Acinetobacter baylyi ADP1 – naturally competent for synthetic biology

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Abstract

Acinetobacter baylyi ADP1 is a non-pathogenic soil bacterium known for its metabolic diversity and high natural transformation and recombination efficiency. For these features, *A. baylyi* ADP1 has been long exploited in studying bacterial genetics and metabolism. The large pool of information generated in the fundamental studies has facilitated the development of a broad range of sophisticated and robust tools for the genome and metabolic engineering of ADP1. This mini-review outlines and describes the recent advances in ADP1 engineering and tool development, exploited in for example pathway and enzyme evolution, genome reduction and stabilization, and for the production of native and non-native products in both pure and rationally designed multispecies cultures. The rapidly expanding toolbox together with the unique features of *A. baylyi* ADP1 provide a strong base for a microbial cell factory excelling in synthetic biology applications where evolution meets rational engineering.

1. Introduction

The latest advances in biotechnology and synthetic biology have enabled the production of a vast number of industrially relevant compounds by genetically tailored microbial cell factories [1]. The strains assigned for the job are expected to possess a set of relevant features, such as genetic tractability, fast growth,

simple growth requirements, and the availability of genetic tools. Due to the stringent requirements for comprehensive carbon recycling and sustainability, the ability to utilize the previously untapped but abundant carbon sources is also considered a highly important trait. Therefore, strains exhibiting interesting metabolic features, such as *Clostridium ljungdahlii* [2], *Rhodococcus opacus* [3], and *Pseudomonas putida* [4, 5], are gaining more ground as industrially relevant hosts. However, as the scope of synthetic biology continuously expands, and the pathways from substrates to products get more complex and wide-ranging [6], the possibility for straight-forward, reliable, and fast genome engineering becomes an increasingly important feature of a microbial cell factory.

Acinetobacter baylyi ADP1 (later ADP1) is a ubiquitous, fast-growing, non-pathogenic, and a strictly aerobic soil bacterium with a diverse metabolism and a compact, tractable genome. The strain exhibits interesting features not existing in the most widely used workhorses, namely *Escherichia coli* and *Saccharomyces cerevisiae*. For example, ADP1 can tolerate and grow on industrially relevant non-sugar carbon sources, such as lignin-derived aromatic compounds and organic acids, and produce both bulk and high-value products from them. The most striking feature of ADP1, however, is its tendency to undergo natural transformation and homologous recombination at exceptionally high rates. This extremely beneficial characteristic has enabled the fast development of smart, efficient, and novel type of engineering approaches and applications discussed in this mini-review.

Despite the distinctive features of *Acinetobacter,* its prevalence in scientific literature and biotechnology applications has remained relatively modest. *E. coli*, for example, was discovered already in 1885, so it became a reference organism for which many genetic tools were developed; ADP1 wasn't discovered until 1960 so it was less amenable to genetic manipulation until a comparable set of genetic tools, described herein, was developed. Nevertheless, the recent increase in the number of the tools and emerging applications of ADP1 shows that the unique features and the potential of this strain are no longer overlooked.

2. Enabling tools developed for and by the ADP1 platform

The research on *A. baylyi* ADP1, or more precisely, its features started when Taylor & Juni isolated its parental strain BD4 from soil using 2,3-butanediol for selective enrichment [7]. The strain BD4 which produced a heavy exopolysaccharide (EPS) capsule was subjected to UV mutagenesis resulting in a strain with a small EPS capsule. This strain, designated as BD413, is currently known as ADP1. The early studies already revealed the extraordinary competence for natural transformation [8, 9], which was later confirmed to be an exclusive trait of *A. baylyi* strains [10]. The sequence of the compact 3.6 Mbp genome with 3325 predicted coding sequences and 40.3% GC-content was published in 2004 [11]. Following the published genome sequence, a genome-wide metabolic model [12], a complete single-gene knock-out mutant library [13], and the experimental annotation of all ADP1 genes by a systematic and phenotypic characterization [14] were established. These studies provided a strong base for further genetic and metabolic investigations, as well as the development of molecular tools and engineering strategies for ADP1 (Figure 1).

