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Metabolic engineering of *Cupriavidus necator* H16 for heterotrophic and autotrophic production of 3-hydroxypropionic acid

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ABSTRACT

3-Hydroxypropionate (3-HP) is a versatile compound for chemical synthesis and a potential building block for biodegradable polymers. *Cupriavidus necator* H16, a facultative chemolithoautotroph, is an attractive production chassis and has been extensively studied as a model organism for biopolymer production. Here, we engineered *C. necator* H16 for 3-HP biosynthesis from its central metabolism. Wild type *C. necator* H16 can use 3-HP as a carbon source, a highly undesirable trait for a 3-HP production chassis. However, deletion of its three (methyl-) malonate semialdehyde dehydrogenases (*mmsA1*, *mmsA2* and *mmsA3*) resulted in a strain that cannot grow on 3-HP as the sole carbon source, and this strain was selected as our production host. A stepwise approach was used to construct pathways for 3-HP production via β-alanine. Two additional gene deletion targets were identified during the pathway construction process. Deletion of the 3-hydroxypropionate dehydrogenase, encoded by *hpdH,* prevented the re-consumption of the 3-HP produced by our engineered strains, while deletion of *gdhA1*, annotated as a glutamate dehydrogenase, prevented the utilization of aspartate as a carbon source, one of the key pathway intermediates. The final strain carrying these deletions was able to produce up to 8 mM 3-HP heterotrophically. Furthermore, an engineered strain was able to produce 0.5 mM 3-HP under autotrophic conditions, using CO_2 as sole carbon source. These results form the basis for establishing *C. necator* H16 as an efficient platform for the production of 3-HP and 3-HP-containing polymers.

1. Introduction

Large scale chemical production is currently reliant on the fossil fuel industry. As a result of the finite nature and negative impact on the environment of fossil fuels, alternative resources are urgently required. Therefore, significant interest has arisen in the production of commodity chemicals using renewable and more sustainable means. In particular, bio-based approaches are gaining traction (Liu et al., 2021).

3-hydroxypropionate (3-HP) is a bifunctional 3-carbon compound regarded as a highly valuable platform chemical (Kumar et al., 2013). In 2004, 3-HP was included on a list of the top twelve chemical building blocks available through metabolic engineering (Werpy and Petersen, 2004). It can act as a precursor to a range of industrially and commercially relevant compounds, including but not limited to acrylic acid, acrylonitrile, and biodegradable polymers (Zhao and Tian, 2021). In nature, 3-HP is found as an intermediate in the autotrophic metabolism of various organisms, and is an antimicrobial agent secreted by certain fungi with activity against medically relevant pathogens, indicating possible future applications in antibiotics (Sebastianes et al., 2012; Zarzycki et al., 2009).

Traditionally 3-HP has been synthesised by chemical routes. These routes are, however, typically costly and involve the use of hazardous chemicals (Jiang et al., 2009; Liang et al., 2019). The advent of metabolic engineering has enabled the production of a range of compounds

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using genetically modified microorganisms, including 3-HP. Various routes to 3-HP have been described (Zhao and Tian, 2021). Of these, the most widely applied involves conversion of glycerol to 3-HP via 3-hydroxypropionaldehyde (3-HPA). This pathway is short, using only two enzymatic steps, and is compatible with the use of a cheap and widely available carbon source. As a consequence, this pathway has formed the basis of numerous studies (Chu et al., 2015; Kim et al., 2020; Tokuyama et al., 2014). A major drawback of 3-HP biosynthesis using this pathway, however, is that the enzyme glycerol dehydratase, required for the conversion of glycerol to the intermediate compound 3-HPA, utilises vitamin B12 as a cofactor. Most bacteria do not synthesize this cofactor. Consequently, vitamin B12 must be added to the growth medium, increasing production costs. Its addition has been avoided through the use of vitamin B12-producing microbes such as *Klebsiella pneumoniae* (Feng et al., 2015; T. Li et al., 2016). However, this bacterium is a known pathogen, a trait which currently represents a major barrier to its exploitation as an industrial chassis (Kumar and Park, 2018).

Another alternative is to use a pathway proceeding via lactate; however, it does not appear to have been tested in host cells yet, likely due to various drawbacks. Firstly, the pathway has an overall positive Gibbs free energy and as such the pathway is thermodynamically unfavourable (Kumar et al., 2013). To overcome this, it is proposed that a high intracellular concentration of lactic acid is maintained while 3-HP production is maintained at a low level, leading to sub-optimal production. Furthermore, lactic acid and 3-HP are structural isomers, which can make their individual detection and separation difficult (Jiang et al., 2009).

On the other hand, the malonyl-CoA pathway has been successfully constructed in host cells (Cheng et al., 2016; Kildegaard et al., 2016;

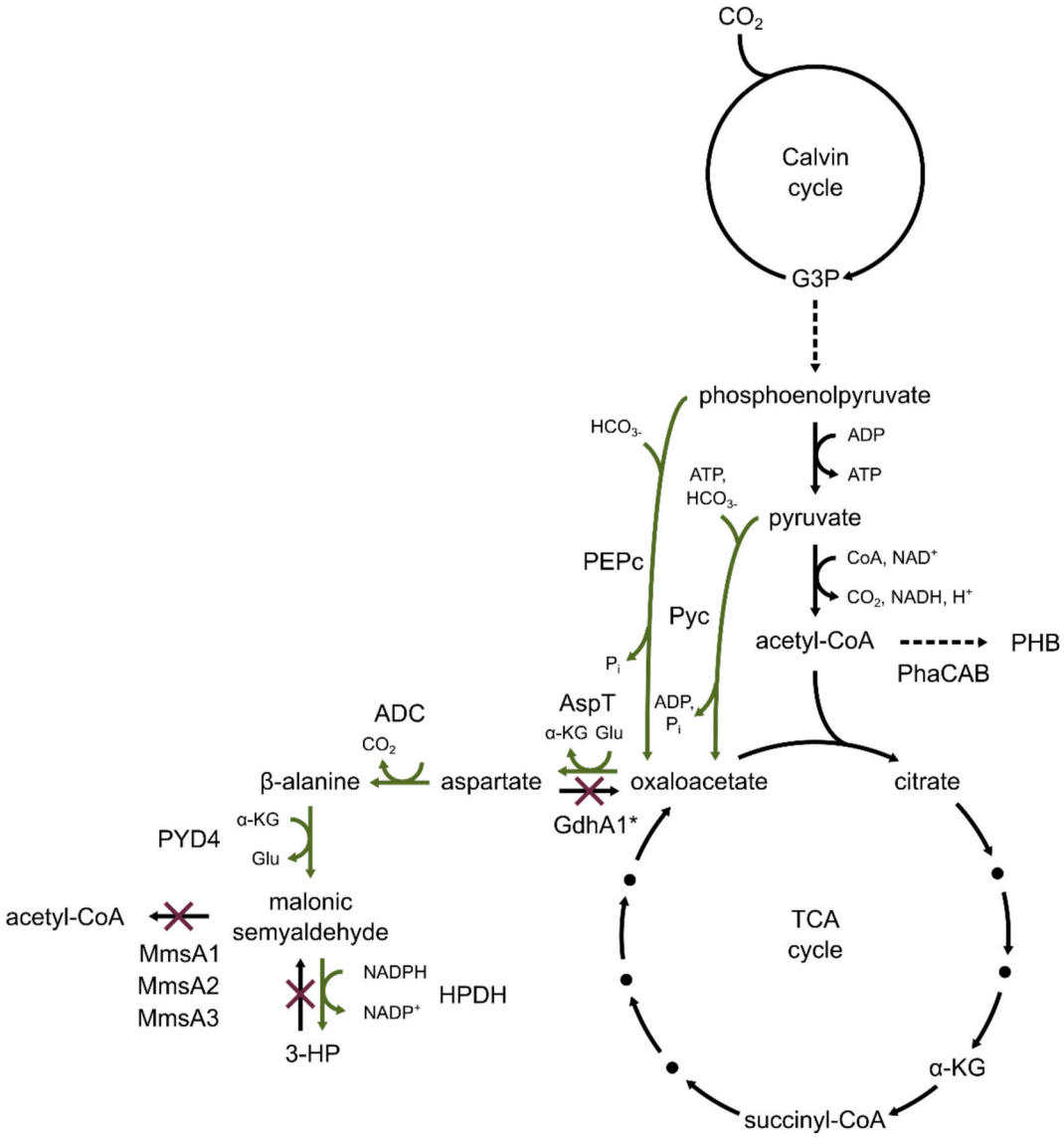

Fig. 1. Metabolic engineering strategy for the production of 3-HP in *C. necator* H16.
Black arrows indicate native enzymatic reactions. Green arrows indicate heterologously expressed enzymes. Burgundy crosses indicate gene knockouts used during this study. Dotted arrows indicate more than one reaction. Enzyme abbreviations: PEPc, phosphoenolpyruvate carboxylase; Pyc, pyruvate carboxylase; AspT, aspartate aminotransferase; ADC, aspartate 1-decarboxylase; PYD4, β-alanine aminotransferase; HPDH, 3-hydroxypropionate dehydrogenase; MmsA1, methylmalonate semialdehyde dehydrogenase A1; MmsA2, methylmalonate semialdehyde dehydrogenase A2; MmsA3, methylmalonate semialdehyde dehydrogenase A3; GdhA1, Glutamate/Aspartate dehydrogenase A1; PhaCAB, *phaCAB* encodes enzymes for PHA production: PHA synthase, 3-ketothiolase and acetoacetyl-CoA reductase. Chemical abbreviations: α-KG, α-ketoglutarate; Glu, glutamate; PHB, poly(3-hydroxybutyrate); 3-HP, 3-hydroxypropionic acid; G3P, glyceraldehyde 3-phosphate. *Proposed activity not validated.

Rathnasingh et al., 2012). The malonyl-CoA pathway is advantageous in that any carbon source can be used, and the pathway is short, typically only comprising of two enzymatic steps from acetyl-CoA. However, both of these steps are tightly controlled by regulatory mechanisms, presenting difficulties for de-bottlenecking these steps, and the acetyl-CoA carboxylase step requires biotin as a co-factor, necessitating supplementation of medium with this compound (Abdel-Hamid and Cronan, 2007; Milke and Marienhagen, 2020). Additionally, the malonyl-CoA route was previously determined to be strongly oxygen-dependent due to a high ATP requirement for acetyl-CoA synthesis (Borodina et al., 2015).

