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Fast Dynamic Response of the Fermentative Metabolism of *Escherichia coli* to Aerobic and Anaerobic Glucose Pulses

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ABSTRACT: The response of *Escherichia coli* cells to transient exposure (step increase) in substrate concentration and anaerobiosis leading to mixed-acid fermentation metabolism was studied in a two-compartment bioreactor system consisting of a stirred tank reactor (STR) connected to a mini-plug-flow reactor (PFR: BioScope, 3.5 mL volume). Such a system can mimic the situation often encountered in large-scale, fed-batch bioreactors. The STR represented the zones of a large-scale bioreactor that are far from the point of substrate addition and that can be considered as glucose limited, whereas the PFR simulated the region close to the point of substrate addition, where glucose concentration is much higher than in the rest of the bioreactor. In addition, oxygen-poor and glucose-rich regions can occur in large-scale bioreactors. The response of *E. coli* to these large-scale conditions was simulated by continuously pumping *E. coli* cells from a well stirred, glucose limited, aerated chemostat ($D = 0.1\ h^{-1}$) into the mini-PFR. A glucose pulse was added at the entrance of the PFR. In the PFR, a total of 11 samples were taken in a time frame of 92 s. In one case aerobicity in the PFR was maintained in order to evaluate the effects of glucose overflow independently of oxygen limitation. Accumulation of acetate and formate was detected after *E. coli* cells had been exposed for only 2 s to the glucose-rich (aerobic) region in the PFR. In the other case, the glucose pulse was also combined with anaerobiosis in the PFR. Glucose overflow combined with anaerobiosis caused the accumulation of formate, acetate, lactate, ethanol, and succinate, which were also detected as soon as 2 s after of exposure of *E. coli* cells to the glucose and O_2 gradients. This approach (STR-mini-PFR) is useful for a better understanding of the fast dynamic phenomena occurring in large-scale bioreactors and for the design of modified strains with an improved behavior under large-scale conditions.

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KEYWORDS: *Escherichia coli*; fermentation metabolism; aerobic-anaerobic metabolism; mixing; BioScope

Introduction

Imperfect mixing in large-scale bioreactors leads to the emergence of spatial gradients. In the case of large-scale fed-batch cultures, substrate gradients are commonly encountered (Enfors et al., 2001; Schmalzriedt et al., 2003; Lara et al., 2006c), and can cause significant effects in cell physiology (Lara et al., 2006c). Cells can travel from glucose-limited conditions in the bulk of the bioreactor, to zones of high glucose concentrations at the substrate feeding point. This in turn, may create an anaerobic region, as the respiration rate of the cells is importantly increased during exposure to glucose-concentrated regions (Enfors et al., 2001; Schmalzriedt et al., 2003; Lara et al., 2006c). This is particularly important in high-cell density cultures. The presence of glucose or dissolved oxygen gradients can have a negative impact on cells, decreasing their productivity. It has also been reported to be a main source of failure during the scale-up of cultures (Bylund et al., 1999, 2000). The

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accumulation of by-products like acetate and formate due to glucose/oxygen gradients is strongly undesirable as it represents a waste of carbon, triggers transcriptional regulation processes and causes loss of productivity.

The effects of environmental gradients and their exposure time on cells can be studied at the laboratory scale using scale-down experimental approaches (Lara et al., 2006c; Palomares et al., in press; Sweere et al., 1987). From the various experimental scale-down configurations, the most commonly used approach to simulate substrate gradients is a two-compartment system in which a stirred tank reactor (STR) is connected to a plug-flow reactor (PFR) (Lara et al., 2006c; Palomares et al., in press). Cells exiting the STR are passed through a PFR where a concentrated glucose solution is injected to simulate the substrate-feeding zone of a large-scale bioreactor. Oxygen depletion can also occur in the PFR if mass transfer is not enough to sustain aerobic conditions. Several reports exist on the study of glucose gradients and the concomitant oxygen limiting conditions in fed-batch cultures of *Escherichia coli* (Bylund et al., 1998; Lin and Neubauer, 2000; Xu et al., 1999). In such studies, the residence time in the PFR was 56 s and the first sample was taken 14 s after cells entered the PFR. A drawback from such studies is that the effects of glucose gradients are not completely decoupled from those of dissolved oxygen tension (DOT), which is of particular importance to better understand the effect of each variable on cell physiology. Moreover, only a limited analysis on cell responses can be derived from the studies mentioned above. Although Neubauer et al. (1995) measured the DOT at the exit of the PFR for an *E. coli* scale-down study, no DOT profiles were shown in the glucose-rich region and only four to five sample points over a period of 56 s were available in the PFR. In the present study, we employed the second generation BioScope (Mashego et al., 2006) as the PFR compartment to evaluate the dynamic response of fermentative metabolism of *E. coli* cells cultured aerobically and under glucose limitation, and subjected to a glucose gradient either under fully aerobic or fully anaerobic conditions. An important difference between the experimental settings of this work and the previously mentioned reports is that in the present study the cells have a steady-state background immediately prior to their exposure to the pulses. This enables the study of *E. coli* response to a single perturbation after a completely defined physiological state, in comparison to other studies in which it has been demonstrated that the age of a culture (for instance, during a fed-batch process) also influences the response of the cells to cyclic environmental oscillations (Enfors et al., 2001). Using the BioScope, 11 samples were taken during a relative short time frame, 92 s (of which 5 samples were taken during the first 20 s), to monitor the extracellular accumulation of mixed-acid fermentation metabolites during exposure to a glucose gradient. This time frame is wider than in other studies using rapid sampling devices (Buziol et al., 2002), but narrower than previous scale-down studies. This generated short time-resolved (in the range of seconds) useful biological information about the characteristic response times of fermentative metabolism during exposure to glucose and oxygen gradients. This information helps to explain the metabolic deviation at different scales of cultivation.

