Effect of ArcA and FNR on the Expression of Genes Related to the Oxygen Regulation and the Glycolysis Pathway in *Escherichia coli* Under Microaerobic Growth Conditions

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Abstract: *Escherichia coli* has several elaborate sensing mechanisms for response to the availability of oxygen and the presence of other electron acceptors. The adaptive responses are coordinated by a group of global regulators, which include the one-component Fnr protein, and the two-component Arc system. To quantitate the contribution of Arc and FNR dependent regulation under microaerobic conditions, the gene expression pattern of the *fnr* the *arcA* and *arcB* regulator genes, and the glycolysis related genes in a wild-type *E. coli*, an *arcA* mutant, an *fnr* mutant, and a double *arcA*, *fnr* mutant, in glucose limited cultures and different oxygen concentrations was studied in chemostat cultures at steady state using QRT-PCR. It was found that ArcA has a negative effect on *fnr* expression under microaerobic conditions. Moreover, the expression levels of the FNR regulated genes, *yfiD* and *frdA*, were higher in cultures of the *arcA* mutant strain compared to the wild-type. These imply that a higher level of the FNR regulator is in the activated form in cultures of the *arcA* mutant strain compared to the wild-type during the transition from aerobic to microanaerobic growth. The results also show that the highest expression level of *aceE*, *pflB*, and *adhE* were obtained in cultures of the *arcA* mutant strain under microaerobic growth while higher levels of *ldhA* expression were obtained in cultures of the *arcA* mutant strain and the *arcA*, *fnr* double mutant strain compared to the wild-type and the *fnr* mutant strain. While the highest expression of *adhE* and *pflB* in cultures of the *arcA* mutant strain can explain the previous report of high ethanol flux and flux through pyruvate formate lyase (PFL) in cultures of this strain, the higher level of *ldhA* expression was not sufficient to explain the trend in lactate fluxes. The results indicate that lower conversion of pyruvate to acetyl-CoA is the main reason for high fluxes through lactate dehydrogenase (LDH) in cultures of the *arcA*, *fnr* double mutant strain.
Keywords: microaerobic conditions; QRT-PCR; pyruvate metabolism; fermentative genes; lactate

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INTRODUCTION

Escherichia coli possesses a large number of sensing/regulation systems for the rapid response to availability of oxygen and the presence of other electron acceptors (Guest et al., 1996; Gunsalus, 1992; Lin and Iuchi, 1991; Lynch and Lin, 1996; Park and Gunsalus, 1995; Unden et al., 1995). Those regulation systems channel electrons from donor to terminal acceptors such that the overall potential difference is maximized for any given growth condition. The adaptive responses are coordinated by a group of global regulators, which include the one-component Fnr (fumarate, nitrate reduction) protein, and the two-component Arc (aerobic respiration control) system.

The Arc system is a two-component regulatory system composed of ArcA, the cytosolic response regulator, and ArcB, the transmembrane histidine kinase sensor. ArcB is activated during the transition from aerobic to microanaerobic growth, and remains in the activated state during anaerobic growth. Upon stimulation ArcB undergoes autophosphorylation, and the phosphoryl group is transferred to ArcA by a His → Asp → His → Asp phosphorelay (Georgellis et al., 1999). Consequently, the increased level of phosphorylated ArcA represses the synthesis of some enzymes, such as the citric acid cycle enzymes, succinate dehydrogenases, fumarase, pyruvate dehydrogenase, and the low oxygen affinity cytochrome bo_3 oxidase, while it activates the expression of other enzymes such as cytochrome *bd* oxidase and enzymes involved in fermentative metabolism (Lin and Iuchi, 1991; Lynch and Lin, 1996; Unden et al., 1995).

The Fnr protein contains a Fe-S cluster that serves as a redox sensor. The concentration of the Fnr protein is similar in both aerobic and anaerobic conditions (Becker et al., 1996; Gunsalus, 1992); however, it is active only during anaerobic growth. Active Fnr protein activates and represses target genes in response to anaerobiosis. Most of the FNR modulon

is concerned with maximizing the capacity for anaerobic energy generation. Thus, the FNR system induces the expression of genes that permit anaerobically growing *E. coli* to transfer electrons to alternative terminal acceptors (Lin and Iuchi, 1991). It acts as a positive regulator of genes expressed under anaerobic fermentative conditions such as aspartase, formate hydrogenase, fumarate reductase, and pyruvate formate lyase (PFL) (Guest et al., 1996). It also represses the aerobic genes, cytochrome *bd*, and bo_3 oxidase.

In a previous work, we compared the metabolic activity of wild-type *E. coli*, an *arcA* mutant, an *fnr* mutant, and a double *arcA*, *fnr* mutant, via the fermentative pathways, in glucose limited cultures and different oxygen concentrations in chemostat cultures at steady state (Shalel-Levanon et al., 2005). It was found that Fnr protein has a minor effect on the fermentative metabolite fluxes under microaerobic conditions (oxygen concentration in the headspace (OCH) higher than 2.5%). However, in the *arcA* mutant strain the cells behave as if a higher level of the FNR regulator is in the activated form compared to the wild-type strain during the transition from aerobic to microanaerobic growth. In particular, a significant increase in the flux through PFL in the presence of oxygen was observed. In cases where both ArcA and Fnr proteins were absent (double mutant) a significant amount of lactate was obtained under microaerobic growth.

In the present study, the expression levels of the glycolysis related genes, genes expressed under anaerobic fermentative conditions, and the oxygen regulation genes were studied, using QRT-PCR, in wild-type *E. coli*, an *arcA* mutant, an *fnr* mutant, and a double *arcA*, *fnr* mutant, at steady state in glucose limited cultures and microaerobic concentrations. Previous work focused on the effect of ArcA and FNR regulation systems on the expression of these genes at complete aerobic and anaerobic conditions (e.g., Lynch and Lin, 1996; Salmon et al., 2003; Unden et al., 1995). In this work, we examined the effect under microaerobic growth.

To confirm that higher levels of the Fnr protein is in the activated state in culture of the *arcA* mutant strain compared to the wild-type under microaerobic conditions, we included the FNR regulated genes, *yfiD* and *frdA*, in our study. To understand the high fluxes through lactate dehydrogenase (LDH) in cultures of the *arcA*, *fnr* double mutant strain under microaerobic conditions (Shalel-Levanon et al., 2005), we also examined *ldhA* expression. Indeed, our results from the study of *yfiD* and *frdA* expression support our previous hypothesis that the Fnr protein is more functionally active in cultures of the *arcA* mutant strain compared to the wild-type. However, the results regarding the *ldhA* expression imply that the main reason for the higher fluxes to lactate in cultures of the *arcA*, *fnr* double mutant strain compared to the *arcA* mutant strain is the lower conversion of pyruvate to acetyl-CoA in the double mutant strain rather than the *ldhA* expression level. Indeed, it was previously shown that *pflA* and *pflB* mutant strains produced a large amount of lactate under microaerobic conditions (Zhu and Shimizu, 2004).

MATERIALS AND METHODS

Strains and Plasmids

The strains and plasmids used in this work are described in Table I. The strains were constructed using the Datsenko and Wanner method (Datsenko and Wanner, 2000) as described in Shalel-Levanon et al. (2005).

Bioreactor Calibration and Growth Conditions

The cells were grown under a glucose limiting condition at a dilution rate of 0.1 ± 0.01/h and variable oxygen concentrations. A 1 L bioreactor (BioFlo 110, New Brunswick Scientific, Edison, NJ) was used, and the working volume was maintained at 0.6 L. The media used was minimal media (7 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 6 mg/L Thiamine, 1 mM $MgSO_4$, and 0.1 mM $CaCl_2$) with glucose as the only carbon source (20 mM). The pH was maintained at 7.0 ± 0.04 by titrating with 3M NaOH. The temperature and gas flow rate were set on 37°C, and 400 mL/min, respectively. The agitation was maintained constant

Table I. List of strains and plasmids that were used in this study.