A more recently described route by which 3-HP can be produced is through the β-alanine pathway (Fig. 1). In this pathway, phosphoenolpyruvate (PEP) or pyruvate can be carboxylated to form oxaloacetate by a PEP carboxylase or pyruvate carboxylase, respectively. Oxaloacetate is subsequently converted to aspartate by the action of an aspartate transaminase. Decarboxylation of aspartate to β-alanine is facilitated by an aspartate decarboxylase. Lastly, β-alanine is converted to 3-HP via malonic semialdehyde. The β-alanine pathway has previously been applied to produce 3-HP and 3-HP-containing polymers in *Saccharomyces cerevisiae*, *Escherichia coli* and the cyanobacterium *Synechococcus elongatus* PCC 7942 (Borodina et al., 2015; Lacmata et al., 2017; Lan et al., 2015; Song et al., 2016; Wang et al., 2014).

The gram-negative bacterium *Cupriavidus necator* H16 (also known as *Ralstonia eutropha*) is a promising host for metabolic engineering. Discovered in the 1960s, *C. necator* H16 has been heavily researched due to its ability to accumulate the biodegradable polymer polyhydroxybutyrate (PHB) and to grow chemolithoautotrophically (Cramm, 2009; Peoples and Sinskey, 1989). The ability of *C. necator* H16 to grow using CO_2 as a sole source of carbon presents an economic advantage when compared to heterotrophic fermentation conditions, as the cost of feedstock in the latter is often comprising more than 60% of the total economic cost of the biobased products (Dürre and Eikmanns, 2015). CO_2 is a relatively cheap and abundant carbon source, available from a range of sources including industrial waste gases, or as a product formed in the gasification of solid waste or biomass. Additionally, use of CO_2 means it is possible to avoid the use of resources to produce sugars for fermentation processes, saving both food and land. The ability to use CO_2 therefore presents an opportunity for cost reduction in microbial processes, compared to sugar-based processes involving *E. coli* and yeast. While cyanobacteria are also capable of using CO_2, their growth rate is typically very slow compared to *C. necator* H16. Furthermore, *C. necator* H16 is amenable to genetic modification, and a broad range of genetic tools have been developed (Alagesan et al., 2018; Bi et al., 2013; Ehsaan et al., 2021; Johnson et al., 2018; Raberg et al., 2018). As a result, *C. necator* H16 has been engineered for the production of various compounds including diverse polymers, ketones, alcohols, and fatty acids (Bommareddy et al., 2020; Budde et al., 2011; Chen et al., 2015; Gascoyne et al., 2021; Ishizaki et al., 2001; Müller et al., 2013; Voss and Steinbüchel, 2006).

In this work we aimed to engineer *C. necator* H16 to produce 3-HP via the β-alanine pathway (Fig. 1). A previously described strain which is unable to use 3-HP as a carbon source was used as a starting strain (Arenas-López et al., 2019). From there, a systematic approach of pathway construction and strain engineering was used to implement a pathway for 3-HP biosynthesis from the central metabolism. Further gene deletions stabilised and increased 3-HP titre. Following successful production of 3-HP from central metabolism using gluconate as the carbon source, the engineered strain was used to demonstrate 3-HP synthesis during autotrophic growth.

2. Materials and methods

2.1. Strains and routine growth conditions

All strains used in this study are listed in Table 1. *E. coli* NEB5α was used for cloning and plasmid propagation. *E. coli* S17-1 was used for plasmid conjugation. *E. coli* strains were routinely grown at 37 °C in lysogeny broth (LB). *C. necator* H16 strains were routinely grown at 30 °C in LB or sodium gluconate minimal medium (SGMM). SGMM contains 4 g/l sodium gluconate, 9 g/l $Na_2HPO_4{\cdot}12H_2O$, 1.5 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.2 g/l $MgSO_4{\cdot}7H_2O$, 0.02 g/l $CaCl_2{\cdot}2H_2O$ and 0.0012 g/l ammonium iron (III) citrate (Trüper and Pfennig, 1981), and 1 ml/l of the modified trace element solution SL7 (Schlegel et al., 1961). If required, 12.5 μg/ml tetracycline was added for selection and plasmid retention, and 15 g/l agar for solid medium preparation. The pH of the media was adjusted to 7.0 using NaOH.

Table 1
List of strains and plasmids used in this study.

Name	Description	Source
Strain		
E. coli NEB5α	*fhuA2 D(argF-lacZ)U169 phoA glnV44 f80D(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs
E. coli S17-1	*recA pro hsdR* RP42Tc::MuKm::Tn*7* integrated into the chromosome	Simon et al. (1983)
C. necator H16	*C. necator* H16 (DSM 428, ATCC 17669)	Little et al. (2019)
C. necator H16 Δ3	*C. necator* H16 Δ*mmsA1* Δ*mmsA2* Δ*mmsA3*	Arenas-López et al. (2019)
C. necator H16 Δ3 Δ*phaCAB*	*C. necator* H16 Δ*mmsA1* Δ*mmsA2* Δ*mmsA3* Δ*phaCAB*	This study
C. necator H16 Δ3 Δ*phaCAB* Δ*gdhA1*	*C. necator* H16 Δ*mmsA1* Δ*mmsA2* Δ*mmsA3* Δ*phaCAB* ΔH16_A0471	This study
C. necator H16 Δ3 Δ*phaCAB* Δ*aspdH*	*C. necator* H16 Δ*mmsA1* Δ*mmsA2* Δ*mmsA3* Δ*phaCAB* ΔH16_B0736	This study
C. necator H16 Δ3 Δ*phaCAB* Δ*asdA*	*C. necator* H16 Δ*mmsA1* Δ*mmsA2* Δ*mmsA3* Δ*phaCAB* ΔH16_A3009	This study
C. necator H16 Δ3 Δ*hpdH*	*C. necator* H16 Δ*mmsA1* Δ*mmsA2* Δ*mmsA3* Δ*hpdH*	This study
C. necator H16 Δ3 Δ*gdhA1*	*C. necator* H16 Δ*mmsA1* Δ*mmsA2* Δ*mmsA3* ΔH16_A0471	This study
C. necator H16 Δ3 Δ*gdhA1* Δ*hpdH*	*C. necator* H16 Δ*mmsA1* Δ*mmsA2* Δ*mmsA3* ΔH16_A0471 Δ*hpdH*	This study
Plasmid		
pNC	pMTL71301-P_{phaC}-eyfp	McGregor et al. (2021)
pBAPAT$_{Cv}$	pMTL71301-P_{phaC}-BAPAT$_{Cv}$-*ydfG*$_{Ec}$	This study
pPYD4$_{Sk}$	pMTL71301-P_{phaC}-PYD4$_{Sk}$-*ydfG*$_{Ec}$	This study
pPanD$_{Cg}$	pMTL71301-P_{trc}-*panD*$_{Cg}$-P_{phaC}-PYD4$_{Sk}$-*ydfG*$_{Ec}$	This study
pADC$_{Aa}$	pMTL71301-P_{trc}-*ADC*$_{Aa}$-P_{phaC}-PYD4$_{Sk}$-*ydfG*$_{Ec}$	This study
pADC$_{Tc}$	pMTL71301-P_{trc}-*ADC*$_{Tc}$-P_{phaC}-PYD4$_{Sk}$-*ydfG*$_{Ec}$	This study
pPEPc$_{Cg}^{N917G}$	pMTL71301-P_{trc}-*ADC*$_{Aa}$-*ppc*$_{Cg}^{N917G}$-*aspT*$_{Cg}$-P_{phaC}-*PYD4*$_{Sk}$-*ydfG*$_{Ec}$	This study
pPyc$_{Cg}^{T132A}$	pMTL71301-P_{trc}-*ADC*$_{Aa}$-*pyc*$_{Cg}^{T132A}$-*aspT*$_{Cg}$-P_{phaC}-*PYD4*$_{Sk}$-*ydfG*$_{Ec}$	This study
pΔ*phaCAB*	pIO3-*phaCAB*LH-*phaCAB*RH	This study
pΔ*gdhA1*	pMTL70641-*gdhA1*LH-*gdhA1*RH	This study
pΔ*aspDH*	pMTL70641-*aspDH*LH-*aspDH*RH	This study
pΔ*asdA*	pIO3-*asd*ALH-*asdA*RH	This study
pΔ*hpdH*	pIO3-*hpdH*LH-*hpdH*RH	Arenas-López et al. (2019)

2.2. Construction of production and deletion plasmids

Plasmids were constructed using NEBuilder® HiFi DNA assembly (New England Biolabs, USA). DNA parts were amplified by PCR using Q5® High-Fidelity DNA Polymerase (New England Biolabs, USA) according to the manufacturer's instructions. All the primers used in this study are listed in Table S1 pMTL71301, a modular plasmid carrying a tetracycline resistance marker (Ehsaan et al., 2021), was used as the backbone for all the production plasmids. P_{phaC}, *BAPAT*$_{Cv}$, and ydfG$_{Ec}$ were amplified by PCR using the plasmid pCNCM0 as the template (McGregor et al., 2021). The genes *panD*$_{Cg}$, *aspT*$_{Cg}$, *ppc*$_{Cg}^{N917G}$ and *pyc*$_{Cg}^{T132A}$

were amplified by PCR using *Corynebacterium glutamicum* colonies as the template. Point mutations for ppc_{Cg}^{N917G} and pyc_{Cg}^{T132A} were introduced in the primers during the PCR amplification of the DNA parts. $PYD4_{Sk}$, ADC_{Aa} and ADC_{Tc} cDNAs were codon optimized for *C. necator* H16 and synthesised by Invitrogen (USA) (sequences in Supporting Information 1.4). Ribosome-binding sites (RBSs), with target translation initiation rates of ~5000, were designed for each gene using the RBS calculator (Salis et al., 2009) and inserted upstream of each gene (Table S2). P_{trc}, TrrnB1 and all the RBSs were added as intervening sequences as part of the primers. Suicide plasmids were constructed using homology arms consisting of ~700 bp upstream and downstream of the gene or operon to be deleted and either the pLO3 or the pMTL70641 plasmids were used as the backbone. Homology arms were amplified by PCR using *C. necator* H16 colonies as the template. The backbones contain a tetracycline resistance marker and the *sacB* gene for counter-selection (Lenz and Friedrich, 1998). The backbones were linearized using SacI and XbaI. All DNA parts were gel purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, United States) according to the manufacturer's instructions. Plasmids were built using the NEBuilder® HiFi DNA Assembly Master Mix according to the manufacturer's instructions. The resulting assembly reactions were used to transform *E. coli* NEB5α chemically competent cells, candidate plasmids isolated using the QIAprep Spin Miniprep Kit (QIAGEN, Germany) according to the manufacturer's instructions, and DNA sequences verified by Source BioScience (UK). All plasmids are listed in Table 1.

