Exploiting unconventional prokaryotic hosts for industrial biotechnology

Bastian Blombach[1,2], Alexander Grünberger[3], Florian Centler[4], Nick Wierckx[5], Jochen Schmid[6]*

[1] Microbial Biotechnology, Campus Straubing for Biotechnology and Sustainability, Technical University of Munich, Straubing, Germany
[2] SynBiofoundry@TUM, Technical University of Munich, Schulgasse 22, 94315 Straubing, Germany
[3] Multiscale Bioengineering, Bielefeld University, Universitätsstr. 25, 33615 Bielefeld, Germany
[4] Department of Environmental Microbiology, UFZ – Helmholtz Centre for Environmental Research, Leipzig, Germany
[5] Forschungszentrum Jülich, Institute of Bio- and Geosciences IBG-1: Biotechnology, Wilhelm-Johnen-Straße, 52425 Jülich, Germany
[6] Institute of Molecular Microbiology and Biotechnology, University of Münster, Corrensstraße 3, 48149 Münster, Germany

*Correspondence: jochen.schmid@uni-muenster.de

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Abstract
The development of cost-efficient biotechnological processes is a major challenge in the substitution of fossil-based industrial production processes. The remarkable progress in genetic engineering ensures efficient and fast tailoring of microbial metabolism for a wide range of bioconversions. However, the improvement of intrinsic properties such as tolerance, handling, growth, and substrate consumption rates is still challenging. At the same time, synthetic biology tools are becoming easier applicable and transferable to non-model organisms. These trends resulted in the exploitation of new and unconventional microbial systems with sophisticated properties which render them promising hosts for the bio-based industry. Here, we highlight the metabolic and cellular capabilities of representative prokaryotic newcomers and discuss the potential and drawbacks of these hosts for industrial application.

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A paradigm change – exploiting novel microbial hosts

The bioeconomy relies on the use of biogenic resources and waste streams such as lignocellulosic hydrolysates, biorefinery or industrial side streams, or off-gases for the production of energy and materials (Figure 1A). This must happen in a cost-efficient way if it is to replace fossil-based processes and thus enable a circular economy. Highly efficient biotechnological conversions are thus required, which places severe requirements to meet on the microbial systems used in industrial biotechnology. Ideally, boundary conditions such as a defined substrate, product, and **bioprocess window** should guide the selection of a suitable microbial system up front (Figure 1B) before performing iterative rounds of strain development to eventually yield an optimized industrial process [1] (Figure 1C).

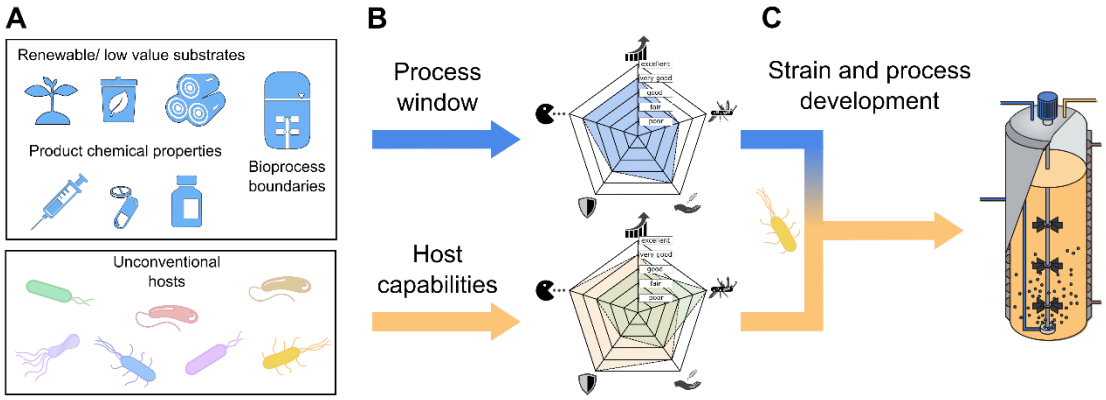

Fig. 1. Exploiting unconventional prokaryotic hosts for industrial biotechnology. (A) Based on the available substrate, process boundaries, and desired product properties, natural strains can be selected that (B) best match the envisioned process window in terms of growth efficiency, tool availability, handling, tolerance, and metabolic capability. This *ab inito* selection ensures the highest chance of success of subsequent strain and process development (C) for envisioned industrial bioprocesses.

Highly robust and tolerant strains towards non-natural or even toxic substrates or products are required which are able to utilize so far untapped carbon and energy sources. Moreover, growth in low-cost media such as prepared with sea-water [2] or a high resistance against phage infections are of growing interest. Flexible use of (seasonally available) feedstocks further relies on versatile microbes which are easy to handle at lab scale as well as in the industrial environment. Ideally, the metabolism of such microbes can efficiently be engineered to further expand the substrate and product spectra. For heterotrophic organisms this approach is generally feasible [3][4], but the metabolic engineering of autotrophic organisms, and the engineering of growth on C1 substrates in heterotrophs, is often much more challenging [5]. In addition, a high growth efficiency

enables the development of more productive processes which are competitive with classical chemical approaches.

Metabolic engineering of microbial strains is a rapidly evolving field that, in combination with synthetic biology approaches and metabolic modeling, ensures fast tailoring of the metabolism [6,7]. This currently leads to an impressive flood of publications describing approaches for the production of valuable natural and non-natural products [8]. However, recent progress also clearly points to obstacles in this field which cannot be easily overcome. The optimization of basic cellular properties such as tolerance to chemical and physical stresses, high growth and substrate consumption rates, or the ability to efficiently utilize C1 carbon sources, are still challenging (See **outstanding questions**). This is mainly due to the complexity of these traits and the lack of underlying biochemical knowledge.

This insight has led to a paradigm shift towards the development and application of **unconventional prokaryotic hosts**. Certainly, many eukaryotic hosts like *Saccharomyces cerevisiae* also have highly advantageous traits, but these will not be in the scope of this opinion article. Historically grown microbial systems such as *Escherichia coli* are established in industrial biotechnology and therefore have the “first mover advantage“, but they are not per se superior catalysts. In fact, wildtype *E. coli* has very few outstanding natural properties, and in our opinion its central position in the biotechnology community is mainly due to the long history of tool development and ease of handling. Nature`s diversity provides an enormous resource to meet the abovementioned challenges with organisms that possess superior trophic levels, growth efficiency, tolerance or handling, and such microbes should be selected already during the first stages of process design [9,10]. Many of these organisms are increasingly coming into focus, often occupying specific industrial and scientific “niches” where established hosts simply cannot perform [1]. Some of these novel microbial hosts will be highlighted here as exemplary case studies, such as the solvent-tolerant *Pseudomonas taiwanensis* (Box 1), the fast-growing *Vibrio natriegens* (Box 2), the metabolically versatile *Paenibacillus polymyxa* (Box 3) or the methylotrophic *Bacillus methanolicus* (Box 4). In our opinion, these and other unconventional hosts possess superior properties that, with the current knowledge, cannot be easily engineered into established biotechnological workhorses.

