. J. M. Stams for advice while designing and testing the oxygen gradients, F. Gillissen for the use of the micro-electrode, and prof. dr. V. de Lorenzo for helpful discussion and hosting SD in his lab for this project.

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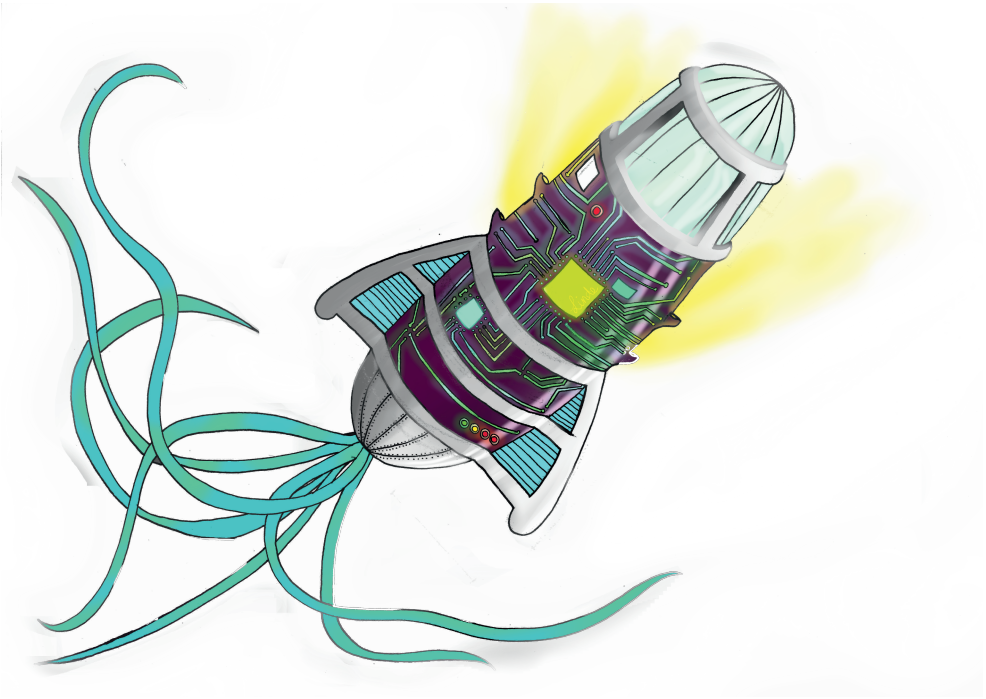
5

A Rational Design of *Pseudomonas putida* KT2440 capable of Anaerobic Respiration



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Submitted for publication

Abstract

Pseudomonas putida KT2440 is a metabolically versatile, HV1-certified, genetically accessible, and thus interesting microbial chassis for biotechnological applications. However, its obligate aerobic nature hampers production of oxygen sensitive products and drives up costs in large scale fermentation. The inability to perform anaerobic fermentation has been attributed to insufficient ATP production and an inability to produce pyrimidines under these conditions. Addressing these bottlenecks enabled growth under micro-oxic conditions, but does not lead to growth or survival under anoxic conditions.

Here, a data-driven approach was used to develop a rational design for a *P. putida* KT2440 derivative strain capable of anaerobic respiration. To come to the design, data derived from a genome comparison of 1628 *Pseudomonas* strains was combined with genome-scale metabolic modelling simulations and a transcriptome dataset of 47 samples representing 14 environmental conditions from the facultative anaerobe *Pseudomonas aeruginosa*.

The results indicate that the implementation of anaerobic respiration in this species would require at least 61 additional genes of known function, 3 externally added vitamins, and through guilt by association, 8 genes encoding proteins of unknown function.

Introduction

Pseudomonas putida KT2440 is a HV1-certified [1], genetically accessible [2–7] and metabolically versatile [8–10] species, which makes it an interesting adaptable industrial workhorse. However, its strict aerobic lifestyle is an industrial disadvantage [4, 11–14] as the strict requirement for dissolved O_2 results in increasing costs of large-scale cultivation and may lead to unstable production rates due to inadequate local oxygen supply due to oxygen fluctuations. Its strict aerobic nature also excludes production of O_2 sensitive enzymes and pathway intermediates as well as the production of O_2-sensitive products.

Most *Pseudomonas* species are facultative anaerobes and can use an inorganic compound such as nitrate as alternate electron receptor. This includes species that are closely related to the *P. putida* KT2440 strain, such as *P. fluorescens* and *P. denitrificans*. Only one *Pseudomonas* species is capable of anaerobic fermentation: *Pseudomonas aeruginosa* [15–18]. *P. aeruginosa* is capable of arginine fermentation and pyruvate fermentation, although the latter only leads to prolonged survival under anoxic conditions, not to growth [16–18].

The relatively short evolutionary distance between *P. putida* KT2440 and facultative anaerobic *Pseudomonas* species suggests that through the implementation of a rational engineering cycle, this strain can be adapted to a facultative anaerobic lifestyle. In earlier work a Design, Build, Test, Learn engineering cycle [19] was performed in an attempt to obtain an anaerobic fermentative *P. putida* KT2440 strain. Using genome metabolic models (GSMs) iJP962 and iJP746 combined with a protein domain comparison analysis (PCA) between six aerobic *Pseudomonas putida* strains including KT2440 and six facultative anaerobic *Pseudomonas* strains, three key enzymes were selected and included in the final design: acetate kinase (encoded by *ackA*), dihydroorororotate dehydrogenase (*nrdD-nrdG*) and ribonucleotide triphosphate reductase class II (*pyrK-pyrD B*). This design was built and the resulting recombinant strain showed growth under micro-oxic conditions (Chapter 4, [20]). Earlier work already described an increase in survival rates upon introduction of solely acetate kinase [4, 12], and since the model predictions used in the design can only consider either full oxic or full anoxic conditions, survival rates of the recombinant

strains under anoxic conditions needs to be tested.

Here, we (*i*) analysed the survival rate of the recombinant strains under anoxic conditions, (*ii*) identified limitations for anaerobic growth through respiration and, (*iii*) composed a new design for a recombinant *P. putida* KT2440 capable of anaerobic respiration. In pursuit of this goal we expanded upon earlier work using the current wealth of genome data available on *P. putida* and other *Pseudomonas* species by inclusion of 1628 strains in an extensive comparative genome and domainome analysis [21]. Transcriptome data of 151 clinical isolates of the facultative anaerobic *Pseudomonas aeruginosa* cultures grown in 14 different conditions [22] was also taken into account and integrated with previous and newly obtained GSM simulation results [4, 20].

Materials and methods

Bacterial strains and cultivation conditions

Bacterial strains and plasmids used can be found in Table S1. For plasmid construction see previous work [20]. *E. coli* CC118λpir was used for cloning procedures and plasmid maintenance, and was routinely cultivated at 37°C in aerated conditions in LB medium (containing 10 g/l tryptone, 10 g/l NaCl and 5 g/l yeast extract), optionally containing antibiotics for selection (50 μg/ml kanamycin or 50 μg/ml ampicillin as indicated). For solid medium, 15 g/l agar was added to the medium. *P. putida* KT2440 was routinely cultivated under oxic conditions at 30°C in LB medium. Experiments were performed in De Bont minimal medium (DBGA) [23] (3.88 g/l K_2HPO_4, 1.63 g/l $NaH_2PO_4 \cdot 2H_2O$, 2.00 g/l $(NH_4)_2SO_4$, 0.1 g/l $MgCl_2 \cdot 6H_2O$, 10 mg/l EDTA, 2 mg/l $ZnSO_4 \cdot 7H_2O$, 1 mg/l $CaCl_2 \cdot 2H_2O$, 5 mg/l $FeSO_4 \cdot 7H_2O$, 0.2 mg/l $Na_2MoO_4 \cdot 2H_2O$, 0.2 mg/l $CuSO_4 \cdot 5H_2O$, 0.4 mg/l $CoCl_2 \cdot 6H_2O$, 1 mg/l $MnCl_2 \cdot 2H_2O$), with 20 g/l gluconic acid as the sole carbon source. In previous work, different carbon sources were tested for optimal performance [20]. Gluconic acid was used for optimal growth by eliminating ATP consumption for substrate uptake due to passive membrane transport. The medium was supplemented with 50 μg/ml kanamycin when indicated. Precultures were prepared aerobically overnight (o/n) at 200 rpm at 30°C.

Anoxic survival experiment

Oxygen gradients served to allow the recombinant strains to grow in micro-oxic conditions [20]. Anoxic cultivation of *P. putida* KT2440 recombinants unpassed or passed over oxygen gradients was performed at 30°C in 50 ml glass 20 mm aluminium crimp cap vials with rubber stoppers (Glasgerätebau Ochs laborfachhandel e.K.) in 30 ml DeBont GA with 1 mg/l resazurin and with 50 μg/ml kanamycin as selection marker for recombinant strains. Were indicated, a 1000x diluted vitamin mix (vitmix) was added (0.02 g/l biotin, 0.2 g/l nicotinamide, 0.1 g/l p-aminobenzoic acid, 0.2 g/l thiamin, 0.1 g/l panthotenic acid, 0.5 g/l pyridoxamine, 0.1 g/l cyanocobalamine, 0.1 g/l riboflavine). Before inoculation, the vials were gas exchanged with CO_2/N_2. Inoculation was done with aerobically pre-cultured bacterial sample at an OD_{600} of 0.05. Approx. 8 h after inoculation, the resazurin became completely colourless, indicating full anaerobic conditions. Samples were taken using sterile CO_2 flushed 1.5" Needles (BD Microlance) and 3-5 ml syringes (ThermoFisher) to avoid O_2 exposure. Anoxic conditions were ensured as the resazurin turned from colourless to bright pink within seconds in extracted samples. Survival rates were analysed by colony forming units (CFU) determination. A dilution series was made and five drops of 10 μl per dilution were applied onto LB-agar plates without selection marker, which were incubated o/n at 30°C. Colonies were counted manually, and photos were taken of the plates. Gram-staining was performed to ensure culture purity, according to manufacturers' instructions (gram-staining kit Machery-Nagel, Germany).

Statistical analysis

The reported experiments were independently repeated six times with biological triplicates in each separate experiment. Figures represent the mean values of corresponding biological triplicates and the standard deviation. The level of significance of the differences when comparing results was evaluated by means of analysis of variance (ANOVA), with α=0.05.

Genome annotation

The information of 16989 different *Pseudomonas* strains was obtained via the Gold database [24]. Per species, extensive literature research was performed to elucidate their aerobicity (Data S5). 1628 Different genomes of facultative anaerobic and strict anaerobic strains from the *Pseudomonas* genus (Data S5) were obtained from the EnsemblBacteria repository in March 2015 [25]. Metadata for all genomes obtained are present in the GOLD database repository as of March 2018 [24] and were de-novo annotated in SAPP [26] using Prodigal for gene prediction (version 2.6) [27], 2010] and with InterProScan version 5.4-47.0 [28] for functional annotation using Pfam. A similar analysis was performed on earlier work in [21].

Protein domain architecture-based clustering

The positions (start and end on the protein sequence) of domains having InterPro/Pfam [28] identifiers were used to extract domain architectures (i.e. combinations of protein domains). Protein domains were retrieved for each protein individually [21].

Persistence and essentiality analysis

The persistence of a gene in a taxonomic group or group of genomes can be defined as

$$Persistence = \frac{N(orth)}{N}$$

where $N(orth)$ is the number of genomes carrying a given ortholog and N is the number of genomes considered [21]. For the set of 1628 considered genomes. Orthologous genes were identify through identity of protein domain architectures taking into account copy number. Resulting protein domain contents were analysed through protein domain comparison (PDC).

Feature selection using random forest

The random forest classification algorithm was used to classify the genome sequences in aerobic and facultative anaerobic species with the goal to identify the domains (features) responsible for the separation in these two groups (feature selection). Three hundred randomly selected genomes from aerobic or anaerobic *Pseudomonas* species were selected to train random forest models. The process was repeated one hundred times. The resulting 100 different models were used to weigh 5831 protein domains from both aerobic and anaerobic *Pseudomonas* species. Variable selection was used to identify the most influential domains for classification in aerobic and facultative anaerobic strains, yielding 100 Gini coefficients, representing the importance of a protein domain for separation per domain. Gini coefficients were combined into a cumulative Gini. The resulting protein domains were separated into aerobic/anaerobic specific protein domains before further analysis.

Transcriptome data analysis

A publicly available *P. aeruginosa* transcriptome data set was retrieved from GEO database (accession number GSE55197) [22]. This dataset contains 47 samples corresponding to 14 environmental conditions, including changes in growth temperature, growth stage, osmolarity, concentration of ions in the media, and surface attachment and anaerobic respiration. For every gene the log_2 fold change of its expression values was calculated in comparing every possible conditions with anaerobic respiration. Missing or infinity values arising from genes with very low counts in some condition(s) were imputed to 0 or $\pm$ 4, according to the significance of the differential expression (False discovery rate, fdr < 0.05). Normalization, fold change computations and differential expression analysis were performed using the R package DESeq [29].

Genome-scale metabolic models

In this study we used the *P. putida* genome-scale metabolic models (GSMs) iJP962, iJN746 and iJN1411 [3, 5, 30]. iJN1411 was obtained directly from the authors [30]. GSM simulations were performed as in [20].

Results

Insertion of *acetate kinase* in *P. putida* KT2440 does not lead to extended strain survival under anoxic conditions

Designs to obtain *P. putida* strains surviving anoxic conditions were conceptually based on a lack of energy conservation and redox balancing [4, 11–14]. Expression of the acetate kinase gene from *P. aeruginosa* and *E. coli* was reported to result in extended survival under anoxic conditions [4, 12]. Expression of the acetate kinase gene (*ackA*) from *E. coli* combined with class I dihydroorotate hydrogenase (*pyrK-pyrD B*) and class III ribonucleotide triphosphate reductase (*nrdD-nrdG*) from *L. lactis* successfully led to growth under micro-oxic conditions (Chapter 4, [20]).

To determine the tolerance of a *P. putida* KT2440 negative control carrying an empty plasmid and the recombinant strain enriched with *ackA*, *pyrK-pyrD B* and *nrdD-nrdG* to anoxic conditions and to analyse the effect of adaptation over oxygen gradients performed earlier [20], a survival experiment of 18 days was performed. After inoculation at a standardized cell density under oxic conditions, the cultures were incubated overnight in capped gas-exchanged vials in oxygen-depleted medium (see Materials and Methods). The survival rate was determined by performing colony forming unit (CFU) counts at set time points over a period of 18 days, with T0 being the start of the experiment in anoxic conditions (Figure 5.1, Figure 5.4). The results showed that in anoxic conditions there is no significant difference in survival rates between the negative control and any of the recombinant strains tested. Under these conditions, only the positive control, *E. coli BW25113* harbouring an empty plasmid, survived.

A rational design of *P. putida* KT2440 capable of anaerobic respiration

The failure of the fermentative design [20] (Chapter 4) to grow under anoxic conditions could be explained by the heavy reliance on the two state of art genome-scale models (GSMs) used in this design, which currently do not include an accurate representation of the complete redox balance and its intricate involvement in the metabolism. Additionally, the analysis was performed using only a few strains

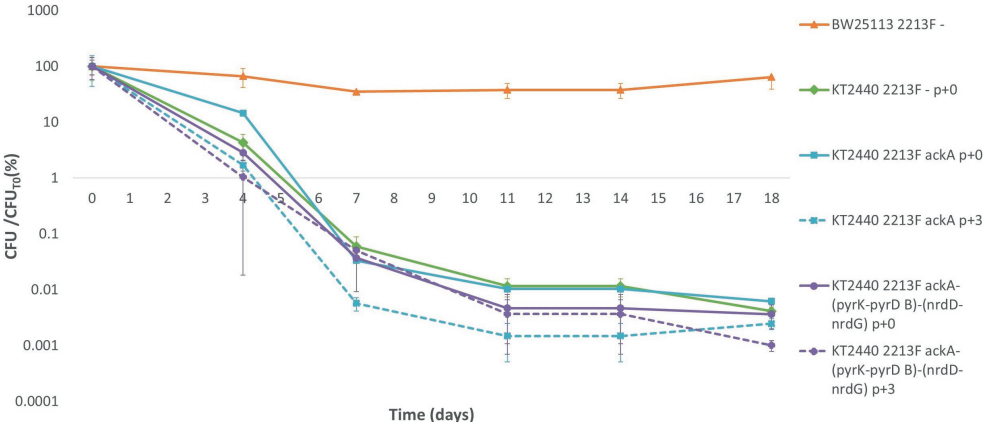

Figure 5.1: Survival experiment of P. putida KT2440 under anoxic conditions Survival under anoxic conditions was determined by comparing the number of colony forming units (CFU) over time with the number of CFU at T0. Escherichia coli BW25113 was used as positive control. Pseudomonas putida KT2440 with an empty plasmid (pS2213 -) was used as a negative control. The tested strains were Pseudomonas putida KT2440 with acetate kinase (pS2213 ackA) unpassed (p+0) or passed three consecutive times over oxygen gradients (p+3), and Pseudomonas putida KT2440 with acetate kinase, dihydroorotate dehydrogenase and ribonucleotide triphosphate reductase II (pS2213 ackA-(pyrK-pyrD B)-(nrdD-nrdG) unpassed (p+0) or passed three consecutive times over oxygen gradients (p+3). Results were processed with a level significance evaluated by analysis of variance (ANOVA), with α=0.05

while the protein domain comparison performed showed apparent differences between aerobic and anaerobic strains in availability of protein domains.

Many facultative anaerobic *Pseudomonas* species are incapable of anaerobic fermentation, but rather perform anaerobic respiration. The close phylogenetic distances between some of these facultative anaerobic *Pseudomonas* species and *P. putida* KT2440 may suggest that acquiring a facultative anaerobic lifestyle via anaerobic respiration would require less genetic changes. To come to a rational design of *P. putida* KT2440 capable of anaerobic respiration, the previous methods were thus expanded upon by i) using significantly more facultative anaerobic and aerobic *Pseudomonas* strains for domain analysis, ii) inclusion of iJN1411, a manually curated metabolic reconstruction of *P. putida* KT2440 [30] and iii) incorporation of an elaborate transcriptome analysis of anaerobic respiration of 115 isolates of *P. aeruginosa* grown under anoxic conditions compared to *P. aeruginosa* grown under 13 other conditions [22]. Inclusion of transcriptome data shows gene regulation,

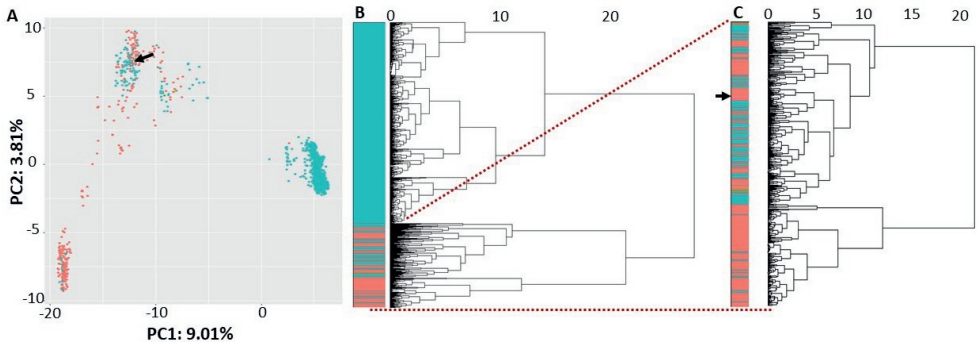

Figure 5.2: Principal component analysis of 1628 Pseudomonas strains. Blue indicates facultative anaerobic strains capable of respiration, red indicates aerobic species. (A) 2D Plot of PCA analysis. (B) Complete dendrogram derived from PCA analysis. (C) Specific cluster from dendrogram including P. putida KT2440, marked with black arrow. (D) Enlarged cluster containing P. putida KT2440, indicated in purple and with a black arrow.

improving the design by complementing the genome based methods.

For the protein domain comparison genomic data from 1628 *Pseudomonas* species was used. For each species oxygen requirements were determined via literature analysis. A principal component analysis was performed, creating a dendrogram based the binary distance (gene domain presence/absence) of the principal component analysis plot (Figure 5.2).

We assumed that domains essential for anaerobic respiration are highly persistent in facultative anaerobic strains, but show a lower persistence in obligate aerobic strains. The strategy to obtain this protein domain core is outlined in Figure 5.3. A "long list" of anaerobic protein domains was generated by comparing domain persistence between aerobic versus anaerobic strains. First a 95% persistence threshold was applied, to obtain a "domain core" of present in at least 95% of the genomes of "aerobic" strains and in the "anaerobic" strains analysed. These aerobic and anaerobic domain cores were used as input for subsequent comparative analysis and first list split into "shared between aerobic and anaerobic species" (Shared domain core), "specific for aerobic species" (Aerobe specific domain core) and "specific for anaerobic species" (Anaerobe specific domain core creating a long list of 427 anaerobe specific protein domains. A second long list was created by the same input but searching for the reverse, a separation based on domains with a very low persis-

tency in aerobic or anaerobic strains. For this threshold of no more than 1% was applied creating the second long list of 167 anaerobe specific protein domains. This process was repeated with a 90% and a 1% persistence threshold for a "restricted list", based on the early branch split between a large group of exclusively anaerobic *Pseudomonas* strains and a mixed group, including *P. putida* KT2440, containing 138 facultative anaerobic and 87 obligatory aerobic *Pseudomonas* strains (Figure 5.2 panel C).

The four different lists enriched in protein domains essential for anaerobic growth were compared to each other and manually further annotated. Results are summarized in Table 5.1 and Figure 5.3. The Random Forest analysis as described in the Materials and Methods section was used to add weight to the generated protein domain lists.

The transcriptomics data obtained [22] from 115 samples of *P. aeruginosa* grown under 14 different conditions was filtered based on extreme (up- or downregulation of ≥4 times in at least 3 of the conditions tested with all other conditions similarly regulated) differential gene regulation in all conditions when compared to anoxic growth. Differential regulation through all conditions versus all other conditions was performed to identify a general stress response. Genome-scale models were used to identify any reactions that could either directly or indirectly not function without the presence of oxygen [20].

All results were annotated by hand and filtered based on function and association. Domains of unknown function (DUFs) were filtered out unless an indirect link with anaerobic respiration could be made through guilt by association. A comparison was made between all different lists obtained from the metabolic models, protein domains analysis, transcriptomics data and previous efforts [4, 11, 12, 20] (Table 5.1) towards a design for anaerobic respiration, resulting in an extensive overview of the many hurdles that need to be overcome towards an *P. putida* KT2440 strain capable of anaerobic respiration (Figure 5.3).

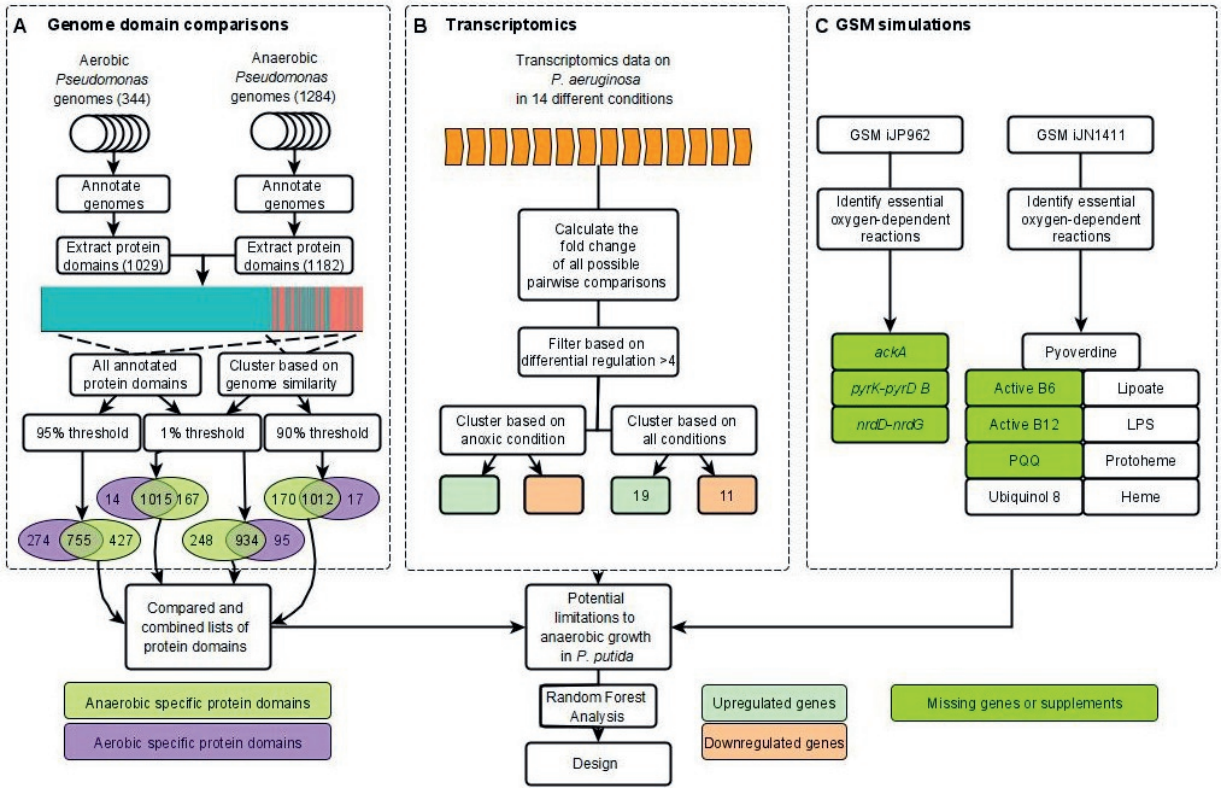

Figure 5.3: Overview of in silico approaches to identify limitations to anaerobic respiration in P. putida. A) Comparative genomics workflow. Genomes of the P. putida group and the anaerobic Pseudomonas group were systematically annotated using SAPP [21, 26], the protein domains were extracted, and both all domains or only the domains common to all anaerobic Pseudomonas species (the core domains) were selected using a 95% confidence interval. Analysis was performed on the whole set of genomes (left) or a genome cluster of closely related strains (right). Each of these methods resulted in a list of protein domains related to an aerobic lifestyle (purple) or an anaerobic life style (light green). B) Transcriptome analysis. C) GSM simulations. GSM iJP962 [5] and iJN1411 [30] were expanded with indicated reaction sets and tested for anaerobic growth under anaerobic conditions. Colours indicate final implementation in the design (green). Model and genome base predictions were combined to obtain a final design.

Table 5.1: Number of unique protein domains obtained from each separate in silico method

Method	# Unique protein domains
Genome domain comparisons	
Input aerobic domain core	1029
Input anaerobic domain core	1182
Long list, 1628 strains [95% threshold]	
Shared domain core	755
Aerobe specific domain core	274
Anaerobe specific domain core	427
Long list [1% threshold]	
Shared domain core	1015
Aerobe specific domain core	14
Anaerobe specific domain core	167
Restricted list, 225 strains [90% threshold]	
Shared domain core	1012
Aerobe specific domain core	17
Anaerobe specific domain core	170
Restricted list [1% threshold]	
Shared domain core	934
Aerobe specific domain core	95
Anaerobe specific domain core	248
Transcriptome analyses	175
GSM simulations	18
RandomForest [input]	5831
Domains with an accumulative influence ≥20	360
Domains with an accumulative influence ≥100	5

Design Considerations

The protein domain analysis of 1628 genomes yielded 1012 protein domains present only in anaerobic respiring *Pseudomonas* species (Figure 5.2, ES Data S6). Transcription data [22] of 5831 genes tested in 15 different conditions (including anaerobic respiration) yielded 87 upregulated genes and 85 downregulated genes (Figure 5.5, ES Data S7). GSM simulations yielded 18 metabolic reactions directly or indirectly requiring oxygen (ES Data S8) [20]. Lists were compared by evaluating the function of each gene starting with the encoded domain annotation, checking for domain co-existence in operonic structures and operon associated non-listed genes, comparing metabolic functions with GSM data, and with gene regulation. The weight of each protein domain was determined using the random forest analysis (Data S9). In this way the list could be brought back to 69 genes and a supplement of 3 vitamins to the medium that are deemed essential for *P. putida* KT2440 to enable anaerobic respiration.

The selected genes can be separated into various categories based on their functions: Nitrogen metabolism (49 domains in 37 genes), Hydrogenases (18 domains in 16 genes), Cytochrome C (3 domains in 3 genes), Pyrimidine and amino acid biosynthesis (4 domains in 2 genes, 3 vitamins added), ATP production (3 domains in 3 genes), and Domains of Unknown Function (indirectly associated with anaerobic respiration) (8 domains).

