Metabolic engineering of *Vibrio natriegens* for anaerobic succinate production

Felix Thoma,1,2 Clarissa Schulze,1 Carolina Gutierrez-Coto,1 Maurice Hädrich,1 Janine Huber,1 Christoph Gunkel,1 Rebecca Thoma1 and Bastian Blombach1,2

1Microbial Biotechnology, Campus Straubing for Biotechnology and Sustainability, Technical University of Munich, Uferstraße 53, Straubing, 94315, Germany.

2SynBiofoundry@TUM, Campus Straubing for Biotechnology and Sustainability, Technical University of Munich, Schulgasse 22, Straubing, 94315, Germany.

Summary

The biotechnological production of succinate bears serious potential to fully replace existing petrochemical approaches in the future. In order to establish an economically viable bioprocess, obtaining high titre, yield and productivity is of central importance. In this study, we present a straightforward engineering approach for anaerobic succinate production with *Vibrio natriegens*, consisting of essential metabolic engineering and optimization of process conditions. The final producer strain *V. natriegens* 

\[
\Delta pfi \Delta ald \Delta dns; pycQg \text{ (Succ1)}
\]

yielded 1.46 mol of succinate per mol of glucose under anaerobic conditions (85% of the theoretical maximum) and revealed a particularly high biomass-specific succinate production rate of 1.33 g\text{Succ} g\text{CDW}^{-1} h^{-1} compared with well-established production systems. By applying carbon and redox balancing, we determined the intracellular flux distribution and show that under the tested conditions the reductive TCA as well as the oxidative TCA/glyoxylate pathway contributed to succinate formation. In a zero-growth bioprocess using minimal medium devoid of complex additives and expensive supplements, we obtained a final titre of 60.4 g\text{Succ} l^{-1} with a maximum productivity of 20.8 g\text{Succ} l^{-1} h^{-1} and an overall volumetric productivity of 8.6 g\text{Succ} l^{-1} h^{-1} during the 7 h fermentation. The key performance indicators (titre, yield and productivity) of this first engineering approach in *V. natriegens* are encouraging and compete with costly tailored microbial production systems.

Introduction

Succinate (1,4-butanedioate) is a member of the 1,4-dicarboxylates and was rated amongst the top twelve bio-based platform chemicals (Werpy and Petersen, 2004; Bozell and Petersen, 2010) since it serves as a precursor for the synthesis of numerous chemical products, e.g. adipate, 1,4-butanediol, tetrahydrofuran, N-methylpyrrolidone, 2-pyrrolidone and \(\gamma\)-butyrolactone (Lee et al., 2019b,c). Its demand is, hitherto, primarily satisfied by the chemical synthesis from the petro-based precursors butane and benzene, which has been in place for decades (Lee et al., 2019b). Motivated by environmental concerns and anticipating a shortage of fossil resources, various studies have demonstrated that the biotechnological production from biogenic resources is feasible and some processes are currently commercialized (Bozell and Petersen, 2010; Ferone et al., 2019; Lee et al., 2019c). Bioprocesses must achieve superior key performance indicators and produce succinate from inexpensive substrates in cheap media to compete with the petrochemistry. Besides high yield and titre that keep the operating costs at a possible minimum, volumetric productivities can significantly reduce the required fermentation time or scale and thus minimize operating and capital expenditures respectively.

Encouraging data were reported for the bio-based succinate production with established bacterial (e.g. *Escherichia coli* and *Corynebacterium glutamicum*) and yeast cell systems (e.g. *Saccharomyces cerevisiae* and *Yarrowia lipolytica*), as well as bacteria that naturally secrete large quantities of succinate, such as *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens* and *Basilia succiniciproducens*. © 2021 The Authors. *Microbial Biotechnology* published by Society for Applied Microbiology and John Wiley & Sons Ltd. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
Excellent reviews summarize the wealth of knowledge about metabolic engineering and fermentation strategies of aerobic and anaerobic succinate production (Wieschalka et al., 2013; Ahn et al., 2016; Ferone et al., 2019; Lee et al., 2019b; Yang et al., 2020; Dai et al., 2020; Kumar et al., 2020). In general, depending on the host’s metabolism and the anticipated production mode, succinate yields were optimized by the inactivation of competing pathways and the elimination of negative regulation (Lin et al., 2005), enhancing e.g. the anaplerotic flux (Vemuri et al., 2002a; Thakker et al., 2012), NADH availability (Kim et al., 2017), the succinate export capacity (Zhu et al., 2014), as well as by rational enzyme engineering within the reductive branch of the TCA cycle (Ahn et al., 2020). These efforts resulted in engineered strains, that converted glucose (as the sole organic carbon and energy source) efficiently to succinate (≥ 1.40 mol of succinate per mol of glucose (molSucc molGlc−1), ≥ 0.90 gSucc gGlc−1), and reached final titres of up to 1.24 M (146 g l−1) in anaerobic batch and fed-batch processes (Lee et al., 2006; Jantama et al., 2008; Okino et al., 2008; Ahn et al., 2016; Lee et al., 2019b; Yang et al., 2020; Dai et al., 2020). However, only few of these studies reported productivities exceeding 22 mmol succinate l−1 h−1 (2.5 g l−1 h−1), which was considered the minimum volumetric productivity to meet economic viability (Werpy and Petersen, 2004).

