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Requirement of ArcA for Redox Regulation in *Escherichia coli* under Microaerobic but Not Anaerobic or Aerobic Conditions

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In *Escherichia coli,* the two-component regulatory ArcAB system functions as a major control system for the regulation of expression of genes encoding enzymes involved in both aerobic and anaerobic catabolic pathways. Previously, we have described the physiological response of wild-type *E. coli* to changes in oxygen availability through the complete range from anaerobiosis to full aerobiosis (S. Alexeeva, B. de Kort, G. Sawers, K. J. Hellingwerf, and M. J. Teixeira de Mattos, J. Bacteriol. 182:4934-4940, 2000, and S. Alexeeva, K. J. Hellingwerf, and M. J. Teixeira de Mattos, J. Bacteriol. 184:1402-1406, 2002). Here, we address the question of the contribution of the ArcAB-dependent transcriptional regulation to this response. Wild-type *E. coli* and a mutant lacking the ArcA regulator were grown in glucose-limited chemostat cultures at controlled levels of oxygen availability ranging from full aerobiosis to complete anaerobiosis. A flux analysis of the distribution of catabolic fluxes over parallel pathways was carried out, and the intracellular redox state (as reflected by the NADH/NAD ratio) was monitored for all steady states. Deletion of ArcA neither significantly altered the in vivo activity of the pyruvate dehydrogenase complex and pyruvate formate lyase nor significantly affected catabolism under fully aerobic and fully anaerobic conditions. In contrast, profound effects of the absence of ArcA were seen under conditions of oxygen-restricted growth: increased respiration, an altered electron flux distribution over the cytochrome *o*- and *d*-terminal oxidases, and a significant change in the intracellular redox state were observed. Thus, the ArcA regulator was found to exert major control on flux distribution, and it is concluded that the ArcAB system should be considered a microaerobic redox regulator.

Catabolic flexibility allows *Escherichia coli* to proliferate under a wide range of environmental conditions. This is most dramatically exemplified by the organism's response to differences in the environment with respect to the availability of oxygen. In environments that provide the cell with excess oxygen, reducing equivalents generated by glycolysis and the Krebs cycle are reoxidized by the respiratory chain, resulting in the buildup of a proton motive force and the subsequent synthesis of ATP by the ATP synthetase. In the absence of oxygen or other external electron acceptors, ATP synthesis occurs at the level of substrate phosphorylation, and the demand for redox neutrality is met by electron transfer from reducing equivalents (such as NADH) to an internal electron acceptor (such as pyruvate or acetyl coenzyme A). When growing on glucose, the latter results in a mixed acid fermentation (7), with ethanol, acetate, lactate, succinate, and formate (or CO_2 and H_2) as its typical products. The relative rate of formation of these products is governed by the demand of redox neutrality (7, 14) and is brought about by the specific activity of a number of catabolic enzymes.

Clearly, aerobic and anaerobic catabolism in *E. coli* require different pathways. Enzymes of each pathway are regulated at the kinetic level as well as at the level of transcription. At least 120 proteins are shown to change expression in response to a shift from aerobic to anaerobic conditions (19). Of these proteins, the expression of at least 40 operons is controlled by the two-component regulatory ArcAB system, and over 30 operons (over 70 genes) are under control of the Fnr regulator (16). These two global regulation systems are the major controlling factors of catabolic gene expression and in most cases operate coordinately to fine-tune catabolism in response to oxygen.

The cytoplasmatic ArcA regulator of the ArcAB system (encoded by the *arcA* gene, previously known as *dye*, *sfrA*, *fexA*, *msp*, or *seg* (see reference 15 and references therein) has a pleiotropic effect on a number of cellular functions. It regulates gene expression in response to deprivation of oxygen and is required for proper expression of catabolic genes, aerobic resistance against redox dyes (methylene blue and toluidine blue), and the expression of F plasmid DNA transfer (*tra*) genes (3, 4). In addition, ArcA is required for efficient *Xer*-based recombination at the *psi* site (in plasmid pSC101), the process that ensures maintenance of the plasmids in a monomeric state and helps to promote stable plasmid inheritance (8). In concert with its cognate sensory kinase ArcB, it represents a global regulation system that negatively or positively controls the expression of operons, such as several dehydrogenases of the flavoprotein class, terminal oxidases, tricarboxylic acid cycle enzymes, enzymes of the glyoxylate shunt, and enzymes of the pathway for fatty acid degradation.