Materials and Methods

Bacterial Strain and Culture Medium

E. coli K-12 derivative W3110 (ATCC 27325) was used and cultured in a mineral medium containing the following components (in g/L): Glucose monohydrate, 7.5; $(NH_4)_2SO_4$, 5.0; KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.5; NaCl, 0.5; NH_4Cl, 2.0; Thiamine, 0.001. A trace elements solution (Verduyn et al., 1992) was added at a proportion of 2 mL/L of medium.

Chemostat Culture

E. coli was cultured in a glucose-limited chemostat at a dilution rate of 0.1 h^{-1} in a 7-L bioreactor with a working volume of 3-L (Applikon, Schiedam, the Netherlands) under aerobic conditions. The bioreactor was operated at an aeration rate of 0.5 vvm and an agitation rate of 500 rpm and overpressure of 0.3 bar. The pH and temperature were controlled at 7.0 and 37°C, respectively. DOT was measured (Mettler-Toledo Greifensee, Switzerland), but not controlled, only to verify that aerobic conditions were maintained at all times in the chemostat (>80% air saturation). The content of O_2 and CO_2 in the inlet and outlet gases was analyzed by an exhaust gas analyzer (NGA 200, Rosemount Analytics, Hasselroth, Germany).

Two-Compartment Bioreactor System

Simulation of Glucose Gradients

Exposure of cells to glucose gradients in large-scale bioreactors was simulated by means of a two-compartment system, shown in Figure 1, which consisted of a mini-PFR connected to a stirred tank bioreactor (glucose-limited chemostat). A broth flow of 1.8 mL/min was derived from the liquid effluent of the chemostat and connected to the PFR. At the entrance of the PFR, the broth was mixed with a concentrated glucose solution of 166.7 mM (30 g/L) flowing at 0.2 mL/min. After mixing with the broth, the concentration of glucose in the liquid entering the PFR was 16.7 mM (3 g/L). In this system, cells experienced a substrate gradient while moving from a glucose-limited (chemostat) to glucose-rich (PFR) regions.

The PFR consisted of a mini-bioreactor called Bioscope, designed for continuous perturbation experiments, and that has been described in detail previously (Mashego et al., 2006). Briefly, it consists of two hemispherical channels with a two-dimensional serpentine geometry, milled in a Perspex

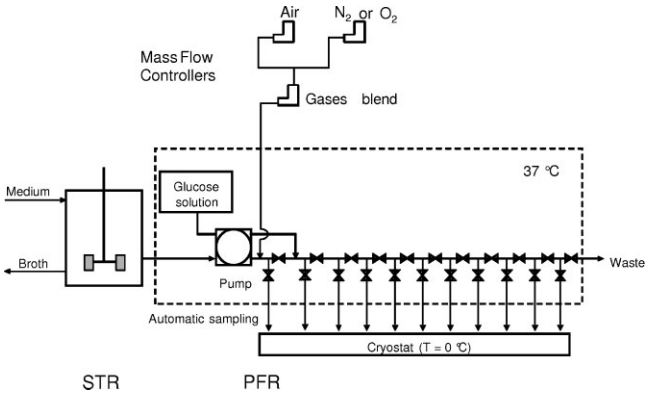

Figure 1. Simplified diagram of the experimental bioreactors and sampling system used to simulate spatial gradients of glucose and dissolved oxygen.

block, and separated by a silicone membrane permeable to O_2 and CO_2. One of the hemispherical channels was used for liquid flow, while the other was used for exchange of gases by countercurrent gas flow. The total volume of the PFR was 3.5 mL and the total length was 6.51 m. The mini-bioreactor was equipped with 11 ports for computer-controlled sampling at different distances from the inlet point, corresponding to increasing residence times of the cells in the PFR under high glucose concentrations. The first port allowed sampling shortly before the addition of the glucose-concentrated solution. The PFR was placed in a chamber at a controlled temperature of 37°C, equal to the STR temperature. Samples were taken from the PFR and instantaneously cooled in a cryostat kept at 0°C (Lauda RK 20 KS, Lauda-Königshofen, Germany). Shortly after sampling (which took around 15 min), the biomass was removed by filtering through 0.45 μm (Millex-HV, Millipore, Cork, Ireland) using pre-cooled syringes ($T = -20$°C). The supernatants were stored at −80°C for further analysis.

