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Respiratory glycerol metabolism of *Actinobacillus succinogenes* 130Z for succinate production

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Abstract *Actinobacillus succinogenes* 130Z naturally produces among the highest levels of succinate from a variety of inexpensive carbon substrates. A few studies have demonstrated that *A. succinogenes* can anaerobically metabolize glycerol, a waste product of biodiesel manufacture and an inexpensive feedstock, to produce high yields of succinate. However, all these studies were performed in the presence of yeast extract, which largely removes the redox constraints associated with fermenting glycerol, a highly reduced molecule. We demonstrated that *A. succinogenes* cannot ferment glycerol in minimal medium, but that it can metabolize glycerol by aerobic or anaerobic respiration. These results were expected based on the *A. succinogenes* genome, which encodes respiratory enzymes, but no pathway for 1,3-propanediol production. We investigated *A. succinogenes*'s glycerol metabolism in minimal medium in a variety of respiratory conditions by comparing growth, metabolite production, and in vitro activity of terminal oxidoreductases. Nitrate inhibited succinate production by inhibiting fumarate reductase expression. In contrast, growth in the presence of dimethylsulfoxide and in microaerobic conditions allowed high succinate yields. The highest succinate yield was 0.75 mol/mol glycerol (75 % of the maximum theoretical yield) in continuous microaerobic cultures. *A. succinogenes* could also grow and produce succinate on partially refined glycerols obtained directly from biodiesel manufacture. Finally, by expressing a heterologous 1,3-propanediol synthesis pathway in *A. succinogenes*, we provide the first proof of concept that *A. succinogenes* can be engineered to grow fermentatively on glycerol.

Keywords Succinate · Glycerol · *Actinobacillus succinogenes* · Microaerobic · Continuous culture · Fermentation · 1,3-Propanediol

Introduction

Succinic acid tops the US Department of Energy's list of value-added products from biomass [49], because it has the potential, if produced economically, to become the feedstock for a bulk chemical industry currently based on the petrochemical maleic anhydride [27, 51]. *Actinobacillus succinogenes* is one of the best natural succinate-producing microorganisms [27], producing succinate as part of a mixed acid fermentation with acetate, formate, and ethanol as co-products. Succinate production by *A. succinogenes* is favored under conditions of high CO_2 availability [28] and reducing power, the latter of which can be provided as H_2 [26], as electricity [30], or as a reduced carbon source [45].

Biodiesel production yields 10 lb of crude glycerol from the trans-esterification of every 100 lb of triglycerides. The growth of biodiesel production has caused a surge in the availability and a steep drop in price of crude glycerol, and it

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has prompted interest in glycerol as a feedstock for the production of valuable fuels and bulk chemicals [2, 29]. Glycerol is an attractive feedstock for fermentative succinate production because of its highly reduced nature (oxidation state, −0.67, calculated using Eq. 1, where n is the number of a particular atom in the molecule).

$$\text{Oxidation state} = -\left[n_{\text{hydrogen}} + \left(n_{\text{oxygen}} \times -2\right) + \left(n_{\text{nitrogen}} \times -3\right)\right] / n_{\text{carbon}} \quad (1)$$

Because microbial biomass (oxidation state, −0.4, assuming a typical microbial biomass composition of $CH_2O_{0.5}N_{0.2}$ [40]) is less reduced than glycerol, bacteria growing fermentatively on glycerol must excrete products with a combined oxidation state lower than that of glycerol (i.e., less than −0.67). All bacterial species that grow well on glycerol by fermentation produce 1,3-propanediol (1,3-PD, oxidation state, −1.33) as one of their fermentation products. As illustrated in Fig. 1a, the 1,3-PD dehydrogenase reaction recycles the NADH produced in the glycerol dehydrogenase reaction to NAD^+, and dihydroxyacetone phosphate (DHAP) production from glycerol is redox-balanced. In contrast, in microorganisms that do not produce 1,3-PD, such as *A. succinogenes*, DHAP production from glycerol is accompanied by the accumulation of one redox equivalent (Fig. 1a). Succinate ($C_4H_6O_4$, oxidation state, 0.5) is less reduced than glycerol. Succinate biosynthesis requires CO_2 fixation, though, and with CO_2 supplied exogenously, the succinate skeleton coming from glycerol is $C_3H_6O_2$ (oxidation state, −0.67). Succinate production from glycerol plus CO_2 is, therefore, redox-neutral (Fig. 1b), and it cannot, alone, support fermentative growth on glycerol, because it cannot recycle the additional redox equivalents produced during biomass production.

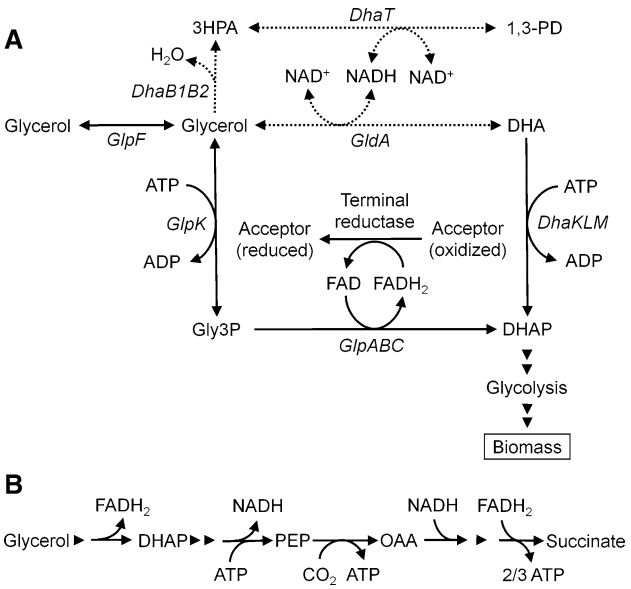

Fig. 1 Pathways for glycerol assimilation into biomass (**a**), 1,3-propanediol production from glycerol (**a**), and succinate production from glycerol (**b**). *Continuous lines* reactions present in *A. succinogenes*; *dashed lines*: reactions absent in *A. succinogenes*. Metabolites: *1,3-PD* 1,3-propanediol, *3HPA* 3-hydroxypropionaldehyde, *DHA* dihydroxyacetone, *DHAP* dihydroxyacetone phosphate, *Gly3P* glycerol-3-phosphate, *OAA* oxaloacetate, *PEP* phosphoenolpyruvate. *Double arrow head*, multiple enzymatic steps

To grow on glycerol, succinate-producing bacteria need to reduce a terminal electron acceptor to recycle the $FADH_2$ produced by glycerol-3-phosphate dehydrogenase (Fig. 1a). This fact is illustrated in Table 1 where succinate production from glycerol has been accomplished either in the presence of a terminal electron acceptor [i.e., dimethylsulfoxide (DMSO), and microaerobic conditions] or in the presence of more oxidized organic medium components (i.e., yeast extract and polypeptone). One exception is a batch culture of *Escherichia coli*. *E. coli* is known for producing low amounts of 1,2-propanediol, which could explain a slow growth and a low succinate productivity in that case, and the non-described atmosphere could have contained some oxygen (Table 1). *A. succinogenes* and all other Pasteurellaceae species tested for growth on glycerol are unable to grow on glycerol in the absence of yeast extract or of an inorganic terminal electron acceptor (Table 1) [22, 24, 34], and glycerol-based succinate production by Pasteurellaceae has only been tested in the presence of yeast extract. How the metabolism of Pasteurellaceae responds to different inorganic terminal electron acceptors in minimum medium has never been characterized.