Vibrio natriegens is an attractive candidate for biotechnological succinate production. Its fast growth and high substrate uptake rates (Hoffart et al., 2017; Hoff et al., 2020; Thoma and Blombach, 2021) set an excellent basis to develop highly productive fermentation processes especially for the bio-based manufacturing of bulk chemicals such as succinate. The genomic sequence of V. natriegens is available and fully annotated (Maida et al., 2013; Wang et al., 2013; Lee et al., 2016). A wealth of molecular biological tools has been established (Lee et al., 2016, 2017; Weinstock et al., 2016; Dalia et al., 2017; Hoffart et al., 2017; Tschirhart et al., 2019), and flux distributions of central carbon metabolism under aerobic conditions were determined during growth on glucose (Eagon and Wang, 1962; Long et al., 2017). This Gram-negative γ-proteobacterium is moderately halophilic and grows prototrophically in minimal medium containing glucose under aerobic (μ = 1.48–1.70 h−1) and anaerobic conditions (μ = 0.92 h−1), and even anaerobically resting cells show a high biomass-specific glucose consumption rate of 1 g g−1 h−1 (Thoma and Blombach, 2021). During anaerobic growth, V. natriegens ferments glucose to acetate, formate, ethanol, succinate and lactate. Non-growing anaerobic cells additionally secrete a remarkable amount of alanine (Fig. 1) (Hoffart et al., 2017). Deducing from the genome annotation (Lee et al., 2016), V. natriegens possesses the enzymatic equipment for aerobic and anaerobic succinate production: glycolysis, reductive branch of the TCA cycle (redTCA), oxidative branch of the TCA cycle (oxTCA) and glyoxylate pathway (Fig. 1). Noteworthy, the anaplerotic flux towards oxaloacetate might be exclusively provided via PEP carboxylation in V. natriegens. The genome annotation features PEP carboxylase and PEP carboxykinase genes, respectively, whereas a pyruvate carboxylase gene is lacking (Fig. 1). The biotechnological potential of V. natriegens has been exemplified recently by demonstrating high productivities of pyruvate-derived alanine and 2,3-butanediol, as well as 1,3-propanediol (Hoffart et al., 2017; Erian et al., 2020; Zhang et al., 2021). However, this bacterium has not been engineered for the production of succinate so far. In this study, we tailored V. natriegens for anaerobic succinate production by inactivation of competing pathways, enhancing the anaplerotic flux and optimizing process conditions. In an anaerobic resting cell approach, we produced 60.4 gSucc l−1 within 7 h of fermentation, achieving a maximum productivity of 20.8 gSucc l−1 h−1. The promising results of this first engineering approach highlight the potential of V. natriegens as future host for succinate production.

Results

Optimum flux distribution for succinate production

Initially, we set up a metabolic network consisting of three equations to describe succinate and acetate production from glucose in V. natriegens (Fig. 2). When glucose is solely routed through the glycolysis and redTCA to produce succinate, the maximum product yield (YP/S) of 2 molSucc molGlc−1 can be achieved on the carbon basis (Fig. 2B). However, this requires additional reducing power, as only 2 molNADH molGlc−1 is supplied in glycolysis (Fig. 2A and B). 1 molSucc molGlc−1 is yielded, if succinate is produced via the oxTCA or glyoxylate pathway, but a net surplus of NADH is generated (Fig 2C and D). Since NADH cannot be recycled by the respiratory chain under anaerobic conditions and alternative electron acceptors are not present, the redox state of the cell must be balanced. In the case that oxTCA and glyoxylate pathway are not active under anaerobic conditions, 1.33 molSucc molGlc−1 can be obtained, accompanied by acetate formation with a yield of 0.66 molAc molGlc−1 (0.50 molAc, molSucc−1). In this scenario, 1 mol pyruvate is decarboxylated by the PDHC to acetyl-CoA which is subsequently converted to 1 mol acetate yielding 1 mol ATP and NADH respectively. Based on the assumption that all pathways are fully operative under anaerobic conditions, the maximum yield of 1.71 molSucc molGlc−1 (Vuoristo et al., 2016) can be achieved by routing 71% of the carbon via the redTCA and 29% via either the oxTCA or the glyoxylate pathway. The oxTCA and glyoxylate pathway perform equally with regard to
succinate formation, reduction equivalents and net CO₂ formation (Fig. 2C and D). Thus, they can functionally compensate each other, and we did not distinguish between the pathways in the following.

**Optimization of production conditions**

Succinate secretion has been observed as a minor product of fermentative glucose metabolism by anaerobically growing and resting cells of *V. natriegens* (Hoffart *et al.*, 2017). In a small anaerobic screening system (50 ml test tubes), we obtained a yield of 0.15 ± 0.04 molₘₒₙₖ_/molₖₑₙₚₑₙ for succinate secretion with *V. natriegens* WT (Fig. 3A). By inoculating the test system from growing precultures, instead of using cells from the stationary phase of overnight (O/N) precultures, we increased the yield by > 60% (Fig. 3A). To provide sufficient HCO₃⁻ for the carboxylation of PEP, we supplemented the VN medium additionally with 100 mM KHCO₃, which further improved the Yₚ_/ᵦₚₑₙ by 50% (Fig. 3A). Under the latter condition 9.7 ± 0.5 mM, succinate was produced by *V. natriegens* WT at a yield Yₚ_/ᵦₚₑₙ= 0.38 ± 0.02 molₘₒₙₖₙₖ/molₖₑₙₚₑₙ. The effect of KHCO₃ supplementation remained marginal, when anaerobic tubes were inoculated from stationary precultures. Consequently, we performed all further experiments under the optimized cultivation conditions (inoculum from growing precultures, supplementation of 100 mM KHCO₃).