Simulation of Dissolved Oxygen Gradients

To study the effects of a sudden up-shift of the glucose concentration without oxygen limitation, the gas channel of the BioScope was continuously flushed with a blend of air/oxygen at 100 mL/min that was controlled with mass flow controllers (Brooks Instruments, Ede, The Netherlands). To assess the effects of an increase in glucose concentration under anaerobic conditions, the gas channel was flushed with pure nitrogen. In this case, a blend of air/nitrogen was sparged in the chemostat to lower its DOT to 15% (ca. 0.03 mmol/L) and thereby shorten the transition to fully anaerobic conditions after the glucose pulse. Such a DOT is considered high enough to maintain aerobic conditions in *E. coli* cultures (Lara et al., 2006a; Sandoval-Basurto et al., 2005). This was confirmed because the concentration of CO_2 in the exhaust gases and the biomass concentration in the broth did not change after the DOT decrease, which indicated that the steady state in the chemostat was not altered (data not shown). During the glucose pulse experiments, the DOT in the BioScope was measured using a single polarographic dissolved O_2 probe (Mettler-Toledo GmbH) mounted onto a flow cell that was consecutively switched to each of the 11 sampling ports. Glucose pulses under aerobic and anaerobic conditions were carried out twice. The data shown are an average of two values, and the differences between both values are also reported.

Analytical Techniques and Calculation Methods

Biomass concentration was determined as dry cell weight of washed pellet samples dried in Falcon tubes at 70°C for at least 24 h. Glucose, lactate, succinate, and formate were analyzed by enzymatic assays (R-Biopharm, Boehringer Mannheim, Darmstadt, Germany). Acetate and ethanol were quantified by gas chromatography (Chrompack CO 9001, Hewlett Packard, Palo Alto, CA) using a flame ionization detector. The reported values of yields and specific rates were calculated with the proper mass balances over the time periods involved.

Results

Characteristics of the Steady State

The chemostat was operated at a dilution rate of 0.1 h^{-1}. Steady state was confirmed by a constant composition of the off-gases and a stable biomass concentration (2.79 ± 0.03 g/L). The residual glucose concentration was 0.14 ± 0.01 mM. The biomass yield on glucose was 0.41 g/g at steady state. The specific uptake rates of glucose and oxygen (q_S and q_{O_2}) were 1.35 ± 0.04 and 3.78 ± 0.14 mmol/g h, respectively. The specific rate of carbon dioxide generation was 3.41 ± 0.10 mmol/g h. Small amounts of acetate and formate were detected in the effluent of the chemostat, namely, 0.08 ± 0.01 and 0.005 ± 0.002 mM, respectively. These values correspond to specific production rates of 0.003 and 0.0002 mmol/g h for acetate and formate, respectively.

Simulation of Oxygen Gradient in the Two-Compartment System

Figure 2 shows the DOT profiles in the PFR during aerobic and anaerobic pulses. It can be seen that the use of an air-oxygen blend in the PFR was enough to maintain aerobic conditions. The DOT in the chemostat was 0.25 mM. During the first 22 s of transit of the cells through the PFR, the DOT rapidly decreased to around 0.14 mM. This value was nearly constant during the remaining residence in the PFR. According to the mass transfer characteristics of the BioScope (Mashego et al., 2006), the q_{O_2} in the PFR was

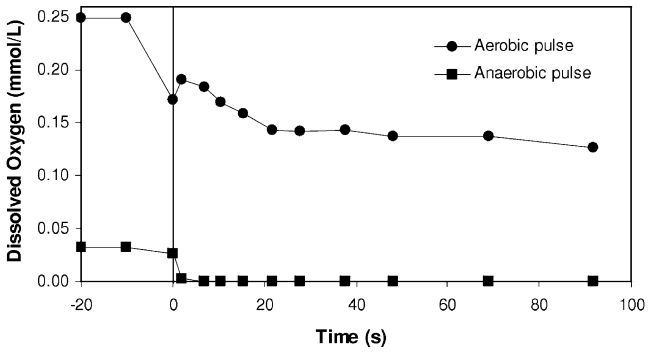

Figure 2. Dissolved oxygen concentration in the PFR during glucose pulses under fully aerobic or anaerobic conditions. The continuous vertical line indicates the samples taken previous to the perturbation of the glucose concentration.

16 mmol/g h, which means a fourfold increase in oxygen uptake rate. A reduced DOT level in the STR and flushing of nitrogen through the BioScope gas channel were enough to deplete oxygen almost at the entrance of the PFR during the glucose pulse under anaerobic conditions (Fig. 2).

