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Research Article

Toward efficient microaerobic processes using engineered *Escherichia coli* W3110 strains

Operational and economic constraints in large-scale bioreactors often result in local or global microaerobic conditions, which lead to less efficient bioprocesses. *Escherichia coli* adapts to microaerobicity by activating fermentation pathways that accumulate acidic by-products, in detriment of growth rate (μ) and biomass yield on glucose ($Y_{X/S}$). In this study, the metabolism of *E. coli* was modified to better cope with microaerobicity. For that purpose, genes coding for global regulators like carbon source responsive B protein and aerobic respiratory control A protein, or for fermentative pathways were inactivated. The performance of a wild-type (W3110) and engineered *E. coli* strains was evaluated in batch cultures at constant low dissolved oxygen tension (3% air sat.). By combining the partial elimination of fermentation pathways and the expression of the *Vitreoscilla* hemoglobin (VHb), a 32% decrease on carbon waste as by-products, 24 % increase on $Y_{X/S}$ and 13% increase of μ were obtained. Flux balance analysis of the best strain estimated major differences in the fluxes through the pentose phosphate pathway and tricarboxylic acid cycle as consequence of VHb presence. Overall, our results show that *E. coli* can be genetically modified to overcome some of the disadvantages of microaerobic growth, which is potentially useful for better bioreactor scale-up and operation.

Keywords: Aerobic respiratory control A protein / Carbon source responsive B protein / Fermentation pathways / Microaerobic processes / *Vitreoscilla* hemoglobin

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1 Introduction

Aerobic high cell-density cultures are well established in industrial biotechnology. Nevertheless, ensuring oxygen availability to the cells is a challenge for scaling-up such type of cultures, since oxygen demand can easily surpass the mass transfer capacity of the bioreactor. In cultures of the facultative anaerobic bacterium *Escherichia coli*, maintaining adequate levels of dissolved oxygen tension (DOT) is important to prevent the activation of fermentative pathways [1]. When the DOT is above 10% of air saturation, aerobic respiration occurs [2], and the carbon source is oxidized to CO_2 with the maximal ATP generation [3]. However, under oxygen limitation, fermentative pathways are activated in *E. coli*, producing a mixture of organic acids (lactate, succinate [SUC], formate, and acetate) and ethanol as waste by-products. The synthesis of each organic acid helps to maintain the redox balance, at least partially, but energy generation is considerably lower than under fully aerobic conditions. In consequence, the activation of this metabolic mode negatively impacts the yield and productivity of the process. Although there are alternatives to increase oxygen transfer to the bioreactor (v. gr., [4–6]), they usually involve additional equipment investment or process costs. A promising, yet less exploited alternative to cope with microaerobicity, is to genetically redesign the cell to make it more efficient under oxygen limitation. The challenge is to develop strains with robust, similar behavior and physiology under aerobic or microaerobic conditions. Such metabolic

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Abbreviations: μ, specific growth rate; **AcCoA**, acetyl coenzyme A; **ArcA**, aerobic respiratory control A protein; **ATP**, adenosine triphosphate; **CreB**, carbon source responsive B protein; **DOT**, dissolved oxygen tension; **NADH**, nicotinamide adenine dinucleotide (reduced); **OAA**, oxaloacetate; **PDH**, PYR dehydrogenase; **PEP**, phosphoenolpyruvate; **PPP**, pentose phosphate pathway; **Pi**, inorganic phosphate; **PYR**, pyruvate; **SUC**, succinate; **TCA**, tricarboxylic acid cycle; **VHb**, *Vitreoscilla* hemoglobin ; $Y_{X/S}$, biomass yield on glucose

engineering approach can result in many benefits for the development and operation of bioprocesses, yet, there are not many examples on this field. In principle, robust strains, less sensitive to scale-up effects could be developed by manipulating the oxygen-related regulatory machinery, by selectively inactivating undesired metabolic pathways or by increasing the efficiency of oxygen uptake of *E. coli*.

One of the major regulators of aerobic–anaerobic metabolism of *E. coli* is the ArcAB two-component system: ArcB is a transmembrane sensor for dissolved oxygen, and aerobic respiratory control A protein (ArcA) is a cytoplasmic regulator that recognizes ArcB signals [7]. ArcA represses the expression of genes of the TCA, like those coding for SUC dehydrogenase (*sdh*), fumarase (*fumA*), pyruvate (PYR) dehydrogenase (*pdh*) [8], malate dehydrogenase (*mdh*) [9], and malate synthase (*ace*) [10]. ArcA also represses the expression of *cyoABCDE* operon, coding for the low O_2-affinity cytochrome *bo* oxidase, and activates the expression of cydAB, coding for the high O_2-affinity cytochrome *bd* oxidase [11], the *pfl* operon [12], as well as enzymes of fermentative metabolism [8] (Fig. 1). Therefore, the general function of ArcA is to slowdown the rate of TCA and the aerobic respiration under O_2 limitation, thus making *arcA* mutants attractive for microaerobic processes. Another important transcriptional regulator in microaerobic conditions is the CreABCD system, which regulates the use of the carbon source. CreABCD is activated when glycolytic carbon sources are being fermented in minimal medium but not in complex media [13]. CreABCD regulon consists of four genes: *creA*, which acts as an open reading frame; *creD*, encoding an intramembranal protein of unknown function; and *creB* and *creC*, which in resemblance to the ArcAB system, encode a cytoplasmic regulator and a sensor kinase, respectively [9]. This system activates seven genes under microaerobic conditions [14], among them, those coding for the acetate kinase and phosphotransacetylase (*ackA* and *pta*; Fig. 1), as well as transaldolase. Accordingly, it has been reported that *creB* mutants accumulated less organic acids and produced more biomass than its parental strain in microaerobic chemostat cultures [9]. The second approach to develop engineered strains with better performance under microaerobic conditions is to inactivate fermentative pathways. Since the fermentative pathways are used to regenerate NAD^+ under oxygen-limited conditions, only some pathways can be eliminated without strong effects on the growth rate. It was previously demonstrated that inactivation of the *ldhA*, *pflB*, and *poxB* genes (coding for enzymes that synthesize lactate, formate, and acetate, respectively, see Fig. 1) improved the growth of *E. coli* under intermittent aerobic–anaerobic conditions [15]. However, such strain was not tested under constant microaerobic conditions.