Nitrogen metabolism

Of the 61 known genes found vital for anaerobic respiration, 37 are either directly or indirectly involved in nitrogen metabolism. With nitrate as the final electron acceptor in anaerobic respiration, the largest amount of energy can be conserved as opposed to other final electron acceptors such as sulfate, iron(III), manganese(II), or selenate [31]. *P. putida* KT2440 lacks the nitrate/nitrite respiration pathway, which was resolved in earlier studies [11] by inserting either a Nir-Nar or Nor plasmid. This resulted in extended survival under anoxic conditions, but not growth. Our transcriptomics and protein domain analysis indicated that the combination of both the Nir-Nar and the Nor operon are required (Table 5.2). The operons include genes required for energy conservation, cofactor biosynthesis, amino acid biosynthesis, nitrogen metabolism, nitrate, nitrite and nitrogen transporters, nitrate, nitrite, nitric oxide and nitrous oxide reductases and several regulatory proteins (Table S3). Of the 49 protein domains or 37 genes we identified within this category, only 15 genes were previously found (*narK1*, *narK2*, *narG*, *narH*, *NarJ*, *narI narX*, *narL*, *nirF*, *nirQ*, *nirM*, *nirS*, *nirJ*, *nirL* within Nir-Nar operon, *norC*, *norB*, *norD*, *nosR* within the Nor operon) [11].

Previously unidentified genes include many transporters, and mechanisms to tap indirect sources of nitrate or nitrite. *Pseudomonas* species capable of anaerobic respiration apply these when nitrate or nitrite is scarce. Depending on the industrial process, it might be interesting if the recombinant *Pseudomonas putida* KT2440 could tap from different nitrogen sources or even use electron acceptors besides nitrate and nitrite, such as sulfate. Numerous genes could aid in alternative nitrogen sourcing. Only the genes which were found uniformly present in species capable of anearobic respiration and strongly associated with those of the nitrogen metabolism were included in the design.

Allantoicase (or allantoate amidinohydrolase) participates in purine metabolism, and facilitates the use of purines as secondary nitrogen sources under nitrogen-limiting conditions, which results in the production of ammonia and carbon dioxide using the uricolytic pathway, which is absent in *P. putida* [32]. A second example of an enzyme required for sourcing secondary nitrogen sources is methylaspartate

ammonia-lyase. This enzyme catalyses the second step of glutamate fermentation, a process in which L-threo-3methylaspartate is converted to mesaconate and ammonia. Ureohydrolases facilitate the ammonia to urea conversion, with urea as the principle product of nitrogen excretion.

Table 5.2: Respiratory design of facultative anaerobic P. putida KT2440. Genes to add for a P. putida KT2440 capable of anaerobic respiration

InterPro	PFAM	Name	Abbreviation	Function	Source
Nitrogen metabolism (37)					
	PF00491	**Ureohydrolases**		Ammonia to urea conversion (principal product of nitrogen excretion)	PDC
	PF00491	Arginase	ArgI	L-arginine + H_2O ↔ L-ornithine + urea	PDC
	PF00491	Agmatinase	SpeB	Agmatine + H_2O ↔ putrescine + urea	PDC
	PF00491	Formimidoylglutaminase	HutG	N-formimidoyl-L-glutamate + H_2O ↔ L-glutamate + formamide	PDC
		Proclavaminate amidinohydrolase	Pah	Amidinoproclavaminate + H_2O ↔ proclavaminate + urea	PDC
IPR015908	PF03561			allantoate + H_2O ↔ (S)-ureidoglycolate + urea	PDC, T
IPR015868	PF04960	Glutaminase		Glutamine + H_2O → Glutamate + NH_3	PDC, T
IPR000292	PF01226	Formate/nitrite transporter		Transport of Formate/Nitrite	PDC, T
IPR025736	PF13556	PucR C-terminal helix-turn-helix domain		PucR-like transcriptional regulators	PDC, T
IPR022665	PF05034	Methylaspartate ammonia-lyase N-terminus		L-threo-3-methylaspartate → mesaconate + NH_3	PDC, T
IPR022662	PF07476	Methylaspartate ammonia-lyase C-terminus		L-threo-3-methylaspartate → mesaconate + NH_3	PDC, T
IPR000825	PF01458	**Uncharacterized protein family (UPF0051)**		Chaperone proteins for nitrogenase production	PDC, T
			NifS	Metallocluster formation nitrogenase	PDC, T
			NifU	Metallocluster formation nitrogenase	PDC, T
IPR005346	PF03658	Ubiquitin	RnfH family	Electron transport	PDC, T
	PF02508	Rnf-Nqr subunit,		Nitrogen fixation membrane protein	PDC, T
	PF03116	**Nqr2 family**		Nitrogen fixation	PDC, T
		RnfD family		Nitrogen fixation	PDC, T
		RnfE family		Nitrogen fixation	PDC, T
	PF03060	Nitronate monooxygenase		Nitrogen metabolism	PDC, T
IPR010349	PF06089	L-asparaginase II		L-asparagine + H_2O → L-aspartate + NH_3	PDC, T
PA3862	PF02423		DauB	NAD(P)H-dependent anabolic L-arginine dehydrogenase	PDC, T, [11]
PA3863	PF01266		DauA	DauBAR operon	PDC, T, [11]
PA3864	PF08348		DauR	Transcriptional regulator of the dauBAR operon	PDC, T, [11]
	PF13309				PDC, T, [11]

Table 5.2: Respiratory design of facultative anaerobic P. putida KT2440. Genes to add for a P. putida KT2440 capable of anaerobic respiration

InterPro	PFAM	Name	Abbreviation	Function	Source
PA14_13750	PF07690	Nitrite extrusion protein (putative)	NarK2	Membrane proteins Transport of small molecules	PDC, T, [11]
PA14_13770	PF07690	Nitrite extrusion protein	NarK1	Membrane proteins Transport of small molecules	PDC, T, [11]
PA3875	PF14710		NarG	ATP generation	PDC, T, [11]
	PF00384				PDC, T, [11]
	PF01568				PDC, T, [11]
PA14_13800	PF13247	Nitrate reductase	NarH	β-subunit, ATP generation	PDC, T, [11]
	PF14711				PDC, T, [11]
PA14_13810	PF02613	Nitrate reductase	NarJ	λ-chain, ATP generation	PDC, T, [11]
PA14_13830	PF02665	Nitrate reductase	NarI	γ-chain, ATP generation	PDC, T, [11]
PA3878	PF02518	Two-component sensor	NarX	Nitrogen metabolism	PDC, T, [11]
	PF00672				PDC, T, [11]
	PF07730				PDC, T, [11]
	PF13675				PDC, T, [11]
PA3879	PF00072	Response regulator	NarL	Two-component response regulator	PDC, T, [11]
	PF00196				PDC, T, [11]
PA14_13850	PF04055	Heme d1 biosynthesis protein	NirJ	Heme d1 biosynthesis	PDC, T, [11]
PA0516	PF02239	Heme d1 biosynthesis protein	NirF	Heme d1 biosynthesis	PDC, T, [11]
PA0514		Heme d1 biosynthesis protein	NirL	Heme d1 biosynthesis	PDC, T, [11]
PA0520	PF07728		NirQ	Regulatory protein	PDC, T, [11]
	PF08406		CbbQ	Post-translational activation of Rubisco – photosynthesis	PDC, T, [11]
			NorQ,	Post-translational activation of Rubisco – photosynthesis	
PA14_06750	PF13442	Nitrite reductase precursor	NirM	Biosynthesis of cofactors, prosthetic groups and carriers	PDC, T, [11]
PA3870	PF00994	Molybdopterin biosynthetic protein A1	MoaA1	Biosynthesis of cofactors, prosthetic groups and carriers	PDC, [11]
	PF03453				PDC, [11]
	PF03454				PDC, [11]
PA14_13260	PF00994	Molybdopterin biosynthetic protein B1	MoaB1	Biosynthesis of cofactors, prosthetic groups and carriers	PDC
	PF00394	**Multicopper oxidase**			PDC, T
PA0519	PF13442	Nitrate reductase	NirS	ATP generation	PDC, T, [11]
		Nitrate reductase	NirS	ATP generation	PDC, T, [11]
	PF02239	Nitrate reductase	NirS	ATP generation	PDC, T, [11]
	PF05940	**NnrS protein**			PDC, T
			NirK	Reduction of nitrite to nitrous oxide	PDC, T
			Nor	Reduction of nitrite to nitrous oxide	PDC, T
PA14_06810	PF00034	Nitric-oxide reductase	NorB-NorC	Subunit B, C	PDC, T, [11]
PA14_06830	PF00115	Nitric-oxide reductase	NorB-NorC	Subunit B, C	PDC, T, [11]
PA14_06840	PF00092		NorD	Putative dinitrification protein	PDC, T, [11]
PA14_20230	PF04205		NosR	Regulatory protein for N_2O reductase	PDC, T

T, Trancriptomics; PDC, Protein Domain Comparisons; GSM, Genome Scale Modelling; [27, 28]

Hydrogenases

Included in the list are 16 hydrogenases. The redox state of the cell and the availability of O_2 are regulatory signals in facultative anaerobic species [33]. [FeFe]- and [NiFe]-hydrogenases are widely distributed under anaerobic species. These hydrogenases are only produced under anoxic conditions, and most [NiFe]-hydrogenases are inactivated by oxygen, only to be re-activated under reducing conditions [34].

Hydrogenases catalyse the reversible oxidation of molecular hydrogen, and thus play a regulatory role and balance the redox state. Hydrogen oxidation is coupled to the reduction of electron acceptors (such as oxygen, nitrate, sulphate, carbon dioxide and fumarate). *P. putida* KT2440 lack hydrogenases necessary for the reduction of nitrogen compounds, and the necessary hydrogenase chaperones, assembly, maturation and formation proteins (Table 5.3).

Of the 16 proteins vital for maintaining the redox balance in anaerobic conditions only 2 have been found in previous work [11].

Table 5.3: Respiratory design of facultative anaerobic P. putida KT2440. Genes to add for a P. putida KT2440 capable of anaerobic respiration

InterPro	PFAM	Name	Abbreviation	Function	Source
Hydrogenases (16)					
IPR027394	PF14720	NiFe/NiFeSe hydrogenase small subunit C-terminal		Oxidation of molecular hydrogen	PDC
IPR001501	PF00374	Nickel-dependent hydrogenase		activation of hydrogen	PDC
IPR006894	PF04809	Hydrogenase expression protein	HupH	Hydrogenase synthesis, C-terminal conserved region	PDC
IPR000671	PF01750	Hydrogenase maturation protease		Hydrogenase maturation	PDC
IPR002780	PF01924	Hydrogenase formation hypA family	HypA-HypF	Hydrogenase formation	PDC, [34]
IPR000688	PF01155	Hydrogenase/urease nickel incorporation	HypA	[Ni,Fe]-Hydrogenase and urease metallochaperone	PDC, [34]
IPR010893	PF07449	Hydrogenase-1 expression protein	HyaE	Hydrogenase assembly	PDC
IPR023994	PF11939	[NiFe]-Hydrogenase assembly chaperone	HybE	[NiFe] Hydrogenases assembly chaperones	PDC
	PF13237	4Fe-4S dicluster domain		Mediate electron transfer	PDC
IPR007038	PF04955	HupE / UreJ protein		Hydrogenase / urease accessory proteins.	PDC
PA0527	PF00027	Transcriptional regulator	DNR	Transcriptional regulators	[11]
	PF13545				[11]

T, Trancriptomics; PDC, Protein Domain Comparisons; GSM, Genome Scale Modelling; [27, 28]

Cytochrome C

Included in the list are 3 C-type cytochromes. C-type cytochromes account for a vital step in ATP bio-generation via the proton motive force (Table 5.4). Aerobically, the cytochrome BC1 complex requires oxygen as electron acceptor, yielding H_2O. Anaerobically, cytochrome C 551 (NirN), C 552 and cytochrome C oxidase CBB 3 transfer electrons to nitrate reductase (NirS) and nitric-oxide reductase (NorB-NorC). The importance of NirN and NirC (the precursor of NirN) was demonstrated in [11]. In addition, cobalamin-independent methionine synthase is important. This methionine synthase is a precursor of C 551 that can be produced without using vitamin B12. This might be a key component for anaerobic growth, since both the protein domain analysis and the GSM iJN1411 [30] predict that, amongst other vitamins, the active form of vitamin B12 can only be bio-generated in the presence of oxygen in *P. putida* KT2440.

The protein domain comparison analysis also indicates the need for cytochrome C 552, and for cytochrome C oxidase CBB 3 and its maturation protein. The enzyme cytochrome C nitrite reductase (C 552) catalyses the six-electron reduction of nitrite to nitrogen as one of the key steps in denitrification, Nitrogen is then reduced to ammonium in the nitrogen fixation pathway, where it participates in the anaerobic energy metabolism of dissimilatory nitrate ammonification. If *P. putida* KT2440 would be enriched with both the denitrification pathway and the nitrogen fixation pathway it could reduce nitrate or nitrite to ammonium, which can then be assimilated to organic compounds, transforming *P. putida* KT2440 in a diazotroph of agronomical importance. Expression of cytochrome CBB 3 oxidase allows agronomically important diazotrophs to sustain anaerobic respiration [35].

Table 5.4: Respiratory design of facultative anaerobic P. putida KT2440. Genes to add for a P. putida KT2440 capable of anaerobic respiration

InterPro	PFAM	Name	Abbreviation	Function	Source
Cytochrome-C (3)					
IPR003321	PF02335	Cytochrome C 552		Proton motive force cytochrome C oxidase biogenesis	PDC
PA0517	PF13442	Probable C-type cytochrome precursor	NirC	Cofactor biosynthesis	PDC, T, [11]
PA0521	PF00510	Probable cytochrome C oxidase subunit		Proton motive force	PDC, [11]

T, Trancriptomics; PDC, Protein Domain Comparisons; GSM, Genome Scale Modelling; [27, 28]

Pyrimidine and amino acid biosynthesis

Included in the list are 2 genes involved in pyrimidine and amino acid synthesis, and additional bottlenecks that can be solved by adding 3 vitamins to the medium. Earlier GSM simulations with iJP962 indicated that alternate genes must be inserted for dihydroorotate dehydrogenase and ribonucleotide triphosphate reductase type II for pyrimidine and ultimately DNA and RNA biosynthesis [20]. Both the protein domain analysis and GSM simulations using the iJN1411 metabolic model predict that cobalamin (vitamin B12), pyridoxal-5-phosphate (vitamin B6) and menaquinone (vitamin K2) cannot be produced under anoxic conditions.

Crespo *et al.* showed that class II RNRs depend on adenosylcobalamin or vitamin B12 (cobalamin) to generate its radical independently of oxygen [36]. Cobalamin is an complex essential cofactor for many enzymes mediating methylation, reduction and intramolecular rearrangements, and for methionine synthase. There is a recognised distinction between aerobic and anaerobic generation of cobalamin [37, 38]. The routes differ in terms of cobalt chelation (via CobNST complex in the aerobic pathway, via precorrin-2 with CbiK in the anaerobic pathway) and oxygen requirements. The enzymes CobI, CobG, CobJ, CobM, CobF, CobK, CobL, CobH, CobB and CobNST form the aerobic pathway. CbiK, CbiL, CbiH, CbiF, CbiG, CbiD, CbiJ, CbiET, CbiC and CbiA form the anaerobic route [28, 37, 39]. Surprisingly, the protein domain comparison yielded none of the enzymes of the anaerobic pathway for Vitamin B12 synthesis, but instead CobT and CbtB, both described important for the aerobic pathway. According to the extensive analysis, these specific protein domains linked to these genes are not present in aerobic species analysed but only in anaerobic species. It was found that in the anaerobic bacterium *E. limosum*, CobT functions as an activator for a range of lower ligand substrates including DMB, determining cobamide diversity. The specific function of CbtB is unknown [37, 38].

Vitamin B6 is required for a wide variety of processes [40]. There are many vitamin B6-dependent proteins involved in amino acid biosynthesis, amino acid catabolism, antibacterial functions, iron metabolism, carbon metabolism, nucleotide utilization, cofactors for biotin, folate and heme, NAD biosynthesis, cell wall metabolism, tRNA modification, regulate gene expression and biofilm formation.

Vitamin K2 is responsible for electron transport during anaerobic respiration. However, knock-out experiments in *E. coli* showed that upon loss of menaquinone and vitamin K1 only 3% of theorethical yield was obtained, but this was instantly revived to 44% upon supplementing of vitamin K1 or vitamin K2 [41], indicating vitamin K1 can partially make up for the loss of vitamin K2.

Rather than inserting all missing genes, in a minimal design setup, these vitamins can be supplemented to the medium (indicated in Table 5.5 with ∗). To determine any immediate effect on growth or survival rates, vitamin supplementation through the medium was tested, monitoring performance of all recombinant strains under anoxic conditions. This was done parallel to a survival experiment without vitamin mix added. No difference in growth rates or survival rates was found (Figure S4, Figure S5, Data S10, Data S11).

Table 5.5: Respiratory design of facultative anaerobic P. putida KT2440. Genes to add for a P. putida KT2440 capable of anaerobic respiration

InterPro	PFAM	Name	Abbreviation	Function	Source
Pyrimidine and amino acid synthesis (2)					
IPR002751	PF01891	Cobalt uptake substrate-specific transmembrane region	Vitamin B12 ∗	VitB12 biosynthesis	PDC
IPR006538	PF06213	Cobalamin biosynthesis protein	CobT	VitB12 biosynthesis	PDC
	PF09489	Probable cobalt transporter subunit	CbtB	VitB12 biosynthesis	PDC
	PF10531	SLBB domain	Vitamin B12	Vit B12 uptake	PDC
		Adenosylcobalamin	Vitamin B12	Cofactor for enzymes and proteins	GSM
	PF02621	Menaquinone	Vitamin K2 ∗	Electron transport	PDC, [41]
		Pyridoxal 5 phosphate	Vitamin B6 ∗	Cofactor for proteins and enzymes	GSM, [40]
		Dihydroorotate dehydrogenase	PyrK-PyrD B	Pyrimidine biosynthesis	GSM, [20]
		Ribonucleotide triphosphate reductase type II	NrdD-NrdG	Pyrimidine biosynthesis	GSM, [20]

T, Trancriptomics; PDC, Protein Domain Comparisons; GSM, Genome Scale Modelling; ∗ can be added as vitamin to medium; [27, 28]

ATP Generation

Of the 61 genes known genes required for anaerobic respiration, 3 are involved in ATP generation. Both the protein domain analysis, transcriptomics data and metabolic modelling with iJP962 and iJN1411 indicate that ATP production remains one of the main bottlenecks to tackle. Earlier work has come to the same conclusion and tackled this by insertion of genes for acetate production or ethanol production [4, 12]. Our protein domain analysis has elucidated specific ATPases that only occur

in anaerobic strains, providing an alternative to ATP production by fermentation (Table 5.6).

Table 5.6: Respiratory design of facultative anaerobic P. putida KT2440. Genes to add for a P. putida KT2440 capable of anaerobic respiration

InterPro	PFAM	Name	Abbreviation	Function	Source
ATP Generation (3)					
IPR002736	PF01874	ATP:dephospho-CoA triphosphoribosyl transferase		Triphosphoribosyl-dephospho-CoA production	GSM
IPR017557	PF10620	Phosphoribosyl-dephospho-CoA transferase	MdcG	Triphosphoribosyl-dephospho-CoA production	GSM
		Acetate kinase	AckA	ADP to ATP conversion by acetate production	PDC, GSM, [20]

T, Trancriptomics; PDC, Protein Domain Comparisons; GSM, Genome Scale Modelling; [27, 28]

Domains of Unknown Function

The protein domains analysis resulted in 270 unique protein domains of unknown function occurring in the genomes of anaerobic strains but not in aerobic strains. Based on contextual information, 8 were identified as important for anaerobic respiration. These were included in the design (Table 5.7). Similarly, 28 protein domains of unknown function were associated with virology factors or immunity, and could be excluded from the design. This leaves 244 protein domains of which the function is unknown and which can thus not be completely excluded from this design.

Table 5.7: Respiratory design of facultative anaerobic P. putida KT2440. Genes to add for a P. putida KT2440 capable of anaerobic respiration

InterPro	PFAM	Name	Abbreviation	Function	Source
Domains of Unknown Function (8)					
	PF09086	Domain of unknown function	DUF1924		PDC
IPR013039	PF07627	Domain of unknown function	DUF1588		PDC
IPR013036	PF07626	Domain of unknown function	DUF1587		PDC
IPR013042	PF07631	Domain of unknown function	DUF1592		PDC
IPR013043	PF07637	Domain of unknown function	DUF1595		PDC
IPR011727	PF09601	Domain of unknown function	DUF2459		PDC
	PF12981	Domain of unknown function	DUF3865		PDC
	PF02026	Domain of unknown function	RyR domain		PDC

T, Trancriptomics; PDC, Protein Domain Comparisons; GSM, Genome Scale Modelling; [27, 28]

Discussion

No extended survival under anoxic conditions after acetate kinase integration

The first rational design that was proposed [20] (Chapter 4) was based on two genome-scale models and genome domain comparison analysis of six anaerobic *Pseudomonas* species compared to six aerobic *Pseudomonas putida* species. Under micro-oxic conditions, the addition of acetate kinase, dihydroorotate dehydrogenase and class II ribonucleotide triphosphate reductase lead to growth. However, we did not find that *P. putida* KT2440 could survive anoxic conditions when introducing acetate kinase via a plasmid. The lack of improvement in survival rates can easily be explained when contemplating the novel design assembled in this research, as numerous factors such as an alternative electron acceptor or an anaerobically active cytochrome-C is missing. However, earlier research did report an increase in survival rate in *Pseudomonas putida* under anoxic conditions upon plasmid integration of acetate kinase alone [4, 12].

Sohn and colleagues tested *P. putida* with or without a plasmid carrying *ackA*. They used conical vials with medium purged with nitrogen and kept under anaerobic conditions 2 days prior to incubation. The cells added were from an aerobic overnight culture washed twice and transferred to the anaerobic chamber for incubation. The bottles were sealed from gas exchange with nitrogen, which only removes oxygen from the headspace, before incubation at 30°C. They then performed sampling with sterile needles every two days over a period of 8 days. Each sampling was followed by recharging the bottles with nitrogen [4]. No additives were used to remove oxygen from the medium, and oxygen concentrations in the medium were not checked. No blanks or positive controls were reported.

Nikel and colleagues tested *P. putida* KT2440 with or without *ackA* or *pdc-AdhB* [12]. They describe the use of modified M9 medium with K_2SO_4 instead of NaCl and yeast extract to supply additional vitamins. An oxic preculture was prepared up to mid-exponential growth phase, after which the cells were washed and incubated at 30°C in Hungate tubes, filled up to 75%. Resazurin was added to monitor oxygen levels only in the medium. Separate tubes were used for each sampling point to

avoid oxygen leakage during sampling. Anoxic biotransformation experiments were performed in separate commercial glass vials with teflon-stoppered metallic screw caps. In screw-cap vials, headspace chromatography was used to verify anoxic conditions only in the headspace. No blanks or positive controls were reported.

In our experiments, we have seen that both the medium and the headspace contain much oxygen. Both must be treated to remove oxygen for anaerobic conditions from the start of the experiment. If only one is treated, oxygen depletion takes up to 12h. The medium can be prepared with L-cysteine or sodium thioglycollate to remove oxygen and counter oxygen leakage. Medium without any supplements is very easily oxygenated in case of an oxygen leak [20]. The headspace must be gas-exchanged to remove oxygen. Resazurin staining indicates when levels drop below a detectable level (determined with micro-electrode at 0.01 g/l dissolved oxygen, as seen in previous work [20], chapter 4), but does not indicate micro-oxic levels of oxygen. Using screw-caps on anaerobic vials, even though a wide applied method, risks oxygen leakage. Oxygen leakage is clearly apparent in anaerobic experiments as CFU count suddenly increases compared to the sampling time before. Additionally, we flushed the sterile syringes used for sampling prior to sampling, to counter oxygen leakage there. Repeating this method of testing six times over a longer period of time (minimally 9 to maximally 31 days). None of the work mentioned applied a blank or a positive control to verify their method.

We showed no extended survival of the strains upon introduction of *ackA*. Even though only Sohn and colleagues and Nikel and colleagues performed this same experiment, anoxic conditions prove so exact that to consider extended survival under anoxic conditions all previous work should be re-evaluated. This proves it is extremely challenging to acquire anoxic conditions.

The importance of the redox balance alone is shown when using bio-electrical systems [13, 14, 42, 43], resulting in sustained metabolic activity and growth upon the introduction of an external redox mediator under oxygen limiting conditions.

Technical difficulties in building a design

Previous designs included between 3 and 24 genes that should be incorporated in the *P. putida* KT2440 genome to enable an anaerobic lifestyle [4, 11, 12, 20], but our *in silico* methods shows this to be almost at least three times that. Novel methods developed specifically for integration of large operons or multiple genes like yTREX [44] allow incorporation of approximately 14 genes at one time in *P. putida*, which are all incorporated in the Tn5 or Tn7 sites in the genome. The 65 genes in our design are excluding 244 unknown genes, which complicate the task even further. Considering the short evolutionary distance between the anaerobic and aerobic *Pseudomonas* species, this research shows that the evolutionary gap is larger than expected.

The new design compared to previous designs

We elucidated that for anaerobic growth both Nir-Nor and Nar operons are vital. Comparing this to the experimental work of Steen and colleagues [11], where the nitrate operon or the nitrite operon was implemented. There do exist *Pseudomonas* species that naturally have but one of these operons and are capable of nitrate to nitrite transformation, but these belong to the category "Aerobic nitrogen respiration": they are shown to be incapable of growth in anoxic conditions [45, 46]. Another element that has not been addressed before is cytochrome C552, identified as specific for anaerobic respiration. Since it is well-known that energy production is already a bottleneck in anaerobic growth [4, 11, 12], this might be one of the key proteins missing from previous designs.

The most prevalent anaerobic dissimilatory nitrate respiration regulator DNR is one of the key hydrogenases obtained from the protein domain comparison. In the facultative anaerobic *E. coli*, knock-out mutants for *dnr* ortholog, *fnr*, were unable to grow under anoxic conditions. By DNA microarray technology it was shown that in *E. coli* 49% of the genes which differ in expression under anoxic and oxic conditions are regulated by FNR [33]. The two-component aerobic respiratory control system (ArcA and ArcB) control gene transcription in *E. coli* under anoxic conditions. Mutations in this system are known to affect expression of over 30 operons. Most of

these are repressed under anoxic conditions, but cytochrome *d* oxidase and pyruvate formate lyase are activated. In *E. coli*, ArcA and FNR are deemed essential for anaerobic activation [47]. In an anaerobic respiratory design of *P. putida* KT2440, it is debatable whether regulatory genes are required. We deem this advisable, in order to maintain optimal functionality of this strain under oxic conditions next to gaining the anaerobic respiration treat. These genes are thus included in the final design.

We argue that for a lifestyle shift from a strict aerobic lifestyle in *P. putida* KT2440 to an anaerobic respirative one, all these genes are required. However, an increase of strain performance under micro-oxic conditions or prolonged survival rates under anoxic conditions significantly improves strain robustness in large scale bioreactors with fluctuating oxygen levels. Hence, each step towards an anaerobic lifestyle may substantially ease processes in large scale bioreactors. For enhanced performance under micro-oxic conditions, it was demonstrated that increasing ATP production alone through acetate production is enough [20]. For prolonged survival rates, however, these key elements include both Nir-Nar and Nor operons for denitrification and nitrogen fixation, cytochrome C 552, and external supplementation of the lacking vitamins. This conclusion is supported by previous findings that energy supply and redox balancing are the main bottlenecks in an anaerobic lifestyle [4, 11–14, 20].