2.3. Transformation of E. coli and C. necator strains

E. coli S17 and *E. coli* NEB5α chemically competent cells were prepared and transformed as described previously (Chung et al., 1989). A heat shock step of 90 s at 42 °C followed by 90 s on ice was performed before adding 950 ml of SOC medium for recovery. For *C. necator* H16 strains, a tube containing 5 ml of SOB medium was inoculated with a loop of *C. necator* cells and grown at 30 °C with shaking at 200 rpm overnight. The cells were centrifuged for 1 min at 13000 rpm and the pellet washed twice using 1 ml of 1 mM $MgSO_4$. The pellet was resuspended in 50 μl of 1 mM $MgSO_4$ and mixed with ~250 ng of plasmid vector. Electroporation was performed using a 0.2 cm gap cuvette at 2.5 kV, 200Ω and 25 μF. Immediately after electroporation, 950 μl of SOC medium was added for recovery. The cells were incubated for 3 h at 30 °C with shaking at 200 rpm, and 100 μl plated on a LB agar plate with 15 μg/ml tetracycline for selection. The plate was incubated for 2–3 days at 30 °C. Four single colonies were streaked on SGMM agar plates, and 15 μg/ml tetracycline and the plate incubated for 2–3 days at 30 °C.

2.4. Construction of deletion strains

A tube containing 5 ml of LB supplemented with 12.5 μg/ml tetracycline was inoculated with a colony of *E. coli* S17-1 carrying the suicide plasmid and cultivated at 37 °C with 200 rpm shaking for 16 h. In parallel, 5 ml of low salt LB (LSLB, 2.5 g/l NaCl) was inoculated with *C. necator* H16 and cultivated at 30 °C for 20 h with shaking at 200 rpm. Conjugation was carried out using a spot mating technique (Simon et al., 1983), and transconjugant selected by plating on SGMM agar plates supplemented with 12.5 μg/ml tetracycline. Single colonies were purified by streaking on the same medium twice and inoculated in LSLB supplemented with 150 g/l sucrose. The culture was grown overnight at 30 °C with 200 rpm and ~10^8 cells plated on LSLB agar supplemented with 150 g/l sucrose and the plate incubated at 30 °C. Single colonies were purified in LB agar plates with and without tetracycline supplementation and strains without antibiotic resistance purified. Deletion strains were confirmed by colony PCR using external and internal primers (Table S1).

2.5. Shake flask cultivation for 3-HP production

C. necator H16 colonies carrying the desired plasmid were used to inoculate tubes containing 5 ml of LB with 15 μg/ml tetracycline. Each tube was incubated at 30 °C overnight with shaking at 200 rpm. Precultures were centrifugated at 8000 rpm for 3 min, washed twice and used to inoculate 250 ml baffled flask containing the production medium. Unless stated nitrogen-limited medium consisting of 2% sodium gluconate (w/v) minimal medium containing 0.61 g/l $(NH_4)_2SO_4$ instead of 1 g/l NH_4Cl was used as the production medium (2%SGMM). For phosphate limitation the phosphate content of 2%SGMM was reduced to 0.14 g/l $Na_2HPO_4x12H_2O$ and 0,02 g/l KH_2PO_4, and $(NH_4)_2SO_4$ increased to 7.5 g/l. For magnesium limitation $MgSO_4 \times 7H_2O$ in 2%SGMM was reduced to 0.025 g/l and $(NH_4)_2SO_4$ increased to 7.5 g/l. Cultivations were carried out using 25 ml of culture at OD600–0.1 supplemented with 15 μg/ml tetracycline and 50 mM β-alanine or 50 mM aspartate if required. The flasks were incubated at 30 °C with shaking at 200 rpm.

2.6. Growth analysis

The inoculum was generated as in section 2.5 excluding the tetracycline and was used to prepare a 48-well flower BioLector plate with 1 ml of culture at OD600–0.1. The plate was incubated at 30 °C in a BioLector (m2p-labs, Germany) for 96 h with shaking at 1200 rpm. Scattered light was recorded every 30 min. OD600 was calculated using a standard curve correlating the measured scattered light values and OD600 values. A modified minimal medium containing 4 g/l $(NH_4)_2SO_4$ and 6.66 g/l aspartate was used.

2.7. Transcriptome analysis

C. necator H16 Δ*mmsA123* Δ*phaCAB* colonies were used to inoculate tubes containing 5 ml of LB. Each tube was incubated at 30 °C overnight with shaking at 200 rpm. Precultures were centrifugated at 8000 rpm for 3 min, washed twice and used to prepare 25 ml of culture at OD600–0.1 in a 250 ml baffled flask. 2%SGMM containing 4 g/l $(NH_4)_2SO_4$ with and without 4 g/l aspartate was used as the culture medium. The flasks were incubated at 30 °C with shaking at 200 rpm. Samples were collected at the early exponential growth phase (OD600 ~1) and treated with RNAprotect Bacteria Reagent (QIAGEN, Germany) according to the manufacturer instructions. Cell pellets were sent to Genewiz (USA) for RNA sequencing and differential gene expression analysis using the DESeq2 algorithm. Raw fastq files were deposited at ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) and are accessible under accession number E-MTAB-11897.

2.8. Autotrophic fermentation

Autotrophic fermentations were carried out as described previously (Bommareddy et al., 2020). Briefly, cultivations were performed in 1.3 l DASGIP parallel bioreactors (Eppendorf SE, Juilich, Germany) with a working volume of 750 ml. CO_2, H_2 and air were supplied via separate mass flow controllers into the bioreactors at a ratio of 1:26:6.3, and total flow rate of 0.75 vvm. The pH of the media was monitored throughout using a pH probe (Mettler Toledo, Leicester, UK) and kept constant at 6.9 by adding 1 M NaOH solution. Dissolved oxygen was kept at 10% minimum by cascading to agitation and monitored using a DO probe (Mettler Toledo, Leicester, UK), calibrated at 0 and 100% pO_2. Off-gas composition was measured using Raman based gas analyser (ARI inc). CO_2 and air compositions were controlled using the mass flow controllers within the DASGIP system and H_2 gas composition was controlled using an integrated Red-y mass flow controller (Voetlin Instruments) via the DASGIP's DASware control.

2.9. Analytical methods

Extracellular 3-HP concentration was determined by HPLC using the method described in (Arenas-López et al., 2019).

3. Results and discussion

3.1. A biosynthetic pathway for 3-HP production from β-alanine

The β-alanine pathway is one of the most promising routes for the industrial production of 3-HP, as it is thermodynamically favourable and is not significantly affected by the degree of aeration (Borodina et al., 2015; Vidra and Németh, 2018). Therefore, it was chosen to be engineered in *C. necator* H16 for the production of 3-HP. In order to identify the best enzymes for each reaction and to identify potential bottlenecks, a stepwise approach was applied. First, a synthetic pathway for the biosynthesis of 3-HP from the intermediate β-alanine was designed. This was followed by the identification of an aspartate decarboxylase suitable for the conversion of aspartate to β-alanine and thus allowing the production of 3-HP from aspartate. 3-HP production from the central metabolism was then assessed by establishing pathways for aspartate production from pyruvate and phosphoenolpyruvate. Finally, based on the previous results additional knockout targets were identified to further improve 3-HP production. The overall metabolic engineering strategy to produce 3-HP in C. necator H16 is shown in Fig. 1Co-expression of a β-alanine-pyruvate aminotransferase from *Chromobacterium violaceum* ($BAPAT_{Cv}$) and a NADPH-dependent 3-hydroxypropionate dehydrogenase from *E. coli* ($YdfG_{Ec}$) in *C. necator* H16 Δ3 was shown to allow 3-HP synthesis from β-alanine (McGregor et al., 2021). However, for each molecule of β-alanine utilized, $BAPAT_{Cv}$ generates one molecule of L-alanine (Fig. 2, A). As L-alanine has been proven difficult to recycle (Borodina et al., 2015), in this study we also studied the use of $PYD4_{Sk}$ as an alternative to CvBAPAT. $PYD4_{Sk}$, a β-alanine aminotransferase from *Saccharomyces kluyveri*, generates one molecule of glutamate per molecule of β-alanine utilized (Fig. 2, A). PYD4Sk was chosen as a second candidate as it has been previously used to produce 3-HP in the cyanobacterium *Synechococcus elongatus* PCC 7942 (Lan et al., 2015). Two plasmids were constructed: $pBAPAT_{Cv}$ and $pPYD4_{Sk}$ (Fig. 2, B). $pBAPAT_{Cv}$ carries $BAPAT_{Cv}$ and $ydfG_{Ec}$ under the control of the native *C. necator* H16 promoter *phaC*, while $pPYD4_{Sk}$ maintains the same architecture having $BAPAT_{Cv}$ replaced by $PYD4_{Sk}$. pNC, carrying an *eyfP* gene under the control of the *phaC* promoter, was included as a negative control. The *phaC* promoter, which controls expression of the *phaCAB* operon in *C. necator* H16 is a well characterized constitutive weak promoter (Alagesan et al., 2018; Delamarre and Batt, 2006) previously used for the production of PHB in *E. coli* (T. Y. Li et al., 2016; Sato et al., 2007), as well as 3HHx-containing- and lactic acid-containing copolymers in *C. necator* (Arikawa and Matsumoto, 2016; Insomphun et al., 2015; Park et al., 2013). Additionally, it was shown previously that expression of $BAPAT_{Cv}$ and $YdfG_{Ec}$ under the *phaC*