Requirements for novel microbial hosts to reach industrial maturity

Microbial biotechnology is highly diverse and it is almost impossible to directly compare for example the production of a secondary metabolite from glucose to the conversion of carbon monoxide into acetate. That said, certain universal parameters such as growth efficiency, tool availability, handling, tolerance, and metabolic capacity, can be defined that are relevant for almost all processes. These parameters can help to guide

the evaluation and selection of a microbial host for a specific industrial application (Figure 2A):

Growth efficiency of the applied microbial host is an essential factor for reducing production costs of a biotechnological process. For this, exploitation of the exceptionally fast-growing *Vibrio natriegens* as novel host for molecular biology and industrial biotechnology is targeted massively (Box 2; [11–13]. Since a high growth rate (μ) goes hand in hand with a high specific substrate consumption rate (q_S), *V. natriegens* shows q_S values which are significantly higher than those of established microbial systems under both aerobic and anaerobic conditions. This property is an excellent prerequisite to reach outstanding productivities as recently shown for alanine production with *V. natriegens* (Box 2; [13]). Growth efficiency also applies to high biomass yields, low maintenance demand, long-term viability, and growth-uncoupled product formation, which all reduce substrate costs for biomass production, especially for high cell density processes. Some of these traits can be enhanced by the engineering of streamlined chassis through genome reduction [14], provided that the required tools are available.

Tool availability is also an important prerequisite to fully exploit a novel microbial system. Model organisms such as *E. coli* particularly excel in this department, nowadays even enabling full-scale genomic engineering [15,16]. Genetic engineering can be a challenge for non-conventional microbes. However, the courage to use novel microbial systems with superior intrinsic properties is promoted by the steadily increasing development of widely applicable genetic engineering toolboxes. This has been impressively demonstrated for example for the fast-growing *V. natriegens*. Within less than five years *V. natriegens* has been made accessible for genetic manipulation, and a variety of tools to manipulate its metabolism were established and have already been applied to engineer **chassis** and production strains [17]. For *Pseudomonas sp*. an impressive amount of synthetic biology tools for the model organism *P. putida* KT2440 were developed within the recent years [6], which are also applicable for the deep engineering of unconventional hosts such as *P. taiwanensis* (Box 1, [14]. Also, for *P. polymyxa* (Box 3), which was regarded for a long time as resistant towards genetic manipulation, tools for genetic and metabolic engineering were rapidly developed by the design of an efficient CRISPR-Cas tool [18], ending up in the first described Cpf1 based multiplexing CRISPRi/a system for prokaryotes within only 3 years [19,20].

Convenient *handling* is not only restricted to lab scale and must also be considered for the industrial environment. Efficient cultivation in simple mineral media lowers production costs and widens the application range of microbes. The use of robust strains or extremophiles as well as recalcitrant substrates enables (semi-) autosterile process development, for example in sea water [2]. Morphology also plays an important role in the handling of microbes. Unicellular growth is usually preferred, but biofilm catalysis can also

provide major advantages in certain niche applications [21,22]. Engineered production strains have to prove their robustness in industrial scale where gradients (e.g., O_2, CO_2) can significantly impact the performance [23]. This issue is often not sufficiently considered but should be addressed already in the early stage of process development especially for non-conventional hosts [24]. Further, unconventional hosts should be safe to handle with a biosafety level 1 classification. A unified biosafety classification is sorely needed in this context, especially in the European Union where large differences exist between member countries. Strains should ideally be Generally Regarded as Safe, although this is rare and often confused for microbes [25].

A high *tolerance* against relevant substrate and product concentrations is essential for industrial application. Pseudomonads such as the non-pathogenic *P. taiwanensis* VLB120 (Box 1) can withstand high oxidative and chemical stresses, enabling more efficient use of toxic substrates, production of higher titers of more toxic chemicals, and application of *in situ* solvent extraction [26,27]. Other microbes such as the pathogenic *Klebsiella* sp., or the non-pathogenic *P. polymyxa* (Box 3) can cope with high concentrations of 2,3-butandiol [28,29]. Also, a tolerance against fluctuating process parameters such as dissolved oxygen concentration, as present in large scale production environments, represents a beneficial attribute of the microbial host. Although **adaptive laboratory evolution** (ALE) is a powerful approach to increase tolerance and titers [30,31], but it often stagnates at a certain level of improvement and can even be detrimental [32]. Exemplary, ALE can increase the thermotolerance of *E. coli* up to 48ºC [33]. Above this temperature most of the proteome of *E. coli* is unstable, making it unfeasible to achieve higher temperature tolerance [34]. In contrast, industrially interesting thermophiles of the *(Geo-)Bacillus* or *Thermus* genera grow at much higher temperatures.

The versatile *metabolic capabilities* of a (mainly heterotrophic) microbial host to utilize various substrates, is a prerequisite for the microbial funneling of complex mixtures such as hydrolysates of lignocellulose or plastics to desired products [35,36]. Pseudomonads (Box 1) are at the forefront of this development. In contrast, but equally important, are the current developments towards the valorization of alternative carbon sources such as C1 substrates, which demand the use of more specialized microbial systems. One example is *Bacillus methanolicus* (Box 4), a Gram-positive and thermophilic bacterium that is able to utilize a limited number of alternative carbon sources, however, has the ability to efficiently utilize methanol [37]. Also, the versatile utilization of terminal electron acceptors under aerobic and anaerobic conditions is an important attribute of a microbial host which impacts the ability to synthesize specific products.

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A

Characteristics	Desired properties	KPIs*
Growth efficiency	- High biomass yield and low maintainance demand - High growth, substrate uptake rate and metabolic flux	- Yield - Rate
Tool availability	- "Engineerability" with efficient and reliable tools for genomic engineering - Knowledge base of annotated genome, metabolic models and systems analysis	- Time to market - Versatility
Handling	- Cultivation to high cell density - Lack of overflow metabolism and by-product formation - Amenable to screening and engineering - Low impact of heterogeneities	- Rate - Scale-up
Tolerance	- Ability to withstand high concentrations of (crude) substrate/product - Broad process windows of pH, temperature, oxygen and salt concentrations - Adaptability to physical/chemical gradients in industrial scale	- Titer - Scale-up - Substrate flexibility
Metabolic capability	- Utilization of different, or specialized, feedstocks including, sugars C1 substrates, lignocellulosic hydrolysates, and waste streams - Production of many different types of chemicals	- Substrate and product flexibility

* KPIs = Key performance indicators

B

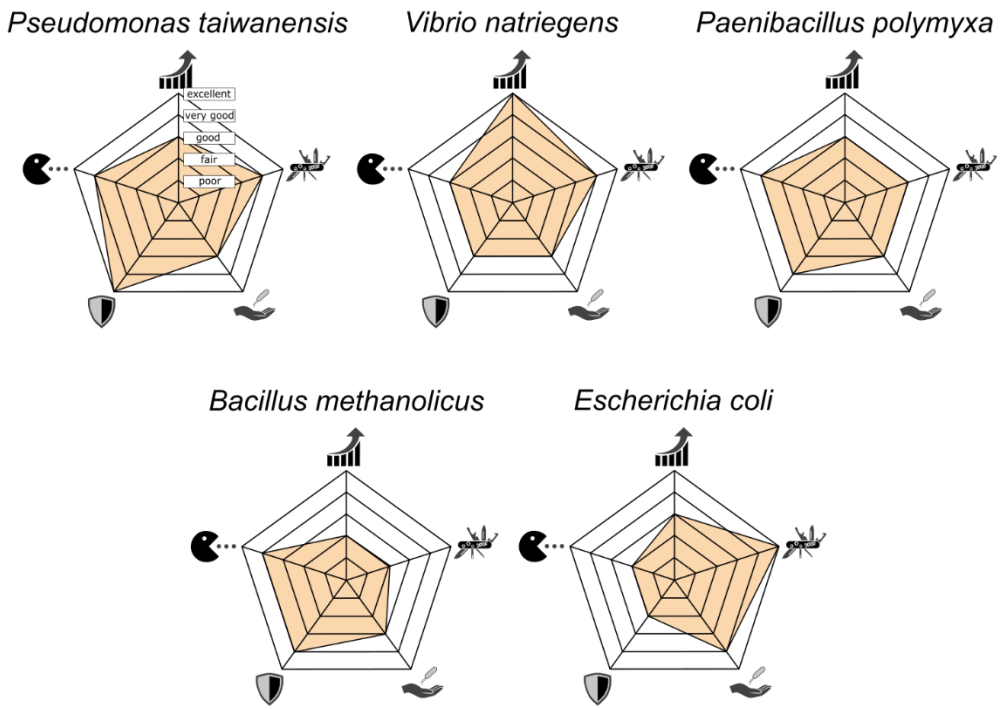

Fig. 2. (A) Characteristics, desired properties, and key performance indicators (KPIs) of novel microbial hosts to reach industrial maturity. (B) Estimation of strain characteristics of emerging production hosts regarding defined key requirements, compared to wildtype *E. coli*.