In this report we combine physiological and biochemical approaches to study the environmental determinants of *A. succinogenes*'s glycerol metabolism in minimal growth medium. We report here that *A. succinogenes* can metabolize glycerol aerobically or while respiring nitrate or dimethylsulfoxide (DMSO) anaerobically. We also demonstrate that under microaerobic conditions, *A. succinogenes* conserves most of the reducing power in glycerol and produces succinate at up to 75 % of the maximum theoretical yield of 1 mol/mol glycerol consumed. Finally, because anaerobic fermentations are easier and less expensive to maintain than microaerobic cultures in industrial production settings, and because they require lower investment costs [50], we also demonstrate that *A. succinogenes* can be engineered to grow fermentatively on glycerol by expressing a heterologous 1,3-PD synthesis pathway in *A. succinogenes*.

Materials and methods

Manual annotation of the *A. succinogenes* genome

The open reading frames (ORFs) described were annotated using the BLAST-based methods and criteria described

Table 1 Glycerol-based succinate production by various bacterial species

Bacterial species	Glycerol input (mM)	Additional organic C/N sources	Atmosphere	System (dilution rate, h^{-1})	Succinic acid yield g/g glycerol (mol/mol glycerol)	Succinic acid productivity ($g\ l^{-1}\ h^{-1}$)	Final succinic acid concentration g/L (mM)	References
Anaerobiospirillum succiniciproducens	71	5 g/l YE, 10 g/l PP	CO_2	Batch	1.33 (1.04)	0.136	4.9 (42)	[23]
A. succiniciproducens	120	25 g/l YE	CO_2	Continuous (0.14)	1.37 (1.07)	2.1	15.5 (131)	[21]
M. succiniciproducens DD1	109[a]	5 g/l YE, 10 g/l PP	CO_2	Batch	1.2 (0.94)	0.9	8.4 (71)	[33]
M. succiniciproducens DD1	55[a]	0.5 g/l YE	CO_2	Continuous (0.18)	1.02 (0.80)	0.094	5.2 (44)	[34]
M. succiniciproducens DD1	109	5 g/l YE, 10 g/l PP	CO_2	Batch	1.24 (0.97)	1.47	35.3 (299)	[35]
E. coli XZ721(Δ*pflB* Δ*ptsI* $pck_{upregulated}$)	540	None	Not described	Batch	1.0 (0.8)	0.084	12 (102)	[52]
E. coli (Δ*adhE* Δ*pta* Δ*poxB* Δ*ldh* Δ*ppc* [pZS-*pyc*])	217	None	Microaerobic, not controlled	Batch	0.69 (0.54)	0.194	14 (119)	[7]
A. succinogenes	401	5–10 g/l YE	CO_2	Batch	0.8 (0.62)	0.27	29.3 (248)	[46]
A. succinogenes	260	10 g/l YE 1.8 % (v/v) DMSO	CO_2	Batch	0.95 (0.74)	2.13	24.4 (207)	[10]
A. succinogenes	366	10 g/l YE 1 % (v/v) DMSO	CO_2	Fed-batch	0.87 (0.68)[b] 0.94 (0.73)[c]	2.31[b] 0.62[c]	24.3 (206)[b] 49.6 (420)[c]	[10]
A. succinogenes	100	None	Microaerobic, not controlled	Batch	0.86 (0.67)	0.087	7.9 (67)	This study
A. succinogenes	150	None	5 % O_2, 95 % CO_2	Continuous (0.02)	0.96 (0.75)	0.139	31.7 (269)	This study

YE yeast extract, *PP* polypeptone

[a] Crude glycerol

[b] Before supplementation

[c] After supplementation with 1 % DMSO plus 272 mM glycerol at 30 and 60 h

in [25] based on similarity to ORFs with experimentally tested functions.

Chemicals, bacteria, and batch culture conditions

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. *Clostridium butyricum* DSM 2478 was obtained from DSMZ (Braunschweig, Germany) and grown in TY basal medium [3] containing 5 g L^{-1} peptone. *E. coli* TOP10 (Invitrogen, Carlsbad, CA) used for plasmid construction and propagation was grown in lysis broth (LB) [4] supplemented with 100 μg ml^{-1} ampicillin when required for plasmid maintenance.

A. succinogenes type strain 130Z (ATCC 55618) was obtained from the American Type Culture Collection. *A. succinogenes* pre-cultures were grown in AM3 defined medium [28] with modifications [26]. AM3-glycerol contained 100 mM glycerol as the sole organic carbon source and 150 mM $NaHCO_3$ as the CO_2 source. To study nitrate (nitrite) respiration and assimilation, NH_4Cl was replaced by $NaNO_3$ ($NaNO_2$) as the sole nitrogen source in AM3. Pure glycerol (>99 %) was from JT Baker (Phillipsburg, NJ). Vegetable and mixed glycerols were provided by Michigan Biodiesel, LLC (Bangor, MI). Vegetable and mixed glycerols originate from biodiesel production using either vegetable oils or a mixture of animal and vegetable oils, respectively. At Michigan Biodiesel, LLC, the glycerols were partially purified by (1) treating crude glycerols with HCl to lower their pH to 4–5, (2) storing in a settling tank (82 °C) for 3−4 days to separate glycerol, fatty acids, and soap by density, and (3) drawing off glycerol from the bottom of the tank and heating it (105 °C) under vacuum to remove the majority of water and methanol. Before adding to AM3, mixed glycerol was autoclaved in a loosely capped Wheaton bottle to decrease the methanol concentration remaining after processing. Ten-mL and 60-mL cultures were grown in 28-mL anaerobic tubes and 150-mL serum vials (Bellco, Vineland, NJ), respectively. Tubes and vials were flushed for 10 min with O_2-free N_2 gas (Airgas, Independence, OH), plugged with rubber bungs, flushed for an additional 10 min, and sealed with aluminum crimps. For all experiments, pre-cultures were collected during exponential phase growth, spun for 3 min at 4,500×*g*, resuspended in 0.15 M phosphate buffer (pH 7.0), and transferred to AM3-glycerol with and without supplementation of electron acceptors (as indicated in the text). The starting optical density at 660 nm (OD_{660}) was between 0.05 and 0.10. Cultures were incubated with shaking (250 rpm) at 37 °C.

Microaerobic batch cultures were grown in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). The gaseous atmosphere was maintained at 1.0 % O_2 by mixing 95 % CO_2 with 5 % air. 125-mL Erlenmeyer flasks containing 60-mL cultures were shaken on a Barnstead Lab Line MaxQ 2000 Benchtop Platform Shaker (Thermo Scientific, Asheville, NC) at 300 rpm for oxygenation. Dissolved O_2 levels were monitored using a Fluorometrix® Non-invasive DO Sensing System (CellPhase™ DO-1, Fluorometrix Corp., Stow, MA). All experimental data are reported as the average of at least three biological replicates ± standard deviation. Samples were considered to be biological replicates after being separately serially transferred (1:40 dilution) to fresh medium at least twice.

Samples (0.8 mL) were withdrawn periodically from batch cultures to quantify biomass, glycerol, electron acceptors, and metabolites.

