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Engineering and Analysis of a Defined Mixed Culture of *Pseudomonas putida* and *Synechococcus elongatus* for Bioplastic Production

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"Yes, and I could show you some figures to prove it easily."
"Oh, figures!' answered Ned. 'You can make figures do whatever you want."

– Prof. Aronnax and Ned in: Jules Verne, 20,000 Leagues Under The Sea

Abstract

Mixed culture cultivations have been used for thousands of years for the production of food, the treatment of solid and liquid wastes, as well as for biogas production. In contrast, cultivation strategies in modern biotechnology mostly rely on pure cultures – mainly because of better process control. During the last years, new efforts have been made to explore the benefits of defined mixed culture cultivations that excel in their robustness and versatility. These new approaches bear the potential for faster growth, better yields and lower risk of contamination compared to conventional processes.

In this work, we present an engineered defined mixed culture, consisting of transgenic *Synechococcus elongatus* PCC7942 and *Pseudomonas putida* KT2440 that were specifically modified to show a collaborative, commensalistic life-style: We used a *S. elongatus* variant that expresses the sucrose permease CscB to secrete sucrose that is accumulated intracellularly to combat salt stress. This sugar was in turn metabolized by *P. putida* strains that were modified to be able to use sucrose as a substrate. The work can be divided in three general parts: We first constructed mini-Tn5-transposon based plasmids containing the *cscAB* genes from *Escherichia coli* W. These sucrose splitting transposon plasmids (pSST) were the first genetic vectors to confer the ability to grow on sucrose as the sole carbon source to *P. putida*. In the second part we established a defined mixed culture with this new *P. putida* strain and the sucrose secreting *S. elongatus cscB* in a 1.8 liter airlift photobioreactor. When both organisms were combined, the production of polyhydroxyalkanoates (PHA) by *P. putida* at a rate of 23.8 mg L^{-1} d^{-1} and a maximal titer of 156 mg L^{-1} out of cyanobacterial carbohydrates could be demonstrated. In the last part, we constructed even superior sucrose-using *P. putida* strains by genomic insertion of the *cscRABY* gene cluster from *Pseudomonas protegens* Pf-5 by means of a Tn7-transposon vector. Special emphasis was given on the *cscY* gene that encodes a sucrose porin missing in the *E. coli* W gene cluster. We found that *P. putida* expressing the *cscRABY* gene cluster grew on sucrose with growth rates of 0.45 h^{-1} which is comparable to the wild-type strain grown on a glucose/fructose mixture. Moreover, the porin was necessary for robust growth of *P. putida*, highlighting the importance of porins and the outer membrane when bacterial substrate metabolism is genetically engineered.

Taken together, the data presented here demonstrates for the first time the efficient production of medium-chain-length polyhydroxyalkanoates from CO_2 and sunlight by a systematically engineered defined mixed culture. Additionally, we also constructed the first *P. putida* phenotypes growing on sucrose as the sole carbon and energy source. This opens up the possibility to grow this organism on cheap substrates like sugar cane molasses. Approaches like this will pave the way for a more sustainable way to produce chemicals in the future with less need for intensive agriculture and fossil resources and their associated shortcomings.

Zusammenfassung

Seit Jahrtausenden nutzen Menschen Mischkulturen zur Herstellung von Speisen und Getränken, für die Aufarbeitung von Reststoffen oder für die Produktion von Biogas. Die Kultivierung von Mikroorganismen in der modernen Biotechnologie hingegen erfolgt meist als Reinkultur – in der Regel aufgrund der besseren Kontrolle über den Prozess. In den letzten Jahren erlebte die Erforschung definierter Mischkulturen jedoch eine Renaissance, in der der Robustheit und Vielseitigkeit von Mischkulturen Rechnung getragen wird. Diese neuen Ansätze haben das Potential für schnelleres Wachstum der Mikroben, höhere Ausbeuten und ein geringeres Kontaminationsrisiko.

Diese Arbeit beschäftigte sich mit dem Design, der Implementation und Analyse einer definierten Mischkultur eines transgenen *Synechococcus elongatus* PCC7942 und *Pseudomonas putida* KT2440 Stammes, die gezielt genetisch verändert wurden, um eine kooperative, kommensalistische Beziehung einzugehen: Der benutzte *S. elongatus* Stamm exprimierte die Saccharosepermease CscB. Diese transportiert Saccharose, die sich unter Salzstress im inneren der Zelle anhäuft, in das Medium. Die sekretierte Saccharose wurde wiederum von *P. putida* Stämmen verwertet, die mit Genen für den Saccharose-Stoffwechsel ausgestattet worden waren. Die vorliegende Arbeit gliedert sich dabei in drei Teile: Zunächst wurden mini-Tn5-basierte Plasmide konstruiert, die die *cscAB* Gene von *Escherichia coli* W enthielten. Diese Saccharose spaltenden Transposon Plasmide (pSST) stellten die ersten bekannten Vektoren dar, die *P. putida* in die Lage versetzen mit Saccharose als alleiniger Kohlenstoffquelle zu wachsen. Im zweiten Teil wurde eine definierte Mischkultur mit diesem neuen *P. putida* Stamm und dem Saccharose sekretierenden *S. elongatus cscB* Stamm in einem 1,8 Liter Airlift-Photobioreaktor etabliert. Bei der Ko-Kultivierung beider Organismen konnte eine Polyhydroxyalkanoatproduktionsrate von 23,8 mg L^{-1} d^{-1} und eine -endkonzentration von 156 mg L^{-1} aus cyanobakteriellen Kohlenhydraten demonstriert werden. Im dritten Teil wurden die Saccharose-nutzenden *P. putida* Stämme noch weiter optimiert, indem die *cscRABY* Gene aus *Pseudomonas protegens* Pf-5 über ein Tn7-Transposonvektor ins Genom von *P. putida* integriert wurden. Ein besonderer Fokus lag dabei auf dem *cscY* Gen, das für ein Saccharoseporin kodiert und im Gencluster von *E. coli* W nicht vorkommt. Es zeigte sich, dass die Expression des *cscRABY* Genclusters in *P. putida* in einer Wachstumsrate des Stammes auf Saccharose von 0,45 h^{-1} resultierte, was in etwa dem Niveau des Wildtyps bei Kultivierung mit einer Glukose/Fruktose-Mischung entspricht. Darüber hinaus konnte festgestellt werden, dass das Porin für verlässliches Wachstum notwendig war. Dies verdeutlicht die Wichtigkeit von Porinen und der äußeren Membrane an sich, wenn das Substratspektrum von Mikroorganismen genetisch erweitert werden soll.

Zusammengenommen, wurde im Rahmen dieser Arbeit das erste Mal die Produktion von Polyhydroxyalkanoaten mittlerer Kettenlänge aus CO_2 und Sonnenlicht durch eine

systematisch konstruierte Mischkultur gezeigt. Zusätzlich wurden die ersten *P. putida* Stämme konstruiert, die Saccharose als alleinige Kohlenstoff- und Energiequelle nutzen können. Dies eröffnet die Möglichkeit günstige Substrate wie Zuckerrohrmolasse als Substrat zu verwenden. Ansätze wie dieser können so eines Tages den Weg ebnen für eine nachhaltigere Art und Weise Chemikalien herzustellen, die mit weniger intensiver Landwirtschaft und fossilen Rohstoffen und all ihren negativen Begleiterscheinungen einhergeht.

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Acronyms

ADP	Adonesine-diphosphate.
AMP	Adonesine-monophosphate.
ATP	Adonesine-triphosphate.
DO	Dissolved oxygen.
ED	Entner-Doudoroff (pathway).
EMP	Emden-Meyerhof-Parnas (pathway).
FACS	Fluorescence-activated cell sorting.
FSC	Forward scatter.
GFP	Green fluorescent protein.
IPTG	Isopropyl β-D-1-thiogalactopyranoside.
LB	Lysogeny broth.
mcl	Medium chain-length.
MCS	Multiple cloning site.
ME	Mosaic end.
NADH	Nicotinamide adenine dinucleotide.
NADPH	Nicotinamide adenine dinucleotide phosphate.
NEB	New England Biolabs.
NR	Nile red.
PCR	Polymerase chain reaction.
PHA	Polyhydroxyalkanoate.
PHB	Polyhydroxybutyrate.
PP(P)	Pentose phosphate (pathway).
PP_i	Pyrophosphate.
PTS	Phosphotransferase system.
scl	Short chain-length.
SSC	Sideward scatter.

ssDNA	Single stranded DNA.
TCA	Tricarboxylic acid cycle.
VAAM	Vereinigung für Allgemeine und Angewandte Mikrobiologie.

1 Introduction

1.1 Historical context of mixed culture cultivation

Since man adopted a sedentary lifestyle and began to sow and harvest edible plants during the Neolithic revolution, food needed to be preserved because crops can only be collected at certain times of the year [1]. Grain could be stored in granary and meat could be cured, but still preservation of food has always been a constant battle against mold, microorganisms and other pests. The invention of fermentation techniques came probably more by chance than reasoning, but can, without a doubt, be regarded as a major breakthrough concerning the preservation of food. Yeast, for instance, could be used to ferment malt to alcoholic beverages – a process that can be dated back to at least ancient Mesopotamia 4000 B.C. [2]. Ethylic alcohol is toxic to most organisms and could thus extend the shelf-life of these beverages and preserve the valuable nutrients within. Alcohol containing beverages can in turn be fermented to vinegar which is an equally old process [3] and can preserve products due to its low pH. Vinegar could be identified in Egyptian urns [3] and is already mentioned in the Old Testament. The same applies to the production of soy sauce or cheese, just to name some examples.

All these processes have in common the use of ***mixed cultures***, i.e. a – mostly undefined – mixture of different microorganisms in the fermentation. This is due to the fact that the knowledge about the underlying microbiology and axenic culturing techniques had not been available at that time.

Figure 1.1: Examples of traditional applications of mixed cultures. A) Production of fermented foods and drinks. B) Composting of wastes for agricultural purposes. C) Production of biogas by anaerobic digestion.

The digestion of organic wastes also has a long history. It is said that people in Assyria and in ancient China already fermented organic wastes to biogas for heating [4]. Anaerobic digestions, as well as water treatment and composting always involve mixed

cultures since it takes a whole repertoire of different metabolisms to efficiently degrade the complex mixture of molecules in wastes. A graphical representation of fermentation processes with ancient roots that rely on mixed cultures is given in Fig. 1.1.

Undefined mixed culture cultivations tend to be quite robust [5] since the organisms are enriched from the environment at conditions under which they outgrow their competitors. However, as the medium changes during a cultivation [6] (e.g. rising alcohol content in yeast fermentations), the distribution of microorganisms can change dynamically [7] which makes process control more challenging and is unwanted in many applications.

With the invention of axenic cultivation techniques in the laboratory of the French scientist Louis Pasteur in the middle of the 19th century, it became possible to use a single strain for cultivation. Nowadays, a lot of modern bioprocesses are based on pure cultures due to the better control over the cultivation process [8]. From an engineering point of view, undefined mixed cultures are normally not desirable because of difficult control over the process, complicated analysis, unwanted contaminations and other reasons (compare Table 1.1).

While these arguments are manageable in traditional food and drink fermentation and might even result in interesting tastes, they are detrimental in the production of medical products like antibiotics or products with a high demand of productivity and yield as in industrial biotechnology.

Another reason for the use of monocultures has been the rise of genetic engineering of microorganisms during the last 50 years that meanwhile comprises a whole tool-kit for bioengineering. Microorganisms that have been modified to reach higher productivities and yield, normally exhibit a slower growth rate compared to the wild-type strains – making the pure culture of the engineered strain mandatory.

1.2 Ecological aspects and opportunities of mixed cultures

In nature, most microorganisms are found in communities with other microbes. Most notable in this regard are biofilms – aggregates of one or more species of microorganism on surfaces in an extracellular matrix. They are a very wide-spread and ecologically successful style of living for bacteria [9] and offer many advantages for their inhabitants, i.e. resistance to mechanical and chemical stress among others. Moreover, some natural biological processes predominantly take place with the action of two different bacteria. One example is lithoautotrophic nitrification, the reaction of ammonia to nitrate, that is usually not performed by a single organism, but is rather done in a two-step fashion: First, the oxidation of ammonia to nitrite by e.g. *Nitrosomonas* strains and in succession the oxidation of nitrate to nitrate by e.g. *Nitrospira* strains. The few isolated organisms that are genetically equipped for both steps (so called "camammox") seem to be restricted to nutrient-limited niches [10]. Another example are methanogens, microbes that can use CO_2 as final electron acceptor and turn it into methane. Most of these organisms depend on small molecules like hydrogen gas, formate, acetate and the like as a substrate, but cannot use glucose or even the complex biopolymer mixture that is

Table 1.1: Comparison of pure and mixed culturing modes

	Undefined mixed culture		**Pure culture**		**Defined mixed culture**
+	high robustness	-	prone to contamination	-	prone to contamination
+	multi-step reactions possible	-	no multi-step reactions	+	multi-step reactions possible
+	broad substrate spectrum possible	-	substrate spectrum of a single organism	+	broad substrate spectrum possible
+	no axenic handling	-	axenic culturing needed	-	axenic culturing needed
-	low control of dynamics of populations	+	high control of the population	±	population control depends on the system[b]
-	complicated to analyze	+	easy to analyze	-	complicated to analyze
-	unwanted side reactions[a]	+	high specificity	+	high specificity
-	genetic engineering not practicable	+	strain optimization by genetic engineering	+	strain optimization by genetic engineering

[a] e.g. sulfate reduction during anaerobic digestion when methane is the product of choice
[b] e.g. populations can be regulated by external factors like temperature or substrates if the populations have different requirements

present in agricultural wastes. They therefore need other microbes that ferment sugars to smaller molecules that are usable by methanogens [11]. To put it in Darwin's words:

"In the long history of humankind (and animal kind, too) those who learned to collaborate and improvise most effectively have prevailed."

– Charles Darwin in: The origin of species

Obviously, this also applies to the smallest living beings: bacteria, archaea and single celled eukaryotes – only microbiology was a young field during Darwin's times and the impact of evolution theory on microbiology was yet to be revealed [12].

In the last years, there has been renewed interest for mixed culture cultivations in the scientific community [6, 9, 5]. They are not only an interesting opportunity to study interactions between microbes and their way of communicating, but they also have some interesting features that make them attractive for biotechnological applications.

The merit of mixed cultures is given credit for example in studies on the conversion of different waste streams to polyhydroxyalkanoates (PHA) [13, 14, 15, 16, 17, 18, 19].

But also most studies on bioelectrochemical systems focus mainly on mixed cultures that perform notoriously better than single culture isolates [20]. Other examples in the recent literature are biohydrogen production [21, 22] and biofuel production [23].

1.3 Motivation and scope of the thesis

To understand and make the synergies of mixed cultures usable, scientist are now constructing synthetic mixed cultures in which two or more organisms are applied instead of an unknown inoculum (see [24] or [6] for a review). These defined mixed culture aim to combine the benefits of undefined mixed cultures with those of pure cultures (see Table 1.1 for a detailed listing).

This work will shed light on the advances in the design, operation and analysis of such a defined mixed culture between the cyanobacterium *Synechococcus elongatus* and the soil bacterium *Pseudomonas putida* that have been achieved during the last 4 years at the Associate Professorship of Systems Biotechnology (Technical University of Munich).

The basic idea was the separation of the cultivation process into two ***biomodules***: The first module is able to fix carbon (i.e. *S. elongatus*) and turn it into a carbon source for the second biomodule (i.e. *P. putida*) – resulting in a kind of artificial commensalism. So why should a mixed culture of these bacteria be cultivated? This new approach was influenced by two inspiring publication of the group of Pamela Silver at the Harvard Medical School [25, 26] and is motivated by a couple of rationales:

- The cyanobacterium *S. elongatus* is a phototrophic organism and can thus use CO_2 and light as the main substrates. This can be regarded as a sustainable source of carbon compared to contemporary biotechnological processes that mainly use (side-)products of extensive agriculture – with all associated downsides [27]. Cyanobacteria have the potential to have a higher areal productivity than conventional crops and don't compete for farmland.

- *P. putida* was chosen as the second biomodule because of its innate robustness to various forms of stress as a soil bacterium [28]. There is an arsenal of genetic tools for this organism [29] that were in part readily available in our lab. Moreover it has been shown to be capable of synthesizing various interesting products [30, 31].

- Last but not least, the combination of two organisms in one fermentation process is an interesting object of study for the methods of Systems Biology: Models for the interaction between microorganisms can be formulated and verified/falsified. Characterizing and calculating metabolic carbon fluxes through two organisms in succession are interesting tasks as well. In any case, the complexity of the system makes it an ideal starting point for modelling even more diverse populations.

In total, this concept results in a two-step process in one pot that is able to convert CO_2 and (sun)light into the desired, valuable product.

Of course setting up a defined mixed culture is more than just mixing two organisms in a vessel and *voilà* – several objectives had to be tackled beforehand that can be seen as

milestones of the project: First it was necessary to find a common cultivation medium. Second, growth and carbon fixation by *S. elongatus* had to be characterized and scaled up into a photobioreactor. Third, *P. putida* needed to be genetically engineered to be able to profit from the fixed carbon of *S. elongatus*. And finally the mixed culture of the two organisms was set up and employed to produce an exemplary product.

The scientific background, the used methods and the material for these tasks will be presented in the following chapters. Lastly, the results will be discussed in the context of the publications by our group that covered the project's progress in the last years.

2 Scientific Background

2.1 Carbohydrate production with cyanobacteria and eukaryotic algae

At the moment, most fermentation processes in industrial biotechnology are based on cheap substrates. Many of them are waste products from other industrial processes like sugars in molasses from the refining of sugar cane or beet, oil-rich waste streams from oil mills, glycerol from biodiesel production, and many more. With the rise of political mandates for ethanol blends to gasoline in countries like the European Union and the United States of America, these wastes do not suffice when compared to the needed amount of ethanol. In consequence, large areas of agricultural land all over the world are just used to produce substrate for biofuels and biogas [32]. Normally, crops do not yield the same amount every year which makes them compete between using them for biotechnology or as food. The prices on the market are therefore coupled, resulting in a very bad situation in countries where food is already less available and expensive – not to speak about investments of industrialized countries in the agricultural land of poorer countries.

2.1.1 Algae and cyanobacteria as a new feedstock for industrial biotechnology

One solution are so called "second generation biofuels" that use those parts of the plants as a substrate that are not edible like straw, wood, and other wastes. But even for the substitution of the non-transport applications of crude oil which account for approximately 10% of the total petroleum, an absurd amount 1 billion tons of biomass would be needed every year for industrial biotechnology [33]. The question if biomass is sufficient to replace crude oil as a feedstock has been extensively studied [34]: Even the most optimistic case comes to the conclusion that to feed the global energy demand of 2011, an area of the size of China would be needed for energy plant cultivation. Moreover, a diet with high meat content could only be sustained by massive deforestation.

To avoid these vast changes of agriculture and ecosystems, algae and cyanobacteria are a possible alternative to crops because of several reasons:

- They tend to have higher areal productivities to a factor of at least 3-5 fold [35, 36].
- As aquatic life forms, they do not compete for the same type of agricultural land as crops.

- Algae and cyanobacteria don't require leaves, stems or other structural components and can therefore direct their carbon flux more specifically to products[1]
- Both groups of organisms can be cultivated in brackish water, waste water [39] or even sea water, saving fresh water resources.
- Since most bioreactors or ponds are not connected to soil, there is no erosion and hence loss of nutrients – making the process more sustainable. This way unlasting nutrients like phosphorous can be more easily recycled [40, 41].

In the recent years, a couple of studies on the production of carbohydrates with eukaryotic algae and cyanobacteria have been published: A lot of algae like *Chlorella* accumulate starch as energy and store carbon as up to 78% of its dry weight under nitrogen limitation [42]. Cyanobacteria, on the contrary, mostly use glycogen or lipids as storage compounds when deprived of nutrients [43].

2.1.2 Pure and mixed cultures of sucrose secreting cyanobacteria

Another noteworthy type of carbohydrate is sucrose which is accumulated by some freshwater cyanobacteria as an osmolyte under salt stress [44]. Already in 2006, researchers at the University of Texas filed a patent (patent number US20080124767A1) that comprises a method for the extraction of sucrose from cyanobacteria without cell lysis (so-called "bacterial milking"). This idea was taken up by the company Proterro inc., New York that aimed for commercialization of the process with a new kind of bioreactor (patent number US8951784B2). Unfortunately the company – like so many other algae-based startups in the U.S. [2] – gave up on producing low value products and eventually completely vanished. Later a patent was filed by the Harvard College (patent number EP2719757A1), claiming a process in which the sucrose is secreted out of the cyanobacteria by genetically implementing a sucrose transporter. The corresponding publication [25] from the group of Pamela Silver at the Harvard Medical School impressively showed the steady production of sucrose under elevated salt levels with a genetically engineered *Synechococcus elongatus* PCC7942 strain. Integration of the sucrose/H^+-antiporter CscB from *Escherichia coli* into the cyanobacterial chromosome resulted in production rates of 36.1 mg L^{-1} h^{-1}. The mechanism of sucrose production and secretion is illustrated in Fig. 2.1.

An interesting trait of this strain is its very high productivity that, even without strain optimization, is on par with sucrose production by sugar cane – the most area

[1] e.g. *Nannochloropsis* sp. can accumulate up to 47% of their cell dry weight as lipids [37]; *Synechococcus* sp. strain PCC 7002 has been shown to store glycogen in its cell up to a content of 60% with respect to cell dry weight [36]. This compares well with sugar-cane that has a reducing sugar content of around 20% [38].

[2] e.g. Joule Unlimited, Solazyme, Algenol, Sapphire Energy and many more; one reason probably is the shale gas and tight oil boom in the U.S., but also a change in the governmental support for renewable energy since the begin of the Trump administration.

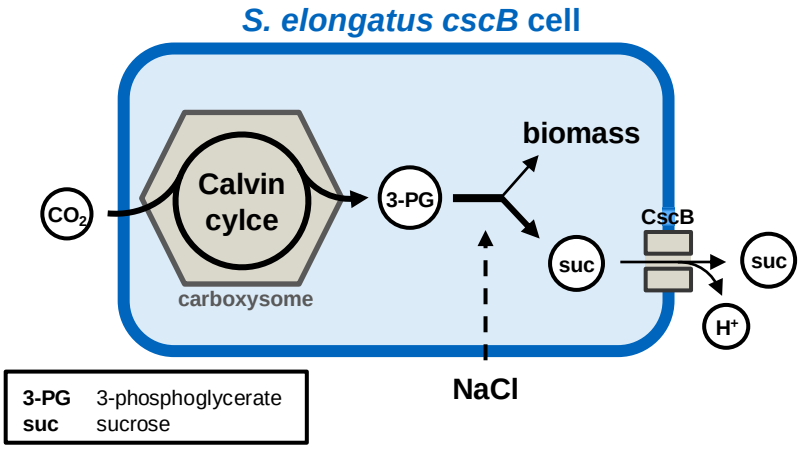


Figure 2.1: Schematic drawing of sucrose accumulation and secretion via CscB in *S. elongatus cscB* cells: CO_2 is first fixed to 3-phosphoglycerate in the Calvin cycle. Under salt stress (NaCl) the major part of it is directed to sucrose instead of biomass production. Sucrose is then secreted with sucrose/H^+-symporter CscB from *E. coli* into the surrounding medium.

efficient process in place for sucrose production. One part of the study also mentioned the possibility to co-cultivate this strain with a sucrose-utilizing heterotroph. In this course, the use of sucrose as substrate was demonstrated with yeast on agar plates. In the follow-up, several studies were published on the topic: The integration of the *cscB* gene into other cyanobacterial strains was demonstrated [45, 46]. Other scientists worked on the combination of *S. elongatus* PCC7942 *cscB* with heterotrophic co-culture partners to form defined mixed cultures [47, 48, 49, 50]: The interactions between *S. elongatus* PCC7942 *cscB* and each *E. coli*, *S. cerevisiae* or *Bacillus subtilis* were characterized. Lipid production and growth dynamics were analyzed in co-culture with various yeast strains in another study [50]. In one publication, an artificial symbiosis between *S. elongatus* PCC7942 *cscB* (as a carbon donor) and *Azotobacter vinelandii* AV3 (as a nitrogen donor) was established.

Many of the defined mixed cultures were set up to produce polyhydroxybutyrate (PHB) by the heterotrophic host [47, 48, 49]. The highest productivity of 28.3 mg L^{-1} d^{-1} was reached in a mixed culture between *S. elongatus* PCC7942 *cscB* and *Halomonas boliviensis*, which compares well with PHB production by genetically engineered cyanobacteria strains [49]. The comparison of PHB production directly by cyanobacteria *versus* in mixed cultures will be given in more detail in section 5.4. This again shows the benefits of defined mixed cultures: Since *S. elongatus* PCC7942 *cscB* is just producing sucrose to reach high osmolarity within the cell, there is little metabolic backlash from product formation – storage compounds like PHA and lipids are mainly formed in stationary phase where carbon fixation is rather low. In our case, the two processes – carbon fix-

ation and product formation – can be split into two species and thus engineered more orthogonally.

PHB is part of a class of linear polyesters called polyhydroxyalkanoates (PHA) that also include longer chain-lengths (see section 2.2.2 for more details). The polymer is also naturally produced by *P. putida* which we chose as a heterotrophic co-culture partner for *S. elongatus cscB* and will be described in the next section.

2.2 *Pseudomonas putida* as a chassis for industrial biotechnology

Pseudomonas putida is a Gram-negative, motile, rod-shaped soil bacterium that exhibits a very diverse, but strictly aerobic metabolism [51]. It is often found in soils contaminated with toxic organic molecules and some strains show exceptional organic solvent resistance. This makes it an ideal candidate for chemical degradation (i.e. bioremediation)[51] or chemical production [52]. One of the best studied strains of this organism is *P. putida* KT2440 which is a plasmid-cured derivative of the toluene degrading strain *P. putida* mt-2 (Fig. 2.2).

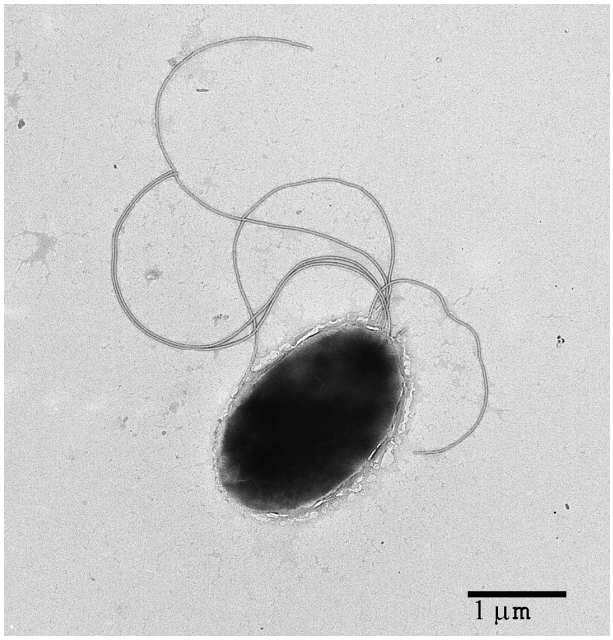


Figure 2.2: Transmission electron micrograph of *P. putida* KT2440. The image was taken at the CNB (CSIC), Madrid, Spain and reprinted in courtesy of Katharina Pflüger-Grau from the Systems Biotechnology group at TUM.

P. putida KT2440 is a popular laboratory organism as it is fast growing, can be easily genetically modified, is certified as GRAS (generally regarded as safe) [53] and very resistant to chemical and oxidative stress [53]. A whole repertoire of tools for genetically engineering *P. putida* has been made available: Among them are conjugative transfer

[54], transposon vectors for site-directed [55] or random [56] insertion into the genome, homologous recombination [57], recombineering [58] and CRISPR/Cas9 selection [59]. With all these methods on hand, *P. putida* is envisioned to have several areas of application:

- Host for chemical production in industrial biotechnology [31]
- Bioremediation due to its resistances and metabolism [60, 61]
- Host for heterologous gene expression [53]
- Agricultural use as bio-based pesticides due to its ability to colonialize the plant rizosphere [62, 63]

In addition, with the help of homologous recombination, the genome could be freed of fractions that are unnecessary for bioengineering purposes [64], yielding streamlined versions of *P. putida*. One of these "cell-factories" is the prophage-free derivative *P. putida* EM178 in which the gene clusters PP3849-PP3920 (prophage 1), PP3026-PP3066 (prophage 2), PP2266-PP2297 (prophage 3) and PP1532-PP1586 (prophage 4) have been deleted. This strain is supposed to have all the key features of the wild-type, but detrimental effects due to prophages can be excluded.

In general, genetic engineering was widely used in *P. putida* to unlock new pathways and to develop the strain toward applications in industrial biotechnology. Apart from deleting unnecessary genomic regions, this also includes implementation of new pathways for product formation [31] or making new substrates accessible by adding corresponding transporters and enzymes [65, 66]. For a dedicated view on the landscape of metabolic engineering in *P. putida*, consider reading a recent summary by Nikel and de Lorenzo (2018) [29]. Finally, even the redesign of central parts of the metabolism like hexose catabolism has been envisioned [67]. In this regard, it is important to understand the way carbohydrates are metabolized in *P. putida* as it is crucial for metabolic engineering the substrate spectrum of this organism. The fundamental carbon catabolism in *P. putida* will be described in the next paragraphs.