Economic and environmental constraints impact host selection

Currently, most biotechnological production processes are carbohydrate-based (e.g. glucose or sucrose; [38]) applying heterotrophs such as *E. coli* or *C. glutamicum* which utilize organic compounds as carbon and energy source. Carbohydrate-based processes generally possess a high technology readiness level and therefore will be preferentially further applied in industry. However, the establishment of more sugar-based processes will increase the demand for this substrate, and the consequent price increase is a major risk for industrial development of bulk processes which have a long return of investment time [39]. The currently applied carbohydrate-based substrates, which derive from starch or sugar-containing plant material, are often viewed as a dilemma for the envisioned bioeconomy since these feedstocks, when used at very large scale, may compete with food production [38]. Even if this point is controversially discussed, at least for ethical reasons the fuel versus food debate also applies for the biotechnological production of chemicals. Perspectivley, bulk bio-based production processes should therefore preferentially be based on non-food feedstocks such as methane, methanol, formate, syngas, and CO_2 (Figure 3; [40]) – carbon sources which do not belong to the natural substrate spectrum of industrially established heterotrophs. Alternatively, more complex and crude substrates such as lignocellulosic or plastic hydrolysates could be used, requiring a higher chemical tolerance and a versatile metabolism of the applied microbial system [41]. Consequently, either heterotrophs are engineered to efficiently utilize such carbon sources [42,43] or novel hosts which are naturally able to assimilate these substrates or show an intrinsic high tolerance towards crude substrates such as *P. taiwanensis* (Box 1) or *B. methanolicus* (Box 4) must be exploited for industrial processes.

The recently approved **Green Deal** by the European Union aims at the reduction of net greenhouse gas emissions to zero by 2050, which will also impact future biotechnological processes. The biotechnological production of chemicals and fuels from biomass is considered atmospheric carbon neutral because as the biomass grows, it absorbs CO_2 which is eventually incorporated in the product. However, biomass-based fermentation processes or anaerobic digesters produce significant amounts of CO_2 as byproduct, which are currently released into the environment. Moreover, taking the whole value chain into account (including e.g. energy for culturing and harvesting of the biomass or reactor cooling and downstream processing), significant net amounts of CO_2 might be emitted. Consequently, to minimize CO_2 emissions, future biotechnological processes have to rely on integrated carbon capture and utilization technologies as well as biotechnological **cascade processes** where a (by-)product (e.g., CO_2, methane, acetate) of one process stage is the substrate for a subsequent (Figure 3).

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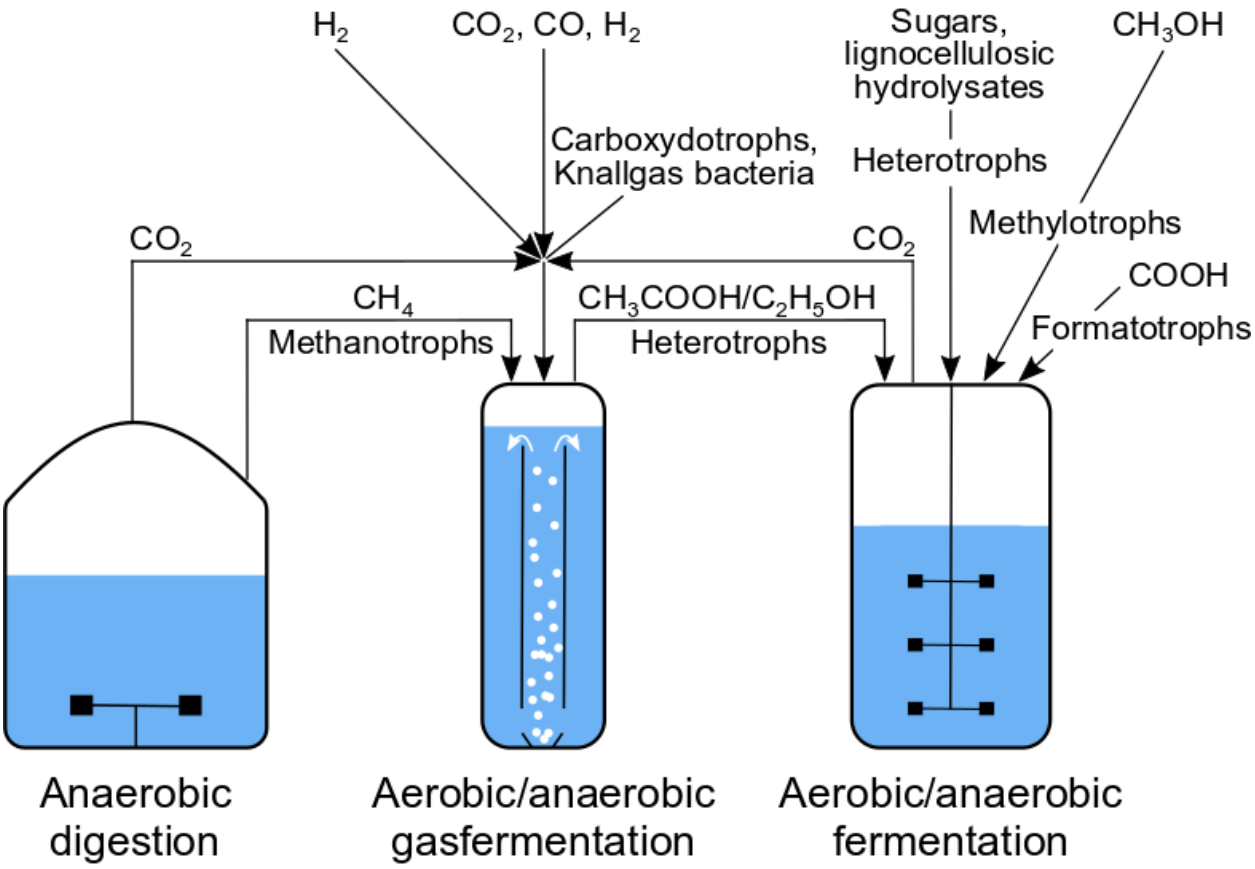

Fig. 3. Valorization of abundant feedstocks and its proposed cascading utilization to reduce greenhouse gas emissions**.**