Increasing O_2 uptake efficiency by the expression of the *Vitreoscilla* hemoglobin (VHb) has been well documented in a variety of organisms, with positive effects on growth, biomass, and product formation [16, 17]. VHb is a transmembranal protein that increases O_2 uptake and intracellular concentration, delivering it directly to the cytochromes, thus increasing the terminal oxidase activity [17, 18]. This would result in a faster regeneration of NAD^+ and the concomitant activation of the TCA [19]. Despite the well-known benefits of VHb, there are very scarce reports on the expression of this hemoglobin in engineered strains with the specific purpose of improving growth performance under microaerobic conditions. In this work, genes coding for ArcA, carbon source responsive B protein (CreB), and fermentation pathways were inactivated in *Escherichia coli*. Although it has been previously reported that the CreB, ArcA, or VAL24 mutants perform better under oxygen-limiting conditions, cultures were performed in chemostats, under uncontrolled DOT conditions, or under oscillatory DOT in scale-down studies [15]. This makes a direct comparison of the results difficult. In the present study, the mutant strains were cultured under controlled microaerobic conditions (DOT = 3% air sat.) in batch mode for unbiased comparison. Furthermore, VHb was expressed in such strains to evaluate the impact of combined genetic modifications and enhanced O_2 uptake, resulting in additional advantages.

2 Materials and methods

2.1 Bacterial strains and plasmids

All *E. coli* mutant strains used in this work are a modified version of wild-type *E. coli* W3110 (ATCC 27325). Strain W3110 *arcA*$^-$ is a single mutant (W3110 Δ*arcA*) in which the *arcA* gene (that encodes for the ArcA cytoplasmic regulator) was inactivated. Strain W3110 *creB*$^-$ is a single mutant with an inactivated *creB* gene that codes for the CreB cytoplasmic regulator (W3110 *creB*::Km). It was constructed by P1 phage transduction into W3110 using a lysate from the corresponding mutant taken from the Keio collection [20]. Kanamycin resistance and colony PCR were used to confirm the mutation using the following primers: 5′ TTGCCGGTGCTGGATAAAG 3′ and 5′ ACTTAACACGGCGGTCAATG3′. VAL24 (W3110 Δ*ldhA*, Δ*pflB poxB:*cat) is a triple mutant unable to produce lactate and formate due to deletions of the *ldhA* and *pflB* genes that code for lactate dehydrogenase and PYR formate-lyase, respectively. Additionally, the *poxB* gene, coding for PYR oxidase, which converts PYR to acetate [15], was inactivated in VAL24 (Fig. 1). All strains were transformed with the pNKD1 and pBS plasmids separately. Plasmid pNKD1 was constructed based on the backbone of pBS (Stratagene, Santa Clara, CA, USA), which contains an ampicillin-resistance gene. The plasmid pNKD1 contains the *vgb* gene coding for VHb (Accession Number: M27061) located adjacent to the *Sma*I restriction site of pBS. The *vgb* gene is located under the transcriptional control of a modified VHb promoter that contains a CRP site 100 bp downstream *vgb*. The *vgb* gene expression is fully induced by microaerobic conditions. The plasmid pNKD1 was kindly donated by Prof. Benjamin Stark, Illinois Institute of Technology. Strains transformed with pNKD1 are denoted in this work as *vgb*$^+$, while those transformed with the pBS plasmid are denoted as *vgb*$^-$ and were used as control without *vgb* expression.

2.2 Preculture and culture media

The medium for preculture (shake flask) and bioreactor cultures had the following composition (in g/L): K_2HPO_4, 17; KH_2PO_4, 5.3; $(NH_4)_2SO_4$, 2.5; $(NH_4)Cl$, 1; NaCl, 1; $MgSO_4 \bullet 7H_2O$, 1; ampicillin disodium salt, 0.1; D-glucose, 7; thiamine 0.01; and 0.5 mL/L of a stock solution of trace elements [21].