2.2.1 Carbohydrate metabolism in *P. putida*

Among the diverse pathways for glucose utilization, the two most common and well known pathways are the Emden-Meyerhof-Parnas (EMP) pathway and the the Entner-Doudoroff (ED) pathway[68]: The EMP pathway, also termed "glycolysis" is present in *E. coli* or mitochondria for instance and yields 2 molecules of ATP and two NADH per molecule of glucose. And second, the ED pathway that seems to be exclusive to prokaryotes and plants [69] and yields only one ATP, but also one NADH and one NADPH (which normally has more reducing power than NADH) *in vivo*.

Of course the prevalence of these pathways is never as black and white as written in the textbooks considering the shear diversity of organism on earth. Instead there are organisms with a combination of both pathways or other modifications. *P. putida*

was long assumed to be using a linear ED pathway. Recent studies, however, show that a special combination of both ED and EMP pathway, together with reactions from the pentose phosphate pathway, are combined in a cyclic manner, forming the so-called EDEMP cycle [70] (depicted in figure 2.3).

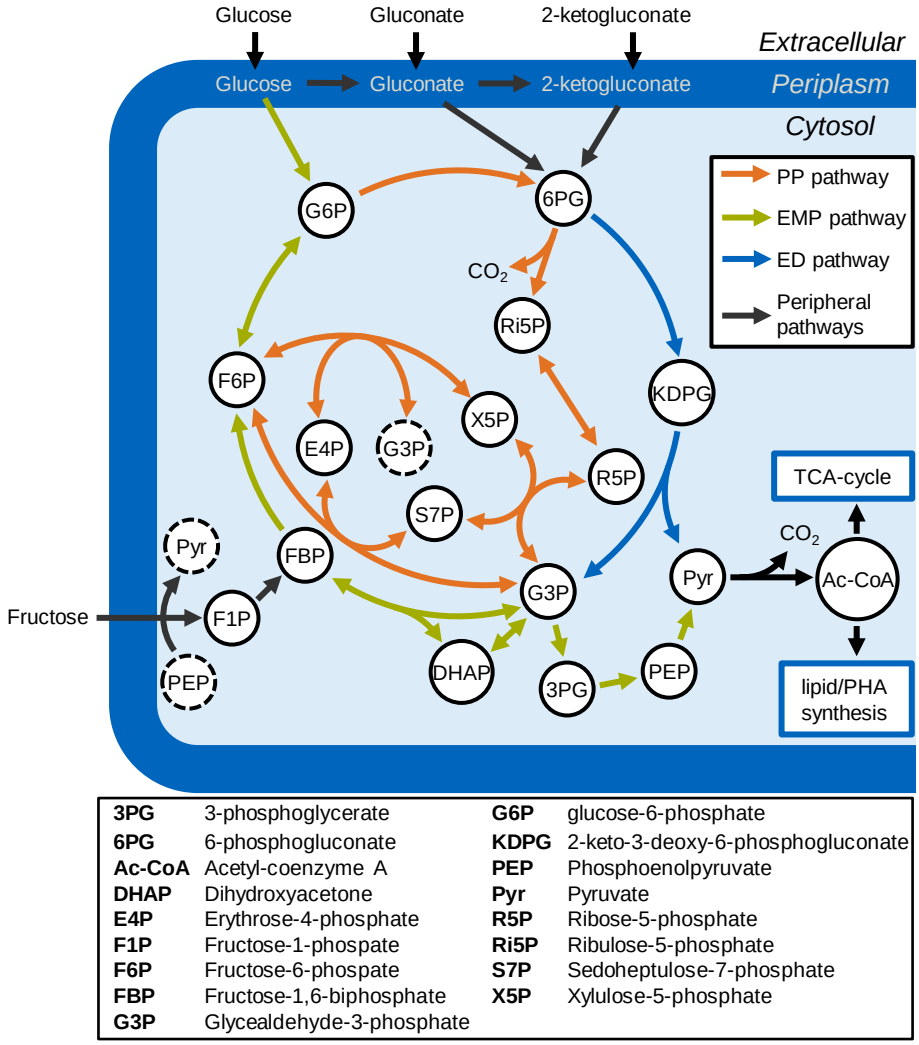


Figure 2.3: Hexose metabolism and EDEMP cycle in *P. putida* depicted in analogy to [70] with slight modifications. The glucose and fructose metabolism in *P. putida* is a mixture of the Entner-Doudoroff (ED) pathway, the Emden-Meyerhof-Parnas (EMP) pathway and the pentose phosphate (PP) pathway. Taken together they form the so called EDEMP cycle. Metabolites in doted circles represent intermediates that are synthesized at a different point in the flux diagram, but are used elsewhere because of better clarity.

After the uptake of glucose into the periplasm, it is oxidized to gluconate or even 2-ketogluconate in the periplasm and then transported into the cytosol. Alternatively, glucose is directly imported into the cytosol and oxidized there [70]. In either way, it will be transformed to 6-phosphogluconate and subsequently split into two C3-molecules from where it follows the ED pathway as usual [70].

Although it seems counter-intuitive, a part of the C3-compounds are recycled back to F6P (the before mentioned EDEMP cycle), even in glycolytic growth regimes [70]. The function of this cycle is supposed to be the synthesis of sugars for polysaccharides in the cell wall or the generation of more reducing power [70] which might also play a role in the outstanding resistance to oxidative stress of this organism.

Fructose, in contrast to glucose, is taken up via a phosphotransferase (PTS) system and is therefore phosphorylated during the transportation process. The phosphate is necessarily transferred to fructose from phosphoenol pyruvate (PEP) and thus the PEP/Pyruvate ratio plays a critical role as control and flux measure for the fructose metabolism [71]. Fructose-1-phosphate is in turn phosphorylated to fructose-1,6-biphosphate and then follows the described EDEMP cycle. It is important to note that there is no phosphofructokinase activity in *P. putida* KT2440 which means that fructose must be imported through the PTS-system to be metabolized [72].

Deletion of the genes encoding the enzymes for oxidation of glucose outside of the cell and thus focusing the pathway on cytosolic oxidation leads to higher PHA-yield [73]. This was attributed to a higher NADPH yield through the entry reactions of the PP pathway. It is also coherent with the observation that biomass yields on fructose seem to be slightly higher than on glucose in *P. putida* (unpublished data). The next section will deal with the biosynthesis of these promising polymers and elucidate the pathway with acetyl-CoA and fatty acids as starting points.

2.2.2 Synthesis of polyhydroxyalkanoates in *P. putida*

In situations where bacteria (and other organisms as well) have a carbon substrate in excess, but run low on another vital resource like a nitrogen, sulfur or phosphorous source, they will prepare for times of starvation: Some organisms like animals store their carbon and energy as lipids [74] or glycogen [75], plants tend to store it as starch, fructosans or sucrose [76]. Storage in form of lipids is also ubiquitous in bacteria, although in general polymeric lipids, called polyhydroxyalkanoates (PHA), are used instead of triacylglyerides [77]. These polymers have gained a lot of attention as bioplastic due to their thermoplastic properties that resemble those of petroleum-based plastics. However, unlike conventional plastics that are derived from oil and gas, these polymers can be produced from renewable sources. Several companies already produce PHA at a tons-per-year scale [78].

PHAs are linear polymers made of 3-hydroxyalkanoic acids that are cross-linked via an esterification of neighboring hydroxyl and carboxyl groups. The monomer units, 3-hydroxyalkanoic acids, are lipids structurally close to alkanoic acids and therefore share the same, high energy density as fats and oils which is about two times as much as carbohydrates or proteins [79].

PHA forms intracellular granules that are visible, even with light microscopy as little dots within the cells. The size, shape and amount of granules is controlled by phasin proteins that can make up around 5% of the cell dry weight under conditions of PHA accumulation in *Cupriavidus necator* [80, 81].

Depending on the chain-length of the 3-hydroxyalkanoic acids, the polymers have different mechanical properties: Most bacteria produce short chain-length PHA (scl-PHA) which consist mainly of 3-hydroxybutyrate monomers and have limited mechanical properties [82] as they tend to be brittle when not combined with other 3-hydroxyorganic acids [83]. Only a few genera can produce longer chain-length PHAs that are more interesting for applications due to their superior and more flexible properties [82, 84, 85].

Among those genera, fluorescent *Pseudomonads* have been extensively studied as producers of mcl-PHA: *P. putida* also is a native producer of medium chain-length PHA (mcl-PHA), either from lipid based substrates or carbohydrates [86] under conditions of one or multiple nutrient starvation [87].

In the metabolism of *P. putida*, synthesis and degradation of PHA is closely linked to fatty acid synthesis or degradation via beta-oxidation by the *phaC1ZC2DFI gene cluster* [88, 89] (compare Fig. 2.4). Carbohydrates are first processed to acetyl-CoA through the EDEMP cycle and then fed into the fatty acid synthesis cycle. One intermediate, (R)-3-hydroxyacyl-ACP can be transformed to (R)-3-hydroxyacyl-CoA which is the starting point for PHA polymerization. Fatty acids, in contrast, enter the network at the beta-oxidation cycle. They are first linked to Coenzyme A and then oxidized. One intermediate is (S)-3-hydroxyacyl-CoA which can be turned into its diastereomer (R)-3-hydroxyacyl-CoA by an epimerase. Alternatively the (R)-diastereomer can be formed from enoyl-CoA with the (R)-selective hydratase PhaJ.

In either way, (R)-3-hydroxyacyl-CoA is polymerized by the PHA synthetases PhaC1 and PhaC2 via esterification and splitting off CoA. The PHA depolymerase PhaZ is needed to mobilize the monomers by hydrolysis to the (R)-3-hydroxyalkanoic acid and a shortened PHA polyester. By esterification of the (R)-3-hydroxyalkanoic acid with Coenzyme A by Acs1, the monomer can reenter the network. It is worth mentioning that this small cycle of synthesis and degradation of PHA by PhaC, PhaZ and Acs1 is always active, even under conditions of PHA synthesis [89] – forming a dynamic cycle that can react on changes in the metabolic status quickly.

Although the carbon and energy footprint of PHA is substantially lower than that of petroleum-based plastics [91], production of PHA is not considered to be commercially viable at the moment. In this context, the cost of the carbon source was found to account for 30-50% of the total production cost and thus has considerable impact on the price of the product [92]. One way to lower the cost of substrate is to explore cheaper carbon sources like waste products or unconventional, innovative carbon sources like cyanobacterial carbohydrates. In the next section, the great potential these carbon sources offer for (defined) mixed culture cultivations will be illuminated.

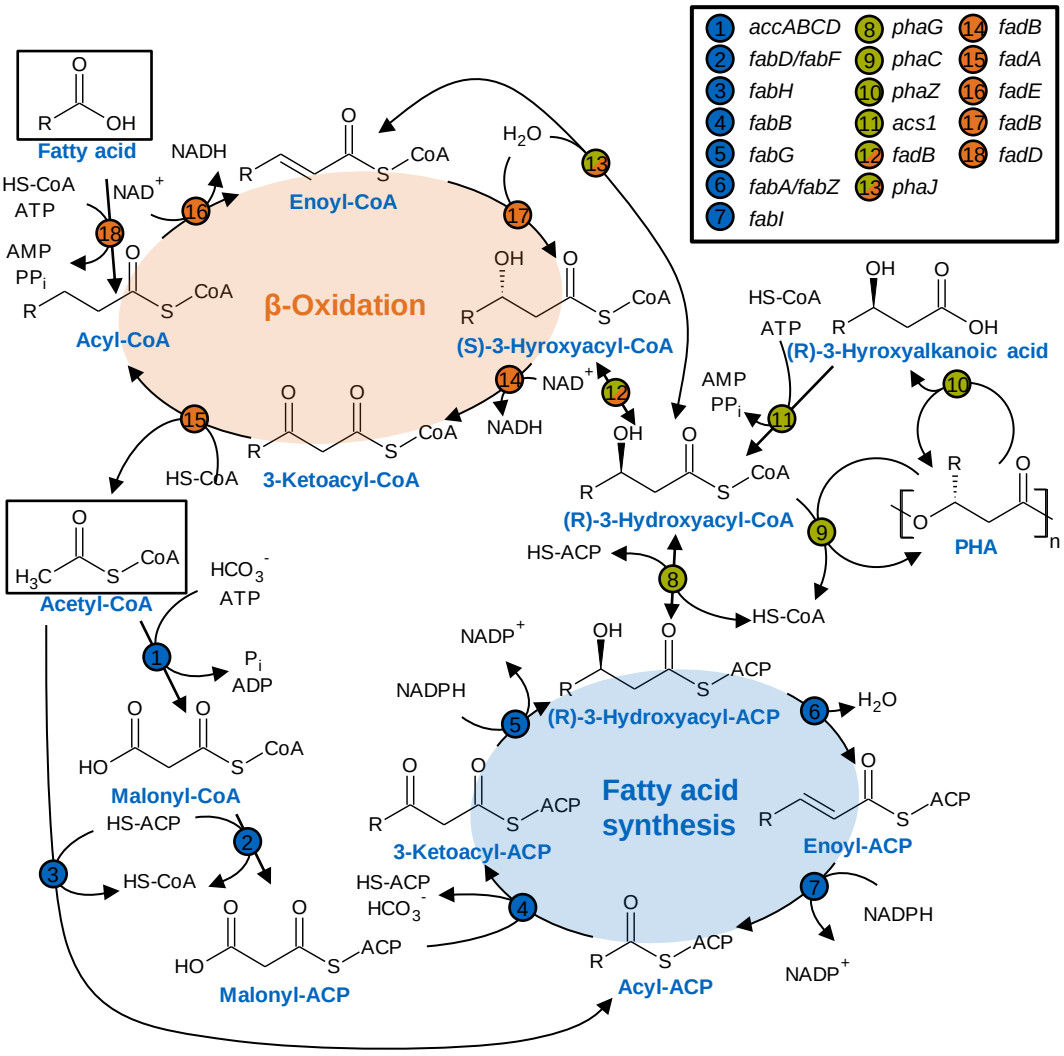


Figure 2.4: PHA synthesis and degradation in *P. putida* as derived from [88], [89] and [90] with slight modifications. PHA synthesis and degradation is closely linked to fatty acid synthesis and degradation cycles (indicated with color). PHA polymerization is a cyclic, dynamic process. Metabolites in frames mark starting metabolites: Acetyl-CoA from the EDEMP cycle (sugar substrates) or fatty acids (lipids as substrate).

2.3 Engineering of defined mixed cultures

To manufacture bulk products, only the cheapest substrates can be considered in industrial biotechnology as many processes have to compete with very well established processes that in part rely on fossil resources. These substrates are often waste products of other industries like waste water or molasses and therefore contain a very complex

mixture of chemicals. One organism alone is normally not sufficient to consume a broad spectrum of substrates, so natural communities display a division of labor.

To make this advantage of mixed cultures – and many others – available for biotechnological applications, scientists have been investigating simplified, defined mixed cultures as a model for interaction and possible synergies [5]. An overview over the different constructed interaction strategies between co-cultured microorganisms is depicted in table 2.1 with representative examples.

In most cases, a positive interaction between the co-culture partners is desired: This can be a higher yield, a faster growth rate or more robust behavior in the process. These interactions in which at least one of the organisms is benefitting, are called *synergistic*. If both organism gain an advantage by the presence of the other, biologists speak of "mutualism" or more precisely:

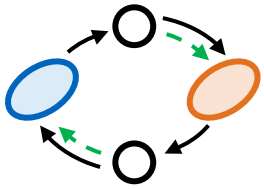
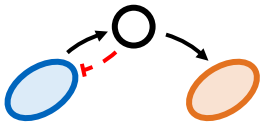
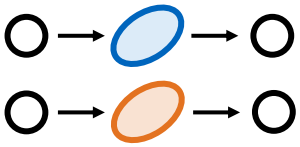
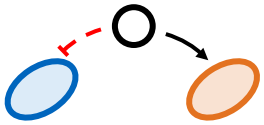
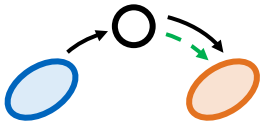
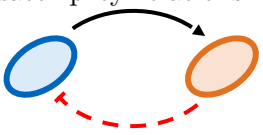
> *"Interactions between individuals of different species that benefit both of them"*,
> (Definition of *mutualism* according to the Encyclopedia of Ecology [102])

A common form of such an interaction is the so-called "cross-feeding" or syntrophy. An early example is the co-culture of *Methanobacillus omelianskii* and the "S organism". *M. omelianskii* is not able to turn ethanol to methane on its own. Instead, ethanol is first oxidized to acetate by the "S organism", accompanied by the release of hydrogen gas. This hydrogen gas is in turn taken to reduce CO_2 to methane by *M. omelianskii* [103]. Interestingly, the reaction of ethanol to acetate and hydrogen is exclusively exergonic at very low partial pressures that can only be reached by the action of the methanogen. In consequence, both organisms profit from each other: *M. omelianskii* gets hydrogen to perform its core metabolism and the "S organism" is rewarded with low hydrogen pressure, so it can oxidize ethanol.

This interaction is just exemplary for the complex substrate chains in the guts of plant degrading animals, in waste water treatment plants or in anaerobic digesters. Bernstein *et al.* [95] simulated cross-feeding by co-culturing two *E. coli* strains, one of them unable to utilize glucose. When both organisms are cultivated in glucose containing medium, the glucose-positive strain is fermenting glucose to acetate, a common behavior of *E. coli*. The second strain cannot use glucose and therefore specializes on acetate as its substrate. This way, acetate and other fermentation products of *E. coli* do not accumulate during the process which results in better biomass productivity than for each of the organisms in pure culture. A possible application for engineered cross-feeding strategies is the complex and notoriously difficult processing of lignocellulosic biomass where defined mixed cultures also perform better than pure cultures [104]. Another form of syntrophy is the elimination of a toxic compound, like an antibiotic, by one of the strains to support the whole group. An artificial system was demonstrated in a mixed culture of *Azotobacter vinelandii*, *Bacillus licheniformis* and *Paenibacillus curdlanolyticus* [94]. Here, *A. vinelandii* provided a nitrogen source for the group, *B. licheniformis* degraded penicillin G and *P. curdlanolyticus* supplied the carbon source.

In cases where two organism are cultivated together, but no interaction between them occurs, the interaction can be regarded as "neutralistic". An example for engineered

Table 2.1: Overview of different modes of interactions in mixed cultures[a] and examples of engineered communities

	Type of interaction & graph[b]	Examples & references
Mutualism/Cooperation	Making substrates available for the interaction partner 	• Mixed culture of adenine-auxotrophic yeast and lysine-auxotrophic yeast [93] • Facilitation of a carbon-source by one organism, fixation of nitrogen by the culturing partner [94, 47] • Division of labor between a glucose-positive and glucose-negative *E. coli* strain [95]
	Scavenging of toxic by-products 	Co-culture of *Scenedesmus obliquues* (carbon donor) and *Candida tropicalis* (respiration of overflowing oxygen) [96]
Neutralism	Division of labor among the organism without interaction 	Co-culture of genetically modified *E. coli* strains, specialized on either glucose or xylose [97]
Commensalism	Maintenance of the cellular environment 	Scavenging oxygen by tolerant organisms to achieve anaerobic conditions for culturing partner [98]
	Substrate facilitation without mutual benefits 	• Secretion of sucrose by *S. elongatus* and take-up by *H. boliviensis* [49] • Sucrose splitting by *B. subtilis* and take-up of glucose/fructose by *C. necator* [99]
Parasitism	Predator-prey relationship 	• Lysis of *P. putida* by transgenic Bdellovibrio bacteriovorus for PHA recovery [100] • Production of ssDNA with phages that infect *E. coli* [101]

[a] adapted and modified from [6] and [5]
[b] straight lines represent mass flow (i.e. production or consumption), dashed lines represent interactions (green arrow: positive, red line with dead end: negative)

neutralism was reported by [97]: One *E. coli* strain able to only use glucose, but not xylose and another strain only able to use xylose (glucose deficient) were co-cultivated in a glucose/xylose based medium for the production of lactate. Together, each strain was readily consuming its corresponding sugar, thereby preventing diauxic growth.

Neutralism is actually difficult to postulate since there are mostly interactions between organism as they will compete for other nutrients, space or interact by quorum sensing. Still, from an engineering point of view, the design of a mixed culture can be neutralistic if no interactions are aimed for.

If only one organism in a mixed culture profits from the other, the interaction is called "commensalistic". Examples include the removal of oxygen by bacteria for efficient hydrogen production by *Chlamydomonas* species [105] or supply of a carbon source by *Bacillus subtilis* for *C. necator* without getting a reward in return [99].

Interactions between organisms are not always positive or neutral, in some cases one culture partner in a mixed culture produces a toxic compound (like antibiotics in some *Penecillium* strains) or they are in a predator-prey relationship. Even these antagonistic, so called "parasitic" relationships have been exploited for biotechnological purposes: PHA, an intracellular compound, was extracted from *P. putida* and *C. necator* for bio-separation by cell lysis via the bacterial predator *Bdellovibrio bacteriovorus* in one study [100]. To prevent PHA degradation by *B. bacteriovorus*, the gene for the PHA depolymerase was deleted beforehand. Another example is the production of single stranded DNA (ssDNA) by phages that use *E. coli* as a host which is one of the most efficient systems for ssDNA production [101].

All these applications of "ecological engineering" [106] offer great potential for the future to make the processes that are now conducted in pure cultures, more efficient, productive and stable. The examples presented here are just a small cross-section of the field of defined mixed cultures [9, 6, 5] A lot of the advances in defined mixed culture studies are due to the progression of analytical methods [5]: For mixed cultures it is not sufficient to simply measure the cell count or the optical density as this will only be a sum of all organisms involved. To selectively count different species, techniques like flow-cytometry, selective plating, or DNA-based methods are used. The analysis of the genome, transcriptome and metabolom of several species at once also is both challenging and rewarding.

The next chapter will cover the methods that were employed for the engineering, the analysis and the co-cultivation itself in this work.

3 Material and Methods

In this section, the methods and the resources that were used for the studies in chapter 4 will be outlined. Each individual publication contains a *material & methods* or *experimental procedures* section. Therefore, only the broader context of the methods will be presented here.

3.1 Bacterial strains, media and maintenance

Over the course of all experiments very different strains were used that are specified in each publication. The basic, unmodified strains and their purpose in this work are listed in table 3.1 with the corresponding source. For engineering sucrose metabolism in Gram-negative bacteria (sections 4.1 and 4.1), genes from *Escherichia coli* W and *Pseudomonas protegens* Pf-5 were used. Those strains were simply taken as a production host for genomic DNA which was extracted with a spin-column based kit (MASCHEREY-NAGEL NucleoSpin® Tissue Kit) from cells grown over night in Lysogeny-Broth (LB) medium.

Pseudomonas putida EM178, *Cupriavidus necator* H16 and *E. coli* DH5α were employed as gene acceptors via conjugation (see section 3.3.2) or transformation (only *E. coli*) to elucidate the functionality of the gene clusters from *E. coli* W and *P. protegens* Pf-5 in these model organisms. Pure cultures of the strains were cultured in MR-medium (*C. necator*) according to [107] or M9 medium with iron and trace-elements (*E. coli*) or without (*P. putida*) according to [108]. Sucrose, fructose, glucose, or citrate (except *E. coli*) were the substrates in the cultivations, depending on the experimental setup. When necessary, antibiotics were added for selection: kanamycin 50 mg/L (30 mg/L for *P. putida*, 300 mg/L for *C. necator*), ampicillin 100-150 mg/L (600 mg/L for *P. putida*), streptomycin 200 mg/L, spectinomycin 50 mg/L, chloramphenicol 37 mg/L (only *E. coli*), gentamycin 10 mg/L, tetracycline 5 mg/L.

Synechococcus elongatus PCC7942 *cscB* was used as a sucrose producing module in pure and mixed cultures under salt stress. Induction of sucrose secretion by *cscB* was achieved by activating the LacI-controlled promoter with 0.1-1 mmol/L IPTG. The standard medium for *S. elongatus* was a modified version of BG-11 medium as described in [109] that lacked carbonate salts to keep the pH value longer at lower levels. The second change was a reduction of the calcium chloride concentration to 1% of the original medium. This was necessary to support growth of *P. putida* as described later in section 4.2. For mixed cultures additionally the phosphate and sulfate salt concentration were increased by a factor of 10 to exclude nutrient limitations due to a greater demand by the mixed culture compared to the pure culture. This enriched medium was denominated BG-11^{+}.

Table 3.1: Original bacterial strains used in this work with explanation and source

Name of the strain	Relevant characteristics	Use in this work	Source & reference
Pseudomonas putida EM178	prophage-free derivative of strain KT2440 (ΔPP3849-PP3920 (prophage 1), ΔPP3026-PP3066 (prophage 2), ΔPP2266-PP2297 (prophage 3), ΔPP1532-PP1586 (prophage 4))	Host for genetic engineering; producer of PHA	Obtained from the lab of Victor de Lorenzo at CNB, Madrid [64]
Escherichia coli W	Wild-type strain with the ability to use sucrose as a substrate with its *csc* gene cluster	Gene donor for cloning to study sucrose metabolism in Gram-negative bacteria	Obtained from the DSMZ [110]
Escherichia coli DH5α	A strain with a lot of genetic modifications to make it suitable as a cloning host	Cloning host	Obtained from the lab of Victor de Lorenzo at CNB, Madrid [111]
Synechococcus elongatus PCC7942 *cscB*	Ability to produce and secrete sucrose autotrophically via the expression of the sucrose permease CscB under salt stress (compare section 2.1.2)	Used as an autotrophic, donor module for sucrose production in mixed cultures	Obtained from the lab of Pamela Silver at Harvard Medical School, Boston, MA [25]
Cupriavidus necator H16	Wild-type strain; ability to use H_2/O_2 as substrate; can grow heteroptrophic and autotrophic; natural producer of PHB; unable to use sucrose or glucose as substrates	Object of study for heterlogous sucrose metabolism	Obtained from the DSMZ [112]
Pseudomonas protegens Pf-5	Wild-type strain; plant symbiont; used as a natural insecticide	Gene donor for cloning to study sucrose metabolism in Gram-negative bacteria	Obtained from the lab of Kirsten Jung at the LMU, Munich [113]

Cells were kept at -80°C in a 1:2 glycerol/LB-medium mixture for long-term storage of the heterotrophic organisms. *S. elongatus* was frozen in modified BG-11 medium with 3-5% DMSO by dropping the cryo-vessels for 5 min in liquid nitrogen before storage at -80°C as described in [114]. Cyanobacterial cultures were kept in liquid medium for maintenance and were transferred approximately every two months in shaking flasks sealed with paper plug and aluminum cap. These cyanobacterial cultures were used as pre-cultures for most experiments. Contaminations were checked by streaking a small volume of these cultures on LB-agar plates at every passage. For medium-term usage, heterotrophs were kept on agar plates made of their specific medium or LB medium with suitable antibiotics and carbon source.

Incubation temperature was set to 30 °C, except for *E. coli* that prefers 37 °C.

3.2 Fermentation at different scales

3.2.1 Culture vials & shaking flasks

Precultures, long-term cultures and cultures for genetic engineering methods were grown in either 10 mL culture vials (2-5 mL culture volume) or in shaking flasks (50-1000 mL). The actual liquid volume in the flasks was 10-20 % of their nominal volume. The flasks were shaken in orbital shakers (Thermo Fisher Scientific, MA, USA or Kuhner AG, Switzerland) at 220 rpm agitation frequency, except *S. elongatus* which was shaken at 100 rpm. For illumination, the shaking incubator was equipped with a 30 W fluorescent tube with a photon flux density of 10-26 µmol/(m^2 s).

3.2.2 Lab-scale photobioreactor

Cultivations under more controlled conditions, in mixed culture or at greater scale were done in the airlift flat-plate photobioreactor Labfors Lux (Infors HT, Switzerland). The reactor and the control panel offer:

- Control of temperature and pH
- Defined gas flow and mixture (air/CO_2)
- High and controlled light influx
- High specific gas-liquid diffusion
- Online measurement and recording of various parameters

The reactor was illuminated by an LED panel with an array of LEDs reaching a photon flux density of up to 3000 µmol m^{-2} s^{-1}. The 2 cm deep cultivation chamber is placed directly in front of the LED panel. A low depth guarantees a short light path which is crucial for efficient cultivation of phototrophic microorganism since the process is basically two-dimensional (i.e. the illuminated surface limits productivity) as soon as a bigger fraction of the incoming light is absorbed by the microorganisms [115].

The temperature of the cultivation chamber is regulated by a similarly shaped cooling chamber right behind it (in direction of the light path). Via an dissolved oxygen (DO) and pH sensor, both sizes can be measured and recorded online. pH was mostly titrated with nitric acid when a BG-11 based medium was used. This way, the nitrate ions – the nitrogen source in BG-11 – could be replenished at the same time. In Fig. 3.1, the schematic buildup of the reactor is illustrated.