Continuous microaerobic cultures

Cultures were grown in AM3 containing 150 mM glycerol and 150 mM $NaHCO_3$ in a working volume of 200 mL in a 1-L fermentor (New Brunswick Scientific Co., New Brunswick, NJ). CO_2 was sparged into the fermentor and into the reservoir medium for 30 min prior to inoculation. The reservoir medium was sparged with CO_2 during the entire continuous culture experiment. The inoculum was grown overnight in 30 mL AM3-100 mM glycerol-20 mM nitrate. The preculture was centrifuged (4,500×*g*, 15 min) and resuspended in 2.5 mL AM3 for inoculation at 1 % vol. After inoculation, the fermentor was sparged with a 5 % O_2–95 % CO_2 (Airgas) mixture and the inlet gas flow was maintained at 2.5 mL min^{-1}. The temperature and agitation speed were maintained at 37 °C and 100 rpm, respectively. pH was measured at regular intervals.

The culture was grown in batch mode until OD_{660} reached 0.7–0.8 before starting the continuous flow. The dilution rate was varied until steady-state growth was established, as determined by a steady OD_{660}. Samples (0.8 mL) were withdrawn periodically to quantify biomass, glycerol, and products. Samples used to calculate carbon balances were harvested at least 6 h apart, starting after 3–4 residence times. A sample from the reservoir medium was also harvested to measure the initial glycerol concentration in the chemostat. Culture purity was checked by microscopy and by plating culture samples at periodic intervals.

Growth measurements and metabolic endproduct analyses

Biomass was estimated by measuring OD_{660} with a DU 650 spectrophotometer (Beckman, Fullerton, CA). Samples (0.1 mL) were diluted ten times in 0.9 % NaCl, and the values were used to calculate growth rates, as well as carbon and electron balances. Culture supernatants were analyzed by high-performance liquid chromatography as described [28]. Glycerol, ethanol, and 1,3-PD were detected with a Waters 410 Differential Refractometer. Organic acids and

DMSO were detected with a Waters 2478 UV detector at 210 nm. CO_2 production was calculated using the equation: CO_2 (mol) = Acetate (mol) − Formate (mol) [14].

Dry cell weight (DCW) in glycerol-grown cultures was measured on steady-state, continuous cultures. At the end of chemostat experiments, 70-mL samples of steady-state cultures were filtered through pre-weighed 0.45 μm HA filters (Millipore, Billerica, MA) and rinsed once with 20 mL of 0.9 % NaCl. The filters were dried to constant weight in an 85 °C oven for 24 h before weighing. The average DCW was calculated based on measurements of seven independent samples (1–3 replicates from each chemostat).

Determination of nitrate and nitrite concentrations

The concentrations of nitrate and nitrite in culture supernatants were determined by ion chromatography coupled to suppressed conductivity with an IonPac AS 11-HC anion exchange column (2 × 250 mm, Dionex Corp., Sunnyvale, CA) in line with a Dionex IonPac AG11-HC Guard column (2 × 50 mm). Fifteen mM NaOH was used as the mobile phase and was pumped at a flow rate of 0.38 mL min^{-1} using a Dionex P680 HPLC pump. The ions were quantified by a Dionex ED electrochemical detector that was suppressed by a 2-mm anion self-regenerating suppressor (ASRS Ultra II, Dionex) set in the external water mode and at 23 mA for analysis. Five μL samples were injected for analysis.

Preparation of cell extracts for enzyme assays

Growth of exponential-phase batch cultures was stopped by submerging in an ice-water bath for 10 min. A volume of cells equivalent to 8 OD_{660} units per mL was spun down for 10 min (5,000×*g* at 4 °C) and resuspended in 1 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM dithiothreitol. The cells were centrifuged for 5 min (5,000×*g* at room temperature) and finally resuspended in 500 μL of the same buffer. Cell suspensions were disrupted by sonication (Branson S-450A probe sonifier, Danbury, CT) with four repetitions, each of 20 s, power level 2, and 50 % duty cycle. The cell extracts were centrifuged for 5 min (2,000×*g*) at room temperature to remove any bubbles introduced by sonication. The suspensions were stored on ice until used.

Enzyme activity assays for terminal oxidoreductases

Enzyme activity assays were conducted in 96-well plates using a TECAN Sunrise™ Absorbance Reader (TECAN, Männedorf, Switzerland). Anaerobic conditions were maintained in a Coy anaerobic chamber. Reactions (200 μL) contained 5 mM benzyl viologen, 0.4 mM $Na_2S_2O_4$, 10 μL cell extract, and 5 mM electron acceptor ($NaNO_3$, $NaNO_2$, sodium fumarate, or DMSO) [12, 45]. All stock solutions were prepared in 50 mM potassium phosphate buffer (pH 7.0). Enzyme activity was calculated from the linear slope of benzyl viologen oxidation recorded at 595 nm. The extinction coefficient of benzyl viologen was 8.65 cm^{-1} mM^{-1} [45]. Total cell protein was quantified using the Bio-Rad (Hercules, CA, USA) protein assay dye reagent concentrate with bovine serum albumin as the standard. Activities were reported as the average of at least three biological replicates, each with three technical replicates.

Cloning and expression of the *Clostridium butyricum* 1,3-PD pathway genes in *A. succinogenes*

C. butyricum genomic DNA was purified as described [25]. The *dhaB1-dhaB2-dhaT* operon encoding glycerol dehydratase, glycerol dehydratase-activating protein, and 1,3-PD dehydrogenase was amplified from *C. butyricum* genomic DNA by PCR using Advantage HD polymerase (Clontech Laboratories, Inc., Mountain View, CA) and primers AATGAGGTGATCTAGATGATAAGTAAAGGAT TTAGTACCCAAA (forward) and CGGCCAGTGAATTCG AGCTCCTTAACTTGTTCTTTGTTGTTAGCC (reverse). The 4.6-kb PCR product was cloned into the shuttle vector pLGZ920 [20] restriction sites *Xba*I and *Sac*I using the In-Fusion cloning kit protocol (Clontech Laboratories, Inc.) and transformed into TOP10 chemically competent *E. coli*. Plasmids containing the *dha* operon were identified by colony PCR. Six recombinant plasmids purified from *E. coli* (Wizard SV miniprep kit, Promega, Madison, WI) were introduced into *A. succinogenes* by electroporation as described [20], and recombinant strains were selected for growth in AM3-glycerol-40 μg mL^{-1} ampicillin in the absence of terminal electron acceptor. The plasmid that generated the highest growth yield was named pCV932.

Results

Genetic reconstruction of the *A. succinogenes* glycerol metabolic pathway

Glycerol enters glycolysis as DHAP. In most glycerol-fermenting bacteria, glycerol is transformed to DHAP by glycerol dehydrogenase and dihydroxyacetone kinase [5, 8] (Fig. 1a), and the NADH produced by glycerol dehydrogenase is recycled through 1,3-PD production [5, 50] (Fig. 1a).