Response of Mixed-Acid Fermentation Metabolism to an Aerobic Glucose Gradient

The extracellular concentrations of glucose and the organic acids produced during an aerobic glucose gradient are shown in Figure 3. The extracellular glucose concentration increased from 0.13 ± 0.01 mM in the chemostat, to 16.5 ± 0.6 mM in the PFR after the glucose pulse and then decreased to 15.45 ± 0.20 mM after 92 s of exposure of *E. coli* to the glucose gradient (Fig. 3A). Therefore, the calculated q_S value in the PFR was 15 ± 1 mmol/g h, which is a 10-fold increase. Compared to the fourfold increase in O_2-uptake this indicates fermentative metabolism. All the samples from the PFR were analyzed for organic acids, but only formate and acetate were detected (Fig. 3). As shown in Figure 3B and C, both metabolites accumulated extracellularly already within 2 s after the glucose perturbation. During the first 10 s after the glucose pulse (phase I), acetate and formate were produced more rapidly than during the remaining 82 s of the experiment (phase II). The acetate concentration in the chemostat was 0.08 ± 0.01 mM and increased to 0.14 ± 0.01 mM during phase I in the PFR (i.e., 0.060 ± 0.001 mM of acetate were produced). During phase II, acetate concentration reached 0.17 ± 0.01 mM (i.e., 0.030 ± 0.002 mM of acetate were produced) (see Fig. 3B).

Formate was also found in very small concentrations in the chemostat (0.005 mM). A very fast accumulation of formate was observed during phase I in the PFR, reaching values of 0.066 ± 0.005 mM. At the end of phase II, formate concentration was 0.076 ± 0.004 mM (Fig. 3C).

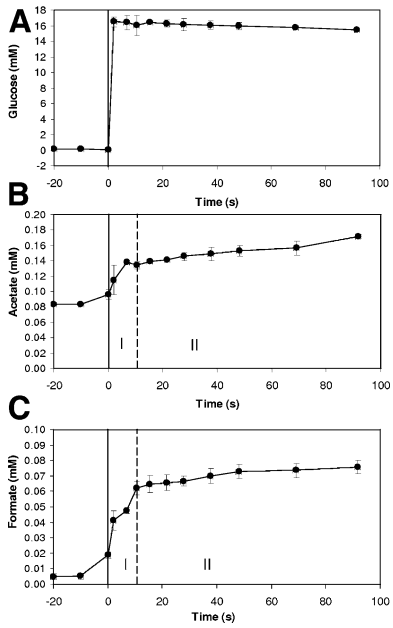

Figure 3. Extracellular concentration of glucose (**A**), acetate (**B**) and formate (**C**) in the PFR during exposure of *E. coli* cells to a glucose gradient under aerobic conditions. Error bars show the difference between duplicate experiments. The continuous vertical line indicates the samples taken previous to the perturbation. The dashed vertical line indicates the division in two distinctive phases of the microbial response to glucose/O_2 perturbations.

Response of Mixed-Acid Fermentation Metabolism to an Anaerobic Glucose Gradient

In order to simulate the combined effects of moving from an aerobic, glucose-limited region to a glucose-rich, anaerobic zone in a large-scale bioreactor, anaerobic conditions in the PFR were achieved as described previously. In contrast with the glucose pulses under aerobic conditions, the glucose excess under anaerobiosis caused the accumulation of all mixed-acid fermentation metabolites. The extracellular profiles of glucose and organic acids during this pulse experiment are shown in Figure 4. The product accumulation was much higher and no evident phases of their production during the glucose pulse were observed.

The glucose concentration increased from 0.14 ± 0.01 mM in the chemostat to 16.84 ± 0.36 mM in the PFR after the pulse to reach 14.63 ± 0.75 mM (Fig. 4A) in the last sampling port. Therefore, 2.22 ± 0.84 mM of glucose was consumed during the perturbation experiments, which is more than twofold the amount of glucose consumed during a glucose pulse under aerobic conditions. Therefore, the q_S value was 31 ± 3 mmol/g h, which is a more than 20-fold increase compared to the steady state. Formate and acetate were the most highly accumulated organic acids in response to a glucose gradient under anaerobic conditions, reaching values of 0.38 ± 0.02 and 0.43 ± 0.02 mM in the last sample point of the PFR (Fig. 4B and C). Ethanol was the third most highly accumulated metabolite, followed by lactate and