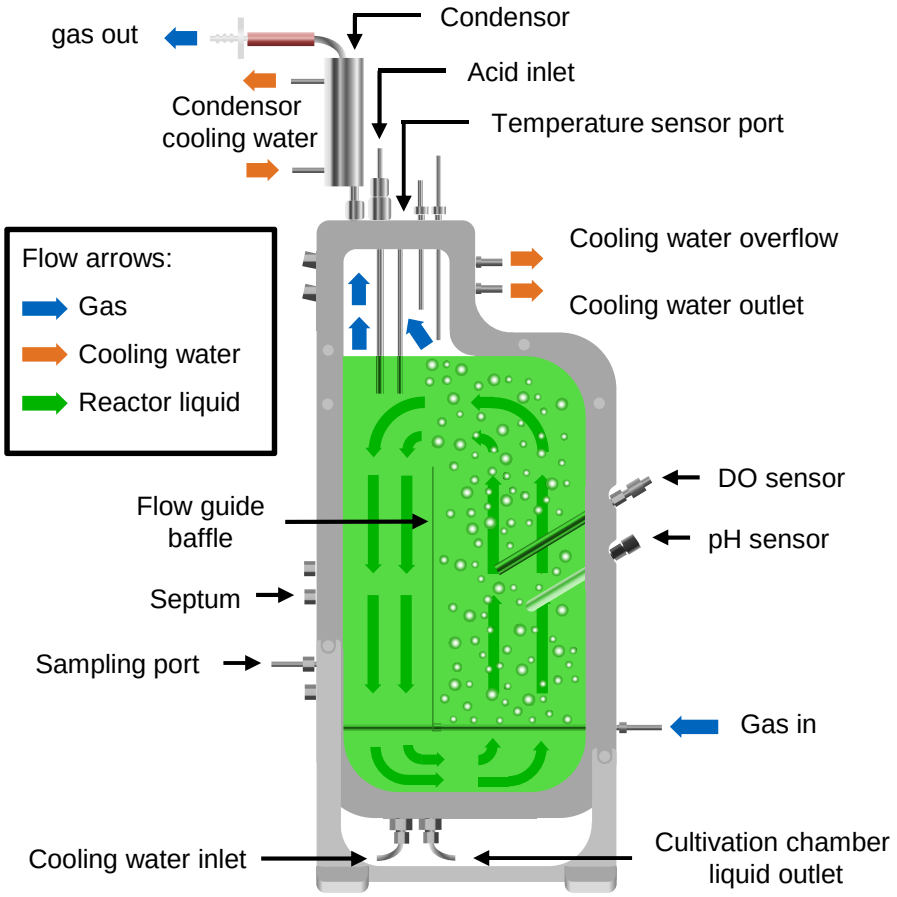


Figure 3.1: Schematic picture of the Labfors Lux airlift photobioreactor (Infors HT, Switzerland). The most important parts for this work are labeled. Green arrows indicate the liquid flow within the reactor, illustrating the cyclic flow that is typical for airlift bioreactors. Note that behind the cultivation chamber (green) lies the cooling chamber in which cooling water flows in and out. Before entering the reactor, the gas mixture passes a sterile filter and is then saturated with water in a 2-5 L bottle. For sampling, a Super Safe Sampler® (Infors HT, Switzerland), was connected to the sampling port before autoclaving.

A normal cultivation of *S. elongatus* started with setting up the reactor and all peripheral tubings and bottles. The reactor was filled with deionized water and sealed

for autoclaving. After autoclaving at 121 °C and 2 bar pressure for 15 min, the peripheral bottles (a foam trap behind the condenser, the bottle for influx gas moistening and the acid/feed bottles) were connected to the reactor with sterilized polypropylene valve connectors (CPC, Minnesota, USA). Gas and cooling water was connected, and the components of the medium were added from stock solutions through the septum with sterile syringes. After equilibration of the pH, the reactor was inoculated with 20-50 mL of stationary *S. elongatus* culture. Sucrose secretion was normally induced at an optical density of 0.3 - 0.6 (measured in a photometer with 1 cm light path and 750 nm wavelength) by adding 0.1 mM IPTG.

In case of a mixed culture, the co-culture partner (mostly *P. putida* was added one or two days after induction of *cscB* to make sure sucrose was already present in the medium and the heterotroph can start to grow right away.

3.3 Molecular biology techniques

To engineer *P. putida* to be a suitable co-culture partner for *S. elongatus*, several genetic constructs were created and screened. The methods for the construction and validation of these new genotypes are described in this section.

3.3.1 Recombinant DNA technology

Since the first time a non-native gene was transferred to a host by Cohen and Boyer [116], recombinant DNA technology has become a wide-spread method to extend or tailor the capabilities of all kinds of organisms. In their experiment, they used the restriction enzyme EcoR1, an enzyme that cuts DNA at specific recognition sequences, to cut out a fragment from a plasmid. They then inserted an equally digested fragment of DNA, containing an antibiotic resistance cassette, into the new gap in the plasmid. A ligase, an enzyme that glues together DNA strands, was used to connect the fragments (plasmid + antibiotics cassette). Then, they transferred the new plasmid to *E. coli* yielding the first artificial transgenic organism.

Today the cloning of DNA by restriction/ligation is still one of the most widely used techniques. Most of the DNA manipulations in the underlying publications of this work have been done with this method. Several suppliers have developed a whole arsenal of restriction enzymes and genetically optimized the proteins to be more compatible, robust and specific. If not stated differently, all enzymes used in this work have been bought at New England Biolabs (NEB), MA, USA. For amplification of DNA via polymerase chain reactions (PCR) to construct new plasmids, a Q5 polymerase was used when accuracy was needed. For the amplification of DNA via PCR for sequencing, the less accurate, but very robust Taq-Polyermase was used (mostly OneTaq-Mastermix by NEB).

A typical protocol for inserting a new gene into *P. putida* was as follows:

1. The DNA donor strain was grown in LB medium (2 mL) over night and the genomic DNA was extracted with the NucleoSpin® Tissue Kit from MASCHEREY-NAGEL

2. A PCR was set up with genomic DNA as a template and primers containing flanking restriction enzymes for insertion into a suitable plasmid
3. The plasmid was extracted from a plasmid-bearing strain out of an 5-10 mL overnight culture in LB and an antibiotic
4. Both the PCR product and the plasmid were digested with two restriction enzymes
5. If necessary, the plasmid was dephosphorylated with antarctic phosphatase (NEB)
6. Both plasmid and PCR product were then extracted and purified from an agarose gel, loaded with the digested DNA
7. The purified fragments were mixed in different ratios and ligated over night with T4 ligase (NEB)
8. The ligation mixtures were transformed into *E. coli* DH5α with the protocol of Chung *et al.* [117]
9. Finally the plasmid was transferred to *P. putida* (or any other host) by conjugation as described in section 3.3.2

The conjugation of the plasmid is the last step in this protocol which delivers the DNA to the final host. Moreover, this technique is rarely used in *E. coli*, the standard host for recombinant DNA. Therefore it will be explained in more detail in the next section.

3.3.2 Conjugation of plasmids

The conjugation of plasmid DNA from one bacterial cell to another is a natural pathway of horizontal gene transfer. After cells get in contact by the action of specialized pili, a pore is formed between the donor and recipient cell. For the mobilization of a plasmid, first the DNA is nicked (i.e. on strand is cut) by the relaxosome at the *oriT* site, the origin of transfer. This sequence is necessary for all plasmids in conjugation. In this work, mostly vectors from the SEVA library [118] and their derivatives were used, all of which contain the needed sequence. The relaxosome guides the DNA to the type IV secretion system T4SS where the protein complex is pumped into the cytoplasm of the recipient cell. Whether or not the DNA is transported along with the protein is not fully understood, there is some evidence, however, that it passes through the T4SS complex [119]. Meanwhile, the remaining DNA strand in the donor cell that was not transferred, is elongated by a DNA polymerase from 5' to 3' in a rolling cycle fashion.

All involved proteins are encoded in the *tra* and *mob* gene clusters. They are part of all natively conjugating plasmids. A frequently used self-conjugating plasmid is pRK600 that can be used to transfer any plasmid with an origin of transfer. To give an *E. coli* cell the ability to transfer a plasmid without having all necessary *tra* and *mob* genes, a self-conjugating plasmid has to be incorporated first into the plasmid donor strain. In a second step, the donor that now has an additional plasmid with the *tra* and *mob* genes, can then itself transport the donor plasmid into the recipient cell. In addition to a donor

and a recipient of a plasmid, there has to be a second donor strain, bearing the pRK600 plasmid. Since this strain is helping the donor and recipient with the transfer it is called "helper strain". As three strains have to be mixed, the method is called "triparental mating".

Conjugation from *E. coli* to *P. putida* was achieved by following the protocol of de Lorenzo and Timmis (1994) [54] in a modified version (see [120, 121]). The three cultures – a donor strain (mostly *E. coli*), a recipient, and the helper strain *E. coli* HB101 (pRK600) – were mixed in equal amounts in a centrifugation tube. After centrifugation and disposal of the supernatant, the pellet was resuspended in the remaining liquid and pipetted on an LB agar plate without antibiotics. The sitting drop was incubated 4-24 hours and then plated on a selective agar plate. The colonies were restreaked to exclude contaminations originating from the conjugation mixture and then used for further purposes.

3.3.3 Genomic integration via Tn5 and Tn7 transposons

For many biotechnological applications, it is a suboptimal situation when recombinant DNA is located on a plasmid inside the cell: There always has to be selective pressure to make sure the plasmids don't get lost. Moreover, the plasmid has to be replicated and its genes are transcribed. Depending on the case, this might consume a significant fraction of the cellular resources, resulting in metabolic burden and hence slower growth and production. While one plasmid is normally manageable, the use of multiple plasmids (for instance if a whole pathway has to be heterologously expressed) often worsens the problem and cells are struggling to grow.

An alternative is the integration of genes in the host cell's chromosome. Here, the natural replication mechanisms of the bacterial cell can be used and the metabolic burden is less pronounced because normally only one copy of the gene will be integrated into the chromosome. A simple and straight-forward method for the integration into the chromosome is the use of transposons. They are mobile DNA-elements that have the ability to "jump" from one site of the chromosome to another, by the action of a nuclease (excision) and a integrase (for integration into the chromosome). Tailored versions of these transposons can be used as integration vectors [55, 56]. They contain a region flanked by two so-called "mosaic end" (ME), the recognition sites for the transposase (see figure 3.2). Any DNA between these flanks will be integrated into the host genome. In this work, mini-Tn5-transposons were used for random integration into the chromosome and mini-Tn7-transposons for site specific insertion into the *att*Tn7 site. The *att*Tn7 site can be found within the *glmS* gene in the genome of many Gram-negative bacteria and is the homing site for Tn7 transposase.

For integration of the transposon, a triparental (Tn5) or quattroparental (Tn7) mating was setup. One strain contained the integration plasmid (derivatives of pBAMD1-2 or pTn7-M) with the insert flanked by mosaic sites and an antibiotic resistance gene. The second strain was the recipient, the third strain contained the helper plasmid (here: *E. coli* HB101 (pRK600)). For Tn7 transposition, a fourth strain was added that shuttled a vector (pTnS1) with the Tn7 transposase. In Tn5-based pBAMD1-x vectors (x = 2,4,6),

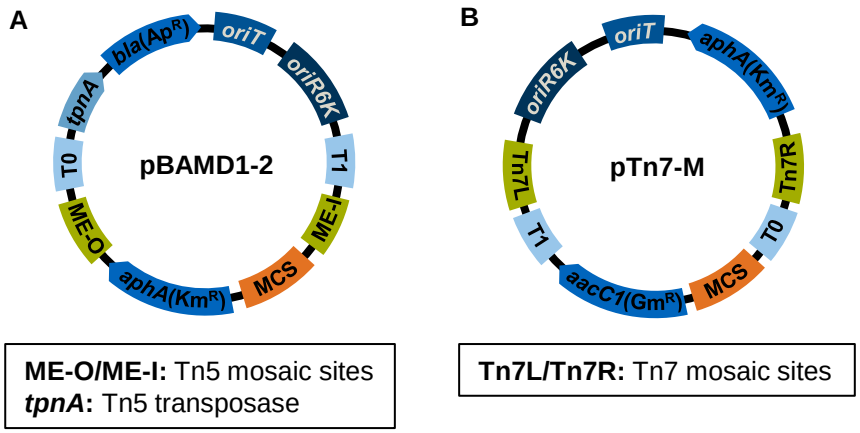


Figure 3.2: Plasmid maps of pBAMD1-2 (panel A, Tn5-transposon vector) and pTn7-M (panel B, Tn7-transposon vector). Both plasmids are similarly structured: Between two insertion flanks (ME-I/ME-O or Tn7L/Tn7R respectively), a multiple cloning site and an antibiotic resistance gene are placed. Transcriptional terminators T0 and T1 are found next to the insertion flanks, either inside the insert or outside. Note that there are additional T_{500} and T_{32} terminators in pBAMD1-2 within the insert next to the insertion flanks. They are not illustrated because of limited space. A read-through at the site of genomic integration is therefore improbable. The Tn5-transposon vector additionally contains a gene for its corresponding, hyperactive transposase TpnA. Both vectors also contain an antibiotic resistance cassette in the insert region for selection of genomic integration. The vector maps have been adapted from [55] or random [56].

the transposase gene is located in the backbone of the plasmid. After conjugation, the mixture of strains was plated on agar plates containing an antibiotic that corresponds to the resistance gene between the mosaic sites. Since all used vectors bear a R6K origin of replication, only the cells that incorporated the insert on the integration vector into their genome will grow on that plate.

With this method, a genomic integration can be done in 3 days with little work, assuming that all plasmids have been constructed.

3.4 Flow cytometry

Among the many analytic methods that were used in this work – like high performance liquid chromatography (HPLC), gas chromatography (GC), fluorospectrometry, and many more – flow cytometry shines as an outstanding method for the analysis of mixed culture cultivations. Therefore, this section will be dedicated to give an overview on this technique. Flow cytometry is a method for cell counting that was originally developed as a tool for histological analysis and is still widely used for the diagnosis of

health disorders like blood cancer. It is, however, also very useful for the discrimination and quantification of different bacterial populations.

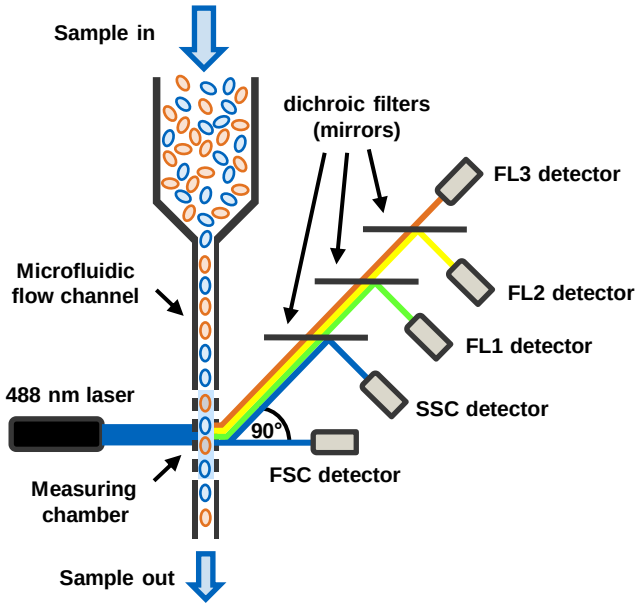


Figure 3.3: Simplified illustration of the flow cytometer (Partec Sysmex GmbH, Görlitz, Germany) that was used in this work. Cells are guided through a microfluidic flow channel and then pass the measuring chamber in which a laser excites every passing cell. Unscattered light is detected to record the forward scatter (FSC); light that was scattered in a 90° angle is split by dichroic mirrors and analyzed at different detectors. Light that did not change its wavelength is recorded as sideward scatter (SSC). Fluorescence of three different wavelengths (FL1, FL2 and FL3) is detected with help of filters. There are many more configurations for a flow cytometer, also including more lasers, more detectors or a sorting function for cells that pass the measuring chamber. The illustration was adapted from Givan: "Flow Cytometry: First Principles" (2013) [122].

A simplified depiction of the flow cytometer (Partec Sysmex GmbH, Görlitz, Germany) that was employed for mixed culture analysis is shown in Fig. 3.3. The basic working principle of a flow cytometer starts with creating a stream of single bacterial cells in a microfluidic channel. The stream of cells is directed through a measuring chamber made of quartz on which a laser beam is focused. After the laser beam passes the measuring chamber, it is split and guided to different detectors: The loss of intensity of the unscattered beam is recorded as forward scatter (FSC) behind the measuring cell. It is a measure that represents size and volume of the cell, but also accounts for absorption at the wavelength of the laser. Light that is scattered by an angle of 90° is recorded as sideward scatter (SSC). SSC accounts for the granularity of passing cells which is caused by intracellular organelles or membrane surrounded compartments like vesicles or membrane stacks. If there are fluorescent compounds in the cell that are excited by

the laser, the emitted light will also be analyzed in a 90° angle. The wavelengths are filtered for example by dichroic filters that act as mirrors and then analyzed with diodes to give fluorescence signals. In the case of the Partec flow cytometer used here, three fluorescence emission wavelengths were available: green fluorescence at 536 nm (FL1), yellow/orange fluorescence at 590 nm (FL2) and red fluorescence at 675 nm (FL3). The flow cytometer can gather all these optical parameters so fast that every passing cell is detected and classified.

For mixed cultures, that means if the bacterial cells have different optical features like pigmentation or size, they can be separately counted by the machine. On top of cell quantification, this also gives dynamic information about cell size, quantity of pigments and sub-populations. If cells are not naturally fluorescent, they can be stained with specific fluorescent dyes that react differently with the bacteria. A widely used dye to stain lipid containing cells is Nile red (NR). It gives strong fluorescence in lipophilic environments like PHA-granules or oil droplets, but is quenched in aqueous solutions. This way concentration of PHA can be quantified qualitatively over the process at the same time [123]. There are however many more dyes that can be specifically applied to gain other information like live/dead-counts or cell type.

In a mixed culture of *S. elongatus* and *P. putida*, the natural pigmentation and thus fluorescence of the phototroph (chlorophylls, carotenoids, phycobilins) can be exploited for flow cytometric quantification. Additionally staining of PHA granules of *P. putida* gives further information about product (=PHA) concentration.

4 Results: Summaries of individual publications

This section will present all advances that have been made over the course of my work at the Associate Professorship of Systems Biotechnology at TUM by means of the key publications in the last years. They form the core of this document and will be outlined with meaningful figures from these publications. Finally the results will be discussed in context of the relevant literature on defined mixed culture cultivation. To make a mixed culture of *P. putida* and *S. elongatus cscB* possible, it was first necessary to genetically engineer *P. putida* to be able to use sucrose as a substrate. The first publication (section 4.1) deals with the creation of sucrose using *P. putida* phenotypes. In the second publication (section 4.2) the mixed culture is set up and analyzed. At last, the third publication (section 4.3) revolves around unanswered questions from the first publication and the role of porins in engineering sucrose metabolism in *P. putida*.

4.1 Metabolic engineering to expand the substrate spectrum of *Pseudomonas putida* toward sucrose

Löwe H., Schmauder L., Hobmeier K., Kremling A., Pflüger-Grau K. (2017). *MicrobiologyOpen* 6(4): e00473. doi:10.1002/mbo3.473

Sucrose is one of the most widely used substrates in modern fermentation processes as it is cheap, highly soluble, untoxic and has a high energy content. However, some of the favorite production organisms in industrial biotechnology like most *E. coli* strains, *C. necator* and *P. putida* cannot use it as a substrate. We therefore set out to construct genetic vectors that confer sucrose metabolism with the focus on *P. putida* as a host.

Since *P. putida* has no broad range PTS uptake system, we chose to implant genes for sucrose utilization from the non-PTS *csc* sucrose gene cluster found in *E. coli* W. It had been recently shown that only the genes *cscA*, encoding a sucrose hydrolase, and *cscB* that encodes a sucrose/proton symporter were needed to restore a sucrose utilizing phenotype in *E. coli*. We therefore chose to express these two genes in *P. putida*. For that purpose, we inserted *cscA* alone or *cscAB* as a polycistronic construct into the expression plasmid pSEVA224. Both constructs were sufficient to allow growth of *P. putida* on sucrose as sole carbon and energy source (see Fig. 4.1). The growth rates of *P. putida* carrying either of the plasmids showed little difference between each other (μ= 0.36 h^{-1} vs. 0.38 h^{-1}) and were on par with the rates of *P. putida* grown on a fructose glucose mixture (μ= 0.45 h^{-1}).

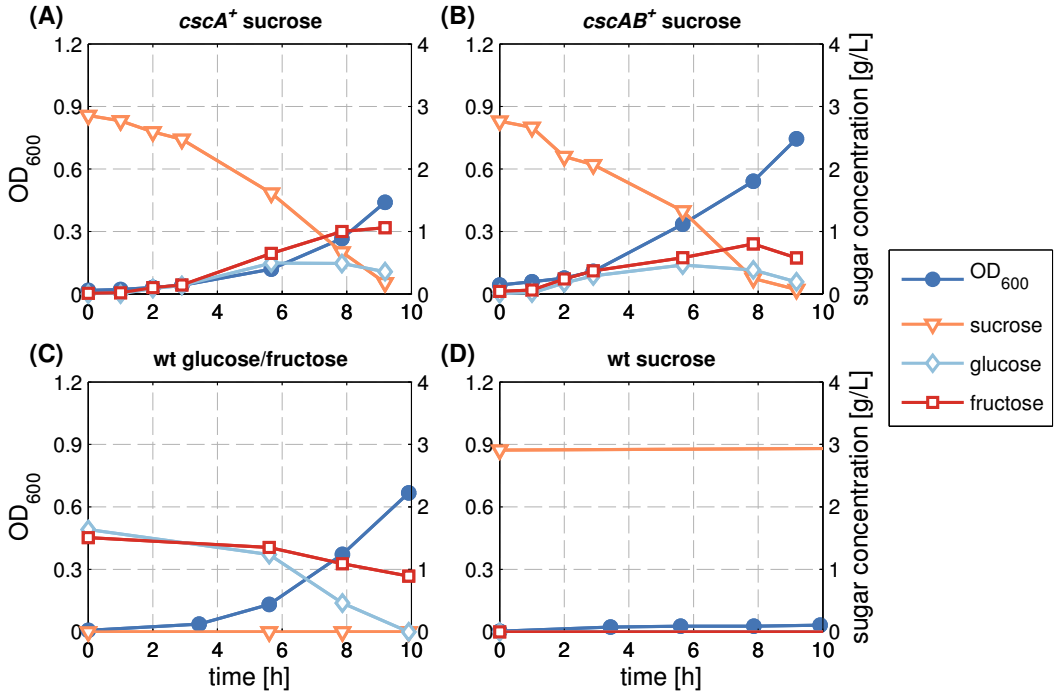


Figure 4.1: Optical densities and sugar concentration in the culture supernatants of different *P. putida* strains grown on sucrose or a mixture of glucose/fructose in shaking flasks. Both *cscA* and *cscAB* strains rapidly grow and consume sucrose with transient accumulation of fructose and glucose (panels **A** and **B**) while the wild-type *P. putida* shows no activity with sucrose (panel **D**). Growth rates are on par with the wild-type strain cultivated on a 1:1 mixture of glucose and fructose (panel **C**). The figure was taken from Löwe *et al.* (2017)[120].

For compatibility with other expression systems, we constructed plasmid-less sucrose consuming phenotypes of *P. putida*. To achieve this, we assembled the broad host-range transposon vectors pSST1 and pSST2 (sucrose splitting transposon) that contained *cscA* alone or *cscAB*, respectively. After integration of the transposon into their genome, *P. putida*, *E. coli* and *C. necator* were able to grow with sucrose as a substrate – showing the universality of the approach. The pSST vectors are based on the mini-Tn5 transposons and hence insert themselves randomly into the host genome. We therefore screened for each transposon ten mutants of *P. putida* containing the integrated transposons for growth rate and location of the insertion. The growth rates varied slightly, but were in general lower than those observed in the plasmid based system. Again, no difference between *cscA* alone or *cscAB* could be detected significantly.

This indicated that *cscB* was not necessary for efficient growth on sucrose which means that the sugar can be either transported natively by *P. putida* or it is split outside the

cell. In fact, the sucrose hydrolase activity in the supernatant was found to be sufficiently high to explain the observation. Still, the major fraction of activity was measured inside the cell which makes a native transport of sucrose in *P. putida* improbable. We could also rule out a lack of expression of *cscB* in *P. putida* by genetically fusing *cscB* to green fluorescent protein (GFP) and subsequently analyzing the expression by confocal fluorescence microscopy.

Taken together, we constructed the first sucrose consuming *P. putida* strains reported so far by means of the plasmid pSEVA224-*cscA(B)* or transposition of sucrose genes into the genome via the pSST vectors. It was found that *cscB* gene showed no apparent activity in *P. putida* toward sucrose transport and that sucrose splitting mainly took place outside of the cells. Nevertheless, the new sucrose phenotypes open up the possibility to cultivate *P. putida* on cheap, sucrose-based substrates that can unlock new products and processes with the promising platform organism.

Personal contributions: The idea to use *S. elongatus cscB* and sucrose utilizing *P. putida* in a mixed culture originated in a literature search I performed at the beginning of my work on the topic. We decided to genetically modify *P. putida* by implanting genes from the *csc* gene cluster of *E. coli* W to make possible the use of sucrose as a carbon source. I constructed the plasmid expressing CscA or CscB in collaboration with my bachelor students, including design of the primers needed for the PCR reactions. All shown growth experiments were conducted by myself and designed with help of my lab supervisor Dr. Pflüger-Grau. The plasmids encoding the CscB-GFP fusion proteins were constructed by my master student Karina Hobmeier with my assistance. Together we examined the cells expressing the fusion proteins under the microscope with aid of Prof. Ramon Torrez Ruiz at TUM. I also conducted all experiments involving *Cupriavidus necator* and cell lysis to measure sucrose splitting activity of *P. putida*. After experiments were finished, I wrote the major part of the manuscript for publication.

Co-author contributions: AK, KPG were involved in the development of the concept of the study. KPG co-designed the experiments. LS, KH assembled a part of the genetic constructs. LS performed some of the cultivation experiments. KH helped in the analysis of the cells by microscopy. KPG wrote a fraction of the draft of the publication. All authors proof-read the text.

4.2 Photoautotrophic production of polyhydroxyalkanoates in a synthetic mixed culture of *Synechococcus elongatus cscB* and *Pseudomonas putida cscAB*

Löwe H., Hobmeier K., Moos M., Kremling A., Pflüger-Grau K. (2017). *Biotechnology for Biofuels* 10:190. doi:10.1186/s13068-017-0875-0

Most processes in industrial biotechnology are based on sugars or lipids as substrates that are originally derived from crops like sugarcane, corn or oil palms. Massive industrial

agriculture is associated, however, with problems such as erosion of soil, competition with the food chain, depletion of water resources and destruction of ecosystems. A upcoming, more area efficient alternative for feedstock production is the culture of cyanobacteria or eukaryotic algae that don't need fertile land, don't compete with food markets, can use salty or brackish water and need less space. Recently, *S. elongatus cscB* was shown to secrete considerable amounts of sucrose into its surrounding medium with efficiencies that are already comparable to sugarcane.

In this study we aimed to use this sugar as a carbon source for *P. putida cscAB* by a two-step cultivation and by setting up a mixed culture between the two organisms in a photobioreactor. Since *P. putida* is natural producer of PHA, our goal was to produce this kind of bioplastic out of cyanobacterial sugars as a proof of concept for the modular approach.

First, the ability of *S. elongatus cscB* to produce sucrose in a photobioreactor with a modified BG-11 medium was assessed at different NaCl concentrations. A maximal productivity of 0.346 $g_{sucrose}$ L^{-1} d^{-1} was achieved at 150 mM NaCl. As a next step, we cultivated *P. putida* in the cyanobacterial medium with glucose/fructose to proof the feasability of the mixed culture. Growth rates μ of up to 0.305 h^{-1} could be reached. This is sufficiently high for the mixed culture because growth and sugar production of *S. elongatus* becomes linear after light saturation is reached and thus heterotrophic exponential growth will catch up after a given time.

Now that a common medium was found in which *S. elongatus cscB* could produce sucrose and *P. putida cscAB* could grow heterotrophically, the mixed culture was set up. For this purpose, *S. elongatus* was grown in the photobioreactor as usual, but two days after induction of sucrose secretion, a *P. putida* pre-culture was added. Over the course of the fermentation, optical densities, colony forming units, cell counts (by flow cytometry), sugar concentrations in the supernatant and PHA-concentration (via GC) were recorded (see Fig. 4.2). While the cell count of *P. putida cscAB* was increasing over the days, the sucrose produced by the cyanobacterium decreased which showed the interspecies carbon transfer. Even though no obvious limitation of nutrients came into play, *P. putida* nevertheless produced PHA at a rate of 3.3 mg L^{-1} d^{-1}, reaching a maximal titer of 19.7 mg L^{-1}.

Finally, the experiment was repeated under nitrogen-limitation that was facilitated by a lower starting concentration of nitrate and a constant nitrate feed during the cultivation of 46 $mg_{nitrate}$ d^{-1}. In this case, PHA production by *P. putida* was significantly improved compared to the unlimited process: A maximal production rate of 23.8 mg L^{-1} d^{-1} and a maximal titer of 156 mg L^{-1} were achieved.