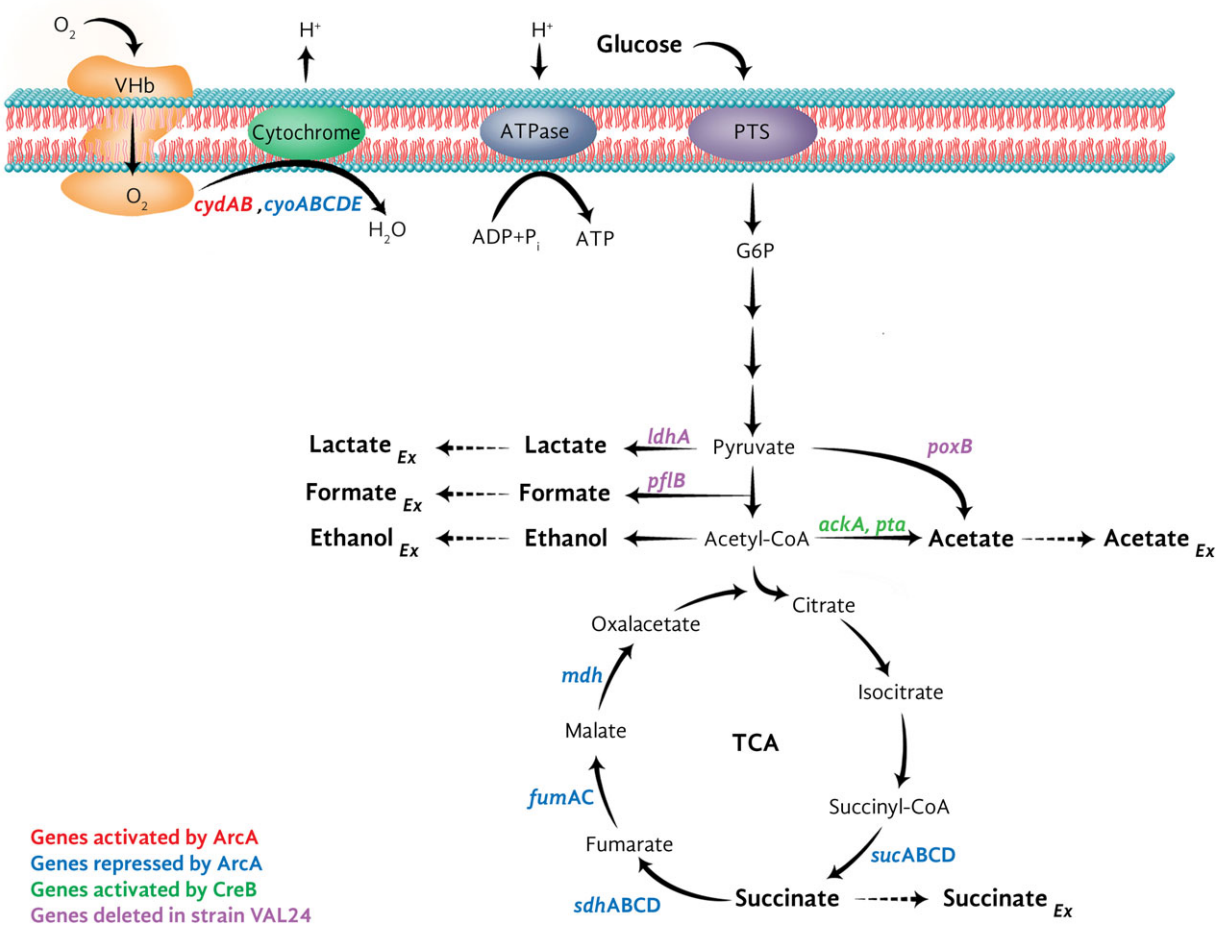

Figure 1. Overview of the metabolism of *E. coli* showing the synthesis of fermentative products and participation of the *Vitreoscilla* hemoglobin. The names in italics refer to the genes coding for the enzymes that catalyze the illustrated reactions. The subscript "Ex" means "extracellular." G6P = glucose 6-phosphate; PTS = phosphotransferase system; Cyt = cytochrome; TCA = tricarboxylic acid cycle.

Precultures were obtained from cryo-preserved transformed *E. coli* cells inoculated in 50 mL of medium and grown for 12 h at 37°C in 250 mL baffled shake flask at an agitation rate 250 rpm.

2.3 Bioreactor cultures

Cultures of all *E. coli* strains were performed in a 1 L Biostat A Plus bioreactor (Sartorius BBI, Melsungen, Germany) with a working volume of 0.5 L and an aeration of 1 vvm. Culture conditions of pH 7.0 ± 0.05 (controlled by addition of a 15% v/v NH_4OH solution) and 37°C. DOT was measured using a polarographic sensor (Hamilton, Reno, NV, USA).The sensor was calibrated by flowing pure nitrogen (for 0% air sat.) or air (for 100% air sat.) at 1 vvm until constant current readings. After the calibration, nitrogen was used again to confirm the calibration at 0% air sat. The DOT sensor was cleaned and filled with clean electrolyte after each culture (Oxylyte, Hamilton, Reno, NV, USA). DOT was controlled at 3% air sat. by a PI controller in agitation cascade mode ($t_i = 50$ s; $x_p = 140\%$; $t_D = 0$ s; dead band = 0.1%) using the MFCS/DA software (Sartorius BBI, Melsungen, Germany). All cultures were carried out by duplicate.

2.4 Analytical methods

Organic acids concentration was determined by HPLC as previously described [21]. Glucose and ethanol concentration were determined with a YSI 2900 biochemical analyzer (Yellow Springs Instruments, Yellow Springs, OH, USA). Dry cell weights were obtained from cell pellet samples dried at 80°C for at least 12 h. Stoichiometric and kinetic parameters were obtained with the proper mass balances over the time periods involved. Carbon wasted was calculated as the sum of C_{mmol} of the by-products at the point of maximum fermentation products concentration, coincident with the time of glucose exhaustion.

2.5 Metabolic model

The experimental physiological data were integrated into a metabolic scale using a metabolic model. The model comprises the pathways of glycolysis, pentose phosphate, TCA, oxidative phosphorylation, and the reactions of mixed-acid fermentation. In total, 97 reactions were included to perform the mass balances and we only considered the external (ex) and cytosolic

(c) compartments. The reactions are listed in the Supporting information. The biomass equation accounted for the primary precursor of the *E. coli* biomass composition [22]. To simulate the effect of the VHb in the model, we associated a stoichiometric reaction for the translocation of protons. In early studies, the presence of VHb was related to the respiratory chain efficiency upon oxygen depletion and that synergistically operates with cytochrome terminal oxidases [23]. The evidence that VHb increases the oxygen uptake rate was observed at different levels of *vgb* expression induced with IPTG [24]. Considering this observation, the simulation of the presence of VHb in the metabolic network through a metabolic reaction must consider the presence of protons, oxygen, NADH, and water. If VHb can reestablish the function of the cytochrome terminal oxidases, then the translocation of protons can be described by the following reaction:

$$4H^+\,[c] + NADH\,[c] + 0.5O_2\,[c] \Rightarrow H_2O\,[c] + NAD^+\,[c] + 4H^+\,[ex]$$

The calculated degree of reduction of this reaction is similar to the reaction that represents the cytochrome *bo* activity. This was the VHb reaction included in the model. With the assumption of the above reaction, we considered that the presence of VHb modifies the P/O ratio.

