Transcriptional and Metabolic Response of Recombinant *Escherichia coli* to Spatial Dissolved Oxygen Tension Gradients Simulated in a Scale-Down System

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Abstract: *Escherichia coli*, expressing recombinant green fluorescent protein (GFP), was subjected to dissolved oxygen tension (DOT) oscillations in a two-compartment system for simulating gradients that can occur in large-scale bioreactors. Cells were continuously circulated between the anaerobic (0% DOT) and aerobic (10% DOT) vessels of the scale-down system to mimic an overall circulation time of 50 s, and a mean residence time in the anaerobic and aerobic compartments of 33 and 17 s, respectively. Transcription levels of mixed acid fermentation genes (*ldhA*, *poxB*, *frdD*, *ackA*, *adhE*, *pflD*, and *fdhF*), measured by quantitative RT-PCR, increased between 1.5- to over 6-fold under oscillatory DOT compared to aerobic cultures (constant 10% DOT). In addition, the transcription level of *fumB* increased whereas it decreased for *sucA* and *sucB*, suggesting that the tricarboxylic acid cycle was functioning as two open branches. Gene transcription levels revealed that cytrochrome bd, which has higher affinity to oxygen but lower energy efficiency, was preferred over cytochrome bO_3 in oscillatory DOT cultures. Post-transcriptional processing limited heterologous protein production in the scale-down system, as inferred from similar *gfp* transcription but 19% lower GFP concentration compared to aerobic cultures. Simulated DOT gradients also affected the transcription of genes of the glyoxylate shunt (*aceA*), of global regulators of aerobic and anaerobic metabolism (*fnr*, *arcA*, and *arcB*), and other relevant genes (*luxS*, *sodA*, *fumA*, and *sdhB*). Transcriptional changes explained the observed alterations in overall stoichiometric and kinetic parameters, and production of ethanol and organic acids. Differences in transcription levels between aerobic and anaerobic compartments were also observed, indicating that *E. coli* can respond very fast to intermittent DOT conditions. The transcriptional responses of *E. coli* to DOT gradients reported here are useful for establishing rational scale-up criteria and strain design strategies for improved culture performance at large scales.

Keywords: recombinant *E. coli*; scale-down; aerobic and anaerobic metabolism; transcriptomics; circulation time; dissolved oxygen gradients; scale-up; scale-down

INTRODUCTION

The design and operation of large-scale fermenters is constrained by practical issues that frequently lead to mixing problems and the concomitant appearance of spatial gradients in culture parameters (Amanullah et al., 2004; Davidson et al., 2003; Palomares and Ramírez, 2000; Schmalzriedt et al., 2003). Under such conditions, cells are continuously exposed to an oscillating environment that can affect their metabolic and physiologic behavior. This explains the different performance generally observed between large-scale cultures and laboratory studies conducted under well-controlled conditions (Bylund et al., 1998, 1999, 2000; Hewitt et al., 2000; Namdev et al., 1993; Onyeaka et al., 2003). The effects of environmental fluctuations on cells can be investigated in scale-down systems as originally proposed by Oosterhuis (1984). Using such approach, the effects of pH, dissolved oxygen tension (DOT), and substrate gradients have been studied on a variety of organisms ranging from bacteria (Amanullah et al., 2001; Bylund et al., 1999; De León et al., 1995; Hewitt et al., 2000; Namdev et al., 1993; Oosterhuis et al., 1985; Sandoval-Basurto et al., 2005; Trujillo-Roldán et al., 2001) and yeast (Cortés et al., 2005; George et al., 1993), to mammalian cells (Osman et al., 2002; Serrato et al., 2004). The study of cell physiology under such fluctuating conditions has been

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regarded as an important challenge for the biochemical engineering science (Larsson et al., 1997).

The presence of DOT gradients, a prevalent problem in large-scale fermenters, can be predicted through regime analysis (Oosterhuis and Kossen, 1984; Sweere et al., 1987). DOT gradients are likely to occur when the characteristic time constant for oxygen consumption (t_{OC}) is lower or within the same order of magnitude than the characteristic circulation time (t_c) (Amanullah et al., 2004; Oosterhuis, 1984; Palomares and Ramírez, 2000; Reuss and Bajpai, 1991; Sweere et al., 1987). The range of mean t_c for a variety of large-scale fermenters, when operated with low-viscosity Newtonian fluids and under conditions typical of *E. coli* cultures is between 3 and at least 60 s (Amanullah et al., 2004; Schügerl, 1993; Sweere et al., 1987; Vrábel et al., 2000). In turn, maximum specific oxygen consumption rates (q_{o_2}) for *E. coli*, either recombinant or wild type are between 5 and 20 mmol/g-h. (Alexeeva et al., 2000; Carlson and Srienc, 2004; Vemuri et al., 2005). Therefore, as long as glucose remains above its critical concentration and even at low or intermediate biomass concentrations (below 20 g/L), there exist a plentitude of realistic conditions where t_{oc} (ratio DOT to oxygen uptake rate) is equal or lower than t_c. Accordingly, DOT gradients can occur in batch cultures of *E. coli* at large scales. A similar regime analysis indicates that the gradients that may occur globally during batch cultures can also occur locally in the feeding zone of fed-batch cultures. That is, t_{OC} in the feeding zone will be lower than t_c as respiration will be at its maximum due to the local high glucose concentration. In contrast, t_{OC} will be higher than t_c throughout the remaining sections of the bioreactor as glucose concentration in the bulk of a fed-batch is close to zero and thus q_{o_2} will also be very low. Although not available for batch *E. coli* cultures, there exist empirical evidence (Griot et al., 1988; Lübbert and Jørgensen, 2001; Oosterhuis and Kossen, 1984) and predictions by computational fluid dynamic analysis (Lübbert and Jørgensen, 2001; Reuss et al., 1994, 2000; Singh et al., 1987) of the presence of DOT gradients for other microbial batch cultures with respiratory and rheological characteristics similar to *E. coli* at low or intermediate cell concentrations.

In spite of its importance, the problem of DOT gradients has not been studied at a sufficient depth for recombinant *E. coli* cultures (Bylund et al., 1999, 2000; De León et al., 1995; Lin and Neubauer, 2000; Namdev et al., 1993; Sandoval-Basurto et al., 2005). As a facultative anaerobe, *E. coli* is able to adapt to limiting DOT conditions, although at the expense of biomass and product yields. Sandoval-Basurto et al. (2005) recently determined the effects of DOT gradients on a recombinant *E. coli* producing pre-proinsulin. Cells were circulated between the aerobic and anaerobic compartments of a scale-down system for simulating circulation times between 0 and 180 s. They showed that transient exposure to anoxic conditions as short as 13.3 s had a detrimental effect on maximum heterologous protein concentration and yield, and was enough to deviate the carbon metabolism to anaerobic pathways as evidenced by the accumulation of undesirable waste products from mixed-acid fermentation.

Most scale-down studies have evaluated the effects of environmental gradients on macroscopic parameters, such as yields, productivities, and by-product accumulation. However, only very scarce reports have determined the effects of gradients at the molecular level. Among such few exceptions are the studies of DOT oscillations in one-compartment scale-down systems for determining the effect on the glycosylation pattern of a monoclonal antibody produced by hybridomas (Serrato et al., 2004) and on the molecular weight distribution of alginate produced by *Azotobacter vinelandii* (Trujillo-Roldán et al., 2001). Gene expression is a molecular event particularly relevant, as it is among the first cellular responses to stress. Such response can have important repercussions in the performance of the bioprocess but may be difficult to analyze at the protein level (Enfors et al., 2001). Nonetheless, to our knowledge only two studies have reported gene transcription measurements under oscillating environmental conditions. Namely, Schweder et al. (1999) and Enfors et al. (2001) analyzed by slot-blotting the relative transcription levels of four stress genes and three genes responding to intermittent oxygen limitation or glucose excess in *E. coli*. Cells were cultured in a 20 m^3 industrial bioreactor and in a scale-down system consisting of a stirred tank reactor connected to a plug-flow reactor. They found that an exposure time to oxygen limitations as short as 13 s was enough to increase twofold the transcription levels of the anaerobic genes *pfl* and *frd* in the scale-down system. Furthermore, the levels of *pfl* mRNA from the middle and the top sections of the large-scale bioreactor were more than twofold higher than at the bottom. Such results demonstrated that a transcriptional induction within seconds can occur in *E. coli* as a response to process-related stresses typical of large-scale cultures.