Previously, we described in detail the oxygen-dependent changes in in vivo catabolic activities in glucose-limited chemostat cultures of wild-type *E. coli* (1). A major finding was that significant changes in cellular makeup with respect to enzyme content and intracellular metabolite concentrations,

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on the one hand, and catabolic activity, on the other, occur when cells are grown at various levels of oxygen availability (1, 2). It seems justified to ascribe these findings to the ArcAB system, as it is well documented that the signaling (phosphorylation) state of the ArcAB system is linked to the oxygen availability by a mechanism that possibly involves the quinone pool (12) and has a severe effect on the induction level of many aerobic and anaerobic catabolic enzymes. However, to date, a quantitative characterization of the impact of the system on the cell's physiological behavior is lacking. To quantitatively determine the contribution of the Arc-dependent regulation in catabolism, we studied the changes in the distribution of catabolic fluxes, redox status, and bioenergetics in a mutant lacking the ArcA regulator and compared them to results for the wild-type *E. coli*. We demonstrate that ArcA's most significant role is to adjust catabolism to oxygen-restricted growth conditions associated with carbon flux via the Krebs cycle and with electron flux via the terminal cytochrome oxidases, rather than to adjust catabolism to fully aerobic or anaerobic conditions. The dramatic effects with regard to the intracellular redox state (NADH/NAD ratio) under microaerobic conditions in a strain lacking ArcA allow us to conclude that ArcA is a microaerobic redox regulator.

MATERIALS AND METHODS

Strains and growth conditions. *E. coli* wild-type strain MC4100 (5) and Δ*arcA* strain RM3133 (1) were used throughout this study.

Cells were grown in chemostat cultures under glucose-limited conditions (New Brunswick Bioflo 3000 and III, Applicon types [21]) at a constant dilution rate (D) of 0.15 ± 0.01 h^{-1} at variable oxygen supply rates. Glucose (45 mM) was used as the single carbon and energy source. A previously described simple salts medium (11) was used, but instead of citrate, nitriloacetic acid (2 mM) was used as chelator. Selenite (30 μg/liter) and thiamine (15 mg/liter) were added to the medium. The pH was maintained at 7.0 ± 0.1 by titrating with sterile 4 M NaOH, and the temperature was set at 35°C. The Δ*arcA* genotype was regularly confirmed by Western blot analysis with polyclonal ArcA antiserum. The strains were maintained in vials in Luria-Bertani medium with 30% (wt/vol) glycerol at −70°C.

Oxygen supply was varied by varying the percentage of oxygen in a gas mixture of air and N_2 while stirring the culture at a constant speed (exactly as described previously [2]). In short, fermentor setups were calibrated with two reference conditions. First, a fully anaerobic condition (no oxygen present) was defined as 0% aerobiosis. The second reference point was determined for glucose-limited conditions in each fermentor setup as the minimum oxygen input rate required for complete oxidation of glucose to CO_2 and was defined as 100% aerobiosis.

Analytical procedures. Steady-state bacterial dry weight was measured by the procedure of Herbert et al. (13). Glucose, pyruvate, lactate, formate, acetate, succinate, and ethanol were determined by high-performance liquid chromatography (LKB) with a REZEX organic acid analysis column (Phenomenex) at a temperature of 40°C with 7.2 mM H_2SO_4 as eluent; an RI 1530 refractive index detector (Jasco) and Borwin chromatography software were used for data integration. CO_2 production and O_2 consumption were measured by passing the eluent gas from the fermentor through a Servomex CO_2 analyzer and a Servomex O_2 analyzer, respectively.

Concentrations of NADH and NAD were determined in extracts obtained by rapid sampling of chemostat cultures into 5 M KOH and 5 M HCl, respectively, and assayed after neutralization and filtration as described previously (21).

Calculation of metabolic fluxes from the levels of PDHc, PFL, and the TCA cycle. The analysis of the specific product formation rates (q values; measured in mmol · g [dry weight]$^{-1}$ · h^{-1}) allowed the calculation of the fluxes (J values; measured in mmol · g [dry weight]$^{-1}$ · h^{-1}) through the pyruvate dehydrogenase complex (PDHc), pyruvate formate-lyase (PFL), and the tricarboxylic acid (TCA) cycle. These calculations are performed exactly as described previously (1).