2.6 Calculation of fluxes distribution

To calculate the metabolic flux distributions by Flux Balance Analysis, an optimization-based algorithm was used [25], restricting the extracellular fluxes to those obtained experimentally. The objective function was to minimize the oxygen uptake rate under the assumption that respiratory capabilities of *E. coli* are the result of the oxygen available per unit of biomass and the cell takes the minimal amount of oxygen to oxidize the glucose to CO_2 and by-products production [26]. Based on the stoichiometry of the enzymatic reactions involved in the electron transport system, the effective P/O ratio for *E. coli* is about 2.7 [27]. We assumed that the expression of the VHb modifies the electron transport systems, by introducing a P/O ratio of 3.75. Such value was calculated representing an increment in the respiratory capacity and allowed a flux calculation for the reaction.

3 Results and discussion

3.1 Growth performance of wild-type and mutant strains without the *vgb* gene

All four *E. coli* strains, expressing or without the *vgb* gene, were cultured in batch mode under controlled microaerobic conditions (DOT = 3% air sat.). The results of the experiments are discussed below using averaged values and their corresponding experimental errors.

Biomass yield on glucose ($Y_{X/S}$), specific rates of growth (μ), glucose consumption (q_S), and fermentation metabolites accumulation were calculated over the exponential growth phase for all cultures. Figure 2A depicts μ and biomass yields on glucose ($Y_{X/S}$) of the different strains studied that did not express the *vgb* gene. The wild-type strain displayed a μ of 0.36 ± 0.01 h and $Y_{X/S}$ $= 0.34 \pm 0.03$ g/g, which are 20 and 30% lower, respectively, than the corresponding values obtained under fully aerobic conditions using the same culture medium [19]. This results from less efficient energy generation and carbon incorporation to biomass, typical of oxygen limitation. The inactivation of the regulator *creB* resulted in a lower μ (0.30 ± 0.01 h), but considerably higher $Y_{X/S}$ (0.48 ± 0.01 g/g; Fig. 2A), compared to the wild-type. It has been reported that *creB* mutants accumulate relatively high levels of intracellular PYR, which can limit the available phosphoenol pyruvate (PEP) for glucose transport through the phosphotransferase system [28]. It is also known that the *talA* gene is repressed in these mutants, which can lead to erythrose-4-phosphate limitations; this metabolite is needed for the synthesis of aromatic amino acids and some vitamins [14]. Such factors could explain the low μ of *creB* mutants under microaerobic conditions. Higher $Y_{X/S}$ in *creB* mutants, compared to parent strains was also observed in glucose-limited chemostats at low dilution rate [9], and could be explained due to lower accumulation of fermentative products, as discussed below. The deletion of *arcA* resulted in growth rate recovery, which reached 0.43 ± 0.01 h. In contrast to the *creB* mutant, the $Y_{X/S}$ of the *arcA* mutant was only 0.38 $\pm$ 0.00 g/g (Fig. 2A). The increase of μ in W3110 *arcA* mutant could be an effect of a more active TCA cycle under microaerobic conditions in comparison with the wild-type strain, supplying more precursors for biomass synthesis and generating NADH used in oxidative phosphorylation [7, 29]. For instance, the expression of *cyoA* coding for the cytochrome *bo* terminal oxidase is higher in an *arcA* mutant than in wild-type strains [30,31], which could result in higher energy generation available for biomass synthesis. Furthermore, Iuchi and Lin [7] reported that in an *E. coli arcA* mutant the anaerobic levels of some enzymes activities like aconitase and isocitrate lyase exceeded the aerobic level. The engineered strain VAL24 displayed the highest μ of all the evaluated strains (0.44 ± 0.01 h; Fig. 2A), which was 22% higher than that of the W3110. Moreover, the $Y_{X/S}$ of VAL24 (0.46 ± 0.02 g/g; Fig. 2A) was also relatively high. These results are in agreement with the observations in bioreactor scale-down studies, in which VAL24 also accumulated biomass faster than its parent strain under DOT oscillations [5]. It was speculated that the decrease in the maintenance coefficient in *poxB* mutants could contribute to the improved culture performance of VAL24 [5].

3.2 Growth performance of wild-type and mutant strains expressing the *vgb* gene

The previously discussed results show that each genetic modification has disadvantages and potential advantages for microaerobic processes. The second group of experiments was performed in order to investigate whether the presence of the VHb could have further benefits for the growth performance of the strains under controlled microaerobic conditions in batch mode. The general positive effect of VHb on microaerobic growth of *E. coli* is well known. Therefore, it was expected that VHb expression by the mutant strains would enhance such positive effect. The