Here we present the relative transcription levels, measured by quantitative RT-PCR, of 21 genes of a recombinant *E. coli* strain subjected to DOT gradients simulated in a two-compartment bioreactor system. Genes were selected from metabolic pathways that directly respond to oxygen availability and that affect bioprocess performance, including TCA cycle, glyoxylate bypass, and mixed-acid fermentation. In addition, genes encoding an anaerobic superoxide dismutase, the most important cytochromes, global regulators of aerobic and anaerobic metabolism, a quorum-signaling synthase, and the heterologous product were analyzed. To our knowledge, this is the first report that incorporates in a single study information on by-product formation, heterologous protein production, and transcriptional response of recombinant *E. coli* to highly dynamic, oscillating DOT conditions. The results obtained are useful for understanding the molecular response of *E. coli* to heterogeneous environments commonly found in large-scale bioreactors. Such information is important for improving bioreactor design and scale-up procedures as well as for constructing robust cell strains that can contend with adverse industrial culture conditions.

MATERIALS AND METHODS

Bacterial Strain, Culture Medium, and Inoculum Preparation

Escherichia coli W3110 (ATCC 27325), transformed with the pV21 plasmid, was used in all fermentations of this study. Construction of the pV21 plasmid was based on plasmid pBCSK+ from Stratagene (La Jolla, CA). It contains a spectinomycin resistance gene and the green fluorescence protein (GFP) gene (*gfp*) inserted between *Nde*I and *Xho*I restriction sites. The heterologous gene was placed under control of the *lacZ* gene promoter. Some *gfp* codons were modified to improve codon usage and to yield a brighter and cytoplasm-soluble GFP variant. (plasmid and *gfp* gene were kindly provided by Dr. J. Osuna and V. Escobar, IBt, UNAM). Cultures were performed in a glucose mineral medium as described by Hewitt et al. (2000) except that spectinomycin was supplemented at 100 mg/L. Medium components together with deionized water were sterilized at 121°C for 25 min. Sterilized magnesium sulphate, trace elements, glucose solution, thiamine, and spectinomycin were added separately after sterilization. Inoculum was prepared by transferring to mineral media 1 mL of cryo-preserved culture on Luria liquid medium and glycerol (40%). Cells were grown in shake flasks to an optical density at 600 nm (OD_{600}) of 3.0–4.0, centrifuged and then resuspended in fresh mineral medium at 4°C for eliminating possible organic acids produced during inoculum propagation. An inoculum volume of 10% of the total culture volume was transferred to either the control bioreactor or to the scale-down bioreactor system. For cultivation in bioreactors, a sterile solution of IPTG was added to a final concentration of 0.5 mM prior to inoculation. All cultures were maintained at 37°C.

Two Compartment Scale-Down Bioreactor System

The oscillating DOT fermentations were performed in a scale-down bioreactor system described previously by Sandoval-Basurto et al. (2005). Briefly, the system consisted of two interconnected stirred tank bioreactors (Virtis, Gardiner, NY), one maintained at a constant DOT of 10% ($B_{aerobic}$) and the other at 0% DOT ($B_{anaerobic}$) to mimic the aerobic and anaerobic regions, respectively, of a typical large-scale bioreactor. Culture broth was continuously pumped between both vessels at an overall circulation time (t_c, calculated as total volume divided by flow rate) of 50 s. Such a t_c is within the range of those typically found in large-scale bioreactors operated under typical conditions for *E. coli* cultures (Palomares and Ramírez, 2000; Sandoval-Basurto et al., 2005). For instance, mean t_c in the range of 3–60 s have been determined for 12 and 30 m^3 non-aerated stirred tank vessels agitated with Rushton or Scaba turbines (Vrábel et al., 2000). It should be noted that, in general, longer t_c may result in aerated systems (Amanullah et al., 2004). Circulation times in the range of 14–46 s have been reported for 0.01–65 m^3 airlift tower loop bioreactors (Schügerl, 1993; Sweere et al., 1987), whereas even higher values exist for other bioreactor configurations and operation modes. For instance, mean t_c in the range of 600–1,200 s have been reported for immobilized bacterial cells in fluidized-bed reactors (Sweere et al., 1987). Furthermore, a distribution of circulation times, rather than a single mean value exists in any vessel, where maximum t_c can be as high as four times the mean value (Amanullah et al., 2004). The selection of t_c in our study was based on previous results by Sandoval-Basurto et al. (2005), and considered a value small enough to mimic an actual situation but large enough to facilitate experimental handling. It should be noted that Sandoval-Basurto et al. (2005) detected that carbon flow was diverted to anaerobic pathways for t_c as low as 20 s. Thus, the results shown here at a t_c of 50 s, although amplified, should also reflect the condition obtained at lower t_c.

The compartment volumes of the scale-down system were established from a regime analysis presented by Amanullah et al. (2004). Using the cumulative circulation time distribution and an estimate of the characteristic time constant for oxygen consumption (t_{oc}) for *B. subtilis*, Amanullah et al. (2004) determined the percentage of cells that may be subjected to conditions of oxygen starvation during batch fermentations at low cell densities. Considering a biomass concentration of 3 g/L and a maximum specific oxygen uptake rate of 17 mmol/g-h, they estimated that 66% and 88% of the cells would be exposed to oxygen-depleted conditions for mean t_c of 20 and 40 s, respectively. Such estimation can be applied as well for *E. coli* cultures, as this bacterium has a similar respiration rate when growing at specific rates between 0.2 and 0.4/h (Alexeeva et al., 2000; Carlson and Srienc, 2004; Vemuri et al., 2005). Accordingly, the working volume of the bioreactors in the scale-down system was set to 0.8 L for $B_{anaerobic}$ and 0.4 L for $B_{aerobic}$. This resulted in an anaerobic to aerobic volume ratio of 2:1, which adequately represents the well to poorly aerated volume ratios of actual large-scale stirred tank conditions (Amanullah et al., 1992, 2004; Oosterhuis, 1984, 1985), so maintaining 66% of the cells under oxygen-depleted conditions. Such percentage is also consistent with the calculated fraction of yeast cells under oxygen limitation in a bubble column reactor (Feijen et al., 1994). DOT of $B_{aerobic}$ was automatically controlled by a proportional-integral-derivative algorithm (PID), whereas $B_{anaerobic}$ was kept at 0% DOT by sparging pure nitrogen through a stainless steel porous diffuser. All DOT values are expressed as percentage of saturation with respect to air. pH was measured and controlled at 7.1 by automatic addition of 2 M NaOH in $B_{aerobic}$, as no differences between the two compartments have been reported at the circulation time used here (Sandoval-Basurto et al., 2005). Some modifications were made to the original circulating loop section of the system. Namely, fermentation broth was circulated with two peristaltic pumps (Masterflex, Cole Palmer) through Tygon® tubing to minimize gas exchange with the atmosphere. The experimental conditions used in this study resulted in mean

cell residence times of 33 and 17 s in the anoxic and aerobic compartments, respectively.

Two different types of control cultures at a constant DOT of 10% were performed. In the first, only the aerobic section of the scale-down system was employed, whereas in the second the two compartments were used at the same DOT and circulating medium between them at a t_c of 50 s. The former controls were used for characterizing the kinetic and stoichiometric (growth rate, glucose consumption, GFP, and by-products production) behavior during fully aerobic conditions. The latter controls allowed the evaluation of possible effects on the cells resulting from mechanical stress generated at the pump heads and circulation loop.