All in all, a defined mixed culture of *S. elongatus cscB* and *P. putida cscAB* was established in a photobioreactor. Simultaneous growth and production of bioplastics out of cyanobacterial sucrose could be shown. This proof of concept opens up the way for the use of cyanobacterial sucrose as a new, beneficial feedstock that can be turned into any valuable product with the help of the platform organism *P. putida*.

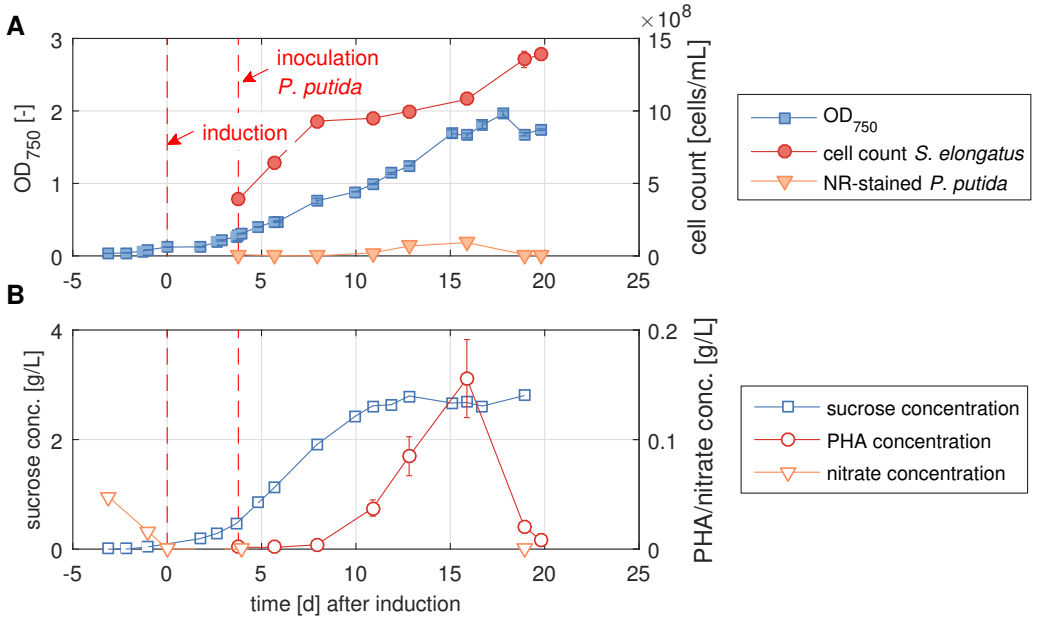


Figure 4.2: Mixed culture cultivtion of *S. elongatus cscB* and *P. putida cscAB* in a photobioreactor using modified BG-11 medium. The reactor was started with a culture of *S. elongatus cscB*. After induction of sucrose secretion, *P. putida* was added. Panel **a** shows optical density of the whole culture and cell counts as measured by flow cytometry as well as counts of Nile red stained cells. In panel **b**, the concentration of sucrose, nitrate in the supernatant, as well as PHA-concentration in the cells is depicted. The figure was taken from Löwe *et al.* (2017)[124].

Personal contributions: For the consolidation of the cyanobacterial medium to support growth of *P. putida*, I designed a new BG-11 derived growth medium based on experiments that are shown in the supplementary information and calculations of medium constraints. I also established a working protocol for the photobioreactor, including the setpoint of the pH, aeration rate, CO_2 partial pressure, time and strength of induction by IPTG, and light intensity by a combination of literature data, good guess and trial-and-error. The initial characterization of sucrose secretion by *S. elongatus cscB* was accessed in various experiments by myself in collaboration with my bachelor and master students. Preliminary mixed culture experiments were done by Manuel Moos with my assistance and the main experiments shown in the publication were conducted in a combined effort of Karina Hobmeier and me. After experiments were finished, I wrote the major part of the manuscript for publication.

Co-author contributions: AK, KPG were in involved in the development the concept of the study. KPG co-designed the experiments. MM, KH performed a part the cultivation experiments. KPG wrote a fraction of the draft of the publication. All authors proof-read the text.

4.3 Engineering sucrose metabolism in *Pseudomonas putida* highlights the importance of porins

Löwe H., Sinner P., Kremling A., Pflüger-Grau K. (2018). *Microbial Biotechnology.* doi:10.1111/1751-7915.13283

Before chemical science emerged, alchemists have been trying for more than a thousand years to transform base metals into gold. Today, in the dawn of the age of biotechnology, there are biotransformation instead of transmutation that convert merely wastes to valuable compounds. Scientists are now trying to efficiently use waste streams, such as molasses from sugar refining or lipid wastes from oil mills and turn them to base chemicals and fuels. In order to digest the complex substrate mixture that is often present in biological waste materials, production organisms must be equipped with a repertoire of enzymes and transporters.

P. putida, an emerging platform strain for industrial biotechnology, has been recently engineered to expand its metabolic menu to substrates such as D-xylose, L-arabinose, D-cellobiose and sucrose. In the latter case, bo uptake of sucrose by the sucrose permease CscB from *E. coli* W could be detected because of unknown reasons. Therefore, we set out to take a closer look at the transport of substrates by implementing a new sucrose operon in *P. putida* from a donor that is genetically closer than *E. coli* W.

Via homology search using the non-PTS sucrose permease *cscB* gene from *E. coli* W as query, we found a sucrose gene cluster in *Pseudomonas protegens* Pf-5, a close relative of *P. putida*. It consisted of 3 orthologues of genes from the *csc* gene cluster: a gene for a sucrose permease (*cscB*), one gene for a sucrose hydrolase (*cscA*) and a gene encoding a LacI-like regulator (*cscR*). Additionally an orthologue to the sucrose porin ScrY from the *scr* gene cluster was located nearby. For consistency reasons, we included this gene in the newly annotated *csc* cluster in *P. protegens* as *cscY*.

As a next step, we extracted the gene cluster from genomic DNA of *P. protegens* and inserted either the whole set of genes, or all genes except the porin into the multiple cloning site of the vector pSEVA221. Cells containing the whole set of genes (*cscRABY*) showed fast growth without a lag phase, whereas those lacking the porin (*cscRAB*) did not start growing after 48 hours (in all replicates). We therefore hypothesized that the porin plays an important role in sucrose transport. To verify this idea, the cells were complemented with an expression vector containing only the porin (pSEVA434-*cscY*). Addition of the porin restored the ability to grow on sucrose (Fig. 4.3).

The lack of growth of *P. putida* EM178 (pSEVA-*cscRAB*) could only be seen when LB medium was used for the pre-cultures, but not if M9 was used. We attributed this effect to the different set of porins that might be present, depending on the used medium. This hypothesis was supported by membrane swelling experiments that indicated that M9-grown cells were more permeable to sucrose than LB-grown cells.

Anyway, using the full gene cluster resulted in fast growth even when genomically integrated via a mini-Tn7-vector – growth rates of up to $\mu = 0.45\ h^{-1}$ were reached. At last, we showed that the newly engineered strain was able to metabolize the sugar in molasses with doubled yield compared to the wild-type strain.

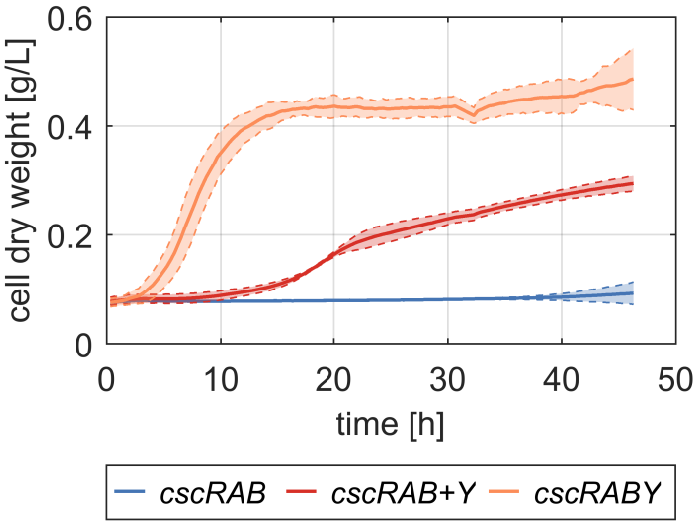


Figure 4.3: Cultivations of *P. putida* EM178 (pSEVA-*cscRAB*) complemented with pSEVA434-*cscY* or with an empty plasmid and a control culture with the whole set of genes (pSEVA-*cscRABY*). Addition of pSEVA434-*cscY* to *P. putida* EM178 (pSEVA-*cscRAB*) complemented growth on sucrose. The cells were cultivated in a microtiter plate in 9 replicates, 3 of which came from the same pre-culture (LB-medium). M9 minimal medium was used with sucrose as sole carbon and energy source. 95 % confidence bands were calculated from all replicates of one strain. The figure was taken from Löwe *et al.* (2018)[121].

Overall, we identified and annotated a new sucrose gene cluster in *P. protegens* Pf-5 that conveyed efficient sucrose metabolism with fast growth to *P. putida* even when integrated into the genome. In addition, the porin CscY was found to be of great importance for sucrose uptake when the pre-cultures were grown in LB medium. This highlights the importance of considering the transport across the outer membrane in metabolic engineering which is overlooked by most studies. The new strain also showed very promising results when cultivated with molasses as carbon source which might make production of typical products by *P. putida* more affordable on the long run.

Personal contributions: During my research stay at the lab of Victor de Lorenzo at CNB, Madrid, I discovered the not properly annotated *csc* gene cluster of *P. protegens* Pf-5 via homology search. Next, I extracted the genes from genomic DNA, provided by Karina Hobmeier at TUM, and transferred them to pSEVA221. The genes were then subcloned into pTn7-M by my master student Peter Sinner with my assistance. All experiments shown in the publication, except for the growth experiment of *P. putida* *att*Tn7::*cscRABY*, were conducted by myself and designed with help of Dr. Katharina Pflüger-Grau. Finally, I wrote the major part of the manuscript for publication.

Co-author contributions: AK, KPG were involved in the development the concept of the study. KPG co-designed the experiments. PS performed a part of the genetic engineering and the cultivation experiments. KPG wrote a fraction of the draft of the publication. All authors proof-read the text.

5 Discussion

5.1 The *csc* gene cluster of *E. coli* W *versus P. protegens* Pf-5

Being a soil bacterium that naturally colonializes the rizosphere of plants, it is surprising that it is unable to utilize sucrose as a carbon source since the disaccharide is the major transport sugar in plants [125]. Fortunately, the closely related *P. protegens* Pf-5 that is also a plant associated organism [113], contains a functional sucrose gene cluster in its genome. The gene cluster could be efficiently extracted from the organism and implanted into *P. putida* that then showed excellent growth in M9 medium with sucrose. Although the *csc* genes from *E. coli* W are highly similar, it is unclear why the *E. coli* permease did not show any effect in *P. putida*. One reason might be the organization of the polycistronic construct that was used in most cases in our work [120]. On the other hand, we also tried to complement the genomically integrated clones (*P. putida* ::mini-Tn5(*cscA*)) with a pTrc-promoter-based expression vector that only contained the permease with no success (unpublished data). Another reason might be the lack of a porin – the main difference between the *csc* genes from *E. coli* W and *P. protegens* Pf-5. It was found, however, that a porin is not necessary in *P. putida* when the cells are already adapted to minimal medium in the pre-culture [121]. Since CscB is membrane bound protein and over-expression of the corresponding genes often leads to problems within the cell [126], one way to optimize the behavior of the original constructs with the *E. coli* genes [120] could be the usage of the native promoter region of the gene. This is however only interesting for studying heterologous gene expression – in fact the *csc* genes from *P. protegens* Pf-5 don't leave much room for improvement concerning sucrose metabolism in *P. putida* in terms of growth rate. Removing the regulatory region of the *csc* genes from *P. protegens* and deletion of *cscR* might lead to a better performance, though. A final step in this regard would be the markerless integration of the genes into a neutral site into the genome of *P. putida*.

5.2 Comparison of engineered and native sucrose metabolism strategies

The most studied gene cluster for sucrose metabolism in Gram-negative bacteria, the *scr* and the *csc* gene clusters, differ in their mode of transport: While the *scr* genes encode a PTS-driven sucrose transporter, CscB is a gradient driven transporter. The choice of the system is tightly bound to the way the pathway is regulated: Since in PTS-driven uptake, the phosphate group has to be transferred from PEP via the PTS which also serves many regulatory functions, the sucrose uptake will be controlled on the metabolic

level by the PTS [127]. When only a gradient-driven permease is used, regulation takes place on a transcriptional level that is easier to control in genetic engineering and makes the strain more accessible to other modifications, but also somehow restricted in its applicability. Whether or not a PTS-based sucrose transporter is compatible with the small PTS in *P. putida* is not known, but seems unlikely: Either the phosphate for the phosphorylation of a PTS-based sucrose transporter would originate from the nitrogen-PTS via cross-talk or from the fructose-PTS that is only expressed in the presence of fructose [128].

Heterologous expression of *scr* or *csc* sucrose modules had been already done in *E. coli* K12 [129, 130, 131] and in *C. necator* [132]. Other industrial relevant strains like *Corynebacterium glutamicum* and *Saccharomyces cerevisiae* already possess the ability to utilize sucrose as a carbon source. In the case of *Zymomonas mobilis*, that produces ethanol from sucrose under anaerobic conditions, sucrose is not only split inside the cell, but the organism also has a sucrose hydrolase that is active in the culture medium [133]. An extracellular sucrose hydrolase was transferred to *C. necator* for the production of PHA in one study [134]. This approach resembles the phenotype of *P. putida cscA* that was observed in our first publication included in this work [120]. It is an open question which of the strategies is superior or if a mixture of intracellular and extracellular sucrose splitting might lead to optimal results. This might be addressed with simple metabolic models in future works to start with.

5.3 Cyanobacterial carbohydrates as a new feedstock for biotechnology

As explained earlier, carbohydrates produced by phototrophic algae or cyanobacteria offer the potential to use area which cannot be used for agriculture like deserts [135] or the marine environments, e.g. in floating bioreactors [136]. Moreover, waste water or salt water can be used for cultivation, saving up precious fresh water. Still, state-of-the-art algae cultivation hardly achieves a positive carbon or energy balance [137], mainly due to costs associated with drying and dewatering (raceway ponds) or pumping and gas influx (photobioreactors). Additionally, reactor and CO_2 costs are still too high for cost-effective production of algae or cyanobacteria [137]. Therefore, it has to be questioned: How do carbohydrates produced by cyanobacteria and algae compare to other kinds of feedstock?

While newer types of photobioreactors that are currently investigated [115, 138] might solve some of the associated problems, the price for algae or cyanobacterial products might never be on par with traditional crops. But then again, agricultural area is limited and algae and cyanobacteria have higher areal productivities [35, 36]. In this study for instance, we used a photobioreactor with a projected area A of about 0.1 m^2 at a photon flux density I of 240 µmol m^{-2} of a white LED with a mean wavelength of around $\lambda = 580$ nm. Using the equation for the energy of a photon:

$$E_{photon} = \frac{h \cdot c}{\lambda_{photon}} \tag{5.1}$$

the energy flow of the incoming light can be estimated as:

$$P_{in} = I \cdot A \cdot N_A \cdot E_{photon} = 4.95W \tag{5.2}$$

with N_A being the Avogadro constant. This is a rough estimate, as normally the photon energy has to be integrated over the wavelength spectrum of the used LED to get the mean energy of photons.

S. elongatus cscB was able to produce sucrose at a rate $\dot{c}_{sucrose}$ of 0.346 g L^{-1} d^{-1} in our experiments [124] which can be used to calculate the fixed chemical energy. With a formation Gibbs-free energy $\Delta G_{sucrose}$ from CO_2 and H_2O of around 5700 kJ/mol [139] and a volume V of the reactor of 1.8 L, the fixed free energy over time of sucrose is:

$$P_{fixed} = \frac{\dot{c}_{sucrose} \cdot \Delta G_{sucrose} \cdot V}{M_{sucrose}} = 0.12W \tag{5.3}$$

This means, in total 2.4 % of the incoming light was fixed to sucrose which does not take into account biomass production that also took a part of the photosynthetic capacity of the organism. Plants, in contrast only turn less than 1 % of the incoming light into chemical energy [35, 36] of which only a small fraction is the product.

Another way to turn light into chemical energy is the use of photovoltaic device to power electrolysis: Modern commercial photovoltaic panels have an efficiency of around 20 % and commercial electrolyzers reach efficiencies of around 73 % [140], resulting in a light-to-chemical conversion efficiency of 14.6 %. Although this is almost an order of magnitude higher than what can be achieved by cyanobacteria and algae, the production of chemicals from hydrogen also is not a particularly efficient process. Synthesis gas fermentation is possible, but at the moment restricted to specific products and has problems of its own [141].

Taken together, the use of algae and cyanobacteria offers higher areal efficiencies than traditional crops and makes the autotrophic production of carbohydrates possible which is hard to achieve by hydrogen-powered processes. Therefore, cyanobacterial carbohydrates are a good compromise between area efficiency and the available products, and might find their way as a future feedstock for biotechnology.

5.4 Phototrophic production of PHA from defined mixed cultures and pure cultures

In the second publication that is part of this work [124] we established a defined mixed culture of *S. elongatus cscB* and *P. putida cscAB*. As a proof-of-concept, we showed the production of PHA out of CO_2 and sunlight. Shortly after our work was published, Weiss *et al.* [49] showed the production of PHB in defined mixed cultures of *S. elongatus cscB* and *H. boliviensis* with even slightly higher PHA production rates than reported by us.

Another approach is the direct production of PHA in wild-type and transgenic cyanobacteria [142, 143, 144, 145, 146] or in transgenic crop plants [147]. The production rates achieved in mixed cultures, however, were on par with these cyanobacterial monocultures [49]. Plants have been shown to accumulate up to 14% of their cell dry weight as PHB without a loss of growth [147]. This is a good start, but probably also to low for industrial applications. *P. putida* in comparison regularly achieves PHA mass-fractions of cell dry weight of around 25% when cultivated on glucose and has been engineered to contain around 35% [73]. *H. boliviensis* reached up to 31% PHA-mass fractions of the cell dry weight [49]. These reported values highly favor the production of PHA/PHB by mixed cultures compared to pure cultures of transgenic phototrophs until now.

When compared to existing, heterotrophic industrial processes [148], yields and productivities appear very low. These commercial fermentations reach PHA mass fraction of cell dry weight of more than 80% in high cell density fermentation with cell dry weights over more than 100 g/L. However, one has to keep in mind that the substrates used in these heterotrophic fermentations are all derived from crop plants that took very long to grow and needed to be sawn, harvested and processed. In addition, these crops cannot compete with the area efficiency of modern cyanobacteria or algae based processes [35, 36].

A central criterion of industrial PHA production is the amount of PHA in the fermentation broth at the time of harvesting. Phototrophic processes lack high cell densities and high mass fractions compared to heterotrophic processes. This could be overcome with newer, high cell density cultivation systems for algae like thin layer cascade reactors [115] or porous substrate reactors [138] that reach cell dry weights an order of magnitude higher than flat plate or tubular reactors. In case of the mixed culture that was characterized in this work, longer sucrose production and hence higher PHA concentrations at the end of the process could be achieved this way. Porous substrate reactors also have the benefit of low energy and water demand compared to state-of-the-art photobioreactors. Another possibility could be in-situ product removal of PHA by facilitated cell lysis as explored in some studies [100, 149, 150] or separation of the two co-culture partners in different compartments [49, 47]. In a far future when fossil resources will become scarce or political incentives promote renewable chemistry, these new technologies will be able to shine.

5.5 Future directions and potential of the technology

The production of PHA by *P. putida* out of cyanobacterial carbohydrates is just one example of many possible processes that could be implemented with *P. putida*, now that the strain has been engineered and a common medium has been found. *P. putida* has been employed as a host for the production of various, industrially interesting products like aromatic chemicals, terpenes and rhamnolipids (for reviews consider [30, 31]). As *P. putida* can easily be genetically modified, other process relevant features could also be engineered, such as induced auto-coagulation or flocculation [151] or in-situ extraction of PHA [100, 149, 150]. For mixed cultures with *S. elongatus cscB*, it might also be

beneficial to rework the EDEMP cycle in *P. putida* as it does not yield as much energy as other forms of glucose splitting pathways [67, 29]. This way the yield of a possible product could be improved. For the production of PHA, the EDEMP cycle might be just right as it provides NADPH that is needed for PHA synthesis instead of NADH. This matter could be investigated in the future.

Since *S. elongatus cscB* is secreting sucrose into the medium, it is making the sugar also available for possible contaminants. The long-term stability of mixed cultures with *S. elongatus cscB* has been assessed with *H. boliviensis* that showed high robustness toward contamination [49]. The key for a low contamination risk is to keep the sucrose concentration at a basal level and to have a strain that takes up sucrose even faster than possible contaminants. This should be considered for engineering approaches for improving the mixed culture. The robustness toward contamination could also be improved under extremophilic conditions like high salt concentration or pH. In this case a different cyanobacterium like the alkaliphile *Arthospira platensis* could be used. This organism accumulates low-molecular-weight carbohydrates, glucosyl-glycerol, and trehalose as osmolytes [152] that might be used as a substrate for a co-culture partner as well. It will be necessary to screen or engineer suitable transporters for the osmolytes, replacing CscB as a transporter in the contemporary host.

Finally, another way to improve the mixed culture might be through directed evolution. At the moment the strain is working very well with just the insertion of the *cscB* gene and without further optimization (although other genetic modifications showed potential in *S. elongatus* [25]). The strain could be evolved by using a heterotroph that uses the sucrose to produce a "reward" like a nitrogen source for *S. elongatus cscB* as demonstrated in [47]. When the two strains are plated on agar, those colonies that grow fastest should be the ones with superior sucrose production capability as they get more nitrogen in return. *S. elongatus* could also be co-cultured with a strain that produces an optical active molecule that could be measured spectrometrically to guess the sucrose production rate at a microplate-reader format for instance.

All these strategies could help this technology to unlock its full potential, ascending it to the most area efficient methods for sunlight to chemical conversion.

5.6 Fuels and chemicals in biotechnology – *Quo vadis*?

The engineering of defined mixed cultures has given bioengineers a whole new toolkit for biochemical reaction engineering. Genetically modifying different bacterial populations (in this case *S. elongatus cscB* and *P. putida*) enables processes that have not been possible before. In this study, continuous photosynthesis by *S. elongatus cscB* could be coupled to starvation-induced accumulation of PHA in *P. putida cscAB*. Sucrose production by cyanobacteria, the foundation of the process, is one of the most area efficient ways to produce sucrose from sunlight. As nowadays more and more area in this world is used for agriculture because of growing populations and economical demands, area efficiency will be crucial in the future.

The invention of the sophisticated mechanisms of photosynthesis by nature, including the water splitting reaction that still is not fully understood [153], was a milestone in the history of life on earth. Nature gave us a remarkable way to fix carbon into complex molecules at an efficiency that is unchallenged by human engineering. This is still the great advantage of the underlying biochemistry and biotechnologists should use this potential to develop technologies that can solve a part of our man-made problems. Instead of trying to just convert them to biofuels and burn this valuable fixed carbon, we should consider capitalizing on their inherent properties: Wood, for instance, is still used as a construction material after thousands of years as nature evolved it to have excellent mechanical properties. Instead of using cellulose wastes for second-generation biofuels, some efforts are being made to turn them into textiles for the fast growing demand of clothes in the world [154].

In the same fashion, biotechnologists should use the strengths of their production hosts. *E. coli* for example is a bacterium found in the intestinal tract of animals. It might therefore not be the most suitable organism to be used in outdoor cultivations or harsh industrial conditions. Its fast growth, the many methods for genetic modification and the deep knowledge about its metabolism, however, make it an ideal strain for the rapid production of a desired protein product at a small or prototype scale. *P. putida*, in contrast, is a natural soil dwelling bacterium and very resistant to chemical and oxidative stress. For that reason, it is a good candidate as a platform strain for industrial biotechnology and many future applications and processes can be envisioned with this outstanding organism. Acetogens are especially suited to efficiently produce acetyl-CoA from sugars or synthesis gas through the Wood-Ljungdahl pathway and are thus the perfect hosts for the production of ethanol, acetate or other acetyl-CoA derived products. For the autotrophic production of sugars, on the other hand, the Calvin cycle found in plants, algae and cyanobacteria seems better suited because the intermediate 3-phosphoglycerate can be directly fed into gluconeogenesis.

A very popular concept in synthetic biology is the design of minimal organisms [155] that can be used as a bacterial-based chassis [156]. Given the many life-styles and pathways that exist in nature, this is rather suboptimal: Even the basic vital functions like electron transport chains or membrane structure are adapted to specific environmental conditions and thus should not be modularly exchanged. Instead we need consequently many platform organisms and different types of chassis: Phototrophic, anaerobic, thermophilic or even halophilic, and many more depending on the application. Finally putting in Leonardo Da Vinci's words:

> *"Human subtlety [...] will never devise an invention more beautiful, more simple or more direct than does nature because in her inventions nothing is lacking, and nothing is superfluous."*
>
> – Leonardo da Vinci in: Leonardo da Vinci's Notebooks

6 Bibliography

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A Appendix

A.1 Full text versions of the key publications

A.1.1 Metabolic engineering to expand the substrate spectrum of *Pseudomonas putida* toward sucrose

Löwe H., Schmauder L., Hobmeier K., Kremling A., Pflüger-Grau K. (2017). *MicrobiologyOpen* 6(4): e00473. doi:10.1002/mbo3.473

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A short summary of article is also given in section 4.1.

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ORIGINAL RESEARCH

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Metabolic engineering to expand the substrate spectrum of *Pseudomonas putida* toward sucrose

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Abstract

Sucrose is an important disaccharide used as a substrate in many industrial applications. It is a major component of molasses, a cheap by-product of the sugar industry. Unfortunately, not all industrially relevant organisms, among them *Pseudomonas putida*, are capable of metabolizing sucrose. We chose a metabolic engineering approach to circumvent this blockage and equip *P. putida* with the activities necessary to consume sucrose. Therefore, we constructed a pair of broad-host range mini-transposons (pSST – sucrose splitting transposon), carrying either *cscA*, encoding an invertase able to split sucrose into glucose and fructose, or additionally *cscB*, encoding a sucrose permease. Introduction of *cscA* was sufficient to convey sucrose consumption and the additional presence of *cscB* had no further effect, though the sucrose permease was built and localized to the membrane. Sucrose was split extracellularly by the activity of the invertase CscA leaking out of the cell. The transposons were also used to confer sucrose consumption to *Cupriavidus necator*. Interestingly, in this strain, CscB acted as a glucose transporter, such that *C. necator* also gained the ability to grow on glucose. Thus, the pSST transposons are functional tools to extend the substrate spectrum of Gram-negative bacterial strains toward sucrose.

KEYWORDS

metabolic engineering, *Pseudomonas putida*, sucrose metabolism

1 | INTRODUCTION

Pseudomonas putida is a well-characterized Gram-negative soil bacterium endowed with many traits that make it a suitable chassis for contemporary, industrially oriented metabolic engineering. This strain is genetically fully tractable and tolerant toward solvent or oxidative stress (Kim & Park, 2014; Ramos et al., 2015). Therefore, this bacterium is a good candidate for hosting harsh redox reactions, which makes it a favorite workhorse for industrial and environmental biocatalysis. *P. putida* possesses a novel and unique metabolic architecture, the so-called EDEMP cycle, which enables the bacterium to recycle part of the carbon sources through activities of the Entner-Doudoroff, the Embden-Meyerhof-Parnas, and the pentose phosphate pathways (Nikel, Chavarría, Führer, Sauer, & de Lorenzo, 2015). This ability allows it to adjust NADPH formation, the key cofactor to combat oxidative stress. It is considered a model organism for biodegradation, as it possesses a remarkable capacity to degrade aromatic compounds, such as m-xylene or toluene (Jiménez, Miñambres, García, & Díaz, 2002) and provides a robust metabolic and biochemical environment, that facilitates recombinant biosynthesis of several valuable natural products, including rhamnolipids, terpenoids, polyketides and nonribosomal peptides (Loeschcke & Thies, 2015). These properties,

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together with a plethora of SynBio tools (Aparicio, Jensen, Nielsen, de Lorenzo, & Martínez-García, 2016; Lieder, Nikel, de Lorenzo, & Takors, 2015; Martínez-García, Aparicio, de Lorenzo, & Nikel, 2014; Martínez-García, Aparicio, Goñi-Moreno, Fraile, & de Lorenzo, 2015) and the availability of genome-wide metabolic models (Belda et al., 2016; Nogales, Palsson, & Thiele, 2008; Puchalka et al., 2008; Sohn, Kim, Park, & Lee, 2010), place *P. putida* on the list of the preferred contemporary SynBio chassis and production platforms (Nikel, Chavarría, Danchin, & de Lorenzo, 2016).