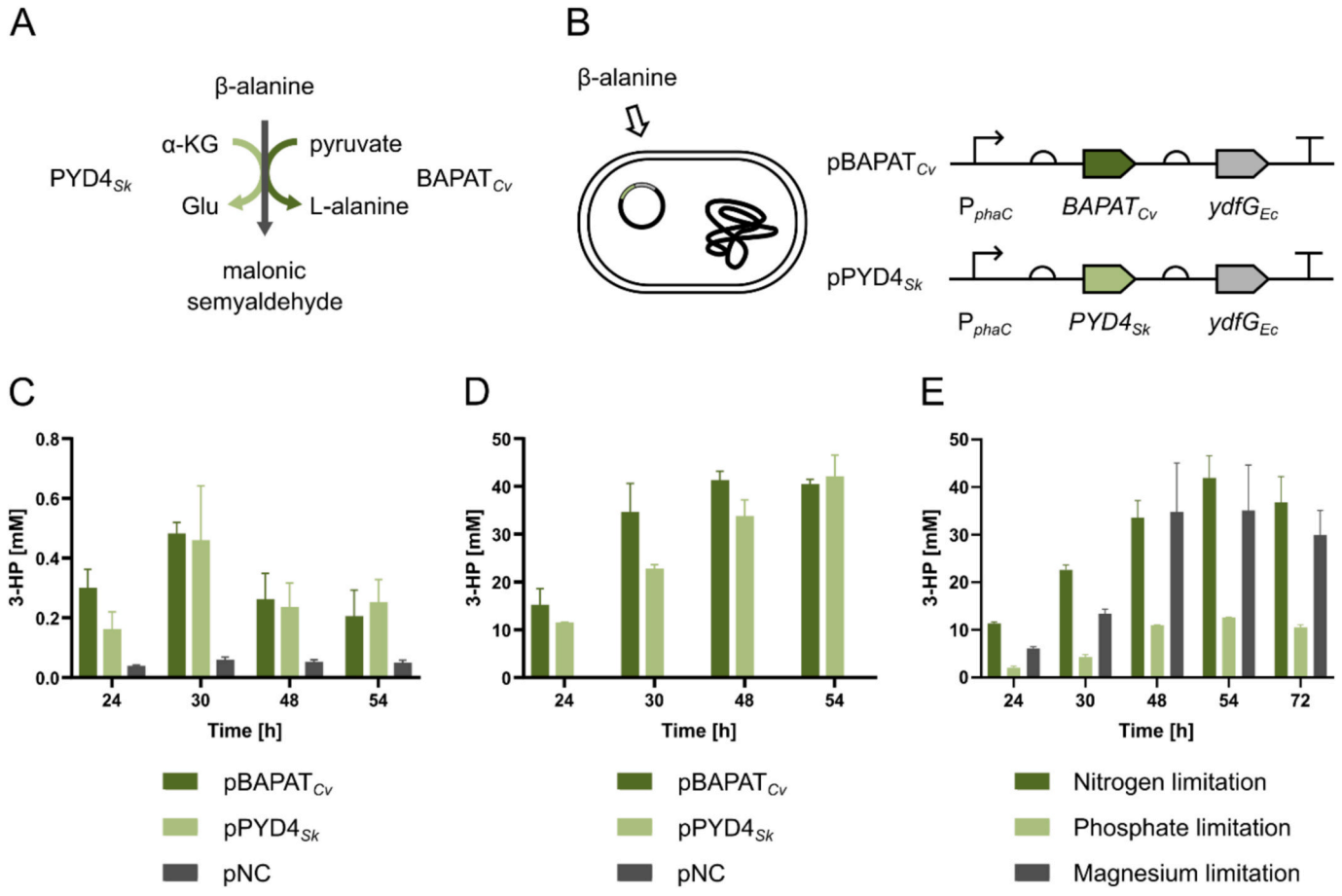

Fig. 2. Recombinant production of 3-HP from β-alanine in *C. necator* H16 and *C. necator* H16 Δ3. Schematic representation of β-alanine aminotransferase reactions (A) and the 3-HP producing constructs (B). P_{phaC}: *phaC* promoter, $BAPAT_{Cv}$: β-alanine-pyruvate aminotransferase from *C. violaceum*, $PYD4_{Sk}$: β-alanine aminotransferase from *S. kluyveri*, and $ydfG_{Ec}$: 3-hydroxypropionate dehydrogenase from *E. coli*. Conversion of β-alanine to 3-HP using *C. necator* H16 (C) and *C. necator* H16 Δ3 (D). Cultivations were carried out using nitrogen limited 2%SGMM supplemented with 50 mM β-alanine. $pBAPAT_{Cv}$: P_{phaC}-$BAPAT_{Cv}$-$ydfG_{Ec}$, $pPYD4_{Sk}$: P_{phaC}-$PYD4_{Sk}$-$ydfG_{Ec}$, pNC: P_{phaC}-*eyfp*. (E) 3-HP production from β-alanine using *C. necator* H16 Δ3 carrying $pPYD4_{Sk}$ under different nutrient limitations. Cultivations were carried out using either nitrogen, phosphate, or magnesium limited 2%SGMM supplemented with 50 mM β-alanine. Error bars indicate standard deviations of 3 biological replicates.

promoter was sufficient for efficient conversion of exogenously supplied beta-alanine to 3-HP (McGregor et al., 2021). The modular vector pMTL71301 (Ehsaan et al., 2021) was used as the backbone in all constructed plasmids.

C. necator H16 and *C. necator* H16 Δ3 were transformed with the three plasmids. Three methyl-malonate semialdehyde dehydrogenases (*mmsA1*, *mmsA2* and *mmsA3*) have been deleted in the latter mutant and *C. necator* H16 Δ3 is therefore unable to grow using 3-HP as sole carbon source (Arenas-López et al., 2019). The strains were grown in nitrogen-limited 2%SGMM supplemented with 50 mM of β-alanine. Nitrogen-limited MM was utilized to simulate the conditions needed to produce PHB, where NADPH is also required for the synthesis of the final product. As expected, when the wild-type strain was used very little 3-HP production was observed (<0.5 mM 3-HP) (Fig. 2, C). On the other hand, when *C. necator* H16 Δ3 was used the strains carrying the plasmids pBAPAT$_{Cv}$ and pPYD4$_{Sk}$ were able to produce considerably higher levels of 3-HP (~40 mM 3-HP) (Fig. 2, D). No 3-HP production was observed when the control plasmid was used. These results confirm that the deletion of *mmsA1*, *mmsA2* and *mmsA3* is required for efficient production of 3-HP using *C. necator* H16. Although similar production levels were observed when cells carried either plasmid pBAPAT$_{Cv}$ or pPYD4$_{Sk}$ the latter was selected for the remainder of the study as the produced glutamate can be used by a heterologously expressed aspartate aminotransferase to produce 3-HP from the central metabolism intermediates (Fig. 1). As β-alanine is required for pantothenate synthesis, a crucial precursor of Coenzyme A, carbon losses are expected when using this pathway. The biosynthesis of pantothenate from β-alanine is catalysed by the pantothenate synthetase PanC, an essential enzyme in *C. necator* H16, and thus it was not possible to eliminate this competing pathway.

As nitrogen is required for some of the intermediates of the β-alanine pathway, the use of other nutrient limited media was investigated. Phosphate- and magnesium-limited media were chosen as these conditions are also used in *C. necator* H16 for PHB production (Asenjo et al., 1995). In addition, the use of magnesium starvation has been shown to be beneficial in *E. coli* to produce 3-HP via malonyl-CoA, another NADPH-dependent pathway (Tokuyama et al., 2019). Accordingly, *C. n*ecator H16 Δ3 cells carrying pPYD4$_{Sk}$ were grown using phosphate- and magnesium-limited MM supplemented with 50 mM β-alanine and 2% (w/v) sodium gluconate (Fig. 2, E). In both cases 3-HP production was observed. The levels of production, however, were only comparable with those achieved using nitrogen limitation when magnesium limitation was used. The low levels of 3-HP accumulation when phosphate limitation was used may be attributed to a reduction in the availability of pyridoxal 5′-phosphate (PLP) caused by PLP phosphatases. PLP is an essential co-factor for the aminotransferase reaction catalysed by PYD4$_{Sk}$ (Andersen et al., 2007). The induction of phosphatases under phosphate starvation conditions, and in particular pyridoxal 5′-phosphate (PLP) phosphatases, has been reported in other microorganisms (Doi and Shioi, 1988; Torriani, 1960). A small reduction in the 3-HP concentration after the peak at 54 h was observed in all cultures