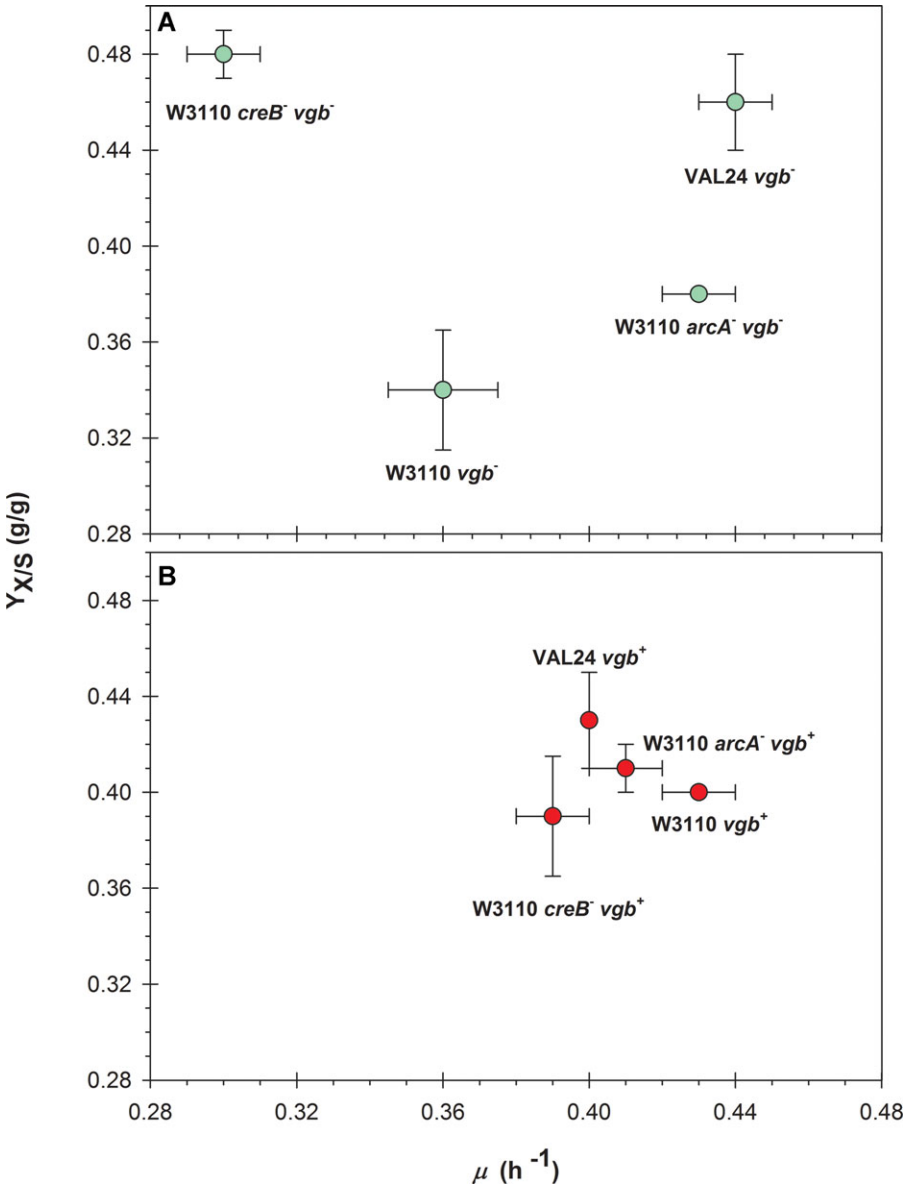

Figure 2. Biomass yield glucose ($Y_{X/S}$) versus specific growth rate (μ) of the different strains nonexpressing (A) and expressing (B) the *vgb* gene. The horizontal and vertical bars represent the experimental deviation between duplicate experiments.

results of growth rate and biomass yields are shown in Fig. 2B. The expression of *vgb* in W3110 increased μ and $Y_{X/S}$ by of 19 and 17%, respectively, compared to the *vgb*$^-$ strain, which is in agreement with the general effect of VHb reported [17]. However, the presence of VHb was not beneficial for all the mutant strains. For example, *vgb* expression increased only slightly the values of $Y_{X/S}$ of the *arcA* mutant (0.41 $\pm$ 0.01 g/g) and μ (0.43 $\pm$ 0.01 h), in comparison with the strain W3110*arcA*$^-$ *vgb*$^-$. In the case of the *creB* mutant, the expression of *vgb* increased μ by 30%, while strongly decreased the $Y_{X/S}$, which fell down to 0.39 $\pm$ 0.02 g/g, which is 19% lower than the strain W3110*creB*$^-$ *vgb*$^-$. When VHb was present in the mutant strain VAL24, little effects were observed: μ decreased 10% (reaching 0.40 $\pm$ 0.00 h) and $Y_{X/S}$ decreased 7% (to 0.43 $\pm$ 0.02 g/g), compared to cultures without *vgb* expression (Fig. 2).

3.3 Accumulation of fermentative by-products

The specific rates for q_S and fermentative by-products accumulation were calculated during exponential growth phase in all cultures. The obtained values are shown in Table 1. The q_S values for the strains without the *vgb* gene were similar, except for W3110*creB*$^-$, which consumed glucose nearly 4% slower than the wild-type strain (Table 1). This is consistent with the lower μ displayed by W3110*creB*$^-$, compared to the other strains (Fig. 2A). Moreover, it has been reported that a *creB* mutant consumed glucose at a rate 47% lower than that of its parent strain in microaerobic chemostats at a dilution rate of 0.1 h [9]. The presence of the VHb resulted in a 62% increase of q_S for the *creB* mutant, while minor changes were observed for the other strains (Table 1). This increase in q_S agrees with the higher μ observed

Table 1. Specific extracellular rates of the different strains not expressing and expressing the *vgb* gene

	q_S	q_{acet}	q_{for}	q_{lac}	$q_{et\text{-}OH}$	q_{suc}
W3110 *vgb*$^-$	5.94 ± 0.35	1.64 ± 0.11	0.79 ± 0.38	0.24 ± 0.04	0.29 ± 0.01	0.87 ± 0.18
W3110 *vgb*$^+$	6.01 ± 0.14	1.00 ± 0.20	0.12 ± 0.03	0.05 ± 0.00	0.14 ± 0.00	1.46 ± 0.06
W3110*arcA*$^-$ *vgb*$^-$	6.26 ± 0.25	1.74 ± 0.22	0.38 ± 0.10	0.09 ± 0.01	0.16 ± 0.02	1.31 ± 0.22
W3110*arcA*$^-$ *vgb*$^+$	5.55 ± 0.04	1.67 ± 0.34	0.40 ± 0.09	0.36 ± 0.06	0.26 ± 0.05	1.41 ± 0.12
W3110*creB*$^-$ *vgb*$^-$	3.45 ± 0.01	0.56 ± 0.09	0.60 ± 0.25	0.30 ± 0.23	0.07 ± 0.02	0.39 ± 0.16
W3110*creB*$^-$ *vgb*$^+$	5.60 ± 0.59	1.12 ± 0.01	0.21 ± 0.04	0.18 ± 0.06	0.12 ± 0.00	1.07 ± 0.20
VAL24 *vgb*$^-$	5.30 ± 0.03	1.70 ± 0.14	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.02	1.04 ± 0.10
VAL24 *vgb*$^+$	5.24 ± 0.22	1.62 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.70 ± 0.03