**Engineering *V. natriegens* for succinate production**

To improve the precursor availability for succinate production and to reduce by-product formation, we engineered *V. natriegens* by inactivating competing enzymes governing aerobic metabolism and fermentative reduction of NAD(P)⁺ by succinate and other organic acids. We thereby obtained a 10-15-fold higher succinate yield of up to 0.38 mol/mol Glc in 50 ml test tubes (Fig. 3A), which was comparable to the succinate production observed in 15 L fermentors (Hoffart *et al.*, 2017). The production of CO₂ (reflected by a decrease in the pH values of the cultures), however, was substantially increased in the engineered strain, presumably due to the inactivated respiratory pathways. Since the succinate production was observed in the absence of oxygen, we conclude that it is generated by anaerobic fermentation with CO₂ as the terminal electron acceptor in the engineered *V. natriegens* strain. The complete metabolic pathway from Glc to succinate is indicated by the red arrows in Fig. 1 and summarized in Table 1.
pathways. *V. natriegens* Δlldh Δdldh Δpfl has been constructed recently to abolish/mitigate lactate, acetate, formate and ethanol formation (Hoffart et al., 2017) and showed here under the tested conditions a Y_P/S of 0.90 ± 0.11 mol\textsubscript{Succ} mol\textsubscript{Glc}⁻¹ (Fig. 3B). Since this strain secreted 0.71 ± 0.08 mol alanine per mol glucose (Fig. 3C), we additionally deleted the ald gene annotated as alanine dehydrogenase, which potentially regenerates NAD⁺ by the reductive amination of pyruvate under anaerobic conditions. The resulting strain *V. natriegens* Δlldh Δdldh Δpfl Δald showed significantly reduced alanine formation (0.07 ± 0.01 mol\textsubscript{Ala} mol\textsubscript{Glc}⁻¹); however, the Y_P/S was only slightly improved to 1.01 ± 0.08 mol\textsubscript{Succ} mol\textsubscript{Glc}⁻¹ (Fig. 3B and C). Instead, 30% of glucose-derived carbon was directed to another so far unknown by-product (Fig. 3C), which could not be detected as amino or organic acid (including pyruvate) in the supernatant. All deletions made in *V. natriegens* led to a potentially elevated intracellular pyruvate availability. However, *V. natriegens* does not possess a pyruvate carboxylase to support the anaplerotic flux from pyruvate.

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possibility that PEP synthase activity, catalysing the pyruvate conversion to PEP, or the subsequent carboxylation was insufficient in *V. natriegens*, we chromosomally integrated the pyc gene encoding pyruvate carboxylase from *C. glutamicum* at the dns gene locus (Weinstock et al., 2016; Dalia et al., 2017). Pyc catalyses the carboxylation of pyruvate to produce oxaloacetate, thus enhancing the anaplerotic flux (Peters-Wendisch et al., 1997, 1998). The resulting strain *V. natriegens* Δlldh Δdldh Δpfl Δald Δdns::pyc*Cg*(=Succ1) yielded 1.46 ± 0.08 mol_{Succ} mol_{Glc}^{-1} (Fig. 3B), which represents 85% of the theoretical maximum, and indicates that under our test conditions the oxidative branch of the TCA cycle and/or the glyoxylate pathway actively contributed to succinate formation. Acetate was secreted as the main by-product (0.38 ± 0.08 mol_{Ac} mol_{Glc}^{-1}), accounting for 12% of the glucose-derived carbon (Fig. 3C), and the combined yield of amino acids (alanine,
valine and lysine) summed up to 0.02 ± 0.01 mol mol\(_{\text{Glc}}^{-1}\) in the final succinate-producing strain. Another 12% of the glucose-derived carbon could not be quantified. A degree of reduction balance was generally in good agreement with the results of the carbon balance (Fig. S2, Table S2). Interestingly, for *V. natriegens* Succ1, the degree of reduction balance was closed to 100%, suggesting that the missing carbon of the carbon balance is redox neutral (Fig. S2, Table S2). It became evident that the missing carbon sink was CO\(_2\), when we estimated the metabolic flux distribution by feeding the experimental data into the reaction equations (1)–(3) and balancing reduction equivalents as before (Fig. 4). The glucose-derived carbon was mainly routed towards succinate via the redTCA (68%), acetate (18%) and 13% contributed to succinate formation via the oxTCA (Fig. 4A).

Eventually, we refined the preliminary carbon balance, with the knowledge about the intracellular flux distribution (Fig. 4B). The theoretical CO\(_2\) release within the oxTCA accounted for 10% of the missing carbon. Another 2% of the missing carbon was identified as succinate, which was produced via the oxTCA. Unlike succinate produced via the redTCA (3 glucose-derived carbon atoms), this fraction contains four glucose-derived carbon atoms (Fig. S1). By calculation of the intracellular flux distribution, we could close the previous gap of the carbon balance to 100% (Fig. 4B), indicating that there was no undetected by-product formed by *V. natriegens* Succ1.

*Vibrio natriegens* Succ1 grew in minimal medium containing glucose as sole carbon and energy source without a lag phase at \(\mu = 1.41 ± 0.02\) h\(^{-1}\), which represents 90% of the WT in the same experiment (\(\mu = 1.57 ± 0.08\) h\(^{-1}\), Fig. S3). Anaerobically resting cells of *V. natriegens* Succ1 consumed glucose at \(q_0 = 1.38 ± 0.19\ g_{\text{Glc}}\ g_{\text{CDW}}^{-1}\) h\(^{-1}\) within 3 h and secreted succinate at \(q_p = 1.33 ± 0.18\ g_{\text{Succ}}\ g_{\text{CDW}}^{-1}\) h\(^{-1}\) within the first 2 h (Fig. 3D).

Last, we attempted to increase the succinate yield through the mitigation of acetate formation, which might be catalysed by the phosphate acetyltransferase and acetate kinase. Both enzymes are annotated by two independent genes, respectively. The deletion of the *pta-ackA* operon on chromosome 1 in *V. natriegens* Succ1 resulted in a strain, that did no longer consume glucose as sole carbon and energy source under aerobic as well as anaerobic conditions although it grew well in complex medium. The deletion of both *ackA* genes in the same background remained without effect and produced the same amount of acetate as *V. natriegens* Succ1 (data not shown).