Gas fermentation will play an important role in this scenario to convert (waste) gases such as CO_2, CO, H_2, methane and mixtures thereof into chemicals and fuels [44]. Carboxydotrophic bacteria generate energy by aerobic or anaerobic oxidation of CO. Aerobic carboxydotrophs use the Calvin-cycle, whereas strictly anaerobic carboxydotrophs, also called acetogenic bacteria or acetogens, harness the Wood–Ljungdahl (WL) pathway for CO_2-fixation. In anaerobic gas fermentations acetogens such as *Acetobacterium woodii*, *Clostridium ljungdahlii*, *Moorella thermoacetica*, and *Clostridium autoethanogenum* convert $CO/CO_2/H_2$-containing gases efficiently to ethanol and acetate [45]. Anaerobic gas fermentation has become a technology of industrial maturity and first production plants have been successfully installed by companies such as LanzaTech [44]. Among the known pathways for CO_2-fixation, the WL is the most efficient one with regard to ATP and H_2/electron requirements [46]. However, due to the anaerobic life style, acetogenic bacteria are energy limited and the production of longer-

chain or ATP-demanding molecules is challenging [46]. To overcome this limitation, recently aerobic carboxydotrophs such as *Oligotropha carboxydovorans* and *Hydrogenophaga pseudoflava* have been proposed for industrial gas fermentation since the aerobic oxidation of CO provides a higher ATP-yield compared to the anaerobic route [45,47,48]. Also, Knallgas bacteria such *Ralstonia eutropha* which are naturally unable to oxidize CO have a high potential to convert CO_2 and H_2 containing gases to chemicals and microbial upgrading of methane with methanotrophs, methanol with methylotrophs or formate with formatotrophs to value-added products might represent another piece of the puzzle in the envisioned circular economy (Figure 3). In this refinery concept, products derived from **C1 substrates**, such as acetate or ethanol, can be further valorized by applying these substrates as feedstock for heterotrophic hosts to produce more complex chemicals (Figure 3; [38]). With these considerations in mind, economic and ecological constraints will help make greater use of microbial diversity for industrial biotechnology.

Challenges to establish novel microbial hosts for industrial biotechnology

Classically, novel microbial hosts were implemented in biotechnological processes for their ability to produce a certain chemical, for example, antibiotics with *Streptomyces* sp., or vitamin B12 with *Pseudomonas denitrificans* [49]. The advent of modern metabolic engineering and synthetic biology has greatly decreased this reliance on novel hosts by allowing the insertion of production pathways in microbial workhorses such as *E. coli* [50]. Since then, the selection of a novel microbial host is more often driven by other limitations, such as the utilization of a specific substrate (as elucidated above). However, besides the obvious advantage, the abovementioned requirements should be carefully considered. Especially, the transfer of a novel host into industrial scale comes with greater risk compared to established microbes, because long-term experience with regard to for example genetic stability or cellular heterogeneity is simply missing **(see Outstanding questions)**. As mentioned above, the ability to grow very fast, to utilize a special carbon or energy source, or to tolerate a toxic product or extreme process conditions, can be enormously advantageous and these beneficial traits might even be utilized in combination for syntrophic cultures [51]. However, driving the fitness of a certain trait to extremes often comes with drawbacks in other areas. Examples for this are the limited substrate and product flexibility of C1 metabolizing strains, the high metabolic energy demand imposed by solvent-tolerance [52], or the relatively poor genetic accessibility of halophilic or thermophilic microbes [53]. These trade-offs can affect the application range or production parameters (yield, titer, rate) of the envisioned biocatalyst, and should therefore be carefully weighed against the advantage of the unconventional host.

On the road from the discovery and development of unconventional hosts endemic to natural habitats into their industrial applications, *in silico* methods can support and guide the process at various stages in a **systems biotechnology** approach [54]. It has become common to reconstruct so-called metagenome-assembled genomes (MAGs)

from environmental samples [55], providing a first glimpse at the metabolism of uncultured organisms and informing isolation strategies [56]. On successful isolation and based on the sequenced genome, several pipelines are available to reconstruct genome-scale metabolic network models (e.g.,[57–59]. Using constraint-based modeling techniques [60], such models enable a first assessment of the host's metabolic capabilities [61], including theoretically achievable product yields. Furthermore, feasible product and substrate ranges can be explored, including the effort in terms of genetic modifications required to redirect carbon flow from a chosen substrate to a desired product. This assessment of the metabolism's flexibility and versatility comes together with concrete genetic engineering strategies for given substrate-product pairs. Once the novel host driven process is established at lab-scale, process optimization and upscaling can be supported by *in silico* optimization strategies such as evolutionary algorithms (e.g., [62]. More recently, due to their ability to easily integrate heterogeneous multiomics data, machine learning techniques have been used to directly predict phenotypic behavior based on such data [63,64]. Especially for novel hosts, for which underlying biochemical mechanisms might be unknown, this might be a worthwhile approach which can also be combined with metabolic modeling [65,66] and employed for metabolic engineering [67].

Conclusion

Industrial biotechnology is considered a key technology for the transition to a bio-based circular economy, which is constantly influenced by rapid scientific developments and changing ecological and socio-economic framework conditions. As discussed here, unconventional microbial hosts with superior intrinsic properties have the potential to meet challenges that cannot easily be overcome with established hosts. Rapid advances in genetic and metabolic engineering further lower the threshold to select such novel hosts, and their characterization will also enable the rational engineering of complex traits into more established microbes. The here discussed examples possess promising features but ultimately still have to prove their suitability for industrial application. Undoubtedly, nature can offer many more so far unexplored microbial candidates with high potential to occupy industrial niches. However, many questions concerning efficient strategies for establishing (the complex traits of) unconventional hosts for industrial applications still remain (see **outstanding questions**). Future studies essentially have to provide an improved genetic engineering and synthetic biology toolbox as available for established microbial systems. This tool development not only fosters expansion of the hosts' application range, it also speeds up the characterization of promising complex features, supported by systematic analysis with readily available omics-technologies. Gas fermentation will also be an important piece of the puzzle in the envisioned circular

economy but economic feasibility strongly relies on efficient mass transfer in large scale bioreactors. Other beneficial traits, e.g., a high growth rate or exceptional tolerance, come along with challenges like a high oxygen demand or low biomass yield. These examples emphasize the need for an integrated approach of strain- and process design, taking the whole process chain into account, when considering an unconventional microbial system for industrial biotechnology.

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Glossary:

Adaptive laboratory evolution: Method to enhance and understand specific microbial traits by long-term selection under specific growth conditions.
Bioprocess window: the boundary conditions for a bioprocess, determined by the technical restriction of the equipment and the biological restrictions of the microbial host.
C1 substrates: Biotechnological feedstocks that contain one carbon atom such as carbon dioxide, carbon monoxide, formate, methanol, and methane
Cascade processes: Stepwise utilisation of different carbon sources or intermediates as well as process variants in a cascading manner.
Chassis: Genetically engineered organism with enhanced basic properties such as growth rate, tolerance, or stability.
Green Deal: Initiative by the European Commission with the overarching aim of making Europe climate neutral in 2050.
Systems biotechnology: The application of omics driven systems biology methods to gain a holistic understanding of, and to engineer, a microbial production host.
Unconventional prokaryotic hosts: Bacteria with specific beneficial features that are not yet established in industrial biotechnology.

Boxes

(Box 1) *Pseudomonas taiwanensis*

Growth efficiency Routine cultures of *P. taiwanensis* VLB120 in mineral glucose medium grow at a rate of 0.6-0.7 h^{-1} [14]. With related Pseudomonads, this can increase above 1 h^{-1} on succinate [68]. The maintenance demand of Pseudomonads is relatively low [69], especially when compared to other oxidative biocatalysts such as *Gluconobacter oxydans*. Biomass yields around 0.5 $g_{CDW}/g_{glucose}$ are generally achieved, but can drop dramatically under solvent-stress conditions [52].

Tool availability A wide and rapidly expanding range or systems- and synthetic biology tools are developed by the *Pseudomonas* community [6]. These tools enable deep genetic engineering aimed at product formation [70] and the generation of streamlined chassis strains [14]. These developments are fundamentally supported by the availability of detailed metabolic models [71,72], and the outstanding genome database www.pseudomonas.com [73].