The viability of any biotechnological process depends on the overall costs to convert a certain substrate into a defined product. Even when the bioconversion yield is high, the process can be financially unfavorable, because of high or unstable feedstock costs. Given the importance of cost-to-benefit ratios in microbial biotechnology processes, there is a need for organisms that can convert the best-suited substrate into the desired product. Still, classical substrates, like starch and sucrose from crops, remain the main substrates for fermentation processes, because of high areal yields and easy accessibility for microorganisms. Sucrose is an important disaccharide used in many industrial applications as a substrate as it is a major component of molasses, a cheap by-product of the sugar industry.

Unfortunately, despite its metabolic diversity, *P. putida* is not able to metabolize sucrose, as transporters and degradation enzymes are missing (Nelson et al., 2002). However, this sugar is an interesting feedstock for industrial applications. Simulations of rhamnolipid production by *P. putida* carrying the genes necessary showed that sucrose and glycerol were superior to glucose with regard to theoretically achievable yields (Wittgens et al., 2011). In order to eliminate this blockage and to broaden the metabolic traits of *P. putida*, we took a metabolic engineering approach to introducing the ability of sucrose uptake and hydrolysis into this strain. Plasmids and plasmid-based transposons were built that carry the genes *cscA* and *cscB* from the *csc* operon of *Escherichia coli W*. The *cscA* gene encodes a sucrose invertase, which hydrolyzes sucrose-yielding glucose and fructose, and *cscB* encodes a sucrose permease, which is thought to be responsible for the transport of sucrose into the cell. The engineered plasmids and transposons were then employed to convey the sucrose using phenotype to *P. putida*. To show the potential of the engineered constructs for other Gram-negative bacteria, they were also used to metabolically engineer *Cupriavidus necator*, formerly known as *Ralstonia eutropha*. Both strains gained the ability to grow with sucrose as the sole carbon source.

2 | EXPERIMENTAL PROCEDURES

2.1 | Organisms, strains, and genetic manipulation

The bacterial strains and their sources used in this work are listed in Table S1. *P. putida* eYFP was constructed by the insertion of a constitutively expressed eYFP-gene via a mini-Tn7 transposon (Lambertsen, Sternberg, & Molin, 2004) into the genome of *P. putida* KT2440 EM178 (obtained from Víctor de Lorenzo). The oligonucleotides for the construction of plasmids can be found in Table S2. All plasmids and genetic manipulations were performed on *E. coli* DH5α or *E. coli* DH5α λ-*pir*. Restriction sites and Shine-Dalgarno sequences (when necessary) were added with the primers during the PCR. Fragments were cloned into the corresponding plasmids with the aid of suitable restriction sites. Plasmids were subsequently transferred to *P. putida* by conjugation with the helper strain *E. coli* HB101 (pRK600) and selected on M9 citrate with suitable antibiotics (de Lorenzo & Timmis, 1994). The pSEVA plasmids and pBAMD1-2 were used as backbones (Martínez-García, Calles, Arévalo-Rodríguez, & de Lorenzo, 2011; Martínez-García et al., 2015; Silva-Rocha et al., 2013) and *cscA* and *cscB* were amplified from genomic DNA of *E. coli* W (DSM 1116). For C-terminal GFP-tagging of CscB, pVLT_gfp was constructed. Therefore, the GFP-encoding gene borne by pGREEN plasmid (accession number AB124780) was amplified with PCR primers and cloned into the polylinker of pVLT31 (de Lorenzo, Eltis, Kessler, & Timmis, 1993), originating in pVLT_gfp. The *cscB* gene was amplified by PCR adding a Shine-Dalgarno sequence, restriction sites and a linker sequence (GSAGSAAGSGEF, (Waldo, Standish, Berendzen, & Terwilliger, 1999)), and cloned upstream of *gfp*, resulting in pVLT_*cscB_gfp*. As control pVLT_gfp_SD (Pflüger-Grau, Chavarría, & de Lorenzo, 2011) was used.

2.2 | Cultivation of microorganisms

In order to culture microorganisms for genetic engineering, bacteria were grown in LB medium with an appropriate antibiotic (standard concentrations: 50 mg/L Kanamycin, 200 mg/L Streptomycin, 50 mg/L Spectinomycin, 10 mg/L Gentamycin).

For cultivation studies, all microorganisms were cultivated in three steps unless stated otherwise: Initially, the cultures were cultivated in 5 ml LB medium in culture vials. The second culture step used M9 medium (Miller, 1972) with glucose, and MR medium (Lee & Lee, 1996) with fructose for *C. necator* in 100 ml shake flasks filled with 10 ml of medium. The main culture was conducted in 500 ml shake flasks, filled with 50 ml of M9 (*P. putida* or *E. coli*) or MR medium (*C. necator*), supplied with sucrose as a carbon source. The standard initial substrate concentration was 3 g/L for each sugar. If necessary, a suitable antibiotic (see above) was added. Cultivation temperatures were 30°C for *P. putida* and *C. necator* and 37°C for *E. coli*. Cultures were shaken in an orbital shaker with a rotation frequency of 220 rpm.

For experiments with a large number of conditions to be tested, a cultivation system in a microplate reader (Tecan, Austria) was used. Sterile plates (Greiner, Germany) were filled with 200 µl medium in each well and inoculated with 4.5–5.6 µl of cultures washed in cultivation medium. Optical density (OD) at 600 nm was measured every 20 min after shaking for 60 s over a period of 72 hr. Nonsterile microtiter plates (Nunc, Germany) were used to measure the OD of cultures in shake flasks in the microplate reader (Tecan, Austria) with 200 µl of sample. The growth experiments were performed in three completely independent setups (different days, different precultures originating from different colonies). Additionally, each experiment was done in triplicates, that is, inoculation of three different wells from the same preculture.

2.3 | Determination and normalization of growth rates in strains with chromosomal inserts

To determine the differences in the growth rates of the individual clones of *P. putida*::miniTn*5-cscAB*, each experiment was performed three times in triplicates in the microplate reader (Tecan, Austria) as described above. The individual growth rate of each culture of one experiment was determined by linear regression of the logarithmized optical density in the exponential growth phase. The relative growth rate μ/μ_{mean} was determined by calculating the ratio of each growth rate and the mean growth rate of the experiment. Therefore, we compared which strain performs better or worse than average. This was done separately for each experiment. The data shown is the mean and standard deviation of the normalized growth rates of the three independent experiments.

2.4 | Determination of sugar concentration via HPLC

Sucrose, glucose, and fructose concentrations during fermentation were determined by high performance liquid chromatography (HPLC). Samples were taken from shake flasks and centrifuged for 5 min at 17,000*g*. The supernatant was frozen and stored for analysis. All samples were thawed prior to measuring, filtered with 0.22 μm regenerated cellulose filter plates and injected (20 μl) into the HPLC machine (Agilent 1,100 series). A Shodex SH1011 column was used at a flow rate of 0.5 ml/min with 0.5 mmol/L H_2SO_4 as the mobile phase. Every hour, a new sample was injected.

2.5 | Confocal fluorescence microscopy

Expression of the GFP-fusion proteins was induced with 0, 0.1 or 1 mmol/L IPTG in LB medium. Cultures were grown overnight with tetracycline and washed in phosphate-buffered saline. After that, 10 μl of these suspensions were pipetted onto a microscope slide, sealed with a cover slip and directly analyzed by microscopy. For confocal microscopy, an Olympus FluoView 1,000 confocal laser scanning microscope was used with an IX81 inverted microscope stand and a UPLSAPO 60× objective. The laser wavelength was set to 488 nm for optimal excitation of GFP and the emission wave length was considered to be 510 nm. A Kalman Line estimator was applied to reduce noise. The gain of the laser was adjusted for every sample to avoid overexposure of cells that might conceal the distribution of fluorescent protein. Several pictures were taken for each condition and representative images were chosen.

2.6 | CscA activity assays

To get insight into the mechanism of sucrose cleavage, different fractions (cell extract, supernatant and up-concentrated cells) of *P. putida* cultures were tested for invertase activity. The experiment was performed in triplicates. Aliquots of *P. putida* PP_0075::*cscA* cultures were harvested by centrifugation for 10 min at 8,000*g* in the exponential growth phase (OD_{600} = 0.81, OD_{600} = 1.03, and OD_{600} = 0.62, respectively). One pellet of each culture was resuspended in 5 mmol/L $MgCl_2$ to obtain about 25-fold up-concentrated whole cell suspensions. To prepare the cell extracts, pellets of an equal amount of cells (*V* = 1.55 ml, *V* = 1.21 ml, and *V* = 2.0 ml, respectively) were resuspended in an equal volume of 5 mmol/L $MgCl_2$, sonicated for 2 min in intervals of 40 s (10 s pulse) with 20% intensity. A quantity of 200 μl of up-concentrated cells, cell extracts, and 5 mmol/L $MgCl_2$ as control were mixed with 1.8 ml of culture supernatant (the native medium) and incubated at 750 rpm in a shaking incubator at 30°C. Sucrose cleavage was followed every hour by taking samples, inactivation for 10 min at 90°C, and subsequent analysis by HPLC. Sucrose depletion was fitted by exponential regression and initial slopes were calculated to obtain the corresponding initial sucrose cleavage rates.

3 | RESULTS AND DISCUSSION

3.1 | Expression of *cscA* and *cscAB* from plasmids yield sucrose-utilizing phenotypes in *P. putida*

In order to broaden the metabolic traits of *P. putida* and thereby increase its relevance in industrial applications, we set out to construct a strain that is able to metabolize sucrose. We chose *cscA*, encoding an invertase and *cscB*, encoding a sucrose permease from *E. coli W*, as candidate genes. To test their functionality in *P. putida*, either *cscA* or both genes were cloned under the control of an inducible promoter in a plasmid of the pSEVA family (Martínez-García et al., 2015), resulting in pSEVA224-*cscA* and pSEVA224-*cscAB* (Fig. S1). These plasmids were introduced into *P. putida*, and the resulting strains were grown in minimal medium with sucrose as the sole carbon source. *P. putida* carrying either *cscA* or *cscAB* was able to grow on sucrose (Figure 1). Both strains metabolized sucrose, while fructose and glucose were transiently detectable in the culture supernatant until the sucrose was completely split. As control, the plasmid-free *P. putida* was grown with a mixture of equal parts of glucose and fructose and on sucrose. As expected, it grew well in the presence of the monosaccharides (Figure 1), but showed no growth in the presence of sucrose. Glucose and fructose were metabolized at the same time although glucose uptake was slightly faster than fructose uptake.

Interestingly, *P. putida* carrying either the *cscA* or the *cscAB*-expressing plasmid did not show any obvious qualitative difference in time courses of sucrose hydrolysis nor in kinetic parameter (Table 1). Growth rate and biomass yield from sucrose were comparable in both cases. The growth rate could not be increased by induction of *cscA* or *cscAB* expression with IPTG. As was the case without IPTG, the sucrose splitting rate was still higher than the uptake of glucose and fructose (data not shown). This indicates that for sucrose consumption by *P. putida*, the expression of *cscB* is dispensable or at least not advantageous. To test this, we performed competition experiments by mixing an equal number of cells of cultures of *P. putida* (pSEVA224-*cscA*) with cultures of *P. putida* (pSEVA224-*cscAB*). At the beginning of

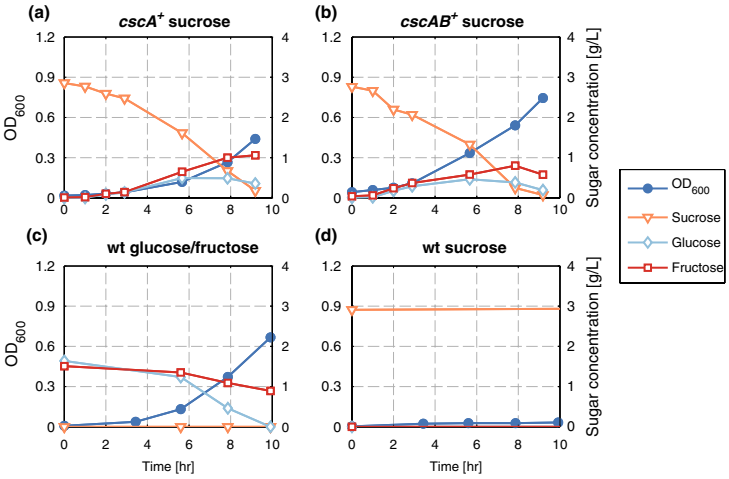

FIGURE 1 Growth and sugar consumption/production of different *P. putida* strains grown in M9 minimal medium in shake flasks. OD (blue circles), sucrose (orange triangles), glucose (light blue diamonds), and fructose (red squares) were measured in each experiment. (a) *P. putida* (pSEVA224-*cscA*) with 3 g/L sucrose. (b) *P. putida* (pSEVA224-*cscAB*) with 3 g/L sucrose (c) *P. putida* eYFP with 1.5 g/L glucose and fructose each (d) *P. putida* eYFP with 3 g/L sucrose

TABLE 1 Kinetic growth parameters: growth rate μ, biomass yield from substrate $Y_{X/S}$, for different *P. putida* strains

Genotype	WT	pSEVA224-*cscA*	pSEVA224-*cscAB*	PP_0075::*cscA*	PP_3398::*cscAB*
Medium[a]	1.36 g/L Glc + 1.5 g/L Fru	3 g/L Sucrose + 15 mg/L Km	3 g/L Sucrose + 15 mg/L Km	3 g/L Sucrose	3 g/L Sucrose
μ [h^{-1}]	0.45 ± 0.02[e]	0.36 ± 0.04[b]	0.38 ± 0.01[b]	0.27 ± 0.05[b]	0.27 ± 0.01[e]
Yxs[d] [g/g]	0.172 ± 0.009[b]	0.253	0.269	0.210 ± 0.005[b]	0.218 ± 0.003[e]
lag phase [h][c]	not determined	4.2 ± 1.4	3.4 ± 0.3	not detectable	not detectable

Glc, glucose; Fru, fructose; Km, kanamycin.
[a]M9 was used as standard medium, only substrates and additives are noted here.
[b]Standard deviations calculated from three biological replicates.
[c]Duration of the lag phases were estimated from the intercept of the exponential growth curve with the initial OD_{600}, for genomic integrated strains the initial OD was a lot higher which led to no observable lag phase.
[d]During exponential growth phase.
[e]Standard deviations calculated from two biological replicates.

the experiment and after each transfer, which corresponds to approximately 6.6 generations, we determined the ratio between both plasmids to check whether one was advantageous for growth of the cells. If this were the case, we would expect that clones carrying this plasmid overgrow the others, which would result in a shift of the pSEVA224-*cscA*/pSEVA224-*cscAB* ratio toward the advantageous one, or even in complete loss of the less advantageous one. However, we could not observe any trend toward one of the plasmids in the ratio of the plasmid abundance in these competition cultures (Fig. S3). This suggests that CscB production is dispensable for sucrose consumption of the engineered *P. putida*.

But how can the sucrose-metabolizing phenotype be obtained? There are mainly two plausible explanations: either CscA is acting outside of the cell, or *P. putida* carries porins and transporters that unspecifically facilitate sucrose transport. In the literature, we could not find any indications that *P. putida* possesses transporters that import sucrose. This, of course, does not exclude the presence of a transport protein that aside from its natural sugar is able to transport sucrose as well, maybe to a lower extent. The transient accumulation of glucose and fructose in the culture supernatant independent of the presence of CscB (Figure 1) suggests that sucrose is split outside of the cell. This phenomenon has already been reported for *E. coli* (Kim, Kim, Lee, & Lee, 2013) and was attributed to periplasmic release of CscA into the surrounding medium. Another source for the transient accumulation of glucose and fructose could be an overflow metabolism produced by the rapid intake and hydrolysis of sucrose, especially as the functions are plasmid-encoded and the proteins should be produced in high amounts due to copy number and induction. One way or the other, the presence of glucose and fructose in the medium could also explain why the additional presence of CscB did not bring any detectable advantage for sucrose-mediated growth of *P. putida*, as the organism preferably takes up glucose and fructose if present in the medium.

3.2 | Construction of a broad-host range shuttle vector and genomic integration of *cscA* and *cscAB* into *P. putida*

As a next step, we introduced *cscA* and *cscAB* into the chromosome of *P. putida*. This was done on one hand to reduce the metabolic burden of plasmid replication and eliminate the need for antibiotics. On

the other hand, after genomic integration, *cscA* and *cscB* are present in a single copy, which should reduce the probability for a potential overflow of certain metabolites due to high amounts of the heterologous proteins. For genomic integration, we constructed a broad-host range shuttle vector, based on the pBAMD1-2 mini-Tn*5* transposon vector (Martínez-García et al., 2011), containing either *cscA* alone or the *cscAB* cassette (Fig. S1). Both cassettes were placed under transcriptional control of the P_{trc} promoter devoid of the LacIq repressor binding site, in order to obtain constitutive transcription. With the resulting plasmids pSST1 (sucrose splitting transposon 1 with *cscA*) and pSST2 (sucrose splitting transposon 2 with *cscAB*), we successfully integrated the genes randomly into the chromosome of *P. putida*. To check whether the integration site, that is, disruption of the gene where the cassette has inserted, has an influence on sucrose consumption, we chose 22 transformants from the genomic integration of the Tn*5*-*cscAB* cassette of pSST2 for a more detailed analysis. Ten of them showed reproducible growth in M9 minimal medium with sucrose. These were compared in parallel growth experiments in a microplate reader to determine the growth rate of each construct (Figure 2). The experiment was performed three times, each in triplicates. We observed that the absolute values for the growth rates varied between different experiments, but the relative differences were conserved. Therefore, growth rates were normalized by the mean growth rate of all single clones in each round of experiments in order to compare the results from all experiments (Figure 2). Clone #17 grew slightly faster in each round of experiments compared to the other clones, which is reflected by the highest relative growth rate (Figure 2).

Next, we mapped the integration site of the Tn*5*-*cscAB* cassette. As expected, the mini-Tn*5* integrated randomly along the chromosome. We could not draw any conclusion regarding the general fitness of the different clones from the gene annotations of the integration sites (Table 2, Fig. S2). Therefore, we decided to use clone #17 for all further studies, in which the gene cassette has inserted into PP_3398, annotated as Curlin-associated repeat protein. We also screened various clones with the integrated Tn*5*-*cscA* cassette. As with the Tn5-*cscAB* cassette, no remarkable difference was observed (data not shown) and

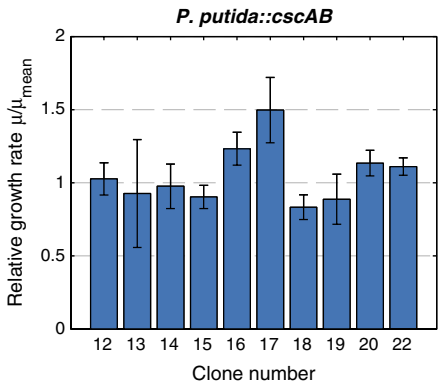

FIGURE 2 Relative growth rates of different *P. putida* eYFP strains with genomic integration of a polycistronic *cscAB* construct via pSST2. Cells were grown in M9 medium with 3 g/L sucrose in a microplate reader and OD_{600} was measured every 20 min. Growth rates were determined in the log-linear parts of the growth curves and normalized to the respective mean growth rate of each experiment. Standard deviations were calculated from these normalized growth rates of three independent experiments

we chose the clone with the highest growth rate for further analysis, in which the cassette had inserted into PP_0075, annotated as sulfate transporter.

Next, both strains, *P. putida* PP_0075::*cscA* and *P. putida* PP_3398::*cscAB*, were analyzed in more detail during growth on sucrose. Both strains showed almost equal growth kinetics with almost identical growth rates and very similar biomass yields from sucrose (Table 1). In both cultures, sucrose was hydrolyzed and glucose and fructose accumulated transiently (Figure 3). The strains with the chromosomal insertion of *cscA* or *cscAB* had about 30% reduced growth rates compared to the plasmid-based systems. The reduced growth rates are a consequence of the lower sucrose hydrolysis, which is reflected by a slower decrease in sucrose compared to the plasmid-bearing cultures. This probably has its origin in different expression

TABLE 2 Integration of pSST2 in *P. putida* eYFP and mapping of insertion sites

Clone	Gene(s)	Genetic Locus/Loci
12	Hypothetical protein/ISPpu13, transposase Orf3	PP_3983/PP_3984
13	Acriflavine resistance protein/transcriptional regulator, GntR family	PP_2065/PP_2066
14	Alcohol dehydrogenase, zinc-containing	PP_1720
15	Nitrate-binding protein NasS, putative/dihydroorotate dehydrogenase	PP_2094/PP_2095
16	DNA-binding response regulator GltR/sensor histidine kinase	PP_1012/PP_1013
17	Curli fiber major subunit CsgA	PP_3398
18	Sensory box histidine kinase/response regulator; transcriptional regulator, GntR family	PP_3544/PP_3545
19	ATP-dependent Clp protease, ATP-binding subunit ClpA	PP_4008
20	S50 ribosomal protein L19/long-chain acyl-CoA thioester hydrolase family protein	PP_1465/PP_1466
22	Polyamine ABC transporter substrate-binding protein	PP_2195

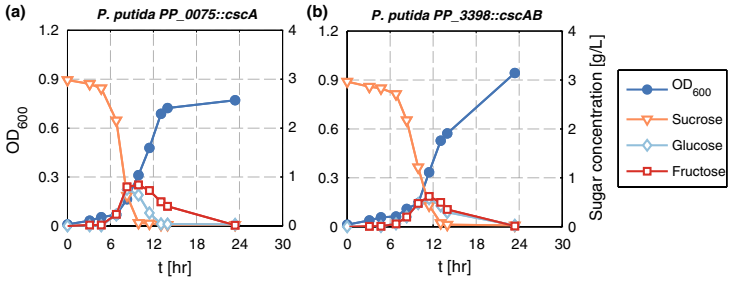

FIGURE 3 Growth and sucrose consumption by (a) *P. putida* PP_0075::*cscA* and (b) *P. putida* PP_3398::*cscAB*. Cells were grown in M9 minimal medium in shake flasks with 3 g/L sucrose. OD (blue circles), sucrose (orange triangles), glucose (light blue diamonds), and fructose (red squares) were measured in each experiment. Shown are representative curves of three independent experiments

levels of the genes due to different promoters and gene copy numbers. As with the plasmid-based strains, a transient accumulation of glucose and fructose was observed. Both sugars started to be detectable in the culture medium when the cells started to grow, and accumulated to concentrations of 0.5 to 1 g/L. With increasing cell number, glucose and fructose disappeared from the medium, as they were taken up and metabolized. These data hint toward the extracellular splitting of sucrose by a fraction of CscA leaking out of the cells and make the accumulation of glucose and fructose as a result of overflow metabolism unlikely.

Still, as with the plasmid-based strains, no difference could be observed between the strain expressing only the invertase and the one additionally producing the sucrose permease. Thus, even at a lower sucrose splitting rate, no significant effect of the presence of *cscB* in the genome could be observed. This raised the question of whether the sucrose permease is functionally produced at all in *P. putida*.

3.3 | CscB is produced and localizes to the cell membrane

As the presence of *cscB* encoding the sucrose permease did not lead to any detectable effect when *P. putida* cells were grown on sucrose, we aimed to show that it is indeed expressed and to compare its production and localization in *P. putida* to the one in *E. coli* where it was shown to be functional (Sahin-Tóth, Frillingos, Lengeler, & Kaback, 1995).

Therefore, we expressed it as an N-terminal fusion to GFP from an inducible plasmid in *P. putida* and in *E. coli*. As control, GFP alone was produced from the same plasmid in both strains. The cellular location of the fluorophore was examined by confocal microscopy. As expected, in the controls, the fluorescent signal is evenly distributed in the cytoplasm (Figure 4a, c). In *P. putida* as well as in *E. coli* expressing a GFP-*cscB* fusion protein, another distribution of the fluorescent signal is observed (Figure 4b, d). In both organisms, a uniformly fluorescing ring surrounding the cell can be seen, which hints toward a localization of the CscB protein to the membrane of both organisms. Additionally, foci with higher fluorescence occur, which might well be inclusion bodies or unspecific aggregates (Landgraf, Okumus, Chien, & Baker, 2012). Thus, we can assume that the CscB protein itself is produced and behaves in a similar fashion as in *E. coli*. However, these data do not explain why the production of the sucrose permease has no effect on growth of *P. putida* on sucrose.

3.4 | CscB acts as glucose transporter in *C. necator*

To show the broad-host range potential of the constructed transposon and the ability to transfer sucrose metabolism to bacteria other than *P. putida*, we chose *C. necator* as the target. *C. necator* is already used industrially in the production of poly-3-hydroxybutyrate (PHB) (Chen, 2009), but the selected strain, *C. necator* H16, is not naturally able to metabolize glucose, sucrose or any other sugar except for fructose and N-acetylglucosamine (Pohlmann et al., 2006; Sichwart, Hetzler, Bröker, & Steinbüchel, 2011). The vectors pSST1 and pSST2 were transferred from *E. coli* to *C. necator* via triparental mating and the transposon-carrying cells were identified by antibiotic selection (Km^R). Both strains, *C. necator*::miniTn*5-cscA* as well as *C. necator*::miniTn*5-cscAB*, were able to grow on sucrose as the sole carbon source, whereas the wild type was not (Figure 5). Thus, the pSST plasmids could be used successfully to bring about the ability to split sucrose not only to *P. putida* but also to *C. necator*. Whether sucrose is extracellularly cleaved by leaking invertase and cells are growing on fructose or whether sucrose is transported into the cell by the activity of CscB cannot be deduced from the data. However, we could show that CscB acts as glucose transporter in this strain. In the growth experiments, the *C. necator* strains were also cultivated with fructose as positive control and glucose as negative control. Surprisingly, *C. necator*::mini-Tn*5-cscAB* was able to metabolize glucose (Figure 5c), whereas *C. necator*::mini-Tn*5-cscA* was not (Figure 5b). The organism is equipped with everything needed for growth on glucose except for transport activity. When a suitable glucose transporter is heterologously expressed, growth is possible (Sichwart et al., 2011). To the best of our knowledge, it has not been shown before that CscB can act as a glucose transporter – in fact (Sugihara, Smirnova, Kasho, & Kaback, 2011) stated that CscB catalyzes the transport of sucrose, fructose and lactulose, but shows no recognition of glycopyranosides, in particular glucose, as this sugar was not able to inhibit sucrose transport in *E. coli*. From our experiments, transport activities other than glucose can neither be confirmed nor excluded. Nevertheless, the functionality of the sucrose permease CscB in *C. necator* shows that, in principle, CscB is transcribed and translated into a functional protein – at least in *C. necator*.

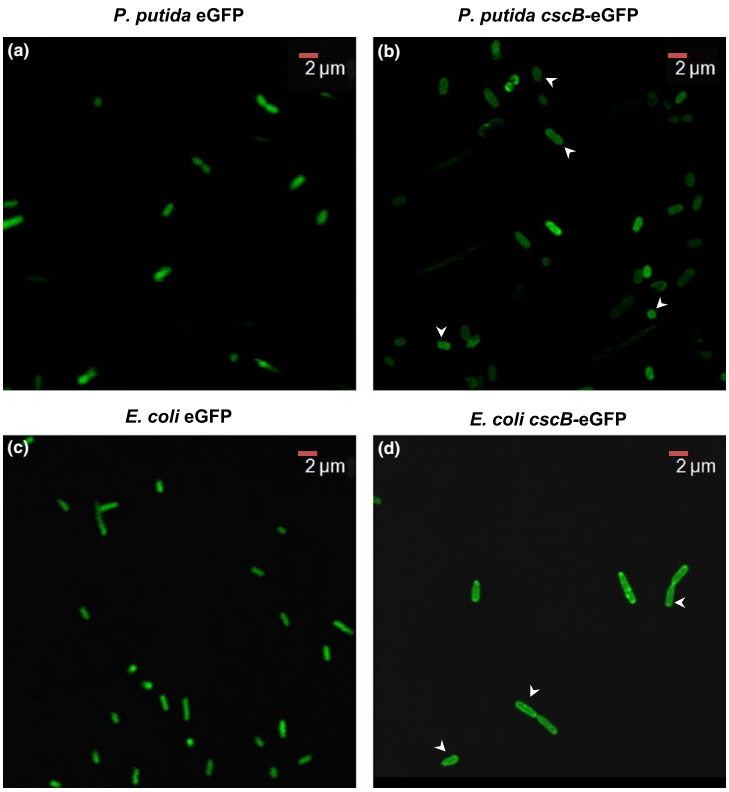

FIGURE 4 CscB production and localization in *P. putida* and *E. coli*. Confocal fluorescence microscopy of (a) *P. putida* (pVLT_gfp), (b) *P. putida* (pVLT_*cscB*-GFP), (c) *E. coli* (pVLT_*gfp*), and (d) *E. coli* (pVLT_*cscB*-GFP). Pictures were taken with an Olympus FluoView 1000. Arrows point to the cells where the membrane localization rings are most clear

However, this does not explain the missing activity for sucrose transport of CscB in *P. putida*.