The role of metals

Many of the genes found in the design are closely linked to metal transport, including many hydrogenases and genes for pyrimidine and amino acid biosynthesis. Before oxygen arose in the Earth's atmosphere, oceans were saturated with metal ions like manganese (Mn^{2+}) and molybdenum (Mo^{6+}), with soluble iron (Fe^{2+}) being the most common. However, oxygen reacted with soluble Fe^{2+} to form insoluble iron oxides as Fe^{3+}, causing a drop in the bioavailability of Fe^{2+}. The reverse occurred with copper, which upon reacting with oxygen passed from insoluble Cu^{+} to soluble Cu^{2+}. In microorganisms, this change is still visible. Since early (anaerobic) life was constrained to the use of Fe^{2+} in an anoxic environment, these bacteria have had to adapt to avoid, adapt, detoxify or use Fe^{3+} or Cu^{2+} in a different way than

aerobic bacteria, leading to main differences between an aerobic and an anaerobic lifestyle. This is extensively reviewed [48]. This adaptation has had a substantial impact on the regulation of the aerobic and anaerobic redox balance. Metal toxicity is mainly caused by Cu^{2+}, which is more toxic than Fe^{3+}. Reasons for this can be summarized as (i) Cu^{2+} has a higher capacity compared to Fe^{3+} to non-specifically bind biomolecules and thus provokes higher oxidative damages, (ii) Cu^{2+} can catalyze the conversion of Fe^{2+} to the more toxic Fe^{3+}, and (iii) through a series of reactions, Cu^{2+} causes the production of large quantities of radical OH. As strict aerobic species are completely wired to resolve these toxicity problems and mainly use Cu^{2+} in their metabolism, a switch to anoxic conditions–and thus a switch to the bioavailability of Fe^{2+} instead of Cu^{2+}–poses serious problems, which so far have not been addressed in an attempt to change the strict aerobic lifestyle to a facultative anaerobic one [4, 11, 12, 20]. Promising results obtained using electrode systems highlight the importance of redox balancing under anoxic conditions [13, 14, 42].

Domains of unknown function are unexplored territory

A major bottleneck of the respiratory anaerobic respiration design could be hidden within the 270 DUFs resulting from the genome domain comparison. Without knowing their exact function, these genes cannot entirely be excluded from the design. Eight of these are somehow associated with survival and/or growth in anoxic conditions [28]. The possible importance of unknown genes was earlier found by Hutchison and colleagues [49], who in their attempt to make a minimal bacterial genome, unexpectedly found 149 genes with unknown biological function to be essential for their cell to grow. However, of over 1000 unique genes of known function initially deemed necessary for anaerobic respiration based on the combined *in silico* methods, only 53 genes (roughly 6%) made it to the final selection based on function, annotation and literature, so we can be confident that not all of the 270 unknown DUFs will be vital for anaerobic growth. Future efforts could include more *in silico* research of the DUFs found followed by systematically knocking out the DUFs in anaerobic species to see if it hampers anaerobic respiration, proving their importance for the design.

Conclusion

Increased ATP generation by insertion of *acetate kinase* via a plasmid does not lead to prolonged survival rates of *Pseudomonas putida* KT2440 under anoxic conditions. This proves that increased performance under micro-oxic conditions does not guarantee prolonged survival under anoxic conditions. A *P. putida* KT2440 strain capable of anaerobic respiration would require the insertion of at least 69 genes into the genome and a supplement of 3 vitamins to the medium. The conversion of a strict aerobic species to a facultative anaerobic lifestyle by anaerobic respiration is a much more elaborate process than was thought before. The function of DUFs and their role in anaerobic respiration must be researched.

Acknowledgements

We gratefully acknowledge dr. P. I. Nikèl and dr. R. A. Weusthuis for insightful discussions regarding the metabolic insights and anaerobic experimentation in this project. We gratefully acknowledge financial support from the European Horizon 2020 project EmPowerPutida (Project reference: 635536). The funders had no role in study design, data collection and analysis, or preparation of the manuscript. All authors have read and approved the manuscript.

Author contributions

Conceived and designed the experiments: LFCK/JJK/MSD Performed the anaerobic experiments: LFCK Performed the GSM simulations: RGAvH Performed Comparative genomics: JJK/LFCK Performed transcriptomics analysis: MSD/LFCK Protein annotation: LFCK Final design construction: LFCK/AJMS Work supervision: AJMS/PJS Wrote manuscript: LFCK Revised manuscript: MSD/AJMS/JJK/PJS Arranged Funding: VAPMdS

Conflict of interest

The authors declare there are no conflicting interests.

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Supplementary data

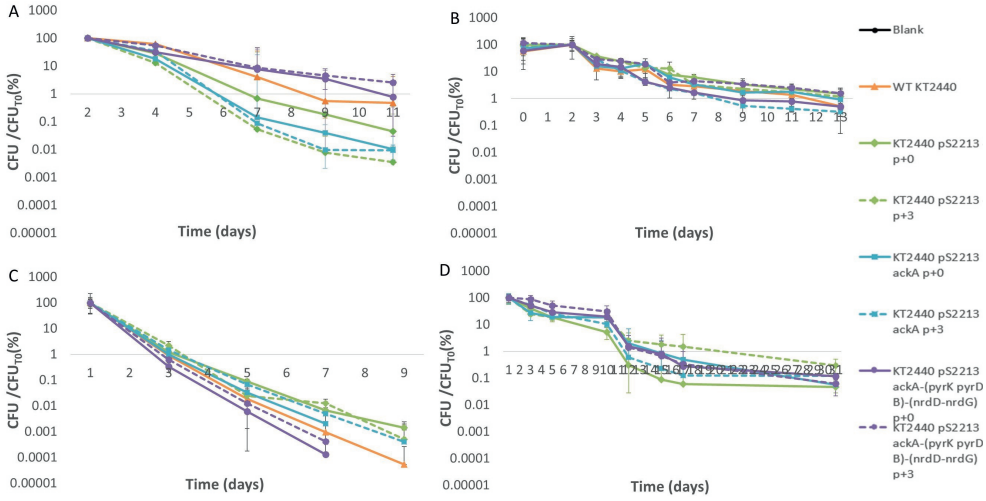


Figure 5.4: Survival experiment of P. putida KT2440 under anoxic conditions. The CFU determination of Pseudomonas putida KT2440 with an empty plasmid (pS2213 -), acetate kinase (pS2213 ackA) or acetate kinase, dihydroorotate dehydrogenase and ribonucleotide triphosphate reductase type II (pS2213 ackA-(pyrK-pyrD B)-(nrdD-nrdG) unpassed (p+0) or passed three consecutive times over oxygen gradients (p+3) survival under anoxic conditions. The experiment was repeated independently six times. All figures share the same legend. (A) Experiment 1 (B) Experiment 2 (C) Experiment 3 (D) Experiment 4

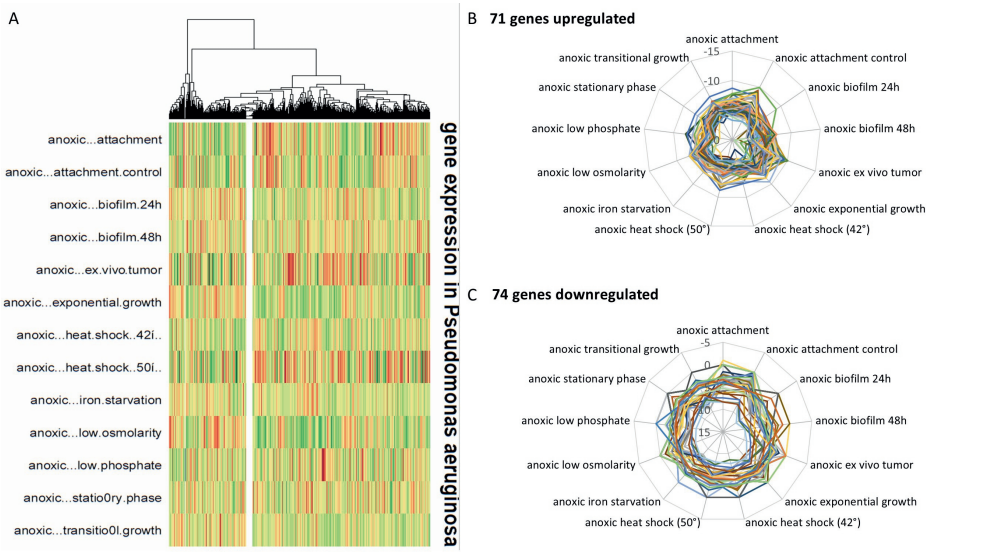


Figure 5.5: Transcriptomics of Pseudomonas aeruginosa PA01 in 15 different conditions. (A) Heatmap of up- (green) or downregulation (red) of all genes per condition. (B) All upregulated genes per condition. (C) All downregulated genes per condition.

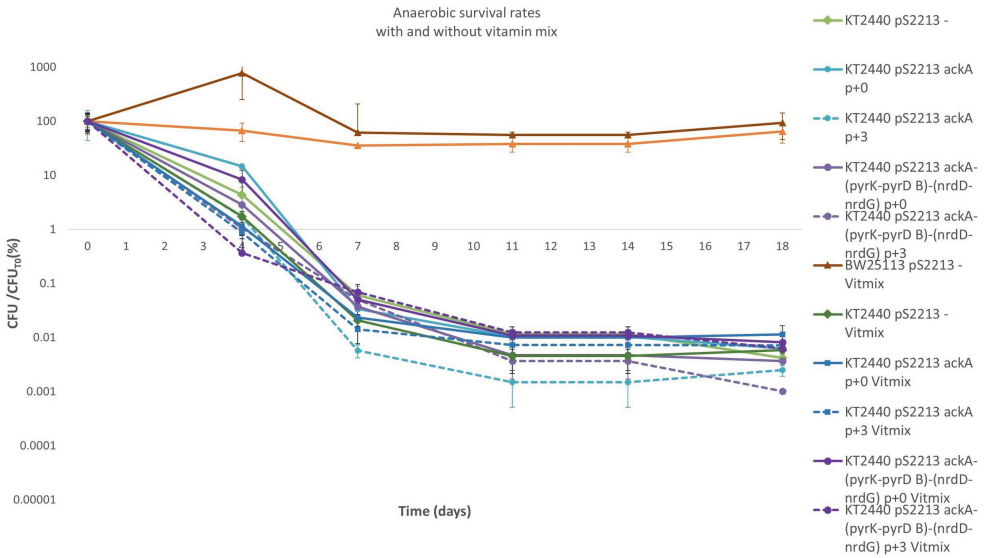


Figure 5.6: Survival experiment of P. putida KT2440 under anoxic conditions. The CFU determination of Pseudomonas putida KT2440 with an empty plasmid (pS2213 -), acetate kinase (pS2213 ackA) or acetate kinase, dihydroorotate dehydrogenase and ribonucleotide triphosphate reductase type II (pS2213 ackA-(pyrK-pyrD B)-(nrdD-nrdG) unpassed (p+0) or passed three consecutive times over oxygen gradients (p+3) with or without vitamin mix.

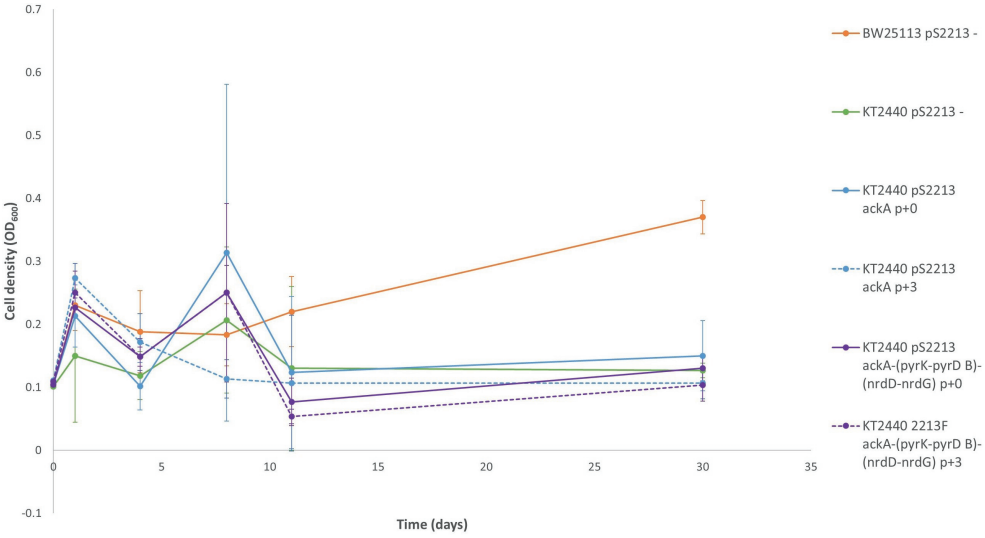
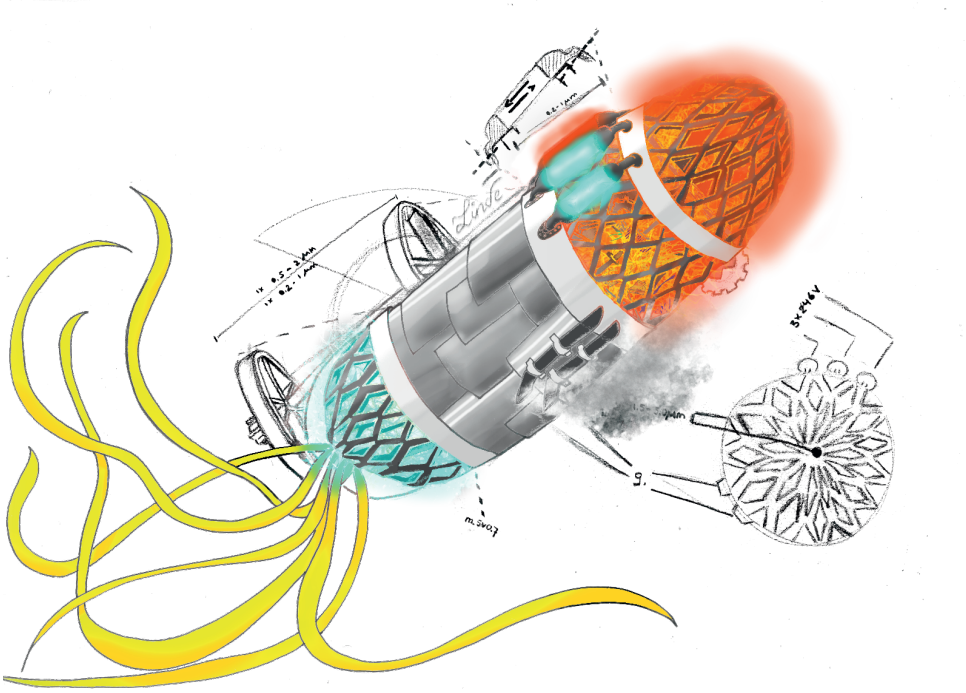


Figure 5.7: Growth experiment of P. putida KT2440 under anoxic conditions. The OD_{600} determination of Pseudomonas putida KT2440 with an empty plasmid (pS2213 -), acetate kinase (pS2213 ackA) or acetate kinase, dihydroorotate dehydrogenase and ribonucleotide triphosphate reductase type II (pS2213 ackA-(pyrK-pyrD B)-(nrdD-nrdG) unpassed (p+0) or passed three consecutive times over oxygen gradients (p+3) with vitamin mix.

6 The Universal Connection in Microorganisms Between NAD^+ Availability and Thermo-Tolerance



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Abstract

The universally applied essential redox factor NAD^+ is involved in internal signalling, defense, detoxification, circadian regulation, chromosome segregation, aerotaxis, sporulation, biofilm formation and dispersal. The redox homeostasis is often the first affected by internal and environmental changes.

Previous work described the thermal stability of thermophilic *L-aspartate oxidase*; the main bottleneck in the aspartate pathway for NAD^+ biosynthesis.

We determined the effect of exchanging the *L-aspartate oxidase* gene in different mesophiles for a thermophilic ortholog. In *E. coli*, the exchange results in a 10 times shorter lag phase compared to a negative control when the growth temperature is shifted to 44°C. In *P. putida* the exponential growth phase becomes twice as steep compared to a positive control when the growth temperature was shifted to 40°C.

To determine how widespread the connection between NAD^+ availability and thermo-tolerance is, the mesophilic or thermophilic aspartate pathway was implemented in yeast, which normally generates NAD^+ via the kynurenine pathway. Not only was *S. cerevisiae* able to use the aspartate pathway for NAD^+ generation, but supplemented with the bacterial pathway the strain proved more robust when grown at 41°C.

Introduction

The redox homeostasis is universally recognised as highly important in all living organisms [2]. In response to donor or acceptor availability, microorganisms adjust their metabolism to balance their redox state. Alterations in redox state often act as a primary signal for developmental or behavioural changes, thus facilitating the survival of the organism. Severe fluctuations in the redox potential, both internally or environmentally, are generally considered as stressors as they bring about major shifts in gene expression, affecting the metabolism and growth cycle, in either the organism itself or the entire population. Recent work in diverse microorganisms reveals that more subtle changes act as signals, eliciting responses in defence and detoxification. Moreover, the redox balance has a large influence on the circadian regulation and chromosome segregation, aerotaxis, sporulation and social behaviours, including biofilm formation and dispersal [3–5]. In redox homeostasis, the widely available redox factor nicotinamide adenine dinucleotide, or NAD^+, plays a major role. NAD^+ is an essential molecule in all living organisms. It functions as an electron carrier or cofactor in redox reactions, and serves as a substrate for the synthesis of cyclic ADP ribose or nicotinic acid adenine dinucleotide phos-

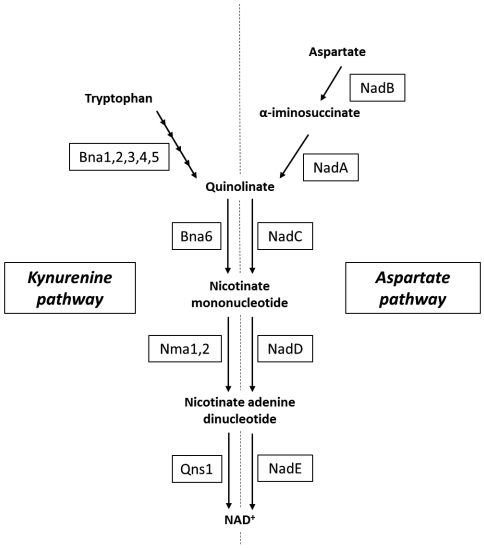

Figure 6.1: Overview of different de novo biosynthesis pathways of NAD^+. Nicotinate-nucleotide dephosphorylase (Bna6, eukaryotic) or decarboxylating quinolinate phosphoribosyltransferase (nadC, prokaryotic), nicotinamide/nicotinic acid mononucleotide adenylyltransferase (Nma1,2, eukaryotic) or nicotinamide mononucleotide synthetase (nadD, prokaryotic), and NAD^+ synthetase (Qns1, eukaryotic or nadE, prokaryotic) overlap in function between eu- and prokaryotes, respectively. In eukaryotes, NAD^+ is synthesizes in the mitochondria [1].

phate.

NAD^+ is generated *de novo* through the complex kynurenine pathway or the aspartate pathway (Figure 6.1). In addition, NAD^+ can be generated through salvage pathways [1, 3, 4, 6–13]. Eukaryotes generate NAD^+ mainly through the kynurenine pathway [14, 15], while most prokaryotes generate NAD^+ through the aspartate pathway [16, 17]. The kynurenine pathway and the aspartate pathway converge in the last three steps, to make NAD^+ from quinolinate.

This research will focus on the aspartate pathway (Figure 6.1). *De novo* biosynthesis of NAD^+ through the aspartate pathway proceeds through the condensation reaction between L-aspartate and dihydroxyacetone phosphate, catalysed by the quinolinate synthase system [18]. This proposed complex is composed of two enzymes: L-aspartate oxidase (NadB) which catalyses the oxidation of L-aspartate to iminoaspartate using O_2 as an electron receptor releasing H_2O_2 [19], and quinolinate synthase, which condenses iminoaspartate with dihydroxyacetone phosphate to produce quinolinate [20]. Despite the important role played by these enzymes in NAD^+ biogeneration, many of their biochemical and structural properties are still largely unknown (Table 6.1). As the rate limiting step in this pathway, NadB will be the focus of this research.

Table 6.1: Enzymes from the Aspartate pathway

Enzyme	Full name	Size	K_m	K_{cat}	Source
NadA	Quinolinate synthase	38.24 kDa	0.08 mM	0.12 s^{-1}	[20]
NadB	L-aspartate oxidase	60 kDa	4.1 mM	32 min^{-1}	[17]
NadC	Quinolinic acid phosphoribosyltransferase	36 kDa	6.4 μM	0.6 s^{-1}	[17]
NadD	Nicotinic acid mononucleotide adenylyltransferase	24.5 kDa	1.5 mM	15 min^{-1}	[17, 21]
NadE	NAD synthase	30.24 kDa	0.6 mM	1.8 min^{-1}	[17]

We set out to elucidate the effect of aspartate oxidase in relation to NAD^+ availability and external stress. As a stress factor, shifts in growth temperature were used. The *nadB* from *Escherichia coli*, *Pseudomonas putida*, thermophilic *Bacillus smithii* or mesophilic psychrophile *Trichococcus flocculiformis* were isolated and expressed via a plasmid system in *nadB* knock-out strains of *E. coli* and *P. putida*. Growth experiments were performed to test strain performance throughout temperature shifts both in oxic and anoxic conditions and diauxic shifts. The bacterial aspartate pathway of either *P. putida*, *B. smithii* or *T. flocculiformis* was also placed in the eukaryote *Saccharomyces cerevisiae* to determine if growth was affected by a temperature shift.

Materials and methods

Strains, cultivation conditions and media

Bacterial strains used are listed in Table 6.2. *E. coli* DH5α was used for cloning procedures and plasmid maintenance, and was routinely cultivated at 37°C under oxic conditions in LB medium (containing 10 g/l tryptone, 10 g/l NaCl and 5 g/l yeast extract), optionally containing antibiotics for selection (10 μg/ml chloramphenicol, 100 μg/ml ampicillin, 50 μg/ml kanamycin or 10 μg/ml gentamycin as indicated). For solid medium, 15 g/l agar was added to the medium. *E. coli* BW25113 and KEIO library strains JW2558, JW0733 and JW0105 were used for experiments. Experiments with *E. coli* strains were performed in LB medium or in M9 minimal medium (5x m9 minimal salts, 1M $MgSO_4$, 1M $CaCl_2$, Thiamin and 100x trace elements containing $(NH_4)_2SO_4$, 0.1 g/l $MgCl_2 \cdot 6H_2O$, 10 mg/l EDTA, 2 mg/l $ZnSO_4 \cdot 7H_2O$, 1 mg/l $CaCl_2 \cdot 2H_2O$, 5 mg/l $FeSO_4 \cdot 7H_2O$, 0.2 mg/l $Na_2MoO_4 \cdot 2H_2O$, 0.2 mg/l $CuSO_4 \cdot 5H_2O$, 0.4 mg/l $CoCl_2 \cdot 6H_2O$, 1 mg/l $MnCl_2 \cdot 2H_2O$), with 20 g/l glucose, 4 g/l L-asparate, or 10 g/l glycerol as sole carbon source as indicated. *P. putida* KT2440 was routinely cultivated under oxic conditions at 30°C in LB medium. Experiments were performed in LB medium or in De Bont (DB) minimal medium [22] (3.88 g/l K_2HPO_4, 1.63 g/l $NaH_2PO_4 \cdot 2H_2O$, 100x trace elements), with 20 g/l glucose, 20 g/l acetate, 4 g/l L-aspartate, or 10 g/l glycerol as sole carbon source as indicated. The medium was supplemented with 10 μg/ml gentamycin when indicated. The optical cell density was analyzed photometrically at 600 nm (OD_{600}).

Precultures were prepared overnight at 200 rpm at 30°C under oxic conditions. *Saccharomyces cerevisiae* BY4741 was routinely cultivated under oxic conditions at 30°C in YPD medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose). For solid medium, 15 g/l agar was added to the medium. Experiments with *S. cerevisiae* strains were performed in YNB medium (6.7 g/l Yeast Nitrogen Base w/o amino acids, 30 mg/l leucine, 20 mg/l methionine and 20 mg/l histidine, 20 mg/l uracil was added when wild type strain BY4741 without any plasmid needed to be cultivated). For solid medium, 20 g/l agar was added to the medium.

Table 6.2: Strains used in this study

Bacterial strains	Relevant characteristics	Optimal growth temperature	Growth temperature range	Source
Escherichia coli	Gram-negative mesophile	37°C	27 – 44°C	
DH5α	Cloning host; *ϕ80lacZΔM15 recA1 endA1 gyrA96* thi-1 hsdR17() supE44 *relA1 deoR Δ(lacZYA-argF)U169*			[11]
BW25113	*lacIq* rrNAT14 Δ*lacZWJ16* hsdR514 Δ*araBADAH33* Δ*rhaBADLD78*			[12]–[14]
JW2558	BW25113 Δ*nadB*			[23]
Pseudomonas putida	Gram-negative mesophile	30°C	25 – 40°C	
KT2440	Wild-type strain, spontaneous restriction-deficient derivative of strain mt-2 cured of the TOL-plasmid pWW0			[24]
KT2440 ΔnadB	KT2440 with a knocked out nadB gene			This study
Bacillus smithii	Gram-Positive motile, spore-forming, rod-shaped, facultative anaerobic, facultative thermophile	55°C	25 – 65°C	
DSM 4216T	Wild type strain			[25]
Trichococcus flocculiformis	Gram-Positive cocci, facultatively anaerobic, mesophilic psychrophile	25 – 30°C	4 – 40°C	
Echt (DSM 2094)	Wild type strain			[26]
Yeast strain				
Saccharomyces cerevisiae	Mesophile	33°C	3 – 45°C	[27]
BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0			[27]

Construction and transformation of plasmids

Plasmids used in this study are detailed in Table 6.2. For plasmids used in bacterial strains, the SEVA plasmid collection was used [28]. DNA segments were amplified by colony PCR using the Phire Green Hot Start II DNA Polymerase kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturers protocol. Clones were regularly checked by colony PCR and sequencing. All primer oligonucleotides used were purchased from Sigma-Aldrich Co. (ES Table 1). Restriction

enzymes were obtained from NEB (New England BioLabs inc.). Using the Standardized SEVA plasmid system [28], the cargo was designed with BamHI and EcoRI restriction sites on the 5' end and 3' end, respectively. DNA fragments were purified from agarose gel using the Machery-Nagel GmbH & Co. KG Gel Purification Kit (Machery-Nagel GmbH & Co. Düren, Germany). Plasmid inserts were verified by gel electrophoresis or DNA sequencing via Lightrun sequencing at GATC Biotech. T4 DNA Ligase (Roche Applied Science Indianapolis, IN USA) was used to ligate the isolated DNA fragments in the pSEVA 638 backbone.

The three plasmids constructed for use in yeast strains, pRV156, pRV157 and pRV178 (ES Table S1, ES Data S2), were constructed using the Yeast ToolKit described by Lee *et al.* [29], which is based on Golden Gate cloning. In short, the *nadABC* genes from both *P. putida* and *B. smithii* were amplified from gDNA using the oligos indicated (ES Table S1). After cloning the individual genes in the entry vector YTK001 with restriction enzyme *BsmBI*, gene cassette vectors were constructed with restriction enzyme *BsaI* in which each gene was combined with a promoter and terminator, both taken from the Yeast ToolKit. Each *NadA* gene was cloned downstream of promoter pHHF2 and upstream of terminator tADH1, each *NadB* gene was cloned downstream of promoter pTEF1 and upstream of terminator tPGK1, each *NadC* gene was cloned upstream of promoter pTEF2 and upstream of terminator tENO2. Finally, plasmids pRV156 containing *NadABC* (with their respective promoters and terminators) from *B. smithii* and and pRV157 containing *NadABC* (with their respective promoters and terminators) from *P. putida* were constructed by cloning them into a level 2 backbone plasmid with restriction enzyme *BsmBI*. pRV178 was contructed by cloning a 44-bp spacer (CTCTAGAGTCGACCTGCAGGCATGCAAGCTTAGGAGGAAAAACA) into the same backbone as pRV156 and pRV157. All three plasmids were transformed to electrocompetent *S. cerevisiae* BY4741 according to the method of Suga and colleagues [30, 31].