	Description	Reference
Strains		
MG1655	Wild-type	
MG1655 [*arcA*, Km^R]	*arcA* mutant which is Km resistant	Shalel-Levanon et al. (2005)
MG1655 [*arcA*]	*arcA* mutant	Shalel-Levanon et al. (2005)
MG1655 [*fnr*, Km^R]	*fnr* mutant which is Km resistant	Shalel-Levanon et al. (2005)
MG1655 [*fnr*]	*fnr* mutant	Shalel-Levanon et al. (2005)
MG1655 [*arcA*, *fnr*, Km^R]	*arcA* and *fnr* mutant which is Km resistant	Shalel-Levanon et al. (2005)
MG1655 [*arcA*, *fnr*]	*arcA* and *fnr* mutant	Shalel-Levanon et al. (2005)
Plasmids		
pKD4	Kanamycin resistant cassette	Datsenko and Wanner (2000)
pKD46	Lambda Red helper plasmid, contains Amp resistance	Datsenko and Wanner (2000)
pCP20	Amp and Cm resistant, temperature-sensitive replication and thermal induction of FLP synthesis	Datsenko and Wanner (2000)
pRZ7382	Contains *fnr* mutation that substitutes His for Leu at position 28, Cm and Amp resistant	Bates et al. (1995, 2000)

(285 rpm). This agitation value was chosen using Alexeeva et al. (2002) calibration for 100% aerobiosis. The idea was to find the minimum agitation required for zero acetate production. The strain MG1655 (wild-type) was used to find this minimum agitation and air was used as the flowing gas.

The oxygen supply was varied by varying the percentage of oxygen in the gas mixture of oxygen and nitrogen (gas cylinder, MATHESON TRI-GAS, Irving, TX). Fermentor conditions were calibrated with two reference conditions. For anaerobic conditions, nitrogen was used as the flowing gas (zero OCH). For aerobic growth, for which a complete oxidation of glucose to CO_2 occurs, air was used as the flowing gas.

RNA Extraction

For RNA extraction, phenol chloroform extraction was performed as follows (using the method in www.microarrays.org/pdfs/Total_RNA_from_Ecoli.pdf). Samples (25 mL) were collected into 50 mL tubes containing 3.2 mL of ice cold stop solution (5% water saturated phenol in ethanol) (McDowall et al., 1994) and immediately centrifuged at 3,030*g* for 10 min at 4°C (in a Sorvall RC-5B Refrigerated centrifuge, Sorvall, Newtown, CT) and the supernatant was removed. Then the cells were lysed by resuspending the pellets in 2 mL of 0.5 mg/mL lysozyme, in TE buffer pH 8 (10 mM Tris-HCl, 1 mM EDTA), mixed with 100 μL of 10% SDS, and placed in a water bath at 64°C for 2 min. After incubation, 220 μL of sodium acetate pH 5.2 and 2.5 mL of water saturated phenol (Sigma P4682, Sigma-Aldrich Co., St. Louis, MO) were added and the mixture was incubated at 64°C for 6 min (hot phenol extraction), placed on ice to chill, and centrifuged (in a Sorvall Biofuge *pico* micro centrifuge) at 16,060*g* for 10 min at 4°C. The aqueous layer was then transferred to fresh microfuge tube containing an equal volume of chloroform, mixed, and centrifuged at 16,060*g* for 5 min at 4°C (chloroform extraction). For ethanol precipitation, the aqueous layer was transferred into microfuge tubes, a 1/10 volume of 3M sodium acetate 1 mM EDTA pH 5.2, and 2 volumes of ice cold 100% ethanol were added and the mixture was incubated for 20 min at −80°C. Then it was centrifuged at 16,060*g* for 25 min at 4°C, and the pellets were washed with 1 mL 80% ice cold ethanol. Finally, the ethanol was removed and the pellets were air dried and resuspended in 20–40 μL of diethyl pyrocarbonate (DEPC) treated water. To verify that the RNA did not contain DNA, the RNA was treated with DNase (RQ1 RNase-Free DNase, Promega, Madison, WI) according to the manufacturer recommendations. The quantity and purity of the RNA were determined by optical density measurements at 260 and 280 nm.

cDNA Synthesis and Q-PCR Amplification

The genes that were studied in this work and their primers are listed in Table II. Reverse transcription reaction was carried out in a RoboCycler Gradient 96 (Stratagene, La Jolla, CA) using Promega Reverse Transcription System. The reaction mixture (containing 1 μg of RNA in 60 μL mixture), was incubated for 10 min at room temperature, 30 min at 50°C for reverse transcription, 5 min at 95°C, and 10 min at 6°C for reverse transcriptase inactivation. For each sample a no amplification control (containing RNA but not reverse transcriptase) was also prepared. The first strand cDNA was diluted to 300 μL with nuclease free water and further diluted tenfold.

Q-PCR reaction was carried out in a 96-well plate in ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (Applied Biosystems). Each well contained 20 μL reaction mixture of 3 μL diluted cDNA, 2 μL mixed primers (1.25 pmol/μL), 10 μL SYBR Green PCR Master mix, and 5 μL nuclease free water. The reaction mixture was incubated 2 min at 50°C, 10 min at 95°C (Taq activation) followed by 40 cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/extension). Each plate included samples for no amplification control as well as samples for no template control (water instead of cDNA).

The comparative C_T (threshold cycle) method ($\Delta\Delta C_T$) for relative quantifying of gene expression was used (ABI Prism 7700 Sequence Detection System, User Bulletins #2). For this method, the *rrsA* gene that encodes the rrnA 16S ribosomal RNA, was used as a control gene. This gene is not subject to variable expression. For the $\Delta\Delta C_T$ calculation to be valid, the efficiency of the target amplification and the efficiency of the control amplification must be approximately equal. A sensitivity test to confirm that the two amplicons have the same efficiency was performed, by testing how the ΔC_T values (i.e., C_T(gene)$-C_T$(*rrsA*)) vary with template dilution. For each gene the plot of ΔC_T versus the log of the input amount resulted in a slope of approximately zero, which suggested that the efficiencies of the two amplicons (the tested gene and the control gene) are approximately the same. The $\Delta\Delta C_T$ values of each gene were determined from the difference between the ΔC_T value in a tested strain (either a mutant strain or the wild-type at 1%–10% OCH) and the ΔC_T value in the wild-type at 10% OCH (calibrator). The comparative expression level (relative to the expression in the wild-type at 10% OCH) was calculated using the formula:

$$\frac{X_s}{X_{cb}} = 2^{-\Delta\Delta C_T} \quad (1)$$

Where X_s is the normalized amount of target gene in a mutant strain or in the wild-type at 1%–10% OCH, and X_{cb} is the calibrator normalized amount of target gene. For better presentation of the results, the formula $2^{-\Delta\Delta C_T}$ was multiplied by 100. Thus, the comparative expression level of each gene in the wild-type at 10% OCH (calibrator) was always 100.

RESULTS

The strains MG1655, MG1655 [*arcA*], MG1655 [*fnr*], and MG1655 [*arcA*, *fnr*] were grown in a chemostat with glucose as the only carbon source at a dilution rate of 0.1/h and pH

Table II. List of genes, primer pairs, and the amplified PCR products.

Gene	Enzyme or function	Primer pairs	PCR products (bp)
ptsG	Part of the phosphoenolpyruvate: sugar phosphotransferase system	5′-GGTAGTTGCGTTTGGCATTT-3′ 5′-GCCATATAACGCGGAATGTC-3′	153
pfkA	6-phosphofructokinase-1	5′-GGTGCCTTACGACCGTATTC-3′ 5′-GGACGCTTCATGTTTTCGAT-3′	150
pykA	Pyruvate kinase II	5′-ACTGACGCTGTGATGCTGTC-3′ 5′-CCACATTGTCGAACTGAACG-3′	151
pykF	Pyruvate kinase I	5′-ACCGCCATTGAAGGTAACAA-3′ 5′-TGTTCGCAACCAAAGATCAG-3′	149
frdA	Fumarate reductase	5′-CGATAAGACCGGCTTCCATA-3′ 5′-CCTTCCATCATGTTCATTGCT-3′	150
ldhA	D-lactate dehydrogenase	5′-AGTCCGTGTTCCAGCCTATG-3′ 5′-CGGTCAGACCTTCCAGAGAG-3′	134
aceE	Pyruvate dehydrogenase	5′-TCTGATCGACCAACTGCTTG-3′ 5′-GGCGTTCCAGTTCCAGATTA-3′	137
pflA	Pyruvate formate-lyase activating enzyme	5′-TACGATCCGGTGATTGATGA-3′ 5′-TCACATTTTTGTTCGCCAGA-3′	151
pflB	Pyruvate formate-lyase (inactive)	5′-GCGAAATACGGCTACGACAT-3′ 5′-CATCCAGGAAGGTGGAGGTA-3′	142
yfiD	Stress-induced alternate pyruvate formate-lyase subunit	5′-ACTAAAGCCGCTAACGACGA-3′ 5′-TTCAATGTCACCCAGTTTGC-3′	138
pflC	Probable pyruvate formate lyase activating enzyme 2	5′-GTCTGCACTGTGCGAAATGT-3′ 5′-GGACGTGCGAAAGAAAATGT-3′	134
pflD	Formate acetyltransferase 2	5′-CCCTTACTGGCTGCTGAAAG-3′ 5′-ATCTGCCCGTTGATGAAATV-3′	150
adhE	Alcohol dehydrogenase/Pfl-deactivase	5′-CTGGCAGGCTTCTCTGTACC-3′ 5′-TACCGCGTCTTCGAAATCTT-3′	141
pta	Phosphate acetyltransferase	5′-TAATCCGGCAGAGATCAACC-3′ 5′-GTTTCGGTCATGCCTTTGTT-3′	144
ackA	Propionate kinase 2/acetate kinase A	5′-CGTTGACGCAATCAACAAAC-3′ 5′-GGTGGCAGTAAACGTCCATT-3′	140
arcA	ArcA transcriptional dual regulator	5′-TGTTTTCGAAGCGACAGATG-3′ 5′-GAACATCAACGCAACATTCG-3′	151
arcB	ArcB sensory histidine kinase	5′-TATTGGTCTGGCCGTTTCTC-3′ 5′-ACGCATCATCGACCTCTTCT-3′	137
fnr	FNR transcriptional dual regulator	5′-TGATCCTGCTGTTGTCGAAG-3′ 5′-AGTTACCGATATCGCCACGA-3′	138
rrsA	rrnA 16S ribosomal RNA	5′-AGGCCTTCGGGTTGTAAAGT-3′ 5′-ATTCCGATTAACGCTTGCAC-3′	147