2.1 Genetic and genomic engineering of ADP1

For more than 30 years, ADP1 served primarily as a model system for studying bacterial genetics, metabolism, and horizontal gene transfer [15-24], which facilitated the development of standard protocols for deletions and insertions in ADP1 using integrative gene cassettes and counterselection methods, such as those based on AZT/*tdk* and sucrose/*sacB* [19]. Notably, the straight-forward methodology and the exceptionally high transformation efficiency have allowed the establishment of automated, high-throughput cloning platforms, as no purification or electroporation steps are required [13, 25]. The widely used CRISPR/Cas9 systems have been also established in ADP1 for scarless genomic integration [26] and dCas9-based gene silencing [27]. Also, the native Type I–F CRISPR/Cas system of ADP1 has been harnessed for genome editing and stabilization, aided by simplified construction of natural multiplex CRISPR-arrays [28]. In addition, elegant tools derived from modern techniques, such as ‘Golden

Transformation' derived from Golden Gate Assembly and 'CRISPR-Lock' also exploiting ADP1's native CRISPR/Cas system to secure the engineered genotype, were recently developed to further facilitate simple multi-component DNA assembly and rapid and reliable genome engineering [29].

2.2 Expression tools

Due to the convenience of the genomic integration, the use of few plasmids in ADP1 has been reported. The most well-characterized expression vectors for ADP1 are based on the efficiently replicating and stable pBAV1 (BioBrick Accepting Vector) backbone [30, 31], although others, such as the cryptic plasmid pWH1277 [32] is maintained in ADP1. In addition, plasmids with RSF1010 origin replicate stably in ADP1 [27], allowing the use of well-established vector platforms, such as the Standard European Vector Architecture (SEVA) [33]. For the regulation of expression, Biggs et al. [26] recently constructed a comprehensive genetic toolset for ADP1 comprising synthetic constitutive promoter and RBS libraries. In addition, 27 chromosomal sites (a greater number than in any other bacterial host) were investigated and validated for protein expression. Also, many of the well-established inducible promoter systems, such as LacI/lacO, AraC/pBAD, and the cyclohexanone-inducible ChnR/pChnB are functional in ADP1 [31, 34].

2.3 Tools for pathway and strain development

The genomic plasticity of ADP1, perceivable as spontaneous mutations and gene duplications or rearrangements during adaptation to new environments, have been harnessed for enhancing enzymatic activities and existing or new pathways. Tumen-Velasquez et al. [35] developed a highly efficient method, Evolution by Amplification and Synthetic biology (EASy), which facilitates beneficial genetic modifications to overcome bottle-necks and inefficiencies of catabolic pathways. The method allows the amplification of specific chromosomal segments and rapid accumulation of beneficial mutations in the target sequences to achieve increased or altered catabolic activity. For example, the EASy method has been employed to introduce and evolve enzymatic activities that enable guaiacol [35] and terephthalic acid [36] utilization in ADP1, thus further expanding the broad substrate range relevant on application.

Despite that the genomic plasticity serves as a valuable asset in targeted application, this feature can also lead to unwanted evolution, which can be counterproductive in production hosts due to the risk of evolutionary failure, i.e. the loss of the engineered functionalities. For example, transposable insertion sequences (IS) have major contribution in spontaneous mutations, genomic rearrangements, and loss of transformability in ADP1 [37]. Suárez et al. [38] deleted all the copies of the IS elements in ADP1, resulting in not only significantly reduced mutation rates, but also increased transformation efficiency in the transposon-free ADP1-ISx strain. Subsequently, the approach was developed into a generalizable method to increase evolutionary stability, CRISPR interference against mobile elements (CRISPRi-ME), by constructing a broad-host-range vector carrying CRISPR interference tools for targeting multiple genetic elements that can contribute to deleterious mutations and evolutionary failure [27].

Insertion sequences are not the only elements that potentially decrease the host performance. Eliminating redundant, unnecessary, or potentially hazardous genes [39] can help to improve the genomic stability and fitness of microbial cell factories. Also, ADP1 genome is widely clustered into operons and 'catabolic islands', which provides a convenient starting point for genome streamlining. Suárez et al. [29] deleted 18 multi-gene regions from the previously constructed transposon-free genome, each ranging from 21 to 183 kb, exploiting the above-mentioned Golden Transformation and CRISPR-Lock system, as well as transposon insertion sequencing (Tn-Seq) to determine gene essentiality. In total, the deleted regions accounted for 23.4% of ADP1 genome. Future work will hopefully entail the combinatorial optimization of deleted regions and the demonstration of their effect on industrially-relevant physiological traits, such as expression of non-native activities, stability, and fitness.