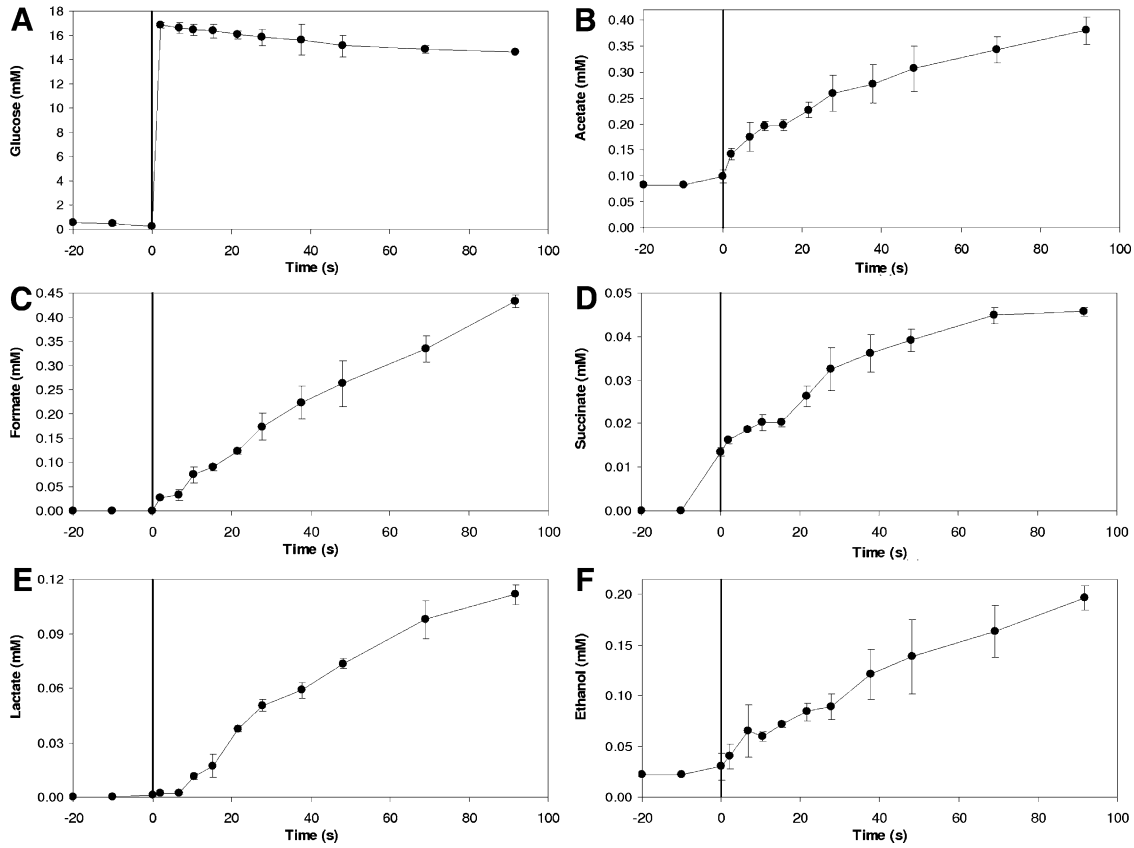

Figure 4. Extracellular concentration of glucose (**A**), acetate (**B**), formate (**C**), succinate (**D**), lactate (**E**), and ethanol (**F**) in the PFR during exposure of *E. coli* cells to a glucose gradient under anaerobic conditions. Error bars show the difference between duplicate experiments. The continuous vertical line indicates the samples taken previous to the perturbation.

succinate, whose maximum concentrations during the perturbation experiment were 0.20 ± 0.01, 0.11 ± 0.01, and 0.05 ± 0.01 mM (Fig. 4C–F), respectively. The total products amounted to about 1 mM.

Discussion

The existence of local zones with elevated glucose concentrations in large-scale bioreactors is often combined with oxygen depletion (Lara et al., 2006c). However, the existence of DOT gradients does not depend on the presence of substrate gradients, as DOT fluctuations can also occur in batch cultures (Lara et al., 2006a). Depending on the operational conditions, DOT gradients can also exist in small-scale fermentors, as shown by Garcia et al. (2009), who used a fluorescence reporter in *E. coli* to detect oxygen limitations in a STR lab-scale bioreactor. The effects of DOT gradients on mammalian (Serrato et al., 2004) and bacterial (Lara et al., 2006a,b; Sandoval-Basurto et al., 2005) cultures have been studied and are known to have a profound influence in the performance of cultures. Despite the relatively well-studied response of cultures to glucose gradients, the scale-down studies published to date have not effectively dissected the effects of glucose gradients from anaerobiosis. Moreover, in such studies, the first sample was typically taken after 14 s of exposure to regions with high glucose concentration (Bylund et al., 1998; Enfors et al., 2001; Xu et al., 1999) and at 28, 36, and 52 s of residence time in the PFR. The typical scale-down system to evaluate the effects of substrate gradients is the STR-PFR configuration (Lara et al., 2006c), in which a glucose pulse is added at the entrance of the PFR. The sudden increase in glucose uptake after a glucose pulse in scale-down studies (for instance, from 2.8 in the STR to 8.9 mmol/g h after 40.5 s in the PFR; Xu et al., 1999) can cause a rapid depletion of oxygen, and hence oxygen limitation has been assumed to occur in the PFR section. However, no direct measurement of oxygen depletion in the PFR section of the scale-down systems has been documented previously, making it difficult to draw conclusions about the direct effect on cells upon exposure to a substrate or oxygen gradient. In the present study, the effects of glucose gradients and anaerobiosis were dissected by employing a STR connected to a mini-PFR in which the concentration of dissolved oxygen can be measured and manipulated. The STR was operated as chemostat in a

steady-state mode at a dilution rate of 0.1 h^{-1}, which was similar to the feeding rates found in typical *E. coli* cultures (Lara et al., 2008). The characteristics of the BioScope allowed the effective exchange of gasses with the culture broth, and fully aerobic or anaerobic conditions were achieved and monitored (Fig. 2).