All data present a carbon balance of 95% ± 3%, as calculated from the glucose consumption and product formation rates. All aerobic and anaerobic redox balances were 95 to 98%, again as calculated from all products and biomass.

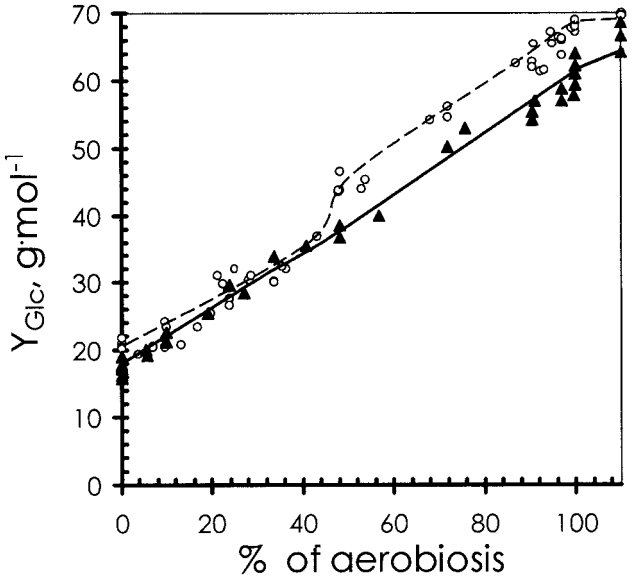

FIG. 1. Effect of the oxygen supply rate on the yield value for glucose (Y_{glc}, calculated as grams of dry weight formed per gram of glucose consumed and expressed as a percentage) in the wild-type (open symbols) and Δ*arcA* (filled symbols) strains.

RESULTS

Effect of an *arcA* deletion on glucose consumption and product formation. Both the wild type and the Δ*arcA* strain were grown in glucose-limited chemostat cultures at a dilution rate of 0.15 h^{-1} with varying availabilities of oxygen expressed as aerobiosis (1, 2). Both strains responded to an increase in oxygen availability with an increasing catabolic efficiency, as indicated by an increase in cell yield per mole of glucose

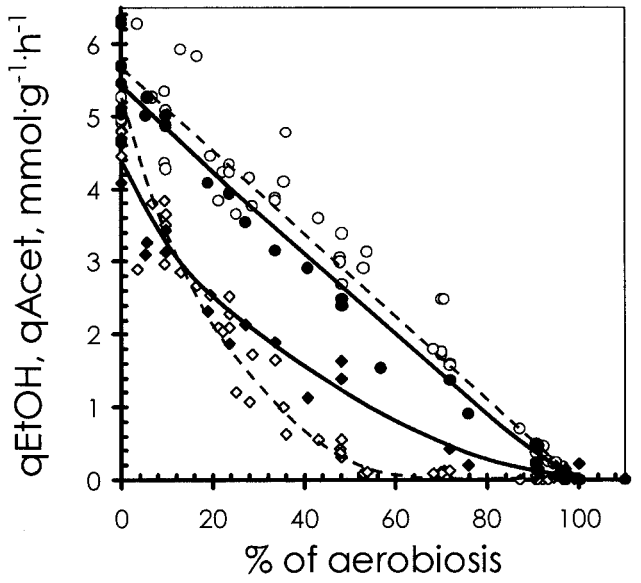

FIG. 2. Effect of the oxygen supply rate on the formation rates of acetate (circles) and ethanol (diamonds) of the wild-type (open symbols) and Δ*arcA* (filled symbols) strains. Data for the wild-type strain are derived from reference 1.