Based on its genome sequence, *A. succinogenes* likely takes up glycerol by facilitated transport through a glyceroporin (GlpF, Asuc_1603) and phosphorylates

glycerol to glycerol-3-phosphate by glycerol kinase (GlpK, Asuc_1604). Because *A. succinogenes* lacks a glycerol dehydrogenase (GldA), phosphorylation by GlpK, followed by dehydrogenation to DHAP by glycerol-3-phosphate dehydrogenase is the sole route to glycolysis and central metabolism (Fig. 1a). Facultative anaerobic bacteria such as *E. coli* have aerobic and anaerobic glycerol-3-phosphate dehydrogenases encoded by *glpD and glpABC*, respectively [9]. *A. succinogenes* has the anaerobic enzyme only (GlpABC, Asuc_203–205).

A. succinogenes lacks the reductive pathway to 1,3-PD. The pathway to 1,2-propanediol is incomplete as well, since *A. succinogenes* lacks methylglyoxal synthase, which catalyzes the committing step of 1,2-propanediol synthesis. These observations suggest that *A. succinogenes* cannot ferment glycerol. They also explain why *A. succinogenes*'s anaerobic growth on glycerol has only been reported in minimum media supplemented with complex carbon sources (Table 1). As expected, all our attempts to grow *A. succinogenes* anaerobically in AM3-glycerol in the absence of terminal electron acceptor failed.

Genomic reconstruction of the *A. succinogenes* respiratory chains

The *A. succinogenes* genome encodes several terminal oxidoreductases (Table S1). Unlike *E. coli,* which has multiple enzyme complexes for the reduction of nitrate, nitrite, and oxygen [11, 31], *A. succinogenes* has a single terminal reductase for each electron acceptor (Table S1). Note that *A. succinogenes*'s genome encodes the high-affinity cytochrome *bd* oxidase (*cydAB*), which is expressed in *E. coli* under hypoxic conditions [42], and not the low-affinity cytochrome *bo* oxidase, which is expressed in *E. coli* under higher O_2 tensions. This observation is not surprising since *A. succinogenes* has only been found in the rumen, where dissolved O_2 is below 2 μM [36]. *A. succinogenes*'s genome encodes the enzymes for nitrate reduction to ammonia, which is the predominant nitrate respiratory mechanism in the bovine rumen microbiota [19]. *A. succinogenes*'s genome encodes the high-affinity, nitrate-scavenging nitrate reductase (Nap), presumably giving *A. succinogenes* a growth advantage during nitrate-limiting conditions in the rumen [32].

The multiple terminal oxidoreductases encoded in *A. succinogenes*'s genome suggest that production of reducing equivalents by glycerol-3-phosphate dehydrogenase can be linked to the reduction of external electron acceptors and that *A. succinogenes* can grow on glycerol via respiration. Fumarate, nitrate, nitrite, DMSO, and oxygen were tested for their ability to support growth of *A. succinogenes* in AM3-glycerol. Fumarate supported anaerobic growth on AM3-glycerol (not shown), but because, like succinate, it is a target value-added chemical for production from biomass [49], it was not studied further. As expected, *A. succinogenes* grew on glycerol by respiring nitrate, nitrite, DMSO, and oxygen and produced a variety of mixed acids during growth.

Nitrate respiration

A. succinogenes was grown in AM3-glycerol with different initial concentrations of $NaNO_3$. Culture supernatants were analyzed after 72 h, after growth had stopped. Acetate and CO_2 were the predominant products, followed by succinate (Fig. 2a). Ethanol is a typical product of *A. succinogenes* glucose fermentations [28], but it was not detected in these conditions. Formate accumulated to at most 3 mM during growth with 40 mM $NaNO_3$. As expected, total glycerol consumption, as well as acetate, CO_2, and succinate production increased with increasing initial $NaNO_3$ concentrations.

The ratio of succinate to acetate (S/A ratio, mol succinate produced/mol acetate produced) compares the production of reduced products with that of oxidized products [23]. A low S/A ratio indicates a loss of electrons to external terminal electron acceptors (i.e., nitrate or nitrite). A high S/A ratio indicates conservation of electrons in succinate. Regardless of the initial nitrate concentration the S/A

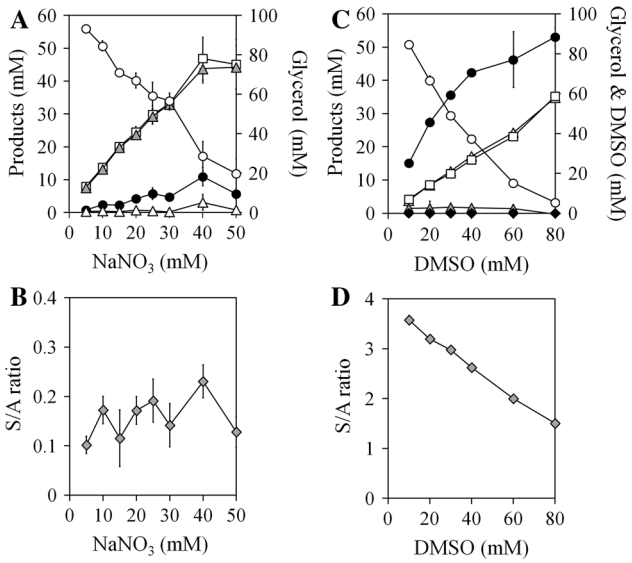

Fig. 2 Effects of initial $NaNO_3$ (**a** and **b**) and DMSO (**c** and **d**) concentrations on glycerol consumption and final product distribution in AM3-glycerol-grown *A. succinogenes* 130Z cultures. Supernatant samples were collected 72 h after inoculation. **a**, **c** Glycerol consumption and product distribution, **b**, **d** succinate/acetate (S/A) ratios. Results are the average of three biological replicates ± standard deviation. *Open circle* glycerol, *filled diamond* DMSO, *filled circle* succinate; *open square* acetate, *open triangle* formate; *shaded triangle* CO_2, *shaded diamond* S/A ratio

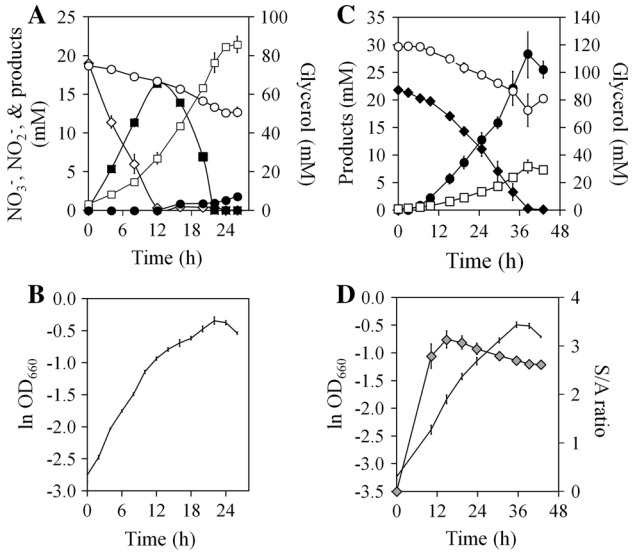

Fig. 3 Time courses of nitrate and DMSO respirations by *A. succinogenes* grown in AM3-glycerol with 20 mM $NaNO_3$ (**a** and **b**) or DMSO (**c** and **d**). **a**, **b** Metabolites, **c**, **d** cell growth and S/A ratio. Data represent the average of three biological replicates ± standard deviation. *Open circle* glycerol, *filled circle* succinate, *open square* acetate, *open diamond* nitrate, *filled square* nitrite, *filled diamond* DMSO, *dash* biomass, *shaded diamond* S/A ratio. CO_2 levels (in **a**) and formate levels (in **b**) were equimolar to acetate and are not shown for clarity

ratio remained below 0.25, with an average of 0.15 ± 0.01 (Fig. 2b). The average succinate yield for glycerol-nitrate cultures was 0.12 ± 0.03 mol succinate/mol glycerol.