When *E. coli* cells were exposed to a glucose gradient under fully aerobic conditions, the strongest effect was the increase in q_S and q_{O_2} and the immediate accumulation of acetate (Fig. 3B). A comparison of the specific glucose uptake rates (q_S) in the chemostat and during pulse experiments is shown in Table I. The value of q_S increased from 1.35 mmol/g h in the chemostat to 15.3 mmol/g h in the PFR following the aerobic glucose pulse, which is an increase of about 11-fold. The latter value of q_S can sustain a specific growth rate higher than 1 h^{-1} (after calculations presented by Carlson and Srienc, 2004), and is well above the critical value for triggering overflow metabolism. For instance, it has been reported (Kayser et al., 2005) that acetate production by *E. coli* in chemostat cultures started when q_S reached a value of 0.64 g/g h (ca. 10.7 mmol/g h). Figure 5A shows a comparison of the specific production rates of organic acids in pulse experiments under aerobic conditions, during phases I and II. The specific production rate of acetate in the chemostat was 0.003 mmol/g h, and it increased to 4.71 ± 0.30 mmol/g h during the first phase of the pulse experiment. During phase II, it decreased to 0.53 ± 0.01 mmol/g h. Acetate is mainly produced in *E. coli* by action of the phosphotransacetylase (PTA) and acetate kinase (ACK) enzymes. Both are constitutive enzymes in *E. coli* (Clark, 1989; Wolfe, 2005), and our results show that this pathway is immediately inducible by glucose overflow. The enzyme pyruvate oxidase (PoxB) converts pyruvate into acetate and also plays an important role in acetate production by *E. coli* W3110, both aerobically (Phue et al., 2005) and under oscillating DOT conditions (Lara et al., 2006a). Therefore, it is possible that PoxB is also present in *E. coli* under aerobic conditions and rapidly induced by a glucose pulse.

Formate also accumulated rapidly (as early as 2 s after the glucose pulse) during the exposure of *E. coli* culture to the aerobic glucose gradient (Fig. 3C). Formate production in *E. coli* is expected under oxygen-limited conditions, as part of the fermentative metabolism (Clark, 1989), but not under aerobic conditions, as in this experiment. The specific production rate of formate in the chemostat was 0.0002 mmol/g h. Other authors have also reported the production of

Table I. Specific glucose (q_S) and oxygen (q_{O_2}) uptake rate during exposure of *E. coli* cells to a glucose gradient under fully aerobic and anaerobic conditions.

Condition	q_S (mmol/g h)	q_{O_2} (mmol/g h)
Chemostat	1.35	3.78
Aerobic pulse	15 ± 1	16
Anaerobic pulse	31 ± 3	—

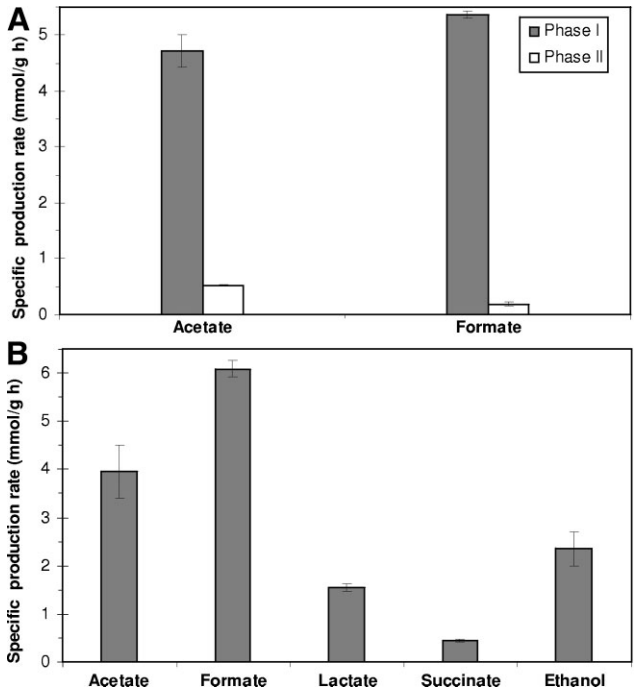

Figure 5. Specific production rates of the mixed-acid fermentation products in response to a glucose gradient under fully aerobic (**A**) or anaerobic (**B**) conditions. Error bars show the difference between duplicate experiments. Specific acetate and formate production rates in the chemostat were 0.003 and 0.0002 mmol/g h.