Table 6.3: Plasmids used in this study

Plasmids for bacterial strains	**Characteristics**	**Source**
pGNW *	Derivative of vector pEMG carrying $p_{14g} \longrightarrow$ *msfGFP*	[32]
pSEVA 6213S *	Helper plasmid; *oriV(RK2)*, $P_{EM7} \longrightarrow$ *I-SceI*, Gm^R	[32]
pSEVA 638 *	Expression vector, *pBBR1*, *xylS-Pm*, Gm^R	[28, 33]
pSEVA 638 nadBEco *	pSEVA 638 *nadB E. coli* BW25113	This study
pSEVA 638 nadBPpu *	pSEVA 638 *nadB P. putida* KT2440	This study
pSEVA 638 nadBBsm *	pSEVA 638 *nadB B. smithii* DSM 4216T	This study
pSEVA 638 nadBTfl *	pSEVA 638 *nadB T. flocculiformis* DSM 2094	This study
Plasmids for yeast strain		
pRV178	URA3, 2-micron ori, 44 bp spacer	This study
pRV156	pRV178 *nadABC B. smithii* DSM 4216T	This study
pRV157	pRV178 *nadABC P. putida* KT2440	This study

* Plasmids that belong to the SEVA (Standard European Vector Architecture) collection [28, 33]

Knock-out generation

For *E. coli* knock-outs, the KEIO library strains were used and verified via genome sequencing. To generate *P. putida* KT2440 knock-out strains, the accelerated genome engineering protocol from Wirth *et al.* was used [32]. The *AMUSER* tool was applied to design primers for amplification of ~100 bp upstream and downstream of the gene of interest. Via USER cloning, the homologous regions were amplified from *P. putida* KT2440 gDNA and assembled into pGNW. The resulting plasmid was accumulated with DH5α-λ*pir* cells before delivery in *P. putida* KT2440. Successful co-integration of the pGNW plasmid was determined by fluorescence. The resulting recombinant cells were transformed with pS6213S. Knock-out of the gene of interest was determined by loss of fluorescence, PCR analysis and genome sequencing.

Growth experiments

Plate-reader experiments were performed to monitor growth through cell density (OD_{600}) at varying temperatures, in different types of media and in oxic or anoxic conditions closely over periods of 24-72h. Recombinant *E. coli* or recombinant *P. putida* strains were precultured at 30°C under oxic conditions on the same medium as applied in the experiment. 96 Wells-plates were inoculated at a starting OD of 0.05. As a control, wells were prepared with un-inoculated medium. The plates were taped on the short sides to prevent condensation at higher temperatures.

Growth experiments with *S. cerevisiae* strains were conducted in glass test tubes with a loose fitting cap to ensure presence of sufficient oxygen. Biological triplicates of wild type and recombinant yeast strains were precultured at 30°C with shaking at 250 rpm in YNB medium. Glass test tubes with 3 ml medium were inoculated to a cell density of 0.2 at 600 nm. Each replicate of each strain was inoculated in YNB medium with and without 30 mg/ml aspartate. Cultures with both media were inoculated at 30°C and 41°C with shaking at 250 rpm. Cell density was measured every hour during the first 12 hours of incubation and then once 24 hours after start of experiment.

Transcriptomics

Cultures of *P. putida* KT2440 wild type (positive control), *P. putida* KT2440 Δ*nadB* *P. putida* KT2440 Δ*nadB* p638 empty (negative control), and *P. putida* KT2440 Δ*nadB* p638 *nadB* from *B. smithii* were harvested at mid-exponential growth phase when grown at 30°C and at 40°C. Harvested samples were washed with 0.9 % saline solution before they were flash-frozen at −80°C. RNA Isolation was performed using a Promega Maxwell machine, using the Maxwell 16 LEV SimplyRNA Purification tissue kit, according to the manufacturer's protocol. Quality control of isolated RNA was done by gelelectrophoresis, and Qsep analysis. Concentration was determined using a NoVix Fluorometer Spectrophotometer. Isolated RNA of high quality was sent to Novogene for sequencing.

Illumina reads were trimmed for low quality and adapters with fastp (v0.20.0) [34] using default settings. rRNA Sequences were removed with BBDuk(v38.35) [35]

using the following parameters k=31 and ref=riboKmers.fa.gz. Transcripts from the reference strain of Pseudomonas putida KT2440 (GCF_000007565.2_ASM756v2) were quantified with Kallisto (v0.46.0)[36] with a bootstrap value of 100. In R [37] transcript abundances were imported using the Bioconductor package tximportp [38] for differential expression analysis. Differential expression analysis was performed with DESeq2 [39] using the biological replicates for each condition. The statistical significance of gene expression differences was evaluated using an adjusted P-value < 0.05 and log2 (fold change)| ≥ log2 (1.5) as a threshold. Graphs were made using Microsoft Excel.

Statistical analysis

The plate-reader experiments were done using biological and technical triplicates. Other growth experiments were conducted with biological triplicates. Figures represent the mean values of corresponding biological and, if applicable, technical triplicates and the standard deviation. Data was normalized by dividing all OD values by OD at T0. The level of significance of the differences when comparing results was evaluated by means of analysis of variance (ANOVA), with α=0.05. The growth rate μ in the exponential phase between T4 and T7 in the yeast growth experiments was calculated as

$$\mu = \frac{ln2}{G} \tag{6.1}$$

where

$$Generation\ time\ \ G = \frac{7-4}{n} \tag{6.2}$$

and

$$Number\ of\ generations\ \ n = \frac{log_{10}OD600_{T7} - log_{10}OD600_{T4}}{log_{10}2} \tag{6.3}$$

Results

Expression of thermophilic *nadB* increases the resilience to high temperatures in mesophilic bacteria

A possible link between biogeneration of NAD^+ through the aspartate pathway and external stress was determined by focusing on the main pathway bottleneck, L-aspartate oxidase (*nadB)*, and exchanging this gene in mesophiles for the heterologous gene of thermophile *B. smithii* or psychrophile *T. flocculiformis*. Then, growth was monitored through a shift in growth temperature to determine strain thermotolerance. Thermo-tolerance is here defined as the temperature range at which a micro-organism sustains normal growth.

To this end, a pSEVA 638 plasmid carrying either the L-aspartate oxidase gene (*nadB*) from *P. putida* KT2440, *E. coli* BW25113, *B. smithii* DSM4216 or *T. flocculiformis* DSM 2094 was introduced in a *P. putida* KT2440 Δ*nadB* strain. A transformant with an empty plasmid was used as a negative control. The pSEVA 638 plasmid contains an inducible XylS-Pm promoter system, which when not induced with 3-methyl benzoate (3MB) results in basal expression levels of the genes (Figure 6.2), and a high expression level when induced with 3MB (Figure 6.3). The performance of the recombinant strains was monitored through a temperature shift from 30°C to 40°C. After grown overnight at 40°C, performance was determined at 41°C (Figure 6.2).

Overexpression of the recombinant genes by XylS-Pm induction with 3MB did not improve strain performance at any temperature tested. When looking at basal expression levels the replacement of the homologous gene with that of a bacterial species with a higher optimum growth temperature, either mesophilic *E. coli* or thermophilic *B. smithii*, caused the maximum growth rate to increase by a factor 2 when shifting the growth temperature from 30°C to 40°C. From 0.0453 to 0.0943 (*E. coli*) or 0.0922 (*B. smithii*). Isolation of the growing strains and increasing the temperature by 1°C to 41°C also resulted in growth in the recombinant strains carrying thermophilic *nadB*, as opposed to the positive control.

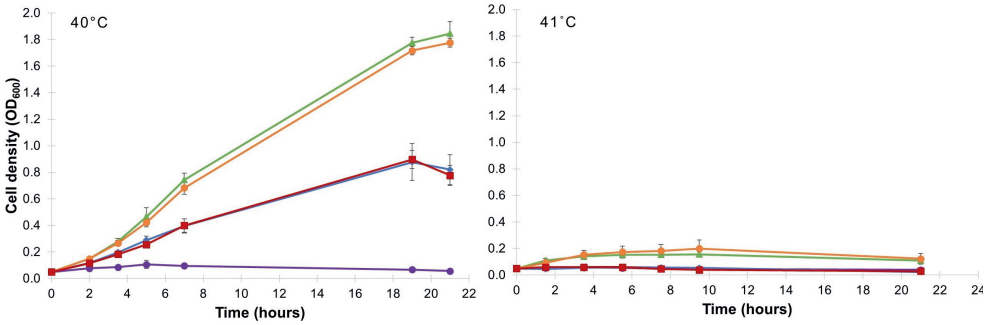

Figure 6.2: Growth experiment of P. putida at 40°C (left) and 41°C (right). Depicted are P. putida KT2440 ΔnadB pS638 - (Purple circle), nadBPpu (Blue diamond), nadBEco (Green triangle), nadBBsm (Orange circle), nadBTfl (Red square). Growth in wells was monitored over 21 hours in a platereader. Blank wells with un-inoculated medium were used to correct for background emission.

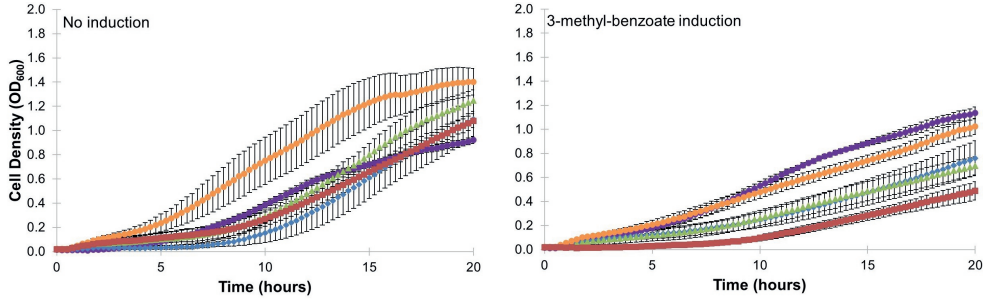

Figure 6.3: Induction experiment. Recombinant strain performance of P. putida KT2440 ΔnadB without induction (left) and with induction using 3-methyl benzoate (right). Depicted are P. putida KT2440 ΔnadB pS638 - (Purple circle), nadBPpu (Blue diamond), nadBEco (Green triangle), nadBBsm (Orange circle), nadBTfl (Red square). Growth in wells was monitored over 21 hours in a platereader. Blank wells with un-inoculated medium were used to correct for background emission.

An overview of differentially expressed genes by thermophilic *nadB*

The assumption is that strains harbouring a thermophilic *nadB* gene exert their effect through an increased NAD^+ availability. To find out which processes could profit from the thermo-tolerance effect, duplicate samples of *P. putida* KT2440 wild type, *P. putida* KT2440 ΔnadB p638 empty and *P. putida* KT2440 ΔnadB nadBBsm were grown at either 30°C or 40°C and harvested mid-exponential phase. RNA was isolated and used for RNA sequencing. An extensive comparative analysis was performed (Figure 6.4).

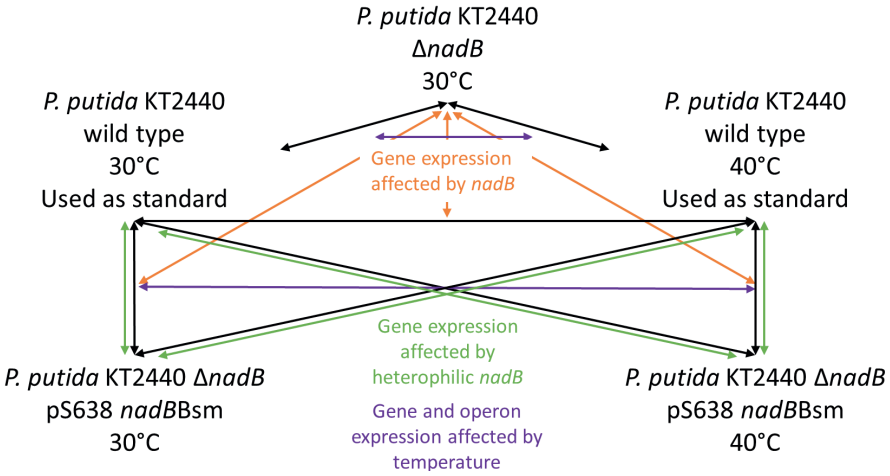

Figure 6.4: Schematic overview of comparisons made in the transcriptomics data. As a standard, P. putida KT2440 wild type grown at its optimum growth temperature of 30°C or P. putida KT2440 wild type grown at 40°C was used. P. putida KT2440 ΔnadB served as the negative control, indicating the genes whose expression was affected by nadB. In purple, the comparisons in expression patterns made to determine gene expression affected by temperature is shown. In green, the comparisons in expression patterns made to determine gene expression affected by the recombinant thermophilic nadB gene are indicated. A final list of all these comparisons was composed, after which all similarly differentially expressed surrounding genes were sought out and sorted into operons.

All gene expression patterns were compared both against *P. putida* KT2440 wild type grown at its optimum growth temperature of 30°C, and at 40°C. Of all 5565 predicted genes, 3304 had a significantly different expression (2log fold change >0.5). Of these, 2854 genes encoded domains of unknown function, leaving 450 genes of known function with a significantly different expression pattern for further analysis. First, genes whose expression were affected by *nadB* were filtered out by comparing the gene expression patterns of *P. putida* KT2440 wild type and *P. putida* KT2440 Δ*nadB* pS638 nadBBsm, both grown at 30°C and at 40°C, with *P. putida* KT2440 Δ*nadB*. Next, the wild type gene expression pattern was compared to the recombinant strain grown at the same temperature. This shows which genes are affected by the change of *nadB* for that of the thermophilic gene from *B. smithii*. Finally, all genes surrounding the genes of interest which were similarly regulated were sorted into operons (Figure 6.4).

When analysing comparative transcription levels of the strain carrying the *nadB* gene of thermophile *Bacillus smithii* versus *P. putida* KT2440 wild type grown at 30°C or 40°C, 90 genes of interest were found. Differential gene expression as a response to the effects caused by the introduction of *nadB* from *B. smithii* compared to the homogenous *nadB* can be divided into few categories. First, genes can be divided to be upregulated (19), downregulated (34) or regulated according to temperature (37) as a response to the effects caused by the recombinant *nadB* introduction, increased temperature, or both. Second, the genes per main category can be placed in functional categories: Electron transfer and redox balance (18), Catalytic activity (28), Regulatory (7), Transporters (14), Translation and transcription machinery (8), Structural proteins (11), Amino acid degradation (2), and Arsenic resistance (2) (Table 6.4).

Table 6.4: Significant differences in gene expression between P. putida KT2440 WT and P. putida KT2440 ΔnadB p638 nadBBsm, both grown at 30°C or 40°C represented as 2log fold change.

Downregulated	Genes	nBBsm30C vs nBPpu30C	nBBsm40C vs nBPpu40C	product	Function
Electron transfer and redox balance	aldB-I	-0.61275	-1.39614	aldehyde dehydrogenase	oxidoreductase
	gdhB	-0.76767	-0.51018	NAD-specific glutamate dehydrogenase	glutamate dehydrogenase (NAD+)
	hmp	-0.80336	-1.6805	flavohemoprotein	NO detoxification
	icd	-1.07328	-0.39898	NADP(+)-specific isocitrate dehydrogenase	oxidoreductase
	katG	-1.16995	-0.58632	catalase-peroxidase	response to oxidative stress
	pydA	-1.62555	-0.70144	NADP-dependent dihydropyrimidine dehydrogenase subunit PreA	oxidoreductase
	pydX	-1.73963	-0.51434	NADP-dependent dihydropyrimidine dehydrogenase subunit	oxidoreductase
Catalytic activity	astA-I	-0.71198	-0.53244	arginine N-succinyltransferase subunit beta	beta
	astA-II	-0.45042	-0.85432	arginine N-succinyltransferase subunit alpha	alpha
	ate	-0.87467	-0.47983	arginyl-tRNA–protein transferase	transferase
	bktB	-0.7084	-1.26317	beta-ketothiolase	transferase
	glgA	-0.981	-0.6891	glycogen synthase	glycogen (starch) synthase
	glgB	-1.07698	-0.62117	1,4-alpha-glucan branching enzyme	glycogen (starch) synthase
	phaC-II	-0.84217	-0.92722	poly(3-hydroxyalkanoate) polymerase	transferase
	ppkB	-0.87588	-1.69535	polyphosphate kinase	transferase
	pydB	-1.95845	-1.45479	bifunctional D-hydantoinase/dihydropyrimidinase	hydrolase
	rlmAA	-1.62008	-0.95897	23S_rRNA_(guanine(745)-N(1))-methyltransferase	transferase
	selA	-0.6985	-0.99765	selenocysteine_synthase	transferase
	ycaC-I	-1.57514	-1.15796	putative hydrolase	hydrolase
	yeaG	-0.77412	-2.84547	protein kinase	protein kinase
	ypfJ	-0.72843	-0.77947	zinc metalloprotease	hydrolase, metalloprotease
Transporter	braF	-0.92812	-0.53246	ABC transporter ATP-binding protein	coenzyme and enzyme regulator
	cbcX	-1.10495	-0.6713	choline/betaine/carnitine ABC transporter substrate-binding protein	trans-membrane transporter
	dppA-I	-0.80455	-0.91172	dipeptide ABC transporter substrate-binding protein	trans-membrane transporter

	dppA-II	-1.01806	-0.65105	dipeptide ABC transporter substrate-binding protein	trans-membrane transporter
	dppA-III	-0.95958	-0.46341	dipeptide ABC transporter substrate-binding protein	trans-membrane transporter
	oprB-I	-0.94002	-0.51506	carbohydrate-selective porin	carbohydrate transport
	pydP	-1.56619	-0.75614	NCS1 family transporter	trans-membrane transporter
	ydcS	-0.98609	-0.59035	polyamine ABC transporter periplasmic polyamine-binding protein	trans-membrane transporter
Translation/transcription	fusB	-0.87057	-0.52957	elongation factor G 2	translation elongation
	rpoS	-1.38063	-0.57261	RNA_polymerase_sigma_S_factor	master transcriptional regulator
Structural proteins	cycH	-0.52745	-0.67556	cytochrome c-type biogenesis protein	cytochrome c-type biogenesis protein
	flgJ	-0.60946	-0.40104	peptidoglycan hydrolase	amidase/hydrolase activity bacterial-type flagellum assembly
	pilJ	-0.44388	-0.60596	twitching motility protein	trans-membrane signal transporter
	TrgI	-0.60946	-0.40104	peptidoglycan hydrolase	amidase/hydrolase activity bacterial-type flagellum assembly
	ycgB	-1.0193	-1.00872	type IV piliation protein	putative regulator of piliation
	ygaU	-1.22305	-1.21294	murein cross-linking regulator	murein (peptidoglycan) cross-linking regulator
Regulation depending on temperature	**Genes**	**nBBsm30C vs nBPpu30C**	**nBBsm40C vs nBPpu40C**	**product**	**Function**
Redox [NAD+] or NAD-dependent	ccoN-II	-0.71101	0.911771	cbb3-type cytochrome c oxidase subunit	oxidoreductase
	dpkA	-0.83474	0.655787	delta 1-piperideine-2-carboxylate reductase	oxidoreductase
	hom	-0.62153	0.488486	homoserine dehydrogenase	oxidoreductase
	nfnB	-0.63445	0.414224	6,7-dihydropteridine reductase	oxidoreductase
	putA	-0.48701	0.481051	proline dehydrogenase 1-pyrroline-5-carboxylate dehydrogenase	oxidoreductase
	queF	0.743845	-1.22909	NADPH-dependent 7-cyano-7-deazaguanine reductase	oxidoreductase
	ssuD	0.829357	-0.6321	alkanesulfonate monooxygenase	oxidoreductase
	stcD	-1.02742	1.156684	N-methylproline demethylase	oxidoreductase
Catalytic activity	allE	-0.53706	0.773632	S-ureidoglycine aminohydrolase	putative hydrolase
	aroF-I	-0.67224	0.456275	tyr-sensitive phospho-2-dehydro-3-deoxyheptonate aldolase	

	aspA	0.664995	-1.45722	aspartate ammonia-lyase	Aspartate ammonia-lyase
	bfr-II	0.741752	-1.49266	bacterioferritin	Iron-storage protein
	dacA	-0.52366	0.564037	D-alanyl-D-alanine carboxypeptidase	hydrolase, protease
	glnL	0.698987	-1.85385	two-component system sensor histidine kinase/phosphatase	kinase/phosphatase
	hsdM	-0.51866	0.64274	type I restriction modification system methylase	methyltransferase
	metG	-0.50943	0.454618	methionine–tRNA ligase	ligase
	proS	-0.4778	0.488282	proline–tRNA ligase	protein biosynthesis
	purM	-0.80853	0.387849	phosphoribosylformylglycinamidine cyclo-ligase	ligase, IMP biosynthesis
	quiP	-0.54027	0.401649	acyl-homoserine lactone acylase	hydrolase, quorum sensing
	thiG	-0.71665	0.717973	thiazole synthase	cofactor biosynthesis
Regulatory	clpV	0.488164	-0.69645	protein ClpV1	ATP-binding chaperone
	ribC	-0.86838	0.583599	riboflavin synthase	riboflavin (vitamin B) synthase
Transporter	brnQ	0.76866	-0.50286	branched-chain amino acid transporter	trans-membrane transporter
	dctA-I	0.679991	-1.79935	C4-dicarboxylate transport protein	symporter
	glpF	0.711528	-0.9711	aquaglyceroporin	trans-membrane transporter
	kgtP	-0.8356	1.150814	alpha-ketoglutarate permease	trans-membrane transporter
	opdH	-1.31127	0.727679	tricarboxylate-specific outer membrane porin	trans-membrane transporter
Translation/transcription	arfB	0.7366	-1.10664	peptidyl-tRNA hydrolase	hydrolase, translation release factor
	fleQ	0.622414	-0.66506	transcriptional regulator	transcriptional regulator
	frr	-0.68134	0.639397	ribosome-recycling factor	Increase efficiency of translation
	sahR	-0.61633	1.009969	methionine metabolism transcriptional regulator	Transcriptional regulator
Structural proteins	cidB	2.794447	-4.68966	holin CidA anti-holin regulator	trans-membrane regulator
	cvpA	0.654254	-0.71377	colicin V production membrane protein	membrane protein required for colicin V production
	pilE	0.650308	-1.4455	type IV pili biogenesis protein	pili biogenesis
	tolR	-0.66342	0.732603	outer membrane stability complex protein	cell membrane integrity during cell division
	yphA	0.97762	-0.73889	inner membrane protein	uncharacterised
Amino acid degradation	speC	-0.93267	0.52923	ornithine decarboxylase	putrescine formation
Upregulated	**Genes**	**nBBsm30C vs nBPpu30C**	**nBBsm40C vs nBPpu40C**	**product**	**Function**
Redox [NAD+] or NAD-dependent	benA	1.394691	1.085338	benzoate 1,2-dioxygenase subunit alpha	oxidoreductase
	dadA-I	0.978802	0.627444	D-amino acid dehydrogenase small subunit	oxidoreductase

	xenA	0.83684	0.586903	protein XenA	oxidoreductase
Catalytic activity	acpP	0.817853	0.856037	acyl carrier protein	fatty acid biosynthesis
	aruC	1.509122	2.907714	acetylornithine aminotransferase	fatty acid biosynthesis
	ilvA-I	0.718564	1.069221	threonine deaminase	L-isoleucine biosynthesis
Regulatory	hscA	0.661744	0.457245	DnaK-like molecular chaperone	chaperone for protein binding
	hscB	0.873016	0.508719	DnaJ-like molecular chaperone	chaperone for protein binding
	ibpA	1.308825	0.490916	small heat shock protein IbpA	stress response
	iscU	0.664131	0.700206	iron-sulfur cluster assembly scaffold protein	iron-sulfur cluster assembly
	yoeB	0.907429	0.958889	YefM-YoeB antitoxin/toxin complex toxin/DNA-binding transcriptional repressor	toxin, transcriptional repressor
Transporter	cbtA	0.811066	0.723701	cobalt transporter subunit A	trans-membrane transporter
	emrB	0.790519	0.691157	multidrug transporter permease	trans-membrane transporter
	gabP-II	0.890862	0.754266	gamma-aminobutyrate permease	trans-membrane transporter
Translation/transcription	rplL	0.763614	0.483952	50S ribosomal protein L7/L12	accurate translation
	rpoE	0.51839	0.661357	RNA polymerase sigma E factor	transcription regulation
Amino acid degradation	puuP	0.980708	0.868704	putrescine permease	trans-membrane transporter
Arsenic resistance	arsB-I	1.987125	2.103638	arsenite/antimonite transporter	trans-membrane transporter
	arsB-II	1.015906	1.393588	arsenite/antimonite transporter	trans-membrane transporter
	arsR-I	2.437894	1.749515	arsenic resistance transcriptional regulator	arsenic resistance, transcription regulation
	arsR-II	2.805632	1.584417	arsenic resistance transcriptional regulator	arsenic resistance, transcription regulation

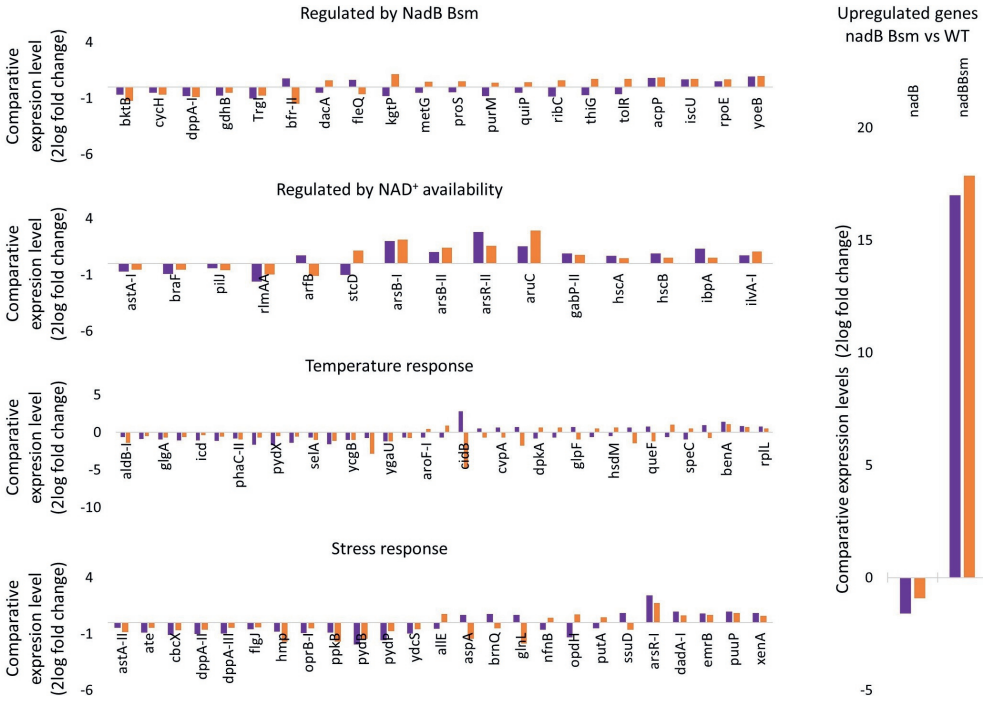

Figure 6.5: Schematic overview of significant differences in gene regulation between P. putida KT2440 WT and P. putida KT2440 ΔnadB p638 nadBBsm, both grown at 30°C *(purple) or* 40°C *(orange). From top to bottom: genes regulated by NadB of Bacillus smithii specifically, regulated by NadB in general, genes responding to temperature and genes responding to stress. Right shows the difference in expression of nadB and nadB Bsm.*

Function analysis of genes differentially expressed by thermophilic *nadB*

An extensive literature search was performed to elucidate the function of each gene or operon of interest. Of the 90 genes of interest, 20 genes were identified as being of highest importance in gene regulation, caused specifically by the insertion of *nadB* from *Bacillus smithii* and the expected resulting increase in the NAD^+-pool. Of these, 5 were downregulated, 4 were upregulated, and 11 were regulated according to growth temperature. Out of the 20, 8 genes are part of 8 different operons. The function of the genes of interest and the effect of their differential regulation is outlined below.

Electron transfer and redox balance

NAD-specific glutamate dehydrogenase (GdhB) is part of the arginine catabolism pathway. Arginine is an important nutrient to *P. putida* [40]. This protein is reported to be allosterically activated by $NADP^+$, but is downregulated in the recombinant strain. This could indicate a minor shift in the redox balance towards NAD^+.

Highest upregulated is the *iscU* gene, part of the isc-operon. This operon regulates the biological assembly of Fe-S clusters and their insertion into apo-proteins. These clusters are important in electron transfer, redox and non-redox catalysis and gene regulation [41]. IscU specifically functions as a scaffold for the assembly of an Fe-S cluster prior to its delivery to apo-proteins. Additionally, two iron-storage cytochrome systems, bacterioferritin (previously identified as cytochrome B1) and the cycH/cmmABCDEFG operon (cytochrome C and protoheme), are downregulated; the first at higher temperatures, the latter consistently. Release of cytochrome C is a typical biochemical characteristic of apoptosis [42, 43]. The lesser degree of expression in the modified strains thus imply that there is less oxidative stress due to the larger redox capacity.