Nitrate is the only abundant nitrogen source in AM3-glycerol-$NaNO_3$ grown cultures. Growth of *A. succinogenes* in this medium confirmed that *A. succinogenes* is able to reduce nitrate to ammonia [39] to satisfy the cellular nitrogen demand. Note that *A. succinogenes* lacks the genes required for denitrification. Nitrite alone also supported growth (data not shown), but nitrite-respiring cultures predominantly produced acetate and were not tested further.

To further understand *A. succinogenes*'s metabolism under nitrate-respiring conditions, growth and products were tracked during a batch culture grown on AM3-glycerol with 20 mM $NaNO_3$. Nitrate was first respired to nitrite, followed by a phase of nitrite reduction (Fig. 3a). Growth was biphasic with an initial generation time of 4.7 ± 0.2 h during nitrate reduction. Once nitrate was exhausted (at approx. 12 h), the generation time increased to 11.7 ± 0.6 h (Fig. 3b). Growth stopped when nitrite was completely depleted. Succinate started accumulating only after 12 h (Fig. 3a), once nitrate was completely reduced to nitrite. Since succinate production by *A. succinogenes* was low in nitrate and nitrite-respiring conditions, these conditions were not studied further.

DMSO respiration

A. succinogenes was grown in AM3-glycerol with different initial concentrations of DMSO. Culture supernatants were analyzed after 72 h, after growth had stopped. As seen with nitrate-respiring cells, glycerol consumption and succinate production increased with increasing initial DMSO concentrations (Fig. 2c). Succinate was the main product, followed by acetate, formate, and CO_2. Ethanol was not detected. The end-point S/A ratio was highest with 10 mM DMSO (3.6) and decreased with increasing initial DMSO concentrations (Fig. 2d).

To further understand *A. succinogenes*'s metabolism under DMSO-respiring conditions, growth and products were tracked during a batch culture grown on AM3-glycerol with 20 mM DMSO. From 0 to 39 h, DMSO was respired while biomass, succinate, formate, and acetate accumulated (Fig. 3c). Growth and metabolite production stopped after 39 h, when DMSO was completely reduced. From 0 to 15 h the S/A ratio increased from 0.0 to 3.1 (Fig. 3d). After 15 h the S/A ratio remained near 3.0, but slowly decreased to 2.6 by the end of incubation.

Having found DMSO respiration to be favorable to succinate production from glycerol, pure glycerol and glycerols originating from biodiesel manufacture were compared as carbon and energy sources for *A. succinogenes* growth and succinate production. The exact glycerol concentrations in vegetable glycerol (0.67 g glycerol g^{-1}) and mixed glycerol (0.56 g glycerol g^{-1}) were taken into account when preparing culture media containing 100 mM glycerol. With 20 mM DMSO, growth curves and maximum OD_{660} were identical for batch cultures grown on pure and on vegetable glycerols (not shown). Cultures grown on mixed glycerol grew faster than on pure glycerol, with a generation time of 8.3 ± 0.4 vs. 9.4 ± 0.5 h (calculated between 11 and 22 h), and their maximum OD_{660} was 20 % higher than on pure glycerol. The S/A ratio in mixed glycerol batch cultures followed the same trend over time as that seen with pure glycerol (not shown).

The metabolism of *A. succinogenes* in DMSO-respiring cultures was similar with all three glycerols. Complete end-point carbon and electron balances were calculated using supernatant samples taken at the beginning and end of these cultures, reflecting *A. succinogenes*'s metabolism throughout the entire growth period (Table 2). Results for vegetable glycerol (not shown) were nearly identical to those for pure glycerol. The molar yields of succinate, formate, and acetate were also very similar on pure and mixed glycerols, with small differences in yields that could be due to experimental variations (Table 2). The faster growth and higher biomass yield in cultures grown on mixed glycerol were not accompanied by a higher DMSO consumption, suggesting that some contaminants in the mixed glycerol

Table 2 Products of glycerol metabolism in 60-mL batch cultures of *A. succinogenes* grown either with DMSO or microaerobically

Electron acceptor	Time points (h)[a]	Glycerol substrate[b]	Products (mmol/100 mmol glycerol consumed)					DMSO reduced (mmol/100 mmol glycerol consumed)	Carbon recovery (%)[d]	Electron recovery (%)[e]	S/A[f] ratio
			Succinate	Formate	Acetate	CO_2	Biomass[c]				
20 mM DMSO	0, 43	100 mM, P	59 ± 2	21 ± 1	23 ± 1	1.3 ± 0.5	43 ± 4	58 ± 2	102 ± 4	105 ± 4	2.6 ± 0.1
20 mM DMSO	0, 29	100 mM, M	62 ± 1	20 ± 0	19 ± 0	0.0 ± 0.0	68 ± 2	51 ± 4	106 ± 3	110 ± 3	3.3 ± 0.0
1 % O_2[g]	0, 90	100 mM, P	67 ± 4	0.4 ± 0.1	25 ± 0	25 ± 0	21 ± 1	N/A[h]	100 ± 5	89 ± 4	2.7 ± 0.1
1 % O_2[g]	18, 66	100 mM, P	76 ± 4	3.9 ± 0.9	23 ± 2	19 ± 3	18 ± 1	N/A	106 ± 1	97 ± 2	3.3 ± 0.4
1 % O_2	0, 90	100 mM, M[i]	62 ± 3	0.8 ± 0.1	34 ± 5	33 ± 5	17 ± 4	N/A	103 ± 5	89 ± 4	1.9 ± 0.4

[a] End points used for growth balance calculations were the start and end of growth. The period between 18 and 66 h for pure-glycerol cultures grown under 1 % O_2 represents the growth period during which succinate production was maximum, with a cumulative S/A ratio starting at 2.0 and ending at 2.9 (Fig. 4b)

[b] Glycerol: *P* pure, *M* mixed

[c] Biomass (mM) was determined using 770 mg L^{-1} dry cell weight per OD_{660} and a cell composition of $CH_2O_{0.5}N_{0.2}$ (24.967 g mol^{-1}) [45]

[d] Carbon in product/carbon in glycerol consumed. The assumption was made that one mole of CO_2 was fixed per mole succinate produced [45]. Therefore, $C_3H_6O_2$ was used as the chemical composition of succinate derived from glycerol consumed

[e] Electron recoveries are based on how much hydrogen is released in the complete oxidation of a molecule (e.g., for glycerol, $C_3H_8O_3 + 3\ H_2O \rightarrow 3\ CO_2 + 14\ H$) [17]

[f] S/A: succinate/acetate ratio

[g] Except for 130Z cultures grown under 1 % O_2 (two biological replicates), all other data are mean ± standard deviation of at least three biological replicates

[h] N/A, not applicable

[i] *A. succinogenes* produced 0.2 ± 0.2 mmol pyruvate/100 mmol glycerol consumed in these conditions

are used as growth substrates and partially offset the need for a terminal electron acceptor.