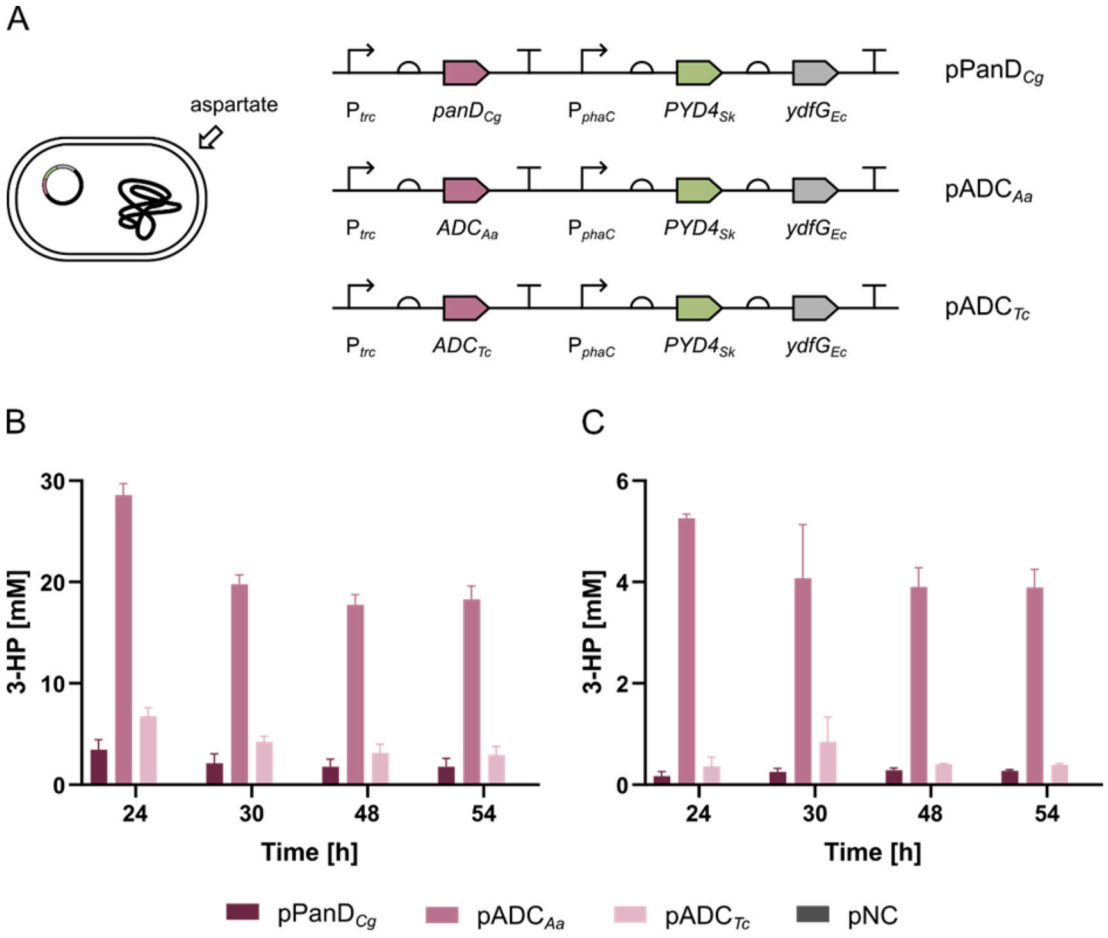

Fig. 3. Heterologous production of 3-HP from aspartate in *C. necator* H16 Δ3. (A) Schematic representation of the 3-HP producing constructs. P$_{phaC}$: *phaC* promoter from *C. necator* H16, P$_{trc}$: *trc* promoter from *E. coli*, *panD*$_{Cg}$: aspartate 1-decarboxylase from *C. glutamicum*, *ADC*$_{Aa}$: aspartate 1-decarboxylase from *A. aegypti*, *ADC*$_{Tc}$: aspartate 1-decarboxylase from *T. castaneum*, *PYD4*$_{Sk}$: β-alanine aminotransferase from *S. kluyveri*, and *ydfG*$_{Ec}$: 3-hydroxypropionate dehydrogenase from *E. coli*. Production of 3-HP from aspartate using *C. necator* H16 Δ3 using nitrogen (B) and magnesium (C) limitation. Cultivations were carried out using 2%SGMM supplemented with 50 mM aspartate. pPanD$_{Cg}$: P$_{trc}$-*panD*$_{Cg}$-P$_{phaC}$-*PYD4*$_{Sk}$-*ydfG*$_{Ec}$, pADC$_{Aa}$: P$_{trc}$-*ADC*$_{Aa}$-P$_{phaC}$-*PYD4*$_{Sk}$-*ydfG*$_{Ec}$, pADC$_{Tc}$: P$_{trc}$-*ADC*$_{Tc}$-P$_{phaC}$-*PYD4*$_{Sk}$-*ydfG*$_{Ec}$, pNC: P$_{phaC}$-*eyfp*. Error bars indicate standard deviations of 3 biological replicates.

(Fig. 2, E), suggesting that enzymes other than the three methyl-malonate semialdehyde dehydrogenases may be involved in 3-HP metabolism.

3.2. Comparison of three aspartate decarboxylases for 3-HP production from aspartate

Three aspartate 1-decarboxylases were assessed for the production of 3-HP from aspartate: $PanD_{Cg}$ from *Corynebacterium glutamicum*, ADC_{Aa} from *Aedes aegypti* and ADC_{Tc} from *Tribolium castaneum*. The aspartate decarboxylase genes were cloned upstream of the P_{phaC}-*PYD4*$_{Sk}$-*ydfG*$_{Ec}$ operon under the control of the *trc* promoter from *E. coli*, generating the plasmids $pPanD_{Cg}$, $pADC_{Aa}$ and $pADC_{Tc}$ (Fig. 3, A). Plasmid pNC was used as a negative control.

C. necator H16 Δ3 cells were transformed with the plasmids and the obtained strains grown in nitrogen- and magnesium-limited MM supplemented with 50 mM of aspartate and 2% (w/v) sodium gluconate (Fig. 3, B–C). Cells containing $pADC_{Aa}$ showed the highest levels of 3-HP production in both nitrogen- and magnesium-limited MM, while those carrying $pPanD_{Cg}$ showed the lowest levels of 3-HP production in both media. These results are in agreement with those reported in *S. elongatus* and *S. cerevisiae*, where ADC_{Aa} and $pADC_{Tc}$ outperformed $PanD_{Cg}$, respectively (Borodina et al., 2015; Lan et al., 2015). Unlike bacterial PanDs, ADCs from insects do not undergo post-translational self--cleavage activation or suffer from turnover-dependent inactivation (Liu et al., 2019; Mo et al., 2018), which may explain these findings. To the best of our knowledge, no prior comparison between ADC_{Aa} and ADC_{Tc} has been reported. Cells carrying all three production plasmids produced considerably higher levels of 3-HP under nitrogen-limited conditions when compared to magnesium-limited conditions. In particular, when $pADC_{Aa}$ was used, 3-HP production was more than 5-fold higher under nitrogen-limited conditions (29 mM) than under magnesium-limited conditions (5 mM). These results suggest that the presence of Mg^{2+} may be beneficial for the aspartate decarboxylases. Supporting this

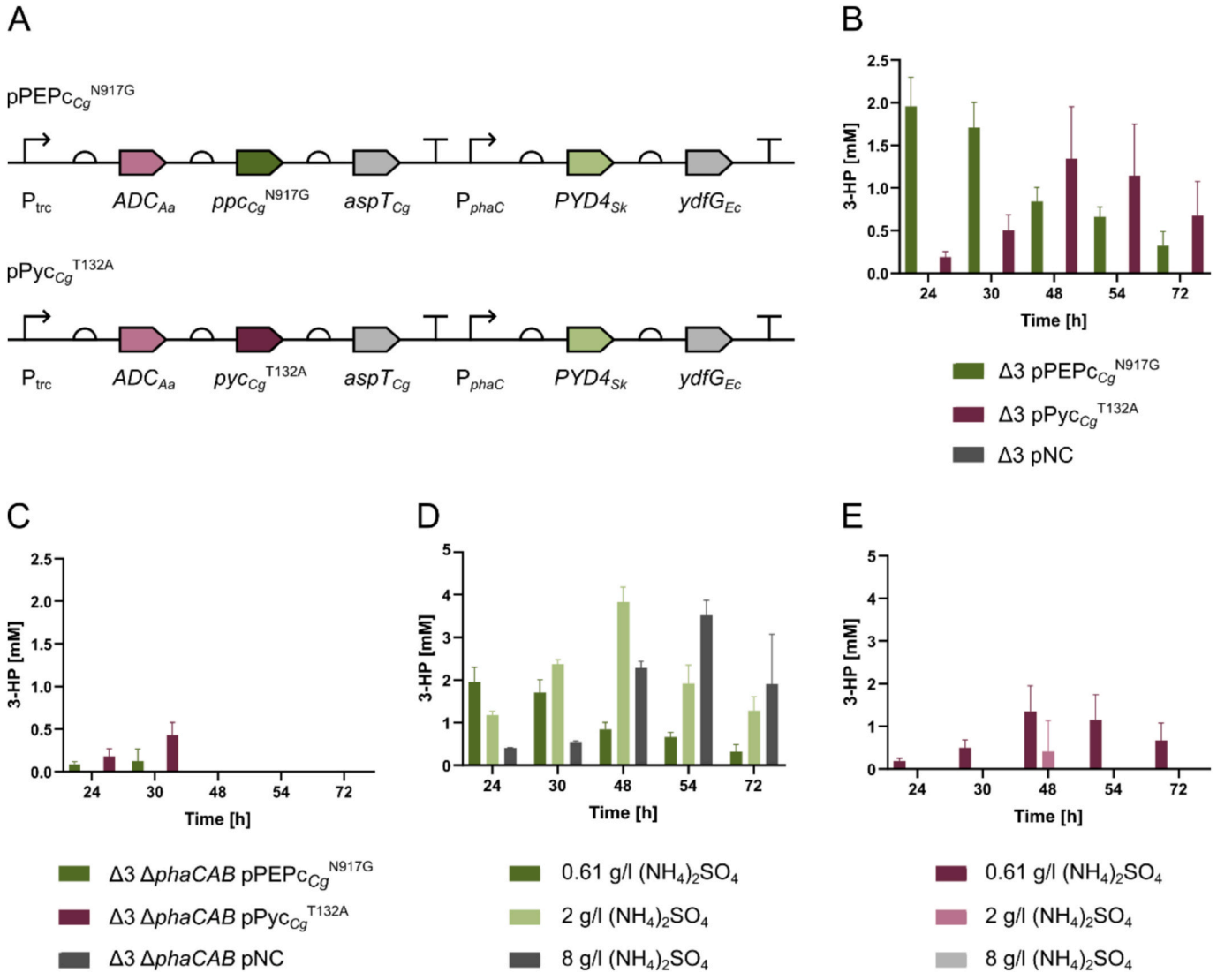

Fig. 4. 3-HP production from the central metabolism using *C. necator* H16 Δ3 and *C. necator* H16 Δ3 Δ*phaCAB*. (A) Schematic representation of the 3-HP producing constructs. P_{phaC}: *phaC* promoter from *C. necator* H16, P_{trc}: *trc* promoter from *E. coli*, ppc_{Cg}^{N917G}: feedback resistant mutant of the phosphoenolpyruvate carboxylase from *C. glutamicum*, pyc_{Cg}^{T132A}: mutant variant of the pyruvate carboxylase from *C. glutamicum*, $aspT_{Cg}$: aspartate aminotransferase from *C. glutamicum*, ADC_{Aa}: aspartate 1-decarboxylase from *A. aegypti*, *PYD4*$_{Sk}$: β-alanine aminotransferase from *S. kluyveri,* and *ydfG*$_{Ec}$: 3-hydroxypropionate dehydrogenase from *E. coli*. 3-HP production using *C. necator* H16 Δ3 (B) and *C. necator* H16 Δ3 Δ*phaCAB* (C). Cultivations were carried out using 2% SGMM containing 0.61 g/l $(NH_4)_2SO_4$. $pPEPc_{Cg}^{N917G}$: P_{trc}-ADC_{Aa}-ppc_{Cg}^{N917G}-$aspT_{Cg}$-P_{phaC}-*PYD4*$_{Sk}$-*ydfG*$_{Ec}$, $pPyc_{Cg}^{T132A}$: P_{trc}-ADC_{Aa}-pyc_{Cg}^{T132A}-$aspT_{Cg}$-P_{phaC}-*PYD4*$_{Sk}$-*ydfG*$_{Ec}$, and pNC: P_{phaC}-*eyfp*. 3-HP production using H16 Δ3 harbouring $pPEPc_{Cg}^{N917G}$ (D) and $pPyc_{Cg}^{T132A}$ (E) under different nitrogen conditions. Cultivations were carried out using 2%SGMM containing the indicated concentrations of $(NH_4)_2SO_4$. Error bars indicate standard deviations of 3 biological replicates.

statement, it has been reported that the activity the L-aspartate-α-decarboxylase from *E. coli* is enhanced by Mg^{2+} (Nakano and Kitaoka, 1971). Considering this, the use of magnesium limitation as a strategy for 3-HP production was discarded and only nitrogen limitation was used in the following experiments. As before, a decrease in 3-HP accumulation was observed after reaching maximum production in all the cultures.