3. Metabolic engineering for improved production of native and non-native products

In addition to the interesting catabolic pathways, ADP1 possesses a fruitful cellular framework for the production of both native and non-native biocompounds from a variety of carbon sources (Figure 2); ADP1

naturally produces storage compounds, such as triacylglycerols (TAG) and wax esters (WE), which can facilitate the overproduction of other acetyl-CoA -derived products. However, one of the first examples of metabolic engineering of ADP1 was related to the improved production of cyanophycin from amino acids. Engineering the arginine metabolism and the PhoB/PhoR phosphate regulon system by gene knock-outs and overexpression resulted in increased cyanophycin content up to 30% of cell dry weight (CDW) [40]. In the next subchapters, the metabolic engineering of both native and non-native pathways in ADP1 is discussed, the focus being on long chain hydrocarbons and lipids.

3.1 Triacylglycerols and wax esters

The storage lipids TAG and WE are fatty acid (FA) derived industrially relevant products, that can be broadly used in for example biofuels, lubricants, cosmetics, coatings, and for nutritional and pharmaceutical purposes. By definition, *Acinetobacter* strains are not oleaginous, which means that their cellular lipid content does not naturally exceed 20%. However, various engineering strategies have been employed to improve the lipid production metrics. For example, the effect of gene knock-outs on TAG synthesis was studied by first conducting metabolic modelling followed by the experimental validation of the model predictions by single and multiple gene knock-outs [41]. As a result, the cellular TAG yield was improved by several folds, but the overall production metrics were still low. In recent studies, the focus has been on the production of WEs, for which ADP1 has notable potential [42].

Wax esters are oxoesters of long-chain fatty acids (FA) esterified with long-chain alcohols. The synthesis pathway in ADP1 involves three steps; first, the fatty acyl coenzyme A (acyl-CoA) is reduced to the corresponding aldehyde by an NADPH-dependent acyl-CoA reductase Acr1 [43]. The long-chain aldehyde is further reduced to the corresponding alcohol by a yet uncharacterized aldehyde reductase. In the last step, the fatty alcohol is esterified with a fatty acyl-CoA by an acyl-CoA:fatty alcohol acyltransferase (wax ester synthase) of a bifunctional enzyme WS/DGAT [44]. ADP1 naturally produces mainly C16-C18 FA and alcohol moieties, the total average size of a WE molecule being C33-34 with a single double bond [45].

144 However, it has been possible to alter the WE profile by replacing the fatty acyl-CoA reductase Acr1 with
145 a fatty acid reductase complex LuxCDE from *Photorhabdus luminescens* [46].

146 The wild type ADP1 typically produces 3-8% WEs of CDW from glucose or gluconate. To study the pathway
147 activity, a bioluminescence-based sensor for real-time monitoring of the WE synthesis pathway was
148 developed [47]. The reduction of fatty acyl-CoA to fatty aldehyde was shown to have a key role in the
149 synthesis pathway, and the sensor was employed for the selection of the most optimal reductase and
150 tuning its expression level [45]. Luo et al. combined the overexpression of the native reductase Acr1 with
151 the elimination of isocitrate lyase AceA, which further increased the production of WEs to 27% of CDW
152 (1.82 g L^{-1}), the highest reported cellular WE yield [48]. In the same study, WE production was also
153 demonstrated from unconventional, nitrogen-rich carbon sources, namely amino acids and yeast biomass
154 [48].

155 Despite the highest WE production metrics thus far have been obtained with glucose, glycolytic substrates
156 in general are not optimal for ADP1 in terms of the growth and production yield. In ADP1, glucose and
157 gluconate enter metabolism through a modified Entner-Doudoroff pathway, where they form one
158 glyceraldehyde-3-phosphate (G-3-P) and one pyruvate molecule, with the former being unable to directly
159 contribute to acetyl-CoA derived products [11, 49]. Instead, G-3-P is readily directed to the synthesis of
160 exopolysaccharides (EPS) which can form up to 20% of CDW. Extensive production of EPS also complicates
161 downstream processing and cell harvesting [50]. However, the complete elimination of the EPS synthesis
162 pathway can result in reduced growth rate and cell aggregation [51]. To prevent the loss of carbon and to
163 improve the growth rate of ADP1 on glucose and gluconate, Kannisto et al. [49] introduced pyruvate
164 kinase pykF from *E. coli* to enable the conversion of phosphoenolpyruvate to pyruvate. The specific
165 growth-rates of pykF-expressing cells increased from 0.18 h^{-1} to 0.42 h^{-1} while simultaneously maintaining
166 the biomass and WE production yields, demonstrating the potential of using ADP1 also in glucose-based
167 applications.