All the values are given in g/g h. The symbol ± represents the experimental deviation between duplicate experiments. *q* denotes the specific rates for: glucose (q_S), acetate (q_{acet}), formate (q_{for}), lactate (q_{lac}), ethanol ($q_{et\text{-}OH}$), and succinate (q_{suc}) production.

in the W3110*creB*$^-$ *vgb*$^+$ strain, compared to W3110*creB*$^-$ *vgb*$^-$ (Fig. 2).

The rates of fermentative by-products accumulation showed several differences between strains. When *vgb* was not present, the specific rate of acetate accumulation (q_{acet}) was similar for all the strains, except for the *creB* mutant, which accumulated acetate around 30% slower than the other strains (Table 1). This could be attributed to a lower transcription of *ackA* and *pta* genes, which are induced by CreB [13] and the lower q_S of the *creB* mutant. SUC was rapidly synthesized by all strains except the *creB* mutant in agreement with a previous report [9]. The deletion of *arcA* resulted in a decreased rate of accumulation of lactate (q_{lac}), formate (q_{for}), and ethanol ($q_{et\text{-}OH}$), compared to W3110 and W3110*creB*$^-$ strains (Table 1). This may be attributed to a more active TCA cycle in *arcA* mutants. Moreover, it is known that the *pfl* operon (coding for the PYR formate-lyase enzyme, responsible for formate synthesis) is activated by ArcA under microaerobic conditions [12]. It is also known that in *arcA* mutants the expression of *pfl* is lower than in its parental strain [32], which also could explain the observed decreased in q_{for}. As expected from its genotype, neither formate nor lactate was produced by strain VAL24 (Table 1). The presence of VHb changed the accumulation patterns of by-products in all strains. Since the production of a given by-product is tuned to maintain the redox balance within the cell, it is clear from results in Table 1 that VHb have an important impact in the redox balance. The presence of VHb in W3110 resulted in a 60% decrease of q_{acet}, which coincides with previous observations [33]. VHb did not change q_{acet} in strains W3110*arcA*$^-$ and VAL24, but strongly increased it in strain W3110*creB*$^-$. This could be an effect of the faster glucose uptake of W3310 *creB*$^-$ *vgb*$^+$, compared to W3110*creB*$^-$ *vgb*$^-$ (Table 1). The presence of VHb in W3110 also resulted in a decrease of q_{for}, q_{lac}, and $q_{et\text{-}OH}$ of ca. 85, 81, and 50%, respectively, compared to W3110 *vgb*$^-$ (Table 1). Such decreased rates, together with the slightly increased growth rate of W3110 *vgb*$^+$ compared with W3110 *vgb*$^-$ suggest a higher activity of the TCA cycle due to the presence of the VHb. This hypothesis is further explored in the next section with the estimation of metabolic fluxes. In contrast to the mentioned results, q_{for} only slightly changed for W3110*arcA*$^-$ *vgb*$^+$, compared to W3110*arcA*$^-$ *vgb*$^-$. VHb increased the specific rate of SUC production q_{suc} for all strains except VAL24 (Table 1). Such an increase has already been observed in *arcA* mutants [7], and may imply that TCA cycle is working as a branched pathway.

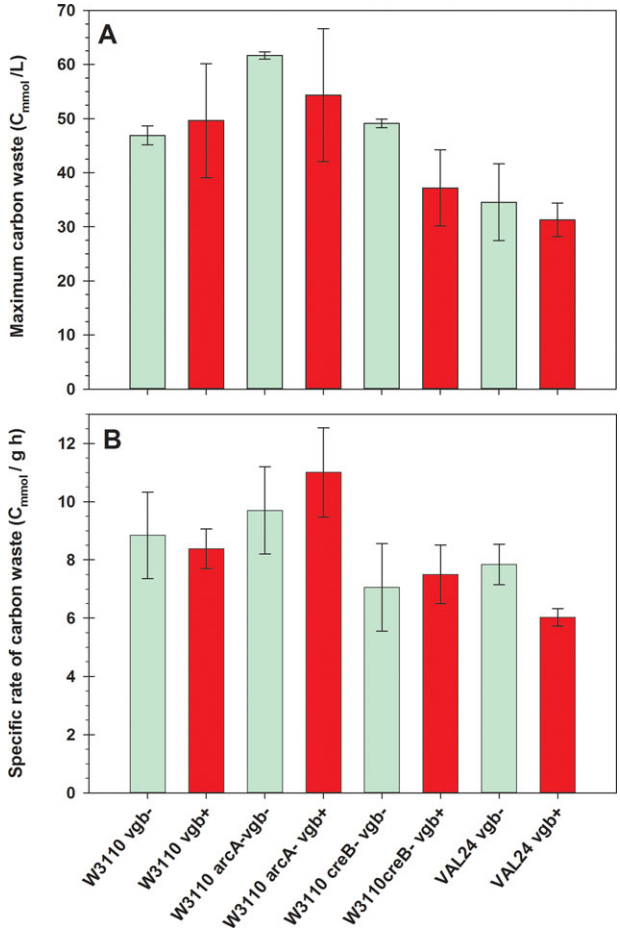

Figure 3. Maximum carbon waste (A) and specific rate of carbon waste (B) of the different strain. Carbon waste is calculated as the sum of C_{mmol} of the fermentative products. The vertical bars represent the experimental deviation between duplicate experiments.