It is worth noting that considerably higher growth levels were observed when aspartate was added to the culture medium compared to when the medium was supplemented with β-alanine (data not shown). This suggest that *C. necator* H16 may be able to use aspartate as a source of carbon, diverting some of the carbon away from the production of 3-HP to biomass formation.

3.3. Production of 3-HP from the central metabolism

To avoid supplementation of expensive precursors for 3-HP production, two strategies for aspartate synthesis from central metabolism were explored. The first strategy involves the carboxylation of phosphoenolpyruvate to oxaloacetate, while the second requires carboxylation of pyruvate to oxaloacetate. In both cases, oxaloacetate then needs to be aminated by an aspartate aminotransferase to form aspartate (Fig. 1). Based on these strategies two plasmids for the production of 3-HP from the central metabolism were constructed: $pPEPc_{Cg}^{N917G}$ and $pPyc_{Cg}^{T132A}$ (Fig. 4, A). To generate $pPEPc_{Cg}^{N917G}$, a feedback resistant mutant of the phosphoenolpyruvate carboxylase from *C. glutamicum* (ppc_{Cg}^{N917G}) (Chen et al., 2014) and an aspartate aminotransferase from *C. glutamicum* ($aspT_{Cg}$) were added downstream of ADC_{Aa} in $pADC_{Aa}$. To generate $pPyc_{Cg}^{T132A}$, ppc_{Cg}^{N917G} from $pPEPc_{Cg}^{N917G}$ was replaced by a mutant variant of the pyruvate carboxylase from *C. glutamicum* (pyc_{Cg}^{T132A}) (Kortmann et al., 2019). Plasmid pNC was used as a negative control.

C. necator H16 Δ3 cells were transformed with the plasmids and the obtained strains grown in nitrogen-limited MM supplemented with 2% (w/v) sodium gluconate (Fig. 4, B). *C. necator* H16 Δ3 $pPEPc_{Cg}^{N917G}$ showed the highest levels of 3-HP production among all tested strains, achieving a maximum concentration of 1.96 mM 3-HP after 24 h which then decreased over time to 0.32 mM after 72 h. The same behaviour was observed when *C. necator* H16 Δ3 $pPyc_{Cg}^{T132A}$ was used, where 3-HP production peaked at 1.35 mM after 48 h and then dropped to 0.68 mM after 72 h. These results confirm that the produced 3-HP is re-consumed by the strains.

Additionally, to investigate whether the carbon used for PHB synthesis could be redirected towards 3-HP production, the plasmids $pPEPc_{Cg}^{N917G}$ and $pPyc_{Cg}^{T132A}$ were used to transform the PHB- strain *C. necator* H16 Δ3 Δ*phaCAB*. The strains were grown using the same conditions as above and 3-HP production studied. Interestingly, deletion of the *phaCAB* operon was detrimental to 3-HP production and considerably lower levels of production were obtained when Δ*phaCAB* strains were used (Fig. 4, C). PHB production can alter global metabolic regulation in microorganisms, including redox and carbon balances (Peplinski et al., 2010; Xu et al., 2016; Zhang et al., 2006). It has previously been shown that co-production of PHAs can have a positive effect on the production of a second metabolite (Kang et al., 2010; Liu et al., 2007; Xu et al., 2016; Zhang et al., 2006). While this phenomenon is not yet well understood, one possible explanation for this could be the effect of NADPH consumption on flux through the TCA cycle. Previously it was shown that deletion of the *zwf* gene from the *E. coli* genome increases flux through the TCA cycle (Nicolas et al., 2007). The reaction catalysed by G6PDH, which is encoded by zwf, is an NADPH-producing reaction. The authors of the study determined that in strains lacking a functional *zwf* gene, flux through the TCA cycle was increased to maintain a sufficient intracellular concentration of NADPH. It was later hypothesised by Kang et al. (2010) that this effect could be mimicked in *E. coli* which had been engineered to produce PHB. Since PHB is a sink for NADPH, Kang et al. suggested that the engineered PHB-producing *E. coli* would also increase flux through the TCA to maintain adequate quantities of NADPH in the cell. Their results showed that the NADPH/$NADP^+$ ratio in PHB-producing and non-producing strains of *E. coli* was the same, suggesting higher carbon flux through TCA to maintain NADPH concentration. Since the target product of that study was succinate, an intermediate of the TCA cycle, increased flux through the TCA was hypothesised to result in increased production of succinate. One possibility is, therefore, that removing PHB biosynthesis in *C. necator* H16 could reduce flux to the TCA cycle, reducing the availability of precursors to 3-HP.

In order to try to improve 3-HP production levels, the use of different concentrations of initial nitrogen was studied. *C. necator* H16 Δ3 cells carrying either $pPEPc_{Cg}^{N917G}$ or $pPyc_{Cg}^{T132A}$ were grown using MM supplemented with 2% (w/v) sodium gluconate and either 2 g/l (C/N: 18.2 mol-C/mol-N) or 8 g/l $(NH_4)_2SO_4$ (C/N: 4.5 mol-C/mol-N) instead of the 0.61 g/l $(NH_4)_2SO_4$ (C/N: 59.2 mol-C/mol-N) used before. When cells carried plasmid $pPEPc_{Cg}^{N917G}$, a delay in 3-HP production was observed when the concentration of initial nitrogen was increased (Fig. 4, D), suggesting that nitrogen limitation may be required for 3-HP production. However, considerably higher levels of 3-HP production were obtained when the C/N ratio was reduced. The highest level of 3-HP production (3.83 mM) was achieved after 48 h of cultivation using 2 g/l $(NH_4)_2SO_4$. Noticeably, a subsequent increase in the initial nitrogen concentration to 8 g/l $(NH_4)_2SO_4$ did not have any further effect on 3-HP accumulation and only retarded its synthesis. No improvement in 3-HP accumulation was observed after increasing the initial nitrogen concentration when using cells carrying plasmid $pPyc_{Cg}^{T132A}$ (Fig. 4, E), suggesting that lower C/N ratios may not promote sufficient accumulation of pyruvate for the pathway. We believe that the different tendencies seen in the two mutants were due to the various C/N ratios triggering different stress responses, and at different times as they were reaching their respective nitrogen-limiting conditions. In the $pPEPc_{Cg}^{N917G}$ mutant grown in the presence of 0.6 g/l $(NH_4)_2SO_4$, the stringent response is triggered earlier, resulting in lower final biomass yields and maximum 3-HP titres of only 1.96 mM, whereas when cultures were grown in the presence of 2 g/l $(NH_4)_2SO_4$, more biomass was produced, and stringent response was triggered at a later stage, resulting in higher 3-HP titres. A similar trend was observed when 8 g/l $(NH_4)_2SO_4$ was present in culture media. The same could not be observed for the $pPyc_{Cg}^{T132A}$ mutant, and we believe that this is because these strains are also producing PHB, and once stringent response is triggered, most of the carbon is pulled from pyruvate towards PHB synthesis, thus limiting the pool of available pyruvate for product synthesis. In line with our previous findings, 3-HP re-consumption was observed following the production peak in all the cultures.

3.4. Effect of gdhA1 and hpdH deletions on 3-HP production

Based on the above results, two targets to enhance 3-HP production in *C. necator* H16 were identified: the utilization of aspartate as a source of carbon and the re-consumption of 3-HP after its synthesis.

To identify enzymes that could allow the use of aspartate as a carbon source, the genome of *C. necator* H16 was analysed (Fig. 5, A). No homologues to known aspartate ammonia-lyases (AspA, EC:4.3.1.1) or aspartate aminotransferases (AspC, EC:2.6.1.1) were found. It has been reported that *Cupriavidus pinatubonensis* (formerly *C. necator*) JMP134 has an L-aspartate dehydrogenase (AspDH, EC 1.4.1.21) which can catalyse the interconversion of aspartate to oxaloacetate (Li et al., 2011). Considering this, we speculated that *C. necator* H16 may also have an AspDH. A BlastP analysis against *C. necator* H16 revealed an enzyme with 90.6% amino acid identity (H16_B0736) to AspDH from *C. pinatubonensis* JMP134. Additionally, a putative aspartate 4-decarboxylase (EC:4.1.1.12) annotated as AsdA (H16_A3009) which can likely convert aspartate to L-alanine was identified.

Two strains were generated to investigate the influence of AspDH and AsdA on the utilization of aspartate as a source of carbon: *C. necator* H16 Δ3 Δ*phaCAB* Δ*aspDH* and *C. necator* H16 Δ3 Δ*phaCAB* Δ*asdA*.