Although glucose is a still an important carbon source in biotechnological processes, due to societal and economical reasons, using more sustainable carbon sources is of interest. Acetic acid, for example, is readily available, inexpensive, and readily metabolized by ADP1. In fact, ADP1 has a higher growth rate on acetate than on glucose [52]. As acetate is primarily directed to growth in ADP1, Santala et al. developed an autonomously regulated switch to decouple growth from WE synthesis and to intensify the production of WEs from acetate [53]. The shift from growth-mode to WE synthesis-mode was achieved by placing a growth-essential gene, isocitrate lyase *aceA*, under an arabinose-inducible promoter. Although arabinose cannot be used as a carbon source in ADP1, it is progressively oxidized to inactive form by a native glucose dehydrogenase causing a gradual and timed decrease of *aceA* expression and growth. This in turn, forces cells to direct the acetate towards the WE synthesis pathway. Bypassing the native regulation of lipid synthesis allowed the engineered strain to accumulate 19% of CDW as WEs, which was a fourfold improvement to wild type cells. Recently, also a kinetic metabolic model was constructed to simulate the function of the constructed dynamic switch [54].

Despite the improvements achieved by the dynamic regulation described above, the engineered cells exhibited reduced growth and volumetric productivity, likely due to the limited supply of energy and reducing equivalents. In order to address the fundamental issues of both unbalanced supply of biochemical resources and the trade-off between growth and production typical in lipid synthesis, Santala et al. used metabolic engineering and synergistic substrate cofeeding to partition the metabolism of ADP1 into two distinct modules, each dedicated to cell growth and WE biosynthesis, respectively (Santala, Santala, Liu, Stephanopoulos: *manuscript under review*). Combining the advantages of each approach, it was possible to maximize the synthesis of WEs during active cell growth. As a result, 7.2- and 4.2-fold improvements in cellular WE content and productivity were obtained, respectively, and the product titer was enhanced by 8.3-fold over the wild type strain. Notably, a yield of 18% C-WE/C-total-substrates was achieved, being the highest reported for WE biosynthesis.

3.2 Alkanes and 1-alkenes

ADP1 can naturally utilize long chain hydrocarbons, such as C12-C18 alkanes as sole carbon sources [55, 56]. As hydrocarbons are desirable products in chemical and fuel industry, this feature was reversed in ADP1 to establish the heterologous production of alkanes. Development of a twin-layer metabolic biosensor which simultaneously monitors the key pathway intermediates, aldehydes, and the final products, alkanes, allowed the construction of a functional pathway employing the fatty acyl-ACP reductase and aldehyde-deformylating oxygenase from *Synechococcus elongatus* [57]. The sensor was subsequently exploited to balance the relative expression levels of the pathway enzymes, resulting in the production of 540 µg/L heptadecane from acetate [58]. More recently, the production of alkanes directly from lignin-related aromatic compounds, namely p-coumarate, was demonstrated in ADP1 [59].

1-Alkanes, or alpha olefins (AO), are interesting platform chemicals as they can be derived to a variety of products. Due to their hydrophobicity and volatility, AOs can be continuously recovered from the culture vessels, thus significantly simplifying the cost-intensive product recovery and purification. ADP1 was previously engineered for the production of AOs, namely 1-undecene, by the heterologous expression of 'TesA from *E. coli* and UndA from *P. putida*. Adaptive laboratory evolution was carried out to improve the growth and tolerance of ADP1 against aromatic compounds, allowing the production 1-undecene using high concentrations of ferulate, another major representative of lignin monomers, as a sole carbon source [34]. The genetic changes in the adapted strains have been investigated to increase the understanding about the mechanisms behind the improved tolerance and growth (Luo et al.; manuscript in preparation). In another study, the direct collection of 1-undecene from a bioprocess using lignocellulosic substrates was demonstrated by a continuous gas-stripping-condensation device, which highlighted the straight-forward recovery and purity of the

produced AOs [60]. However, like in the case of alkanes, the production metrics for AOs have been thus far modest, up to 700 μg L^{-1} [34].