fixed at 7. The growth was performed with different oxygen concentrations (1%, 2.5%, 5%, and 10% OCH). For each culture the mRNA was extracted and the level of expression of the glycolysis related genes, genes expressed under anaerobic fermentative conditions (Fig. 1), and the oxygen regulation genes were studied using QRT-PCR. In the following results, the relative expression levels of each gene are compared in cultures of the wild-type *E. coli*, the *arcA* mutant the *fnr* mutant, and the double *arcA*, *fnr* mutant at different oxygen concentrations. However, the expression level of different genes cannot be compared using these results since the absolute concentration of each mRNA was not determined. The results are given as an average of four samples that were prepared using the same RNA. Thus, experimental errors as a result of RNA isolation were not considered. However, expression levels of genes that should not be subject to variable expression at the different growth conditions (i.e., *rrsA* and *dnaA*) show that the errors involving RNA isolation should be lower than 3%.

Oxygen Regulation Genes

The expression level of *fnr*, *arcA*, and *arcB* in the different mutant strains during microaerobic growth is shown in Figure 2. For each gene the level of expression was calculated relative to the expression observed in the wild-type at OCH of 10% as described in the Materials and Methods section. The *fnr* expression levels are shown in Figure 2A. No *fnr* expression was detected in cultures of the *fnr* mutant and the *arcA*, *fnr* double mutant strains, which confirmed the nature of the mutations. The expression of *fnr* was constant at all OCH in the wild-type. Indeed, it was previously suggested that the concentration level of the Fnr protein is similar under both aerobic and anaerobic condition (Becker et al., 1996; Gunsalus, 1992). The constant expression level of *fnr* observed in the present study suggests that the experimental errors as a result of RNA isolation are not significant. Higher expression levels of *fnr* were observed in cultures of the *arcA* mutant strain compared to the wild-type at OCH 1%–10%

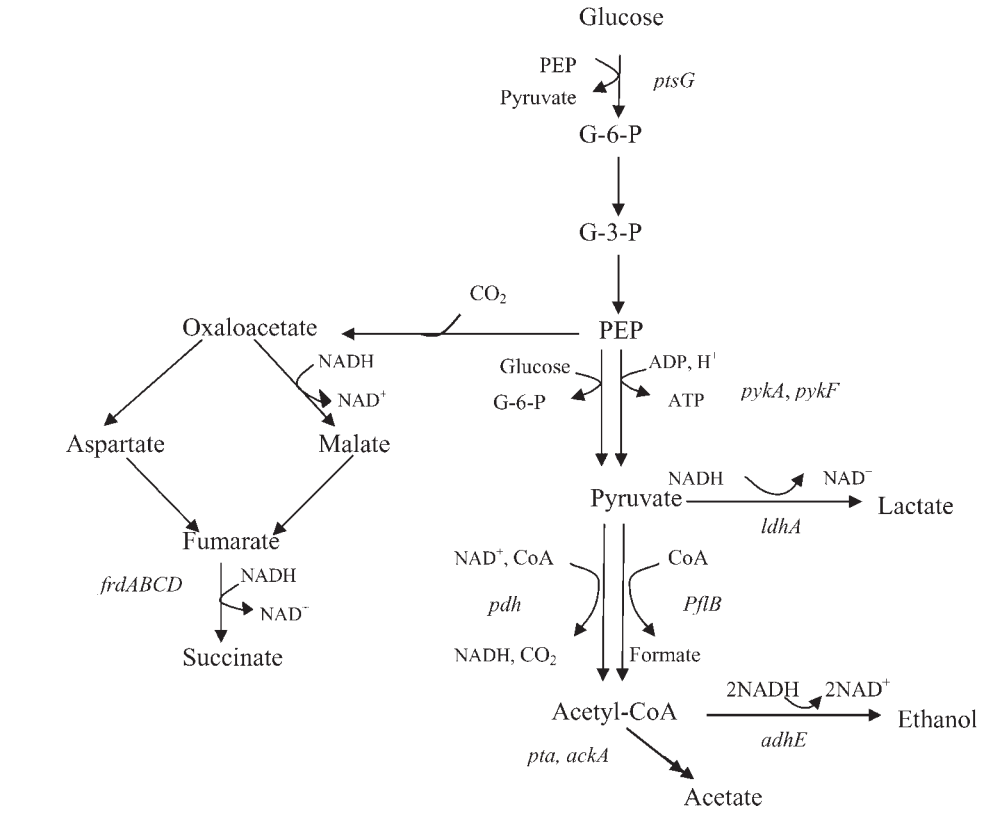

Figure 1. Major anaerobic catabolic pathways in *E. coli*. The definition of the genes can be found in Table II.

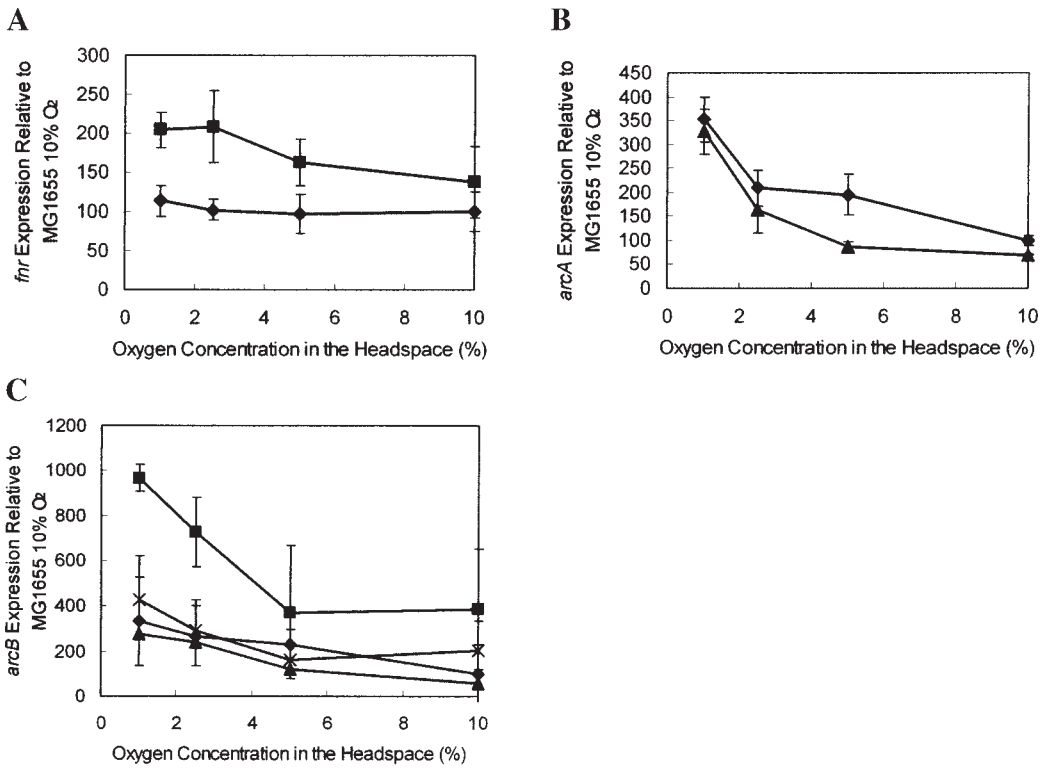

Figure 2. Expression of oxygen regulation gene as function of oxygen concentration in the headspace at steady state. The level of expression was calculated relative to the expression in cultures of the wild-type at 10% OCH as described in the Materials and Methods section. (**A**) *fnr*, (**B**) *arcA*, (**C**) *arcB*. (◆) MG1655, (■) MG1655 [*arcA*], (▲) MG1655 [*fnr*], (×) MG1655 [*arcA*, *fnr*]. The error bars indicate the standard deviation of four samples taken from the same RNA sample.