3.5 | CscA activity is mainly intracellular

A possible scenario for explaining the similar phenotype of strains producing just the invertase CscA or additionally the permease CscB, is a high extracellular sucrose splitting rate of CscA. In this case, sucrose would be hydrolyzed fast enough outside of the cell to make CscB superfluous. This implies that CscA should either be associated with the membrane or occur extracellularly. However, at least in its native host *E. coli* W, it is a cytoplasmic protein, cleaving sucrose intracellularly (Sabri, Nielsen, & Vickers, 2012). To test this scenario and to localize the main activity of CscA, we determined the sucrose cleavage rates of *P. putida* PP_0075::*cscA* and *P. putida* PP_3398::*cscAB* in the culture, the culture supernatant, and in cell extracts (Figure 6). In either

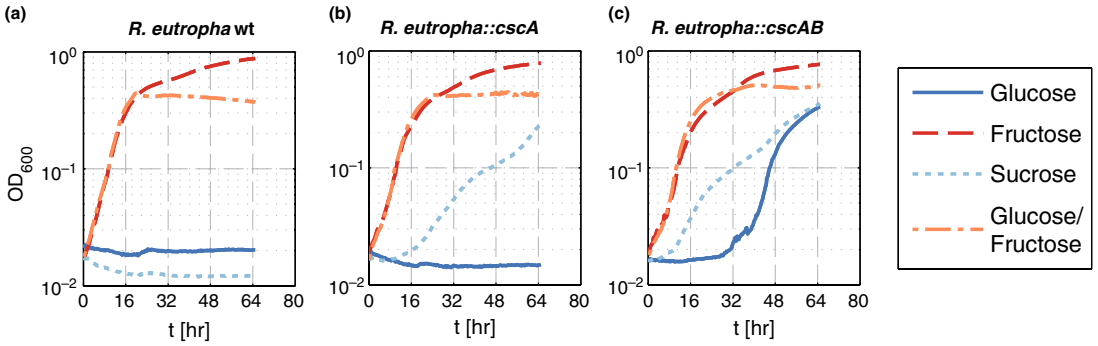

FIGURE 5 Growth of *C. necator* wt (a), *C. necator*::miniTn*5-cscA* (b), and *C. necator*::miniTn*5-cscAB* (c) with either fructose (red dashed line), sucrose (light blue dotted line), or glucose (solid dark blue line), or glucose/fructose (orange dotted and dashed line) as the single carbon source. Note that the presence of CscA is sufficient to allow growth on sucrose, whereas the expression of *cscB* additionally allows growth on glucose

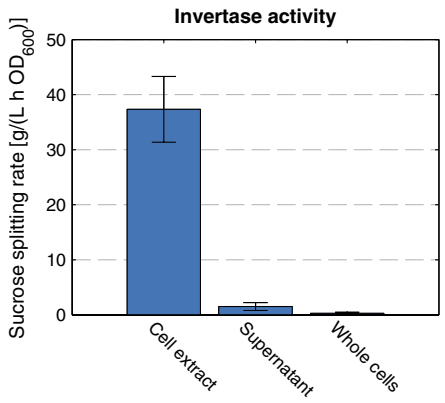

FIGURE 6 CscA activity of *P. putida* PP_0075::*cscA*. Sucrose cleavage rates were determined from the cell extracts, the culture supernatants, and the whole cells of *P. putida* PP_0075::*cscA*. The mean activity of the supernatants is shown in relation to the optical density at the time of harvest, mean activity of the whole cells and cell extracts in relation to the optical density of the cells used for the assay. The mean values and the corresponding standard deviations of three independent experiments are depicted

strain, the vast majority of activity (>90%) could be found intracellularly and only a small fraction was detected in the culture supernatant. Another, even smaller fraction seems to be associated with the cell, as cleavage rates were marginally higher in the culture compared to the supernatant. We assume that the extracellular activity is a result of nonspecific leaking of CscA out of the cell or of freed CscA after cell lysis. Interestingly, this small fraction of CscA seems to be sufficient for the sucrose-metabolizing phenotype of *P. putida*. This is a good starting point for further optimization of the sucrose-metabolizing *P. putida* strains, which is currently in progress in our laboratory.

4 | CONCLUSION

With the pSST transposon-mediated integration of the *csc* genes conferring sucrose metabolism, the first step toward a sucrose-consuming *P. putida* was undertaken. This opens a new field of potential applications for this otherwise remarkably versatile organism by adding sucrose to the repertoire of substrates of *P. putida*. Further attempts will be aimed at increasing the growth rate either by introducing a functional sucrose transporter into the membrane for efficient sucrose uptake and thus exploitation of the full potential of the intracellular the invertase activity, or by exporting CscA out of the cell for extracellular cleavage. We expect to reach growth rates equal to those observed with glucose and fructose in either case as the cleavage rate of CscA is not the liming step, but the access to sucrose is. The pSST transposons can be used to transfer the sucrose-consuming phenotype also to other Gram-negative organisms as well, which was demonstrated here with *C. necator*. However, the functionality of the CscB permease remains somewhat ambiguous: although in *P. putida*, no effect could be assigned to the presence of the protein, in *C. necator* there is a strong indication that it does not only transport sucrose but also glucose across the membrane.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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A Appendix

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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A.1.2 Photoautotrophic production of polyhydroxyalkanoates in a synthetic mixed culture of *Synechococcus elongatus* cscB and *Pseudomonas putida cscAB*

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A short summary of article is also given in section 4.2.

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Biotechnology for Biofuels

RESEARCH Open Access

Photoautotrophic production of polyhydroxyalkanoates in a synthetic mixed culture of *Synechococcus elongatus cscB* and *Pseudomonas putida cscAB*

Hannes Löwe, Karina Hobmeier, Manuel Moos, Andreas Kremling and Katharina Pflüger-Grau*

Abstract

Background: One of the major challenges for the present and future generations is to find suitable substitutes for the fossil resources we rely on today. Cyanobacterial carbohydrates have been discussed as an emerging renewable feedstock in industrial biotechnology for the production of fuels and chemicals, showing promising production rates when compared to crop-based feedstock. However, intrinsic capacities of cyanobacteria to produce biotechnological compounds are limited and yields are low.

Results: Here, we present an approach to circumvent these problems by employing a synthetic bacterial co-culture for the carbon-neutral production of polyhydroxyalkanoates (PHAs) from CO_2. The co-culture consists of two *bio-modules*: *Bio-module I*, in which the cyanobacterial strain *Synechococcus elongatus cscB* fixes CO_2, converts it to sucrose, and exports it into the culture supernatant; and *bio-module II*, where this sugar serves as C-source for *Pseudomonas putida cscAB* and is converted to PHAs that are accumulated in the cytoplasm. By applying a nitrogen-limited process, we achieved a maximal PHA production rate of 23.8 mg/(L day) and a maximal titer of 156 mg/L. We will discuss the present shortcomings of the process and show the potential for future improvement.

Conclusions: These results demonstrate the feasibility of mixed cultures of *S. elongatus cscB* and *P. putida cscAB* for PHA production, making room for the cornucopia of possible products that are described for *P. putida*. The construction of more efficient sucrose-utilizing *P. putida* phenotypes and the optimization of process conditions will increase yields and productivities and eventually close the gap in the contemporary process. In the long term, the co-culture may serve as a platform process, in which *P. putida* is used as a chassis for the implementation of synthetic metabolic pathways for biotechnological production of value-added products.

Keywords: Carbon neutral bioplastics, Polyhydroxyalkanoates (PHA), Synthetic co-culture, *Pseudomonas putida cscAB*, *Synechococcus elongatus cscB*, Cyanobacteria, CO_2 fixation

Background

For a long time, natural polymers like wood or wool have been used by humans to craft weapons and tools or to protect against the cold. This enhanced our ability to survive and allowed us to build cultures and to live in places that are hostile to our biology. From the nineteenth century, with the invention of modern polymer chemistry, many of these natural materials were complemented and/or replaced by modern plastics [1]. Plastics found application in all areas of our daily life. In 2015, the global plastics material production was estimated to reach 250 million tons per year [2] and most of the plastics produced were derived from petroleum. Because of the inevitable finiteness of fossil resources and the massive pollution caused by plastic wastes [3], contemporary research in this field is directed towards the exploration

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of renewable and biologically degradable sources of plastics. Advanced natural polymers like polyhydroxyalkanoates (PHA) that show thermoplastic, polypropylene-like properties, could be a valuable substitute for some applications. Under natural conditions, PHA, a linear polymer of 3-hydroxy fatty acids, serves as both energy and carbon storage in certain bacteria, among them *Pseudomonas putida*. In industrial production of PHA, substrate price is a key factor [4] as bio-based plastics have to compete with those made from fossil resources. Therefore, efforts are directed towards alternative feedstocks to reduce substrate costs in the overall process.

A newly discussed, potentially cheap source of substrates are carbohydrates produced by microalgae and cyanobacteria, for example, starch production by eukaryotic algae or sucrose production by recombinant cyanobacteria [5–8]. Compared to conventional crops, microalgae and cyanobacteria have the potential to reach higher areal yields and, additionally, their products do not interfere with the food markets. Further benefits include the ability to use salty or brackish water and bioreactors can be placed on non-arable land. The genetically engineered cyanobacterial strain *Synechococcus elongatus cscB* has recently been shown to export sucrose on a level comparable to sugar cane [6]. This was achieved by the introduction of only one heterologous gene encoding the sucrose permease CscB from *Escherichia coli* ATCC 700927. Under salt stress, *S. elongatus cscB* accumulates remarkable amounts of sucrose as a compatible solute, which are released into the surrounding medium by the activity of the heterologous permease CscB [6]. However, when these sugars are produced on a large scale, limitations will arise: These include the risk of contamination, as a carbon source is provided that can be used by heterotrophs, and economic aspects, e.g., the cost of sugar recovery from the fermentation broth [9, 10]. A recent approach to circumvent these problems is to convert the cyanobacterial feedstock directly into value-added products in a multispecies microbial factory in a so-called "one-pot" reaction [11]. By co-inoculation of both strains, the sugar-producing strain together with the product accumulating strain, several barriers can be overcome. Thus, the costs for sugar recovery from the cyanobacterial fermentation broth as well as the potential loss due to contamination are saved. This contributes to making the overall process economically more competitive. Furthermore, there are cases in which a positive effect of synthetic consortia on the productivity have been described [12, 13]. Along that line, two studies have been published recently that aimed to produce polyhydroxybutyrate (PHB) in a mixed culture of *S. elongatus cscB* with *Escherichia coli* or *Azotobacter vinelandii*, respectively [9, 14]. Even though the final titers reached were quite low (around 1 mg/L), which might be a result of slow growth rates and suboptimal media composition, this shows the feasibility of the general approach and leaves room for improvement.

In this work, we aimed to produce biodegradable plastic from light and CO_2, tackling two of the major challenges of modern times: global warming and pollution by plastics. Strategies to combat these problems are the fixation of CO_2 to avoid its emission into the atmosphere and to find economically competitive processes for the production of plastic substitutes like PHA. Here, we present an approach in which the sucrose production of *S. elongatus cscB* is directly coupled to PHA accumulation by *P. putida cscAB* in a synthetic co-culture. Thus, CO_2 and sunlight are converted into carbon neutral bioplastics (Fig. 1). *P. putida* is known for its innate stress resistance and robustness and is therefore an excellent candidate as a mixed culture partner. The strain *P. putida cscAB* is genetically modified to be able to metabolize sucrose [15, 16], and is the strain of choice in this co-culture. We present the steps undertaken towards a functional mixed culture and the first improvements made to considerably increase the PHA content of the cells (to about 150 mg/L at the end).

Results and discussion

Sucrose production of *S. elongatus cscB* in BG-11$^+$ medium

As *S. elongatus* accumulates sucrose as a compatible solute in response to the external salt concentration, we set out to identify the optimal NaCl concentration that would allow maximal sucrose excretion without severely inhibiting growth. Recently, it was shown that in normal BG-11 medium, *S. elongatus cscB* showed the highest sucrose production in the presence of 150 mM NaCl [6]. However, as the medium in this study was modified to meet the needs of both co-culture partners (see "Methods") and the cultivation conditions were different, the influence of NaCl on sucrose production and growth had to be assessed again in the newly defined medium in a 1.8 L photobioreactor at controlled pH. Therefore, *S. elongatus cscB* was cultivated in BG-11$^+$ medium in the absence or presence of NaCl in concentrations ranging from 150 to 250 mM. Growth of the cells and excretion of sucrose were monitored by measuring the optical density and determining the concentrations of sucrose in the supernatants by HPLC (Fig. 2). The growth of *S. elongatus cscB* is clearly reduced by an increase in the external salt concentration, ranging from a biomass production rate of 0.236 g CDW/L d without NaCl to 0.059 g CDW/L d in the presence of 250 mM NaCl during light-limited, linear growth (Table 1). The highest sucrose productivity, however, was observed with 150 mM NaCl, which is in accordance with what was reported by Ducat

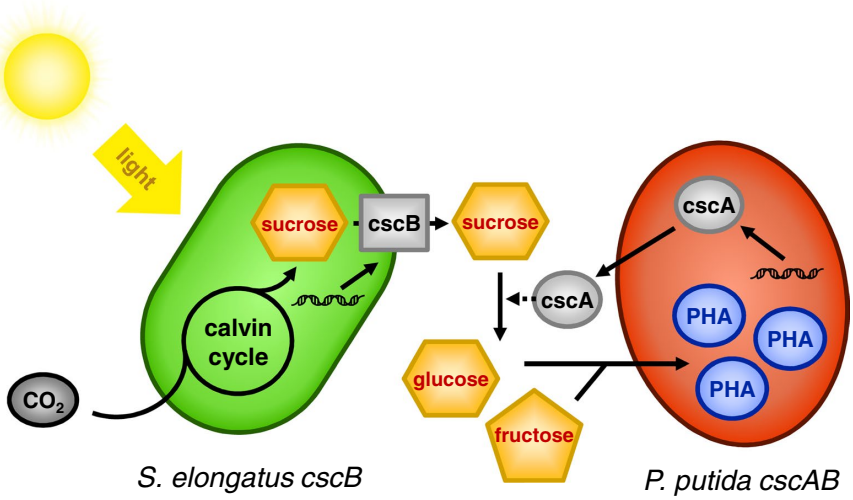

Fig. 1 Concept of the synthetic co-culture of *S. elongatus cscB* and *P. putida cscAB* for the production of PHA from CO_2 and light. CO_2 is fixed via the Calvin cycle to make sucrose, which in turn is secreted into the surrounding medium by the activity of the heterologous sucrose permease CscB. CscA produced by *P. putida cscAB* leaks out of the cell, where it splits sucrose extracellularly [16]. The monomers (glucose and fructose) are metabolized by *P. putida cscAB*, and polyhydroxyalkanoates (PHA) are accumulated in the cytoplasm [15]

et al. for normal BG-11 medium [6]. We reached a production rate of 0.346 ± 0.014 g/(L day) and a maximal titer of 2.63 g/L after 12 days (Table 1). By comparing the amount of sucrose produced under salt stress to the biomass production without NaCl, the carbon flux in the cyanobacteria is mirrored: At 150 mM NaCl, the produced mass of sucrose per unit time is higher than the amount of biomass produced without NaCl, indicating that a large fraction of the primary production is directed towards the synthesis of the compatible solute sucrose.

Growth of *P. putida cscAB* in modified BG-11 media

Next, we had to confirm that the metabolically engineered *P. putida cscAB* is able to grow in the modified BG-11 medium in the presence of 150 mM NaCl, the optimal salt concentration for sucrose production by *S. elongatus cscB*. Additionally, the influence of the changes in the medium composition had to be examined by comparing both BG-11 derived media. Therefore, *P. putida cscAB* was grown in BG-11[–$NaCO_3$, $CaCl_2$/100] and BG-11^+, in the presence of 150 mM NaCl and a mixture of glucose and fructose (each 1.5 g/L) as the carbon source (Fig. 3). We chose the monomers of sucrose as the carbon source to assay solely the effect of the medium composition on the growth of *P. putida cscAB* and not the influence of the efficiency of sucrose splitting. The strain grew well in both media with a growth rate of 0.239 ± 0.001/h for BG-11[–$NaCO_3$, $CaCl_2$/100] and 0.305 ± 0.011/h for BG-11^+. The slight increase in the growth rate in BG-11^+ can most likely be attributed to the higher nutrient availability as the concentrations of potassium phosphate and magnesium sulfate were increased tenfold in this medium. This suggests that one or both of these nutrients are limiting factors in the original medium. The growth rate obtained in BG-11^+ was in the same range as the one determined in M9 medium [16], which is the standard minimal medium for *P. putida*. Thus, only minor adjustments in medium composition were necessary to achieve comparable growth of *P. putida cscAB* in BG-11 as well. This reflects the broad metabolic versatility and robustness of this bacterium. Other bacteria and eukaryotic culture partners seem to be less suited for co-cultivation in a photosynthetic consortium as they have higher nutritional demands. Recently, Hays et al. reported co-cultivation of three heterotrophs with *S. elongatus cscB*. However, in their process, the medium was also supplemented with ammonium salts and buffer [9]. The same seems to be true for oleaginous yeast as described by Li et al. [17]. As outlined above, supplementation of the medium with additives was not necessary with *P. putida cscAB* as co-cultivation partner. This underlines the versatility and suitability of *P. putida* as a chassis for industrial biotechnology [18].

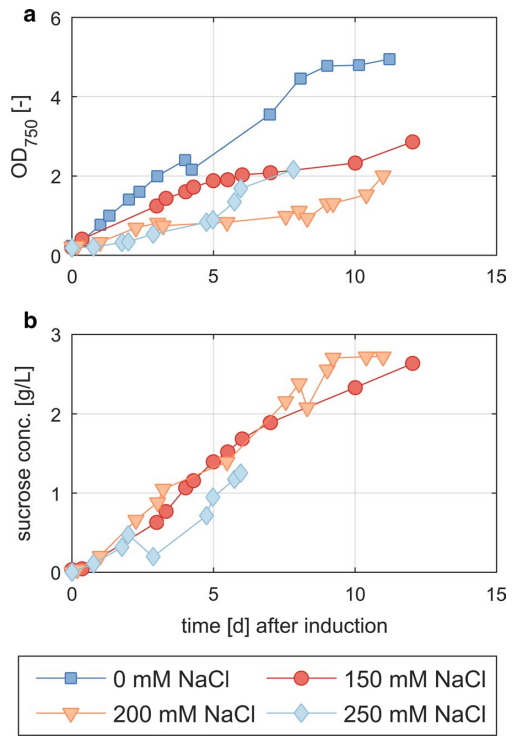

Fig. 2 Growth and sucrose concentration from fermentations of *S. elongatus cscB* in a photobioreactor in BG-11$^+$ in the presence of different salt concentrations. Cells were grown in BG11$^+$ medium with the NaCl concentration indicated, and growth and sucrose secretion were monitored. CscB production was induced with 0.1 mM IPTG at an OD of 0.1–0.2

Mixed culture of *S. elongatus cscB* and *P. putida cscAB*

Having two strains with complementary functions, one producing sucrose from CO_2, the other consuming sucrose, and a common medium allowing the growth of both, we set out to grow them simultaneously in the same cultivation vessel. Each strain represents a functional bio-module, which is linked to the other by sucrose transfer. To start the synthetic mixed culture, first *S. elongatus cscB* was inoculated in a photobioreactor in BG-11$^+$ in the presence of 150 mM NaCl to promote sucrose accumulation right from the beginning. The heterotrophic organism was inoculated at least 1 day after induction of sucrose export with 0.1 mM IPTG to ensure that sucrose was readily available for *P. putida cscAB*. The total OD of the culture was monitored, i.e., the sum of *S. elongatus cscB* and *P. putida cscAB* cells, as well as the concentration of sucrose in the culture supernatant (Fig. 4). To have an approximation of the contribution of each strain to the overall OD, the colony forming units (CFUs) of each strain, and the cell counts of Nile red-stained *P. putida cscAB* were determined. Nile red predominantly stains lipophilic residues like PHAs [19] therefore, the number of cells reported represents only the proportion of *P. putida cscAB* cells that accumulated PHA in their cytoplasm. The overall OD of the mixed culture increased over a period of 7 days reaching a plateau of about OD = 2.3 at the end of the process (Fig. 4a). The growth behavior of *S. elongatus cscB* was similar to the process in pure culture at 150 mM NaCl (compare Figs. 2, 4a), hence there seems to be no severe negative effect from the presence of the commensal *P. putida cscAB*. Recently, co-cultivation of *S. elongatus cscB* with other organisms even showed a positive effect on the growth of the cyanobacterium [9]. Sucrose accumulated steadily up to 1.5 g/L in the culture supernatant until day 6. This is when *P. putida cscAB* reached a critical mass, and when sucrose started to be metabolized more rapidly than it was built. As the cell counts of *P. putida cscAB* increased, sucrose concentrations decreased, but no extracellular glucose or fructose accumulation was detected. Transient accumulation of the sugar monomers was observed in earlier studies, when *P. putida cscAB* was grown in M9 medium with sucrose as the sole carbon source [16]. It was attributed to the extracellular cleavage of sucrose by invertase CscA that was leaking out of the cells. Therefore, sucrose splitting seems to be the limiting factor for the growth of *P. putida cscAB*, as no sugar monomers were accumulated, and as with sucrose as C-source the growth rate was markedly reduced compared to a mixture of glucose and fructose in BG-11$^+$ (compare to Fig. 3). Along the same line, pure cultures of *P. putida cscAB* in BG-11$^+$ medium with sucrose as the sole carbon source showed inconsistent and very slow growth (data not shown). Thus, when sucrose is used as carbon source, the medium composition clearly has an influence on the growth of *P. putida cscAB*. However, the controlled process parameter in the photobioreactor and maybe the presence of *S. elongatus cscB* seem to have a stabilizing effect on *P. putida cscAB*, so that reliable growth in the co-culture was achieved.

Table 1 Maximal linear growth and sucrose production rates of *S. elongatus cscB* at different NaCl concentrations in BG-11$^+$ medium

NaCl (mM)	Biomass production rate[a] [g_{CDW}/(L day)]	Sucrose production rate[a] [g/(L day)]
0	0.236 ± 0.00400	n.d.
150	0.134 ± 0.004	0.346 ± 0.014
200	0.116 ± 0.008	0.282 ± 0.012
250	0.059 ± 0.005	0.20 ± 0.03

[a] Standard deviations are regression errors, not derived from replicates

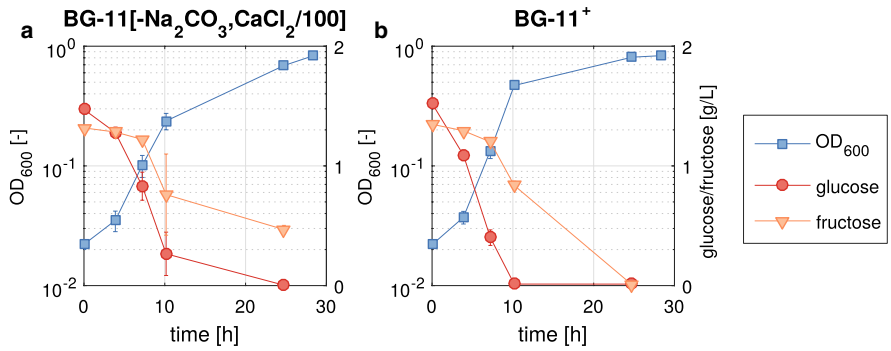

Fig. 3 Growth of *P. putida cscAB* in modified cyanobacterial BG-11 medium supplemented with glucose, fructose (each 1.5 g/L), and 150 mM NaCl. Experiments were conducted in 250 mL unbaffled shake flasks with an effective volume of 25 mL. Temperature was set to 30 °C, and agitation rate to 220 rpm. Shown are the means and standard deviations calculated from three biological replicates

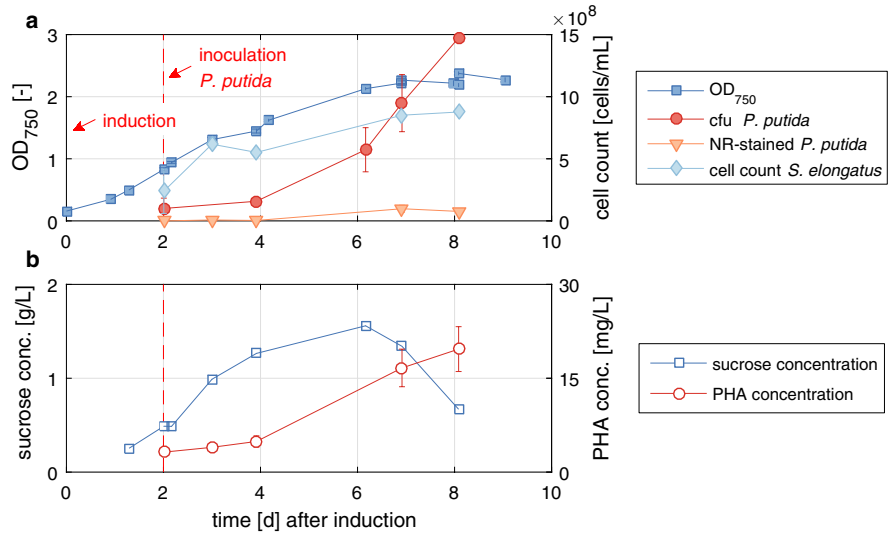

Fig. 4 Co-cultivation of *S. elongatus cscB* and *P. putida cscAB* in the photobioreactor. Optical density, Nile red-stained cell count of *P. putida cscAB*, cell count of *S. elongates cscB* and colony forming units (CFU) determined from plating are depicted over time in **a**. *Arrows* indicate the induction of sucrose export by the addition of 0.1 mM IPTG and inoculation of *P. putida cscAB*. Sucrose and PHA concentrations are plotted in **b**. Uncertainties in PHA concentrations were estimated from the propagation of errors of the PHA standards

Nevertheless, we assume that the growth of *P. putida cscAB* can be enhanced when sucrose is metabolized more efficiently. The sugar was detectable in the supernatant during the whole co-cultivation period, thus it was not consumed completely. This shortcoming will be tackled by the development of new sucrose splitting variants of *P. putida* with a higher splitting rate, which can be obtained for instance by active secretion of the invertase or screening for other sucrose permeases.

We also analyzed the PHA content of the cells, as *P. putida* accumulates these polymers to a small extent even under non-standard PHA accumulating conditions [20, 21]. *P. putida cscAB* produced PHA in the mixed culture at an approximate maximal production rate of 3.3 mg/(L day) and a maximal titer of 19.7 mg/L was reached 6 days after inoculation with *P. putida cscAB* (Fig. 4b). It can be excluded that *S. elongatus cscB* is responsible for PHA formation since it lacks the corresponding genes for

PHA synthesis [22]. Moreover, the distribution pattern of 3-hydroxyalkanoic acids is typical for *P. putida* (Table 2), with 3-hydroxydecanoic acid being the most abundant monomer [23]. Apparently, PHA was accumulated in only a small fraction of the cells, which can be deduced when comparing the CFU/mL to the cell counts of Nile red-stained cells. On day 8, when the highest PHA titer was detected, only a very small fraction of the *P. putida cscAB* cells were stained with Nile red, i.e., accumulated PHA in their cytoplasm. To provide absolute numbers, further experiments, including counting cells with PHA granules under a microscope and comparing the numbers to counts gained by flow cytometry, are necessary to determine the efficiency of the staining method with Nile red. The flow cytograms, however, do provide qualitative information about the specific fluorescence and thus the PHA content of the stained cells, clearly showing the presence of PHA (see Additional file 1: Figure S2).

This way, the general process parameters for co-cultivation of *S. elongatus cscB* and *P. putida cscAB* for the production of PHAs from CO_2 and light were set and the feasibility of the process was confirmed. Without adding any other carbon source than CO_2 to the process, PHAs were produced by *P. putida*, however, yield and production rate were low.

Polyhydroxyalkanoate production in the co-culture under nitrogen-limiting conditions

One way to improve the PHA accumulation is to alter the C/N ratio of the growth conditions. Although not strictly necessary for PHA accumulation, nitrogen limitation in general increases the PHA content of the cells [24]. Therefore, we conducted the mixed culture experiment under nitrogen-limiting conditions. An initial batch phase was performed in which *S. elongatus cscB* was grown with a starting nitrate concentration of 48.0 mg/L and a nitrate feed of 9.2 ± 0.4 mg/day. Four days after induction of sucrose export with 0.1 mM IPTG and upon inoculation of the co-culture partner *P. putida cscAB*, the HNO_3-feed was increased to a rate of 46 ± 2 mg/day (NO_3^-). The nitrogen concentration was chosen so that it was just below the threshold that allows unlimited growth of *S. elongatus cscB*. Ideally, this should keep the steady-state concentration of nitrogen low enough to promote PHA formation by *P. putida cscAB*.