Experimental Approach

Samples for comparing gene transcription levels were obtained from *E. coli* cultures in the scale-down bioreactor system. Cultures were initiated with medium circulation and maintaining a DOT of 10% in the two compartments ($B_{aerobic}$ and $B_{anaerobic}$) until an OD_{600} of 1.0 was reached. After sampling, the DOT in $B_{anaerobic}$ was changed step-wise to 0% while maintaining $B_{aerobic}$ at 10% DOT. The culture was then operated under oscillatory DOT conditions for ca. 2 h until a complete cell doubling was obtained (OD_{600} of 2.0). At this moment, two samples were taken simultaneously, one from $B_{aerobic}$ and another from $B_{anaerobic}$. Such a strategy was followed for two independent cultures and allowed a direct comparison of gene transcription levels of cells from the same culture at different (constant or oscillating), but well defined, pseudo steady-state environmental conditions. Samples for qRT-PCR analysis were immediately poured on an ice-cooled tube containing RNAlater® (Ambion, Austin, TX) to protect and stabilize RNA, and centrifuged at 4°C.

Analytical Procedures

Concentration of glucose and organic acids was determined by high pressure liquid chromatography (Waters) with an Aminex HPX-87H column (Bio-Rad) equipped with a refractive index detector and a photo-diode array. A mobile phase of 5 mM H_2SO_4 was used at 0.8 mL/min, run at 60°C. Cell growth was followed by optical density reading at 600 nm (OD_{600}). Biomass was determined as OD_{600} values measured in Beckman DU 610 spectrophotometer. Fluorescence intensity was monitored with a Perkin Elmer LS55 Luminescence Spectrometer. Fluorescence was monitored at wavelengths of 480 nm for excitation and of 507 nm for emission. Fluorescence intensity (FU) was directly proportional to GFP concentration, as determined from densitometric analysis of SDS–PAGE gels of sonicated cell pellets.

RNA Extraction, cDNA Synthesis, and RT-PCR

Total RNA extraction and cDNA synthesis was performed as described by Flores et al. (2005a). Briefly, RNA was extracted with hot phenol equilibrated in water and precipitated with 3 M sodium acetate and ethanol, and treated with DNAse kit (DNAse free™, Ambion). RNA integrity was tested in 1.2% agarose gels and its concentration measured by densitometry and by 260/280 nm absorbance ratio. cDNA was synthesized using RevertAid™ H first strand cDNA Synthesis kit (Fermentas, Inc., Hanover, MD), using a mixture of specific pairs of DNA primers. The sequences of the primers used for cDNA synthesis were those reported by Flores et al. (2005a), except for *gfp* (5′-TGGCGAAAAC-AATGAACACC and 5′-ATGGATCCCTCTTTCTGGCA) and *luxS* (5′-CGTGGCCAACCCTAGTCACT and 5′-GGGCATGGCACTCTTGAAAA) genes. cDNA obtained in this way was used as template for real-time PCR assays.

Quantitative RT-PCR was used to evaluate gene transcription levels. qRT-PCR was performed in an ABI Prism 7000 Sequence Detection System (Perkin-Elmer/Applied Biosciences, Boston, MA), using SYBR green PCR Master Mix™ and amplification conditions described by Flores et al. (2005a). The final concentration of primers was 0.2 μM in a total volume of 15 μL. An estimated 5 ng of total target cDNA were added to the reaction mixture. The size of all amplimers was 101 bp. RT-PCR was performed in triplicate for each sample, and standard deviations below 0.3 cycles were obtained in all cases. A control reaction without template was included for each gene.

Mathematical Treatment of qRT-PCR Data

Data from qRT-PCR plots were treated using the $2^{-\Delta CT}$ or $2^{-\Delta\Delta CT}$ methods after Livak and Schmittgen (2001). Data were normalized using the *ihfB* gene as an endogenous control (housekeeping gene), which showed the same expression level in all the culture conditions assayed. The analyzed genes were compared against *ihfB* and using the $2^{-\Delta CT}$ method for internal comparison of expression levels (i.e., comparison of expression levels of different genes in the same sample). Moreover, for analyzing each gene under the different culture conditions, data were treated using the $2^{-\Delta\Delta CT}$ method. In this case, the transcription level of the circulated culture at constant DOT was set as control to normalize data (an assigned expression level value of one).

RESULTS AND DISCUSSION

Control Cultures at Constant DOT

Cultures were performed at 10% DOT in $B_{aerobic}$ without medium circulation to characterize the behavior of *E. coli* under constant conditions. A DOT of 10% was selected as such value is high enough to support growth near its maximum rate, but low enough to allow meaningful DOT gradients in the scale-down system (De León et al., 2003; Sandoval-Basurto et al., 2005). Typical kinetics of growth, consumption of glucose, production of mixed-acid fermentation metabolites, and profiles of pH and DOT are shown in Figure 1. Duplicate cultures showed variations of less than 10% with respect to data shown in Figure 1. Data referred to

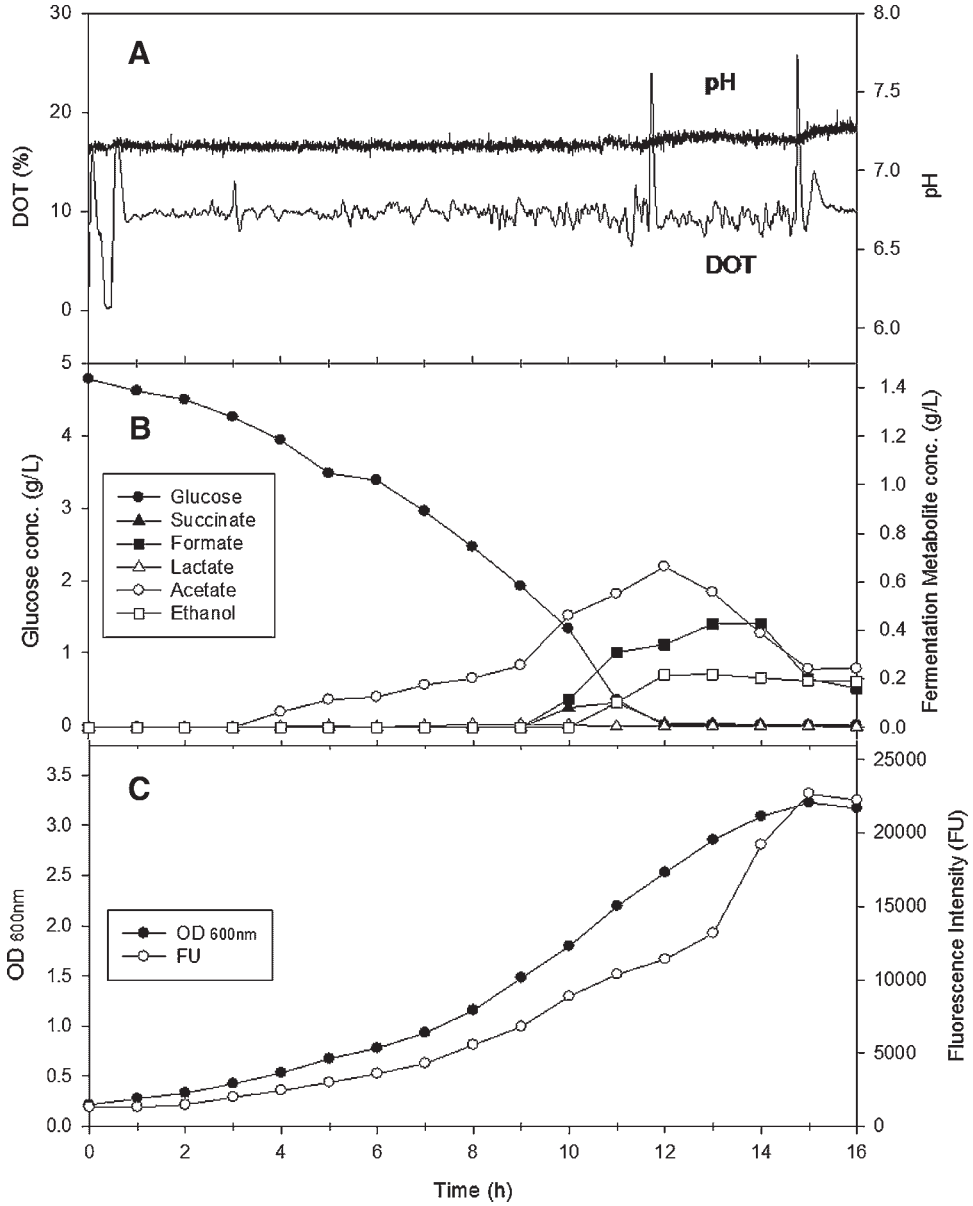

Figure 1. Typical kinetics of recombinant *E. coli* cultures performed at constant 10% DOT in $B_{aerobic}$ without medium circulation. **A**: pH and DOT control; (**B**) concentration of glucose and mixed-acid fermentation metabolites; (**C**) biomass and GFP fluorescence intensity.