### Zero-growth anaerobic bioprocess

Finally, we scaled up the anaerobic resting cell approach with *V. natriegens* Succ1 to a lab-scale bioreactor, which we inoculated to a starting biomass concentration of 18 g l\(^{-1}\). We used VN minimal medium lacking any complex additives and expensive nutrients. The anaerobic process was highly reproducible and reached within 7 h a final succinate titre of 520.7 ± 7.7 mM (i.e. 60.4 g l\(^{-1}\)) from glucose and KHCO\(_3\) which corresponds to a \(Y_{P/S}\) of 1.14 ± 0.02 mol\(_{\text{Succ}}\) mol\(_{\text{Glc}}^{-1}\). \(Y_{P/S}\) remained constant during the entire fermentation. Acetate was the major by-product with a yield of 0.18 ± 0.02 mol\(_{\text{Ac}}\) mol\(_{\text{Glc}}^{-1}\), followed by alanine with a yield of 0.02 mol\(_{\text{Ala}}\) mol\(_{\text{Glc}}^{-1}\) (Fig. 5). Considering these products, 64% of the initial glucose was recovered by carbon balancing and 73% by a degree of reduction balance. Other carbon sinks (including organic acids, amino acids and alcohols, which we analysed routinely) were not detected, indicating a considerable fraction of unknown products. The biomass-specific productivity started at levels, that were almost as high as in the small anaerobic cultivation system (1.15 ± 0.8 g Succ g\(_{\text{CDW}}\) h\(^{-1}\)), and decreased with

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**Fig. 4.** Metabolic flux distribution (A) and refined carbon balance (B) of *V. natriegens* Succ1 (\(\Delta\ildh \Delta\ddh \Delta\pdf \Delta\ald \Delta\dns \Delta\pyc\)). As outlined above, we could not distinguish between carbon flux via the oxidative branch of the TCA cycle and glyoxylate pathway (A). For simplicity, only the oxTCA route is highlighted.

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the overall process performance. The anaerobic process achieved an overall volumetric productivity of $74.4 \pm 1.1 \text{ mmol succinate l}^{-1} \text{ h}^{-1}$ (i.e. $8.6 \pm 0.1 \text{ g l}^{-1} \text{ h}^{-1}$). During the first hour of the process with *V. natriegens* Succ1, the maximum volumetric productivity of $179.3 \pm 1.9 \text{ mmol succinate l}^{-1} \text{ h}^{-1}$ (i.e. $20.8 \pm 0.2 \text{ g l}^{-1} \text{ h}^{-1}$) was reached.

**Discussion**

In this study, we increased the succinate yield of *V. natriegens* 10-fold through optimization of the anaerobic cultivation conditions and applying a minimal metabolic engineering approach. Under optimized conditions, the $Y_{P/S}$ of *V. natriegens* WT reached about 55% of the designated succinate producer *M. succiniciproducens* WT (Lee et al., 2006). The final production strain *V. natriegens* Succ1 maintained wild type-like fitness under aerobic conditions (growth rate reduced by only 10% compared with the WT, Fig. S3) and yielded $1.46 \pm 0.08 \text{ mmol succinate g}_{\text{CDW}}^{-1}$, which represents 85% of the theoretical maximum with glucose as sole organic substrate. Particularly, the high yield and biomass-specific productivity of $1.33 \pm 0.18 \text{ g succinate g}_{\text{CDW}}^{-1} \text{ h}^{-1}$ place *V. natriegens* Succ1 amongst the best performing succinate producers reported so far (Ahn et al., 2016, 2020; Kim et al., 2017; Lee et al., 2019b; Yang et al., 2020; Dai et al., 2020). Carbon and degree of reduction balancing indicated that in a small-scale cultivation of *V. natriegens* Succ1 acetate was the only major by-product beside CO$_2$. We calculated the intracellular carbon flux distribution under these conditions by feeding a metabolic network with the experimental data (glucose, succinate and acetate concentrations). Accordingly, succinate was produced by both pathways – redTCA and oxTCA (or glyoxylate pathway).

The very high biomass-specific productivity of *V. natriegens* Succ1 could finally be translated into an exceptionally high overall volumetric productivity ($8.6 \text{ g succinate l}^{-1} \text{ h}^{-1}$ during the production phase), when we performed the production under pH-controlled bioreactor conditions. Within only 7 h, we achieved final succinate titres that compete with commonly reported literature values (Table 1). However, higher titres have been achieved with various organisms under optimized fermentation conditions (Ahn et al., 2016; Lee et al., 2019b; Yang et al., 2020; Dai et al., 2020) and must be improved significantly with *V. natriegens* Succ1 in the future, to facilitate downstream processing. Our maximum volumetric productivity during the initial production phase ($20.8 \text{ g succinate l}^{-1} \text{ h}^{-1}$) is at the same level as a recently developed process using engineered *M. succiniciproducens* of $20.9 \text{ g succinate l}^{-1} \text{ h}^{-1}$ (i.e. $21.3 \text{ g succinic acid l}^{-1} \text{ h}^{-1}$) in an identical set-up (Ahn et al., 2020). In contrast to the latter and many other studies (Vemuri et al., 2002a; Sánchez et al., 2005; Zhu et al., 2014; Lee et al., 2019b; Ahn et al., 2020; Dai et al., 2020), our production process did not require the addition of vitamins, amino acids and other expensive supplements. The succinate yield of the bioreactor cultivation was significantly lower than that of the small test system. Carbon and degree of reduction balancing suggested a considerable proportion of glucose was converted to a reduced product. However, we could not identify the carbon sink in this set-up, as about 50% less acetate was formed in the larger set-up and no other organic acids, alcohols or amino acids were detected, except for alanine that made up about 1% of the glucose-derived carbon.

Consequently, future work is essential to elucidate the physiological response of *V. natriegens* to the bioreactor environment. The re-direction of carbon fluxes might be triggered by quorum sensing stimuli at high cell densities (Thoma and Blombach, 2021). Process intensification strategies are needed to increase titre and yield further and enable industrially relevant dual-phase fermentations by combining biomass and product formation in a single vessel and using only minimal media (Lange et al., 2016; Kumar et al., 2020; Raj et al., 2020). The development of such approaches is challenging since the physiological state of the cells entering the transition from aerobic to anaerobic conditions can significantly impact product formation as shown for succinate production with *E. coli* (Vemuri et al., 2002b; Martínez et al., 2010). Thus, system-level analysis in combination with process optimization will probably be required also for *V. natriegens* to optimally adjust the enzymatic machinery for the anaerobic production phase.

Interestingly, the succinate yield of *V. natriegens* WT was higher, when the production was started with a
The anaplerotic flux from 
various organisms (Sauer and Eikmanns, 2005) and the enzyme is responsible for about 90% of the anaplerotic carbon flux in *M. succiniciproducens* during succinate production under anaerobic conditions (Lee et al., 2006). A pyruvate carboxylase gene is not encoded in the genome of *V. natriegens* and pyruvate accumulates frequently, when the oxygen demand is insufficient (Thoma and Blombach, 2021). Therefore, the positive effect of *pyc* overexpression on the succinate yield was consistent with the outcome of similar engineering strategies that have been applied to other host organisms in the past (Vemuri et al., 2002a).