Handling As an obligate aerobe, *P. taiwanensis* doesn't produce typical overflow metabolites such as acetate or lactate. Pseudomonads are also robust against oxygen heterogeneities associated with large scale cultivation [23]. One drawback is posed by a highly active periplasmic glucose dehydrogenase (GCD), which rapidly converts glucose to gluconate [74]. Although this doesn't affect biomass yield or growth rate much, it does require titration and back-titration in batch cultures. This can be avoided by disruption of the *gcd* gene, but depending on the strain this can significantly affect the growth rate [75]. The use of glycerol as an alternative substrate is advantageous in this respect, and it has the further benefit of enabling higher yields in engineered aromatics-producing strains [76].

Tolerance *P. taiwanensis* VLB120 and related non-pathogenic strains such as *P. putida* S12 and *P. putida* DOT-T1E are best known for their high tolerance to organic solvents with a $\log P_{o/w}$ between 2.5 and 4 [26,27]. Toxicity of more hydrophilic chemicals such as phenol or (*S*)-styrene epoxide can be alleviated through *in situ* product removal in biphasic cultivations [77,78], where solvent-tolerance enables a wider degree of freedom in the selection of the optimal extractant [79,80]. Pseudomonads are also well equipped to withstand oxidative stress [81].

Metabolic capability Based on their broad carbohydrate substrate spectrum, Pseudomonads have been identified as key hosts for the biological funneling of complex mixtures of lignin aromatics [35,82], and plastic-derived monomers [83] into useful products. Their versatile metabolism towards aromatics also provides a wealth of biocatalytic potential that can be exploited for metabolic engineering [84,85] and for whole-cell biotransformations [86,87]. However, this versatility also has a downside, and often degradation pathways of intended products and precursors have to be identified and disrupted [70].

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(Box 2) *Vibrio natriegens*

Growth efficiency Among the non-pathogenic organisms, *V. natriegens* shows currently the highest growth rate on our planet [13]. In complex medium this Gram-negative bacterium grows with a generation time of 9.4 – 9.8 min. Under aerobic batch conditions in minimal medium with glucose, *V. natriegens* grows with a μ of 1.48 – 1.70 h^{-1}, exhibits a biomass yield ($Y_{X/S}$) of 0.5 g_{CDW} g_{Glc}^{-1} and a biomass specific glucose consumption rate (q_S) of 3.5 – 3.9 g_{Glc} g_{CDW}^{-1} h^{-1} [13,88]. Even in the absence of oxygen, this facultatively anaerobic bacterium shows a μ of 0.92 h^{-1} and a q_S of 7.81 g_{Glc} g_{CDW}^{-1} h^{-1} and ferments glucose mainly to acetate, succinate, formate, lactate, and ethanol [13]. μ and q_S under both conditions are at least two times higher compared to established microbial systems [13].

Tool availability The genome of *V. natriegens* is sequenced and genetic, synthetic biology, and metabolic engineering tools such as synthetic promoters, expression and (multiplex) genome editing systems as well as metabolic flux analysis have been developed. These tools have already been applied to engineer first genome-reduced derivatives and production strains [17].

Handling *V. natriegens* grows rapidly on common liquid and solid complex media when supplemented with sufficient sodium ions [11,13]. However, storage for a prolonged time at 4 °C negatively impacts the viability and should be omitted [11]. Due to its easy handling in the lab, the Weinstock group [11] and Lee and colleagues [12] proposed to utilize the fast-growing *V. natriegens* as novel host for routine applications in molecular biology and biotechnology.

Tolerance *V. natriegens* is a marine bacterium, requires sodium ions (Na^+) for growth and consequently tolerates sea salts. It has an exceptionally high tolerance towards selenium, an attribute which was already utilized for efficient production of selenium nanoparticles [89].

Metabolic capability *V. natriegens* utilizes several industrially relevant substrates including carbohydrates, amino, carboxylic and aromatic acids, polyamines, alcohols and the chitin monomers glucosamine and N-acetylglucosamine as well as starch. This nutritional flexibility is an important prerequisite for the flexible feedstock concept in industrial biotechnology [17]. Besides the facultatively anaerobic lifestyle, *V. natriegens* can fix N_2 [90] and is able to perform extracellular electron transfer [91].

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(Box 3) *Paenibacillus polymyxa*

Growth efficiency Cultures of *P. polymyxa* DSM 365 in glucose or sucrose medium grow at a rate of 0.7-0.76 h^{-1} [28] under aerobic conditions whereas growth rates of 0.3 – 0.6 h^{-1} are described for microaerophilic conditions [92]. *Paenibacillus* as most Bacilli is a mesophilic Gram-positive spore forming bacterium with an ubiquitarian appearance.

Tool availability For a long time, Paenibacilli were not really genetically accessible, since only a limited number of genetic engineering tools were available. Just recently, a rapidly expanding range of synthetic biology tools were developed. These tools enable in-depth genetic engineering for product formation [18,92] as well as targeted pathway modelling via multiplexed pathway perturbation towards streamlined chassis strains [93]. These developments are fundamentally supported by the availability of initial metabolic models which can be optimized in the near future [94].

Handling As a facultative anaerobe, *P. polymyxa* tends to produce typical overflow metabolites such as acetate or lactate, which can be reduced via metabolic engineering [92]. Based on their native production of the antibiotic polymyxin [95] as well as further antimicrobial peptides such as for example fucariscidin [96], Paenibacilli are very robust against contaminants and non-sterile complex media.

Tolerance *P. polymyxa* DSM 365 and related strains such as *Bacillus licheniformis,* or *B. ameloquefaciens* are known for their high tolerance to green organic solvents such as 2,3-butandiol, with concentration tolerances up to 115 g/L [97–99].

Metabolic capability Based on their broad carbohydrate substrate spectrum, Paenibacilli can be applied as production host of a great variety of carbohydrate polymeric substrates in biorefinery approaches [95,96], including lignin degradation [100,101]. Their versatile metabolism towards valuable products such as exopolysaccharides and for example enantiomeric pure R,R-2,3-butandiol or secondary metabolites such as antibiotics or antimicrobial peptides makes them highly interesting for metabolic engineering as well as agricultural applications in its native form [96]. Especially its ability to fix nitrogen and to convey growth promoting properties to plants makes *P. polymyxa* highly interesting for commercial applications.

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(Box 4) *Bacillus methanolicus*

Growth efficiency *Bacillus methanolicus* MGA3 is a Gram-positive, aerobic, thermotolerant and relatively fast growing facultatively methylotroph which shows a μ of 0.65 and 0.53 h^{-1} and q_S values of 30 and 36 C-mmol g_{CDW}^{-1} h^{-1} on methanol and D-mannitol as sole carbon and energy source, respectively [102,103]. The optimal growth temperature for *B. methanolicus* was determined to be around 50 – 55 °C [104,105].

Tool availability In contrast to other Bacilli, the genetic engineering toolbox for *B. methanolicus* is quite limited and thus restricts it efficient engineering [106]. Recently, a CRISPR interference (CRISPRi) system based on a catalytically deactivated Cas9 protein (dCas9) has been developed [107]. However, an efficient and robust gene deletion system is still pending. The whole genome sequence of *B. methanolicus* is available [108] and transcriptomics as well as metabolomics have already been applied to study the physiology of this promising bacterium [102,109].

Handling As a thermotolerant, restricted methylotroph *B. methanolicus* can be cultivated at elevated temperatures on methanol. Toxicity of methanol in combination with elevated temperature on the one hand favors auto sterility of the processes, and on the other hand complicates handling by necessary consideration of safety issues [110].

Tolerance *B. methanolicus* can cope with methanol concentrations of up to 2 M and grows at temperatures from 35 up to 60 °C which makes this bacterium highly interesting for industrial production [111].