4. Multi-species approaches employing ADP1

Synthetic microbial consortia are a promising new approach in synthetic biology as they exhibit improved robustness and functional diversity in terms of task performance and substrate utilization. Several studies have demonstrated the benefits and potential of employing ADP1 in synthetic cocultures and other multispecies approaches (Figure 3). For example, the synthetic cocultures of *E. coli* and ADP1 have shown that the rationally engineered consortia can improve growth, production, and stability of engineered systems [61, 62]. In addition, ADP1 has been shown to be a convenient bacterial host for the detoxification of lignocellulosic hydrolysates; in a coculture of ADP1 and *Clostridium butyricum*, the sugar-utilizing pathway of ADP1 was eliminated by removing the glucose dehydrogenase encoded by *gcd*. The engineered ADP1 rapidly consumed acetate, formate, 4-hydroxybenzoate, and oxygen, allowing the subsequent fermentation of lignocellulosic sugars to hydrogen by the strict anaerobe *C. butyricum* [52]. In another example, the pathways responsible for the aromatics catabolism were engineered in two glucose-negative ADP1 strains to simultaneously remove common growth inhibitors, 4-hydroxybenzoate and benzoate, prior to the fermentation of glucose to ethanol by *Kluyveromyces marxianus* [63].

The multispecies approach has been also employed in the production of WEs and alkanes using CO2 as a sole carbon source; in the two-stage system, acetogenic bacteria first produced acetate from CO2 and electricity (or hydrogen), followed by the upgrading of acetate to WEs [64] or alkanes [58] by ADP1. Conversely, Salmela et al. showed that strictly aerobic and anaerobic bacteria can be cultivated in 'one-pot' to optimize the energy and carbon recovery. In the coculture, *C. butyricum* produced hydrogen from glucose while glucose-negative ADP1 consumed the remaining acetate and butyrate for the production of WEs [65]. By a similar approach, Mangayil et al. produced 1,3-propanediol and WEs from crude glycerol

by *C. butyricum* and ADP1, respectively [66]. Recently, the production of AOs was established from cellulose and authentic lignin hydrolysate by a two-stage culture of *C. cellulolyticum* and ADP1 [60].

Some native ADP1 features can be counterproductive in some coculture applications. For example, Cooper et al. showed that when cells are in physical contact (e.g. in biofilms), ADP1 uses a contact-dependent type-VI secretion system (T6SS) to lyse and acquire genes from neighboring cells [67]. This feature is potentially detrimental for the cocultured cells, and the unintended exchange of genes can cause further problems. However, these issues can be readily overcome by the elimination of T6SS and for example one of the competence-essential genes by single gene deletions [20, 67].

5. Conclusions and future outlook

A. baylyi ADP1, also rightfully referred as 'the sexiest organism on earth' [68] (for being highly competent and a soil bacterium), is slowly but steadily emerging as a new alternative microbial cell factory. In recent years, the molecular toolset for the strain improvement and engineering has significantly expanded, facilitating the further development of ADP1 as a relevant host. The major strengths of ADP1 are the highly versatile metabolism and the unique genome engineering opportunities, which can contribute to the emergence of novel applications involving previously unattainable substrates and products. Moreover, the fast growth and divergent substrate preference justify the use of ADP1 in synthetic cocultures and other multispecies approaches. The production of non-native products, such as alkanes and 1-alkenes, has been demonstrated in ADP1, but the reported production metrics have been low compared to more established hosts. However, the strain has shown strong potential for the production of wax esters; the cellular yields in engineered strains well exceed 20%, and sustainable and abundant non-sugar carbon sources can be used as the substrates for the production. Nevertheless, scale-up cultivations are yet to be conducted to evaluate the strain's potential in industrial applications. Therefore, bioprocess development and optimization would be the logical next steps in promoting ADP1 as a host for the applications of

synthetic biology and industrial biotechnology, for which the developed strains, tools, and engineering strategies serve as an excellent starting point.

Summary points

- The metabolic diversity and straight-forward genome engineering promote the use of *Acinetobacter baylyi* ADP1 as a microbial cell factory
- Broad range of tools for *A. baylyi* ADP1 engineering and strain development has been recently established
- *A. baylyi* ADP1 shows promise for the production of lipids and other acetyl-CoA derived products from challenging carbon sources, such as lignin-derived aromatics and organic acids
- *A. baylyi* ADP1 is well-suited for applications employing a multispecies approach
- Bioprocess development and scale-up are the next steps in advancing *A. baylyi* ADP1-based systems

Author contributions

SS and VS structured and wrote the manuscript. Both authors approved the final version of the manuscript.