Activity of terminal oxidoreductases during anaerobic respiratory glycerol metabolism

To explain the large differences in succinate production by nitrate- and DMSO-respiring cultures, the in vitro activities of fumarate reductase and other terminal oxidoreductases were tested under these different respiring conditions (Table 3). Activity levels in aerobic and anaerobic glucose-grown cultures were used as controls. Fumarate reductase activity was highest in the two culture conditions (anaerobic-glucose and glycerol-DMSO) that allow high succinate yields. Maximal DMSO reductase activity was detected in DMSO-grown cultures. Maximal nitrate and nitrite reductase activities were detected in nitrate-grown cultures. Nitrite reductase activity remained high in late glycerol-nitrate cultures, when nitrite became the only available terminal electron acceptor. Nitrate and nitrite reductase activities were elevated in glycerol-DMSO cultures as well.

Microaerobic growth on glycerol: batch cultures

Growth of *A. succinogenes* on pure glycerol under microaerobic conditions was first studied in batch conditions in a microaerobic chamber containing 1 % O_2. The background gas was CO_2 to prevent the loss of bicarbonate from the cultures. Growth was exponential between 3 and 9 h ($\ln OD_{660} = 0.20\ t – 3.34$, $R^2 = 0.999$) with a growth rate of 0.20 h^{-1}, but then steadily slowed down and stopped after 48 h (Fig. 4a). Dissolved O_2 in the cultures quickly dropped to undetectable levels within 5 h (data not shown). Succinate was the predominant product. Acetate and CO_2 were produced in nearly equimolar amounts. Formate accumulated in small amounts early in the culture but was consumed starting at approximately 68 h. Ethanol was not produced.

Metabolite production changed over time under 1 % O_2. As the culture density increased, the cells produced more succinate and less acetate (Fig. 4a), with the S/A ratio reaching 2.8 at 40 h (Fig. 4b). All the glycerol was consumed at 90 h (Fig. 4a). The microaerobic succinate yield at the end of growth was in the same range as that of DMSO-respiring cells (Table 2). Succinate production was the highest between 18 and 66 h, with a yield during that period reaching 76 % of the maximum theoretical yield, and an S/A ratio of 3.3, suggesting that continuous microaerobic conditions can be established that favor even higher succinate yields.

In the same microaerobic conditions, cultures grown on mixed glycerol had a lower S/A ratio than on pure glycerol (Table 2), largely because these cultures had increased acetate and CO_2 yields.

Table 3 Activity of terminal oxidoreductases in *A. succinogenes* crude cell extracts from various culture conditions

Growth condition	Specific activity (μmol/min mg protein)			
	DMSO reductase	Fumarate reductase	Nitrate reductase	Nitrite reductase
Glucose (aerobic)[a]	0.05 ± 0.03	1.10 ± 0.40	2.32 ± 0.50	7.71 ± 2.35
Glucose (anaerobic)	0.15 ± 0.01	3.97 ± 0.08	2.56 ± 0.29	10.1 ± 0.4
Glycerol-$NaNO_3$ (early)[b]	0.22 ± 0.03	1.62 ± 0.05	13.8 ± 2.5	47.9 ± 9.0
Glycerol-$NaNO_3$ (late)[c]	0.05 ± 0.03	1.35 ± 0.23	6.49 ± 0.72	28.3 ± 3.3
Glycerol-DMSO	0.32 ± 0.05	2.80 ± 0.43	12.7 ± 3.7	41.2 ± 11.5

[a] Cultures were grown aerobically in lysogeny broth containing 50 mM glucose in Erlenmeyer flasks shaken at 250 rpm

[b] Early phase of culture, during which nitrate is reduced to nitrite (concentrations at sampling time: $[NO_3^-] = 18.8 \pm 0.5$ mM; $[NO_2^-] = 8.2 \pm 0.4$ mM)

[c] Late phase of culture, when all nitrate is depleted and nitrite is being reduced to ammonia (concentrations at sampling time: $[NO_3^-] = 0.1 \pm 0.1$ mM; $[NO_2^-] = 15.5 \pm 3.7$ mM)

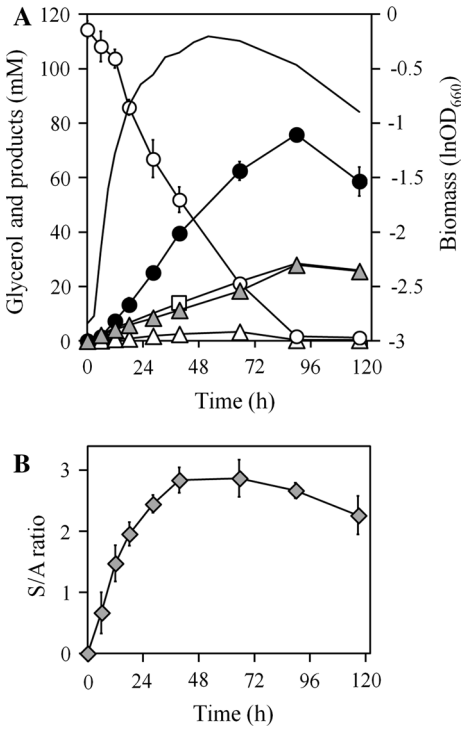

Fig. 4 Time course of microaerobic respiration by *A. succinogenes* in AM3-glycerol with a 1.0 % O_2 headspace. **a** Metabolites and cell growth, **b** S/A ratio. Data are the averages of two biological replicates ± standard deviation. *Open circle* glycerol, *filled circle* succinate, *open triangle* formate, *open square* acetate, *shaded triangle* CO_2, *dash* biomass, *shaded diamond* S/A ratio

Microaerobic growth on glycerol: continuous cultures

To get away from uncontrolled transient oxygen concentrations, continuous cultures in microaerobic conditions were established in a chemostat. Continuous cultures grown on glycerol under sparging with 5 % O_2/95 % CO_2 (0.0125 vvm) stabilized at OD_{660} ~ 1.1–1.2 with a dilution rate of 0.02 h^{-1}. In these conditions the succinate yield reached 75 % of the maximum theoretical yield (Table 4), the succinate productivity was 0.139 g l^{-1} h^{-1}, and the S/A ratio reached 5.8. Ethanol was produced at detectable levels, and formate accumulated to significantly higher levels than in microaerobic batch cultures.

1,3-PD-dependent fermentative growth on glycerol

Anaerobic fermentations have the advantage over microaerobic processes that they are less capital-intensive and their operation is easier and less expensive. A previous study showed that expressing the *C. butyricum dha* operon in *Clostridium acetobutylicum* allowed the recombinant strain to produce 1,3-PD and to ferment glycerol [16]. In our study, expression of the *C. butyricum dha* genes is under control of the strong *A. succinogenes pckA* promoter in plasmid pCV932. As expected, *A. succinogenes* (pCV932) was able to grow fermentatively on glycerol by producing 1,3-PD (Figure S1). 1,3-PD and acetate productions were favored over succinate and biomass production, with 60 and 25 % of the glycerol being funneled into 1,3-PD and acetate productions, respectively (Table 5). Compared to 25 mM NaHCO3, growth with 100 mM $NaHCO_3$ had a small negative effect on succinate production. Growth in the presence of H_2 as a source of reducing power had no significant effect on succinate or 1,3-PD production (Table 5).