Table 2 Distribution of chain-lengths per mass fraction in PHA produced by *P. putida cscAB* in the mixed culture at maximal PHA concentration during the process

Chain-length (carbon number)	6	8	10	12	12:1
Mass fraction (%)	4.2	25.2	58.4	4.4	7.8

The results of this cultivation are illustrated in Fig. 5. After an initial period of 4 days, nitrate was no longer detectable in the photobioreactor, and is therefore, considered completely consumed by *S. elongatus cscB*. This was the time point of induction of *cscB* expression and is defined as day 0. The optical density started to rise, i.e., *S. elongatus cscB* started to grow, and on day 4, *P. putida cscAB* was inoculated. As expected, *S. elongatus cscB* grew linearly (dOD/dt = 0.105 ± 0.005/day during days 4–8, Fig. 5), although at a lower rate than under unlimited conditions (dOD/dt = 0.38 ± 0.03/day during days 1–4, Fig. 4). Sucrose was secreted constantly, reaching a maximal production rate of 0.316 g/(L day) and a maximal titer of 2.8 g/L 10 days after induction. The production rate reached was only marginally lower than the one obtained under non-limiting conditions (0.346 g/(L day), compared in Table 1). *P. putida cscAB* was apparently very stressed, which was manifested in non-reliable growth on LB agar plates when determining the CFUs (data not shown). Therefore, we could not use the cell count data from plating as an approximation of cells present in the co-culture. This behavior may be a result of the cumulative stress of a poor nitrogen source, nutrient limitation, low carbon availability because of low sucrose splitting rate, and possibly salt stress due to the presence of 150 mM NaCl in the medium. However, counting the Nile red-stained cells of *P. putida cscAB* by flow cytometry gave a qualitative estimate, and cell counts showed a steady course over time (Fig. 5a), which correlated nicely with the PHA concentration measured (Fig. 5b). The actual number of *P. putida cscAB* cells might be higher, assuming that only a fraction of the culture accumulates the polymer or are stained by the staining method. PHA accumulating cells as well as the PHA concentration increased until day 16, and then both decreased again. One possible explanation for the decrease might be cessation of sucrose metabolism and the consumption of intracellular reserves of PHAs, since it is possible that the actual number of *P. putida cscAB* cells continued to increase. PHA production reached a maximal production rate of 23.8 ± 6 mg/(L day) and a maximal titer of 156 ± 40 mg/L after 16 days. Thus, by applying nitrate limitation, we could increase the production rate about 7.3-fold.

To place these numbers in context: The achieved PHA level in the nitrogen-limited process exceeded the values reported for the mixed cultures of *S. elongatus cscB* with *E. coli* or *A. vinelandii* about a 150-fold [9, 14]. There are also efforts to produce PHB directly with recombinant cyanobacteria [25, 26], in which a high-cell dry weight fraction of PHB is already reached. However, there is no information about productivity available, and hence it cannot be determined how those strains compare to the process presented in this work.

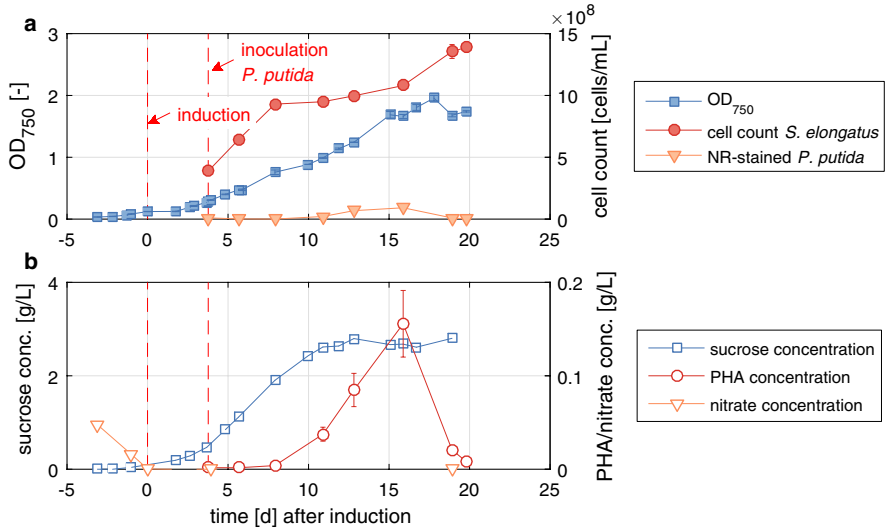

Fig. 5 Nitrate-limited co-cultivation of *S. elongatus cscB* and *P. putida cscAB*. Optical density, Nile red-stained cell count of *P. putida cscAB* and cell count of *S. elongatus cscB* are depicted over time in **a**. Sucrose, PHA and nitrate concentrations are plotted in **b**. Uncertainties in PHA concentrations were estimated from the propagation of errors of the PHA standards. Nitrate was fed at a constant rate of 46 ± 2 mg/day after inoculation with *P. putida cscAB*

A general challenge when working with phototrophic organisms is to reach the high-cell densities needed for efficient downstream processing. One problem is self-shading, which in the approach presented here could be solved by a compartmentalization of *S. elongatus cscB* and *P. putida cscAB*. In the process presented here, a major fraction of sucrose was left untouched by *P. putida cscAB*, which leaves room for the production of even more PHA. This might be facilitated by the construction of *P. putida* strains that split sucrose more efficiently. Furthermore, PHA production by *P. putida* can also be increased by metabolic engineering to direct more carbon flux to PHA formation [27] and thus reach higher PHA weight fractions of the cell dry weight, or by increasing the fraction of the population that actually accumulates PHAs. These attempts are currently under investigation in our laboratory.

Conclusion

In the work presented here, a mixed culture of *S. elongatus cscB* and *P. putida cscAB* was established in a lab-scale photobioreactor for the production of PHAs, a green plastic substitute. The concept of coupling a photosynthetic organism to a heterotrophic bacterium with the aim to produce an industrially relevant compound was successful and makes room for the implementation of a wide repertoire of bioproducts that can be produced by *P. putida* [18, 28]. Only slight adjustments in the cyanobacterial growth medium composition were necessary to promote growth and PHA production by *P. putida cscAB*, showing the universality and robustness of this organism. When evaluating the productivity and yields of the co-culture, it has to be kept in mind that neither the process nor the organisms were optimized yet, which leaves great potential for optimization: One possible target is to increase the sucrose production by *S. elongatus cscB* through metabolic engineering, optimizing light influx or the use of higher photon flux densities. Indeed, under natural conditions at well-suited sites, photon flux densities are higher than the ones used in this work [29], which could result in an increased photosynthetic carbon fixation rate. Other potential targets include the improvement of sucrose consumption by *P. putida cscAB* or the more efficient channeling of the carbon flux towards PHA formation to achieve a higher weight fraction of PHA relative to the cellular dry weight. Here, we reached a maximal concentration of ~150 mg/L PHA, which is quite low, compared to pure cultures. However, this was achieved with a small number of cells compared to the cell densities reached in pure cultures, which are normally cultivated in fed-batch, high-cell density processes [23]. Nevertheless, it is crucial for efficient downstream processing to obtain a high concentration of PHA. One way to accomplish

higher cell densities in coupled cultures is to separate the autotrophic and heterotrophic processes into different compartments [30] or to extend the process duration. In a possible future scenario, sunlight will be collected by a large area of photobioreactors and product formation by *P. putida* will be mainly limited by the sucrose production of *S. elongatus cscB*. For efficient downstream processing, *P. putida* could be either upconcentrated, e.g., by cultivation in a membrane bioreactor or hydrogel as suggested by Smith and Francis [14], or it could be auto-aggregated as described for other bacteria [31] and collected by decanting. Consequently, titers and production rates here cannot, and maybe do not have to, be compared to those of contemporary studies on PHA production in pure cultures. Future experiments with set-ups closer to a possible application scenario will elucidate the feasibility of this technology. In the future, the concept of the modular co-culture might also serve as a platform process with *S. elongatus cscB* fixing CO_2 and converting it to sucrose and *P. putida cscAB* serving as chassis for the implementation of synthetic pathways. This way, the product spectrum could be amplified tremendously.

In the long run, the applicability of commodities produced by microalgae will stand or fall depending on many factors: the development of cheaper and more efficient photobioreactors, the long-term dominance of cultures versus contaminations, the genetic streamlining of organisms, energy costs, the development of power-to-chemical processes and the price of oil, just to name a few. Whichever renewable technology will be feasible in the future, all research efforts in this area are important, as one fact is evident: availability of fossil resources will come to an end.

Methods

Bacterial strains and batch cultivation

Two organisms were used in the mixed culture: The autotrophic host, *S. elongatus cscB,* was kindly provided by Pamela Silver [6]. The heterotrophic commensal was *P. putida* EM178 *att*::miniTn7(eYFP) PP_3398::*cscAB*, a derivative of the prophage-free KT2440 strain EM178 [16]. For reasons of simplicity the strain will be called *P. putida cscAB.* Pre-cultures of *S. elongatus cscB* were grown in 100 mL shaking flasks with 40 mL of BG-11[–$NaCO_3$, $CaCl_2$/100] medium at 100 rpm, at 30 °C and a photon flux density of approximately 10–26 µmol/(m^2 s). A 30 W tubular fluorescent lamp was used for lighting, and air was the only source of CO_2. Experiments with *P. putida* alone were conducted in 250 mL flasks with 25 mL of BG-11[–$NaCO_3$, $CaCl_2$/100] or BG-11$^+$ at 30 °C under shaking. Pre-cultures for *P. putida* experiments were grown in LB-medium overnight and washed once in the cultivation medium of the main culture prior to inoculation.

Media

As a first step towards a functional co-culture between *S. elongatus cscB* and *P. putida cscAB*, a common growth medium for both organisms had to be defined, using original BG-11 (ATCC Medium 616) medium for blue-green algae [32] as starting point. The pH was shifted towards neutral pH, by omitting sodium carbonate, and $CaCl_2$ was reduced 100-fold to 3.4 µM, as it interfered with the growth of *P. putida* (see Additional file 1: Figure S1). This medium is referred to as BG-11[–$NaCO_3$, $CaCl_2$/100]. For its preparation four stock solutions were made, filter sterilized and stored at −20 °C in appropriate aliquots: solution 1 [100×]: 150 g/L $NaNO_3$, 3 g/L K_2HPO_4; solution 2 [1000×]: 75 g/L $MgSO_4{\cdot}7H_2O$, 5 g/L citric acid, 6 g/L iron–ammonium citrate; 1.1 g/L disodium ethylen ediaminetetraacetate·$2H_2O$; solution 3 [1000×]: 0.36 g/L $CaCl_2$; trace element solution A5 [1000×]: 2.86 g/L H_3BO_3, 1.81 g/L $MnCl_2{\cdot}4H_2O$, 0.222 g/L $ZnSO_4{\cdot}7H_2O$, 0.39 g/L $NaMoO_4{\cdot}2H_2O$, 0.079 g/L $CuSO_4{\cdot}5H_2O$, 49.4 mg/L $Co(NO_3)_2{\cdot}6H_2O$. The medium was prepared by adding each stock solution to autoclaved deionized water to reach 1× concentration (10 mL/L of solution 1, and 1 mL/L of solutions 2–4, respectively).

Additionally, based on this, a second medium was designed by increasing the phosphate and sulfate concentrations tenfold to exclude limitations that might occur when adding an additional microbe to the culture.

For this purpose, the components K_2HPO4 and $MgSO_4{\cdot}7H_2O$ were increased 10 times. They were added as separate, sterile stock solutions (300 g/L K_2HPO_4, 246.5 g/L $MgSO_4{\cdot}7H_2O$). This enriched medium is designated herein BG-11$^+$.

Cultivation in a photobioreactor

Liter-scale cultivations of *S. elongatus cscB* and mixed cultures were performed in a Labfors 5 Lux flat panel airlift photobioreactor (Infors AG, Switzerland) at a photon flux density of approximately 240 µmol/(m^2 s). The pH was controlled at 7.5 with 1 mol/L HNO_3 and an airflow of 2 L/min, enriched with 2% CO_2, was used as a carbon supply for autotrophic growth. The reactor was filled with 1.8 L of water containing the desired NaCl concentration, and autoclaved, and the medium ingredients were added sterilely through a septum from stock solutions. Twenty mL of a stationary *S. elongatus cscB* culture were used as inoculum and cells were grown until an optical density (OD_{750}) of 0.1–0.2 (equals a CDW of about 0.04–0.08 g/L) was reached; then 0.1 mmol/L IPTG was added to induce expression of *cscB* and thus sucrose export. For mixed culture cultivation, an over-night culture of *P.*

putida cscAB grown in BG-11$^+$ medium supplemented with 3 g/L glucose H_2O was then washed once with 5 mL reactor medium and added to the reactor.

In the case of the nitrate-limited process, the initial nitrate concentration was reduced to 50 mg/L NO_3^-. Upon inoculation with *S. elongatus cscB* a constant nitrate feed of 9.2 ± 0.5 mg/day was implemented by pumping a 0.03 mol/L HNO_3 solution into the reactor vessel. After inoculation with *P. putida cscAB,* the nitrate feed was increased to 46 ± 2 mg/day.

Samples were taken to measure the optical density, and culture supernatants were frozen and stored for subsequent HPLC analysis. At least every third day cells were diluted and the composition of mixed cultures was assessed by flow cytometry. The pellets of 5 mL of every sample were frozen at −80 °C for GC analysis. Growth of heterotrophic populations was additionally followed via plating in suitable dilutions on LB-medium agar plates.

Optical density, cell counting and determination of cell dry weight

The optical density of 200 µL culture was measured with an Infinity® microplate reader (Tecan, Austria) at wavelengths of 600 (*P. putida cscAB* cultivation) and 750 nm (*S. elongatus cscB* and mixed cultures). Sucrose, glucose, and fructose concentrations were determined by high performance liquid chromatography (HPLC) using an Agilent machine (Agilent 1100 series). Sugars were separated via a Shodex SH1011 column and a mobile phase of 0.5 mmol/L H_2SO_4 at a flow rate of 0.5 mL/min and a temperature of 30 °C. For flow cytometry, cultures were diluted to reach a cell count of about 800–1400 at a flow rate of 1–5 µL/s and injected into a CyFlow® instrument (Sysmex Partec GmbH, Germany) equipped with a laser (488 nm excitation wavelength). Fluorescence was measured at 536, 590, and 630 nm emission wavelengths. One mL of PHA-containing cells was centrifuged, stained with 10 µL Nile red solution (1 g/L in DMSO) and incubated for 5 min prior to dilution and followed by PHA-mediated Nile red fluorescence at 590 nm emission wavelength.

For cell dry weight measurement, an appropriate volume of cells was centrifuged at 8000×*g* for 10 min. The pellet was resuspended in phosphate buffered saline (PBS) and centrifuged again in a pre-dried and weighted 1.5 mL centrifuge tube. The supernatant was carefully discarded as completely as possible and the tube was dried at 60 °C for at least 3 days until the weight remained constant. The weight difference represented the dry weight. A correlation between optical density and cell dry weight was made for both organisms (data not shown) and cell dry weights of the other experiments were estimated from this correlation.

PHA determination

PHA content and composition were determined by gas chromatography (GC). A modified version of the propanylation protocols by Riis and Mai [33] and Furrer et al. [34] was applied. Samples were centrifuged at 17,000×*g* for 5 min and pellets were frozen and stored at −80 °C. To remove residual water, samples were freeze-dried for at least 3 days. The samples were dissolved in 2 mL of chloroform in an Ace® overpressure glass tube, and subsequently 1 mg of Poly-3-hydroxybutanoate (Sigma-Aldrich) and 0.2 mg of 3-methylbenzoic acid (Sigma-Aldrich) were added as internal standards. Two mL of an 80% (v/v) solution of 1-propanol and 37% HCl were pipetted into the mixture and the tube was sealed tightly. The bottom third of the tube was placed in an oil bath at 80 °C and mixed using a magnetic stirrer. After 16–24 h the tube was cooled to room temperature and 4 mL of bidistilled water were added. After shaking the tube vigorously, the tube was left at room temperature until the phases separated. The upper, aqueous phase was removed carefully, and the remaining liquid was dried with Na_2SO_4 and neutralized with Na_2CO_3. The remaining organic layer was transferred to a GC vial and injected into the GC machine (injection volume 1 µL). The samples were separated with a fused silica Stabilwax® column (Restek AG, Fuldabrueck, Germany) and measured with a flame ionization detector (detector temperature 245 °C). A temperature of 240 °C was set for the split/splitless injector (split ratio 1:10). Hydrogen gas was used as carrier gas at a flow rate of 3 mL/min. The different 3-hydroxyalkanoic acid esters were separated by applying a temperature gradient, starting at 80 °C (1 min), which then increased 5 °C every minute, stopping at 240 °C (hold time 5 min).

3-hydroxydecanoic acid (Sigma-Aldrich) and PHB were used as external standards. The response factors of the remaining 3-hydroxyalkanoic and 3-hydroxyalkenoic acids were inter- or extrapolated linearly from the two standards, according to Tan et al. [35].

Measurement of nitrate concentration

The nitrate/nitrite concentration of culture supernatants was determined using a colorimetric assay (Nitrite/Nitrate colorimetric method, Roche Diagnostics GmbH, Penzberg, Germany) in a microplate reader based on the enzymatic reduction of nitrate to nitrite. Nitrite reacts with a combination of dyes to form a diazo dye that can be measured at a wavelength of 540 nm and correlates linearly with the original nitrate/nitrite concentration.

Additional file

Additional file 1: Figure S1. Reduction of the $CaCl_2$ concentration promotes growth of *P. putida* EM178 in BG-11 [–$NaCO_3$] medium. Growth of *P. putida* EM178 with different concentrations of $CaCl_2$. Experiments were performed in 100 mL, unbaffled shake flasks filled with 10 mL of medium at 30 °C and an agitation rate of 220 rpm. Note that at 3.4 µM $CaCl_2$, the concentration chosen for $BG11^+$, no limitation in growth of *P. putida* EM178 is observed. **Figure S2.** Exemplary flow cytogram of Nile red-stained cells during nitrate-limited mixed culture of *P. putida cscAB* and *S. elongatus cscB* at the maximal concentration of PHA. The cells marked in the red circle only appeared upon staining with Nile red and are not found in the unstained control (data not shown). Cells below are unstained cells of both strains and undefined background.

Abbreviations
PHA: polyhydroxyalkanoate; PHB: polyhydroxybuturate; CFU: colony forming units; GC: gas chromatopraphy; PBS: phosphate buffered saline; HPLC: high performance liquid chromatography; CDW: cellular dry weight; DMSO: dimethyl sulfoxide.

Authors' contributions
HL, AK, and KPG designed the experiments. HL, KH, and MM performed the experiments. HL and KPG wrote the manuscript. AK, KH, and MM carefully proofread the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
All data generated during this study are included in this published article and in Additional file 1.

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A Appendix

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A.1.3 Engineering sucrose metabolism in *Pseudomonas putida* highlights the importance of porins

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A short summary of article is also given in section 4.3.

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Engineering sucrose metabolism in *Pseudomonas putida* highlights the importance of porins

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Summary

Using agricultural wastes as a substrate for biotechnological processes is of great interest in industrial biotechnology. A prerequisite for using these wastes is the ability of the industrially relevant microorganisms to metabolize the sugars present therein. Therefore, many metabolic engineering approaches are directed towards widening the substrate spectrum of the workhorses of industrial biotechnology like *Escherichia coli*, yeast or *Pseudomonas putida*. For instance, neither xylose or arabinose from cellulosic residues, nor sucrose, the main sugar in waste molasses, can be metabolized by most *E. coli* and *P. putida* wild types. We evaluated a new, so far uncharacterized gene cluster for sucrose metabolism from *Pseudomonas protegens* Pf-5 and showed that it enables *P. putida* to grow on sucrose as the sole carbon and energy source. Even when integrated into the genome of *P. putida*, the resulting strain grew on sucrose at rates similar to the rate of the wild type on glucose – making it the fastest growing, plasmid-free *P. putida* strain known so far using sucrose as substrate. Next, we elucidated the role of the porin, an orthologue of the sucrose porin ScrY, in the gene cluster and found that in *P. putida*, a porin is needed for sucrose transport across the outer membrane. Consequently, native porins were not sufficient to allow unlimited growth on sucrose. Therefore, we concluded that the outer membrane can be a considerable barrier for substrate transport, depending on strain, genotype and culture conditions, all of which should be taken into account in metabolic engineering approaches. We additionally showed the potential of the engineered *P. putida* strains by growing them on molasses with efficiencies twice as high as obtained with the wild-type *P. putida.* This can be seen as a further step towards the production of low-value chemicals and biofuels with *P. putida* from alternative and more affordable substrates in the future.

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Introduction

Second-generation biofuels have received much attention in recent years. Using waste biomass instead of sugar from edible crops, it has been possible to uncouple biofuel production from food production. Therefore, the development of an affordable process using agricultural waste material is generally perceived to be one of the 'holy grails' of industrial biotechnology (Sparks and Payne, 2010; Money, 2018). Consequently, it is of great interest to make these carbon sources available to production strains like *Saccharomyces cereivisiae*, *Escherichia coli* or *Pseudomonas putida* in order to maximize the overall yield.

Pseudomonas putida is an emerging chassis for industrial biotechnology and a promising host for the production of biofuels and chemicals due to its intrinsic robustness to various sources of stress and its solvent resistance (Ramos *et al*., 2015). Recently, *P. putida* was successfully employed to use aromatic, lignin-derived compounds (Olivera *et al*., 1998; García *et al*., 1999). Metabolic engineering approaches to expand the substrate spectrum of this organism have already unlocked the hemicellulose monomers D-xylose and L-arabinose (Meijnen *et al*., 2008; Dvorak and de Lorenzo, 2018), D-cellobiose (Dvorak and de Lorenzo, 2018) as well as sucrose (Löwe *et al*., 2017b), the main sugar of molasses from sugarcane and beet, to be used as a carbon source. To confer sucrose metabolism to *P. putida,* genetic constructs were designed based on the *csc* operon from *E. coli* W. *P. putida* EM178 showed reasonable growth on sucrose, when the genes were expressed from a plasmid (Löwe *et al*., 2017b). The *csc* gene cluster in *E. coli* W consists of four genes: *cscA* encoding an invertase (CscA), *cscB* coding for a sucrose/H^+ symporter (permease CscB), *cscR* encoding a regulator protein (CscR) and *cscK* coding for a fructokinase (CscK). It was shown that *cscA* and *cscB* were sufficient for efficient utilization of sucrose in *E. coli*

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(Sabri *et al*., 2013). However, when these two genes were integrated into the *P. putida* genome via a mini-Tn5 transposon, growth on sucrose was slower ($\mu = 0.27\ h^{-1}$) compared with growth on a glucose/fructose mixture ($\mu = 0.45\ h^{-1}$). This effect was attributed to poor transport across the membrane of *P. putida* (Löwe *et al*., 2017b). We hypothesized that this might be the result of a lack of sucrose diffusion through the outer membrane or incompatibility of the *E. coli* sucrose permease CscB with *P. putida*. In order to circumvent this bottleneck and to confer efficient sucrose metabolism to *P. putida*, we set out to explore genes from donors closer to *P. putida* than *E. coli*.

For any metabolic engineering strategy aiming to make a new substrate available to Gram-negative bacteria, one has to consider three barriers that the new substrates have to pass: (i) crossing the outer membrane, (ii) uptake into the cytoplasm and (iii) entry into the metabolism. Most studies focus on the latter two points because transport across the outer membrane is rarely regarded as a problem in model organisms like *E. coli*. However, the outer membrane of *Pseudomonads* is structured differently: Instead of relying on constitutively expressed generalistic porins like *E. coli*'s OmpF and OmpC, *P. putida* and *P. aeruginosa* have a more specialized set of porins and the outer membrane is generally less permeable (Yoshimura and Nikaido, 1982; Nakae *et al*., 1989; Saravolac *et al*., 1991).

In this study, we describe a new approach to tackle all three barriers mentioned above at once. This is achieved by integrating the genes of a so far unannotated operon from *Pseudomonas protegens* Pf-5 containing a sucrose hydrolase, permease and a sucrose-specific porin into *P. putida*. We subsequently evaluated the growth behaviour of the resulting strains with sucrose as substrate, thereby paving the way to using sugarcane molasses as a cheap carbon source. In the conclusion, we discuss some general aspects of strategies for metabolic engineering in *Pseudomonads*, taking the outer membrane into account.

Results and Discussion

Identification and cloning of an unannotated gene cluster from P. protegens P*f-5*

The most intensively studied operons for sucrose metabolism in Gram-negative bacteria are the *scr* genes in the pUR400 plasmid from *Salmonella* (Ebner *et al*., 1988) and the *csc* genes from the enteric bacterium *E. coli* W. The gene clusters are depicted in Fig. 1A. Comparing both sucrose uptake systems, two major differences are observed: First, the ScrAY uptake system transports sucrose via the phosphotransferase system (PTS), thereby phosphorylating the sugar during uptake. In contrast, the transport system of the *csc* genes is driven by the sucrose/H^+ symporter CscB that facilitates gradient-driven sucrose uptake. Second, the *scr* gene cluster contains a gene encoding the sucrose-specific porin ScrY while a homologue is missing in the *csc* genes of *E. coli* (Fig. 1A).

A gene cluster was identified in *P. protegens* Pf-5 comprising the genes PFL_3236 to PFL_3239 via BLAST homology search (Altschul *et al*., 1990) using *cscB* as query. The genes are annotated as a LacI-like repressor (PFL_3236), a sucrose hydrolase (PFL_3237), a sucrose or galactoside permease (PFL_3238, with homology to *cscB*), and a sucrose porin (PFL_3239, with homology to *ScrY*). Interestingly, this newly identified gene cluster of *P. protegens* Pf-5 carries features of both known gene clusters because a gene encoding a non-PTS permease resembling the CscB protein of *E. coli* W, as well as a gene coding for a porin resembling ScrY, is present. Due to the high similarity between PFL_3236 and *cscB*, we hypothesized that the corresponding proteins carry out the same function and that the whole operon is responsible for sucrose uptake and hydrolysis. Apart from *P. protegens* Pf-5, there are a few other *Pseudomonas* strains that carry orthologues of the *cscB* gene encoding the proton-gradient-driven sucrose transporter in their genomes. For instance, *Pseudomonas fluorescence* strains AU13852 and AU20219 posses a gene cluster with the same genetic organization as found in *P. protegens* Pf-5 (Fig. S3). As an analogy to the *csc* operon of *E. coli* W, we will denominate the genes of the *P. protegens* Pf-5 cluster in the rest of the article as *cscR* (repressor, PFL_3236), *cscA* (sucrose hydrolase, PFL_3237), *cscB* (permease, PFL_3238) and *cscY* (porin, PFL_3239) making the whole cluster *cscRABY* (Fig. 1A).

To test the above mentioned hypothesis that the *cscRABY* operon is responsible for sucrose uptake and hydrolysis and to elucidate in more detail the function of the porin CscY of the *P. protegens* Pf-5 gene cluster, the whole fragment (~6 kB) was cloned into pSEVA221 without the addition of any promoter sequence, as the native promoters are predicted to be in the middle of the fragment (Fig. 1B). This should leave the regulation as in its native host, thereby avoiding the need to express the operon in the presence of sucrose and preventing the associated metabolic stress when other substrates are used. In scenarios where only sucrose is to be used, removal of the regulation or use of tailored promoters should be considered.

Additionally, the same fragment without the last gene encoding the porin CscY was likewise inserted into pSEVA221 (Fig. 1B; see Experimental Procedures for details). For complementation experiments, an expression vector (based on pSEVA434) containing only the

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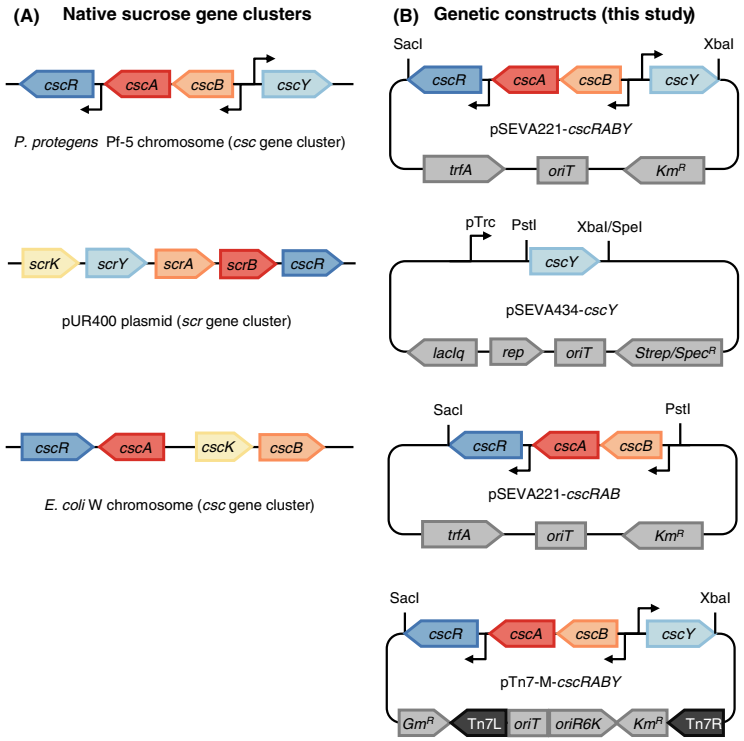

Fig. 1. Organization of well-characterized, native sucrose operons (panel A) and genetic constructs created in this study (panel B). The arrangement of the *csc* gene cluster of *P. protegens* Pf-5 was taken from pseudomonas.com (Winsor *et al.*, 2016), the pUR400 plasmid from (Ebner *et al.*, 1988) and the *E. coli* W chromosome from (Sabri *et al.*, 2013). A complete list of genetic constructs and their features are listed in Table S1.

gene c*scY* was created. We also constructed the pTn7-M-based plasmid carrying the whole operon to test the functionality of the *cscRABY* operon of *P. protegens* Pf-5 when present in single copy and to allow stable integration into *P. putida* without the need for selective pressure. Conjugation and integration of this mini-Tn7-based transposon into *P. putida* EM178 yielded *P. putida attTn7::cscRABY*.