in text correspond to mean of replicate cultures. As seen in Figure 1B, acetate accumulated to a maximum concentration of 0.67 g/L. It is well known that the acetate produced during fully aerobic cultures of *E. coli* originates from the metabolic overflow caused by glucose excess. Minor amounts were detected of other mixed-acid fermentation products, including formate (0.343 g/L), succinate (0.005 g/L), lactate (0.01g/L), and ethanol (0.184 g/L). Interestingly, as far as we know, ethanol has not been detected in other scale-down studies when forcing *E. coli* to oxygen limitation or even anoxic environments (Sandoval-Basurto et al., 2005, Xu et al., 1999). Upon glucose depletion, a net consumption of acetate and succinate was observed. As expected, GFP was produced from the beginning of the culture since IPTG was added at inoculation. Specific growth rate during exponential phase and maximum GFP fluorescence were 0.22/h and 22,700 FU, respectively. Similar results to those shown in Figure 1 were obtained for control cultures with medium circulation (data not shown), although a longer lag phase (2 h) and a slightly higher acetate concentration (0.790 g/L) were observed. Such results indicate that mechanical stress to *E. coli* in the pump heads and circulation loops was not relevant, which is in agreement with previous reports (Onyeaka et al., 2003; Sandoval-Basurto et al., 2005).

Cultures Under Oscillating DOT Conditions

E. coli was continuously circulated between the aerobic and anaerobic compartments of the scale-down system to mimic the effect of DOT gradients present in large-scale bioreactors.

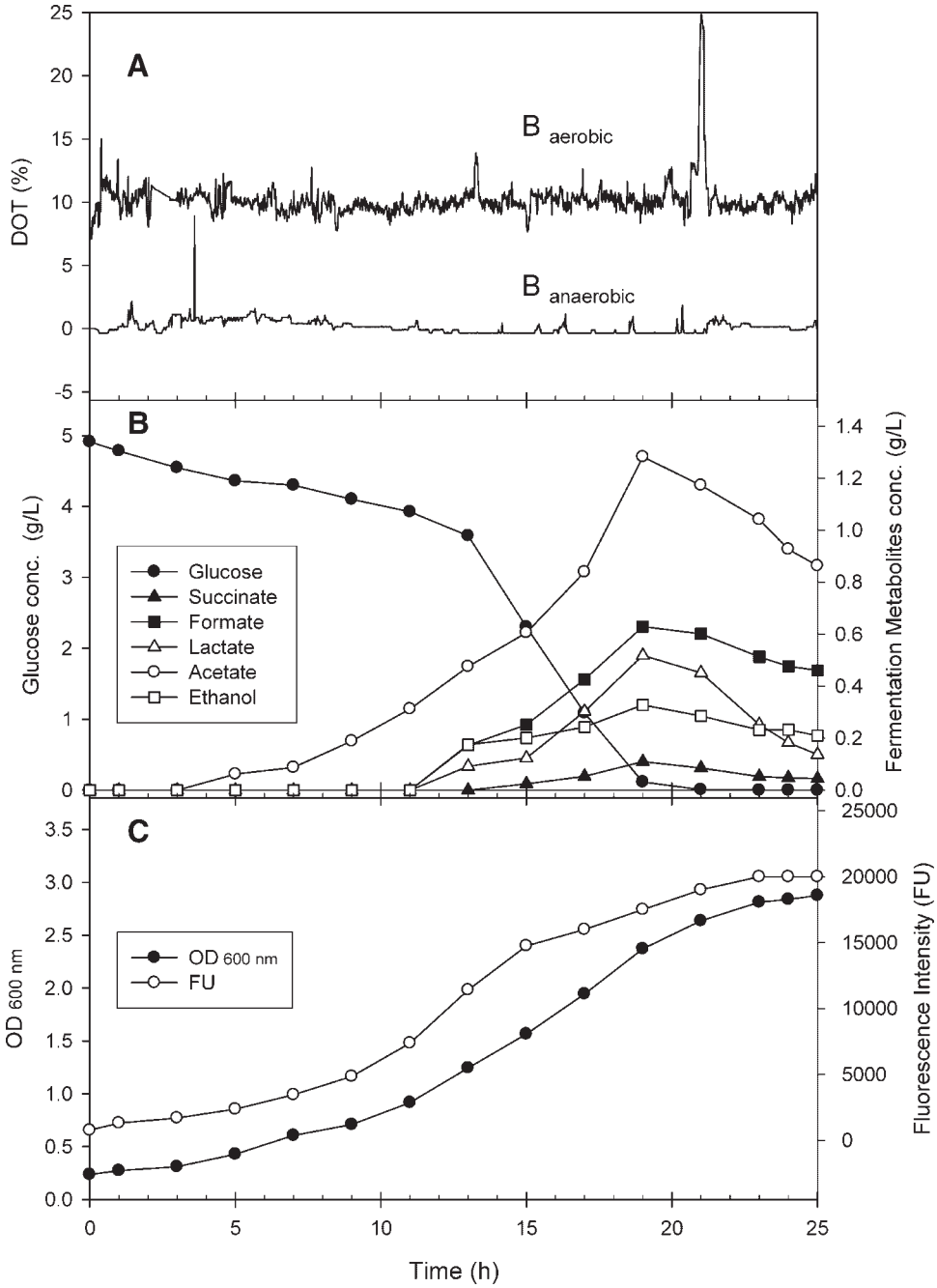

Figure 2. Typical kinetics of recombinant *E. coli* at oscillating DOT conditions. **A**: DOT control in each compartment; (**B**) concentration of glucose and mixed-acid fermentation metabolites; (**C**) biomass and GFP fluorescence intensity.

Cultures were performed in triplicate and typical results are shown in Figure 2 (on average standard deviation of less than 10% were observed between experiments). DOT gradients of at least 9% were maintained in all cases (Fig. 2A). Major differences were observed with respect to cultures maintained at constant DOT. Namely, maximum specific growth rate decreased 30%, whereas specific glucose uptake rate (q_S) increased threefold (2.42 g/g-h). Furthermore, the maximum concentration of acetate, formate, succinate, lactate, and ethanol increased on average among replicates 2, 2.15, 21, 53, and 1.8 times respectively, compared to constant DOT cultures (Fig. 2B). Such behavior indicates that cells deviate their metabolism to anaerobic pathways, with the concomitant reduction in the efficiency of energy generation (Cronan and Laporte, 1996) when exposed to transient DOT limitations as short as 33 s. The concentration of all fermentation metabolites, produced to maintain the redox balance during growth under transient anaerobic conditions declined when glucose concentration decreased below 0.15 g/L.

Maximum concentration of GFP decreased on average 17% in the oscillated cultures compared to controls (Fig. 2C). This is in agreement with Sandoval-Basurto et al. (2005) and

Namdev et al. (1993) who observed a loss in recombinant protein productivity and plasmid stability, respectively, in *E. coli* cultures subjected to fluctuating conditions. In the former study, the heterologous protein was under control of the *trp* operon promoter, thus a delayed induction due to a slower consumption of tryptophan was at least partially responsible for the observed decrease in recombinant pre-proinsulin productivity. In our case, induction conditions (IPTG concentration and time of its addition) were constant in both control and oscillating cultures. Accordingly, the observed loss in recombinant protein productivity was a direct result of the simulated DOT gradients. Oxygen limitation downregulates the TCA cycle, leading to an unfavorable energetic state of the cells and a reduced production of amino acids such as isoleucine, valine, and leucine (Konz et al., 1998). Moreover, downregulation of genes for amino acid biosynthesis on *E. coli* under anaerobic conditions, like *trpB* and *pheA*, as reported by Salmon et al. (2003), may also affect recombinant protein production capability. This can explain the reduced heterologous protein productivity in the scale-down system. However, no general conclusions can be drawn as other examples exist of improved recombinant protein productivity and quality in cultures under limiting or oscillating DOT (Bylund et al., 2000; De León et al., 2003). Further insights on the mechanisms that limit recombinant protein production in the scale-down system can be obtained from the transcriptional analysis discussed in the following sections.