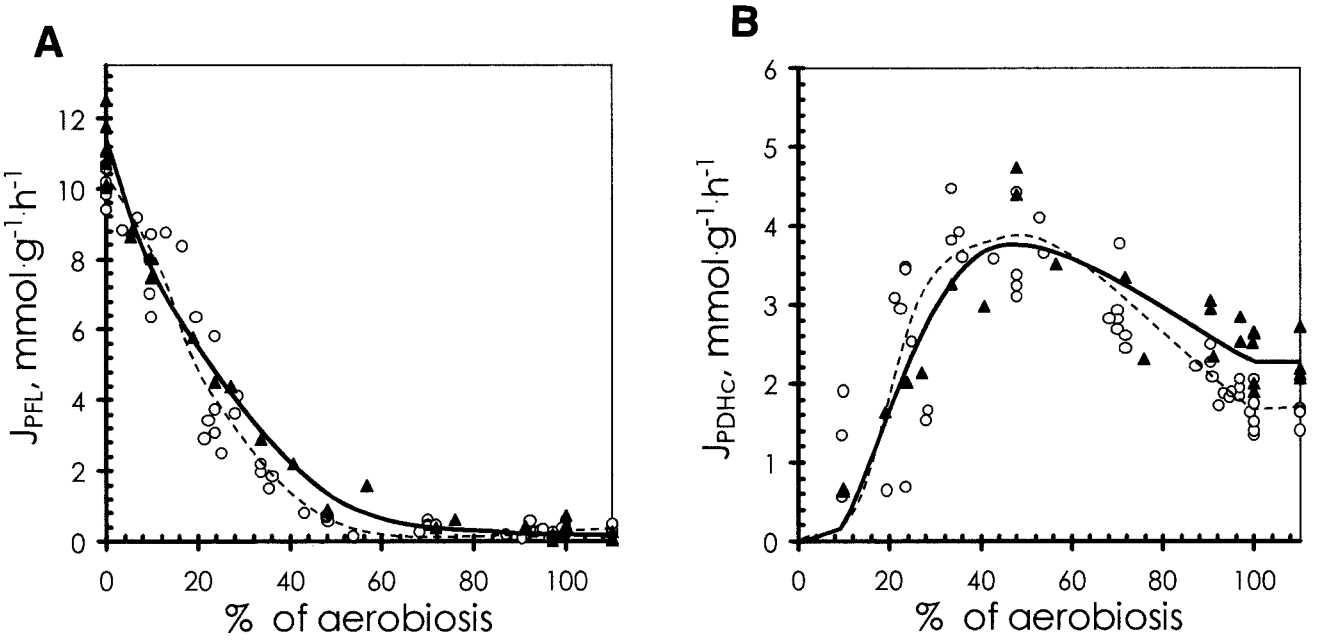

FIG. 3. Effect of the oxygen supply rate on the in vivo fluxes via PFL (A) and PDHc (B) of the wild-type (open symbols) and Δ*arcA* (filled symbols) strains.

consumed (molar yield value, Y_{glc}) (Fig. 1). The lack of the ArcA regulator did not result in significant differences in catabolic efficiency under fully aerobic or fully anaerobic conditions, nor in the lower microaerobic range. In contrast, higher Y_{glc} values were found invariably in the higher microaerobic range (50% and higher) for the wild type (Fig. 1).

Much as in the wild-type strain, an increase in the extent of aerobiosis led also to a gradual decrease of the rate of fermentation product formation in the Δ*arcA* strain, but the pattern of fermentation product formation was slightly changed (Fig. 2). In wild-type cells, ethanol (EtOH) production ceased at 70% aerobiosis ($q_{EtOH} < 0.05$ mmol · g^{-1} · h^{-1}); in contrast, the mutant cells continued to produce measurable amounts of ethanol up to almost 100% aerobiosis, accompanied by a specific acetate production rate that was not significantly different from that of the wild-type strain in the range above 50% aerobiosis. Although these differences in acetate and ethanol production seem to be minor (Fig. 2), it should be realized that these data reflect a strong effect on the distribution of the electron flow to internal and external acceptors due to the large difference in the degrees of reduction between acetate and ethanol. Whereas for the wild-type cells the acetate/ethanol ratio increased from just over 1 to 17 at 70% aerobiosis, in the mutant strain this ratio remained more or less constant (2 ± 0.5) for all microaerobic conditions.