formate by *E. coli* in fully aerobic cultures (Castan and Enfors, 2002; Lara et al., 2006a,b; Sandoval-Basurto et al., 2005). The exact mechanism for this phenomenon is not yet known, but authors have related formate accumulation to DNA release into the media, probably due to cell rupture (Castan and Enfors, 2002). It should be noted that the *pfl* gene, that codes for the enzyme responsible for formate production from pyruvate (pyruvate–formate lyase, PFL), is reported to be expressed only under anaerobic conditions. The regulation of PFL is rather complex, however, it is known that there exists a basal level of PFL in *E. coli* even under aerobic conditions (Pecher et al., 1982). Our results show that formate accumulates in response to glucose overflow under fully aerobic conditions and imply that there is a basal level of PFL in the *E. coli* strain used under aerobic conditions. This might occur due to a similar mechanism of overflow metabolism which leads to acetate production. The sudden increase in glucose uptake could have caused a transient accumulation of intracellular pyruvate, which in turn could have led to formate by PFL. Formate accumulation during the aerobic glucose pulse can also be divided into two phases. During the first phase, it accumulated at a rate of 5.36 ± 0.06 mmol/g h, but then the specific production rate rapidly decreased during phase II to a value of 0.19 ± 0.03 mmol/g h. Formate production in the aerobic conditions of the experiment must have been originated from the basal PFL present in

cells. This indicates that PFL must be present at relatively high amounts, and that it is very sensitive to glucose overflow, as formate was produced at similar rates as acetate during phase I of the pulse experiment (Fig. 5A). Formate can be converted to CO_2 and H_2 by the formate hydrogenlyase (FHL) enzyme complex (Clark, 1989), which is formed of four enzymes (Sawers, 1994). Selenium acts directly as cofactor of the FHL complex (Axley et al., 1991; Gladyshev et al., 1994). It has been demonstrated that a lack or inadequate amount of this metal in the culture medium has conducted to formate accumulation, whereas the addition of this cofactor effectively prevented formate accumulation (Sandoval-Basurto et al., 2005; Soini et al., 2008).

As summarized in Table I, the q_S value increased 11 times, whereas the oxygen uptake rate increased fourfold from 3.78 in the STR to 15.6 mmol/g h in the PFR during the aerobic glucose pulse. This latter value corresponds to the maximum oxygen uptake rate for the W3110 strain, according to data compiled elsewhere (Carlson and Srienc, 2004). During exposure to the glucose gradient under anaerobic conditions all the mixed-acid fermentation final products were detected. Formate and acetate accumulated to the highest concentrations. The specific production rates of formate and acetate were 6.09 ± 0.18 and 3.95 ± 0.55 mmol/g h, respectively (Fig. 5B). Glucose overflow, combined with the step-change to anaerobic conditions enhanced the production of formate and acetate, as their specific production rates were much higher than under aerobic conditions (Fig. 5A and B). This is in agreement with the higher glucose uptake rate during anaerobic glucose pulses, which was almost threefold higher than during aerobic pulses (Table I). It is well known that *E. coli* consumes glucose at higher rates under anaerobic than under aerobic conditions. This is due to the fact that under anaerobic conditions, the bulk of energy is obtained from the glycolysis (Gennis and Stewart, 1996). Furthermore, formate and lactate are directly produced from pyruvate, whereas acetate can be produced also from pyruvate under anaerobic conditions by PoxB (Clark, 1989; Lara et al., 2006a). Also, an increased demand for pyruvate may increase the glucose uptake rate, since one mole of phosphoenol pyruvate (PEP) is converted to pyruvate per mole of glucose that is internalized through the phosphotransferase system in *E. coli* (De Anda et al., 2006). The value of q_S for the anaerobic glucose pulse is well within the values reported by Carlson and Srienc (2004). Ethanol and lactate also accumulated rapidly in the PFR during the anaerobic glucose pulse at rates of 2.35 ± 0.36 and 1.56 ± 0.08 mmol/g h, respectively (Fig. 5B). Succinate was produced at a relatively low specific rate of 0.45 ± 0.03 mmol/g h.

The residence time of cells in the PFR (92 s) can be considered insufficient for completion of induction, transcription, translation, and activation events necessary for enzyme synthesis. Altogether, these processes can take place in a time frame of several minutes (Stephanopoulos et al., 1998). During the exposure of fully aerobically, glucose-limited cultivated *E. coli* cells to a glucose gradient under anaerobic conditions, all the mixed-acids fermentation metabolites were immediately detected (in <5 s). This means that organic acids were produced by enzymes already present in the cells even under fully aerobic conditions, as was the situation prevailing in the chemostat. Ethanol was also detected early after the glucose pulse in the PFR. The *adhE* gene, coding for the fermentative ADH is known to be induced only under anaerobic conditions (Clark, 1989). However, our results suggest that at least a low basal level of ADH should be present in *E. coli* even under fully aerobic conditions. The enzyme responsible for lactate synthesis, the lactate dehydrogenase (LDH) is known to be present in *E. coli* under both, aerobic and anaerobic conditions (Mat-Jan et al., 1989). Induction of LDH under aerobic conditions requires acidification of the medium. It is possible that during the glucose pulse under aerobic conditions, the amounts of acetate and formate produced did not significantly decrease the pH of the culture and in consequence, LDH was not induced and lactate was not detected.