Our metabolic engineering efforts to mitigate acetate formation revealed some interesting insights in the physiological peculiarities of *V. natriegens*. In the Succ1 strain background, acetate seems to be formed from acetyl-CoA, since a gene encoding a pyruvate oxidase is not annotated in the genome. In accordance with Lee et al., who showed that both genes of the *pta-ackA* operon on chromosome 1 are essential in the wild type during aerobic growth on glucose (Lee et al., 2019a), *V. natriegens* Succ1 Δ(*pta-ackA*) did not consume glucose as sole carbon and energy source. Apparently, neither the gene duplicates on chromosome 2 nor other annotated CoA-transferases or acetaldehyde/alcohol dehydrogenases, that could theoretically catalyse the same reaction, compensated the deletion of the *pta-ackA* operon in this strain background. In this regard, *V. natriegens* differs from other organisms, in which the disruption of the *pta-ackA* pathway was a successful engineering approach to abolish or reduce acetate formation under anaerobic conditions (Sánchez et al., 2005; Lee et al., 2006; Litsanov et al., 2012). The deletion of both annotated *ackA* genes in *V. natriegens* Succ1 remained without effect on the acetate yield. Altogether these results suggest, that acetyl phosphate is exclusively formed by the phosphate acetyltransferase (encoded on chromosome 1) and might play an indispensable physiological role in *V. natriegens*. The acetate generation from acetyl phosphate might be catalysed by the acylphosphatase, if both acetate kinase genes are deleted. The corresponding gene is annotated in the genome of *V. natriegens* (Long et al., 2017).

**Conclusion**

Here, we present the first approach to engineer *V. natriegens* for the biotechnological production of succinate with remarkable key performance indicators.

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**Table 1**: Selected anaerobic succinate production processes with genetically modified organisms in fed-batch mode (yield calculated as mol succinate per mol glucose equivalent (carbon basis), maximum theoretical yield for the given set-up (considering substrates + reduction degree) without consideration of complex compounds and organic supplements; calculated from data for succinic acid).

| Organism                  | Substrate(s)                                                                 | Complex/organic supplements | Volumetric productivity (maximum) g<sub>Succ</sub> g<sub>Glc</sub>⁻¹ h⁻¹ | Biomass-specific productivity (maximum) g<sub>Succ</sub> g<sub>Cow</sub>⁻¹ h⁻¹ | Final succinate titre g l⁻¹ | Yield (%) theoretical maximum yield mol<sub>Succ</sub> mol<sub>Glc</sub>⁻¹ | Duration h | Reference                      |
|---------------------------|----------------------------------------------------------------------------|------------------------------|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------|-----------------------------|--------------------------------------------------------------------------------|-----------|---------------------------------|
| *Vibrio natriegens* Δald Δsdh Δpfd Δald Δsdh Δpfd *Actinobacillus succinogenes* NJ113 | Sucrose + CO<sub>2</sub> + NaHCO<sub>3</sub> (base titration) | Yeast extract + corn steep liquor | 2.1 (-)<sup>a</sup> | 0.06 (0.23)<sup>b</sup> | 143.6<sup>b</sup> | 1.40 (82%) | 46 | Litsanov et al. (2012) |
| *Corynebacterium glutamicum* ΔflaDH (pCRA717) | Glucose + HCO<sub>3</sub> | Biotin + thiamin | 3.2 (11.6)<sup>a</sup> | 133.8 | 1.67 (84%) | 53 | Litsanov et al. (2012) |
| *Escherichia coli* AFBP111 (pTrc99A-pyc) | Glucose + formate + NaHCO<sub>3</sub> | Yeast extract + tryptone + biotin | 1.3 (-) | 97.5 | 1.78 (105%) | 75 | Vemuri et al. (2002b) |
| *Marinimicrobium succiniciproducens* PALK cgmR | Glucose + glycerol + NaHCO<sub>3</sub> + CO<sub>2</sub> | Biotin, Ca-pantothenate, pyridoxine-HCl, thiamine, ascorbic acid, nicotinic acid + amino acids (Ala, Asn, Asp, Cys, Met, Pro, Ser) | 10.2 (20.9)<sup>a</sup> | 1.17 (2.40)<sup>b</sup> | 132<sup>b</sup> | 1.25 (71%) | 13 | Ahn et al. (2020) |
Scope for improvement remains particularly in the process intensification and scale-up. Especially, the final titre needs to be increased to realize cost-efficient purification of succinate. Future studies have to develop sophisticated dual-phase processes to combine the high growth rate and metabolic activity for rapid biomass and product formation in a single industrially relevant bioprocess. However, the high biomass-specific productivity of our engineered strain in cheap minimal medium devoid of expensive supplements sets already a benchmark for succinate production with *V. natriegens*.

### Experimental procedures

**Bacterial strains, plasmids and cultivation media**

An overview of the bacterial strains and plasmids utilized during this study is provided in Table 2. Permanent cultures of all strains were maintained at −80 °C in 30% (v/v) glycerol. *E. coli* S17-1 λpir cells were cultivated in 2x YT medium containing per l: 16 g Bacto tryptone, 10 g yeast extract and 5 g NaCl (Sambrook and Russell, 2001). *V. natriegens* strains were precultured in 2x YTN containing an elevated concentration (15 g l⁻¹) of NaCl and 50 mM MgCl₂. Main cultures (growth experiments and succinate production) were set up in VN minimal medium containing per l: 5 g (NH₄)₂SO₄, 21 g 3-(N-morpholino)propanesulfonic acid (MOPS), 1 g KH₂PO₄, 1 g K₂HPO₄, 15 g NaCl, 10 mg CaCl₂ ∙ H₂O, 250 mg MgSO₄ ∙ 7 H₂O, 16.4 mg FeSO₄ ∙ 7 H₂O, 10 mg MnSO₄ ∙ H₂O, 1 mg ZnSO₄ ∙ 7 H₂O, 0.3 mg CuSO₄ ∙ 5 H₂O, 0.02 mg NiCl₂ ∙ 6 H₂O (Hoffart et al., 2017). The medium pH was adjusted to 7.5 with 5 M KOH. The indicated amount of glucose and KHCO₃ was added aseptically. When appropriate, 15 µg chloramphenicol ml⁻¹ was used. MOPS buffer was omitted from media, when pH-controlled bioreactor cultivations were performed.