Regime analysis, empirical observations, and simulation studies indicate that DOT gradients may occur in batch cultures of *E. coli* under typical conditions that prevail at large-scale for a variety of bioreactor configurations (Griot et al., 1988; Lübbert and Jørgensen, 2001; Oosterhuis and Kossen, 1984; Reuss et al., 1994, 2000; Singh et al., 1987). For instance, Griot et al. (1988) using a regime analysis predicted DOT gradients and experimentally verified them in a 4,500 L fermentor with *Bacillus subtilis* growing in batch and at very low cell concentrations (less than 3 g/L). Situations like these (low cell concentrations in batch and Newtonian fluids) can be mimicked directly in the scale-down system presented here. It can also be useful for studying DOT gradients that originate around the feed zone of fed-batch cultures when a high glucose concentration solution is added. For such cases, the scale-down systems employed to date generate DOT oscillations indirectly through changes in oxygen uptake rate induced by oscillating glucose concentration in a one- or two-compartment arrangement (Bylund et al., 1999, 2000; Enfors et al., 2001; Hewitt et al., 2000; Lin and Neubauer, 2000; Neubauer et al., 1995; Onyeaka et al., 2003; Schweder et al., 1999; Xu et al., 1999). Such studies are certainly important and very relevant as they closely simulate the actual situation. Yet, it has been recognized that in these cases the oxygen limitation following glucose overflow, rather than glucose overflow itself, is the critical parameter (Bylund et al., 2000). Accordingly, scale-down studies as the one presented here with independent control of DOT and without glucose addition, constitute a valuable tool as data interpretation is facilitated by allowing the dissection of the sole effect of oxygen limitation from that of glucose excess. In addition, rapid, relevant, and controllable DOT oscillations around the critical oxygen concentration are possible with the scale-down system employed here. Such features are not always attainable in systems based on glucose feeding. For instance, in some cases only relatively slow DOT oscillations are obtained in a range above the critical concentration and thus inconsequential for cell physiology and metabolism (Lin and Neubauer, 2000). In other cases, no information on DOT profiles is presented (Bylund et al., 2000).

Relative Gene Transcription Levels

For the purpose of this study, 21 selected genes were analyzed by quantitative PCR (qRT-PCR). qRT-PCR was used as the transcription levels of multiple genes can be quantified in parallel by a relatively simple, rapid, accurate, sensitive, and reproducible assay (Schweder and Hecker, 2004). Such characteristics overcome many of the drawbacks of slot-blotting, northern-blotting, and optical DNA-chips. Selected genes included those related to branches of metabolism directly affected by oxygen availability in the environment, such as fermentative metabolism and TCA cycle. Figure 3 is a simplified metabolic map showing the reactions catalyzed by enzymes coded by some of the selected genes. The other genes analyzed encode an anaerobic superoxide dismutase (*sodA*), the most important cytochromes (*cydA* and *cyoB*), global regulators of aerobic and anaerobic metabolism (*fnr*, *arcA*, and *arcB*), a quorum-signaling synthase (*luxS*), and the heterologous product (*gfp*).

Relative transcription levels of all genes analyzed are shown in Figures 4–8. A significant difference on transcription levels between samples taken simultaneously from the aerobic and anaerobic compartments of the scale-down system was found for most genes quantified of mixed-acid fermentation and TCA cycle (Figs. 4–6). This shows that the circulation time studied was slower than the transcriptional induction response time of pathways affected by oxygen, but faster than the time needed for transcribing the selected genes. In addition, it can be assumed that a delay will occur from the time cells enter the anaerobic compartment to the moment where genes are induced. As shown in Table I, the calculated time needed for synthesizing a single mRNA chain of some of the studied mixed-acid fermentation genes is longer than the average time cells are exposed to anoxic conditions (i.e., 33 s) in $B_{anaerobic}$ (with the exception of *frd*D and *ldh*A). Accordingly, PCR amplification products of genes induced under anaerobic conditions are detected only after a time delay that is sufficient for cells to circulate to the aerobic compartment. In the scale-down system, cells are continuously transported back to $B_{anaerobic}$ after a mean residence time of 17 s in $B_{aerobic}$. Therefore, the difference in gene transcription levels between compartments also implies that a very rapid and selective mRNA degradation process is occurring in the cultures under oscillatory DOT for most

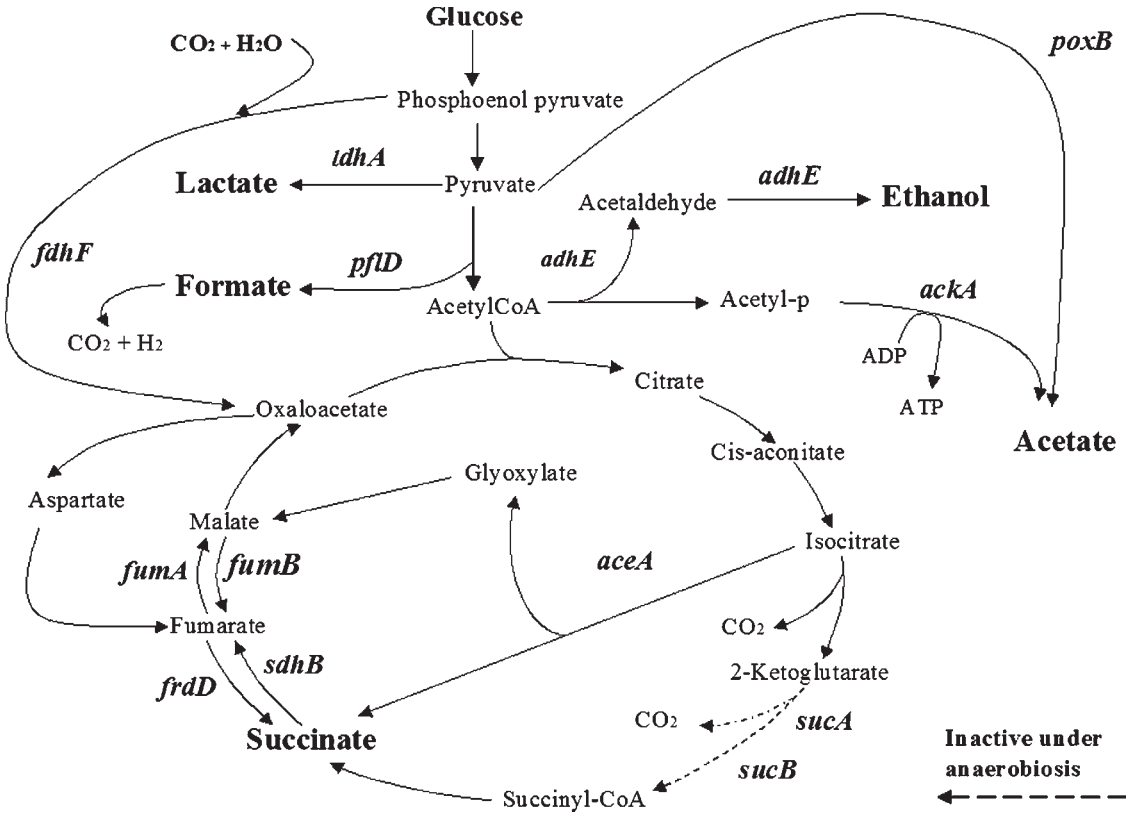

Figure 3. Simplified metabolic map of *E. coli* showing key metabolites and analyzed genes of central carbon metabolism that are affected by oxygen availability. Only the studied reactions are shown.