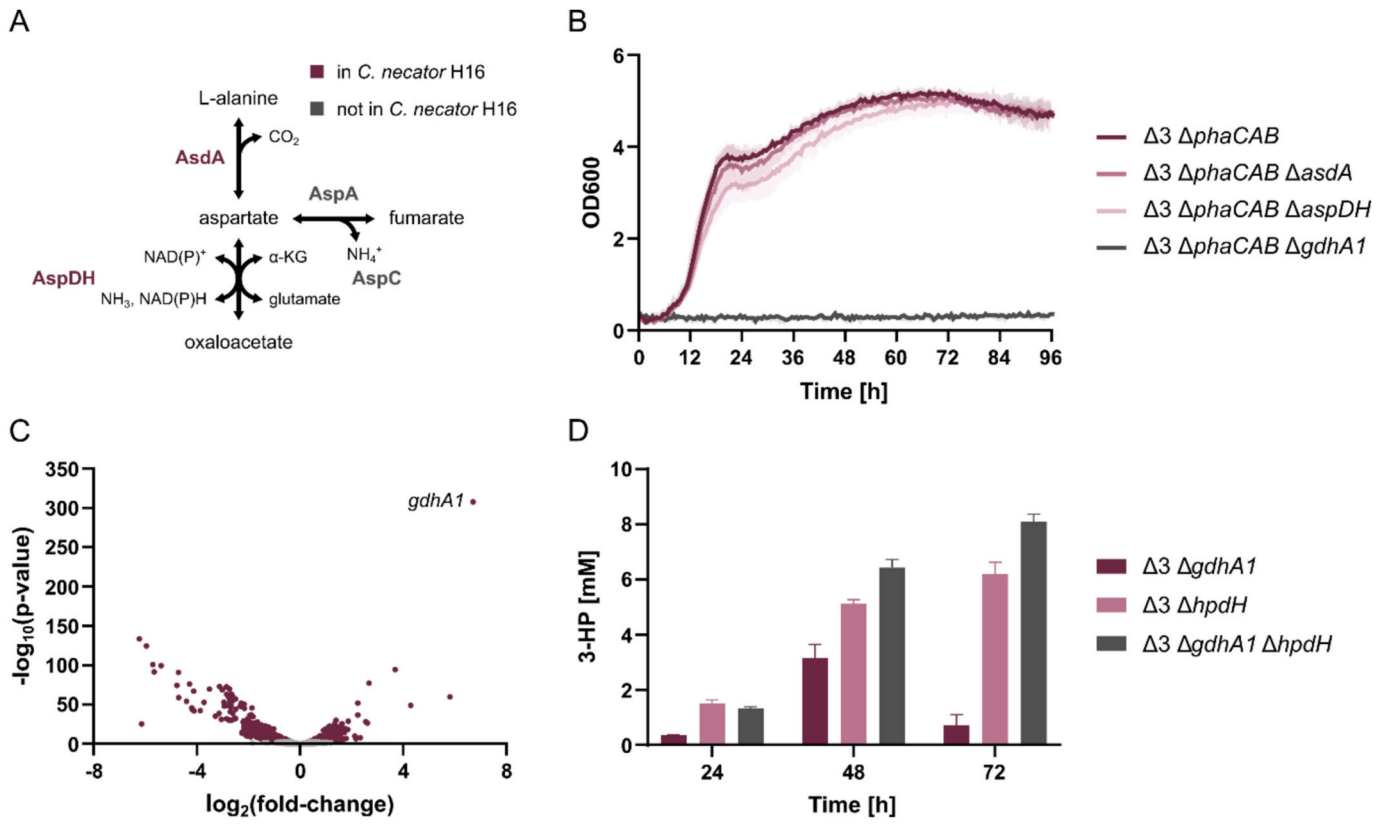

Fig. 5. Identification of enzymes involved in aspartate utilization and 3-HP re-consumption in *C. necator* H16. (A) Main reactions involved in the consumption and production of aspartate in bacteria. Enzyme abbreviations: AspA, aspartate ammonia-lyase; AspC, aspartate aminotransferase; AsdA, aspartate 4-decarboxylase; and AspDH, aspartate dehydrogenase. (B) Growth of *C. necator* H16 strains on aspartate as sole carbon source. MM containing 4 g/l $(NH_4)_2SO_4$ with 50 mM aspartate as the sole carbon source was used as the culture medium. Error shaded areas indicate standard deviations of three biological triplicates. (C) Differential gene expression analysis of *C. necator* H16 Δ3 Δ*phaCAB* grown with a without aspartate. Cultivations were carried out in triplicate using 2%SGMM containing 4 g/l $(NH_4)_2SO_4$, with and without supplementation of 50 mM aspartate. RNA-seq analysis was performed using samples obtained during the early exponential phase of the cultivation. *gdhA1*: glutamate dehydrogenase A1. P-values were calculated using Wald's test (violet dots: $p < 0.05$, grey dots: $p \geq 0.05$). (D) 3-HP production using *C. necator* H16 Δ3 Δ*gdhA1*, *C. necator* H16 Δ3 Δ*hpdH* and *C. necator* H16 Δ3 Δ*gdhA1* Δ*hpdH* carrying $pPEPc_{Cg}^{N917G}$. Cultivations were carried out using 2%SGMM containing 2 g/l $(NH_4)_2SO_4$. Error bars indicate standard deviations of 3 biological replicates.

C. necator H16 Δ3 Δ*phaCAB* was used as the parental strain to avoid the unreliability caused by PHB production on the optical density readings when comparing the growths of the strains. All three strains were grown in a BioLector micro bioreactor platform using nitrogen-rich MM containing 4 g/l $(NH_4)_2SO_4$ supplemented with 50 mM aspartate as sole carbon source (Fig. 5, B). Although a slight reduction in cell growth rate was observed, the analysed deletions did not result in a strain unable to grow on aspartate. This indicated that other enzymes may be involved in the utilization of aspartate as sole carbon source in *C. necator* H16.

In order to identify these enzymes, RNA-seq analysis was performed. *C. necator* H16 Δ3 Δ*phaCAB* cells were grown using 2%SGMM containing 4 g/l $(NH_4)_2SO_4$, with and without supplementation of 50 mM aspartate, and samples collected during the early exponential phase of cultivation. Differential gene expression analysis identified *gdhA1* (H16_A0471) as the gene with the highest upregulation when aspartate was added to the culture medium (Log_2 fold change (Log_2FC) = 6.71; Fig. 5, C). GdhA1 is annotated as a putative glutamate dehydrogenase. In addition, 12 more genes showed a $Log_2FC > 2$, including several genes coding for proteins potentially involved in the utilization of aspartate, such as: 3 C4-dicarboxylate ABC transporters (H16_A0693, H16_A3718, H16_A0299), a porin (H16_A2378), an L-asparaginase (H16_A1910), a D-amino acid aminotransferase (H16_A2521), 2 tripartite tricarboxylate transporter components (H16_A3719, H16_A3720), and an Asp/Glu racemase (H16_A0923) (Fig. 5, C; Supplementary Table S3). No significant upregulation of the expression of *aspDH* and *asdA* was detected. Of all highly upregulated genes, *gdhA1* seemed to be the best candidate to participate in the utilization of aspartate as a carbon source. Thus, *C. necator* H16 Δ3 Δ*phaCAB* Δ*gdhA1* was generated and its ability to grow using aspartate as sole carbon source was examined (Fig. 5, B). No growth was observed within the tested timeframe of 96 h. This suggests that GdhA1 is the main enzyme involved in the utilization of aspartate as a carbon source. Glutamate dehydrogenases catalyses the oxidation of glutamate to α-ketoglutarate. The side chains of glutamate and α-ketoglutarate are extended by only one methylene group compared to those of aspartate and oxaloacetate, the substrate and product of aspartate dehydrogenases. Considering all the above and the lack of aspartate aminotransferases in *C. necator* H16, GdhA1 is most likely mis-annotated and correspond to an aspartate dehydrogenase. In addition, *C. necator* H16 possesses two other putative glutamate dehydrogenases, GdhA2 (H16_B1945) and GudB (H16_A1356). A phylogenetic analysis showed that GdhA1 does not cluster with any experimentally characterized aspartate or glutamate dehydrogenase from bacteria, while GdhA2 and GudB did (Fig. S1). These results imply that GdhA1 may belong to a novel family of aspartate dehydrogenases. Biochemical characterization of the enzyme to confirm this assumption is, however, still required.

Inactivation of all three methyl-malonate semialdehyde dehydrogenases from *C. necator* H16 allowed considerable higher levels of 3-HP production compared to the wild-type strain (Fig. 2). The use of this strain, however, did not completely abolish 3-HP re-consumption under 3-HP production conditions (Figs. 3 and 4). In addition to *mmsA1, mmsA2* and *mmsA3*, two genes encoding for putative dehydrogenases (*hpdH* and *hbdH*) were shown to be upregulated in the

presence of 3-HP (Arenas-López et al., 2019). In particular, *hpdH* is co-expressed with *mmsA2* and it has been hypothesised to encode a 3-HP dehydrogenase (Arenas-López et al., 2019). Deletion of *hpdH* delays growth on 3-HP for more than 100 h (Arenas-López et al., 2019), making this gene an attractive candidate to try to prevent 3-HP re-consumption.

To analyse the influence GdhA1 and HpdH on 3-HP production, three strains were generated: *C. necator* H16 Δ3 Δ*gdhA1*, *C. necator* H16 Δ3 Δ*hpdH*, and *C. necator* H16 Δ3 Δ*gdhA1* Δ*hpdH*. The strains were transformed with $pPEPc_{Cg}^{N917G}$, and 3-HP production was assessed using MM supplemented with 2% (w/v) sodium gluconate and 2 g/l $(NH_4)_2SO_4$ (Fig. 5, D). 3-HP production in *C. necator* H16 Δ3 Δ*gdhA1* behaved very similarly to that of *C. necator* H16 Δ3, where 3-HP was almost entirely re-consumed after reaching a peak at 48 h. This behaviour was not observed in the Δ*hpdH* strains, where the produced 3-HP did not appear to be re-consumed, allowing higher levels of 3-HP accumulation. Of all the strains, *C. necator* H16 Δ3 Δ*gdhA1* Δ*hpdH* showed the highest levels of 3-HP production, achieving ~8 mM 3-HP after 72 h. This demonstrated that both knockouts, Δ*gdhA1* and Δ*hpdH,* are beneficial for the accumulation of 3-HP.