**Genetic manipulation**

Standard techniques of molecular biology (e.g. agarose gel electrophoresis and PCR amplification) were applied as described in the literature (Sambrook and Russell, 2001). Chromosomal DNA and plasmid DNA were isolated using the NucleoSpin Microbial DNA and the NucleoSpin Plasmid kit (Macherey-Nagel, Düren, Germany) respectively. PCR fragments were purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) respectively. PCR fragments were purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany).

### Table 2. Bacterial strains and plasmids used in this study.

| Strain / plasmid | Relevant characteristics | Source / reference |
|------------------|--------------------------|-------------------|
| *E. coli* S17-1 λpir | thi pro hsdR hsdM1 recA RP4-2-Tc::Mu-Km::Tn7 λpir | Simon et al. (1983) |
| *V. natriegens* | wild type (WT) strain DSM 759 (ATCC 14048) | German Collection of Microorganisms and Cell Cultures (Hoffart et al., 2017) |
| *V. natriegens* Δildh Δldh Δptf | *V. natriegens* WT with deletion of the ildh, dlith, and ptf genes encoding 3-lactate dehydrogenase, 1-lactate dehydrogenase and pyruvate formate lyase (locus tags: PN96_167985, PN96_16800 and PN96_08455) | This work |
| *V. natriegens* Δildh Δldh Δptf Δald | *V. natriegens* Δildh Δldh Δptf Δald with additional deletion of the ald gene encoding alanine dehydrogenase (locus tag: PN96_09745) | This work |
| *V. natriegens* Δildh Δldh Δptf Δald Δdns::pyc | *V. natriegens* Δildh Δldh Δptf Δald with genomic integration of the pyc gene from *C. glutamicum* ATCC 13032 encoding pyruvate carboxylase into the dns gene (locus tag: PN96_00865) | This work |
| *V. natriegens* Succ1 Δ(pta-ackA) | *V. natriegens* Succ1 with additional deletion of the pta-ackA operon encoding phosphate acetyltransferase and acetate kinase (locus tag: PN96_03360-65) | This work |
| *V. natriegens* Succ1 ΔackA1 ΔackA2 | *V. natriegens* Succ1 with additional deletion of both ackA genes encoding acetate kinase (locus tags: PN96_03365 and PN96_21510) | This work |
| *C. glutamicum* ATCC 13032 | | American Type Culture Collection, Klopp et al. (1967) |
| pDM4 | oriR6K, oriT, sacB, CmR | Milton et al. (1996) |
| pDM4-Δald | pDM4 derivative carrying 500 bp homologous sequences for the deletion of the ald gene | This work |
| pDM4-Δdns::pycc | pDM4 derivative with an integration construct of the pyc gene from *C. glutamicum* under control of the tac promoter and followed by the mbl terminator; dns gene is disrupted | This work |
| pDM4-Δ(pta-ackA) | pDM4 derivative carrying 500 bp homologous sequences for the deletion of the pta-ackA operon | This work |
| pDM4-ΔackA1 | pDM4 derivative carrying 500 bp homologous sequences for the deletion of the ackA1 gene | This work |
| pDM4-ΔackA2 | pDM4 derivative carrying 500 bp homologous sequences for the deletion of the ackA2 gene | This work |
| pJC4-P_tac-egfp | oriVC_cop, PCG1 oriVC_cop, gltaminum, KanR, for egfp expression under tac promoter, followed by the mbl terminator | Müller et al. (2020) |

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Nagel) according to the manufacturer’s instructions. Enzymes were purchased from New England Biolabs (Frankfurt, Germany), and oligonucleotides were ordered from Sigma-Aldrich (Steinheim, Germany). Chromosomal modifications in V. natriegens were made using pDM4-derived plasmids allowing markerless integration and deletion by selection on chloramphenicol and sacB-based counter-selection (Milton et al., 1996). For the deletion, 500 bp sequences flanking the ald gene (PN96_07945) were PCR amplified with the primer pairs ald1/ald2 and ald3/ald4, respectively (Table S1) and simultaneously inserted into the XbaI- and SalI-linearized pDM4 plasmid by isothermal assembling (Gibson, 2011).

Accordingly, homologous sequences for the deletion of the pta-ackA operon (PN96_03360-65), the ackA1 gene (PN96_03365) and the ackA2 gene (PN96_21510) were PCR amplified with the primers as indicated in Table S1. Chromosomal integration of the pyc gene was targeted to the dns gene locus (PN96_00865) by amplifying 500 bp sequences with the primer pairs dns1/dns2 and dns3/dns4, and the expression module P\text{pta-ycT}\text{mB} was placed in between the two flanks. The pyc gene was amplified from the chromosomal DNA of C. glutamicum ATCC 13032 using the primer pair pyc1/pyc2. The native GTG start codon was replaced with the stronger ATG during the amplification step with a primer encoded point mutation. Promoter and terminator elements were amplified from pJC4-P\text{pta-egfp} (Müller et al., 2020) using P\text{tacl/Ptacl2} and TrmB1/TrmB2 respectively. All fragments were simultaneously assembled with the XbaI- and SalI-linearized pDM4 plasmid as described before.

Competent cells of E. coli S17-1 \text{pir} were transformed with the resulting plasmids by electroporation following the protocol described by (Lange et al., 2018). Plasmids were amplified and isolated, and the insert was verified by sequencing (Eurofins, Constance, Germany) before conjugating it into V. natriegens as described previously (Hoffart et al., 2017). We used 2\times YTN medium during the conjugation protocol instead of BHIN. Insertion/deletion mutants were generated by two homologous recombination steps (Milton et al., 1996) and eventually verified by colony PCR.