mixed-acid fermentation and TCA genes. This is consistent with recent studies showing that bacteria can adapt to changes in growth environment by a controlled and rapid degradation of mRNA (Kushner, 2002; Takayama and Kjelleberg, 2000; Wang et al., 2004). In particular, oxygen has been found to regulate stability of mRNA molecules. For example, Georgellis et al. (1993) observed that anaerobiosis produced a general decrease in RNA turnover during slow growth compared to aerobic conditions. Selective RNA degradation is also a cellular strategy for reducing energy waste and avoiding translation of proteins not used under the current growing conditions. Interestingly, genes such as *gfp*, *cyoB*, *cydA*, *luxS*, *soda*, and global regulators (Figs. 7 and 8), did not show significantly different transcription levels between the two compartments of the scale-down system. This suggests that under fluctuating DOT a rapid induction

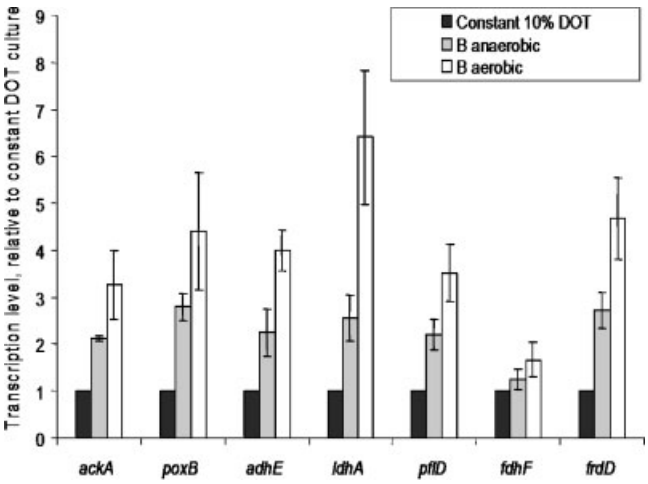

Figure 4. Transcription levels of selected fermentation genes. Values are normalized relative to the constant 10% DOT stage prior to operation under oscillatory DOT conditions.

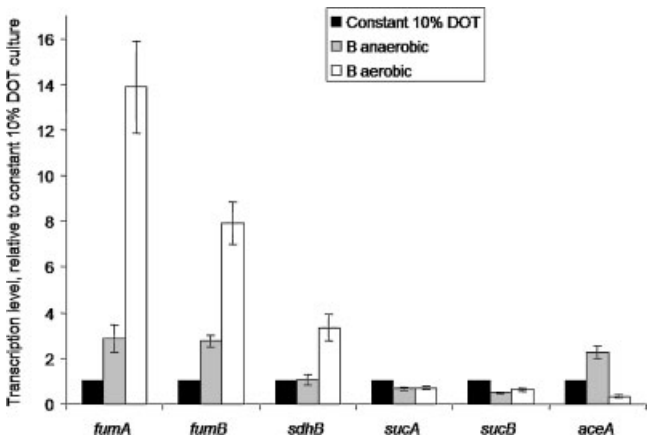

Figure 5. Transcription levels of selected genes from TCA cycle. Values are normalized relative to the constant 10% DOT stage prior to operation under oscillatory DOT conditions.

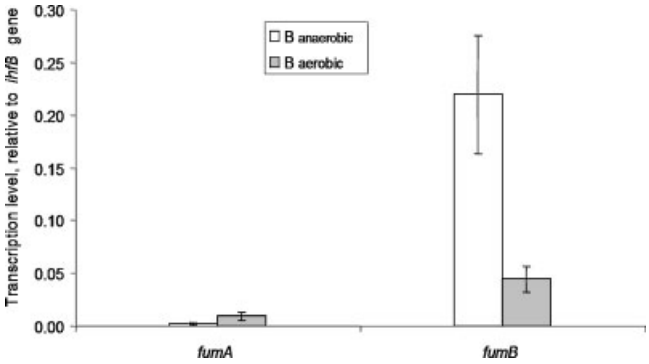

Figure 6. Transcription levels of genes coding for fumarase A and fumarase B on each compartment of the scale-down system. Values are normalized relative to the level of expression of *ihfB* gene.

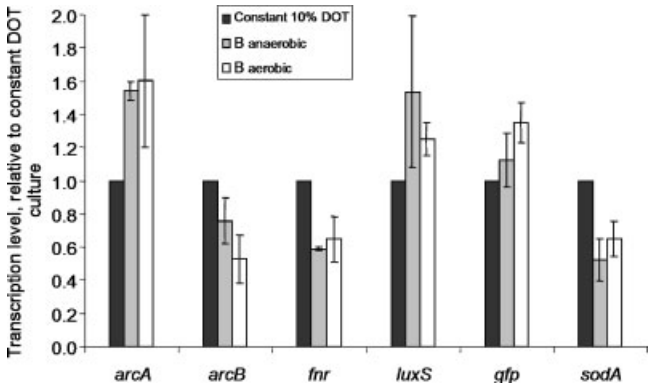

Figure 8. Transcription levels of genes coding for global aerobic/anaerobic metabolism regulators and of other monitored genes. Values are normalized relative to the constant 10% DOT stage prior to operation under oscillatory DOT conditions.

and turnover dynamics only occurs for the most meaningful genes, for instance those that redirect carbon flux and energy efficiency in fermentation pathways and TCA cycle. In the following sections, the relative expression levels by group of metabolically related genes are discussed in detail.

Mixed-Acid Fermentation Genes

There was a generalized increase in transcription levels of all mixed-acid fermentation genes under oscillatory DOT, both in $B_{aerobic}$ and $B_{anaerobic}$, compared to constant DOT conditions (Fig. 4). This result is consistent with the observed increase in products from the anaerobic metabolic branches (Fig. 2). When aerobic cultures are shifted to anaerobic conditions, pyruvate dehydrogenase is rapidly inactivated by the build-up of NADH due to cessation of the respiratory chain. The resulting pyruvate can be disposed to either lactate by lactate dehydrogenase (LDH) or to acetyl-CoA plus formate by pyruvate formate lyase (encoded by *pfl*). The only LDH, out of three in *E. coli*, involved in fermentation is that coded by *ldhA*. *ldhA* is one of the shortest fermentation genes (991 nucleotides), it is not under control of FNR, and its product is present both under aerobic and anaerobic conditions (Linch and Lin, 1996). In contrast, *pfl* is under partial anaerobic control of FNR. Accordingly, *ldhA* can be one of the first fermentation genes induced under anaerobic conditions and one of the most rapidly synthesized. This explains why *ldhA* had the highest expression level (6.4-fold in $B_{aerobic}$, Fig. 4). In comparison, relative expression levels of *pfl* (3.5- and 2.2-fold in $B_{aerobic}$ and $B_{anaerobic}$, respectively, Fig. 4) were about half of *ldhA*. Nonetheless, maximum formate concentration was about 1.5 times higher than maximum lactate concentration. Such result can be explained by the different affinities of the enzymes for pyruvate (2 mM for PFL and 7.2 mM for LDH) (Yang et al., 1999). It should also be considered that *E. coli* synthesizes fermentation

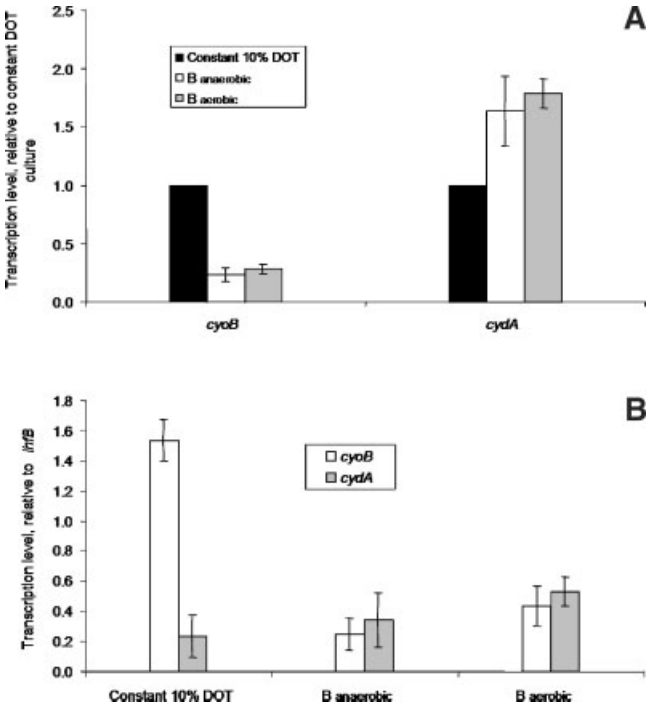

Figure 7. Transcription levels of genes coding for selected cytochromes. Values are normalized relative to the constant 10% DOT stage prior to operation under oscillatory DOT conditions (**panel A**), and relative to *ihfB* gene expression (**panel B**).