Catalytic activity

The enzymes beta-ketothiolase (BktB), acetoacetyl-CoA reductase (Aacs) and PHA synthase (PhaC) make up the metabolic pathway for polyhydroxyalkanoates (PHA) synthesis. PHAs function for energy storage [44, 45]. In the recombinant strain, this pathway is downregulated compared to the control strains, indicating more energy is fuelled into survival and growth than in storage.

Quorum sensing is the intrabacterial communication system to determine population density via the secretion of small diffusible signal molecules called auto-inducers. Interference with this system is an anti-bacterial defense mechanism, also applied by non-quorum sensing strains. The protein QuiP degrades long-chain auto-inducers, using them as growth substrate instead, and thereby quenches quorum sensing [46]. In the recombinant strain, this gene is downregulated at 30°C but upregulated at 40°C. This seems to indicate that bacterial defense mechanisms are also upregulated at higher temperatures.

Regulatory proteins

Analysing the genes that were affected by an increased NAD^+ availability in general, most remarkable was the upregulated expression of the small heat shock protein IbpA, which adds to the explanation of the thermo-tolerance effect observed.

The RibC operon and the THI operon for riboflavin (vitamin B_2) and thiamine pyrophosphate (vitamin B_1) synthesis respectively, [47, 48], are upregulated upon *B. smithii nadB* expression. Increased production of these important regulators and growth factors also adds to the explanation for improved growth of the modified strains under increased stress levels.

Transport

Genes from the Dpp operon, encoding for binding-protein-dependent ABC transporters for dipeptides were downregulated upon expression of *nadB* from *B.smithii*. This superfamily of transporters mediate the overall nutrient uptake capacity [49]. Overall, at higher temperatures (and thus higher stress levels), these genes are upregulated, which could mean the cells increase expression of transporters since they require more nutrients for more energy to deal with stress.

Transcription and translation machinery

Multiple genes required for the transcription and translation machinery were upregulated in the recombinant strain at higher temperatures. These genes include RNA polymerase RpoE, nucleotide biosynthesis operon Pur (PurM, PurL), translation-dependent operon regulators YoeB toxin-YefM antitoxin system for cleavage of translated mRNA, and ligases proline-tRNA ligase ProS and methionine-tRNA ligase metG to generate amino acid specific tRNAs [50, 51]. Another gene related to cell division is *dacA* coding for D-alanyl-D-alanine carboxypeptidase, which is required for trimming D-alanyl residues from the carboxy terminal of peptidoglycan pentapeptides [45]. Expression of this particular gene is upregulated in the recombinant *P. putida* strain at 40°C but repressed at low temperatures (30°C), indicating a more active cell division machinery at raised temperatures. Similarly regulated is the trans-membrane transporter of α-ketoglutarate and protons, α-ketoglutarate

permease (*kgtP*) [52]. α-Ketoglutarate is a source of glutamate and glutamine in the Krebs cycle that stimulates protein synthesis and inhibits protein degradation.

Structural proteins

There are many structural components specifically reacting to the expression of *nadB* from *B. smithii*. Solvent tolerance protein TrgI was downregulated. TrgI was found to be downregulated in response to solvent stress induced with toluene [53]. When TrgI is knocked out, solvent tolerance is significantly increased in *P. putida* S12. Although its exact function is unknown, the results obtained by Volkers *et al.* indicate that TrgI positively affects membrane stability, which is an important trait in temperature resistance [53]. Membrane stability proteins from the Tol operon are upregulated, which explains the increased robustness of the strain to external stress. Similarly, the acyl carrier protein AcpP is upregulated in the recombinant strain. AcpP is a key component for fatty acid biosynthesis, required for the formation of membranes. This component carries fatty acyl intermediates by different enzyme active sites [54].

Similarly, genes from the FLI operon for flagelli bioformation are downregulated in the recombinant strain compared to the wild type. This indicates a shift from energy sources; the 31-gene large operon for flagelli biogenesis is energy expensive, and this energy is redirected towards maintenance, growth and stress-tolerance. Additionally, flagelli influence the membrane integrity of a cell. Less flagelli could indicate a more stable membrane.

The universal link between NAD^+ availability and thermo-tolerance

To see if the observed effect of increased thermo-tolerance at higher temperatures caused by exchange of *nadB* for a thermophilic heterolog is not specific for *Pseudomonas putida*, a *nadB* knock-out of *E. coli* JW2558 was tested using the same plasmids and methods as *P. putida* KT2440 Δ*nadB*. This was done by reinserting a thermophilic *nadB* gene from *B. smithii* DSM 4216 via pSEVA plasmid 638 into a *nadB* knock-out strain of the *E. coli* KEIO library, *E. coli* JW2558. As a control, an empty pSEVA 638

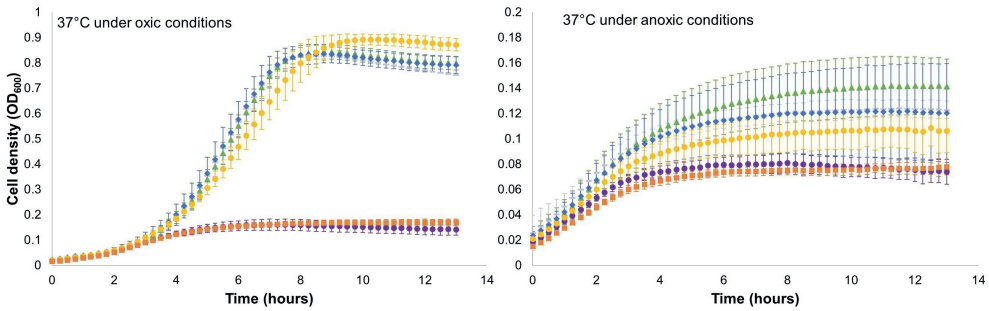

Figure 6.6: Growth experiment under anoxic conditions at 37°C, in LB medium (left) or DBG minimal medium (right). Depicted are E. coli BW25113 WT, (Lilac stripe) E. coli JW2558 ΔnadB pS638 - (Purple circle), nadBPpu (Blue diamond), nadBEco (Green triangle), nadBBsm (Yellow circle), nadBTfl (Orange circle). Growth in a platereader was monitored over 13 hours. Blank wells with uninoculated medium were used to correct for background emission.

plasmid or pSEVA 638 with endogenous *nadB* from *E. coli* BW25113, and *nadB* from the mesophile *P. putida* KT2440 were tested in the *E. coli* knock-out strain. Growth rates of recombinant strains through temperature shifts were monitored by platereader experiments monitoring the increase in cell density over time. Already at ideal growth temperature 37°C, a benefit can be observed from the *nadB* from *B. smithii* (Figure 6.6). When grown under anoxic conditions, this benefit is lost: no significant difference between strains can be observed. When shifting the growth temperature from 37°C to 44°C, the replacement of the inherent gene by the thermophilic *B. smithii nadB* in *E. coli* results in a 10 times shorter lag phase compared to a wild type positive control (Figure 6.7).

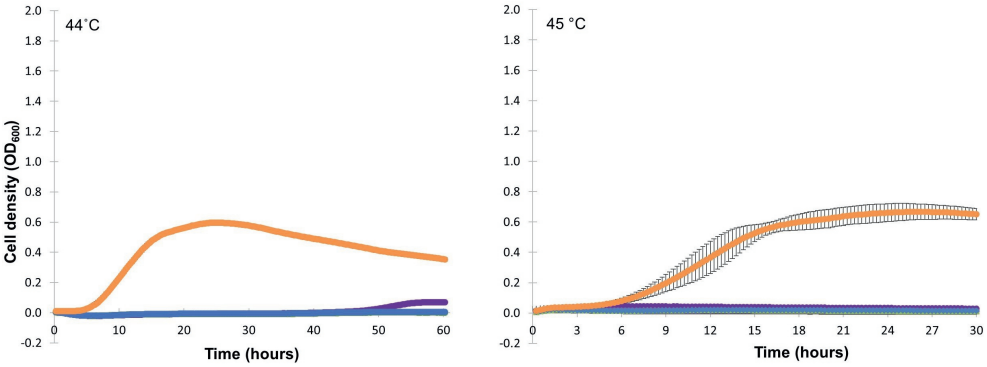

Figure 6.7: Growth experiment of E. coli JW2558 at 44°C (left) and 45°C (right). Depicted are E. coli BW25113 WT (Light Purple), E. coli JW2558 ΔnadB pS638 - (Dark Purple), nadBPpu (Blue), nadBEco (Green), nadBBsm (Orange). Growth in flasks was monitored over 60 hours and 30 hours, respectively. Blank wells with uninoculated medium were used to correct for background emission.

A larger NAD+ pool in yeast increases thermo-tolerance

So far, the thermo-tolerance effect has been observed in prokaryotes which have the aspartate pathway for NAD+ biogeneration. Eukaryotic *Saccharomyces cerevisiae* generates NAD+ through the kynurenine pathway. If the increase in thermo-tolerance in bacteria is indeed caused by the resolution of the main bottleneck (*nadB*), resulting in an increased availability of NAD+, this effect should also occur in an organism that has the kynurenine pathway. To this end, the prokaryotic biosynthesis genes were expressed on a plasmid in *S. cerevisiae* BY4741. This resulted in a yeast strain expressing both its inherent kynurenine pathway and the bacterial aspartate pathway for NAD+ biogeneration (Figure 6.1).

Growth of the wildtype strain and three strains harbouring either *NadABC* from *P. putida* or *B. smithii* or an empty plasmid was monitored at 30°C and 41°C in YNB minimal medium, YNB supplemented with aspartate, which is the substrate of the prokaryotic NAD+ biosynthesis pathway, or YNB supplemented with tryptophan, which is the substrate of the native yeast NAD+ biosynthesis pathway (Figures 6.8 and 6.9, Data S1).

While the growth rates of the wild type strain and the empty plasmid strain (pRV178) are similar, the pRV178 strain grows to a higher cell density from 7 hours onwards at 30°C (Figure 6.8 left and Suppl Data). This effect is most likely caused by

the difference between the concentration of uracil in the medium that the wild type grows in and the amount of uracil that is produced by the pRV178 strain through the URA3 gene that is present on the plasmid. Culturing in YNB medium supplemented with aspartate leads to a higher cell density in pRV157 than in wildtype or pRV156, compared to culturing in YNB medium and YNB medium supplemented with aspartate (Figure 6.8 middle and right). In medium with tryptophan, pRV156 ends with a higher cell density then pRV157 or wild type.

At 41°C in YNB medium all three strains with plasmids perform better with respect to cell density and growth rate than the wild type, especially pRV157 (*NadABC* from *P. putida*) and pRV178 (Figure 6.9 left). This is most likely caused by the effect of uracil that is also seen in the empty plasmid strain at 30°C. Supplementation with either aspartate or tryptophan does not influence growth of the wild type strain at 41°C (Figure 6.9 middle and right).

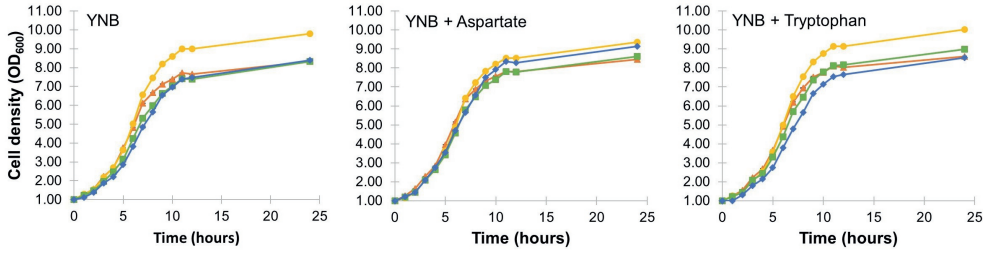

Figure 6.8: Saccharomyces cerevisiae at 30°C. From left to right YNB medium, YNB + Aspartate medium and YNB + Tryptophan medium. Depicted are S. cerevisiae BY4741 WT (Orange triangle), pRV156 empty plasmid (Yellow circle), pRV156 nadABC Bsm (Green square), pRV157 nadABC Ppu (Blue diamond). Growth in glass test tubes was monitored for 24 hours. Test tubes with uninoculated medium were used to correct for background emission.

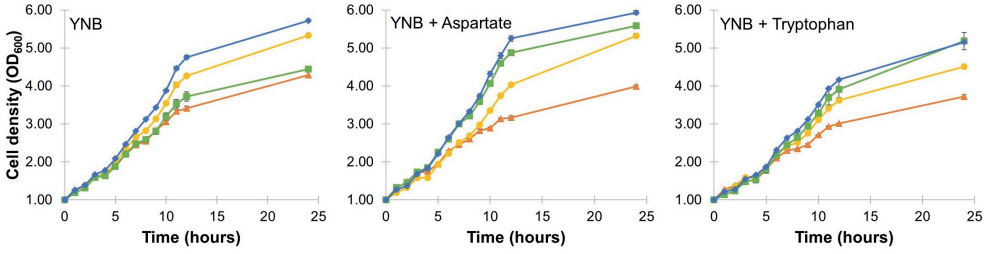

Figure 6.9: Saccharomyces cerevisiae at 41°C. From left to right YNB medium, YNB + Aspartate medium and YNB + Tryptophan medium. Depicted are S. cerevisiae BY4741 WT (Orange triangle), pRV156 empty plasmid (Yellow circle), pRV156 nadABC Bsm (Green square), pRV157 nadABC Ppu (Blue diamond). Growth in glass test tubes was monitored for 24 hours. Test tubes with uninoculated medium were used to correct for background emission.

Remarkably, yeast expressing *NadABC* from either *B. smithii* or *P. putida* cultured in the presence of aspartate or tryptophan (both strains) or YNB (*P. putida* genes) performed the best at the high temperature of 41°C, compared to the empty plasmid strain. This indicates that indeed a higher availability of NAD^+ at elevated temperatures causes thermo-tolerance in yeast. The *NadABC* genes from *P. putida* perform better in yeast than the genes from *B. smithii*, since they do not need supplementation of aspartate in order to have an effect on growth.

All plasmid-bearing strains grow to higher cell densities in asparate and tryptophan medium than the wildtype. Next to that, it seems that supplementation of the medium with tryptophan negatively influences cell density and in some cases also growth rate, in all strains except strain pRV156, compared to YNB.

Discussion

A link between NAD^+ availability and thermo-tolerance

The focus of this work was NAD^+ biogeneration through the aspartate pathway. In general, bacteria have the aspartate pathway, and complex eukaryotes generate NAD^+ through the kynurenine pathway. However, this is not universally correct: there are both bacterial species which apply the kynurenine pathway and eukaryotic species which apply the aspartate pathway. In this research, the aspartate pathway was enhanced and added in yeast which inherently uses the kynurenine pathway to determine if the effect observed between NAD^+ availability and thermo-tolerance is universal. Although the effect does indeed occur in both bacteria which apply the aspartate pathway and in yeast which is forced to employ the kynurenine pathway or the aspartate pathway, the explanation for this effect may vary between pathways and species.

It was briefly mentioned that the link between NAD^+ availability and ROS accumulation in temperature stress is considered the main cause for this effect. This hypothesis is supported by the observation that the thermo-tolerance effect does not occur when the experiment is performed under anoxic conditions. It also explains that upon testing this effect in prokaryotes through the aspartate pathway and in eukaryotes via both an inserted aspartate pathway and the inherent kynurenine path-

way, the thermo-tolerance effect is maintained. Other methods to enlarge NAD(P)+ availability are thus expected to have a similar effect on the thermo-tolerance of a species.

Does thermophilic L-aspartate oxidase increase the NAD+ pool?

In this study, a connection was determined between the exchange of mesophilic *nadB* for thermophilic *nadB* and thermo-tolerance. The extent and limitations of this effect were examined.

All genes were expressed via the pSEVA 638 plasmid, carrying a XylS-Pm inducible promoter system. Only when not induced with 3-methyl benzoate (3MB), thermo-tolerance was improved in recombinant *P. putida* or *E. coli* strains (Figures 6.2, 6.3). Induction with 3MB did not improve strain performance at any temperature tested. The lack of thermo-tolerance upon promoter induction suggests that there is a balance present between the benefit obtained through transcription of *nadB* and the plasmid burden. In other words: the NAD+/ATP balance is precarious. Due to the low basal expression of the XylS-Pm promoter compared to other inducible systems, this system has been applied extensively in both applied and fundamental research. Basal expression dramatically increases with the copy number if the regulatory elements are plasmid-borne, ranging from ~10-fold in a medium copy plasmid to ~100-fold low copy plasmid in *E. coli* with oriV (RK2), and 20 ±10 with oriV (RK2) or 30 ±7 with oriV pBBR1 in *P. putida* [55–59]. In this case, the uninduced XylS-Pm system appears to offer basal expression levels, which transcribe exactly the right amount of *nadB* to improve the NAD+ biogeneration pathway.

The recombinant *P. putida* KT2440 strain carrying *nadB* from *B. smithii* via the pSEVA 638 plasmid was able to grow at 41°C. However, this growth was only minor. This indicates that although the thermo-tolerance of the recombinant *P. putida* KT2440 is improved at higher temperature, 41°C is a hard physiological constraint. Similarly, in the recombinant *S. cerevisiae* strains 42°C proved a physiological constraint (Suppl Data). This suggests that *nadB* and the presumed NAD+ availability linked to it is the main bottleneck towards growth at high temperatures, but as expected, not the only one.

The improvement of thermo-tolerance in yeast upon introduction of the aspar-

tate pathway in addition to its kynurenine pathway for NAD^+ biogeneration supports the claim that in eukaryotes thermophilic *nadB* increases the NAD^+ pool which causes the thermo-tolerance. The yeast strain harbouring *nadABC* from *P. putida* grows better at 41°C in all media and at 30°C in medium with aspartate. This suggests that the *P. putida* KT2440 genes function well in yeast compared to those of *B. smithii* DSM 4216T and that they are very well able to convert aspartate into NAD^+ promoting growth.

What should be considered is that the kynurenine pathway and the aspartate pathway converge at quinolinate phosphoribosyl transferase: NadC in the aspartate pathway, Bna6 in the kynurenine pathway. Some level of cross-functionality can not be excluded. However, since NadC and Bna6 both are no bottlenecks in their respective pathways (Table 6.1), [17, 60], any effect of cross-functionality would be unexpected.

The addition of tryptophan in the yeast strain had a negative effect on growth rates at higher temperatures. This could be explained by results obtained in earlier research by Jarolim *et al.* [61], who found improved survival of *S. cerevisiae* through heat shocks in the absence of tryptophan. This might indicate that the presence of tryptophan suppresses the heat shock resistance or stress survival response in yeast. When the recombinant yeast strain has the aspartate pathway of *B. smithii*, either aspartate or tryptophan are required to improve growth over the empty plasmid control, while this is not required when the pathway of *P. putida* is implemented. Reasons for this are unknown, but it is expected that the *B. smithii* pathway does not function as well in yeast due to structural differences of the proteins. Additional substrate feeding into the pathway (either aspartate for the aspartate pathway or tryptophan for the inherent kynurenine pathway) supposedly increases the NAD^+ pool insofar the plasmid burden can be overcome.

Based on the assumption that due to the thermophilic *nadB* there is an increase in NAD^+ at higher temperatures, the loss of effect under anoxic conditions can also be explained. The availability of NAD^+ combats the formation of reactive oxygen species (ROS). In plants, a close association has been discerned between ROS and the heat stress-response signal transduction pathways and defense mechanisms [62]. In fact, several studies have linked heat shock transcription factors with ROS sensing

[63, 64]. The growth experiments under anoxic conditions prove that the thermo-tolerance effect that occurs upon introduction of a thermophilic *nadB* is lost when repeating the temperature shift without oxygen presence. This could mean that, similarly to seen in plants, an indirect increase of the NAD^+ upon better performance of *nadB* inhibits the buildup of ROS due to temperature stress.

To determine what exactly causes the thermo-tolerance, RNA sequencing was performed. When looking at gene expression patterns, we found that 90 genes were differentially expressed due to the effects caused by the exchange of *nadB* from *B. smithii* for the homogeneous *nadB*. The genes most influenced by this exchange are involved in iron cluster assembly. Iron is essential to maintain the redox balance during the growth of animals, plants and most microorganisms. However, when grown aerobically, iron can pose problems of bioavailability and cytotoxicity. Iron reacts with oxygen, generating toxic free radical. In its oxidised state, it is relatively insoluble. Bacteria overcame this problem with specific mechanisms for iron uptake mechanisms and creating iron storage, which allow growth in an iron-deficient environment [65]. Downregulation of the storage mechanism but upregulation of the uptake mechanism as a response to the effects caused by introduction of *nadB* from *B. smithii* at higher temperatures is thus closely linked to oxidative stress, although its specific role remains controversial.

An overall upregulation of a heat shock protein was observed when NAD^+ availability is increased. Furthermore, the *nadB* gene from *B. smithii* appears to have a strong regulatory effect from rerouting energy from demanding processes such as flagelli biosynthesis and energy storage to stimulate cellular growth, to regulating important factors affecting the redox balance. All together, 19 genes were linked to the specific presence of *nadB* from *B. smithii*, and all help explain the thermo-tolerance effect observed. It is difficult to indicate which genes are cause and which are effect of the thermo-tolerance in this complex system. Are the genes for the transcription/translation machinery upregulated because it grows better, or do they cause it to grow better? More research could aid towards pinpointing the exact pipeline of cause and effect.

Alternative pathways to generate NAD^+ are via the salvage pathway, or the production of lactate. Through these alternative pathways, the *nadB* knock-out negative

control strain is still viable. Metabolic increase in use in these pathways would be recognisable by an upregulation of vital enzymes of these pathways, such as nicotinate phosphoribosyltransferase (PncB) for the salvage pathway [66] or lactate dehydrogenase (ldhA) for lactate production. Transcriptomics data does not reflect any upregulation of these genes, including in the negative control, which reflects the poor growth observed in this strain overall.

As the rate limiting step in the aspartate pathway for *de novo* NAD^+ synthesis (Table 6.1, [17, 20, 21]), the large impact gained by replacement of *nadB* can be explained. Noteably, however, multiple adaptive laboratory evolution (ALE) experiments based on temperature tolerance have not resulted in the adaptation of this single gene [67]. In fact: *nadB* has never before been identified in thermo-tolerance.

Conclusion

An increased thermo-tolerance is a widely sought-after goal in industry. In microorganisms, more stability at higher temperatures means less costs during fermentation due to less need for cooling, a higher product yield since the micro-organism can better handle temperature fluctuations occurring at large scales, and it opens up the possibility to apply thermophilic enzymes in mesophilic species.

The NAD^+ biogeneration pathway is one of the main bottlenecks for thermostability. Prokaryotes and eukaryotes can gain a higher thermo-tolerance upon enlarging the NAD^+ pool, either by the introduction of additional pathways to overproduce NAD^+ or by introduction of thermophilic enzymes which function better at higher temperatures.

The connection between NAD^+ availability and thermo-tolerance could be explained upon transcriptomics analysis, which showed that upon a larger availability the heat shock protein IbpA is upregulated. Furthermore, specifically upon expression of *nadB* from *B. smithii*, it was observed that this L-aspartate oxidase enzyme has a regulatory role, as it redirects energy from demanding non-essential purposes like flagelli biogeneration towards cellular growth by activating the transcription and translation machinery, it works to stabilize the cellular membrane and it largely affects the redox balance. This finding was patented (Appendix).

Acknowledgements

We gratefully acknowledge dr. P. I. Nikèl for hosting part of the research at de Novo Nordisk Foundation, DTU Denmark. We gratefully acknowledge Dr. I. Mougiakos and Dr. N. Strepsis for respectively providing genomic DNA of *B. smithii* DSM 4216T and *T. flocculiformis* DSM 2094 for gene isolation. We gratefully acknowledge financial support from the European Horizon 2020 project EmPowerPutida (Project reference: 635536). The funders had no role in study design, data collection and analysis, or preparation of the manuscript.

Conflict of interest

The invention presented here was patented under PCT/EP2019/080645: Microbial Hosts engineered for Increased Tolerance to Temperature Shifts (see supplement).

Author contributions

Conceived the research: LFCK Conceived the experiments: LFCK/RJMV/PJS Experimental design: LFCK/RJMV Performed the experiments: LFCK/RJMV/DH/AD Transcriptomics: LFCK/BN Data analysis: LFCK/RJMV Work supervision: LFCK-/RJMV/PJS Wrote Manuscript: LFCK/RJMV Proofread Manuscript: RJMV/PJS Arranged Funding: VAPMdS

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Appendix: PCT/EP2019/080645 Microbial Hosts engineered for Increased Tolerance to Temperature Shifts

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Peter J. Schaap, Vitor A. P. Martins dos Santos

The presented invention relates to the field of molecular microbiology, metabolic engineering, and fermentation technology. In particular, the invention relates to microbial host cells that have been engineered for increased tolerance to temperature shifts, for increased performance at temperatures different from the microorganisms optimal temperature and/or for changing at least one of the microorganisms cardinal temperatures by replacing an endogenous NAD^+ biosynthesis gene by a heterologous gene encoding a corresponding enzyme with another temperature profile.

Introduction

Mesophiles are the preferred organisms for industry. They are genetically accessible, easy to culture, and have established methods to adapt the metabolism for production optimization. However, it is often beneficial to use bacteria which are robust against a broad temperature range. Fermentation at high temperature offers several advantages not common with mesophilic microorganisms, including higher growth and metabolic rates, lower cellular growth yield, increased physicochemical stability of enzymes and organisms and facilitated reactant activity and product recovery [1, 2]. Downsides to use thermophiles include the genetic inaccessibility, but also the high costs that are associated to culturing only at such high temperatures. To adapt a mesophile to have more resilience to higher temperatures would provide the benefits of both. This would open up many different applications for mesophilic industrial workhorses, including increasing the production yield, robustness of the production process, the possibility of inducement with temperature shifts, or applying thermophilic new enzymes that are much more stable than their counterparts from mesophiles [3, 4].

Nicotinamide Adenine Dinucleotide (NAD^+) is a cofactor essential for survival in all living organisms, by balancing the redox balance. In prokaryotes, de novo biosynthesis of NAD^+ proceeds via a condensation reaction of L-aspartate and di-

hydroxyacetone phosphate, catalysed by the quinolinate synthase system [5]. This proposed complex is composed of two enzymes: L-aspartate oxidase (NadB) which catalyses the oxidation of L-aspartate to iminoaspartate using O_2 as an electron receptor, releasing H_2O_2, and quinolinate synthase, which condenses iminoaspartate with dihydroxyacetone phosphate to produce quinolinate.

It is an object of the present invention to provide for microbial host cells that have been engineered for increased tolerance to temperature shifts and/or for increased performance at temperatures different from their strain specific optimal temperature.

Summary of the invention

In a first aspect, the invention pertains to a microbial host cell comprising a nucleotide sequence encoding a heterologous NAD^+ biosynthesis enzyme, wherein at least one of:

1. The heterologous NAD^+ biosynthesis enzyme is from a microbial donor organism with an optimum growth temperature that is different from the optimum growth temperature of the microbial host cell, or from a microbial donor organism that has a wider range of growth temperatures than the microbial host cell.

2. The heterologous NAD^+ biosynthesis enzyme has a higher activity than the corresponding endogenous NAD^+ biosynthesis enzyme of the host cell at a temperature that differs from the optimum growth temperature of the host cell, as determined in an assay for activity of the NAD^+ biosynthesis enzyme wherein the activity of the endogenous and heterologous NAD^+ biosynthesis enzymes is determined over a period of time of at least 10 minutes.

Preferably, the heterologous NAD^+ biosynthesis enzyme encoded by the nucleotide sequence comprised in the microbial host cell is selected from the group consisting of L-aspartate oxidase, quinolinate synthase and quinolinate phosphoribosyl-transferase, and wherein preferably the microbial host cell comprises nucleotide sequences encoding two or all three of the NAD^+ biosynthesis enzyme from the group consisting of L-aspartate oxidase, quinolinate synthase and quinolinate phosphoribosyl-transferase. A preferred microbial host cell according to the invention is a host cell wherein the temperature difference in at least one of a) and b) above, is at least 2°C. More preferably, a microbial host cell according to the invention is a host cell, wherein at least one of:

1. The heterologous NAD^+ biosynthesis enzyme has a higher activity than the corresponding endogenous NAD^+ biosynthesis enzyme in the host cell at a temperature that is higher than the optimum growth temperature of the host cell.