Metabolic capability *B. methanolicus* is a so-called restricted methylotroph, which means that it can utilize beside methanol only D-mannitol, D-glucose, and D-arabitol as sources for energy and growth. In contrast to other strict methylotrophs the genome of *B. methanolicus* is equipped with the whole set of genes for the citric acid as well as the glyoxylate cycle, which are only weakly transcribed during growth on methanol [102]. Furthermore, *B. methanolicus* can grow in seawater [112] and has the ability to efficiently produce L-lysine and L-glutamate from methanol at elevated temperatures [104].

References

1 Volmer, J. *et al.* (2015) Guiding bioprocess design by microbial ecology. *Curr. Opin. Microbiol.* 25, 25–32

2 Yue, H. *et al.* (2014) A seawater-based open and continuous process for polyhydroxyalkanoates production by recombinant *Halomonas campaniensis* LS21 grown in mixed substrates. *Biotechnol. Biofuels* 7, 108

3 Lenzen, C. *et al.* (2019) High-Yield Production of 4-Hydroxybenzoate From Glucose or Glycerol by an Engineered *Pseudomonas taiwanensis* VLB120. *Front. Bioeng. Biotechnol.* 7, 130

4 Li, W.-J. *et al.* (2020) Unraveling 1,4-Butanediol Metabolism in *Pseudomonas putida* KT2440. *Front. Microbiol.* 11, 382

5 Claassens, N.J. *et al.* (2016) Harnessing the power of microbial autotrophy. *Nat. Rev. Microbiol.* 14, 692–706

6 Nikel, P.I. and de Lorenzo, V. (2018) *Pseudomonas putida* as a functional chassis for industrial biocatalysis: From native biochemistry to trans-metabolism. *Metab. Eng.* 50, 142–155

7 García-Granados, R. *et al.* (2019) Metabolic Engineering and Synthetic Biology: Synergies, Future, and Challenges. *Front. Bioeng. Biotechnol.* 7, 36

8 Lee, S.Y. *et al.* (2019) A comprehensive metabolic map for production of bio-based chemicals. *Nat. Catal.* 2, 18–33

9 Straathof, A.J.J. *et al.* (2019) Grand Research Challenges for Sustainable Industrial Biotechnology. *Trends Biotechnol.* 37, 1042–1050

10 Lee, S.Y. and Kim, H.U. (2015) Systems strategies for developing industrial microbial strains. *Nat. Biotechnol.* 33, 1061–1072

11 Weinstock, M.T. *et al.* (2016) *Vibrio natriegens* as a fast-growing host for molecular biology. *Nat. Methods* 13, 849–851

12 Lee, H.H. *et al.* (2019) Functional genomics of the rapidly replicating bacterium *Vibrio natriegens* by CRISPRi. *Nat. Microbiol.* 4, 1105–1113

13 Hoffart, E. *et al.* (2017) High Substrate Uptake Rates Empower *Vibrio natriegens* as Production Host for Industrial Biotechnology. *Appl. Environ. Microbiol.* 83, e01614-17

14 Wynands, B. *et al.* (2019) Streamlined *Pseudomonas taiwanensis* VLB120 Chassis Strains with Improved Bioprocess Features. *ACS Synth. Biol.* 8, 2036–2050

15 Wang, H.H. *et al.* (2009) Programming cells by multiplex genome engineering and accelerated evolution. *Nature* 460, 894–898

16 Isaacs, F.J. *et al.* (2011) Precise manipulation of chromosomes in vivo enables genome-wide codon replacement. *Science* 333, 348–353

17 Thoma, F. and Blombach, B. (2021) Metabolic engineering of *Vibrio natriegens*. *Essays Biochem.* DOI: 10.1042/EBC20200135

18 Rütering, M. *et al.* (2017) Tailor-made exopolysaccharides—CRISPR-Cas9 mediated genome editing in *Paenibacillus polymyxa*. *Synth. Biol.* 2, ysx007

19 Schilling, C. *et al.* (2020) Novel Prokaryotic CRISPR-Cas12a-Based Tool for Programmable Transcriptional Activation and Repression. *ACS Synth. Biol.* 9, 3353–3363

20 Meliawati, M. *et al.* (2021) Recent advances of Cas12a applications in bacteria. *Appl. Microbiol. Biotechnol.* 105, 2981–2990

21 Hoschek, A. *et al.* (2019) Mixed-species biofilms for high-cell-density application of *Synechocystis* sp. PCC 6803 in capillary reactors for continuous cyclohexane oxidation to cyclohexanol. *Bioresour. Technol.* 282, 171–178

22 Halan, B. *et al.* (2012) Biofilms as living catalysts in continuous chemical syntheses. *Trends Biotechnol.* 30, 453–465

23 Ankenbauer, A. *et al.* (2020) *Pseudomonas putida* KT2440 is naturally endowed to withstand industrial-scale stress conditions. *Microb. Biotechnol.* 13, 1145–1161

24 Noorman, H.J. and Heijnen, J.J. (2017) Biochemical engineering's grand adventure. *Chem. Eng. Sci.* 170, 677–693

25 Kampers, L.F.C. *et al.* (2019) *Pseudomonas putida* KT2440 is HV1 certified, not GRAS. *Microb. Biotechnol.* 12, 845–848

26 Schwanemann, T. *et al.* (2020) Pseudomonas as Versatile Aromatics Cell Factory. *Biotechnol. J.* 15, 1900569

27 Lisa Bitzenhofer, N. *et al.* (2021) Towards robust Pseudomonas cell factories to harbour novel biosynthetic pathways 2. *Essays Biochem.* in press,

28 Häßler, T. *et al.* (2012) Enhanced fed-batch fermentation of 2,3-butanediol by

Paenibacillus polymyxa DSM 365. *Bioresour. Technol.* 124, 237–244

29 Song, C.W. *et al.* (2019) Microbial production of 2,3-butanediol for industrial applications. *J. Ind. Microbiol. Biotechnol.* 46, 1583–1601

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

31 Mohamed, E.T. *et al.* (2020) Adaptive laboratory evolution of *Pseudomonas putida* KT2440 improves p-coumaric and ferulic acid catabolism and tolerance. *Metab. Eng. Commun.* 11, e00143

32 Cheng, C. *et al.* (2019) Laboratory evolution reveals a two-dimensional rate-yield tradeoff in microbial metabolism. *PLOS Comput. Biol.* 15, e1007066

33 Blaby, I.K. *et al.* (2012) Experimental Evolution of a Facultative Thermophile from a Mesophilic Ancestor. *Appl. Environ. Microbiol.* 78, 144 LP – 155

34 Jarzab, A. *et al.* (2020) Meltome atlas—thermal proteome stability across the tree of life. *Nat. Methods* 17, 495–503

35 Linger, J.G. *et al.* (2014) Lignin valorization through integrated biological funneling and chemical catalysis. *Proc. Natl. Acad. Sci.* 111, 12013 LP – 12018

36 Wendisch, V.F. *et al.* (2016) The flexible feedstock concept in Industrial Biotechnology: Metabolic engineering of *Escherichia coli*, *Corynebacterium glutamicum*, Pseudomonas, Bacillus and yeast strains for access to alternative carbon sources. *J. Biotechnol.* 234, 139–157

37 Müller, J.E.N. *et al.* (2015) Methylotrophy in the thermophilic *Bacillus methanolicus*, basic insights and application for commodity production from methanol. *Appl. Microbiol. Biotechnol.* 99, 535–551

38 Kiefer, D. *et al.* (2021) From Acetate to Bio-Based Products: Underexploited Potential for Industrial Biotechnology. *Trends Biotechnol.* 39, 397–411