The cscRABY *genes conferred the ability to metabolize sucrose, but not maltose or lactose*

First, we tested whether the new gene cluster was able to give *P. putida* the ability to grow on sucrose as the sole carbon source. As the putative sucrose permease CscB (PFL_3238) also shows homology to a galactoside permease (Accession number EFK49434.1, *E*-score 4e-71) from *E. coli* and belongs to the LacY super-family, we also tested whether the common disaccharides lactose and maltose could serve as a carbon source in the presence of pSEVA221-*cscRABY*. All three substrates cannot be metabolized by native *P. putida* EM178, albeit they might be transported by CscB. The results of cultivations in M9 medium using 3 g l^{-1} of either of the three disaccharides or citrate as positive control are illustrated in Fig. 2. The only disaccharide able to support the growth of *P. putida* was sucrose. Moreover, the high growth rate of 0.292 $\pm$ 0.016 h^{-1} indicates sucrose as the preferred substrate. In contrast, the strain bearing only the three genes *cscRAB* was not able to grow after 3 days of cultivation in M9 with sucrose (data not shown). Therefore, it was tempting to speculate that the porin CscY carried out a vital role during the consumption of sucrose by *P. putida*, which will be addressed in the following section. Citrate was used at the same (mass) concentration as sucrose, however, it showed lower yields in this experiment, which can be attributed to its higher degree of oxidation.

The permease CscB also has high similarity to a raffinose permease from *E. coli* (Accession number EGI19556.1, *E*-score 9e-81). As raffinose and sucrose are structurally related and share the same sucrose (sub)unit, we cannot exclude that the gene cluster might also be able to transport and cleave raffinose. However,

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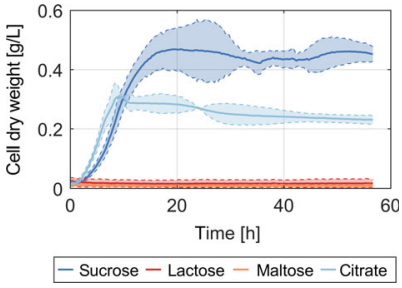

Fig. 2. Cell dry weights of *P. putida* EM178 (pSEVA221-*cscRABY*) on sucrose over time. *P. putida* EM178 (pSEVA221-*cscRABY*) was grown in the presence of 3 g l^{-1} of sucrose (dark blue), lactose (red), maltose (orange) or citrate (light blue) in a microplate reader in M9 minimal medium. The shaded areas represent the 95% confidence bands, which were estimated from three replicates.

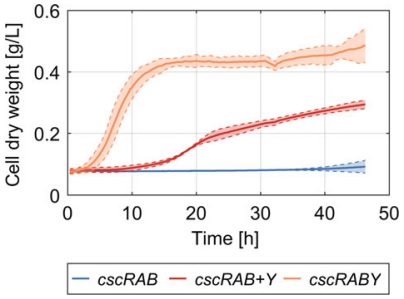

Fig. 3. Complementation studies with *P. putida* EM178. The strain without the porin (pSEVA221-*cscRAB*) was complemented with a LacI/P_{trc} promoter-controlled expression vector containing the gene encoding the porin CscY (pSEVA434-*cscY*). Growth on sucrose of the different strains was monitored in a microplate reader. The plasmid pSEVA221-*cscRABY* was used as a positive control and the empty expression vector pSEVA434 as a negative control. The mean values of nine replicates (three biological with three technical replicates each) and 95% confidence bands were estimated from all replicates.

a second hydrolase may be needed for efficient cleavage of raffinose for the D-galactopyranosyl-(1→6)-α-D-glucopyranoside moiety.

Expression of CscY complemented the loss of growth in P. putida *pSEVA221*-cscRAB

The next step was to analyse the role of the porin CscY in more detail, as we had indications from earlier studies that the transport of sucrose across the membrane might be the limiting step (Löwe *et al.*, 2017b). To test this assumption, we compared the growth of *P. putida* (pSEVA221-*cscRABY)* and *P. putida* (pSEVA221-*cscRAB*) on sucrose (Fig. 3). In fact, no growth could be observed on sucrose in the absence of CscY. To confirm that this effect was caused by the absence of the porin and was not a consequence of changes in the promoter region of *cscRAB* as a cloning artefact, *P. putida* (pSEVA221-*cscRAB*) was complemented with the expression plasmid pSEVA434-*cscY*. As can be seen in Fig. 3, the plasmid pSEVA434-*cscY* was indeed able to restore the loss of growth associated with a lack of the porin in pSEVA221-*cscRAB*, albeit at a slower rate than with the full set of genes. The experiment in Fig. 3 was performed in a microplate reader with LB as the preculture medium. The same experiment with identical precultures was also carried out in shaking flasks yielding similar results (Fig. S1 and Table 1). Following the sugar concentration in the medium over time revealed that sucrose was taken up and split only by strains expressing the porin (Fig. S1).

The unbalanced expression of the porin might provide an explanation for the slower growth rate of the strain complemented with CscY (see Fig. 3): The backbone plasmid pSEVA434 with its pBBR1 ori (moderate copy number) and the control of expression by the leaky LacI/P_{trc} promoter (Balzer *et al.*, 2013) might produce CscY at an unfavourably high rate. Furthermore, the artificial expression of membrane proteins is known to have a negative influence on cell vitality (Wagner *et al.*, 2007). In fact, formation of flocks – probably biofilm formation, a common stress response in *P. putida* – was observed in the strains expressing CscY from pSEVA434-*cscY* in shaking flasks. This was accompanied by a reduction in the cell dry weight of the planktonic cells (compare Fig. S1).

From these experiments, we concluded that, at least under these experimental conditions, the native outer membrane of *P. putida* was not permeable to sucrose. Compared to *E. coli* where transport of small molecules across the outer membrane is mainly facilitated by rather unspecific porins, *P. putida* has an outer membrane that more closely resembles the membrane of *P. aeruginosa*: homologues of the unspecific porins from *E. coli* (OpmF and OmpC) are not present. Instead, the general porin is OprF, which has a diffusion rate of two orders of magnitude lower than OpmF and OmpC of *E. coli* (Saravolac *et al.*, 1991; Nikaido, 2003; Eren *et al.*, 2012). This lower permeability is believed to be a major contributor to the remarkable resistance to toxic agents in fluorescent *Pseudomonads* (Nikaido, 2003) and could also be important for *P. putida*'s resistance to chemical stresses.

Sucrose transport across the outer membrane was dependent on preculture medium

While carrying out the complementation experiments, we realized that, when using M9 medium-grown precultures instead of LB-grown precultures for otherwise identical experiments, the growth of *P. putida* (pSEVA221-

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A Appendix

Table 1. Relevant growth parameters of strains constructed in this study.

Genotype *P. putida* EM178	Substrate	Growth rate μ [h^{-1}]	Biomass yield[a] $Y_{X/S}$ [g/g]	Substrate uptake rate[b] q [mmol g_{CDW}^{-1} h^{-1}]
Wild type	Sucrose	n.d.	n.d.	n.d.
*att*Tn7::*cscRABY*	Sucrose	0.45 ± 0.02	0.23 ± 0.02	5.9 ± 0.7
Wild type	Sugarcane molasses	0.202 ± 0.018	0.052[c]	n.m.
*att*Tn7::*cscRABY*	Sugarcane molasses	0.265 ± 0.017	0.125[c]	n.m.
pSEVA221-*cscRABY*	Sucrose (LB glucose preculture)	0.3945 ± 0.0001	0.277 ± 0.013	4.16 ± 1.9
pSEVA221-*cscRAB*	Sucrose (LB glucose preculture)	n.d.	n.d.	n.d.
pSEVA221-*cscRAB* + pSEVA434-*cscY*	Sucrose (LB glucose preculture)	0.059 ± 0.013	0.17 ± 0.04	1.0 ± 0.3
pSEVA221-*cscRABY*	Sucrose (M9 glucose preculture)	0.515 ± 0.005	n.m.	n.m.
pSEVA221-*cscRAB*	Sucrose (M9 glucose preculture)	0.470 ± 0.019	n.m.	n.m.
pSEVA221-*cscRAB* + pSEVA434-*cscY*	Sucrose (M9 glucose preculture)	0.38 ± 0.02	n.m.	n.m.

n.d., not detectable; n.m., not measured.
a. Yields were calculated from three averaged replicates of cell dry weight and substrate concentrations; errors are calculated from regression.
b. Sucrose uptake rates were calculated from growth rates and biomass yields; errors were calculated with Gaussian propagation of uncertainty. The values in this table consider the total amount of sucrose metabolized – glucose and fructose in the supernatant were considered to be not taken up.
c. Biomass yields in experiments using sugarcane molasses as a substrate were calculated from maximal cell dry weights and are related to the concentration of molasses (10 g l^{-1}) and not to the sugar content as molasses are constituted by three different sugars.

cscRAB) was not impaired on M9 sucrose, but the cells grew at almost the same rate as *P. putida* (pSEVA221-*cscRABY*) (Fig S2 and Table 1). This unexpected finding might be explained by the different metabolic regimes of the cultures: Cells grown in minimal medium with only glucose as a carbon source have a glycolytic regime whereas those grown in LB need to carry out gluconeogenesis. For these different lifestyles, a different set of proteins and also a different composition of the outer membrane is required, which depends on the culture environment (Thompson *et al*., 2010; Choi *et al*., 2014). We speculate that the outer membrane of cells grown in M9 medium has a different set of outer membrane proteins and might already possess sucrose transport activity, whereas the one of LB-grown cells does not. To test this hypothesis, we conducted a periplasm swelling experiment that is based on a method described by Nakae *et al.* (1989). Cells were grown in different media (LB, M9 with glucose, or M9 with citrate) and subsequently washed with a hypertonic solution containing glycerol (which should pass the outer membrane), sucrose and xylose. The substances that passed the outer membrane should be present in the periplasm after this first washing step.

Subsequently, these compounds now present in the periplasm were washed out again in a second washing step with deionized water. Then, the sugar concentrations in the supernatant were determined, which allowed us to draw conclusions on the permeability of the outer membrane for the detected sugars. As expected, the glycerol concentration was independent of preculture conditions, corroborating the assumption that it can diffuse freely across the membrane. However, sucrose was twice as concentrated in the supernatants of cells grown in M9 medium (Fig. 4). This can be explained by the faster diffusion rate of sucrose through the outer membrane of M9-grown cells compared with LB-grown cultures and was a further sign that using LB as preculture medium led to a lower permeability of the outer membrane for sucrose. The negative values for Xylose in Fig. 4 are an artefact from the calculation (Eq. 2) and indicate that xylose is metabolized by the cells. In fact, the loss of xylose in the chromatogram is accompanied by the appearance of another peak close by, which is very likely an oxidized intermediate (data not shown). This behaviour of xylose was also reported by (Dvorak and de Lorenzo, 2018) and makes it an unsuitable internal standard for swelling experiments like this. Therefore, GFP and glycerol were used as internal standards.

The reason for the differences in the uptake of substrates through the outer membrane can probably be attributed to a different set of porins as a result of the preculture medium. In fact, previous proteome studies showed a strong dependency of most outer membrane porins on medium composition and carbon source in *P. putida* F1 and KT2440 (Thompson *et al*., 2010; Choi *et al*., 2014). From our experiments, we cannot conclude which particular porin might be responsible for sucrose uptake, but outer membrane fractions and purified porins of other *Pseudomonads* have been shown to be able to

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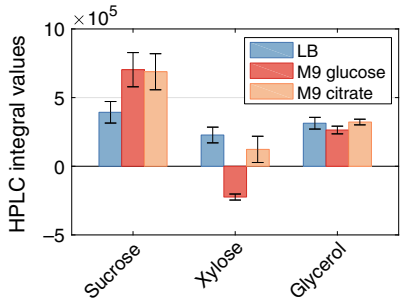

Fig. 4. Permeability of the outer membrane of *P. putida* EM178 estimated from the swelling experiment using a hypertonic solution of 0.15 M sucrose, xylose and glycerol in M9 medium. The bars represent the estimated substrate amounts (as quantified by their peak areas in HPLC) after washing the cells with deionized water, normalized with the fluorescence signal of GFP in supernatants according to Eqs 1 and 2. They should therefore represent the amount of substrate that is able to permeate through the outer membrane. Mean values and standard deviations are derived from three replicates. From left to right: blue bars: precultures grown in LB; red bars: precultures grown in M9 glucose; orange bars: precultures grown in M9 citrate.

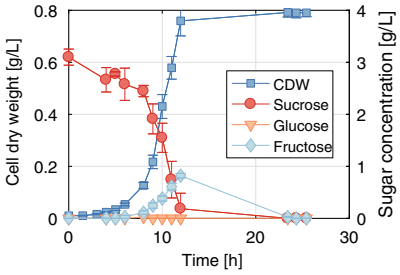

Fig. 5. Cell dry weight and sugar concentrations of a culture of *P. putida* EM178 *att*Tn7::*cscRABY* over time. Cells were grown in M9 medium supplemented with 3 g l^{-1} sucrose. Mean values and standard deviations of three independent biological replicates are shown. Experiments were performed in shaking flasks filled with 50 mL liquid and at an agitation rate of 220 rpm at 30°C.

transport sucrose to a certain extent (Trias *et al.*, 1988; Shrivastava *et al.*, 2011; van den Berg, 2012). The major outer membrane porin of *P. aeruginosa* is OprF. As *P. putida* and *P. aeruginosa* share similar features in the organization of their outer membrane (Saravolac *et al.*, 1991) and OprF is able to transport molecules as large as raffinose (Nikaido, 2003), it might well be responsible for sucrose transport in this work.

In any case, the whole *cscRABY* operon was used to construct a sucrose-consuming *P. putida* strain to avoid phenotypic variations and to ensure sucrose uptake independent of the preculture medium.

Genomic integration of cscRABY *genes resulted in stable and rapid sucrose-dependent growth*

As a next step, we aimed to integrate the gene cluster into the chromosome of *P. putida* in order to avoid the need for a plasmid and the associated antibiotic selection pressure. Using antibiotics may not be desirable or suitable for some applications. Therefore, we integrated the *cscRABY* genes into the *att*Tn7-site of *P. putida* EM178 via the mini-Tn7 transposon vector pTn7-M and evaluated the growth of the resulting strain on sucrose (Fig. 5). In shaking flasks growth rates of 0.45 ± 0.02 h^{-1} and sucrose uptake rates of 5.9 ± 0.7 mmol g_{CDW}^{-1} h^{-1} (calculated from three replicates) could be reached. For a more detailed overview of the growth rates, yields and substrate uptake rates, the key growth parameters are listed in Table 1. This compares well to growth rates measured with the monomers glucose and fructose and is around 60% higher than previously reported growth rates (Löwe *et al.*, 2017b). Time courses of cell dry weight and sugar concentration in the supernatant are depicted in Fig. 5. Interestingly, fructose accumulated in the growth medium during fermentation, which might be explained by leakage of fructose after intracellular splitting of sucrose or by extracellular hydrolase activity. The latter option is not very likely because no splitting activity could be measured when the porin was lacking (Fig. S1). Leakage of fructose is also supported by the fact that *P. putida* does not show any fructokinase activity (Sawyer *et al.*, 1977). Instead, fructose is phosphorylated during uptake through the native phosphotransferase system (PTS^{Fru}) (Velázquez *et al.*, 2007). In fact, the absence of FruB, the cytoplasmatic component of the PTS^{Fru}, completely abolishes growth on fructose (Velázquez *et al.*, 2007). Thus, after the intracellular cleavage of sucrose into glucose and fructose, glucose could be metabolized directly by glucokinase Glk, whereas fructose has to be secreted and taken up again via the fructose-PTS system in order to be accessible to *P. putida*'s metabolism. Alternatively, glucose could also be secreted and then oxidized in to gluconate before uptake. We did not measure any relevant amount of intermediates of glucose metabolism via HPLC, which indicates that glucose either did not accumulate because of rapid oxidation and re-uptake or never left the cell.

For future applications, it might be worth considering the addition of a functional fructokinase to the metabolism of *P. putida*. This would result in a hybrid system consisting of the *csc* genes from *P. protegens* Pf-5 and the ones of *E. coli* W that also comprise a fructokinase, and could be a way to make sucrose metabolism more efficient. Engineering the central carbon metabolism of *P. putida* was recently demonstrated (Sánchez-Pascuala *et al.*, 2017) and offers the potential to replace

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biomodules that are inefficient in an industrial context with streamlined versions for the desired application.

Cultivation of P. putida *EM178* att*Tn7*::cscRABY *in sugarcane molasses*

We cultivated *P. putida* EM178 *att*Tn7::*cscRABY* in M9 medium with untreated sugarcane molasses as a carbon source (Fig. 6) to prove the functionality of the *cscRABY* gene cluster on the industrially relevant waste product sugarcane molasses. *P. putida* EM178 was used as negative control. For the first 5 h, both strains grew nearly identical, but after 9 h the wild-type strain stopped growing and the final cell dry weight of the engineered strain was more than twice as high as the cell dry weight of the wild type. Sugarcane molasses mainly consist of the carbohydrates sucrose, glucose and fructose. The latter two sugars can also be used by wild-type *P. putida*, explaining the initial growth of the negative control. Sucrose can only be metabolized by the strain carrying the *cscRABY* genes, which is reflected by higher final cell dry weights (Fig. 6A), as well as almost complete depletion of sucrose in the supernatant (Fig. 6B) and thus higher biomass yields (Table 1).

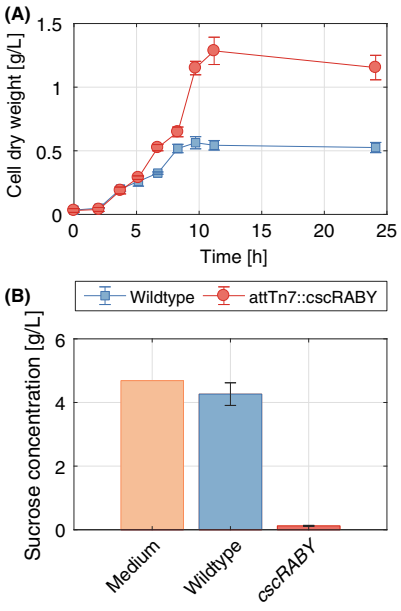

Fig. 6. Growth of *P. putida* EM178 *att*Tn7::*cscRABY* and *P. putida* EM178 on molasses. A M9 molasses (10 g l^{-1}) medium was used, and experiments were performed in shaking flasks with 50 mL volume in three biological replicates; agitation rate 220 rpm; temperature 30°C.
A. Cell dry weight over time and (B) sucrose consumption of both strains, compared with the original concentration in the medium after 24 h.

Sugarcane molasses are a very cost effective carbon source, which might help new bioprocesses using *P. putida* to come to life.

Conclusion

Although a sucrose porin does not seem to be necessary in *E. coli* to efficiently metabolize sucrose (Sabri *et al*., 2013), we found that sucrose is not reliably transported across the outer membrane in *P. putida* unless a suitable porin is expressed. An explanation for that can be found in the lower permeability of the outer membrane of *Pseudomonads* compared with *E. coli*. This remarkable difference has to be taken into account when engineering new pathways in *P. putida* and any other Gram-negative bacteria where a substrate needs to enter the cell or a product is to be secreted out of the cell. Porins normally do not receive much attention in metabolic engineering, but, depending on the desired metabolites, they might be of great importance. By introducing a suitable porin, productivity can be increased if the uptake of the substrate is the limiting factor. In other cases, a porin might improve cell vitality if it facilitates the passage of a possible toxic product through the outer membrane. In the work described here, we were able to show that additionally to the genes *cscRAB,* the porin CscY was needed in *P. putida* for efficient growth on sucrose when precultures were grown in LB medium.

This highlights the phenotypic instability when the outer membrane is neglected in the engineering process. When the *csc* genes were fully implemented, *P. putida* showed excellent growth with sucrose as the sole carbon and energy sources, even when genomically integrated and thus present in single copy. In addition, biomass yield increased more than twofold when grown on sugarcane molasses compared with the wild type, opening up the possibility to grow *P. putida* at low substrate costs. This is especially interesting for products that have to compete with nonrenewable alternatives like bioplastics or biofuels, but also for products that are already produced with *P. putida* like hydroxystyrene (Verhoef *et al*., 2009), phenylalanine (Molina-Santiago *et al*., 2016) or catechols (Robinson *et al*., 1992) (for an overview, see (Poblete-Castro *et al*., 2012; Loeschcke and Thies, 2015)). Medium-chain-length polyhydroxyalkanoates, a promising type of bioplastics, can be produced by *P. putida* from fatty acids (Huisman *et al*., 1992) that are a major part of waste streams from oil mills. However, they can also be produced by *P. putida* using glucose (Huijberts *et al*., 1992; Acuña *et al*., 2014) or sucrose (Löwe *et al*., 2017a) as carbon source and metabolic engineering is already applied to improve these processes (Acuña *et al*., 2014). This and other efforts to streamline *P. putida* as a production organism

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will open up the way for new processes and products that can contribute to making a bio-based and renewable economy finally come to life.

Experimental procedures

Organisms, strains and cultivation

Escherichia coli DH5α λ-*pir* was used for the extraction of plasmids, transformation and as the plasmid donor in conjugation. *E. coli* HB101 (pRK600) and *E. coli* DH5α (pTnS1) served as helpers in conjugation and Tn7 transposition respectively. *P. putida* EM178, a prophage-free derivative of *P. putida* KT2440 (created at Victor de Lorenzo's lab at CNB, Madrid), was used as a working strain. The complete list of organisms in this study and their origin are given in Table S2.

Cultivation was performed as described previously (Löwe *et al.*, 2017b). In brief, LB medium was used in all precultures for genetic manipulations and precultures for shaking flasks experiments if not noted otherwise. M9 minimal medium (Miller, 1972) was used for cultivation experiments with specific carbon sources. Shaking flasks were incubated in an orbital shaker at 220 rpm agitation at 30°C for *P. putida* and 37°C for *E. coli*. Antibiotics were used for selection when necessary (standard concentrations: 50 mg l^{-1} kanamycin, 200 mg l^{-1} streptomycin, 10 mg l^{-1} gentamicin).

A microplate reader (Tecan, Austria) was used for the cultivation at the 200 µl scale. Every 20 min, the microtitre plate with the cultures was shaken for 1 min and the optical density at 600 nm was measured. The temperature was controlled to 30 ± 1°C. Cell dry weights were calculated from optical densities with correlation factors that were determined beforehand in growth experiments of *P. putida* EM178 with M9 glucose medium in shaking flasks.

Genetic manipulations

All genetic constructs were created by restriction/ligation cloning and are listed in Table S1. The *cscRABY* gene cluster was amplified from genomic DNA of *P. protegens* Pf-5 using the primers fwP_I_scr_P_pro and rvP_scr_P_pro (compare Table S3). The PCR product was cut with restriction enzymes SacI and XbaI (New England Biolabs, Ipswich, MA, USA) for the full gene cluster and with SacI and PstI to obtain only the region spanning the genes *cscRAB.* These fragments were subsequently ligated into the multiple cloning site of the likewise digested pSEVA221. Plasmid pTn7-M-*cscRABY* was created by the digestion of pSEVA221-*cscRABY* with restriction enzymes SacI and XbaI and insertion into the identically cut multiple cloning site of pTn7-M. To create pSEVA434-*cscY*, pSEVA221-*cscRABY* was digested with PstI and XbaI and ligated into the multiple cloning site of pSEVA434 that was previously cut with PstI and SpeI.

Constructs cloned in *E. coli* were transferred to *P. putida* EM178 via conjugation as described by others (de Lorenzo and Timmis, 1994). The method was simplified as follows: first, 200–300 µl of overnight grown cultures (LB medium) of plasmid donor, helper strain [*E. coli* HB101 (pRK600)] and recipient (*P. putida* EM178) was mixed and subsequently centrifuged (10 min at 8000 *g*), the supernatant was discarded, and the pellet was resuspended in the remaining droplet. It was transferred to LB agar plates without antibiotics and incubated as a sitting drop for 6–10 h. The cells were then simply streaked onto an M9 citrate agar plate with suitable antibiotics.

Sugar and alcohol determination by HPLC

High-performance liquid chromatography was used to quantify the substrates and intermediates of sucrose metabolism: sucrose, glucose and fructose. Standards of 2 g l^{-1} were used of each substrate. Glycerol and xylose were only determined semiquantitatively by considering their peak areas. Samples were prepared as follows: Cells were separated from samples by centrifuging at least 400 µl of cultivation broth at 17 000 *g* for 5 min. Supernatants were filtered through 0.22-µm regenerated cellulose filter plates and injected (20 µl) into the HPLC (Agilent 1100 series, Waldbronn, Germany). Analytes were separated in a Shodex SH 1011 column at a flow rate of 0.45 ml min^{-1} with 0.5 mM sulfuric acid as the mobile phase at 30°C. Concentrations were calculated by integration of the peak area of each peak and correlation to the corresponding standards.

Outer membrane swelling experiments

Sucrose import into the periplasm of *P. putida* was evaluated with a method relying on membrane swelling experiments, similar to Nakae *et al.* (1989): *P. putida* grown with either LB, M9 glucose or M9 citrate medium was upconcentrated to an optical density of about 200 at 600 nm in a volume of 500 µl. Cells were first washed with 500 µl of phosphate-buffered saline and in a second step with M9 medium containing 0.15 M of sucrose, xylose and glycerol. Xylose and glycerol were added as internal standards as these two substances could be well separated from sucrose by the HPLC method described above. Additionally, purified eGFP, which should be unable to enter the cell and is easily measurable via its fluorescence, was added at a concentration of 0.0332 g l^{-1} as an internal standard. After washing the culture with this complex mixture, cells should have taken up those substances that could diffuse through the

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outer membrane because the solution was hypertonic. Next, the cells were washed with 500 μl deionized water which should extract the content of the periplasm into the washing solution. Both, the supernatants after washing with the hypertonic solution and deionized water were taken as samples to measure sucrose, xylose, glycerol and eGFP.

With such a big number of cells, there was an unneglectable amount of residual water (V_{residual}) after centrifugation which might still contain the components of the hypertonic solution. This was corrected with the residual eGFP signal (F1: signal after hypertonic shock, F2: signal after hypotonic shock):

$$V_{\text{residual}} = \frac{F_2}{F_1 - F_2} 500\mu l \tag{1}$$

This residual volume contained the concentrations of sucrose, xylose and glycerol as determined by HPLC. After washing the cells with deionized water and centrifugation, the supernatants included the sugars that were washed out of the periplasm and those that were left in the residual water after washing with the hypertonic solution. The concentrations had to be corrected with the volume of the residual water (Eq. 1) and the concentrations of the substances before (Index 1) and after washing (Index 2) in order to calculate the concentration in the periplasm only. The contents of the periplasm after a hypertonic shock could then be calculated:

$$c_{i,\,\text{periplasm}} = c_{i,\,2} - \frac{V_{\text{residual}}}{V_{\text{residual}} + 500\mu l} c_{i,\,1} \tag{2}$$

These were the concentrations that we assumed to be in the periplasm after the hypertonic shock and should give information on the extent of diffusion across the outer membrane. All experiments were performed in three biological replicates.

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Figure S1. Cell dry weights and sugar concentration over time of different *P. putida* strains in M9 sucrose (3 g l^{-1}) in shaking flasks derived from LB pre-cultures.
Figure S2. Cell dry weights over time of different *P. putida* strains in M9 sucrose (3 g l^{-1}) in shaking flasks derived from M9 pre-cultures.
Figure S3. Organization of closely related gene clusters in other *Pseudomonads* that also contain a putative sucrose/H+-symporter CscB.
Table S1. Plasmids used and constructed in this work.
Table S2. Bacterial strains that were used in this work.
Table S3. Oligonucleotides used for PCR reactions in this study with name, sequence and function.

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A.2 List of contributions to publications by the author

Peer-reviewed publications

Löwe H., Kremling A., Marin-Sanguino A. Time Hierarchies and Model Reduction in Canonical Non-linear Models. *Frontiers in Genetics* 7:166, 2016. doi:10.3389/fgene.2016.00166

Pfaffinger C. E., Schöne D., Trunz S., **Löwe H.**, Weuster-Botz D. Model-based optimization of microalgae areal productivity in flat-plate gas-lift photobioreactors. *Algal Research* 20: 153-163, 2016. doi:10.1016/j.algal.2016.10.002

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Löwe H., Sinner P., Kremling A., Pflüger-Grau K. Engineering sucrose metabolism in *Pseudomonas putida* highlights the importance of porins. *Microbial Biotechnology*, 2018. doi:10.1111/1751-7915.13283

Wagner G. S., Ziegler M., **Löwe H.**, Kremling A., Pflüger-Grau K. pTRA – A reporter system for monitoring the intracellular dynamics of gene expression. *PLoS ONE* 13(5): e0197420, 2018. doi:10.1371/journal.pone.0197420

Other publications

Löwe H., Kremling A., Pflüger-Grau K. Bioplastik aus Licht und Luft – das Konzept einer synthetischen Ko-Kultur. *Biospektrum* 23(3): 338–340, 2017. doi:10.1007/s1226

(written by Katharina Pflüger-Grau)