Effect of an *arcA* deletion on in vivo catabolic fluxes through the TCA cycle, PFL, and PDHc. Analysis of the catabolic fluxes revealed that the deletion of *arcA* does not affect the in vivo catabolic fluxes through PFL and PDHc (Fig. 3). For both strains, it was calculated that increasing the oxygen supply rate resulted in a gradual shift from PFL-dependent catabolism to PDHc-dependent catabolism (Fig. 3), and in the lower microaerobic range, the two enzymes are active simultaneously, as was observed previously (1). This observation is rather surprising, since PFL and PDHc, the enzymes that catabolize pyruvate under anaerobic and aerobic conditions, respectively, are known to be under transcriptional control of the ArcAB two-component system (1, 6, 10, 15, 18, 22). In contrast to the similar carbon flux distributions over these two pyruvate-degrading systems, it was calculated that the in vivo TCA cycle activity in the Δ*arcA* strain was increasing continuously with increasing oxygen supply, and this was even more so in the microaerobic range above 30% (Fig. 4), where the TCA cycle

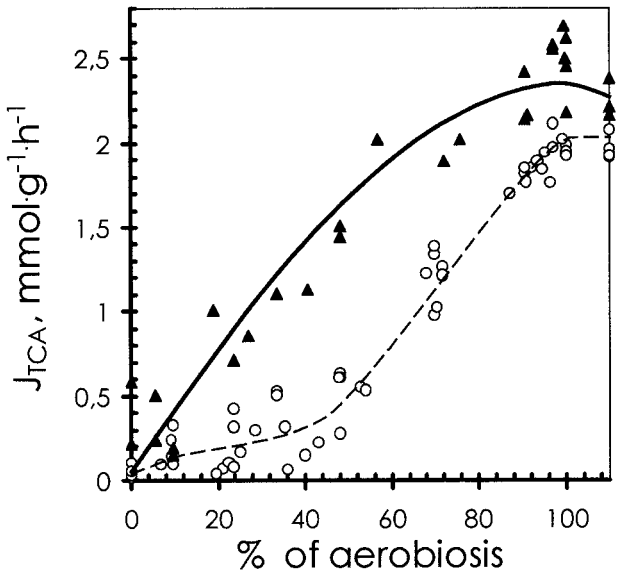

FIG. 4. Effect of the oxygen supply rate on the in vivo TCA cycle activities in the wild-type (open symbols) and Δ*arcA* (filled symbols) strains. Data for the wild-type strain are derived from reference 1.