(up to twofold higher, $P < 0.05$, with the exception of 10% OCH). This suggests that ArcA has a slightly negative effect on *fnr* expression.

The *arcA* expression levels are shown in Figure 2B. No *arcA* expression was detected in the *arcA* mutant and the *arcA*, *fnr* double mutant strains, which was consistent with the expected expression phenotype of the mutant strains. The level of *arcA* expression was increased with the decrease in oxygen concentration in the wild-type strain as well as in the *fnr* mutant strain (3.5-fold higher at 1% OCH compared to 10% OCH in the wild-type, and 4.7-fold higher at 1% OCH compared to 10% OCH in the *fnr* mutant strain, $P < 0.05$). Lower levels of *arcA* expression were obtained in the *fnr* mutant strain compared to the wild-type at OCH 5%–10% (1.4- to 2.3-fold lower, $P < 0.05$) however, the expression levels of *arcA* were similar in both strains at 1%–2.5% OCH. This suggests that Fnr has a minor positive effect or no effect on *arcA* expression under our conditions. These results are in contrast to the previous report that shows a positive effect of Fnr on *arcA* expression under anaerobic conditions (Guest et al., 1996). Since Fnr also affects the Arc system by mediation of metabolic responses which affect ArcB function (Lynch and Lin, 1996) and since active ArcA has also a positive effect on its own expression (Guest et al., 1996) it is possible that the effect of Fnr on *arcA* expression is more pronounced under anaerobic conditions. Indeed, it was previously shown that the Fnr protein has a minor effect on the fermentative metabolite fluxes under microaerobic conditions (OCH higher than 2.5%) (Shalel-Levanon et al., 2005).

The *arcB* expression levels are shown in Figure 2C. The level of *arcB* expression was increased with the decrease in oxygen concentration in all strains (3.3-fold higher at 1% OCH compared to 10% OCH in the wild-type, 2.5-fold higher at 1% OCH compared to 10% OCH in the *arcA* mutant strain, 4.8-fold higher at 1% OCH compared to 10% OCH in the *fnr* mutant strain, and 2.1-fold higher at 1% OCH compared to 10% OCH in the *arcA*, *fnr* double mutant strain $P < 0.05$).The expression levels of *arcB* were the highest in cultures of the *arcA* mutant strain (1.6- to 3.8-fold higher than the expression in the wild-type, $P < 0.1$). Similar expression levels were observed in the wild-type, the *fnr* mutant, and the *arcA*, *fnr* double mutant.

Glycolysis Related Genes

The expression levels of *ptsG*, *pfkA*, *pykA*, and *pykF* in the different mutant strains during microaerobic growth are shown in Figure 3. The expression levels of *pfkA*, *pykA*, and *pykF* (Fig. 3B,C,D) were the highest in cultures of the *arcA* mutant strain at 1%–5% OCH ($P < 0.1$), while the levels of *ptsG* (Fig. 3A) expression in cultures of the *arcA* mutant strain were similar to the levels obtained in the wild-type. Similar expression levels of each of the four genes were observed in cultures of the *fnr* mutant strain and the *arcA*, *fnr*

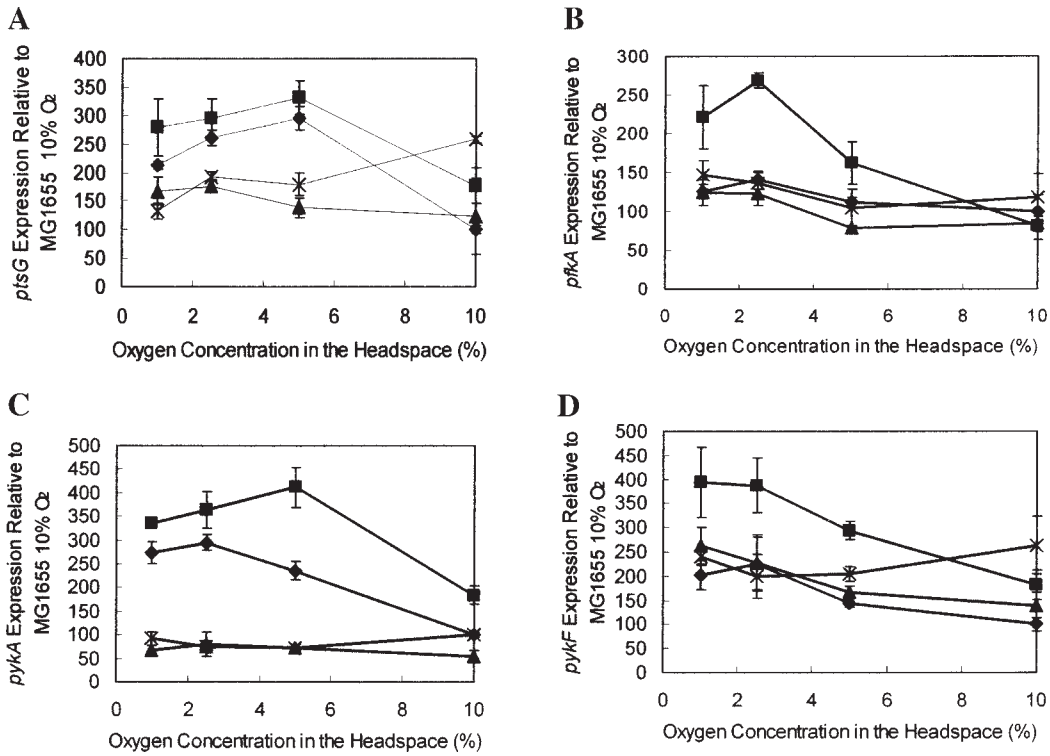

Figure 3. Glycolytic pathway gene expression as function of oxygen concentration in the headspace at steady state. The level of expression was calculated relative to the expression in cultures of the wild-type at 10% OCH as described in the Materials and Methods section. (**A**) *ptsG*, (**B**) *pfkA*, (**C**) pykA, (**D**) *pykF*. (◆) MG1655, (■) MG1655 [*arcA*], (▲) MG1655 [*fnr*], (×) MG1655 [*arcA*, *fnr*]. The error bars indicate the standard deviation of four samples taken from the same RNA sample.

double mutant strain at OCH 1%–5%. The expression levels of both *pfkA* (Fig. 3B) and *pykF* (Fig. 3D) in cultures of the *fnr* mutant and the *arcA*, *fnr* double mutant strains were also similar to that obtained in the wild-type at OCH 1%–5%. However, the levels of both *ptsG* (Fig. 3A) and *pykA* (Fig. 3C) expression were higher in the wild-type compared to the *fnr* and the *arcA*, *fnr* double mutant strains at OCH 1%–5% ($P < 0.05$). These results suggest that Fnr has a minor or no effect on *pfkA* and *pykF*, while it has a positive effect on *ptsG* and *pykA*.

Pyruvate Metabolism

Pyruvate can be metabolized to lactate by LDH, or converted to acetyl-CoA by pyruvate dehydrogenase or PFL. The expression levels of *ldhA*, *aceE*, and PFL related genes were studied. The levels of *ldhA* expression are shown in Figure 4. As can be seen similar expression levels were obtained in cultures of the *fnr* mutant strain and in the wild-type at OCH 1%–10%, and in cultures of the *arcA* mutant strain and the *arcA*, *fnr* double mutant strain at OCH 1%–5%. The levels of *ldhA* expression in cultures of the *arcA* mutant and the *arcA*, *fnr* double mutant strains were higher compared to the wild-type at OCH 1%–5% (2.2- to 4.5-fold higher, $P < 0.05$). These results suggest that Fnr has a minor or no effect on *ldhA* expression, while ArcA has a negative effect.