When VHb was present, VAL24 did not accumulate formate and lactate, and remarkably, ethanol was not detected.

A global view of fermentation products accumulation is presented in Fig. 3. It can be seen that VHb did not change the maximum amount of carbon wasted. The strains W3110*creB*$^-$ and VAL24 wasted less carbon than the other strains

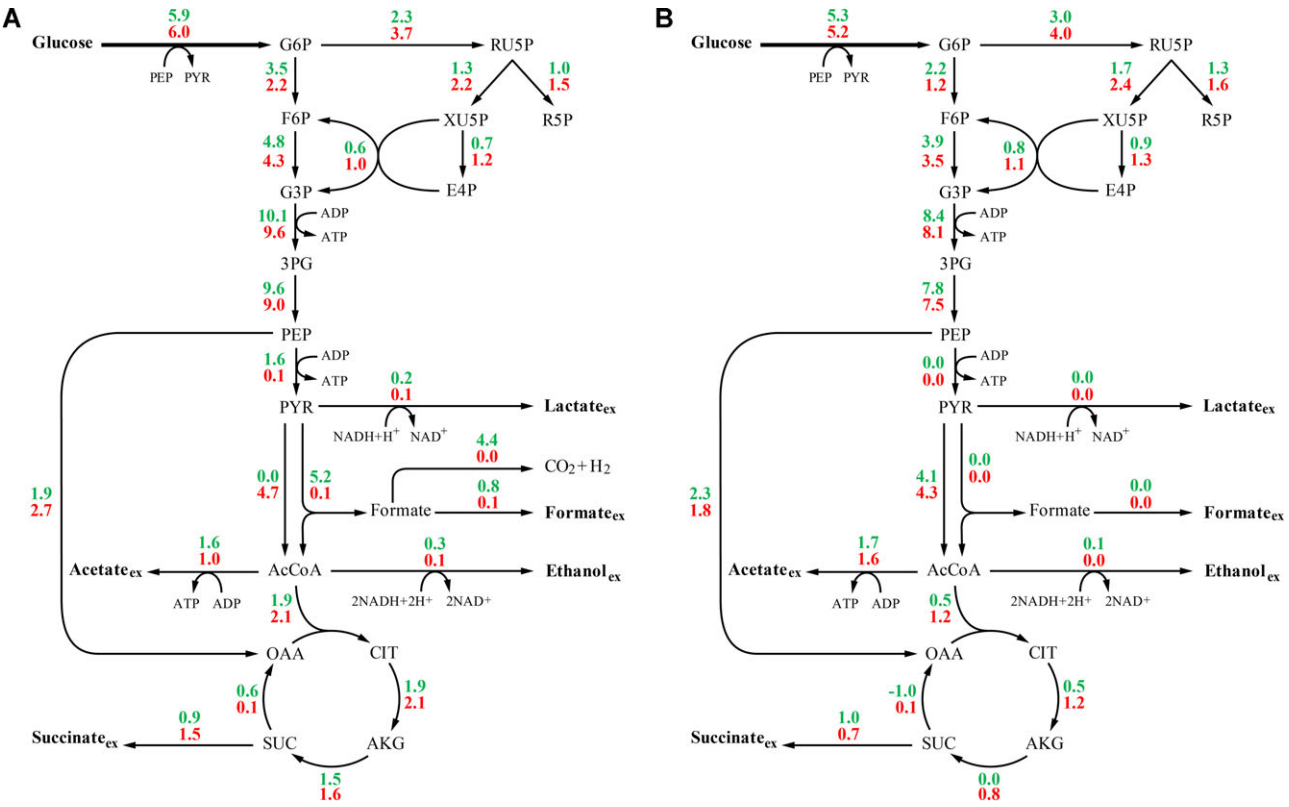

Figure 4. Estimated metabolic fluxes of strains W3110 (A) and VAL24 (B) not expressing (upper values) and expressing (bottom values) the *vgb* gene. Subscript "ex" stands for "extracellular."

(Fig. 3A). Furthermore, the specific rate of carbon waste (the sum of the specific rates of fermentation products accumulation expressed in C_{mmol}/g h) was lower for W3110*creB*$^-$ and VAL24, compared to the other strains (Fig. 3B). Such information, together with the relatively high μ and $Y_{X/S}$ makes VAL24 *vgb*$^+$ an interesting candidate for microaerobic processes and large-scale cultures. Therefore, this strain was selected for further analysis and compared to the wild-type strain as discussed in the next section.

3.4 Estimation of the metabolic fluxes in strains W3110 and VAL24

To better understand the effect of VHb on the engineered strain, the metabolic fluxes were estimated by flux balance analysis during the exponential growth of strains W3110 and VAL24. The simulations estimated values of μ close to the experimental values (data not shown) and the specific oxygen uptake rates estimated were in agreement with previously published values [34]. The results are depicted in Fig. 4. Previous reports have studied the intracellular carbon fluxes in strains expressing the *vgb* gene using a different host (v. gr., MG1655), fed-batch schemes, and uncontrolled DOT [33, 35], which limits the comparison with the present results.