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

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Figure legends

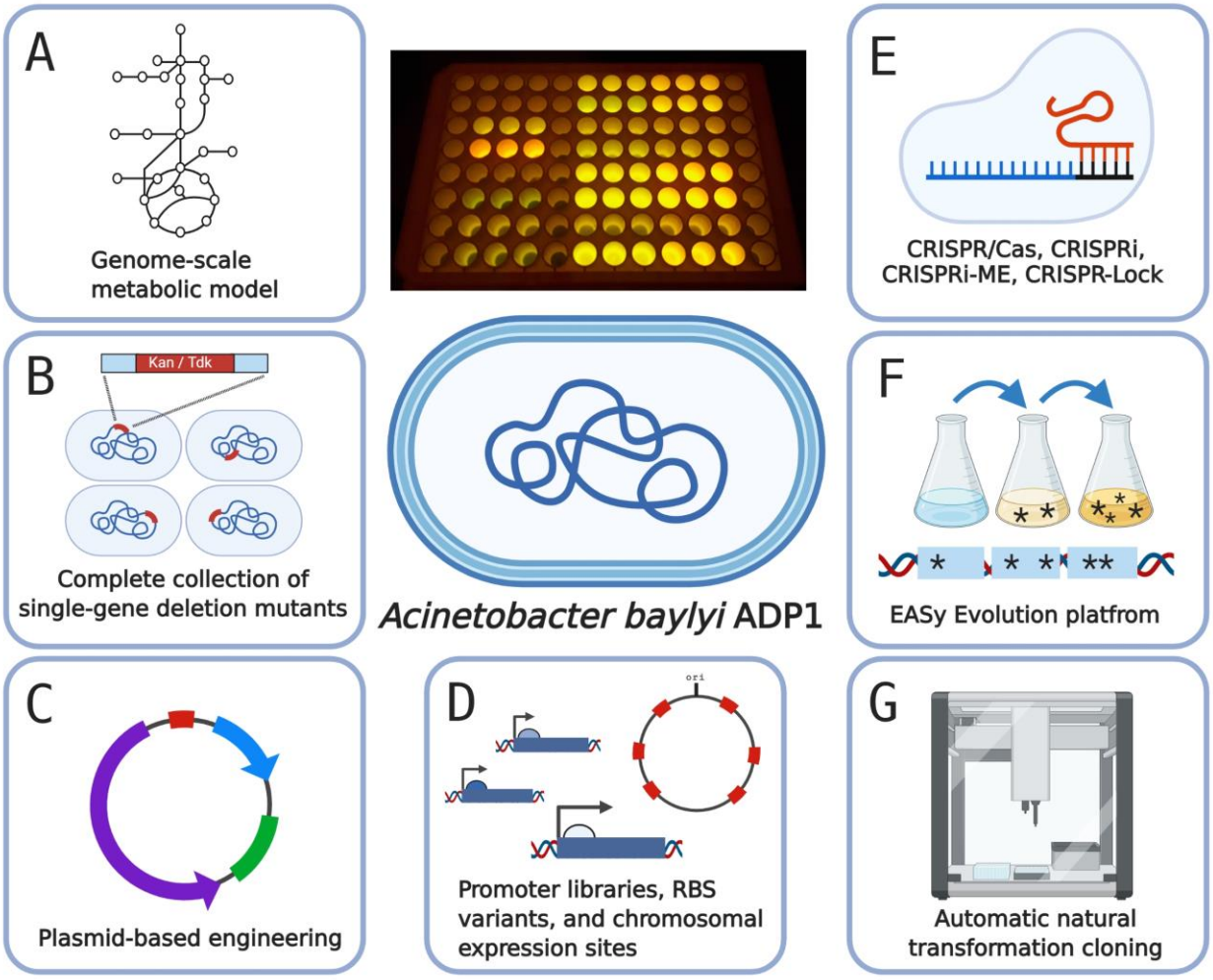
Figure 1. Tools for A. baylyi ADP1 engineering and development. *Based on the genome sequence [11] of ADP1, A) genome-scale metabolic model [12] was constructed and later enhanced by experimental annotation of genes [14]. B) Complete collection of single-gene knock-out mutants [13] utilizing genomic integration cassettes [19] was built to provide further information about gene essentiality and function. C) Plasmid-based engineering tools (such pBAV1 system [30, 31]) are available for ADP1, although the tool development has largely focused on genomic engineering; For expression purposes, great number of chromosomal sites have been tested and validated, along with the construction of a promoter and RBS libraries [26]. In addition, several E) CRISPR-based tools have been designed and implemented in ADP1, such as a one-step CRISPR/Cas9 for constructing scarless genomic modifications and CRISPRi for gene silencing [26]. In addition, for genetic stabilization and streamlining of ADP1, methods called CRISPRi-ME [27] targeting mobile elements and CRISPR-Lock [29] for securing genomic deletions have been developed among other tools exploiting the ADP1's natural CRISPR array [28]. F) Evolution by Amplification and Synthetic biology (EASy) platform has been utilized for developing enzymes and catabolic pathways for enhanced and novel activities [35]. G) The high efficiency of natural transformation and homologous recombination have allowed the establishment of automated cloning platforms for high-throughput genome engineering [25].*