Discussion

Although not the first report of succinate production from glycerol by a natural succinate-producing bacterium [10, 23, 33, 46], this study is the first to use a minimal medium to investigate the important role of respiration in the process. *A. succinogenes, Anaerobiospirillum*

Table 4 Products of glycerol metabolism in *A. succinogenes* continuous microaerobic cultures grown with 150 mM glycerol and 150 mM $NaHCO_3$ under constant sparging (0.0125 vvm) with 5 % O_2–95 % CO_2

Products (mmol/100 mmol glycerol consumed)[a]						Carbon recovery (%)[c]
Succinate	Formate	Acetate	Ethanol	CO_2	Biomass[b]	
75 ± 3	8 ± 1	13 ± 2	3.3 ± 1.9	9 ± 3	37 ± 3	103 ± 5

[a] Averages from three independent chemostats, 9–16 time points in each chemostat

[b] Biomass (mM) was determined using 770 mg L^{-1} dry cell weight per OD_{660} and a cell composition of $CH_2O_{0.5}N_{0.2}$ (24.967 g mol^{-1}) [45]

[c] Carbon in product/carbon in glycerol consumed. The assumption was made that one mole of CO_2 was fixed per mole succinate produced [45]. Therefore, $C_3H_6O_2$ was used as the chemical composition of succinate derived from glycerol consumed

Table 5 Fermentation balances of *A. succinogenes* 130Z (pCV932) grown in AM3-glycerol under N_2 or H_2 atmosphere

Gas phase	$NaHCO_3$ (mM)	Products (mmol/100 mmol glycerol consumed)						Carbon recovery (%)[b]
		Succinate	Formate	Acetate	1,3-PD	CO_2	Biomass[a]	
N_2	25	8.3 ± 0.5	6.0 ± 0.4	24.5 ± 2.9	59.6 ± 3.1	18.5 ± 3.1	5.3 ± 0.6	94.2 ± 4.1
N_2	100	6.8 ± 0.2	12.3 ± 0.7	23.8 ± 1.0	60.2 ± 1.8	11.5 ± 0.7	7.4 ± 0.2	93.3 ± 2.9
H_2	25	8.6 ± 0.2	8.6 ± 0.8	24.2 ± 0.6	59.3 ± 2.1	15.5 ± 0.2	6.6 ± 0.4	94.3 ± 3.0
H_2	100	7.0 ± 0.3	17.0 ± 0.5	26.1 ± 1.0	63.2 ± 3.6	9.1 ± 0.5	10.9 ± 2.3	99.9 ± 5.2

Cultures were grown in 28-ml serum vials containing 10 ml AM3-glycerol supplemented with $NaHCO_3$ and flushed with either N_2 or H_2

[a] Biomass (mM) was determined using 770 mg L^{-1} dry cell weight per OD_{660} and a cell composition of $CH_2O_{0.5}N_{0.2}$ (24.967 g mol^{-1}) [45]

[b] Carbon in product/carbon in glycerol consumed. The assumption was made that one mole of CO_2 was fixed per mole succinate produced [45]. Therefore, $C_3H_6O_2$ was used as the chemical composition of succinate derived from glycerol consumed

succiniciproducens, and *Mannheimia succiniciproducens* required an unidentified component of yeast extract for anaerobic growth on glycerol [23, 33, 34, 46], but all three produced high yields of succinate (Table 1). Manual annotation of the *A. succinogenes* genome justified the use of external electron acceptors, since *A. succinogenes* cannot produce 1,3-PD, the best-known metabolic determinant of glycerol fermentations. The near 100 % carbon and electron recoveries of DMSO-respiring cultures grown in AM3 confirmed the need for external electron acceptors.

Like many facultative anaerobic bacteria, *A. succinogenes* reduces a variety of electron acceptors. The differences in succinate production by nitrate-respiring, DMSO-respiring, and microaerobic cultures of *A. succinogenes* are likely due to the combined effects of transcriptional regulation and redox potentials. The main products of *A. succinogenes* grown in AM3-glycerol-$NaNO_3$ were acetate and CO_2, with succinate as a minor product. Most of the succinate produced accumulated in the nitrite-reducing phase, after nitrate was completely depleted. In *E. coli*, nitrate inhibits the expression of fumarate and DMSO reductases through the actions of the sensor-regulator systems NarXL and NarPQ [15, 43], and it induces the expression of formate dehydrogenase (FDH-N) [41]. A similar type of regulation seems to take place in *A. succinogenes* where fumarate and DMSO reductases are downregulated by nitrate (Table 3). Upregulation of FDH-N (encoded by Asuc_1262–1266) by nitrate likely explains the equimolar amounts of acetate and CO_2 produced in these growth conditions, since pyruvate dehydrogenase is typically downregulated in anaerobic conditions [11].

It is generally understood that facultative anaerobic bacteria regulate their respiratory and catabolic capabilities to maximize energy production [44]. DMSO and fumarate are both low-potential electron acceptors, with potentials of +160 and +30 mV for DMSO/dimethyl sulfide (DMS) and fumarate/succinate, respectively. While the transcription of *E. coli* DMSO and fumarate reductases is repressed in the presence of O_2, nitrate, or nitrite, there seems to be no preferential transcription of one versus the other in the absence of higher potential electron acceptors. For example, *E. coli* fumarate reductase and DMSO reductase activities are similar in cultures grown on glycerol-DMSO and on glycerol-DMSO-fumarate [6]. Nitrate reductase is also expressed at high levels in glycerol-DMSO-grown *E. coli* [6], suggesting that if DMSO has a regulatory effect on respiratory enzymes, it might be as an activator only. *A. succinogenes* seems to respond to DMSO similarly to *E. coli*: both DMSO and fumarate reductase activities are elevated, and nitrate and nitrite reductases have activity levels almost as high as in nitrate-respiring conditions (Table 3).

Because DMSO and fumarate reductase activities are both elevated in DMSO-respiring cultures, the difference in the S/A ratio between conditions of high and low DMSO

availability (Fig. 2d) is likely due to the combined effects of the two enzymes' relative kinetics and of the relative redox potentials of DMSO/DMS and fumarate/succinate. At high DMSO concentrations, enzyme kinetics likely favor the recycling of excess reducing equivalents through DMSO reductase over fumarate reductase, as suggested by the K_m values of *E. coli* DMSO reductase for DMSO (0.18 mM) [48] and *E. coli* fumarate reductase for fumarate (0.42 mM) [13]. As DMSO concentration decreases during growth, enzyme kinetics start to favor fumarate reduction. Because of DMSO respiration, the [DMS]/[DMSO] ratio increases dramatically during growth. In contrast, the $[NAD^+]$/[NADH] ratio is likely to remain more constant. The redox potential of DMSO reduction—which is a linear function of log[DMS][NAD^+]/[DMSO][NADH] at constant temperature—becomes less positive and closer to that of fumarate reduction as DMSO gets depleted, also increasingly favoring fumarate reduction.