The expression of *aceE* (part of the pyruvate dehydrogenase complex) is shown in Figure 5. The expression levels of *aceE* were the highest in cultures of the *arcA* mutant strain (2.4- to 5.4-fold higher than the expression in the wild-type) and the lowest in the wild-type at OCH 1%–5% ($P < 0.05$). Similar expression levels were observed in cultures of the *fnr* mutant strain and the *arcA*, *fnr* double mutant strain at OCH 1%–5%. These results suggest that both ArcA and Fnr have a negative effect on *aceE* expression. However, since similar expression levels were obtained in cultures of the *fnr* mutant and the *arcA*, *fnr* double mutant strains, it seems that the

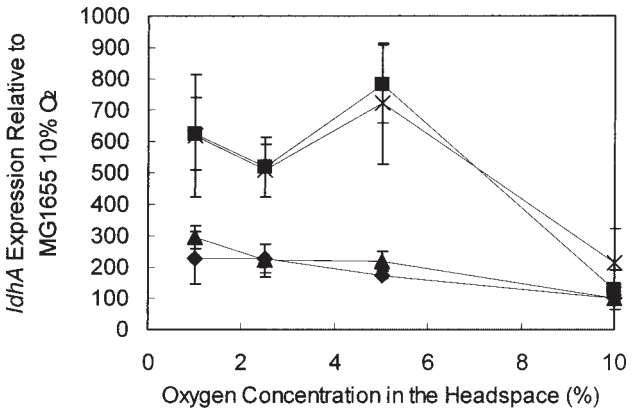

Figure 4. *ldhA* expression as function of oxygen concentration in the headspace at steady state. The level of expression was calculated relative to the expression in cultures of the wild-type at 10% OCH as described in the Materials and Methods section. (◆) MG1655, (■) MG1655 [*arcA*], (▲) MG1655 [*fnr*], (×) MG1655 [*arcA*, *fnr*]. The error bars indicate the standard deviation of four samples taken from the same RNA sample.

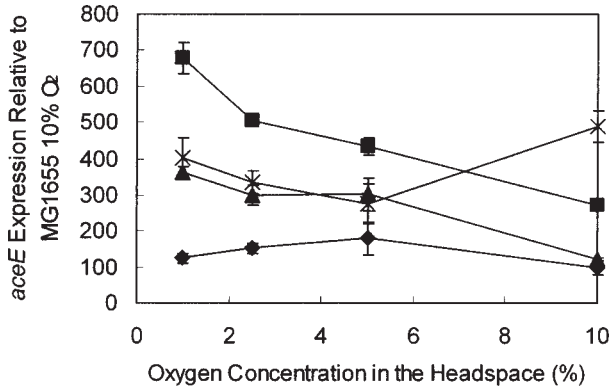

Figure 5. *aceE* expression as function of oxygen concentration in the headspace at steady state. The level of expression was calculated relative to the expression in cultures of the wild-type at 10% OCH as described in the Materials and Methods section. (◆) MG1655, (■) MG1655 [*arcA*], (▲) MG1655 [*fnr*], (×) MG1655 [*arcA*, *fnr*]. The error bars indicate the standard deviation of four samples taken from the same RNA sample.

negative effect of ArcA and Fnr on *aceE* is not additive. Indeed, Quail et al. (1994) suggested that Fnr both activates and represses *aceE* expression (activates the *pdh* promoter and represses the *ace* promoter) under anaerobic conditions.

The expression levels of PFL related genes are shown in Figure 6. The *pflB* gene expresses a protein (Pfl) that is inactive without activator. The Pfl protein is activated by the Act protein (encoded by *pflA*). The active form of the Pfl protein is a radical form and is sensitive to oxygen. The effect of oxygen is irreversible (Kessler and Knappe, 1996). However, recently it was found that the YfiD (encoded by *yfiD*) protein can reactivate the Pfl protein in the presence of oxygen by a special mechanism (Wagner et al., 2001; Wyborn et al., 2002).

The levels of *pflB* expression in the different mutant strains are shown in Figure 6A. As can be seen the levels of *pflB* expression were the highest in cultures of the *arcA* mutant strain at OCH 1%–10% (less than twofold higher compared to the wild-type, $P < 0.09$) and the lowest in cultures of the *arcA*, *fnr* double mutant strain at OCH 1%–5% (2.1- to 2.7-fold lower compared to the wild-type, $P < 0.05$). The levels of *pflB* expression in the *fnr* mutant strain were lower compared to the wild-type at OCH 5%–10% (1.7- to 1.9-fold lower, $P < 0.05$), and similar at 1%–2.5% OCH. These results suggest that both ArcA and Fnr are essential for *pflB* activation. Indeed, it was previously suggested that *pflB* is transcribed from seven promoters and that both ArcA and Fnr are essential for anaerobic activation of promoter 7 transcription (Sawers and Suppmann, 1992). This may explain why the effect of ArcA and Fnr on *pflB* expression is more significant in cultures of the *arcA*, *fnr* double mutant strain compared to a single *arcA* or *fnr* disruption.

The levels of *pflA* expression are shown in Figure 6B. It seems that the expression of *pflA* was independent of ArcA and FNR regulators as the expression levels were similar (within the experimental errors) in all strains at all OCH.

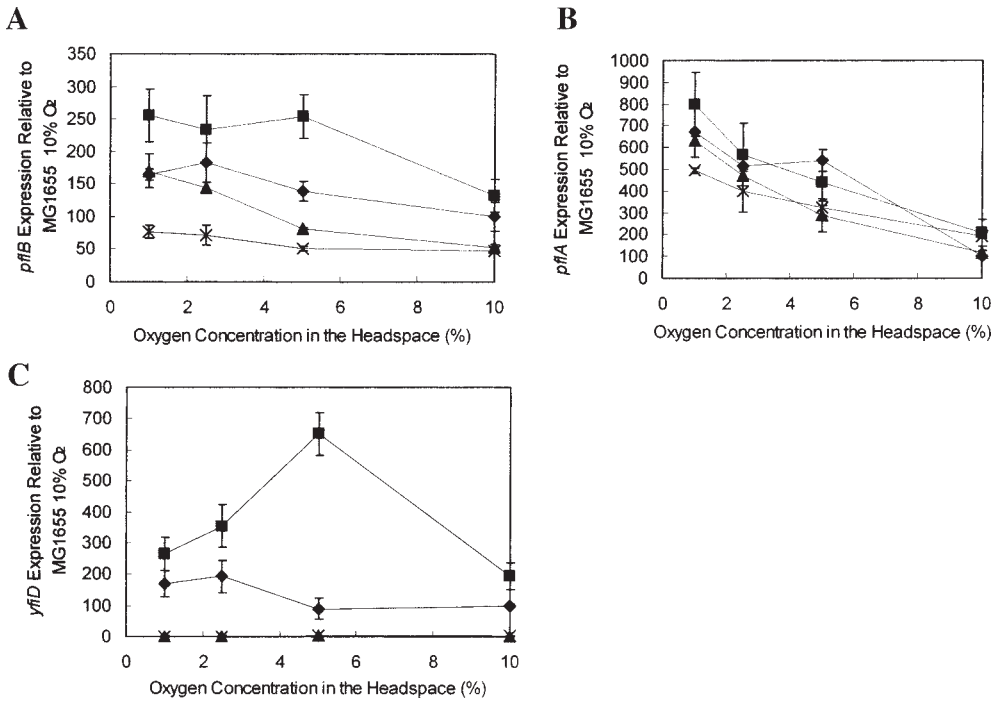

Figure 6. PFL related gene expression as function of oxygen concentration in the headspace at steady state. The level of expression was calculated relative to the expression in cultures of the wild-type at 10% OCH as described in the Materials and Methods section. (**A**) *pflB*, (**B**) *pflA*, (**C**) *yfiD*. (◆) MG1655, (■) MG1655 [*arcA*], (▲) MG1655 [*fnr*], (×) MG1655 [*arcA*, *fnr*]. The error bars indicate the standard deviation of four samples taken from the same RNA sample.

Indeed Sauter and Sawers (1990) also suggested that the expression of *pflA* is independent of the transcriptional regulator, FNR. In addition to *pflA*, Pfl enzyme has other activators such as *pflC* (encodes PFL activating enzyme 2) and *pflD* (encodes formate acetyltransferase 2). The expression levels of both *pflC* and *pflD* were also independent of the ArcA and FNR regulators (data not shown).

The expression levels of *yfiD* are presented in Figure 6C. As can be seen the levels of *yfiD* expression in the *fnr* mutant and the *arcA*, *fnr* double mutant strains were significantly lower compared to the wild-type (comparative expression level were lower than 2 for both mutants). This suggests that the expression of the *yfiD* gene is under a positive control of the Fnr protein. The levels of *yfiD* expression in the *arcA* mutant strain were higher than the expression in the wild-type at 2.5%–10% OCH (1.8- to 7.3-fold higher, $P < 0.01$, with the exception of 2.5% for which the *P*-value was 0.1).

Fermentative Genes

Under anaerobic conditions, acetyl-CoA forms either ethanol or acetate instead of combining with oxaloacetate to form citrate as it does under aerobic conditions, while succinate dehydrogenase is replaced by fumarate reductase. In the present study, the expression levels of the fermentative genes, *adhE*, *pta*, *ackA*, and *frdA*, were studied under microaerobic conditions.