2. The heterologous NAD^+ biosynthesis enzyme is from a microbial donor organism with an optimum growth temperature that is higher than the optimum growth temperature of the microbial host cell.

In one embodiment, a microbial host cell according to the invention comprises a genetic modification that reduces or eliminates the specific activity of an endogenous NAD^+ biosynthesis enzyme that corresponds to the heterologous NAD^+ biosynthesis enzyme encoded by the nucleotide sequence comprised in the host cell, wherein preferably, the nucleotide sequence encoding a heterologous NAD^+ biosynthesis enzyme replaces the endogenous nucleotide sequence encoding the corresponding endogenous NAD^+ biosynthesis enzyme. A microbial host cell according to the invention preferably is a yeast, a filamentous fungus, a eubacterium or an archaebacterium, more preferably the host cell is a Gram-positive or a Gram negative bacterium. A microbial host cell according to the invention can e.g. be a host cell of a genus selected from the group consisting of: *Escherichia*, *Anabaena*, *Actinomyces*, *Acetobacter*, *Caulobacter*, *Clostridium*, *Gluconobacter*, *Gluconacetobacter*, *Rhodobacter*, *Pseudomonas*, *Paracoccus*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Rhizobium Sinorhizobium*, *Flavobacterium*, *Kleb-*

siella, Enterobacter, Lactobacillus, Lactococcus, Streptococcus, Oenococcus, Leuconostoc, Pediococcus, Carnobacterium, Propionibacterium, Enterococcus, Bifidobacterium, Methylobacterium, Micrococcus, Staphylococcus, Streptomyces. Zymomonas, Streptococcus, Bacteroides, Selenomonas, Megasphaera, Burkholderia, Cupriavidus, Ralstonia, Methylobacterium, Methylovorus, Rhodopseudomonas, Acidiphilium, Dinoroseobacter, Agrobacterium, Sulfolobus, Sphingomonas, Acremonium, Aspergillus, Aureobasidium, Cryptococcus, Filibasidium, Fusarium, Humicola, Magnaporthe, Mucor, Myceliophthora, Neocallimastix, Neurospora, Paecilomyces, Penicillium, Piromyces, Schizophyllum, Talaromyces, Thermoascus, Thielavia, Tolypocladium, Trichoderma, Ustilago, Saccharomyces, Kluyveromyces, Candida, Pichia, Schizosaccharomyces, Hansenula, Kloeckera, Schwanniomyces, Yarrowia, Cryptococcus, Debaromyces, Saccharomycecopsis, Saccharomycodes, Wickerhamia, Debayomyces, Hanseniaspora, Ogataea, Kuraishia, Komagataella, Metschnikowia, Williopsis, Nakazawaea, Torulaspora, Bullera, Rhodotorula, and *Sporobolomyces*.

The heterologous NAD^+ biosynthesis enzyme encoded by the nucleotide sequence that is comprised in a microbial host cell of the invention, preferably is an NAD^+ biosynthesis enzyme that is obtained or obtainable from a microbial donor organism, which is a psychrophilic, a psychrotrophic or a thermophilic organism. In a preferred embodiment, the microbial host cell is a mesophile.

In one embodiment of a microbial host cell according to the invention, the heterologous NAD^+ biosynthesis enzyme is a modified version of an enzyme that is endogenous to the host cell, which modified version enzyme comprises at least one modification in its amino acid sequence as compared to the endogenous enzyme, and wherein the modified version has a higher activity than the endogenous enzyme at a temperature that differs from the optimum growth temperature of the host cell, in an assay for activity of the NAD^+ biosynthesis enzyme wherein the activity of the endogenous and the modified enzymes is determined over a period of time of at least 10 minutes.

The heterologous NAD^+ biosynthesis enzyme encoded by the nucleotide sequence that is comprised in a microbial host cell of the invention, preferably is an NAD^+ biosynthesis enzyme comprising an amino acid sequence selected from the group consisting of:

1. An amino acid sequence that is at least 45% identical to NadB *Bacillus smithii*.
2. An amino acid sequence that is at least 45% identical to NadB *Trichococcus flocculiformis*.
3. an amino acid sequence that is at least 45% identical to NadB *Pseudomonas putida*.
4. An amino acid sequence that is at least 45% identical to NadB *Escherichia coli*.
5. An amino acid sequence that is at least 45% identical to NadA *Bacillus smithii*.
6. An amino acid sequence that is at least 45% identical to NadA *Trichococcus flocculiformis*.
7. An amino acid sequence that is at least 45% identical to NadA *Pseudomonas putida*.
8. An amino acid sequence that is at least 45% identical to NadA *Escherichia coli*.
9. An amino acid sequence that is at least 45% identical to NadC *Bacillus smithii*.
10. An amino acid sequence that is at least 45% identical to NadC *Trichococcus flocculiformis*.
11. An amino acid sequence that is at least 45% identical to NadC *Pseudomonas putida*.
12. An amino acid sequence that is at least 45% identical to NadC *Escherichia coli*.

In a second aspect, the invention relates to a process for producing a fermentation product, the process comprises the steps of:

1. Culturing a microbial host cell of the invention in a medium, whereby the host cell converts nutrients in the medium to the fermentation product.

2. Optionally, recovery of the fermentation product.

Preferably, the process comprises a shift in temperature, wherein preferably the shift in temperature is a shift of at least 2, 5, 7 or 10°C. In a third aspect, the invention relates to the use of a nucleotide sequence encoding a NAD^+ biosynthesis enzyme that is heterologous to a microbial host cell, wherein the nucleotide sequence is used for at least one of:

1. Changing at least one of the minimum, maximum and optimum growth temperature of the microbial host cell.

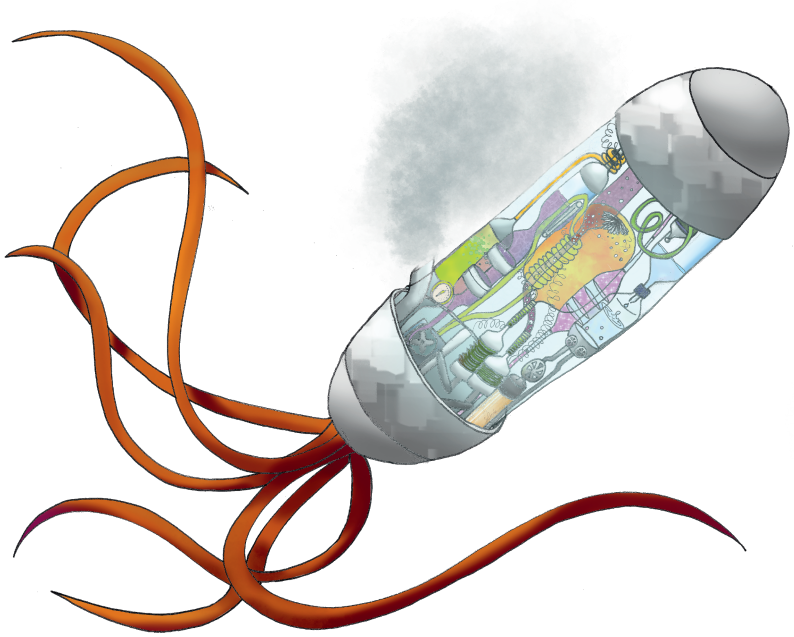
2. Improving resistance of the microbial host cell to a shift in temperature, wherein preferably the resistance of the microbial host cell to a shift to a higher temperature is improved.

Preferably, in the use of a nucleotide sequence encoding a NAD^+ biosynthesis enzyme that is heterologous to a microbial host cell, at least one of the microbial host cell and the nucleotide sequence encoding a heterologous NAD^+ biosynthesis enzyme is as defined herein. Preferably in this aspect, at least one of:

1. At least one of the minimum, maximum and optimum growth temperature of the microbial host cell is changed by at least 1°C.

2. The lag phase of the microbial host cell upon a shift in temperature of at least 2°C, is reduced by at least a factor 1.1.

7 Prokaryotic Thermo-Tolerance in a Down-Scaled System



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Abstract

Fluctuations in temperature due to overheating of large-scale industrial set-ups pose a challenge for stable microbial production. Strain robustness in this respect is highly appreciated, but difficult to establish on a laboratory scale. The here developed millifluidics device enables for testing such fluctuations on a small scale, mimicking industrial conditions during the research phase. In previous work, the universal link between NAD^+ availability and thermo-tolerance was elucidated. Recombinant *P. putida* KT2440 Δ*nadB* was enriched with either the homologous, a psychrophilic or a thermophilic L-aspartate oxidase (nadB) gene. Growth was monitored at 40°C and 41°C. Equipped with the thermophilic *nadB*, thermo-tolerance of the strains increased significantly.

Presently, we analyse the recombinant strains performance through temperature fluctuations that occur in large scale bioreactors. A down-scaled millifluidics system was built to this purpose which at room temperature can expose the cells to near instantaneous temperature fluctuations ranging from 30°C to 50°C. The system was applied to monitor the effect of such fluctuations on the performance of the recombinant *P. putida* strains.

When exposed to stochastic temperature fluctuations ranging up to 50°C, *P. putida* KT2440 Δ*nadB* carrying a plasmid with *Bacillus smithii nadB* performed equally to the unburdened wild type, and significantly better then a positive control with the inherent *nadB* supplied through a plasmid or a negative control with an empty plasmid. Tolerance to (near) instantaneous temperature fluctuations is a desired industrial trait. The results show that this trait is directly linked to the *nadB* gene, and can be transferred between species, substantially improving strain robustness in an industrial set-up.

Introduction

Transition costs from laboratory scale to manufacturing scale, including pilot and demo scales, can run up to $100 million to $1 billion US dollars [1]. Even though it was estimated that it takes over 10 000 experiments for a single bioprocess development project from primary screening to pilot-scale trials [2, 3], this far exceeds the costs of the microbial development and lab-scale testing combined.

Generally, microbial research is performed at relatively perfect conditions compared to industrial production scale: the small scale allows for homogeneous distribution of nutrients, dissolved oxygen, microorganisms and temperature. The scale-up process that follows the research and development phase is considered challenging at best for industrial applications [1, 4–6]. Poor scaling up results in low yields during fermentation, which is often the most expensive step. Scale-up issues include maintaining optimum and homogeneous reaction conditions, minimizing microbial stress and enhancing metabolic accuracy [4]. A literature study by Geisler and colleagues indicated that the broad range of technical correlations used to build large-scale setups is based on data obtained at laboratory scale [7], which leads to discrepancies in the scaling up process. Scaling up any process quickly encounters both practical and theoretical conflicts. When considering important factors like temperature input, equipment shape, and stirring power, choices must be made on what to maintain most true to form [1]. The biological properties of the micro-organism used combined with the fluid properties of the medium and the inconsistencies during the scale up render all laboratory predictions on production yield and stability poorly inconsistent [4, 7].

An alternative to perfecting the manufacturing scale to try to remove fluctuations that occur is to try to mimic them at a laboratory scale to offers more reliability for industrial set-ups. This is referred to as down-scaling, which was indicated as a vital necessity for successful scale-up [1, 2, 5]. It was shown that microfluidics can be used to mimic industrial scale fluctuations. Zanzotto and colleagues built a microbioreactor with a volume of mere micro-litres, with well defined dissolved oxygen and pH conditions. When comparing the microbioreactor with a 500 mL bench-scale bioreactor, similarity were found in growth conditions, dissolved oxy-

gen profile within the vessel over time, pH profile over time, the final number of cells and the cell morphology [8]. They proved that microbioreactors can successfully scale-up to at least laboratory scale.

Microfluidics and millifluidics thus offer a cheap and easy solution during the development stage to mimic the imperfections that occur at industrial scale [9–12]. Micro-reactors can be used to simulate the industrial process in small channels using continuous-flow processes.

In this research, we focus on temperature fluctuations within large-scale systems, and mimic them on a millifluidic scale. Variations in temperature in large scale systems are mainly caused by uneven cooling and heat produced during fermentation by microorganisms [13], and deviate as much as 10°C [14, 15]. Using embedded scaffold removing open technology, or ESCARGOT, a 3D printed acrylonitrile butadiene styrene (ABS) scaffold can be applied to make a micro or milli-channel device in a single block of polydimethylsiloxane (PDMS). PDMS is transparent, gas permeable and heat resistant, with low thermal conductivity (0.15 W m^{-1} K^{-1}).

Combined with heating elements such as nichrome wire [16] or Peltier elements, thermal zones can be created in the device. the elasticity of the PDMS allows for additional use of pumps, valves and thermistors directly in the device [9]. Such micro or millifluidic devices pave the way for a straight-forward and consistent scale-up pathway, clearly highlighting instability in the production process at the earliest stage possible.

In Chapter 6 [17], the universal link between NAD^+ availability and thermo-tolerance is described in *P. putida* KT2440, where the L-aspartate oxidase (nadB) gene was replaced with either the homologous, a psychrophilic or a thermophilic *nadB* gene. Growth was monitored at 40°C and 45°C, respectively. Equipped with the thermophilic *nadB*, strain thermo-tolerance increased significantly [17] (Chapter 6). It was hypothesized that this increase in thermo-tolerance would aid production process stabilization on an industrial scale.

Presently, the thermo-tolerance effect of the previously developed recombinant strains was tested through temperature fluctuations that occur in industrial scale bioreactors, to analyse the recombinant strain value as opposed to the wild-type for large scale application. A unique down-scaled millifluidics system was designed

and built for this purpose. The system was applied to monitor recombinant strain performance through high temperature fluctuations long term.

Materials and methods

Millifluidics

To mimic the temperature fluctuations that occur in a large scale bioreactor, a millifluidics down-scaled system was designed and built. Via the ESCARGOT method [9], a 21 by 6 cm PDMS construct with straight embedded acrylonitrile butadiene styrene (ABS) cylindrical scaffolds with a diameter of 2.85 mm was first cured at 70°C for 4 hours and then and treated with acetone to remove the ABS, creating eight millifluidic channels in one system. Based on a diameter of 2.85 mm and a length of 21 cm the channels each have an effective volume of $\simeq$ 5 mL. Via a reservoir, rubber tubing and a Schenchen LabV1.6 YZ1515X peristaltic pump, bacteria are looped through the system at various speed rates. At a flow speed of 0.5 mL/min the bacteria remain in the channel for 10 minutes (Figure 7.1).

Temperature fluctuations are regulated at will in the millifluidic channels. Peltier elements with attached dissipators were placed on either side of the millifluidic device. The top side is equipped with fans and used for quick cooling of the system, the bottom side is used for heating the system. Copper plating on both sides evenly distributes the temperature over the length of the millifluidics. A thermistor is applied inside one micro-channel to monitor the temperature at the start and end of the channels. The thermistor is linked to an Arduino Uno micro-controller board [18] which was programmed to regulate the temperature of the microchannels using the Peltier elements. A wiring schematic can be found in Figure 7.2. A 3D printed holder was designed to apply accurate contact between the Peltier elements, the copper plate and the millifluidic devices, stabilizing the system.

The schematics for the electronics and the components needed for the Arduino Flow-reactor as well as the code used can be found in the supplementary information. Schematics were drawn using Fritzing 4 and licensed under CC Attribution-ShareALike [19].

Bacterial strains and cultivation conditions

Bacterial strains and plasmids used in this study can be found in Table 7.1. *P. putida* KT2440 was routinely cultivated under oxic conditions at 30°C in LB medium (containing 10 g/l tryptone, 10 g/l NaCl and 5 g/l yeast extract). For solid medium, 15 g/l agar was added to the medium. Experiments were performed in De Bont minimal medium (DBG) [20] (3.88 g/l K_2HPO_4, 1.63 g/l $NaH_2PO_4 \cdot 2H_2O$, 100x trace elements consisting of $NH_4)_2SO_4$, 0.1 g/l $MgCl_2 \cdot 6H_2O$, 10 mg/l EDTA, 2 mg/l $ZnSO_4 \cdot 7H_2O$, 1 mg/l $CaCl_2 \cdot 2H_2O$, 5 mg/l $FeSO_4 \cdot 7H_2O$, 0.2 mg/l $Na_2MoO_4 \cdot 2H_2O$, 0.2 mg/l $CuSO_4 \cdot 5H_2O$, 0.4 mg/l $CoCl_2 \cdot 6H_2O$, 1 mg/l $MnCl_2 \cdot 2H_2O$), with 20 g/l glucose as the sole carbon source. Medium was supplemented with 10 μg/mL gentamycin to maintain the plasmids. The cell density prior to the experiment was determined photometrically at 600 nm (OD_{600}). Optical cell densities in the system were monitored automatically, using a 600 nm LED light and a light-to-frequency sensor positioned opposite the LED source. A standard curve was made to translate frequencies to cell density. The frequencies obtained were corrected using no-light measurements and calibrated using cultures of known OD_{600}. The data was fitted via a non-linear fitting procedure in MATLAB 2018b (The MathWorks, Natick, MA, USA), with a 95% confidence interval.

Table 7.1: Strains and plasmids used in this study

Strains	Characteristics	Optimal growth temperature	Growth temperature range	Source
Pseudomonas putida	Gram-negative mesophile	30°C	25 – 40°C	
KT2440	Wild-type strain, spontaneous restriction-deficient derivative of strain mt-2 cured of the TOL-plasmid pWW0			[21]
KT2440 ΔnadB	KT2440 with a knocked out nadB gene			[17]
Plasmids for bacterial strains				
pSEVA 638 nadBEco	pSEVA 638 *nadB E. coli* BW25113			[17]
pSEVA 638 nadBPpu	pSEVA 638 *nadB P. putida* KT2440			[17]
pSEVA 638 nadBBsm	pSEVA 638 *nadB B. smithii* DSM 4216T			[17]
pSEVA 638 nadBTfl	pSEVA 638 *nadB T. flocculiformis* DSM 2094			[17]

All plasmids belong to the SEVA (Standard European Vector Architecture) collection [22, 23]

Statistical analysis

Experiments were independently repeated three times. Figures represent the mean values of corresponding biological triplicates and the standard deviation. The level of significance of the differences when comparing results was evaluated by means of analysis of variance (ANOVA), with α=0.05.

Results

The millifluidics device

As bacterial cultures suffer stochastic temperature fluctuations in an industrial scale bioreactor, a millifluidics device was designed to expose a random subsampling of bacterial culture to near instantaneous temperature fluctuations (Figure 7.1).

The system is designed to flow a loop of bacteria culture through the channels during fermentation. An external culture reservoir, kept at optimal growth temperature, is pumped through the heated (or cooled) millifluidic channel for a certain amount of time depending on the pump speed, and then flowed back into the reservoir. The external sample is kept in a open 50 mL syringe using the tip of the syringe as inlet for the millifluidics and the open top for flowing back the culture, so that over time, all sample will have gone through the device. A peristaltic pump is used to pump round the same sample so that the effect of the temperature fluctuations can be seen over a long time-span. The peristaltic pump also makes the system easily adaptable: by changing the flow speed, the temperature fluctuation exposure can be prolonged or decreased. The double row of Peltier elements were used for efficient and fast heating and cooling. Copper plating between the Peltiers and the PDMS was used for an even temperature distribution over the length of the millifluidic device. Multiple channels allow for strains to be tested in parallel.

The Arduino Uno micro-controller board regulates the temperature or the different temperatures in time by adjusting the current flown in the heating and cooling Peltier elements. A thermistor was used directly at the end of one channel in the millifluidic device in such a way that the temperature of the fluid is measured at the outlet. With all wiring and programming in place, the device was tested

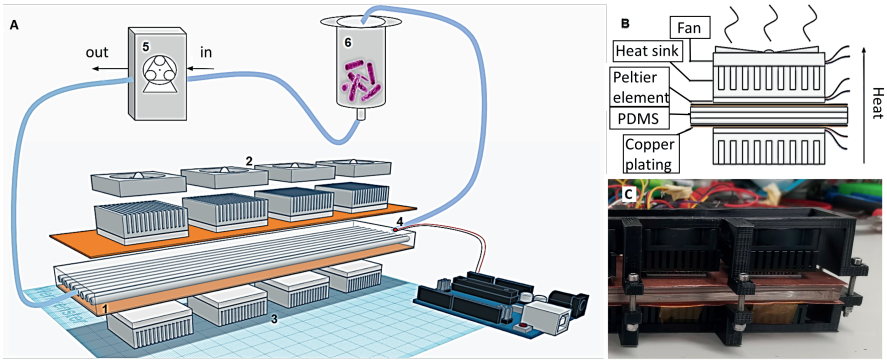

Figure 7.1: Schematic overview of millifluidics device A) depicts the millifluidics device, ***1.*** *the PDMS gel through which eight microchannels run.* ***2.*** *Top Peltier elements, in charge of cooling the system. The fans are placed on top for heat dispersion.* ***3.*** *Bottom Peltier elements, for heating the system.* ***4.*** *Thermistor for temperature registration, which via the Arduino Uno is then used to regulate the Peltier elements, placed within the bacterial sample where the PDMS meets the tubing.* ***5.*** *Peristaltic pump.* ***6.*** *External bacterial sample in a 50 mL syringe. B) shows a side view of the device, C) shows a picture of the actual device.*

for its ability to regulate the temperature when medium is pumped through the channels, and the stability of that temperature (Figure 7.3). A solution containing a thermochromic dye was used to measure the temperature synchronisation and hysteresis of each millifluidic channel (supplementary information). Thermistor data is directly fed into the Arduino Uno, which is programmed to either keep one stable temperature or any time/temperature fluctuation desired. A schematic of the wiring can be found in Figure 7.2.

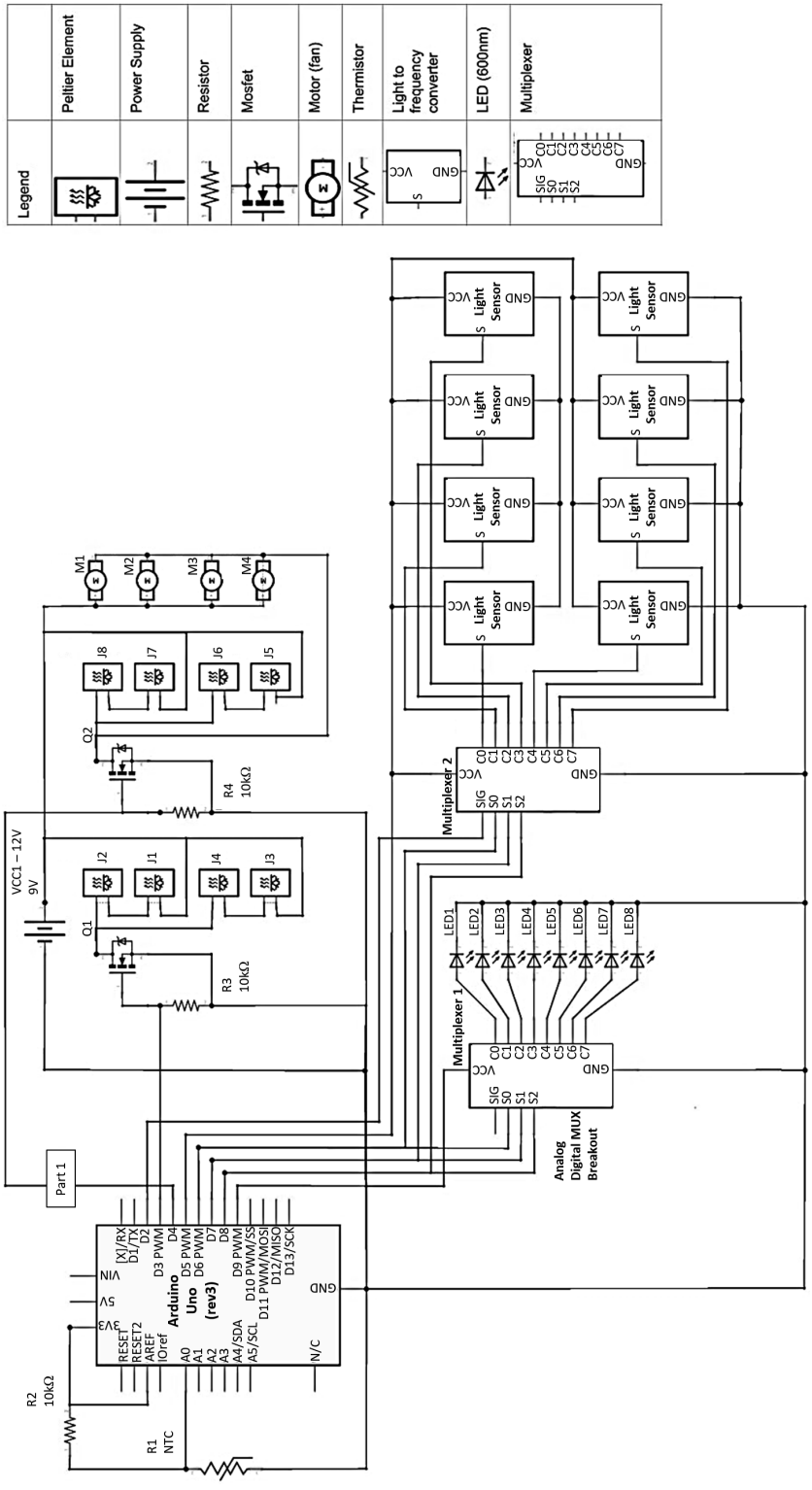

Figure 7.2: Schematic overview of Arduino Uno wiring. The electrical routing of the device.

The side view shows the temperature of media being pumped through four channels (visible as circles within the PDMS) when the Peltier elements are set to heat to 45°C. The temperature reached approximately 40°C, indicating a fluctuation of 5°C between the Peltier and the actual cells. The top view shows that the medium actually reaches this temperature at two-thirds of the PDMS. Due to this hysteresis, cells are exposed to the programmed maximal and minimal temperature for 3.33 minutes per round with a flow speed of 0.5 mL/min.

The temperature determination using the thermistor located at the output of the gel (Figure 7.3 C, D) shows good stability of the device, although temperatures nearing room temperature (21°C) are more difficult to maintain.

For a monitoring of the culture, an automatic OD measurement was integrated at the outlet of the eight channels, before the culture is flown back to the reservoir. The OD measurement work on a 600 nm LED light and a light-to-frequency sensor positioned at 180 degrees from the LED source. The LED and the sensors are connected to the same Arduino UNO which records the OD data for the 8 channels every 10 minutes. To translate frequencies obtained from the light-to-frequency sensor to cell density, a standard curve was made. An overnight culture of *P. putida* KT2440 wild type was prepared and diluted to a cell density measured with the spectrophotometer ranging from OD_{600} 0.1 to 2.6. Blank medium was used to correct for background. A standard curve was determined for each separate sensor (Figure 7.4).

The device should mimic the irregularities within large bioreactors, which are also not maintained at perfect temperature for long periods of time. With the external sample placed at 30°C, and the device set at room temperature with a flow speed of 50 μL/min, the millifluidics device can stably expose the bacteria to temperatures fluctuations between 25°C and 50°C for 3.33 minutes per 10 minutes. This millifluidic device thus is capable, via an Arduino microprocessor, to control and monitor the temperature of the bacteria culture flown into the device, monitor the OD of the culture and record the outcome of the experiment over long periods of time.