The behavior of *A. succinogenes* under a 1 % O_2 headspace is similar to its behavior in the presence of DMSO. In the first 10 h of growth, the S/A ratio is below 1.0. As the cell density increases and less O_2 is available per cell, the cells increasingly rely on succinate production to maintain the redox balance, and the S/A ratio increases to ~3.0. These changes in metabolite production at different O_2 tensions have been well characterized in *E. coli* by Alexeeva et al. [1], who highlighted the changes in fluxes to acetate, formate, and ethanol across a range of aeration conditions from anaerobic to aerobic. The Fnr protein is present in *E. coli* at similar concentrations in aerobic and anaerobic conditions, but it becomes activated only when oxygen is depleted. At O_2 concentrations below 2.5 %, Fnr is partially activated in *E. coli* [37, 38], and it participates in inducing the expression of fumarate reductase. The progressively decreasing oxygen concentration in our *A. succinogenes* microaerobic batch cultures likely also led to an increasingly activated Fnr protein and to an increased induction of fumarate reductase expression, explaining the increasing S/A ratio during growth.

DMSO-respiring and batch microaerobic cultures differed in the production of formate and CO_2. DMSO-respiring cultures produced nearly equimolar concentrations of acetate and formate with minimal CO_2 production, whereas microaerobic cultures produced nearly equimolar concentrations of acetate and CO_2 with minimal formate production. Flux from pyruvate to acetyl-CoA can go through either pyruvate dehydrogenase (PyrDH) or PFL (and then possibly FDH-N). The high formate/CO_2 ratio in DMSO-respiring *A. succinogenes* 130Z cultures suggests that nearly all pyruvate-dissimilating flux goes through PFL. The absence of CO_2 production suggests that FDH-N expression is not induced in DMSO-respiring conditions and that formate does not act as an electron donor for DMSO reductase in *A. succinogenes*. Because FDH-N is unlikely to be expressed in microaerobic conditions, the low formate/CO_2 ratio observed in cultures under 1 % O_2 is likely the consequence of most of the pyruvate being converted to acetyl-CoA by PyrDH. PFL is irreversibly inactivated by O_2, but the PFL-activating enzyme, encoded by *yfiD*, is able to reactivate PFL in the presence of O_2 [47]. *E. coli* Fnr was shown to strongly induce the expression of YfiD in microaerobic conditions [37]. The small amount of formate detected in our microaerobic cultures might be due to a combination of the partially activated Fnr not fully activating PFL expression, and YfiD maintaining PFL in its active form.

Continuous microaerobic cultures of *A. succinogenes* on glycerol had higher succinate yields and higher S/A ratios than the microaerobic batch cultures, indicating that the steady-state microaerobic conditions in the chemostat were more favorable to succinate production than most of the transient oxygen concentrations experienced in the microaerobic batch cultures. Lower dissolved oxygen concentration in the chemostat would explain this result. It would also explain why continuous microaerobic cultures accumulated more formate than microaerobic batch cultures did, as flux through PFL would increase in these conditions. Our conclusion that PFL is active in the chemostat microaerobic conditions is supported by the observation that PFL is the main pyruvate-dissimilating enzyme active in *E. coli* during microaerobic glycerol metabolism [14].

The Gonzalez [50] and Ingram [52] groups have made significant progress in establishing *E. coli*-based platforms for the conversion of glycerol to succinate using different mutagenic approaches. Still the two *E. coli* engineered strains had succinate yields, productivity, and titers in batch cultures on glycerol that were in the same range as that of wild-type *A. succinogenes* (Table 1). The succinate yield and titer of our continuous microaerobic cultures were the highest among studies that did not use additional carbon and nitrogen sources (Table 1). The succinate productivity was in the same range as that of engineered *E. coli* in the absence of additional organic source of carbon or nitrogen and of *M. succiniciproducens* in the presence of 0.5 g yeast extract only (Table 1). These results suggest that succinate yield and productivity would be even higher with genetically engineered *A. succinogenes* strains.

The ultimate aim of this work was to develop a process for the conversion of crude glycerol to succinate. Crude glycerols of different origins can vary widely in composition, especially in methanol and fatty acid concentrations. For this reason, we focused first on mixed glycerol, the composition of which is more easily standardized than that of crude glycerol. Mixed glycerol is 99 % free of fatty acids and methanol and costs \$0.04–0.12 lb^{-1} (average \$0.05 lb^{-1}, John Oakley, Michigan Biodiesel, LLC).

The S/A ratios of DMSO-respiring cultures were the same with pure and mixed glycerol, but the S/A ratio was higher on pure glycerol than on mixed glycerol under 1 % O_2 (Table 2). Which components of the mixed glycerol affect succinate production will be investigated in future studies. Because *A. succinogenes* did not grow on mixed glycerol in the absence of O_2 or DMSO, the unidentified components are unlikely to act as terminal electron acceptors. However, mixed glycerol may contain amino acids or other growth factors that allow cells to grow faster and to a higher density than pure glycerol.

Anaerobic fermentations have the advantage over microaerobic conditions that they do not require a tight regulation of gas flow and oxygen content. *A. succinogenes* strain 130Z(pCV932) was able to grow fermentatively in AM3-glycerol, albeit at a slow growth rate and to a low growth yield. In all growth conditions tested, approximately 60 % of the glycerol consumed was diverted toward 1,3-PD production, with 25 % of the glycerol used to produce ATP through acetate production. The pull of 1,3-PD production was so strong that increasing the $NaHCO_3$ concentration in the culture did not increase succinate production as it does in glucose-fermenting cultures [26]. These results are not highly surprising given that the 1,3-PD pathway is a major fermentation pathway in *C. butyricum* and that the *C. butyricum dha* operon is expressed on a plasmid under control of a strong *A. succinogenes* promoter in the recombinant strain. Even though succinate yields were low with this first *A. succinogenes* recombinant strain, our results represent the first proof of concept that *A. succinogenes* can be engineered for fermentative growth on glycerol. We recently developed a gene knockout method for *A. succinogenes* [18], which should allow us to insert the *dha* operon in a gene deletion directly in the chromosome. Changing the strength of the promoter controlling the *dha* operon should also help with engineering a strain that shows strong fermentative growth on glycerol and produces succinate in high yields.

This study is the first to demonstrate several metabolic features of *A. succinogenes* including (1) *A. succinogenes* can use DMSO, nitrate, nitrite, and oxygen as external electron acceptors for respiratory growth with glycerol as the sole carbon source [all other studies of anaerobic growth on glycerol were in the presence of yeast extract (Table 1)]; (2) *A. succinogenes* can completely reduce nitrate to ammonia as the primary nitrogen source for biomass production; (3) the succinate yield on glycerol is strongly influenced by the nature and concentration of external electron acceptors, which control the expression of fumarate reductase; (4) *A. succinogenes* can microaerobically convert partially refined crude glycerol, an inexpensive and abundant feedstock, to succinate in high yields; and (5) *A. succinogenes* can be engineered to grow fermentatively on glycerol. More work is required to demonstrate the industrial importance of these findings, namely, to demonstrate that the microaerobic process can be scaled up to increase productivity while maintaining high succinate yields or that a robust glycerol fermentation process can be established with an *A. succinogenes* strain engineered to produce just enough 1,3-PD to allow anaerobic growth without draining all the redox power of glycerol. In all, we have demonstrated that with further engineering and process optimization, *A. succinogenes* will be a promising biocatalyst for the conversion of glycerol to succinate.

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Conflict of interest We do not have any financial relationship with the organizations that sponsored the research. We do not have any conflict of interest.

Ethical standards The experiments described in this manuscript comply with the current laws of the country in which they were performed.

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