During the 92 s exposure of *E. coli* cells to a glucose gradient under aerobic conditions in the PFR, 1.06 mM of glucose were consumed (6.38 CmM), whereas 0.08 mM of acetate (0.15 CmM) and 0.06 mM of formate (0.06 CmM) were produced. This means that 3.29% of the consumed carbon was wasted as fermentation by-products due to the glucose gradient. In contrast, when the glucose gradient was combined with anaerobiosis, 2.22 mM of glucose (13.29 CmM) were consumed in the PFR. In this case, 0.43 mM of formate (0.43 CmM), 0.28 mM of acetate (0.56 CmM), 0.16 mM of ethanol (0.33 CmM), 0.11 mM of lactate (0.33 CmM), and 0.03 mM of succinate (0.13 CmM) were produced. Overall, this means that 13.4% of the carbon consumed was diverted to fermentation products during simultaneous oxygen depletion and glucose excess. The consequences of these effects in large-scale cultures where cells are continuously encountering glucose and dissolved oxygen gradients are obvious. It has been estimated that the region of oxygen limitation resulting from a high glucose concentration field in a large-scale, fed-batch bioreactor is of $\sim$10% of the total working volume (Enfors et al., 2001). If we assume a mean circulation time of 50 s as is commonly found in large-scale microbial cultures (Lara et al., 2006c), then it is reasonable to establish that cells will spend at least 10% of the circulation time (i.e., 5 s) in an oxygen-depleted, glucose-rich region. The experimental set-up used in this work, and the design of the PFR allowed us to observe that overflow metabolism due to a glucose gradient under fully aerobic conditions resulted in acetate accumulation as soon as 2 s after exposure to high glucose concentrations. Importantly, formate also accumulated within 2 s. When the glucose gradient was combined with anaerobiosis, all the other fermentation metabolites were also detected after 2 s of exposure of cells to the gradients. This means that even if the circulation time in a large-scale bioreactor could be decreased to 20 s (which correspond to a mixing time of around 80 s; Lara et al., 2006c), the presence of glucose (and probably dissolved oxygen) gradients will

still cause diversion of carbon fluxes towards fermentative products, and a concomitant waste of the substrate. The accumulated fermentative products also will trigger transcriptional responses. Moreover, the local accumulation of organic acids can also lead to a local drop of pH, which can also negatively affects the performance of *E. coli* cultures (Amanullah et al., 2001).

If practical constraints do not allow improving mixing at the large scale, the use of cellular engineering strategies can form an alternative solution to this problem. For instance, *E. coli* with a modified substrate transport system with a much lower uptake rate has been successfully cultured under very high glucose concentrations, displaying a minimal overflow metabolism (Lara et al., 2008). Due to slower glucose uptake rate, compared to wild-type strains, such modified strains are expected to perform better under glucose gradients, as it can be expected that the high glucose concentration lead to less increase in their glucose transport rate (De Anda et al., 2006; Lara et al., 2008).

Another approach to overcome the effects of glucose gradients under aerobic or anaerobic conditions using cellular engineering is to block the undesired pathways that might not be essential for the cells due to the temporal exposure to changes in environmental conditions. Lara et al. (2006b), inactivated the genes responsible for the production of formate, lactate and acetate in *E. coli*. The modified strain showed a much better growth, recombinant protein production, and less by-products accumulation when cultured under DOT oscillations. Our CSTR/PFR set-up is well suited to evaluate such engineered strains on small scale before their application on large scale.

Conclusions

In the present study, the rapid (<2 s) dynamic response of fermentative metabolism of *E. coli* exposed to a glucose gradient under aerobic and anaerobic conditions was analyzed. The use of a special mini-PFR (BioScope) allowed the sampling in shorter time frame than had been possible in previous studies. In addition, the effect of glucose gradients was successfully dissected from dissolved oxygen depletion. It was found that the fully aerobic transit from a glucose limiting to a glucose-rich region triggered the overflow metabolism very rapidly, and acetate and formate were detected as early as 2 s after exposure to the glucose-rich condition. When the glucose gradient was combined with anaerobiosis, many other mixed-acid fermentation metabolites were detected after 2 s of exposure to the gradients and their production rates were higher. Under aerobic conditions, the accumulation of by-products was faster during the first 10 s of residence in the glucose-rich region, which demonstrated that even very short exposures of cells to gradients in large-scale bioreactors can have a profound effect in the fermentation pathways and thus a detrimental outcome to process performance.

The study of cellular response to substrate gradients, in fully aerobic and fully anaerobic conditions, during short time frames is relevant, since it can help to better understand the metabolic effects of industrial fermentation scale-based broth heterogeneity and to establish leads for genetic interventions in order to design strains that show a better performance in large-scale cultures. Future studies on the transcriptional profiling of cell response to these environmental stresses in short time frames will be important to further understand the physiological effects in large-scale bioreactors. Finally, the combination of CSTR/PFR (BioScope) was shown to be successful in controlled application of glucose/(an)aerobic condition and is envisaged to be suitable for testing of engineered strains.

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