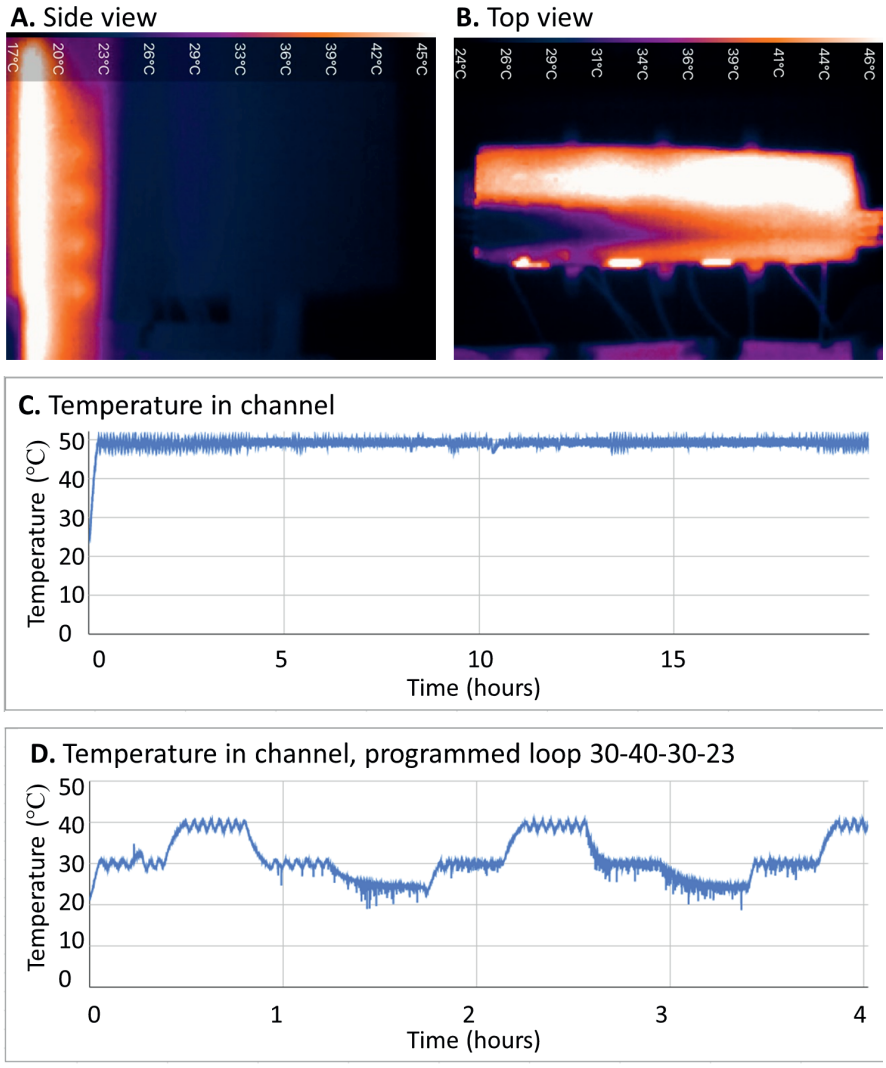

Figure 7.3: Temperature stability within the millifluidics device A) Thermal camera image of the side view of the millifluidics channel. B) Thermal camera image of the top view of the millifluidics channel. C) Temperature in channel as determined with the thermistor at output when the temperature is set at 50°C. *D) Channel temperature as determined with the thermistor at output when the temperature regulation is set to a loop of* 30 – 40 – 30 – 23°C.

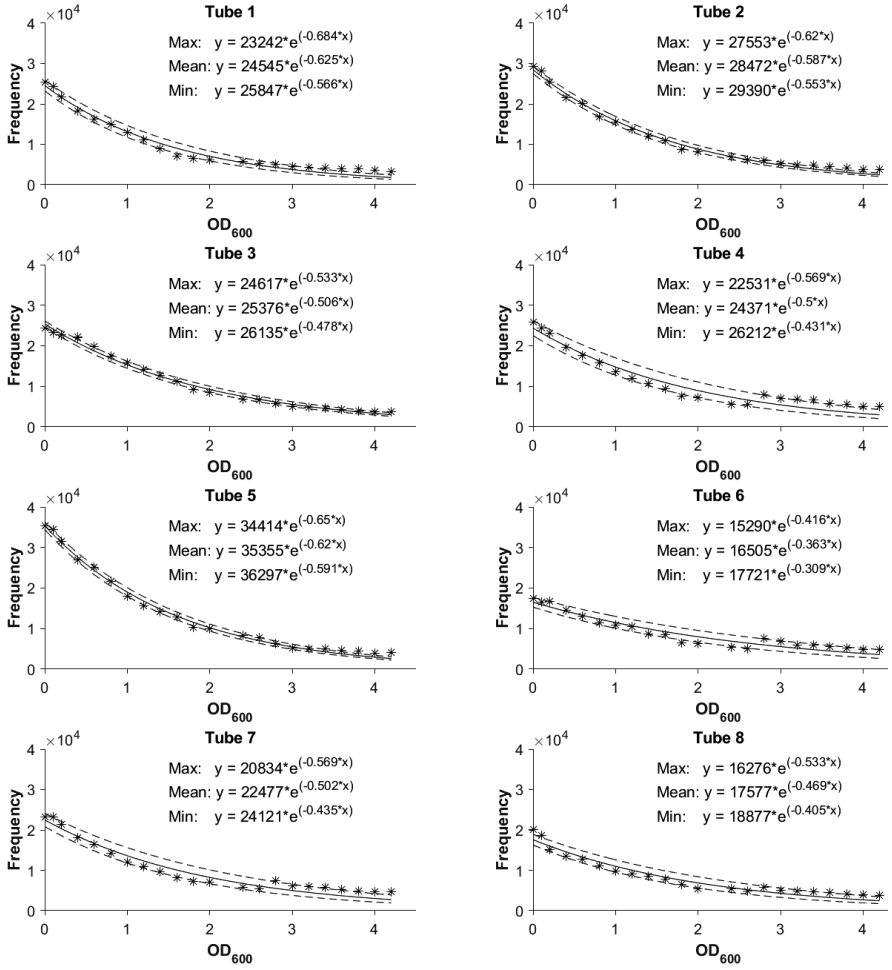

Figure 7.4: Calibration curves for cell density. The non-linear fitted exponential relationship between the frequency measured and cell density, by standard at OD_{600}, was determined using a series of cultures of P. putida KT2440 wild type with known cell density. Frequencies were first corrected with no-light measurements before calibration. The fitted lines were used to extract the formula required for translation of data to cell density. Per tube, a separate standard curve was prepared.

Thermophilic *nadB* in mesophiles increases the resilience to temperature fluctuations

In Chapter 6, the universal connection between NAD^+ availability and thermo-tolerance was established. For this, L-aspartate oxidase (*nadB*) was obtained from either *P. putida* KT2440 itself, the thermophilic *B. smithii* DSM4216T or the psychrophilic mesophile *T. flocculiformis* DSM 2094. The thermo-tolerance of each strain is depicted in Figure 7.5.

Bacterial strain	Optimal	Range	Source
Pseudomonas putida KT2440	30°C	25 – 40°C	[21]
Bacillus smithii DSM 4216T	55°C	25 – 65°C	[22]
Trichococcus flocculiformis DSM 2094	25 – 30°C	4 – 40°C	[23]

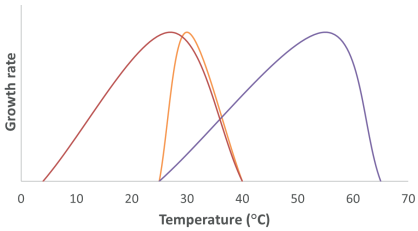

Figure 7.5: Thermo-Tolerance of P. putida, B. smithii and T. flocculiformis. Depicted in a table of optimum growth temperature and temperature range of strains of which the nadB gene originated (right), and a schematic representation of temperature tolerance of P. putida KT2440 (Orange), B. smithii DSM 4216T (Purple) and T. flocculiformis DSM 2094 (Red) (left).

To determine the performance of the recombinant strains in an industrial set-up, the recombinant strains were tested using the millifluidics device. *Pseudomonas putida* KT2440 Δ*nadB* strains carrying *nadB* from *Bacillus smithii* (pS638 nadBBsm) was tested against the wild-type strain, a negative empty plasmid control (pS638 empty) and a positive control with *nadB* from *P. putida* (pS638 nadBPpu) (Figure 7.6).

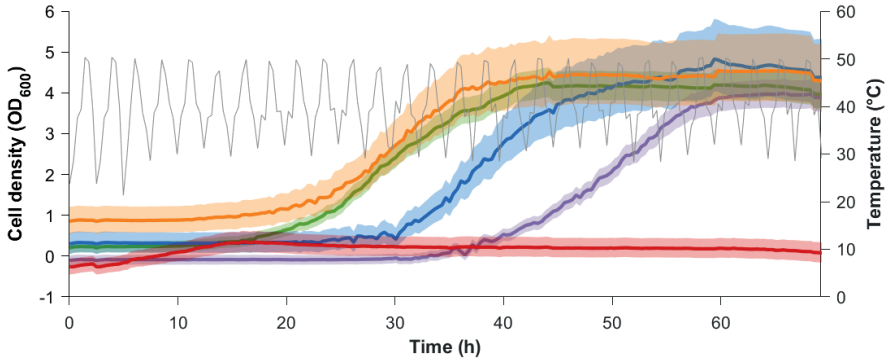

Figure 7.6: Thermo-Tolerance of recombinant strains in a downs-scaled system. P. putida KT2440 wild type (Green), P. putida ΔnadB pS638 empty (Purple), P. putida nadBPpu (Blue), nadBBsm (Orange), and nadBTfl (Red) were tested for growth performance through exposure to stochastic temperature fluctuations in a millifluidics system. Temperature is set to loop 50 – 40 – 30 – 40°C*. Cell growth was monitored over 65 hours using the automatic light-to-frequency sensor set-up, and translated to cell densities using the standard curves.*

The cell density was automatically monitored over 65 hours. Only the lag phase and the exponential growth phase were considered to determine the strain performance. It was seen that with a temperature fluctuation regime of 50 – 40 – 30 – 40°C, *P. putida* KT2440 Δ*nadB* pS638 nadBBsm equipped with the thermophilic ortholog from *B. smithii* grows better than the other strains, mimicking the wild type *P. putida* KT2440 which is unburdened by a plasmid.

The plasmid burden can be determined clearly by comparing *P. putida* KT2440 wild type and the positive control, *P. putida* KT2440 Δ*nadB* pS638 nadBPpu.

Discussion

Millifluidics compared to industrial scale

With the trend of down-scaling, the inability thus far to properly compare results obtained at perfect laboratory scale with what industry can expect at imperfect industrial scale is being tackled. By designing experiments which integrate foreseen variations and fluctuations, the irregularities that occur during the process of scaling up can be caught already during development stage, and can be solved beforehand. This could save much money in an industrial process in development and could make it easier for new products and techniques to enter the market [1, 5].

Earlier research focused on modelling of large-scale conditions to design a scale-down bioreactor [24–26]. These models show a multitude of other factors that influence microbial growth at large scale, and including both chemical gradients such as dissolved oxygen, pH, or nutrient fluctuations, and physical gradients such as pressure, viscosity, or temperature. Additionally, physiological effects on bacteria caused by flow and peristaltic mixing are not taken into account. In the millifluidic system there is a laminar flow, similar to blood-flow in capillaries, as opposed to the turbulent flow expected in large-scale bioreactors. Physiological effects can have great impact on bacterial growth and interactions, which we are only beginning to understand [27–29].

This down-scaled millifluidics system is thus only a small step towards this ambitious goal. The device was designed to only integrate one type of fluctuation–temperature–where many more exist. This choice was made since the recombinant strains were adapted to increased thermo-tolerance. Since the general strain performance at industrial scale is already known, the recombinant strain only had to be tested for increased temperature tolerance through the temperature fluctuations that exist within industrial set-ups.

The millifluidics device was specifically designed to expose multiple different samples to long-term experiment using temperature fluctuations. For the fabrication of the millifluidic device we opted for PDMS and ESCARGOT method. Although PDMS is not specifically good for heat transfer, this method is easy, cheap and fast for experimenting and proof of concept works. Once the characteristics of the millifluidic channels are determined, the millifluidic can be ordered and purchase in aluminium or glass without the need of changing the rest of the system.

The ease and adaptability of the device ensures a multi-purpose use in further research, which will focus on adding different types of external stress to improve the millifluidics device for down-scaling. Additionally, the strains should be tested at truly large scale to be able to make the correlation between the millifluidics and industrial scale, and evaluate the value of these predictions.

Conclusion

The designed millifluidics device proves an easy and adaptable way to estimate the effect of temperature fluctuations that occur in large-scale bioreactors on a lab-scale. Recombinant mesophiles equipped with the thermophilic ortholog for their *L-aspartate oxidase* gene perform better through high temperature fluctuations in a down-scaled system when compared to a positive control or even the wild-type strain, indicating that a strain equipped with thermophilic L-aspartate oxidase actually benefits from fluctuations. This research proves both the value of this adaptation for industrially applied mesophilic strains, and the importance of down-scaled systems to validate research.

Acknowledgements

We gratefully acknowledge K. J. A. Martens for fruitful discussions and help with data analysis on the project. We thank T. Schonewille for his help and all the lab-equipment we have cost him. We gratefully acknowledge financial support from the European Horizon 2020 project EmPowerPutida (Project reference: 635536). The funders had no role in study design, data collection and analysis, or preparation of the manuscript.

Conflict of interest

The authors declare there are no conflicting interests.

Author contributions

Conceived and designed the experiments: LFCK/PJS/VS Performed the experiments: MG/LFCK Data analysis: LFCK/MG Work supervision: LFCK/PJS/VS Wrote Manuscript: LFCK/MG Arranged Funding: VAPMdS

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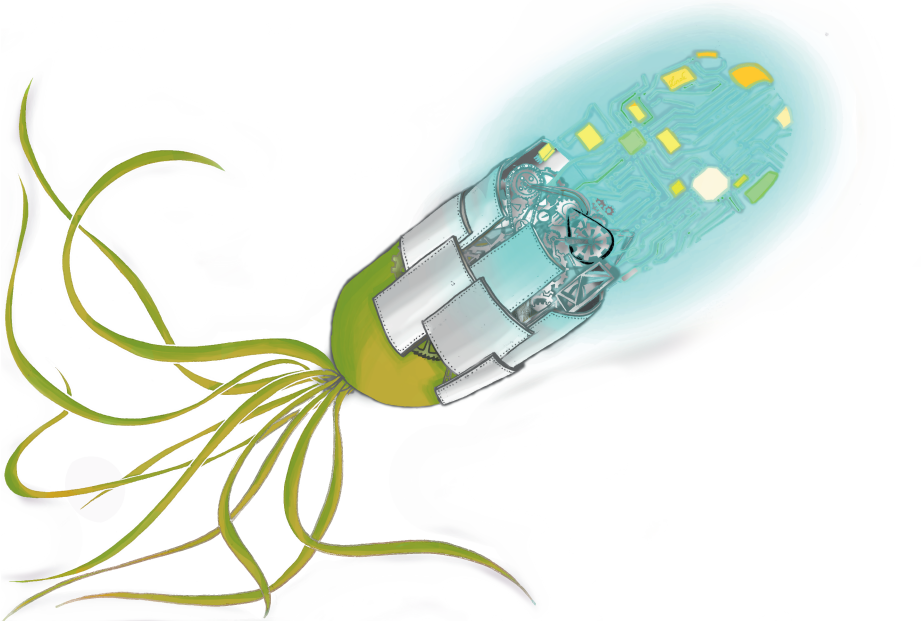
8 General Discussion and Future Perspectives



Linde F. C. Kampers

Synthetic biology for feasible sustainable industry

According to the European Association for Bioindustries, the contribution of the industrial biotech market in the European Union is expected to be between €57,5 billion and €99,5 billion to the European economy by 2030 [1]. In response to Europe's desire to develop a sustainable circular economy, it is foreseen that this growth will be largely driven by a step-wise replacement of fossil carbon materials by renewable feedstock [2, 3].

In this research, the widespread application of microbes was appointed as the way to achieve sustainable industry. However, in order to change completely from a petrochemical to a bio-based economy, many bottlenecks will have to be overcome.

To consider the true potential of a micro-organism, its full growth and production process and its ability to substitute or compete with a fossil-based production line have to be considered. In the U.S., for example, the total demand of crude oil is over one billion annually, or 10^9 metric tons per year. Electricity generation and the production of biofuels that can thus far be achieved using microbes is about 190 million tons [4, 5], which means their yield has to drastically improve to have a chance at replacing fossil fuels completely. The total potential of sustainable biomass in the U.S. has been estimated to be at least 1.2 billion tons per year within 50 years. The weight of sustainable biomass, however, does not equal its stored energy. As fossil fuels contain more energy and carbon density, it was estimated that at least seven times the amount of biomass is needed to equal the energy yield of crude oil. Industry wants to move towards sustainability, but the tools and sources are simply not available yet for a complete rigorous change. In addition, much innovative research never makes it through the Valley of Death to industrial application and market commercialisation. Besides technical restrictions, this study has helped to elucidate other crucial sector-based and social factors play a role **Chapter 2**.

The rapid increase in the ability to modify microbial genomes has revolutionized biotechnology and allowed for a substantial increase in innovation for industrial use [6]. This fast development of industrial biotechnology is now also known as the third industrial revolution, the first being the industrial revolution of machinery and the second of electricity. Currently, the internet breaks the fourth industrial rev-

olution, with everything connected to everything and automatic smart phones, cars and homes are becoming a reality. The impact of the fourth industrial revolution on the field of biotechnology cannot be ignored. Due to the ability to collect, store and share massive amounts of data computational methods have breached research, yielding much more information than we know how to handle.

This available data can be used to further focus research towards altering bacterial genomes using directed approaches to gain or lose characteristics and traits as wanted. If the right tools are available, the fourth industrial revolution may turn microorganisms into plug-and-play systems. The valuable ability to change anything via an ordered approach has made both new and old techniques much safer to use. With classical evolution methods, microbial traits can be cultured by enforcing them with a strict environmental control. Forward genetics, genome sequencing and transcriptomics analysis–and comparing the results to previously obtained data from the organism of choice or any other organism that shares this trait–offers insight in what has changed and all the effects this change has had. This insight has lead to more predictability in the effect of modifications to the genome of microorganisms, gradually leading to safer cell factories. This study has made progress in this field by taking one industrially applied bacterial strain, *Pseudomonas putida* KT2440, as a model organism, and increase its environmental robustness. Both classical evolution methods and available computational data through an ordered approach were applied towards this goal, systematically taking on two main bottlenecks. First, the strict aerobic nature of *P. putida* KT2440 was tackled in two different ways (Chapters 4, 5, [7]). Next, the thermo-tolerance of *P. putida* KT2440 and other species was improved, which was shown first on lab-scale, and next in a down-scaled millifluidics system to indicate strain improvement on a large scale (Chapters 6, 7, [8]). This finding was patented for application in industry (Appendix chapter 6).

Here, I will discuss the how this research has impacted aiding sustainable industry, and science in general.

The importance of keeping your end-user in mind

In Chapter 2, it became apparent that although the general aim of industry and academia align, academics are expected to make more substantial headway by taking on riskier projects.

Industry and academia agreed that industry rarely comes with a clear demand, but rather visit conferences and await interesting invitations into consortia so they can cherry-pick the most applicable and easily adaptable novel research in their production process. The reasons for this are clear: not only does industry fund academic research, but as they make products for a final consumer, introducing sector-based and social factors like government-enforced restrictions, patenting and public perception.

At all times, the biotech industry has to keep their end-user in mind. This simple claim was shown to have far-reaching implications. Production has to be cost-effective, sustainable, and safe, so industry has adapted microorganisms for a more sustainable production process, increasing the titre-rate-yield to improve cost-effectiveness, and has to follow severe safety-regulations for their production hosts. For food, feed, or food additives, only microbes that are generally regarded as safe (GRAS, [9]) can be used. To oblige these strict regulations and protect their signature production process, patents are invaluable, but restrictive in their own way. The introduction of novel microbes is only considered as long as the production process remains mainly the same, or if the titre-rate-yield is improved so much that it will make up for the required changes to the production process. As the value of the products increase, so does the flexibility of the industry. These same restrictions are simply not applicable in academic research, and rightly so. Academics should have the freedom to perform more fundamental research and keep a curiosity-driven approach to discover the unexpected. However, if the researcher aims for industrial and thus market application of their research or invention, industrial restriction must at least be kept in mind to increase chances of making it through the Valley of Death. Close collaboration from an early stage on is key.

Additionally, the existing gap between academia and industry has naturally filled with the rise of small companies. Start-ups companies are companies that

are formed directly from an academic background to commercialise a promising academic innovation. Some fear that start-up companies have increased the gap between academia and industry. However, the case can be made that start-up companies are a great opportunity to close the gap altogether, by easing the transition of academic research to actual industrial application, and opening up room for academics to focus on education and research. Collaborations between academia and industry should include some attention for the possibility of start-ups to develop, leading to more attention to industrialising promising results and growing them into market applications already from the development stage.

The GRAS myth and the status of KT2440

The American Food and Drug Administration (FDA) regulates market introduction for new Food ingredients or food additives by approving them for specific uses as GRAS (generally recognized as safe) substances [9–11]. The GRAS status serves as an official status valued highly in industry, as it indicates a validated safety for consumption. A substance may be GRAS only if its safety is based on the views of experts. GRAS status may be based on a history of safe use in food prior to 1958, or on scientific procedures, which require the same quantity and quality of evidence as would be required to obtain a food additive regulation. The base for any given GRAS status can be viewed via the application number on the GRAS website, `https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices`, although it is often indicated simply as "scientific procedures". The FDA response notices available for GRAS evaluations offer somewhat more information on how the GRAS status was awarded.

GRAS statuses are awarded based on colony forming units (CFU)/serving, aimed use and/or user, strain occurrence, and characteristics. It is specified that a strain is non-pathogenic and non-toxigenic, although it is not further proof or indication of the nature of any proof is not offered in the FDA notice. The processing of the strain is discussed. It is indicated that there exists prior peer-reviewed publications on use of the strain and safe consumption of a strain. Finally, the notice indicates the limitations on the GRAS status (if awarded) [9].

A strain without a GRAS status therefore does not mean it is not safe for consumption. It simply lacks enough proof for an official status. Additionally, GRAS statuses seemingly tend to be given out to well-established strains, which means that years of research and trials, and most importantly, a specific aim for strain use, are required before a GRAS status can even be contemplated. In addition, the limitations to a GRAS status are strictly defined. If the strain is not used according to its intended use or dose described, the GRAS status does not cover it. Toxicity, in any case, is a matter of quantity, not quality.

> The dose makes the poison, Paracelcus, 1538 [12].

In the same way, it can be said that pathogenicity is a matter of opportunity, not inherent to a strain. Examples of this can be found in our own gut. There is a growing academic interest in the involvement of previously labelled commensal gut microbes turned opportunistic pathogen in diseases or defects [13, 14]. Even though these microbes can be found naturally in the human gut, and have no negative effects in most, if the right opportunity arises these bacteria can help or harm.

Since 2015, almost 4000 papers were published on *P. putida* KT2440. Besides fundamental research and genomics, research has generally focused on increasing the industrial value of this species. This includes building new metabolic pathways, for instance to allow *P. putida* to degrade cellulose, lignin [15–18] or plastics [19, 20], redirecting its metabolism for industrial relevance [21–27], engineering adaptable surface-display [28], elucidation and perfecting its ability to produce selenium nanoparticles [29], tuneable biofilm formation to increase robustness [30, 31], biosensor development [32], increasing tolerance to micro- or anoxic conditions [33–37], and increasing strain thermo-tolerance [38].

Although over 40 unique first authors described *P. putida* KT2440 as GRAS, in chapter 3 [39] it was rediscovered not as GRAS, but as HV1 certified [40]. This certification indicates the strain is safe to work with in an ML1 lab environment, as long as the microbe is not ingested.

But what are the implications of this, and more importantly–what should they be? I argue that (strain) safety should consistently be handled on a case-to-case basis. Due to its extensive use in academic literature, the improvements in synthetic

biology tools which can help increase strain safety and the lack of information on the dosis and pathogenicity of this strain, the HV1-status of *P. putida* KT2440 should be seen as a promising step towards safe application of this strain in food, feed or food additives.

Evolution plus ordered approach equals increased changes of success

Classical genome engineering methods like adaptive laboratory evolution (ALE) experiments have been employed towards lifestyle engineering [41–43]. This method allows for a close replication of natural evolution to push microbes towards a desired lifestyle. This top-down approach allows for random changes to occur; any mutation that improves cellular performance under stressful conditions will thrive and can be isolated and characterized afterwards. A substantial benefit to this method is that no *a priori* knowledge of the limiting factor(s) is required. Room is left in the research process to use and possibly uncover those factors that have an effect on the strain performance under the selected environmental conditions. The major downsides of the application of random evolution methods are the time and work intensity. The conditions in which an ALE experiment are performed are crucial, since it concerns an iterative selection cycle. Any deviation from the desired conditions can lead to loss of the carefully evolved bacteria before they can adapt, or wild type bacteria still present might overgrow the evolved bacteria, both resulting in loss of progress. Additionally, random evolution can yield unwanted mutations, with only a few actually contributing to the desired characteristics. This means that ALE experimentation must always be followed by the careful and extensive analysis of all mutations that occurred.

The Design-Build-Test-Learn-cycle is a rational alternative approach. Prior knowledge available on the organism of interest is applied through *in silico* methods towards systematical lifestyle engineering. This not only saves time and resources beforehand, but offers the additional benefit that any changes made in the wild type strain towards improvement are exactly known, offering a safety aspect. The DBTL-cycle substantially shortens the time required for development by tackling foreseen

problems head-on.

However, computational methods used in the Design-phase of the DBTL-cycle have one main advantage, which is at the same time their main disadvantage: they rely heavily on the underlying data. Metabolic models represent simplified bacteria still capable of growth. However, models exclude many factors and gene or protein interactions that are poorly understood or unknown. Models can be applied to attempt to elucidate unknown interaction, by fitting a certain growth curve. However, that does not necessarily mean the fit found is the correct one, due to the simplification gap between model and reality [44]. Proper training models are thus invaluable to assess the reliability of computational methods. Another downside of this approach is that problems are specified beforehand, and answers are only sought in known factors. This leads to obvious solutions or discoveries, possibly missing fundamental discoveries or new gene or protein interactions. As much on the specific functions and workings of microorganisms remains unknown, computational methods should be handled carefully and critically.

An ordered approach to lifestyle engineering was applied in Chapters 4 and 5, where an attempt was made to adapt the strict aerobic nature of *P. putida* KT2440 to a facultative anaerobic lifestyle through anaerobic fermentation [7] or anaerobic respiration. In both attempts, various computational methods were applied to pinpoint bottlenecks. In chapter 4, some of these bottlenecks were addressed and evolutionary methods were applied to determine and improve strain performance first under micro-oxic conditions, then under anoxic conditions. To speed up the development process, the DBTL-cycle was applied to develop a design of *P. putida* KT2440 capable of anaerobic fermentation. For this, two metabolic models were employed. *In silico*, the adapted strain was able to grow under anoxic conditions. After building the design, the recombinant strains were tested under micro-oxic conditions in oxygen gradients, and after multiple rounds of ALE using these oxygen gradients the recombinant strains were able to grow, showing the benefit of using the DBTL-cycle and ALE experiments combined. While saving time and resources by using an ordered approach, you still benefit from the freedom and unexpected solutions offered by adaptation experiments. However, the ultimate short-comings of relying too much on computational methods was shown in Chapter 5, when the designs

made and adapted in Chapter 4 were tested under anoxic conditions, but proved unable to grow.

In **Chapter 5**, genomics, transcriptomics and metabolic modelling were applied to *in silico* design and test different recombinant strains. Together, these methods proved most valuable. It was determined that to enable growth in anoxic conditions through respiration, 69 genes of known function would have to be added to the genome, and 5 vitamins to be supplemented through the medium. In addition, the study revealed a possible implication for a number of genes of unknown function.

To add almost 70 genes to the genome is a Herculean task. Genome engineering and predictable gene expression were identified as topics of interest by both academia and industry (Chapter 2), which is mirrored by the fast rise of methods to allow for the programming gene expression like CRISPR [45]. Still, state-of-the-art methods like yTREX [46] for integration of large operons or multiple genes allow incorporation of approximately 14 genes at one time in *P. putida*. This would have to be repeated 5 times to include the amount of genes required for anaerobic respiration. Moreover, heterologous gene introduction can have unexpected complications. Introduced genes might not function, which can be due to incorrect regulation, incorrect placement in the genome, incompatibility with the host transcription and translation machinery, incorrect folding, and due to a myriad of unidentified problems [47, 48].

Another bottleneck to this design is that it excludes 244 domains of unknown function that on statistical grounds could play a role in anaerobic respiration. Although there is much data available on both genomics and transcriptomics, these uncharacterised genes of unknown function proved the main bottleneck to come to a complete design for **Chapter 5**.

Thermo-tolerance and down-scaled methods

The temperature range in which a microbial strain can grow is referred to as its thermo-tolerance. *P. putida* KT2440 is a mesophilic strain with a relatively small temperature range of 25°C to 40°C with an optimal growth temperature of 30°C [49]. In small scale laboratory conditions, this optimal growth temperature can be

established. However, in large industrial-scale bioreactors, temperatures can deviate as much as 10°C due to uneven mixing and varying conditions [50, 51]. Thermo-tolerance of an industrial strain, thus its ability to cope with such temperature variations, are thus an important aspect in industry. The strain thermo-tolerance was tackled in Chapter 6. The largest bottleneck of *P. putida* KT2440 in NAD^+ biogeneration via the aspartate pathway, L-aspartate oxidase, was replaced by the same gene from the thermophilic strain *Bacillus smithii* DSM 4216T via a plasmid. As a result, the exponential growth phase was twice as steep when compared to a positive control when growing the bacteria at 40°C. In *E. coli* JW2558, this exchange decreased the lag-phase ten times compared to a positive control when grown at 44°C. The redox balances was thus proven to be a major bottleneck in the temperature resilience of any strain. This finding was patented (Appendix chapter 6).