39 Lange, L. *et al.* (2021) Developing a Sustainable and Circular Bio-Based Economy in EU: By Partnering Across Sectors, Upscaling and Using New Knowledge Faster, and For the Benefit of Climate, Environment & Biodiversity, and People & Business. *Front. Bioeng. Biotechnol.* 8, 1456

40 Claassens, N.J. *et al.* (2018) Towards sustainable feedstocks: A guide to electron donors for microbial carbon fixation. *Curr. Opin. Biotechnol.* 50, 195–205

41 Jayakody, L.N. *et al.* (2018) Thermochemical wastewater valorization via enhanced microbial toxicity tolerance. *Energy Environ. Sci.* 11, 1625–1638

42 Kim, S. *et al.* (2020) Growth of *E. coli* on formate and methanol via the reductive glycine pathway. *Nat. Chem. Biol.* 16, 538–545

43 Müller, J.E.N. *et al.* (2015) Engineering *Escherichia coli* for methanol conversion. *Metab. Eng.* 28, 190–201

44 Köpke, M. and Simpson, S.D. (2020) Pollution to products: recycling of 'above ground' carbon by gas fermentation. *Curr. Opin. Biotechnol.* 65, 180–189

45 Takors, R. *et al.* (2018) Using gas mixtures of CO, CO_2 and H_2 as microbial substrates: the do's and don'ts of successful technology transfer from laboratory to production scale. *Microb. Biotechnol.* 11, 606–625

46 Fast, A.G. and Papoutsakis, E.T. (2012) Stoichiometric and energetic analyses of non-photosynthetic CO_2-fixation pathways to support synthetic biology strategies for production of fuels and chemicals. *Curr. Opin. Chem. Eng.* 1, 380–395

47 Grenz, S. *et al.* (2019) Exploiting *Hydrogenophaga pseudoflava* for aerobic syngas-based production of chemicals. *Metab. Eng.* 55, 220–230

48 Siebert, D. *et al.* (2020) Genetic Engineering of *Oligotropha carboxidovorans* Strain OM5—A Promising Candidate for the Aerobic Utilization of Synthesis Gas. *ACS Synth. Biol.* 9, 1426–1440

49 Demain, A.L. (2000) Small bugs, big business: The economic power of the microbe. *Biotechnol. Adv.* 18, 499–514

50 Baeshen, N.A. *et al.* (2014) Cell factories for insulin production. *Microb. Cell Fact.* 13, 141

51 Ghosh, S. *et al.* (2016) Mixed consortia in bioprocesses: role of microbial interactions. *Appl. Microbiol. Biotechnol.* 100, 4283–4295

52 Blank, L.M. *et al.* (2008) Metabolic response of *Pseudomonas putida* during redox biocatalysis in the presence of a second octanol phase. *FEBS J.* 275, 5173–5190

53 Straub, C.T. *et al.* (2018) Biotechnology of extremely thermophilic archaea. *FEMS Microbiol. Rev.* 42, 543–578

54 Lee, S.Y. *et al.* (2005) Systems biotechnology for strain improvement. *Trends Biotechnol.* 23, 349–358

55 Chen, L.-X. *et al.* (2020) Accurate and complete genomes from metagenomes. *Genome Res.* 30, 315–333

56 Karnachuk, O. V *et al.* (2020) Targeted isolation based on metagenome-

assembled genomes reveals a phylogenetically distinct group of thermophilic spirochetes from deep biosphere. *Environ. Microbiol.* n/a,

57 Seaver, S.M.D. *et al.* (2021) The ModelSEED Biochemistry Database for the integration of metabolic annotations and the reconstruction, comparison and analysis of metabolic models for plants, fungi and microbes. *Nucleic Acids Res.* 49, D575–D588

58 Machado, D. *et al.* (2018) Fast automated reconstruction of genome-scale metabolic models for microbial species and communities. *Nucleic Acids Res.* 46, 7542–7553

59 Karp, P.D. *et al.* (2021) Pathway Tools version 23.0 update: software for pathway/genome informatics and systems biology. *Brief. Bioinform.* 22, 109–126

60 Lewis, N.E. *et al.* (2012) Constraining the metabolic genotype-phenotype relationship using a phylogeny of in silico methods. *Nat. Rev. Microbiol.* 10, 291–305

61 Zielinski, D.C. *et al.* (2020) The Expanding Computational Toolbox for Engineering Microbial Phenotypes at the Genome Scale. *Microorganisms* 8,

62 Cankorur-Cetinkaya, A. *et al.* (2017) CamOptimus: a tool for exploiting complex adaptive evolution to optimize experiments and processes in biotechnology. *Microbiology* 163, 829–839

63 Zelezniak, A. *et al.* (2018) Machine Learning Predicts the Yeast Metabolome from the Quantitative Proteome of Kinase Knockouts. *Cell Syst.* 7, 269-283.e6

64 Costello, Z. and Martin, H.G. (2018) A machine learning approach to predict metabolic pathway dynamics from time-series multiomics data. *npj Syst. Biol. Appl.* 4, 19

65 Helmy, M. *et al.* (2020) Systems biology approaches integrated with artificial intelligence for optimized metabolic engineering. *Metab. Eng. Commun.* 11, e00149

66 Antonakoudis, A. *et al.* (2020) The era of big data: Genome-scale modelling meets machine learning. *Comput. Struct. Biotechnol. J.* 18, 3287–3300

67 Presnell, K. V and Alper, H.S. (2019) Systems Metabolic Engineering Meets Machine Learning: A New Era for Data-Driven Metabolic Engineering. *Biotechnol. J.* 14, 1800416

68 Otto, M. *et al.* (2019) Targeting 16S rDNA for Stable Recombinant Gene Expression in Pseudomonas. *ACS Synth. Biol.* 8, 1901–1912

69 Ebert, B.E. *et al.* (2011) Response of *Pseudomonas putida* KT2440 to increased NADH and ATP demand. *Appl. Environ. Microbiol.* 77, 6597–6605

70 Wynands, B. *et al.* (2018) Metabolic engineering of *Pseudomonas taiwanensis* VLB120 with minimal genomic modifications for high-yield phenol production. *Metab. Eng.* 47, 121–133

71 Nogales, J. *et al.* (2020) High-quality genome-scale metabolic modelling of *Pseudomonas putida* highlights its broad metabolic capabilities. *Environ. Microbiol.* 22, 255–269

72 Nies, S.C. *et al.* (2020) High titer methyl ketone production with tailored *Pseudomonas taiwanensis* VLB120. *Metab. Eng.* 62, 84–94

73 Winsor, G.L. *et al.* (2016) Enhanced annotations and features for comparing thousands of Pseudomonas genomes in the Pseudomonas genome database. *Nucleic Acids Res.* 44, D646–D653

74 Wierckx, N. *et al.* (2009) Metabolic flux analysis of a phenol producing mutant of *Pseudomonas putida* S12: Verification and complementation of hypotheses derived from transcriptomics. *J. Biotechnol.* 143, 124–129

75 Borrero-de Acuña, J.M. *et al.* (2014) Production of medium chain length polyhydroxyalkanoate in metabolic flux optimized *Pseudomonas putida*. *Microb. Cell Fact.* 13, 88

76 Otto, M. *et al.* (2019) Rational Engineering of Phenylalanine Accumulation in *Pseudomonas taiwanensis* to Enable High-Yield Production of Trans-Cinnamate. *Front. Bioeng. Biotechnol.* 7, 312

77 Volmer, J. *et al.* (2019) Constitutively solvent-tolerant *Pseudomonas taiwanensis* VLB120Δ CΔ ttgV supports particularly high-styrene epoxidation activities when grown under glucose excess conditions. *Biotechnol. Bioeng.* 116, 1089–1101