Small-scale cultivation experiments

The previously established seed train (Hoffart et al., 2017) was slightly modified in this study by replacing BHIN medium with 2\times YTN medium. Experiments were started by streaking out cells from cryogenic cultures (\textdegree -80 °C) on 2\times YTN agar plates, which were incubated for 6–8 h or overnight (O/N) at 37 °C. 5 ml liquid 2\times YTN medium in test tubes was inoculated with a single colony from the plate and incubated O/N at 37 °C shaking (180 rpm), before cells were pelleted by centrifugation (10 min, room temperature (RT), 4347 g). For aerobic shaking flask experiments, the cell pellet was re-suspended in 0.9% (w v\textsuperscript{-1}) NaCl and the optical density at 600 nm (OD\textsubscript{600}) of the suspension was adjusted, in order to inoculate the main culture (VN minimal medium) to a starting biomass concentration of 26 mg\textsubscript{CDW} l\textsuperscript{-1} (OD\textsubscript{600} = 0.1). Experiments were performed in 500 ml baffled shaking flasks containing 50 ml VN medium and 55 mM glucose as sole carbon and energy source (Hoffart et al., 2017).

Routinely, anaerobic succinate production was performed in 50 ml test tubes with screw caps (to prevent aeration during the experiment) containing 50 ml VN minimal medium with 27.5 mM glucose as sole organic carbon and energy source and 100 mM KHCO\textsubscript{3} as inorganic carbon source to provide the essential substrate for the anaplerotic carboxylation reactions. With the biomass-specific oxygen consumption rate (28 mmol\textsubscript{O\textsubscript{2}} g\textsubscript{CDW} h\textsuperscript{-1}, Thoma and Blombach, 2021) the residual oxygen present in 50 ml liquid phase and 5 ml gas of the head space was exhausted after approximately 34 s, and strictly anaerobic conditions established thereafter. The initial glucose concentration was lower in anaerobic experiments, in order to reduce the medium acidification through organic acid secretion.

Two-stage anaerobic bioprocess

The final anaerobic succinate production processes were performed in 2 l glass vessels of a DASGIP bioreactor system (Jülich, Germany). To obtain enough biomass, precultures were set up in 3\times 1.5 l 2\times YTN medium and incubated at 37 °C until an OD\textsubscript{600} of about 5 was reached. Cells were harvested as before, re-suspended, and anaerobic tubes were inoculated with an initial biomass concentration of 1.3 g\textsubscript{CDW} l\textsuperscript{-1} (OD\textsubscript{600} = 5). The inoculum from stationary phase cells was taken from 5 ml O/N cultures straight away and processed likewise.
production, bioreactors were not aerated and the stirrer speed was set to 180 rpm. The culture pH was monitored with a standard pH probe (405-DPAS-SC-K85/325, Mettler Toledo, Giessen, Germany) and maintained at 7.5 by a two-side regulation using 13.3 M NH₄OH and 1.1 M H₃PO₄. A linear KHCO₃ feed was applied at −10% v/v external standard ranging from 1 to 200 mM (Siebert concentrations were calculated by comparing the peak with a refractive index detector (RID). Analyte

| Concentration (cCDW) | Monitored by measuring the | Exponentially growing cells for the spectrophotometer |
|----------------------|---------------------------|------------------------------------------------------|
|                      |                           |                                                      |

### Analytics

#### Determination of growth parameters.

The biomass concentration (cCDW) was monitored by measuring the optical density at 600 nm (OD₆₀₀). The correlation factor to convert c_CDW to g_CDW l⁻¹ had been determined from exponentially growing cells for the spectrophotometer (Ultriospec 10 cell density meter, Harvard Biochrom, Holliston, MA, USA): $\text{c}_{\text{CDW}} = \text{OD}_{600} \times 0.26$. The growth rate ($\mu$) was determined in the exponential phase through a regression curve, fitting the slope of the biomass concentration in a semilogarithmic plot over time.

#### Quantification of sugars, organic acids and alcohols.

Glucose, succinate, pyruvate, acetate, lactate, formate and ethanol were quantified by high-performance liquid chromatography (HPLC) using an Agilent 1260 infinity II series device (Agilent Technologies, Waldbronn, Germany) equipped with a Hi-Plex H column ($7.7 \times 300$ mm, 8 $\mu$m) and Hi-Plex H guard cartridge ($3.0 \times 5$ mm, 8 $\mu$m) for protection. Analytes were eluted at 50 °C isocratically using 5 mM H₂SO₄ (mobile phase, flow = 0.4 ml min⁻¹) and detected with a refractive index detector (RID). Analyte concentrations were calculated by comparing the peak area of the sample with the peak area of a 7-point external standard ranging from 1 to 200 mM (Siebert et al., 2021).

#### Quantification of amino acids.

Amino acids were quantified by HPLC using an identical device as described before equipped with an AdvanceBio Amino Acid Analysis (AAA) column ($4.6 \times 100$ mm, 2.7 $\mu$m) and AdvanceBio AAA guard column ($4.6 \times 5$ mm, 2.7 $\mu$m) for protection. Primary amino acids were online derivatized with o-phthaldialdehyde (OPA) prior to separation through a gradient of polar (10 mM Na₂B₄O₇, 10 mM Na₂HPO₄, pH 8.2) versus non-polar phase (45% v v⁻¹ acetonitrile, 45% v v⁻¹ methanol and 10% v v⁻¹ water) by increasing the linear gradient stepwise from 2–43% non-polar fraction within 10 min and 43–100% within 2:30 min, at a total flow rate of 1 ml min⁻¹, 40 °C. The analytes were detected with a fluorescence detector (FLD, excitation wavelength: 340 nm, emission wavelength: 450 nm and PMT gain: 10). Peak areas were normalized to the internal standard (100 µM norvaline), and concentrations were finally calculated by comparing the peak area of the sample with the peak area of a 7-point external standard ranging from 1 to 400 µM (Siebert et al., 2021).