Table I. Calculated time needed to synthesise one molecule of mRNA of the mixed-acid fermentation genes.

Gene name	Transcription time (s)
aceA	35.2
ack	32.5
adhE	72.3
fdhF	58.0
frdD	9.7
fumA	44.5
fumB	44.5
ldh A	26.8
pflD	62.1
poxB	46.4

Time necessary for induction was not considered. Values were calculated from known gene lengths and mRNA elongation rates as a function of specific growth given by Bremer and Dennis (1996). Only one RNA polymerase per gene was considered.

products in a tight selection to maintain its redox balance, and that expression levels do not necessarily correspond to the amount of metabolite produced. Formate can be broken down further by the FHL-FDH complex into hydrogen and carbon dioxide. These products follow different fates and their generation is important for the redox balance of the cell (Clark, 1989). Formate dehydrogenase (FDH) is associated with an 80-kDa selenopolypeptide encoded by the *fdhF* gene, which presented an increased expression level of about 1.7-fold in $B_{aerobic}$ during oscillatory DOT (Fig. 4).

Acetate was the most important fermentation metabolite accumulated during both, constant and oscillating DOT. Transcription levels of *poxB* and *ackA* were very similar and increased more than 3.3-fold in $B_{aerobic}$ under oscillatory DOT (Fig. 4). Both genes encode enzymes that produce acetate but from different substrates. As shown in Figure 3, acetate is produced from either pyruvate or acetyl-CoA, suggesting a possible reason for its important accumulation under heterogeneous DOT conditions (Bylund et al., 1999; Enfors et al., 2001; Sandoval-Basurto et al., 2005; Xu et al., 1999). Acetate production from acetyl-CoA is important under anaerobic conditions as it generates 1 mole of ATP per mole of acetate produced, which is significant since only 2 ATP moles per mole of glucose are produced by glycolysis. Schweder et al. (1999), when cultivating *E. coli* in a scale-down bioreactor found that *ack* transcription was more than twofold after remaining 54 s in a PFR section under oxygen limitation. Such data agrees well with the results in our scale-down system, as we found a relative expression level of 2.12 in $B_{anaerobic}$ and of 3.25 in $B_{aerobic}$ (Fig. 4). During the oxidation of pyruvate to acetate by *poxB*, energy is wasted compared to its conversion into acetyl Co-A. Under aerobic conditions, pyruvate dehydrogenase converts pyruvate to acetyl Co-A, and under strictly anaerobic conditions this is done by PFL. However, it has been suggested that under microaerobic conditions pyruvate oxidase bridges the gap in providing a source of energy from pyruvate catabolism (Chang et al., 1994). Consistent with this suggestion, it has been demonstrated that expression of *poxB* is maximal in early stationary phase and is strictly dependent on the *rpoS* gene (Chang et al., 1994), which is involved in heat shock and other general stress responses. This may explain the increased expression of *poxB* observed in this study (Fig. 4).

An alternative fate for acetyl-CoA during fermentation is its conversion to ethanol in two steps catalyzed by alcohol dehydrogenase (encoded by *adhE*), which is one of the largest enzymes produced by *E. coli* (Clark, 1989). Production of ethanol and lactate is important under anaerobic conditions, as these are the only ways to regenerate the NAD+ consumed in glycolysis and so maintain redox balance. As mentioned before, a 1.8-fold increase in maximum ethanol concentration was observed in the scaled-down cultures compared to fully aerobic conditions. In agreement with such results, transcription level of *adhE* increased 2.3- and 4-fold in $B_{anaerobic}$ and $B_{aerobic}$, respectively. *E. coli* can use fumarate as a terminal electron acceptor and preferably catalyses the reaction to reduce fumarate to succinate as part of the reverse TCA cycle that operates under oxygen limitations (discussed below). Anaerobic regulation of *frdABCD* operon is mediated by FNR. We tested the *frdD* gene that codes for fumarate reductase (FrdD), which is responsible for electron transfer from quinone to FrdB. A relative transcription level of *frdD* of 2.7-fold in the anaerobic compartment and 4.7-fold in the aerobic compartment, compared to fully aerobic conditions, was obtained (Fig. 4). This is in agreement with Schweder et al. (1999) who reported that *frd* (no information of which subunit was monitored) was transcribed about twofold higher after a residence time of 54 s in the oxygen-limited section of their scale-down system.

Tricarboxylic Acid Cycle and Glyoxylate Bypass Genes

TCA cycle is essential in central carbon metabolism of *E. coli*, since it is responsible for total oxidation of acetyl-CoA and its intermediates are required for the biosynthesis of amino acids. The expression levels of TCA cycle enzymes respond primarily to the presence of oxygen (Cronan and Laporte, 1996). Under anaerobic conditions, TCA cycle functions as two biosynthetic pathways, a reductive branch that produces succinyl-CoA and an oxidative branch producing 2-ketoglutarate (Fig. 3). These pathways fail to form a cycle because 2-ketoglutarate dehydrogenase (encoded by *sucA* and *sucB*) is virtually absent during anaerobic growth (Cronan and Laporte, 1996). As shown in Figure 5, *sucA* and *sucB* were found to have a reduction (ca. 30%) of transcription levels in both compartments of the scale-down system compared to fully aerobic conditions. During anaerobic growth, *frdD* replaces succinate dehydrogenase (encoded by *sdhB*) to allow reductive production of succinyl-CoA. As mentioned previously, an important increase in the transcription level of *frdD* was found during oscillatory DOT (Fig. 4). The fumarase enzymes are also importantly regulated by oxygen availability (Tseng et al., 2001). In the complete TCA cycle, fumarase A (encoded by *fumA*) catalyzes the formation of malate from fumarate, whereas in the branched TCA cycle, fumarase B (encoded by *fumB*) acts in the exactly opposite reaction. In the latter case malate is utilized as an anaerobic electron acceptor under the prevailing anaerobic conditions (Cronan and Laporte, 1996). The relative expression levels of *fumA* and *fumB* (calculated with the $2^{-\Delta\Delta CT}$ method) during oscillatory DOT increased between 13.9- and 7.9-fold (in $B_{aerobic}$), compared to their levels under fully aerobic conditions (Fig. 5). By normalizing the transcription levels of *fumA* and *fumB* with respect to the *ihfB* gene, which was found to have a constant expression level under all studied conditions (data not shown), their relative values (calculated with the $2^{-\Delta CT}$ method) under the same condition can be obtained. Performing such normalization, it can be seen that *fumB* is much more transcribed than *fumA* in cells cultivated under oscillating DOT conditions (Fig. 6). Based on data of transcription levels of

frdD, *sucA*, *sucB*, *fumA*, and *fumB*, it can be concluded that under oscillating DOT conditions *E. coli* preferentially employs TCA cycle as two separate branches, and thus presents a behavior typical of anaerobic cultures. As shown in Figure 5, the transcription level of *aceA* (that codes for isocitrate lyase of the glyoxylate bypass) increased more than twofold in $B_{anaerobic}$. The glyoxylate bypass is induced when *E. coli* is grown on acetate or fatty acids (Clark and Cronan, 1996). Furthermore, *aceA* has been related to starvation or stress response (Flores et al., 2005b). Accordingly, the augmented transcription level of *aceA* in $B_{anaerobic}$ can indicate either a stress state of the cells, the onset of acetate consumption as a source of carbon despite glucose availability, or the use of the glyoxylate shunt as an anaplerotic pathway to replenish intermediates of TCA cycle used for amino acid synthesis.