Although the levels of 3-HP production achieved in this study are not comparable to those obtained in well-studied model microorganisms such as *E. coli* and *S. cerevisiae* (Borodina et al., 2015; Song et al., 2016), these may be sufficient for the synthesis of 3-HP-containing copolymers. PHB thermal and mechanical properties are not ideal, and even a low 3-HP molar fraction can improve the properties of poly(3-hydroxybutyrate-*co*-3-hydroxypropionate) (poly(3-HB-*co*-3-HP)) compared to PHB. Recently, *C. necator* H16 was engineered to synthesize poly (3-HB-*co*-3-HP) with variable monomer composition using β-alanine as a 3-HP precursor (McGregor et al., 2021). Using this strain, the molar fraction of 3-HP incorporated in the copolymer could be adjusted based on the availability of β-alanine in the culture medium. The addition of as little as 5 mM β-alanine had a significant impact on 3-HP molar fraction in the copolymer. As the supplementation of precursors is not suitable to keep manufacturing costs low, having a strain able to produce 3-HP from the central metabolism is essential. Here we were able to produce ~8 mM 3-HP directly from the central metabolism. Although PHA production and 3-HP content in the polymer were not examined, based on previous studies (McGregor et al., 2021), a low 3-HP molar fraction in the copolymer is expected. Combining the 3-HP producing strain and the poly(3-HB-*co*-3-HP) strain may allow the production of 3-HP-containing copolymers from a variety of carbon sources without the requirement of exogenous β-alanine.

3.5. Autotrophic production of 3-HP

C. necator is able to grow using CO_2 and H_2 as carbon and energy sources. Considering this, we explored the possibility of producing 3-HP using CO_2 as the sole carbon source. To do so, *C. necator* H16 Δ3 Δ*gdhA1* Δ*hpdH* carrying $pPEPc_{Cg}^{N917G}$ was cultivated in a 1.3 l bioreactor with a working volume of 750 ml, MM using 2 g/l $(NH_4)_2SO_4$, and a constant supply of CO_2, H_2 and air. Surprisingly, although the strain grew well, no 3-HP production was observed using these conditions (data not shown). In contrast, equivalent fermentation using the same strain but harbouring $pPyc_{Cg}^{T132A}$, produced 0.5 mM 3-HP (Fig. 6), with a total yield ($Y_{p/s}$) of 2.5 C-mmol-3-HP/C-mol-CO_2. Under heterotrophic growth conditions *C. necator* H16 Δ3 harbouring the feedback insensitive PEPc produced 3-HP at a higher rate compared to the $pPyc_{Cg}^{T132A}$ harbouring strain (Fig. 4). However, under autotrophic conditions the $pPEPc_{Cg}^{N917G}$ harbouring strain did not accumulate 3-HP, whereas the cells carrying $pPyc_{Cg}^{T132A}$ did. Under heterotrophic conditions, it would seem that PEP is available at concentrations sufficient for 3-HP production from the onset of the cultivation. We attribute this to the feedback insensitive PEPc, converting PEP to oxaloacetate. It is well known that *C. necator* H16 accumulates pyruvate under nitrogen-limited heterotrophic conditions (Bommareddy et al., 2020). In the $pPyc_{Cg}^{T132A}$ harbouring strain, we speculate that when nitrogen becomes limited, more pyruvate is

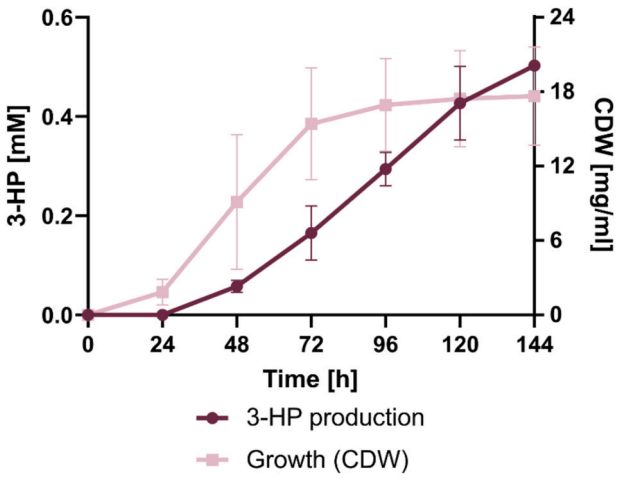

Fig. 6. 3-HP production and cell growth on CO_2 using *C. necator* H16 Δ3 Δ*hdpH* Δ*gdhA1* harbouring $pPyc_{Cg}^{T132A}$. Cultivations were carried out in 1.3 l bioreactors with a working volume 750 ml using MM containing 2 g/l $(NH_4)_2SO_4$ and constant supply of CO_2, H_2 and air. Error bars indicate standard deviations of 3 biological replicates.

available as a precursor, thus 3-HP production was observed after 48 h. Under autotrophic conditions, the PEPc strain did not accumulate any 3-HP which is contrary to what was observed under heterotrophic conditions. This different scenario compared to the heterotrophic conditions may be attributed to the fact that PEP is a negative regulator of CBB cycle in *C. necator* H16 (Grzeszik et al., 2000). It has been observed that excessive concentrations of PEP cause repression of the *cbb* operon. Although the $PEPc_{Cg}^{N917G}$ is feedback insensitive, it did not allow 3-HP production under autotrophic conditions, which suggest that PEP concentration in *C. necator* H16 is tightly regulated during autotrophic growth. These findings show that results obtained under heterotrophic conditions cannot always be extrapolated to autotrophic conditions, and appropriate enzyme selection for each growth condition is essential.

This is the first time that 3-HP has been produced using CO_2 as sole carbon source in a non-photosynthetic bacterium. According to previous publications (Kohlmann et al., 2011; Schwartz et al., 2009), unlike other facultative lithoautotrophs, all enzymes of the TCA cycle can be detected in *C. necator* H16 under conditions of autotrophic growth. However, according to Trüper (1965), the TCA cycle enzymes show reduced activity in lithoautotrophically grown cells compared to heterotrophically grown cells. As some of our precursor molecules, such as oxaloacetate and α-ketoglutarate directly derive from the TCA cycle, this might explain, at least in part, the low yields seen under autotrophic conditions. A similar trend was observed when *C. necator* was used to produce alka(e)ne under heterotrophic and autotrophic conditions (Crépin et al., 2016). The authors found that the strain produced 670 mg/l under heterotrophic conditions versus only 4.4 mg/l under autotrophic conditions. The low yields under autotrophic conditions were explained by suboptimal fermentation conditions. We believe that with further engineering of the TCA cycle enzymes the 3-HP titres can be potentially improved under both autotrophic and heterotrophic conditions.

Although the carbon sources used in this study are highly refined, *C. necator* H16 can grow on a wide range of substrates, including less expensive ones such as used oils and CO_2-rich waste gases (Garcia--Gonzalez and de Wever, 2017; Martino et al., 2014). The use of waste streams in conjunction with the strains presented in this work, which do not require supplementation of 3-HP precursors, may help reduce 3-HP production costs.

Despite successfully producing 3-HP under both heterotrophic and autotrophic conditions using engineered *C. necator* H16, the titres achieved here are below those seen in *E. coli* and *S. cerevisiae* (Borodina et al., 2015; Song et al., 2016). A variety of factors are likely to

contribute to this decreased production. Firstly, a relatively low nitrogen concentration (2 g/l of $(NH_4)_2SO_4$) was used compared to what has been used during 3-HP production in *E. coli* and *S. cerevisiae* using the β-alanine pathway (9 g/l and 7.5 g/l of $(NH_4)_2SO_4$, respectively) (Borodina et al., 2015; Song et al., 2016). In our case, higher nitrogen concentrations did not improve 3-HP production (Fig. 4, D). Additionally, nutrient-limiting conditions are associated with higher NADPH concentration (Lee et al., 1995), which is beneficial for 3-HP production as $YdfG_{Ec}$ is NADPH-dependent. As a result, however, this means less nitrogen is available for the precursors to 3-HP. Secondly, the flux through the TCA cycle, a source of precursor molecules for the β-alanine pathway, is reduced in *C. necator* H16 under nitrogen-limiting conditions (Pearcy et al., 2022). Additionally, in the study by Song et al. (2016) in which 3-HP was produced using the β-alanine pathway in *E. coli*, the TCA cycle was engineered. The *fumAC*, *fumB* and *iclR* genes were deleted, while the native promoters of *sdhC* and *aspA* were swapped for stronger promoters to reinforce flux to fumarate and aspartate respectively. In our work we did not attempt to engineer the TCA cycle, which likely contributed to reduced yield compared to *E. coli*. Furthermore, in our engineered strain, the 3-HP production pathway must compete with the PHB biosynthetic pathway. In *E. coli* and *S. cerevisiae* this is not an issue as the PHB biosynthetic pathway is not present. We attempted to delete the PHB pathway to direct more carbon flux towards 3-HP; however, our results showed that deletion of the PHB biosynthetic pathway had a negative effect on 3-HP production. Further strain engineering, particularly of the TCA cycle, and process optimization are still required for the development of a competitive strain for 3-HP production using *C. necator* H16.

4. Conclusions

In this study, *C. necator* H16 was engineered to produce 3-HP via β-alanine under heterotrophic and autotrophic growth conditions. Accumulation of 3-HP required deletion of the methyl-malonate semialdehyde dehydrogenases *mmsA1, mmsA2* and *mmsA3*. The strain was further optimized by preventing both the utilization of aspartate as a carbon source and the re-consumption of the produced 3-HP. This was achieved through the deletion of *gdhA1* and *hpdH*, respectively. To the best of our knowledge, this is the first time that 3-HP has been produced from intermediates of the central metabolism in *C. necator* H16. This study lays the groundwork for future work to consolidate *C. necator* H16 as an efficient platform for 3-HP and 3-HP-containing polymers.

Author contributions

Conceptualization: A.S., C.M., and K.K.; methodology: A.S., C.M., V. I., and R.R.B.; investigation: A.S., C.M., V.I., and C.A.L.; resources: N.P. M.; data curation, K.K.; writing —original draft preparation, A.S., C.M., V.I., R.R.B., K.W., N.P.M., and K.K.; supervision: N.P.M. and K.K.; funding acquisition: N.P.M., K.W. and K.K. All authors have read and agreed to the published version of the manuscript.

Availability of data and materials

The transcriptomic dataset generated in the present study was uploaded to the ArrayExpress database (https://www.ebi.ac.uk/arrayexpress/) and is accessible under accession number E-MTAB-11897.

Declarations of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2022.10.014.

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