The levels of *adhE* expression in the different mutant strains are shown in Figure 7A. As can be seen the levels of a*dhE* expression were the highest in cultures of the *arcA* mutant strain at 1%–5% OCH (2.9- to 3.5-fold higher than the wild-type, $P < 0.05$). Similar levels of *adhE* expression were observed in cultures of the *fnr* mutant strain, the *arcA*, *fnr* double mutant strain, and the wild-type, which suggest that Fnr has a minor or no effect on *adhE* expression. Indeed, Leonardo et al. (1993) also reported that a mutation in *fnr* had no effect on *adhE* expression.

The levels of *pta* expression are shown in Figure 7B. Similar levels of *pta* expression were obtained in cultures of the *arcA* mutant strain and the wild-type at 1%–5% OCH, while similar expression levels were also obtained in cultures of the *fnr* mutant and the *arcA*, *fnr* double mutant strains at 1%–5% OCH. The levels of *pta* expression in cultures of the *fnr* mutant and *arcA*, *fnr* double mutant strains were lower than the levels in the wild-type at 1%–5% OCH (1.7- to 2.4-fold lower, $P < 0.05$). These results suggest that ArcA has no effect on *pta* expression while Fnr has a slightly positive effect.

The levels of *ackA* expression are shown in Figure 7C. The expression levels of *ackA* were the highest in the wild-type ($P < 0.1$) and the lowest in cultures of the *arcA*, *fnr* double mutant strain (4.3- to 5.9-fold lower than the wild-type, $P < 0.06$) at 1%–5% OCH. The levels of *ackA* expression were 1.4- to 1.6-fold lower in cultures of the *arcA* mutant strain compared to the wild-type ($P < 0.1$) and 2.5- to 3.3-fold lower in cultures of the *fnr* mutant strain ($P < 0.05$) at 1%–5% OCH. These results suggest that ArcA as a minor positive effect while Fnr has a positive effect on *ackA* expression. Since the levels of *ackA* expression were the lowest in cultures of the *arcA*, *fnr* double mutant strain it

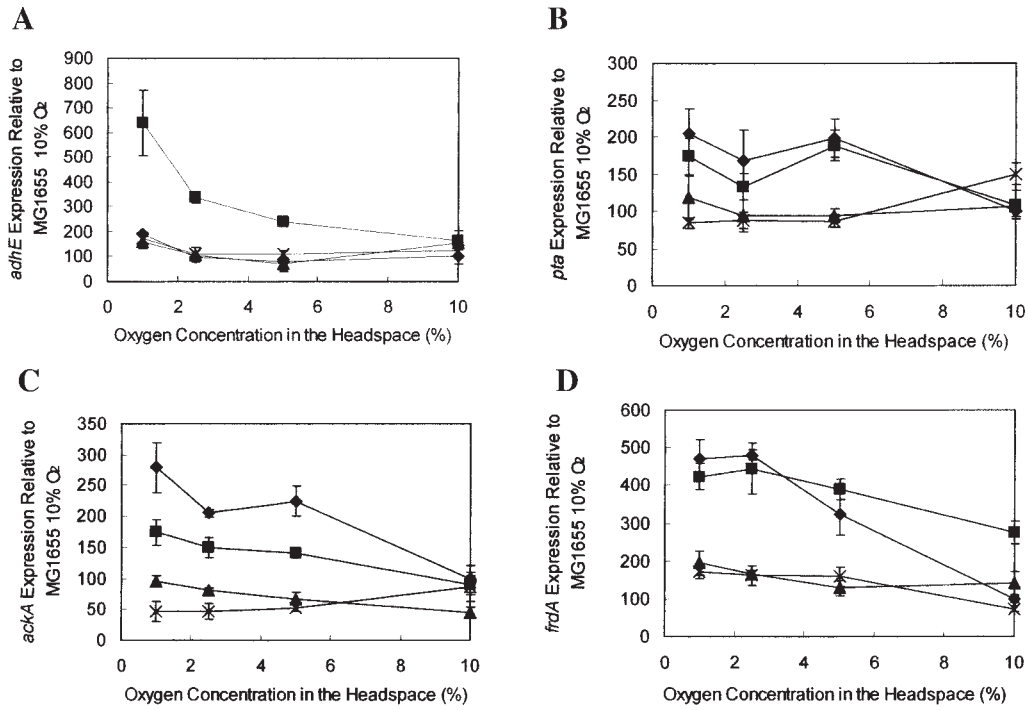

Figure 7. Fermentative gene expression as function oxygen concentration in the headspace at steady state. The level of expression was calculated relative to the expression in cultures of the wild-type at 10% OCH as described in the Materials and Methods section. (**A**) *adhE*, (**B**) *pta*, (**C**) *ackA*, (**D**) *frdA*. (◆) MG1655, (■) MG1655 [*arcA*], (▲) MG1655 [*fnr*], (×) MG1655 [*acrcA*, *fnr*]. The error bars indicate the standard deviation of four samples taken from the same RNA sample.

seems that the positive effect of ArcA and Fnr on *ackA* expression is independent.

The levels of *frdA* expression are shown in Figure 7D. As can be seen the levels of *frdA* expression were higher in cultures of the *arcA* mutant strain compared to the wild-type at 5% and 10% OCH (1.2- and 2.7-fold higher, respectively, $P < 0.05$), while they were similar at 1% and 2.5%. Similar expression levels of *frdA* were obtained in cultures of the *fnr* mutant and the *arcA*, *fnr* double mutant strains, and they were lower than the levels in the wild-type at 1%–5% OCH (two- to threefold lower, $P < 0.05$). A lower expression of *frdA* in cultures of an *fnr* mutant strain compared to a wild-type *E. coli* (*E. coli* MC4100) was previously observed under microaerobic conditions (Tseng et al., 1996). In their study, the effect of Fnr protein on *frdA* expression was more pronounced. However, the differences may result from different experimental conditions.

DISCUSSION

The aim of this study was to examine the effect of ArcA and Fnr on gene expression during microaerobic conditions. This has been done by comparing the level of genes expression at steady state in a wild-type *E. coli*, an *arcA* mutant, an *fnr* mutant, and a double *arcA*, *fnr* mutant, in glucose limiting cultures and oxygen concentrations in the headspace of 1%–10%. Table III summarizes the effect of ArcA and FNR

Table III. The effect of ArcA and FNR on the glycolysis related genes, and genes expressed under anaerobic fermentative conditions.

		Oxygen concentration in the headspace (%)		
Gene	Regulator	1–2.5	2.5–5	5–10
ptsG	ArcA	−	−	−
	FNR	+	+	+
	Dominance	FNR	FNR	FNR
pykA	ArcA	−	−	−
	FNR	+++	+++	+++
	Dominance	FNR	FNR	FNR
ldhA	ArcA	−−	−−	−−−
	FNR	NE	NE	NE
	Dominance	ArcA	ArcA	ArcA
aceE	ArcA	−−−	−−−	−−
	FNR	−−	−	−
	Dominance	ArcA	ArcA	ArcA
pflB	ArcA	−	−	−
	FNR	+	+	NE
	Dominance	Both	Both	Both
pta	ArcA	NE	NE	NE
	FNR	+	+	+
	Dominance	FNR	FNR	FNR
ackA	ArcA	+	+	+
	FNR	++	++	++
	Dominance	FNR	FNR	FNR

−, −−, −−− indicates weak (less than twofold), medium (two- to threefold), and strong (higher than threefold) suppressive effect, respectively.

+, ++, +++ indicates weak (less than twofold), medium (two- to threefold), strong (higher than threefold) inductive effect, respectively.

NE, negligible effect.

regulators on the glycolysis related genes, and the genes expressed under anaerobic fermentative conditions (Fig. 1). In this table, the individual effect of ArcA and FNR is summarized in addition to the dominant effect. For example, ArcA and Fnr have opposite effects on *pykA*; ArcA weakly represses the expression of *pykA* while FNR strongly activates it. Thus, FNR has a dominant effect on *pykA* expression.

In a previous work it was shown that in the *arcA* mutant strain the cells behave as if a higher level of the FNR regulator is in the activated form compared to the wild-type strain during the transition from aerobic to microanaerobic growth (Shalel-Levanon et al., 2005). In particular, a significant increase in the flux through PFL in the presence of oxygen was observed. Moreover, in cases where both ArcA and Fnr proteins were absent (double mutant) a significant amount of lactate was obtained under microaerobic growth. These high lactate fluxes were correlated with the low conversion of pyruvate to acetyl-CoA in this strain. To further support these conclusions the present discussion will be focused on the expression levels of the FNR regulated genes: *frdA* and *yfiD*; the PFL related genes: *pflA*, *pflB*, *yfiD*, and *adhE*; and the gene responsible for high lactate flux: *ldhA*.