Figure 2. *Production of native and non-native products by engineered A. baylyi ADP1. A variety of substrates, including lignin derived molecules (ferulate, p-coumarate, caffeate), organic acids (acetate, lactate, butyrate, gluconate), glucose, and amino acids have been used for the production of native (cyanophycin [40], triacylglycerols [41], wax esters [45, 46, 48, 49, 51, 53, 59]) and non-native (alkanes [57, 59], 1-alkenes [34]) products. For pathway optimization, decoupled growth and synthesis, and*

controlled distribution of resources, new metabolic engineering approaches have been developed, including intracellular metabolic biosensors [47, 57], autonomously regulated switch [53], and partitioning of metabolism.

Figure 3. Multi-species approaches utilizing A. baylyi ADP1. *A) Rationally engineered synthetic coculture of E. coli and glucose-negative A. baylyi ADP1 was shown to improve the culture performance in terms of substrate utilization, growth, and production [62]; When both strains were engineered to be glucose-negative (by deleting genes encoding for phosphotransferase system enzyme I (ptsI) and gluconate permease (gntT) in E. coli and ADP1, respectively), interconnected carbon cross-feeding allowed controlled population growth and stabilized production [61]. B) Glucose-negative ADP1 strains have been employed for the detoxification of lignocellulose hydrolysates or respective model compounds: utilization of formate, acetate, and 4-hydroxybenzoate by engineered ADP1 allowed the growth and hydrogen production by Clostridium butyricum [52], while the simultaneous removal of benzoate and 4-hydroxybenzoate by a consortium of engineered ADP1 strains enabled the fermentation of glucose to ethanol by Kluyveromyces marxianus [63]. C) Multispecies approaches have enabled the upgrading of substrates not attainable by ADP1 alone: In a two-stage cultures of acetogenic strains and ADP1, carbon dioxide and electricity/H2 were upgraded to wax esters [64] or alkanes [58]; crude glycerol was fermented to acetate and butyrate (along with 1,3-propanediol) by C. butyricum and further upgraded to wax esters by ADP1 [66]; C. cellulolyticum fermented cellulose to glucose, acetate, and lactate, which were subsequently upgraded to 1-alkenes by engineered ADP1 [60].*