For this innovation to be considered industrially interesting, it must be shown how the thermo-tolerant recombinant strains weather the fluctuations that occur on a large scale. Scale-up was deemed vitally important by both industry and academia (Chapter 2) for innovations to survive through the so-called "Valley of Death" to market commercialisation, since tests on laboratory scale are not reliable for industrial scale and thus for industrial applications [52–56]. Instead of applying the recombinant strains obtained (Chapter 6, [8]) in large-scale bioreactors, costing much time, money and effort, it was decided to focus on down-scaling instead (Chapter 7).

Millifluidics and microfluidics are a well-known concept rising in importance as their toolkit grows. However, these methods are mainly applied to perform fundamental single-cell research [57], affordable chemical synthesis [58], therapeutic applications in the form of nanoparticles for easy drug delivery [59] or to develop a lab-on-a-chip [60].

However, relatively few scientists seem to be working on using down-scaled systems to imitate large-scale conditions on a lab-scale [61], even though this was recognised as vital for survival through the "Valley of Death" [52–54]. To determine the industrial thermo-tolerance of the adapted *P. putida* KT2440, a down-scaled millifluidics system was built. This system was specifically designed to expose cell cultures to realistic cyclic temperature fluctuations that occur during large scale fermentation. Due to its modular design this system can be expanded easily, incorporating

different types of fluctuations to mimic industrial scale fluctuations better. Novel techniques make aeration and mixing of fluids possible [62–68], offering an opportunity to test gradients of oxygen or medium components. So rather than perfecting large-scale bioreactors, the fluctuations are simply applied during the development stage so that their effects can be taken into account. As such, down-scaling offers the answer to scale-up problems, speeding up the costly development stage by offering reliable results through scale-up.

Concluding remarks

Microbial cell factories can have a large impact towards sustainable society, but their application is currently limited for a multitude of different reasons. Lifestyle engineering to improve microorganisms for industrial applications using *in silico* methods in combination with novel and classical genome engineering methods, followed by testing the novel strains in down-scaled systems, offers an improved chance for new research to make it through the Valley of Death. *In silico* methods can in the long run enable the predictability of gene insertion that would streamline the DBTL-cycle into a linear process and thereby revolutionize industrial biotechnology. Down-scaling offers a way to test strains in the early development stage to more reliably mirror large scale production outcomes.

The main aim for this research was to increase the robustness of industrially applied *P. putida* KT2440 by applying an ordered approach and lifestyle engineering towards feasible sustainable industry. The work performed in this thesis has not only attempted to elucidate the main bottlenecks by active outreach to industry and academia, but has contributed to solving important industrial bottlenecks of *P. putida* KT2440 by combining *in silico* research with wet-lab experiments and method development of down-scaled systems. This research has yielded an industrially applicable outcome in the form of a patent. This contribution has made steps towards application of more research in industry by making attainable adjustments in any micro-organism, thus hopefully aiding the final goal of approachable sustainability.

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9 Summary

Linde F. C. Kampers

English and Dutch

Due to the devastating effects environmental change can cause, sustainability has occupied top priority worldwide for years past. Bacteria aid the environment and battle environmental change by removing toxic pollutants. Industrial application of microbes could thus improve sustainability in industry. However, not all microbes are attractive for industrial application. The field of Systems and Synthetic Biology in Biotechnology offers a vast array of tools to change this.

This thesis, entitled "Microbial Lifestyle Engineering", aims to improve the lifestyle of industrially applied microorganisms. Due to their inherent robustness, large redox capacity, adaptivity and genomic accessibility, the gram-negative, prokaryotic bacteria *Pseudomonas putida* was used as a proof-of-principle, but was extended to *Escherichia coli* and the eukaryotic yeast *Saccharomyces cerevisiae*.

Since this research was aimed to make improvements for industrial purpose from an academic point of view, **Chapter 2** describes a series of interviews which were conducted to determine how far those two worlds are apart. We uncovered the main points of miscommunication and opportunities between both fields. While industry focuses on today's problems, academia focuses on uncovering solutions to tomorrow's problems. Both fields agree that in general better communication is required so that more findings end up as applications.

In **Chapter 3**, a common misconception was eradicated. While the microorganism of interest *Pseudomonas putida* strain KT2440 is often characterised as Generally Regarded as Safe, or GRAS, this description is incorrect. Extensive literature search yielded the original food and drug administration report which describes that *Pseudomonas putida* KT2440 is HV1 certified, not GRAS. The HV1 certification entails that the strain is safe to use at a P1 or ML1 laboratory without further safety measures, but should not be ingested. This does not change its many industrial applications, but does withhold it from use in the food industry.

Pseudomonas putida is a metabolically versatile, genetically accessible, and stress-robust species with outstanding potential to be used as a workhorse for industrial applications. Due to the importance of robustness under micro-oxic conditions for a stable production process, the obligate aerobic nature of *P. putida* severely limits its use for biotechnology applications. This was attributed to an inability to produce enough ATP and maintain its redox balance without molecular oxygen.

In **Chapter 4**, a combination of genome-scale metabolic modelling and comparative genomics is used to pinpoint essential O_2-dependent processes. These explain the inability of the strain to grow under anoxic conditions: a deficient ATP generation and an inability to synthesize essential metabolites. Based on this, several *P. putida* recombinant strains were constructed harbouring acetate kinase from *Escherichia coli* for ATP production, and a class I dihydroorotate dehydrogenase and a class III anaerobic ribonucleotide triphosphate reductase from *Lactobacillus lactis* for the synthesis of essential metabolites. Initial computational designs were fine-tuned by means of adaptive laboratory evolution. We demonstrated the value of combining *in silico* approaches, experimental validation and adaptive laboratory evolution for microbial design by making the strictly aerobic *Pseudomonas putida* able to grow under micro-oxic conditions.

Chapter 5 continues on this research, and shows that although the previously recombined strains did growth better under micro-oxic conditions, they did not lead to growth or better survival under anoxic conditions. Subsequently, a semantic systems biology approach was used to design a derivative of *P. putida* KT2440 capable of anaerobic respiration. We combined data from comparative genomics of 1624 different *Pseudomonads* with transcriptome data from the facultative anaerobe *Pseudomonas aeruginosa* and genome-scale metabolic modelling simulations. The results indicate that to enable anaerobic respiration, at least 69 additional genes and 3 externally added vitamins are required.

Chapter 6 was aimed at a different way of lifestyle engineering for industrial improved use. The universally applied essential redox factor NAD^+ is involved in internal signalling, defense, detoxification, circadian regulation, chromosome segregation, aerotaxis, sporulation, biofilm formation and dispersal. The redox balance is often the first response to internal and environmental changes. Previous work described the thermal stability of thermophilic *L-aspartate oxidase*; the main bottleneck in the aspartate pathway for NAD^+ biosynthesis.

We determine the effect of exchanging the *L-aspartate oxidase* gene in different mesophiles for a thermophilic ortholog. In *E. coli*, the exchange results in a 10 times shorter lag phase when grown at 44°C. In *P. putida* the generation time doubled when grown at 40°C. To determine how widespread the connection between NAD^+ availability and thermo-tolerance is, the mesophilic or thermophilic aspartate pathway was implemented in yeast, which generates NAD^+ via the kynurenine pathway. Not only was *S. cerevisiae* able to use the aspartate pathway for NAD^+ generation, but supplemented with the bacterial pathway the strain proved more robust when grown at 41°C.

To determine what effect this increased thermo-tolerance could have on an industrial scale, **Chapter 7** describes the development of a millifluidics device. A down-scaled microfluidics system was built for this purpose. When kept at room temperature, the device can expose the cells to temperature fluctuations ranging from 25°C to 50°C. The system was applied to monitor long-term recombinant strain performance with respect to these fluctuations. The recombinant thermo-tolerant strains were tested in this system over a three-day span. It was seen that with a temperature fluctuation regime of 50−40−30−40°C, *P. putida* KT2440 Δ*nadB* pS638 nadBBsm equipped with the thermophilic ortholog from *B. smithii* grows better than the other strains, including the wild type *P. putida* KT2440 which is unburdened by a plasmid.

The discovery of the universal link between the NAD^+ availability in microbes and their thermo-tolerance was patented. **Appendix chapter 6** shows the patent summary. This invention relates to the field of molecular microbiology, metabolic engineering, and fermentation technology. In particular, this relates to microbial host cells that have been engineered for increased tolerance to temperature shifts, for increased performance at temperatures different from optimal temperature of a micro-organism and/or for changing at least one of the cardinal temperatures of a micro-organism by replacing an endogenous NAD^+ biosynthesis gene by a heterologous gene encoding a corresponding enzyme with another temperature profile. With the patent, a full trajectory from lab to test to application was completed.

Samenvatting

Door de drastische gevolgen die klimaatverandering teweeg kan brengen is duurzaamheid wereldwijd al jaren van hoge prioriteit. Microben hebben een groot aandeel in de klimaatbalans omdat ze toxische verontreinigingen uit de natuur kunnen filteren. Microben worden in industriële processen toegepast om de industrie te verduurzamen. Niet elk micro-organisme is aantrekkelijk voor industriële applicatie. Het veld van Systeembiologie in Biotechnologie biedt nieuwe methoden om hier verandering in te brengen.

Deze scriptie, diens titel vrij vertaalt naar "Het Aanpassen van Microbiele Levenswijze", is erop gericht om industrieël toegepaste micro-organismen te verbeteren. Door zijn inherente robuustheid, grote redox capaciteit, aanpassingsvermogen en vrij verkrijgbaar genoom wordt de gram-negatieve prokaryote bacterie *Pseudomonas putida* gebruikt als model-organisme, maar het onderzoek is uitgebreidt met *Escherichia coli* en het eukaryote gist *Saccharomyces cerevisiae*.

Hoofdstuk 2 omschrijft het industriële landschap ten tijde van dit onderzoek. Dit onderzoek was erop gericht om bacteriën te verbeteren vanuit een academisch oogpunt, maar voor industriële toepassing. Om te bepalen hoe ver deze velden uit elkaar liggen werd er een serie interviews gehouden met participanten zowel uit de academia als uit de industrie. Door hun compleet verschillende doelstellingen komen de meningen en prioriteiten van academia en industrie niet altijd overeen. De voornaamste punten van miscommunicatie en verschillende kansen zijn blootgelegd in dit hoofdstuk. Terwijl de industrie voornamelijk focust op de problemen van vandaag zoekt academia oplossingen voor de problemen van morgen. Beide partijen zijn het erover eens dat communicatie met rasse streden verbeterd zou moeten worden, zodat meer uitvindingen vanuit het onderzoek toegepast worden.

In **Hoofstuk 3** wordt een veelvoorkomende misconceptie rechtgezet. Ondanks dat de bacterie van interesse, *Pseudomonas putida* KT2440, vaak wordt omschreven als "generally regarded as safe"(GRAS), is deze omschrijving incorrect. Dit officiële label wordt uitgegeven door het Amerikaanse Voedsel en Medicijn Administratiebureau (FDA) en geeft aan dat stoffen of microben veilig geïngesteerd kunnen worden. Echter, intensief literatuuronderzoek bracht het officiële FDA rapport naar boven waarin *Pseudomonas putida* KT2440 gecertificeerd wordt als HV1, niet als GRAS. Deze certificatie houdt in dat er met de bacterie veilig op een p1 of ML1 lab gewerkt kan worden zonder verdere veiligheidsmaatregelen, maar niet geïngesteerd kan worden. Dit verandert niets aan de vele hedendaagse toepassingen van dit micro-organisme, maar weerhouden het gebruik ervan in de voedselindustrie.

Pseudomonas putida is een metabolisch veelzijdig, genetisch toegankelijk en stress-robuuste bacteriële soort met een uitstekende potentie om gebruikt te worden als werkpaard voor industriële toepassingen. Terwijl industrie het belang ondervindt van stabiele bacteriële groei onder micro-zuurstof condities om het productieproces consistent te houden, weerhoudt de obligaat aerobe natuur van *P. putida*, die stamt uit het onvermogen om genoeg ATP te produceren en de redox-balans te onderhouden in afwezigheid van moleculaire zuurstof, de bacterie van een doorbraak voor weidse toepassing in biotechnologie. In **Hoofdstuk 4** wordt een combinatie van genoom-schaal metabolische modellen en vergelijkende genomics gebruikt om essentiële zuurstof-afhankelijke reacties te vinden. Deze verklaren waarom de bacterie niet kan groeien onder zuurstofloze omstandigheden: een tekort aan ATP generatie en het onvermogen om essentiële metabolieten te synthetiseren. Gebaseerd op deze uitkomst zijn meerdere *P. putida* recombinanten gegenereerd, die acetaat kinase (van *Escherichia coli*) voor ATP productie, een klasse I dihydroorotaat dehydrogenase, een klasse III anaerobe ribonucleotide trifosfaat reductase (beiden van *Lactobacillus lactis*) voor de synthese van essentiële metabolieten dragen, of alledrie. Deze computationele ontwerpen werden getest met behulp van adaptieve laboratorium evolutie. We demonstreerden hiermee de waarde van het combineren van verschillende *in silico* methoden met experimentele validatie en adaptieve lab evolutie om een microbieël ontwerp te maken via een gerichte aanpak, door de strict aerobe *Pseudomonas putida* KT2440 succesvol te laten groeien onder micro-oxische

condities.

Hoofdstuk 5 omschrijft het vervolg van dit onderzoek, en toont dat alhoewel de eerder gemodificeerde bacteriën groeiden onder micro-oxische omstandigheden, ze groei noch beter uithoudingsvermogen laten zien onder anoxische condities. Vervolgens werd een semantische systeembiologie aanpak gebruikt om een ontwerp te maken voor een afgeleide van *P. putida* KT2440 die in staat is tot anaerobe groei door respiratie. We combineerden data van vergelijkende genomics van 1624 verschillende *Pseudomonas* stammen met transcriptomics data van de facultatief anaerobe *Pseudomonas aeruginosa*, en met genoom-schaal metabolische model simulaties. De resultaten tonen aan dat om *Pseudomonas putida* KT2440 het vermogen te geven om anaerobe respiratie uit te voeren, minstens 69 additionele genen en 3 extern toegevoegde vitamines nodig zijn.

Hoofdstuk 6 schakelt over naar een andere manier om de microbiële levenswijze te verbeteren voor industriële toepassingen. De universeel toegepaste essentiële redox factor NAD^+ speelt een rol in interne signalering, verdediging, detoxificatie, circadiaanse klok regulatie, chromosoom segregatie, aerotaxatie, spoorvorming, biofilm formatie en verspreiding. De redox balans is vaak de eerste om te reageren op interne en externe veranderingen. Eerder onderzoek omschreef de thermostabiliteit van thermofiele L-aspartaat oxidase, een enzym essentieel voor de biogeneratie van NAD^+ via de aspartaat route in *P. putida*. L-aspartaat oxidase is het voornaamste knelpunt in de route.

In dit hoofdstuk bepaalden we het effect van NAD^+ beschikbaarheid op thermotolerantie door L-aspartaat oxidase van verschillende mesofiele soorten te vervangen voor een thermofiele ortholoog. In *E. coli* resulteerde deze uitwisseling in een 10 keer kortere lag-fase wanneer de bacteriën gegroeid worden op 44°C. In *P. putida* leidt dit tot een verdubbeling van de generatietijd wanneer de bacteriën op 40°C groeien. Om te bepalen hoe universeel de verbinding tussen NAD^+-beschikbaarheid en thermo-tolerantie is werden zowel de mesofiele als thermofiele systeem in de gist *Saccharomyces cerevisiae* gezet, die normaliter NAD^+ genereert door middel van de kynurenine route. Niet alleen werkte de aspartaat route in de gist, *S. cerevisiae* toont hogere robuustheid tegen hogere temperaturen tot 41°C wanneer deze is uitgerust met het aspartaat NAD^+ biogeneratie systeem.

Om te bepalen welk effect deze verhoogde thermo-tolerantie heeft op industriële schaal omschrijft **Hoofdstuk 7** de ontwikkeling van een millifluidica systeem, welke gebruikt wordt om de recombinante cellen over een lange termijn bloot te stellen aan temperatuurfluctuaties van minimaal 25°C tot maximaal 50°C. De recombinante cellen met het L-aspartaat oxidase gen van de thermofiele *B. smithii* groeiden significant beter dan de controlestammen wanneer ze tijdens de fermentatie aan stochastische temperatuurfluctuaties werden blootgesteld.

De ontdekking van de universele link tussen de beschikbaarheid in NAD^+ in microben en hun thermo-tolerantie is gepatenteerd. **Appendix hoofdstuk 6** presenteert de samenvatting van het patent. Deze uitvinding relateert aan het veld van moleculaire microbiologie, metabolische bouwkunde, en fermentatie technologie. De uitvinding betreft specifiek microbiële gastheercellen die aangepast zijn om een hogere tolerantie tegen temperatuur fluctuaties te hebben, voor een verbeterde prestatie op temperaturen verschillend van de optimum-temperatuur van een micro-organisme en/of voor het veranderen van minimaal één van de kardinale temperaturen van een micro-organisme door het vervangen van een endogeen NAD^+ biosynthese gen door een heteroloog die codeert voor hetzelfde enzym met een verschillend temperatuurprofiel. Met dit patent is een volledig traject van laboratorium tot test tot applicatie afgerond.

10 Acknowledgements, About the Author

Linde F. C. Kampers

The Road not Taken

Robert Frost

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I-
I took the one less traveled by
And that has made all the difference.

Acknowledgements

Against my nature, I'll try to keep it short. Without all of your help, I could not have brought this work to such great completion. I'd like to thank all of you for your contributions, be it at the start, at the end, or throughout.

Dear **Vitor**, thank you for the opportunity to work in SSB. You never failed to come up with new ideas, and your insights and enthusiasm in consortia or project discussions was highly valued.

Dear **Peter**, thank you for maintaining the open door policy I requested at the start of my project. I always valued our interactions, even though I'm sure you tired of the countless times I stopped by. Still, you ventured down into my office, a daring move into the unknown.

Dear **Rita**, I really enjoyed our many discussions together concerning the technicalities and future plans of our experimental work. I am infinitely glad that you were appointed my daily supervisor; you helped me through the good times and the bad.

Dear **Jasper**, you endured my endless sea of questions when working together on computational things. I thoroughly enjoyed the many insights, discussions and ideas we've had. Through you, I gained much understanding in the gap that exists between dry- and wetlab, with which I could build a bridge (albeit a small one).

Dear **Maria**, thank you for helping me get through tougher times. I thoroughly enjoyed taking your course at the start of my PhD and know it has helped me a lot. I made thankful use of your seemingly infinite patience, especially when debating computational methods.

Dear **Ruben**, I dropped into the project you had kickstarted, and although it did not work out as you'd envisioned, I hope I did you proud. Your calm demeanour helped me tons during my PhD, and the gamenights and dinners were much appreciated throughout! Thank you so much for all the great talks we've had (although some sure must have felt endless to you).

Dear **Enrique**, we have shared many discussions, lunches, coffees, dinners, talks and wet-lab projects together. I thoroughly enjoyed your enthusiastic and overall positive attitude towards PhD life and beyond. With that attitude I know you'll be

successful in the future.

Dear **Tom**, thank you for the many (emergency aid) discussions we've shared, the equipment you helped me build, but above all the broken equipment you helped me fix or replace. Early on you seemed to know to only give me the rusty old equipment for my experiments, and I'm glad you did!

Dear **Ruud, Vittorio, Fons, Annemarie, Maria, Edoardo, Frits, Jacques** and **Sacco**; Thank you so much for freeing up time to collaborate with me, lending me equipment or discussing my work. Your effort has helped me thoroughly throughout my PhD, improving my work from sometimes unexpected angles.

Dear EmPowerPutida consorts, **Víctor, Ian, Bernhard, Ralf, Sven, Cecília, Andrea, Mechthild, Andreas, Max-Philipp, Robert, Alex, Max, Huseyin, Bastian, Lutz, Rebecca, Naveed, Jennifer, Stephen, Wendy, Dominika**; the EPP consortium meetings held every six months not only seriously helped advance my project each time, but were also amazing on top of that. Thank you all for your scientific and cultural input: you've taught me well!

Dear **Pablo**, thank you for your help during the early stages in my experimental work, and graciously hosting me at your lab. The time there has certainly aided towards finishing this thesis.

Dear DTU SEM lab; **Nico, Daniel, Patricia, Antonin, Ekaterina, Lorena, Silvia** and **Georgios**; you have made my stay amazing. Thank you for the warm welcome and the immediate incorporation in your tight group, I learned so much and had a great time.

Dear **Sonja, Ben** and **Femke**. Each in your own way has helped me procure this position in the first place, and I hope I did you proud. I cannot thank you enough for helping me start my career.

Dear officemates: **Rita, Tom, Marta, Nong, Ruben, Enrique, Lyon, Maria, Luis, Nhung, Erika and Niru**; thank you all for the great times we've shared! I thoroughly enjoyed bothering you all to death with questions, remarks, chats, drawings, activities and chocolate! I have warm feelings to the annual Easter Egg search, and hope you will keep some of our proud traditions alive where possible.

Dear students: **Max, Min, Claudia, Andrea, Arianna, Arsa, Tom** and **Tijmen**. As I hopefully taught you, in turn each of you taught me valuable lessons I will

gratefully apply in my future.

Dear SSB, old and new, in no particular order: **Edoardo, Rob, Tom, Rita, Marta, Nong, Dorett, Rik, Wasin, Luis, Lyon, Maria, Tjerko, Sabine, Bart, Benoit, Erika, Nhung, Sarah, Christos, Jasper, Emma, Sanjeev, Anna, Willemijn, Maarten, Bastian, Mirella, Niru, Anna, Christina**; together, you make SSB a great place to be! I have enjoyed the dinners, the game nights, Christmas events, high chocolates, Easter breakfast, plain ol' lunches and coffee times shared. I am grateful to have met all of you!

Dear **Martijn, Emmy** and **Joyshree**, I highly enjoyed organising the MIB and SSB labtrip with you, and all the awesome chats, lunches and coffees we've had since.

Dear **Carrie, Marie** and **Jara**; the many coffees, sporting nights (or cheating them), dinners and other activities we've shared kept me sane and happy. I am glad to have such amazing friends; each of you inspire me in your own way. Thank you so much for being there for me these last years, and I hope I can do the same for you.

Dear **Merel, Iris, Violet**. Our sisterhood (and the Whatsapp group that comes with that) is invaluable to me. Even though we each pursue our own dreams, moving to different countries every now and then, it is amazing to be able to be so sure of support when it's needed most. Thank you **Simon** and **Dries**, who also (first or secondhand) had to hear about all the hardships of PhD life. I'm glad you're part of our family.

Dear **Frans** and **Lily**. My amazing parents. You always believed in my abilities, and never shied away from asking critical questions. You are the reason our family bond is so strong (and not only because of the great hospitality, silly humour, fantastic advice or awesome cooking skills). You inspire me every day.

Dear **Koen**. I am still grateful we met so early on. We've shared the (my) hardships of college life and those of PhD candidacy together. You painstakingly read everything, listened to every story, and offered your highly valued honest opinion every step of the way. The best part of this PhD was our wedding, without a doubt. Thank you for the amazing times so far, and cheers to those to come!

List of Publications

Linde F. C. Kampers[1], Max Goessens[1], Vittorio Saggiomo*, Peter J. Schaap*. "Prokaryotic Thermo-Tolerance in a Down-Scaled System." *Manuscript prepared for submission*

Linde F. C. Kampers, Rita J. M. Volkers, Daan Hooijenga, Andrea Daveri, Bart Nijsse, Vitor A. P. Martins Dos Santos, Peter J. Schaap. "The Universal Connection in Microorganisms Between NAD^+ Availability and Thermo-Tolerance." *Manuscript prepared for submission*

Linde F. C. Kampers, Enrique Asin-Garcia, Peter J. Schaap, Annemarie Wagemakers, Vitor A. P. Martins dos Santos. "Navigating the Valley of Death: Perceptions of Industry and Academia on Production Platform and Opportunities." *Manuscript prepared for submission*

Linde F. C. Kampers, Jasper J. Koehorst, Ruben G. A. van Heck, Maria Suarez-Diez, Alfons J. M. Stams, Peter J. Schaap. "A Rational Design of *Pseudomonas putida* KT2440 capable of Anaerobic Respiration." *Manuscript submitted for publication*

Kampers, Linde FC, Ruben GA Van Heck, Stefano Donati, Edoardo Saccenti, Rita JM Volkers, Peter J. Schaap, Maria Suarez-Diez, Pablo I. Nikel, and Vitor AP Martins Dos Santos. "In silico-guided engineering of Pseudomonas putida towards growth under micro-oxic conditions." Microbial cell factories 18, no. 1 (2019): 1-14.

Kampers, Linde FC, Rita JM Volkers, and Vitor AP Martins dos Santos. "Pseudomonas putida KT 2440 is HV 1 certified, not GRAS." Microbial biotechnology 12, no. 5 (2019): 845-848.

Embregts, C. W. E., D. Rigaudeau, L. Tacchi, G. P. Pijlman, **L. F. C. Kampers**, T. Vesely, D. Pokorova, P. Boudinot, G. F. Wiegertjes, and M. Forlenza. "Vaccination of carp against SVCV with an oral DNA vaccine or an insect cells-based subunit vaccine." Fish & shellfish immunology 85 (2019): 66-77.

Overview of completed training activities

Discipline specific activities	Organised by	Year
3rd GA EmPowerPutida	EPP Consortium	2016
4th GA EmPowerPutida	EPP Consortium	2017
5th GA EmPowerPutida	EPP Consortium	2017
Evening course Wageningen Dialogue	SSB	2017
6th GA EmPowerPutida	EPP and P4SB consortia	2018
Stay in Denmark	SSB and Novo Nordisk Foundation	2018
Syn City Festival	SSB	2018
7th GA EmPowerPutida	EPP Consortium	2018
8th GA EmPowerPutida	EPP Consortium	2019
GrassRoots symposium 1.0	Dechema/iAMB/Aachen University	2018
GrassRoots symposium 2.0 co-organiser	Leiden University/WUR	2019
Symposium "Brave New World of Smart Data"	SSB	2019
General Courses		
PhD week	VLAG	2016
Competence assessment	WGS	2016
PhD Carousel	WGS	2017
Attune Flow Cytometry Course	ThermoFisher	2017
Supervising BSc MSc Students	WGS	2018
Career Orientation	WGS	2019
Workshop Safety by Design	WUR	2019
Interview Instruction	WUR, HSO	2019
Optionals		
Preparation of research proposal	SSB	2016-2017
Weekly group meetings	SSB	2016-2020
Seminars SSB	SSB	2016-2020
Seminars MIB	MIB	2016-2019
Retreat SSB WUR	SSB	2016
Retreat SEM DTU Denmark	DTU Biosustain	2018
VLAG PhD Council member/chair	VLAG	2016-2020
VLAG Board, PhD candidate representative	VLAG	2019-2020
Molecular Systems Biology	WUR	2017

About the author

Linde Francisca Cornelia Kampers was born on the 17th of August 1992 in Arnhem, the Netherlands. Driven by curiosity into the workings and applications of all living things and drawn by the wide application possibilities, she opted for studying Biotechnology in 2011. She decided to specialise in medical biotechnology, ending her BSc and starting her MSc with a combined project between the Virology and Immunology groups at Wageningen University, focused on the development and application of an oral fish vaccine against VHSV and SVCV. She completed her MSc with an internship at Merial (now Boehringer-Ingelheim) in Lelystad, the Netherlands , where she performed reverse genetics research on Foot and Mouth Disease Virus in a high containment environment.

In pursuit of a leading capacity, she entered a PhD candidacy in 2016 in Systems and Synthetic Biology, aimed at improving the bacteria *Pseudomonas putida* KT2440 for industrial applications within the EmPowerPutida H2020 project.

Throughout her studies and her PhD, she engaged in many extracurricular activities including Chair of the VLAG PhD Council and PhD candidate representative of the VLAG Board.

Within 3.5 years, her work resulted in this thesis.

Colophon

The research described in this thesis was financially supported by the European Union Horizon2020 projects EmPowerPutida (Project reference: 635536). Printed by: ProefschriftMaken