### Statistical analysis

All experiments were performed at least in independent biological triplicate on different days. Seed trains of all replicates were started by streaking cell material of a cryogenic culture on a fresh agar plate. Data represent mean values of at least triplicate, and error bars indicate standard deviations. If appropriate, standard deviations were calculated by error propagation.

#### Carbon and degree of reduction balancing.

The carbon balance for the anaerobic resting cell approach was calculated on the C-molar basis in accordance with Buchholz et al. (2014). C-molar concentrations of the HPLC analytes were summed up, and the individual fraction deriving from the initial glucose was determined. Due to the lack of biomass formation in the resting cell approach, it was not considered in the carbon balance. According to the succinate production mode, we assumed initially that succinate was produced via the reductive branch of the TCA cycle. Consequently, only three carbon atoms of the C₄-molecule were considered as ‘glucose-derived’. The gap between products and substrates was designated as ‘unknown’. All standard deviations were calculated by error propagation of the biological triplicate. The carbon balance of V. natriegens Succ1 was refined after the estimation of carbon fluxes for succinate production. Four carbon atoms of succinate were considered as glucose-derived, when it was produced via the oxidative arm of the TCA cycle (considerations are visualized in Fig. S1). CO₂ release was not quantified, but integrated in the theoretical considerations, when flux passed the decarboxylation reactions (pyruvate dehydrogenase complex (PDHC), isocitrate dehydrogenase and α-ketoglutarate dehydrogenase complex).

In addition to this, a degree of reduction balance was set up, in order to account for the individual contribution of each product independently of CO₂/HCO₃⁻ evolution. In accordance with Villadsen et al. (2011), redox levels of all quantified metabolites (glucose = 24, succinic acid = 14, acetic acid = 8, alanine = 12, ethanol = 12, formic acid = 2, lactic acid = 12, valine = 24 and lysine = 28) and the reduction degrees ($\kappa_i$) per C-mole were calculated (Villadsen et al., 2011). The redox contribution of each product was calculated through multiplying $\kappa_i$ by the C-molar concentration. All products were summed up and expressed as fraction of glucose, in analogy with the carbon balance.
Metabolic flux analysis. A metabolic network of all relevant fluxes for succinate and acetate (as the most prominent by-product of succinate formation) production from glucose in V. natriegens Succ1 was set up in accordance with the genome annotation (ATCC 14048) and KEGG database (Kanehisa and Goto, 2000; Lee et al., 2019a). The network comprises glycolysis, acetate metabolism (considering only the Pta-AckA and Acs catalysed reactions in V. natriegens Succ1), redox branch of the TCA cycle (redTCA), oxidative branch of the TCA cycle (oxTCA) and glyoxylate pathway (Fig. 2). Based on the overall reactions of these modules, equations (1)–(3) describe the conversion of glucose to either succinate or acetate via a combination of glycolysis and redTCA (1), glycolysis and oxTCA/ or glyoxylate pathway (2), and converting glucose to acetate (3).

\[ \text{Glc} - 2 \text{CO}_2 - 2 \text{NADH} + 2 \text{Succ} = 0 \]  
\[ \text{Glc} + 2 \text{CO}_2 + 5 \text{NADH} + \text{Succ} = 0 \]  
\[ \text{Glc} + 2 \text{CO}_2 + 4 \text{NADH} + 2 \text{Ac} = 0 \]

The theoretical environment was an anaerobic production scenario with resting cells, premising no biomass generation from glucose and full conservation of reduction equivalents. Reduction equivalents were considered as the aggregate of NAD(P)H and quinones. We assumed that all pathways were fully operative under the given conditions, and reduction equivalents were interconvertible. ATP energetics were not considered in the calculations, as we could not experimentally verify the carbon flux through either of the energetically different reactions (i.e. PEP carboxylase/pyruvate carboxylase vs. PEP carboxykinase). The optimum flux distribution for a maximum succinate yield from glucose was calculated by balancing the consumption/production of reduction equivalents (1) and (2). In addition, the maximum yield was calculated in a second scenario considering a potentially inactive oxTCA under anaerobic conditions (contribution of (2) = 0).

The metabolic flux distribution during the small-scale experiment (Succ1) was estimated by feeding experimental data of glucose, succinate and acetate quantification into equations (1)–(3) and balancing the reduction equivalents as before. Only mean values of the analytes were used.

Author contributions

BB acquired funding of the project. FT, CS and BB conceived and designed the experiments. FT and RT performed the metabolic modelling. FT, CS, CGC, CG, JH and MH performed the experiments and analysed the data. FT and BB drafted the manuscript. All authors reviewed and agreed with the final manuscript.

Conflict of interest

The authors declare that there are no competing interests associated with this work.

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Supporting information

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

**Fig. S1.** Illustration of the succinate formation and its discrepancy regarding the origin of carbon atoms. Depending on the production pathway, all four carbon atoms of succinate originate from glucose (oxidative branch of the TCA cycle, green). Alternatively, only three carbon atoms of succinate originate from glucose and the fourth carbon atom is provided by CO$_2$/HCO$_3^-$ during the carboxylation of PEP and/or pyruvate (reductive branch of the TCA cycle, magenta). With regards to their origin, glucose-derived carbon atoms are shown as black circles, whereas CO$_2$/HCO$_3^-$ - derived carbon atoms are highlighted as yellow circles.

**Fig. S2.** Degree of reduction balance of anaerobic succinate production in test tubes containing 50 mL VN minimal medium with 27.5 mM glucose. Reduction degree times C-molar concentration ($\kappa_i^*c_i$) of each product ($i$) is described as fraction of glucose ($\kappa_{Glc}^*c_{Glc}$)$^-1$.

**Fig. S3.** Aerobic shaking flask cultivations of *V. natriegens* WT and *V. natriegens* Succ1 in VN minimal medium containing 10 g glucose l$^{-1}$. Data points represent the mean and error bars indicate the standard deviation of three independent biological replicates.

**Table S1.** Oligonucleotides used in this study.

**Table S2.** Comparison of the carbon fraction recovered by balancing carbon atoms and degree of reduction.