To test the hypotheses that higher level of the FNR regulator is in the activated form in the *arcA* mutant strain compared to the wild-type, the expression level of *fnr* was first studied. Indeed it was found that higher levels of *fnr* were expressed in the *arcA* mutant strain compared to the wild-type at OCH 1%–10% (Fig. 2A). However, higher expression of the *fnr* gene in cultures of the *arcA* mutant strain does not necessarily indicate that the Fnr protein is more active in this strain compared to the wild-type. To confirm that the Fnr protein is more active in cultures of the *arcA* mutant strain, one must compare the expression level of FNR regulated genes. The expression of *frdA* gene is commonly used to study the activity of the Fnr regulator protein (see for example Becker et al., 1996). Indeed, the levels of *frdA* expression were lower in cultures of the *fnr* mutant strain and the *arcA*, *fnr* double mutant strain at OCH of 1%–5% compared to the wild-type (Fig. 7D). These results suggest that *frdA* expression is activated by the Fnr regulation protein.

Table IV. *fnr* relative expression level at 1%–10% OCH in a wild-type *E. coli*, an *arcA* mutant, and a double *arcA*, *fnr* mutant strain that carries the pRZ7382 plasmid.

OCH (%)	MG1655	MG1655 [*arcA*]	MG1655 [*arcA*, *fnr*] + pRZ7382
10	100 ± 24	138 ± 31	5083 ± 260
5	97 ± 25	162 ± 24	7441 ± 805
2.5	102 ± 13	208 ± 18	14056 ± 890
1	114 ± 20	204 ± 23	10579 ± 24

The level of expression was calculated relative to the expression in cultures of the wild-type at 10% OCH as described in the Materials and Methods section.

Higher levels of *frdA* expression were obtained in cultures of the *arcA* mutant strain compared to the wild-type at 5%–10% OCH while at lower OCH (1%–2.5%) similar expression levels were obtained in both strains. The higher expression of *frdA* in cultures of the *arcA* mutant strain may suggest that a higher level of Fnr protein is in the activated state in cultures of the *arcA* mutant strain compared to the wild-type.

To test the effect of the Fnr protein on *frdA* expression, we transferred a plasmid that expresses a mutated Fnr protein under the control of the *lac* promoter (pRZ7382) into the *arcA*, *fnr* double mutant strain. This mutated Fnr is active even in the presence of oxygen (Bates et al., 1995, 2000). Moreover, the expression levels of the *fnr* gene were higher in cultures of the *arcA*, *fnr* double mutant that carries the pRZ7382 plasmid compared to the non-plasmid bearing, wild-type, and *arcA* mutant strains (Table IV). The expression levels of *frdA* in the wild-type and in cultures of the *arcA* mutant strain, and the *arcA*, *fnr* double mutant strain that carries the pRZ7382 plasmid are shown in Figure 8A. As can be seen the expression levels of *frdA* in cultures of the *arcA*, *fnr* double mutant strain that carries the pRZ7382 plasmid were higher than the levels obtained in the wild-type and in cultures of the *arcA* mutant strain at 2.5%–10% OCH (4.8-fold higher at 10% and less than twofold higher at 2.5%–5% OCH compared to the wild-type, $P < 0.05$). At 1% OCH, the expression level of *frdA* in cultures of the *arcA*, *fnr* double mutant strain that carries the pRZ7382 plasmid dropped to a value even slightly lower than that obtained in cultures of the

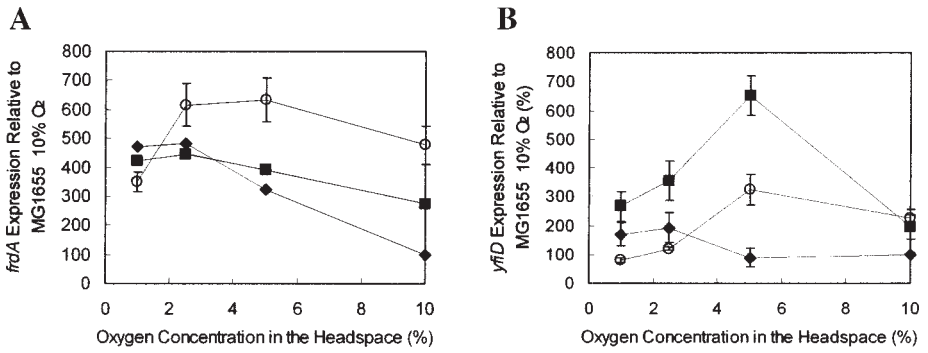

Figure 8. Effect of an active Fnr or *frdA*, and *yfiD* expression. The level of expression was calculated relative to the expression in cultures of the wild-type at 10% OCH as described in the Materials and Methods section. (**A**) *frdA*, (**B**) *yfiD*. (◆) MG1655, (■) MG1655 [*arcA*], (○) MG1655 [*arcA*, *fnr*] + pRZ7382. The error bars indicate the standard deviation of four samples taken from the same RNA sample.

arcA mutant strain and in the wild-type. These results suggest that the effect of higher levels of active Fnr protein (compared to the wild-type) on *frdA* expression is most significant at 5% and 10% OCH. Thus, it is possible that a higher level of Fnr protein is in the activated state in cultures of the *arcA* mutant strain compared to the wild-type.

To further confirm that a higher level of the Fnr regulator protein is in the activated state in culture of the *arcA* mutant strain compared to the wild-type, the expression of *yfiD* gene was also studied. This gene was chosen since it was previously suggested that its expression is under the control of FNR regulator (Green et al., 1998; Marshall et al., 2001; Wyborn et al., 2002). Indeed, a significantly lower expression of *yfiD* was observed in cultures of the *fnr* mutant and the *arcA*, *fnr* double mutant strains compared to the wild-type at 1%–10% OCH (more than 50-fold lower) (Fig. 6C), which suggests that the effect of Fnr on *yfiD* expression is much more pronounced than its effect on *frdA* expression. The levels of *yfiD* expression were higher in cultures of the *arcA* mutant strain compared to the wild-type at all OCH. At 5% OCH, the expression level of *yfiD* in cultures of the *arcA* mutant strain was 7.3-fold higher than the levels in the wild-type. However, at 2.5% OCH, the level of *yfiD* expression dropped in cultures of the *arcA* mutant strain while it increased in cultures of the wild-type.

The effect of an active Fnr protein on *yfiD* expression under our microaerobic conditions was studied in cultures of the *arcA*, *fnr* double mutant strain that carries the pRZ7382 plasmid. The expression levels of *yfiD* in the wild-type and in cultures of the *arcA* mutant strain, and the *arcA*, *fnr* double mutant strain that carries the pRZ7382 plasmid are shown in Figure 8B. As can be seen the expression levels of *yfiD* in cultures of the *arcA*, *fnr* double mutant strain that carries the pRZ7382 plasmid were higher than the levels obtained in the wild-type at 10% and 5% OCH (2.2- and 3.6-fold higher, respectively, $P < 0.05$), however they were lower than the levels obtained in cultures of the *arcA* mutant strain at 5% OCH (twofold lower, $P < 0.05$). At 1%–2.5% OCH, the levels of *yfiD* expression in cultures of the *arcA*, *fnr* double mutant strain that carries the pRZ7382 plasmid were lower than the levels obtained in the wild-type (1.6- to 2.1-fold lower, $P < 0.05$). Thus, it seems that the positive effect of an active Fnr protein on *yfiD* expression in cultures of the *arcA* mutant strain is most significant at 5% and 10% OCH. The lower expression of *yfiD* in cultures of the *arcA*, *fnr* double mutant that carries the pRZ7382 plasmid compared to the expression in cultures of the *arcA* mutant strain may result from a dual effect of Fnr protein on *yfiD* expression. It was previously suggested that the promoter of the *yfiD* gene contains two FNR sites; activation of *yfiD* expression by Fnr was shown to be mainly mediated by the FNR regulator bound at the promoter proximal FNR site, while Fnr occupation of the distal FNR site downregulates *yfiD* expression (Green et al., 1998). In addition it was suggested that the FNR-activated site is a high-affinity site while the FNR-repressed site is a lower affinity site (Marshall et al., 2001). The dual effect of Fnr protein on *yfiD* expression can also explain the decrease in *yfiD* expression levels in cultures of the *arcA* mutant strain at OCH 2.5% and 1%. Thus, it seems that a higher level of the Fnr protein is in the activated state in cultures of the *arcA* mutant strain compared to that found in the wild-type.