78 Wierckx Nick J.P. *et al.* (2005) Engineering of Solvent-Tolerant *Pseudomonas putida* S12 for Bioproduction of Phenol from Glucose. *Appl. Environ. Microbiol.* 71, 8221–8227

79 Heerema, L. *et al.* (2011) In situ phenol removal from fed-batch fermentations of solvent tolerant *Pseudomonas putida* S12 by pertraction. *Biochem. Eng. J.* 53, 245–252

80 Heipieper, H.J. *et al.* (2007) Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. *Appl. Microbiol. Biotechnol.* 74, 961–973

81 Nikel, P.I. *et al.* (2021) Reconfiguration of metabolic fluxes in *Pseudomonas*

putida as a response to sub-lethal oxidative stress. *ISME J.* DOI: 10.1038/s41396-020-00884-9

82 Kohlstedt, M. *et al.* (2018) From lignin to nylon: Cascaded chemical and biochemical conversion using metabolically engineered *Pseudomonas putida*. *Metab. Eng.* 47, 279–293

83 Utomo, R.N.C. *et al.* (2020) Defined Microbial Mixed Culture for Utilization of Polyurethane Monomers. *ACS Sustain. Chem. Eng.* 8, 17466–17474

84 Verhoef, S. *et al.* (2009) Bioproduction of p-hydroxystyrene from glucose by the solvent-tolerant bacterium *Pseudomonas putida* S12 in a two-phase water-decanol fermentation. *Appl. Environ. Microbiol.* 75, 931–936

85 Verhoef, S. *et al.* (2007) Bioproduction of p-hydroxybenzoate from renewable feedstock by solvent-tolerant *Pseudomonas putida* S12. *J. Biotechnol.* 132, 49–56

86 Wery, J. *et al.* (2000) A genetically modified solvent-tolerant bacterium for optimized production of a toxic fine chemical. *Appl. Microbiol. Biotechnol.* 54, 180–185

87 Karande, R. *et al.* (2018) Biocatalytic conversion of cycloalkanes to lactones using an in-vivo cascade in *Pseudomonas taiwanensis* VLB120. *Biotechnol. Bioeng.* 115, 312–320

88 Long, C.P. *et al.* (2017) Metabolism of the fast-growing bacterium *Vibrio natriegens* elucidated by 13C metabolic flux analysis. *Metab. Eng.* 44, 191–197

89 Fernández-Llamosas, H. *et al.* (2017) Speeding up bioproduction of selenium nanoparticles by using *Vibrio natriegens* as microbial factory. *Sci. Rep.* 7, 16046

90 Coyer, J.A. *et al.* (1996) N_2 fixation in marine heterotrophic bacteria: dynamics of environmental and molecular regulation. *Proc. Natl. Acad. Sci.* 93, 3575 LP – 3580

91 Conley, B.E. *et al.* (2020) A Hybrid Extracellular Electron Transfer Pathway Enhances the Survival of *Vibrio natriegens*. *Appl. Environ. Microbiol.* 86, e01253-20

92 Schilling, C. *et al.* (2020) Engineering of the 2,3-butanediol pathway of *Paenibacillus polymyxa* DSM 365. *Metab. Eng.* 61, 381–388

93 C Schilling, MAG Koffas, V Sieber, J.S. (2020) Novel Prokaryotic CRISPR-Cas12a Based Tool for Programmable Transcriptional Activation and Repression. *ACS Synth. Biol.* 9, 3353–3363

94 Adlakha, N. *et al.* (2015) Insight into metabolic pathways of the potential biofuel producer, *Paenibacillus polymyxa* ICGEB2008. *Biotechnol. Biofuels* 8, 159

95 Grady, E.N. *et al.* (2016) Current knowledge and perspectives of Paenibacillus: a review. *Microb. Cell Fact.* 15, 203

96 Jeong, H. *et al.* (2019) Chronicle of a Soil Bacterium: *Paenibacillus polymyxa* E681 as a Tiny Guardian of Plant and Human Health. *Front. Microbiol.* 10, 467

97 Song, C.W. *et al.* (2021) CRISPR-Cas9 mediated engineering of *Bacillus licheniformis* for industrial production of (2R,3S)-butanediol. *Biotechnol. Prog.* 37, e3072

98 Yang, T. *et al.* (2013) Improved Production of 2,3-Butanediol in *Bacillus amyloliquefaciens* by Over-Expression of Glyceraldehyde-3-Phosphate Dehydrogenase and 2,3-butanediol Dehydrogenase. *PLoS One* 8,

99 Yan, P.-F. *et al.* (2017) Production of High Levels of Chirally Pure d-2,3-Butanediol with a Newly Isolated Bacillus Strain. *ACS Sustain. Chem. Eng.* 5, 11016–11023

100 Weselowski, B. *et al.* (2016) Isolation, identification and characterization of *Paenibacillus polymyxa* CR1 with potentials for biopesticide, biofertilization, biomass degradation and biofuel production. *BMC Microbiol.* 16, 244

101 Pasari, N. *et al.* (2019) Genome analysis of *Paenibacillus polymyxa* A18 gives insights into the features associated with its adaptation to the termite gut environment. *Sci. Rep.* 9, 6091

102 Delépine, B. *et al.* (2020) Charting the Metabolic Landscape of the Facultative Methylotroph *Bacillus methanolicus*. *mSystems* 5, e00745-20

103 Bozdag, A. *et al.* (2015) Growth of *Bacillus methanolicus* in 2 M methanol at 50 °C: the effect of high methanol concentration on gene regulation of enzymes involved in formaldehyde detoxification by the ribulose monophosphate pathway. *J. Ind. Microbiol. Biotechnol.* 42, 1027–1038

104 Brautaset, T. *et al.* (2007) *Bacillus methanolicus*: a candidate for industrial production of amino acids from methanol at 50°C. *Appl. Microbiol. Biotechnol.* 74, 22–34

105 Brautaset, T. *et al.* (2004) Plasmid-dependent methylotrophy in thermotolerant *Bacillus methanolicus*. *J. Bacteriol.* 186, 1229–1238

106 Irla, M. *et al.* (2016) Genome-Based Genetic Tool Development for *Bacillus methanolicus*: Theta- and Rolling Circle-Replicating Plasmids for Inducible Gene Expression and Application to Methanol-Based Cadaverine Production. *Front.*

Microbiol. 7, 1481

107 Schultenkämper, K. *et al.* (2019) Establishment and application of CRISPR interference to affect sporulation, hydrogen peroxide detoxification, and mannitol catabolism in the methylotrophic thermophile *Bacillus methanolicus*. *Appl. Microbiol. Biotechnol.* 103, 5879–5889

108 Heggeset, T.M.B. *et al.* (2012) Genome sequence of thermotolerant *Bacillus methanolicus*: features and regulation related to methylotrophy and production of L-lysine and L-glutamate from methanol. *Appl. Environ. Microbiol.* 78, 5170–5181

109 Carnicer, M. *et al.* (2016) Quantitative metabolomics of the thermophilic methylotroph *Bacillus methanolicus*. *Microb. Cell Fact.* 15, 92

110 Chen, A.Y. and Lan, E.I. (2020) Chemical Production from Methanol Using Natural and Synthetic Methylotrophs. *Biotechnol. J.* 15, 1900356

111 Drejer, E.B. *et al.* (2020) Methanol-based acetoin production by genetically engineered *Bacillus methanolicus*. *Green Chem.* 22, 788–802

112 Komives, C.F. *et al.* (2005) Growth of *Bacillus methanolicus* in seawater-based media. *J. Ind. Microbiol. Biotechnol.* 32, 61–66