Genes Coding for Cytochromes

E. coli has two respiratory oxidases, cytochrome *bd*, and cytochrome bo_3 that are not structurally related and have distinct physiological roles (Gennis and Stewart, 1996). The low-affinity cytochromes o oxidase (encoded by *cyoB*) is pre-dominant during oxygen-sufficient growth, whereas the high-affinity cytochromes d oxidase (encoded by *cydA*) prevails under oxygen-limiting conditions (Poole and Ingledwe, 1987). Consistent with these properties, transcription of the *cydAB* operon is activated when oxygen becomes limiting. Under fully anaerobic conditions, *cydAB* transcription is subsequently repressed to an intermediate level relative to microaerobic and oxygen-rich conditions. This control is mediated by the ArcA and Fnr proteins (Gennis and Stewart, 1996). *cydAB* expression reaches its maximum at a DOT of approximately 2% (Tseng et al., 1996). The question is if *E. coli* employs different cytochromes in different zones of large-scale bioreactors (those limited and not limited by oxygen) as a fast adaptation strategy. In Figure 7A, it can be seen that cytochrome d oxidase (coded by *cydA*) was preferred over cytochrome o oxidase (coded by *cyoB*) during oscillatory DOT culture. That means that under the oscillating DOT cultures, *E. coli* uses the less energy efficient, but with higher oxygen affinity cytochrome. Moreover, by analyzing the expression level of these genes relative to *ihfB* (as shown in Fig. 7B), it can be seen that *cydA* was not upregulated in the compartments of the scale-down system, compared to the constant DOT culture. In contrast, *cyoB* did show a significant decrease in its transcription level during the scaled-down cultures. Accordingly, *E. coli* apparently adapts to oscillating DOT conditions by repressing the expression of *cyoB* gene. Yoon et al. (2003) found that, in the stationary phase of fed-batch cultures at DOT controlled above 30%, *cydA* was overexpressed and *cyoB* was downregulated. They related such change to a downregulation of the TCA cycle enzymes and to a severe competition for oxygen at high cell density. A similar conclusion can also apply to our scale-down cultures during the continuous transient aerobic-anaerobic shifts.

Genes of Global Regulators of Aerobic/Anaerobic Metabolism

Genes coding for global regulators of aerobic (ArcAB) and anaerobic (FNR) metabolism in *E. coli* were also tested and their transcription levels are shown in Figure 8. No differences between compartments were observed for *arcA*, *arcB*, and *fnr*. However, *arcB* (that codes for the autophosphorylating signal membrane enzyme that senses oxygen availability) and *fnr* (coding for FNR protein, the main aerobic respiration regulator) showed a slight decrease in their transcription levels compared to aerobic cultures. For *arcB* the decrease was between $24.1\% \pm 13.9\%$ and $47.1\% \pm 14.6$ % for $B_{anaerobic}$ and $B_{aerobic}$, respectively, whereas for *fnr* the decrease was between $41.1\% \pm 1.0\%$ and $35.0\% \pm 13.8\%$ for $B_{anaerobic}$ and $B_{aerobic}$, respectively. In contrast, *arcA*, which codes for the cytoplasmic protein that binds DNA to control aerobic metabolism had a slightly increased transcription level of $54.1\% \pm 5.5\%$ and $60.2\% \pm 40.1\%$ for $B_{anaerobic}$ and $B_{aerobic}$, respectively. It should be noted that replicate qRT-PCR expression values determined here for each gene differ around 30% (Flores et al., 2005a). Accordingly, differences in transcription levels within 30% should be taken with caution because they are within the limits of experimental error. As global regulators are usually expressed at a constant level in *E. coli*, no changes in the transcription levels of *arcA*, *arcB*, and *fnr* would be expected. This was the general trend observed as only small differences above the experimental error were observed (Fig. 8).

Quorum Sensing, *sodA*, and Heterologous Gene

The heterologous gene, *gfp*, presented very little variation on expression level between both compartments and between oscillated and fully aerobic conditions (Fig. 8). Nonetheless, as mentioned previously, an important decrease in GFP productivity was observed in the scale-down cultures. Such results indicate that although there was no direct effect of DOT oscillations at a pre- or post-transcriptional level (for instance loss of plasmid) there was an important detrimental post-transcriptional effect. The finding that a post-transcriptional event is limiting heterologous protein production in the scaled-down system is fundamental for exploring the design of improved operation strategies at large scales where DOT gradients are inevitable. That TCA cycle was functioning as two open branches could have caused an amino acid limitation that in turn resulted in a decrease of recombinant protein production. Such a problem can be easily alleviated by a suitable amino acid supplementation strategy, although this remains to be studied.

Quorum sensing enables intracellular signaling among bacteria for triggering synchronous community-level responses. Quorum sensing in *E. coli* is promoted by production, release, and response to an extracellular factor termed autoinducer-2 (AI-2) (Huisman and Kolter, 1994), synthesized by AI-2 synthase, coded by *luxS*. De Lisa et al. (2001)

demonstrated that overexpression of heterologous proteins results in attenuation of AI-2 signaling and that AI-2 production is regulated by growth conditions, such as glucose level, DOT, pH, and osmolarity. Although mechanisms of quorum sensing in *E. coli* are not clear to date, De Lisa et al. (2001) have suggested that quorum sensing gene expression may play a central role in cellular physiology. As seen in Figure 8, *luxS* presented a small increase in expression level in the scale-down cultures, although within the 30% experimental error of the qRT-PCR technique employed. Accordingly, this shows that cells are not increasing significantly, at least at the transcriptional level, the community communication during oscillating DOT conditions. *E. coli* synthesizes two different superoxide dismutases (SOD), one containing iron (coded by *sodB*) and the other containing manganese (coded by *sodA*). Manganese-SOD is only present under aerobic conditions (Touati, 1988). Accordingly, results shown in Figure 8 indicate that under oscillatory DOT conditions the time cells are exposed to anaerobiosis do not induce a significant relevant transcriptional downregulation of *sodA*.

CONCLUSIONS

To our knowledge, this is the first bioreactor scale-down investigation to present in a single study data on molecular phenomena (transcription) and macroscopic information (kinetic and stoichiometric parameters, by-product formation, etc.) for a recombinant expression system. The results obtained here explain what occurs to a recombinant *E. coli* strain under typical large-scale conditions, as the circulation time simulated in this study mimicked conditions present in industrial fermenters. During oscillating DOT cultures, all the products of mixed-acid fermentation were produced. Such a behavior closely agreed with the transcriptional profiling. Different transcription levels between the aerobic and anaerobic compartments of the scale-down system were found. This indicates that the transcriptional response and adaptation to changing environmental conditions were very fast and that were possibly regulated by rapid and selective mRNA degradation. Kinetic studies of the transcriptional and proteomic responses upon step changes in DOT are currently under way to explain the observed differences between compartments. Gene expression level of *gfp* did not change in the scaled-down cultures, indicating that post-transcriptional limitations and not transcriptional events limited the production of the recombinant protein. Altogether, the results of this study demonstrate that important transcriptional changes can occur in genes of central carbon metabolism while other relevant genes remain unaffected when *E. coli* is subjected to a fluctuating DOT environment. These results are important for establishing rational scale-up criteria and improving bioreactor operation strategies, as well as for designing bacterial strains of enhanced performance when cultured under typical industrial conditions. Our group is currently working on metabolic engineering approaches to address the latter aspect.

NOMENCLATURE

$B_{aerobic}$	Aerobic (DOT 10%) compartment of the scale-down bioreactor system
$B_{anaerobic}$	Anaerobic (DOT 0%) compartment of the scale-down bioreactor system
C_L	Dissolved oxygen concentration in the liquid phase
CTD	Circulation time distribution
DOT	Dissolved oxygen tension
TCA	Tricarboxylic acid
t_c	Circulation time
t_{OC}	Characteristic time constant for oxygen consumption $= C_L/[X\ q_{o_2}]$
q_{o2}	Specific oxygen uptake rate
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
X	Biomass concentration

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