Three functionally different forms of FNR have been previously described (Khoroshilova et al., 1995; Lazazzera et al., 1996; Sutton et al., 2004; Tran et al., 2000). The active Fnr protein is a dimer and carries one $[4Fe\text{-}4S]^{2+}$ cluster per monomer which is liganded by four cysteine residues ([4Fe-4S] FNR). In the presence of oxygen, the $[4Fe\text{-}4S]^{2+}$ cluster is converted to a $[2Fe\text{-}2S]^{2+}$ cluster which inactivates FNR as a transcriptional regulator. After prolonged incubation with O_2, the [2Fe-2S] cluster further disintegrates and FNR devoid of Fe and sulfide is formed (apoFNR). It was shown that [4Fe-4S] FNR can be reconstituted in vitro using cysteine, cysteine desulfurase, Fe(II) ion, dithiothreitol, and glutathione as reducing agent (Green et al., 1993; Khoroshilova et al., 1995; Lazazzera et al., 1996; Unden et al., 2002). In addition, glutathione was shown to affect the functional state of Fnr (Tran et al., 2000) in vivo. Tran et al. (2000) showed that the O_2 tension for the regulatory switch for FNR-dependent gene (*nirB* and FFp*mel*R) transcription was decreased by a factor of 4–5 in cultures of a mutated *E. coli* that cannot express glutathione compared to the wild-type.

The standard state redox potential of glutathione was previously reported to be −240 mV (Åslund et al., 1997). In a previous study, we have shown that the internal redox potential, as reflected by the $NADH/NAD^+$ ratio, was significantly higher in cultures of the *arcA* mutant strain compared to the wild-type (Shalel-Levanon et al., 2005). The redox potential of NADH is well established at −309 mV (Clark, 1960). Thus it is possible that the higher redox potential in cultures of the *arcA* mutant strain compared to the wild-type affects the oxidation state of glutathione which in turn can affect the activity of the Fnr protein.

It was previously shown that inactivation of the electron transfer chain genes, *cyd* and *ubiA*, did not result in a significant decrease of the half-maximal expression values ($pO_{0.5}$) for the FNR regulated gene (*frdA*) (Becker et al., 1996). These results led to the conclusion that the $NADH/NAD^+$ ratio has no specific effect on Fnr activity. However, to study the effect of the electron transfer chain genes on Fnr activity Becker et al. (1996) examined the expression levels of *frdA* gene using β-galactosidase assay. In the present study it was shown that the effect of Fnr on *frdA* expression is not significant at OCH 1%–5% (Fig. 8A). On the contrary lower expression of *frdA* was observed in cultures of the *arcA*, *fnr* mutant strain that carries the pRZ7382 plasmid compared to the wild-type at 1% OCH. Indeed, Becker et al. (1996) also detected lower levels of *frdA* expression in cultures of a *cyd* mutant strain compared to the wild-type at low oxygen concentrations in the medium but the expression levels were higher in the mutant strain compared to the wild-type at oxygen concentrations higher than ∼8 mbar. Thus, to solve this conflict a further study would be valuable to do on the effect of the redox potential in general, and the glutathione

redox state and NADH/NAD^+ ratio in particular on the activity of the Fnr protein in vivo.

The higher level of active Fnr protein in the *arcA* mutant strain compared to the wild-type under microaerobic conditions mainly results in a significant increase in the fluxes through PFL in the presence of these low levels of oxygen (Shalel-Levanon et al., 2005). One possible mechanism by which the Fnr protein could affect the activity of the Pfl enzyme is by the activation of *yfiD*, which activates the Pfl protein in the presence of oxygen (Wagner et al., 2001; Wyborn et al., 2002). Higher fluxes through PFL can also result from higher expression of *pflB* and/or *pflA*. The expression of *pflA* seems to be independent of ArcA and FNR regulators (Fig. 6B), however, somewhat higher expression levels of *pflB* were obtained in cultures of the *arcA* mutant strain compared to the wild-type (Fig. 6A). This higher expression of *pflB* in cultures of the *arcA* mutant strain compared to the wild-type is in contrast to Alexeeva et al. (2000) report of a significantly lower expression of *focApfl* expression in cultures of the *arcA* mutant strain compared to the wild-type. However, they monitored the expression of *focApfl* as a chromosomal *lacZ* fusion while here we measured the *pflB* expression using QRT-PCR.

The Pfl protein can be deactivated by alcohol dehydrogenase (the product of *adhE*), however, CoA and NAD^+ are required for the glycyl radical removal by AdhE (Kessler and Knappe, 1996). Thus, although the highest expression levels of *adhE* were obtained in cultures of the *arcA* mutant strain (Fig. 7A) it is possible that the Pfl protein is more active in this strain as the NADH/NAD^+ ratio was significantly higher in cultures of the *arcA* mutant strain at 1%–10% OCH (even higher than the ratio at complete anaerobic conditions) compared to the wild-type (Shalel-Levanon et al., 2005). The high NADH/NAD^+ ratio in cultures of the *arcA* mutant strain may be responsible for the higher expression levels of *adhE* in this strain, since NADH/NAD^+ ratio was previously suggested to be a signal for transcriptional regulation of *adhE* (Leonardo et al., 1993). However, a high NADH/NAD^+ ratio was also observed in cultures of the *arcA*, *fnr* double mutant strain (Shalel-Levanon et al., 2005) while the expression level of *adhE* in this strain was lower than the expression in cultures of the *arcA* mutant strain (Fig. 7A). The lower levels of *adhE* expression in the *arcA*, *fnr* double mutant strain compared to the *arcA* mutant strain also suggest that ArcA by itself cannot account for the highest expression levels of *adhE* in cultures of the *arcA* mutant strain. Thus, further study should be done to understand the high expression levels of *adhE* in cultures of the *arcA* mutant strain.

As mentioned above, in a previous study we observed significantly higher fluxes toward lactate in cultures of the *arcA*, *fnr* double mutant strain compared to all other strains at 1%–10% OCH (Shalel-Levanon et al., 2005). To examine whether these high lactate fluxes result from higher expression of *ldhA*, the expression of this gene was compared in the different strains. The levels of *ldhA* expression were similar in cultures of the *arcA*, *fnr* double mutant strain and in cultures of the *arcA* mutant strain at OCH 1%–5% (Fig. 4) and they were higher than the levels observed in the wild-type. These results suggest that ArcA has a negative effect on *ldhA* expression. However, since significantly lower fluxes to lactate were observed in cultures of the *arcA* mutant strain compared to the *arcA*, *fnr* double mutant strain at OCH 1%–5% (Shalel-Levanon et al., 2005), the expression level of *ldhA* cannot by itself explain the higher fluxes to lactate in cultures of the *arcA*, *fnr* double mutant.

The main reason for the high fluxes to lactate in cultures of the *arcA*, *fnr* double mutant strain is probably the lower conversion of pyruvate to acetyl-CoA. Pyruvate can be converted to acetyl-CoA either by PFL or PDH. The levels of *pflB* expression were the lowest in cultures of the *arcA*, *fnr* double mutant strain (Fig. 6A), while similar levels of *aceE* (part of the pyruvate dehydrogenase complex) expression were obtained in cultures of the *arcA*, *fnr* double mutant strain and in cultures of the *fnr* mutant strain and they were higher than the levels obtained in the wild-type (Fig. 5). Although the levels of *aceE* expression were higher in cultures of the *arcA*, *fnr* double mutant strain compared to the wild-type, lower fluxes through the PDH pathway are expected in cultures of this strain under microaerobic growth. The reason is that the Pdh enzyme is inhibited by NADH (Nelson and Cox, 2000), and a significantly higher NADH/NAD^+ ratio was previously observed in cultures of the *arcA*, *fnr* double mutant compared to the wild-type at 1%–10% OCH (Shalel-Levanon et al., 2005).

CONCLUSIONS

In this work, the expression levels of the glycolysis related genes, genes expressed under anaerobic fermentative conditions, and the oxygen regulation genes were studied, using QRT-PCR in a wild-type *E. coli*, an *arcA* mutant, an *fnr* mutant, and a double *arcA*-fnr mutant, at steady state in glucose limited cultures and microaerobic concentrations. The results indicate that in the *arcA* mutant strain, the cells behave as if a higher level of the FNR regulator is in the activated form compared to the wild-type strain during the transition from aerobic to microanaerobic growth. The expression levels of the FNR regulated genes, *yfiD* and *frdA*, were higher in cultures of the *arcA* mutant strain compared to the wild-type. Thus, the higher level of active Fnr protein in cultures of the *arcA* mutant strain compared to the wild-type must be taken into consideration when studying the expression level of different genes in this mutant strain under microaerobic conditions. The activity of FNR regulated pathways in the *arcA* mutant strain may result from the high redox potential obtained under microaerobic growth in this strain.

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