493 Figure 1.



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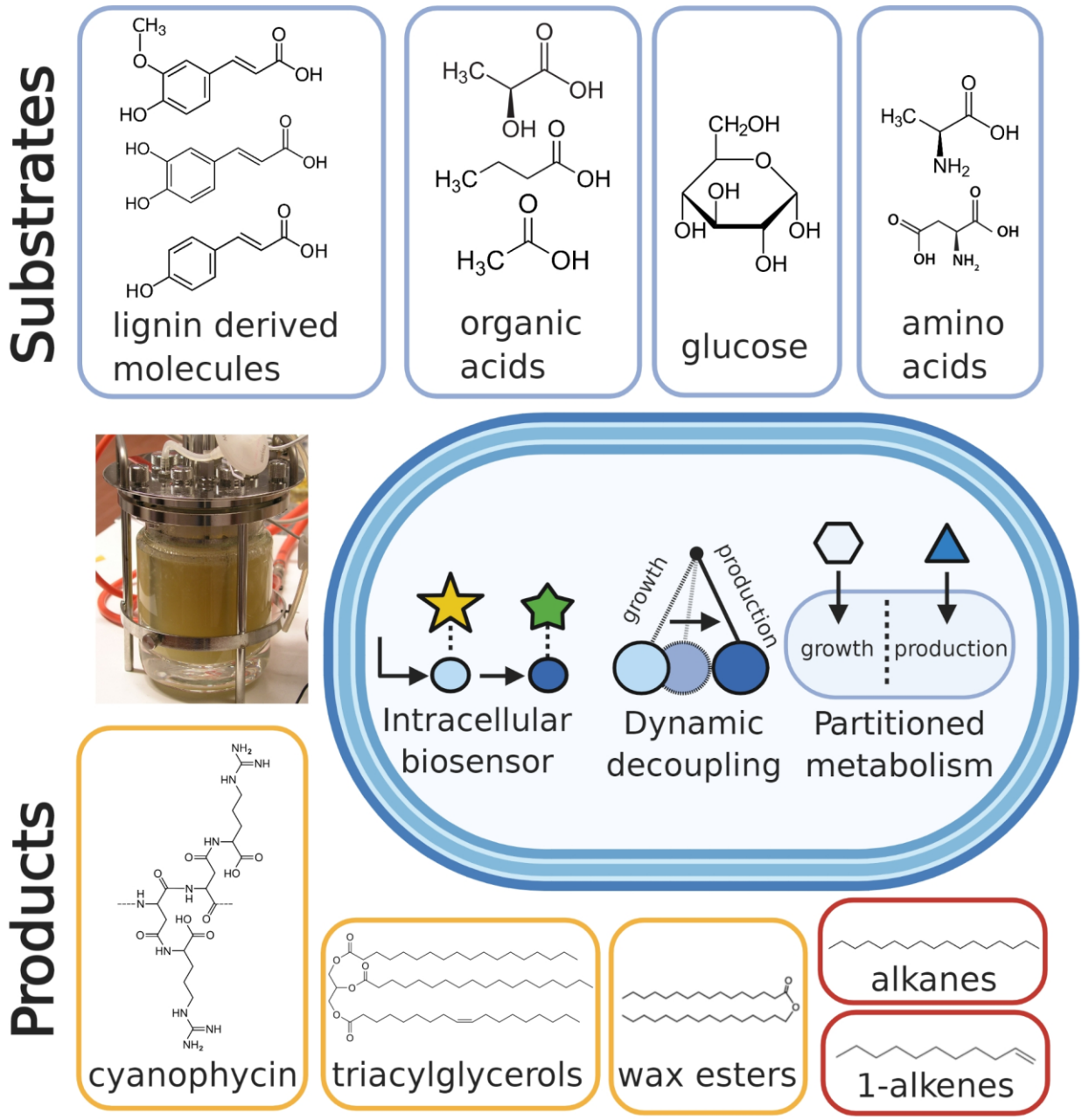
496 Figure 2.



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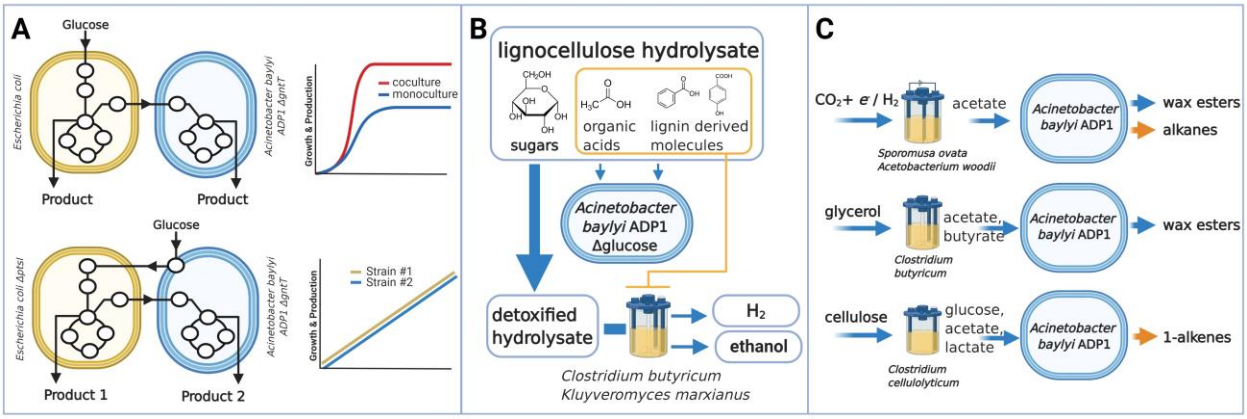
499 Figure 3.



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