While the presence of the VHb had no effect on q_S, the flux to the pentose phosphate pathway (PPP) was 60% higher when VHb was present in W3310 (Fig. 4A). This is consistent with previous observations [35], and together with the lower accumulation of organic acids, explains the higher $Y_{X/S}$ observed when VHb was present (Fig. 2). A considerably higher amount of PEP is converted to oxaloacetate (OAA) through the PEP carboxylase by the strain expressing *vgb*, compared to the one without it. A negligible formation of PYR from PEP for the strains expressing *vgb* was estimated, meaning that PYR is mainly supplied by the transport of glucose through the phosphotransferase system (PTS). This would decrease the production of ATP by substrate level phosphorylation, which is relevant under microaerobic conditions. Nevertheless, the net result is more ATP-synthesized in presence of VHb, due to the greater μ and $Y_{X/S}$ when *vgb* is expressed in W3110. The flux from PYR to acetyl coenzyme A (AcCoA) of strain W3110 *vgb*$^-$ was negligible, in agreement with the low activity of the PYR dehydrogenase (PDH) activity under oxygen-limited conditions [36]. Therefore, AcCoA is mainly produced by the PYR formate-lyase in the strain without *vgb*, with the concomitant production of formate (Fig. 4A), which is in agreement with experimental studies [37]. This contrasts with the relatively high flux through the PDH predicted for strain W3110 *vgb*$^+$ (Fig. 4A). It is interesting to note that the model predicts an important conversion of formate to CO_2 and H_2 in the strain W3110 *vgb*$^-$, but not in W3110 *vgb*$^+$. This might be advantageous, since less acidic by-products are accumulated, nevertheless, CO_2 can accumulate in large-scale bioreactors and form acid species that can also contribute to alter

the host physiology [38]. The estimated distribution of AcCoA also showed changes due to the presence of VHb. The fluxes to acetate and ethanol were 60 and 300% higher in the strain *vgb*$^-$, compared to the *vgb*$^+$ one (Fig. 4A). In consequence, the presence of VHb decreased the formation of ATP and NAD^+ directly from AcCoA. An interesting finding is that the model estimates a closed (not branched) TCA cycle for both strains, expressing or without *vgb*. Recent studies in strains MG1655 demonstrated that TCA cycle operates completely under microaerobic conditions [39]. On the other hand, it has been reported that the TCA cycle works in a branched fashion in strains expressing or without *vgb* [33]. Such discrepancies may be attributed to the mode of operation of the culture and intracellular concentration of active VHb. Actually, Tsai et al. [24] reported that intracellular fluxes from α-ketoglutarate to OAA in a strain expressing *vgb* were maximal when the intracellular VHb concentration was 1.2 μmol/g_{DCW}. The concentration of VHb is dependent on the μ, promoter, and strain used, limiting the comparability of the different studies.

As shown in Fig. 4, the estimated flux through TCA cycle from citrate to SUC is similar in both strains. However, more SUC is excreted by the *vgb* expressing strain. This is in agreement with the known fact that decreased lactate dehydrogenase and PYR formate-lyase activities increase SUC production under anaerobic conditions [40]. In general, the proposed model produced estimations that are consistent with previous experimental observations, while different results in the TCA cycle activity cannot be regarded as inconsistencies, since the published experimental results are not conclusive.

The comparison of the fluxes distributions in the strain VAL24 is shown in Fig. 4B. The flux of the PPP pathway in strain VAL24 *vgb*$^-$ (Fig. 4B) was greater than that of W3110 *vgb*$^-$ (Fig. 3A), while the presence of VHb in VAL24 resulted in the highest flux to the PPP of the four analyzed strains (Fig. 3B), despite the fact that VAL24 *vgb*$^+$ displayed the lowest q_S. The fluxes from fructose 6-phosphate to PEP were slightly lowered by the presence of VHb in strain VAL24 (Fig. 4B). In contrast with the estimations for W3110, the presence of VHb decreased the flux from PEP to OAA in strain VAL24 and did not change the flux through the PYR kinase (Fig. 4B). Therefore, all PYR is provided by the phosphotransferase system, regardless the presence of VHb. The flux through the PDH is only slightly higher in VAL24 *vgb*$^+$ compared to VAL24 *vgb*$^-$. The operation of the TCA cycle showed a major difference as a result of VHb presence. When VHb was not present, TCA was branched, while it worked as a cycle when VHb was present (Fig. 4B). It is possible that the NADH + H^+ demand of VAL24 *vgb*$^+$ could not be satisfied by fermentative pathways, leading to the closed mode of the TCA. The operation of the TCA as a cycle in VAL24 should provide all the required NADH, since ethanol production was not detected in cultures of VAL24 *vgb*$^+$, notwithstanding the fluxes through the TCA cycle in this strain were lower than those of W3110 *vgb*$^+$ and W3110 *vgb*$^+$ (Fig. 4A and B). Overall, the metabolism of VAL24 *vgb*$^+$ under microaerobic conditions was closer to an aerobic mode than to a microaerobic one, with the exception of low PYK activity and SUC accumulation. From the estimated fluxes it can be seen that the flux to SUC is linearly proportional to the flux through the PEP carboxylase enzyme, which agrees with previous observations [40]. Therefore, it could be possible to decrease the SUC accumulation by tuning the PEP to OAA through molecular approaches.

4 Concluding remarks

The results presented show that the performance of *E. coli* under microaerobic conditions can be improved by deletion of selected genes, and that the expression of the VHb further enhances such results. This represents a practical example of metabolic engineering applications to solve bioprocess issues. Particularly, decreasing the flux of PYR toward fermentation products by deleting the *pflB*, *poxB*, and *ldhA* genes is beneficial for improving *E. coli* strains, since the engineered strain VAL24 expressing *vgb* displayed relatively high growth rates, biomass yields and abolished the accumulation of lactate, ethanol, and formate. Thus, this strain is potentially useful for microaerobic processes that could operate under reduced power supply for mixing, simplifying scale-up procedures. The performance of such strain expressing a product of interest in high cell-density cultures should be further tested.

Practical application

Oxygen transfer often limits the operational conditions of bioreactors. The modified strains presented here could be used under transient or constant microaerobic conditions. This could help to obtain higher cell densities in shake flasks and stirred bioreactors, and to operate bioreactors using lower energy inputs.

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