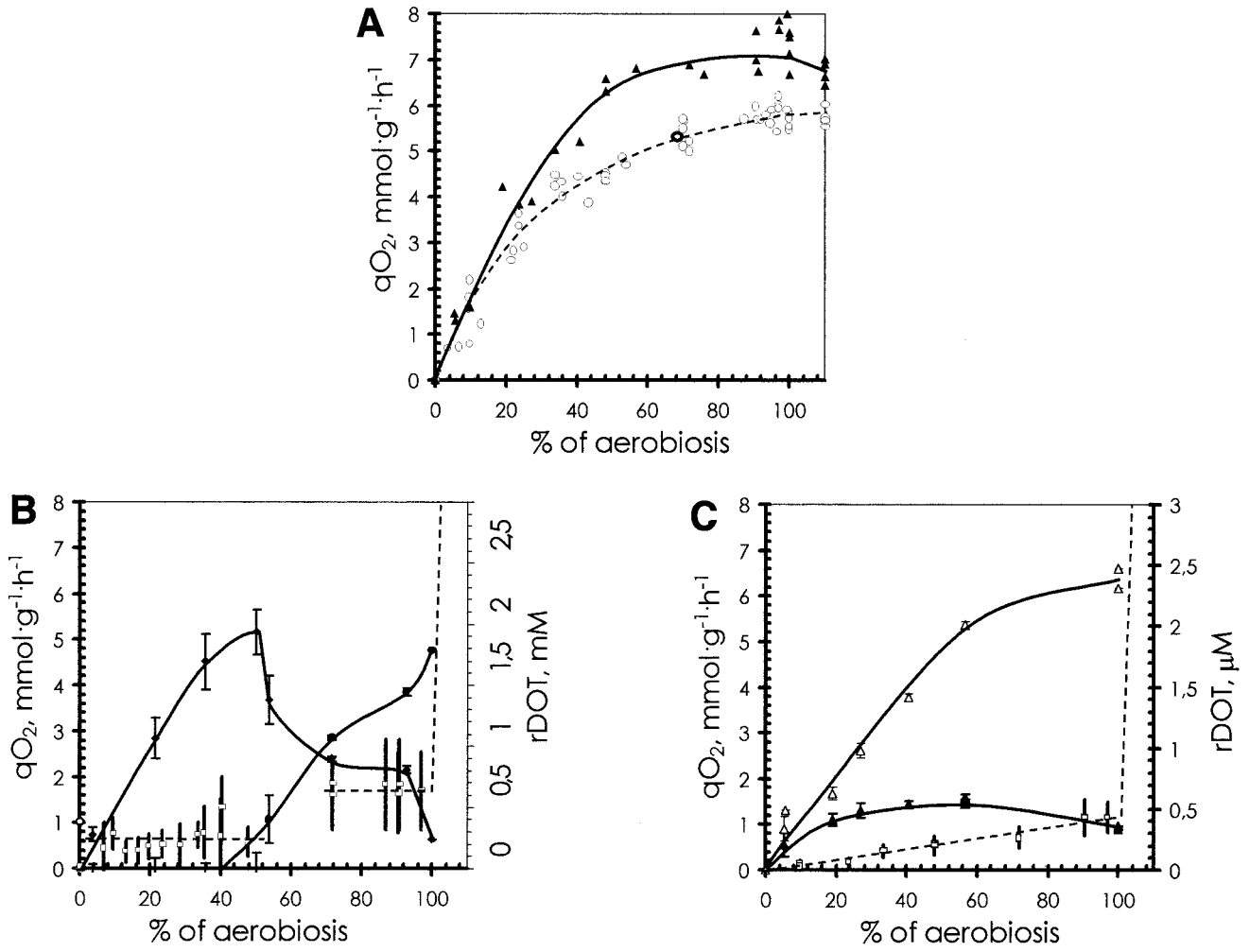

FIG. 5. (A) Effect of the oxygen supply rate on the total oxygen consumption rates of the wild-type (open symbols) and Δ*arcA* (filled symbols) strains. (B and C) The calculated electron flux through cytochrome *bo* oxidase (filled circles and open triangles, respectively), cytochrome *bd* oxidase (filled diamonds and filled triangles), and rDOT (open squares with dotted line) in the wild-type (B) and Δ*arcA* (C) strains.

activities in cultures of the mutant were significantly higher than those in the wild type.

Effect of an *arcA* deletion on distribution of respiratory flux between alternative terminal oxidases. Oxygen consumption could already be observed with the lowest oxygen supply rate applied and, with the exception of the very low microaerobic range, higher q_{O_2} values were observed for the mutant strain (Fig. 5A). In addition, the residual dissolved oxygen (rDOT) concentrations in these Δ*arcA* cultures were different from those in the wild-type cultures (Fig. 5B and C). Whereas no significant changes in rDOT concentrations were observed in the ranges from 0 to ~45% aerobiosis and from 70 to ~99% aerobiosis in the wild-type cultures with step changes in between, rDOT concentrations in the Δ*arcA* cultures showed an increasing trend throughout the full range of aerobiosis, and overall rDOT concentrations were lower. Using Michaelis-Menten kinetics according to the method we applied previously (2), it was possible to calculate the distributions of the respiratory flux from the levels of the cytochrome *bd* and cytochrome *bo* terminal oxidases. This calculation is based on measured respiration rates (q_{O_2}), the cytochrome *bd* content (1), residual oxygen concentrations (Fig. 5), and kinetic parameters determined earlier for the two terminal oxidases in whole cells (for cytochrome *d*, K_m = 0.024 μM O_2 and V_{max} = 42 μmol of O_2 · nmol of cytochrome $o^{-1} \cdot h^{-1}$; for cytochrome *o*, K_m = 0.2 μM O_2 and V_{max} = 66 μmol of O_2 · nmol of cytochrome $o^{-1} \cdot h^{-1}$) (17). The flux via cytochrome *o* was calculated by subtracting the calculated flux via cytochrome *d* from the total flux of electrons to oxygen (q_{O_2}). From this calculation, it follows that the electron flux through respiration is profoundly altered in the mutant strain (Fig. 5B and C). Whereas in the wild type, cytochrome *bd* is the sole terminal oxidase that contributes to respiratory activity up to 50% aerobiosis, a substantial flux through cytochrome *bo* oxidase is seen with the mutant for all conditions.

Effect of an *arcA* deletion on the intracellular redox state. For all growth conditions, the steady-state internal redox state, as reflected by the NADH/NAD ratio, was determined for the cultures lacking the ArcA regulator. Aerobic and anaerobic NADH/NAD ratios in the Δ*arcA* cultures were virtually identical to the corresponding ratios in the wild type (Fig. 6). Moreover, the change in the NADH/NAD ratio in response to

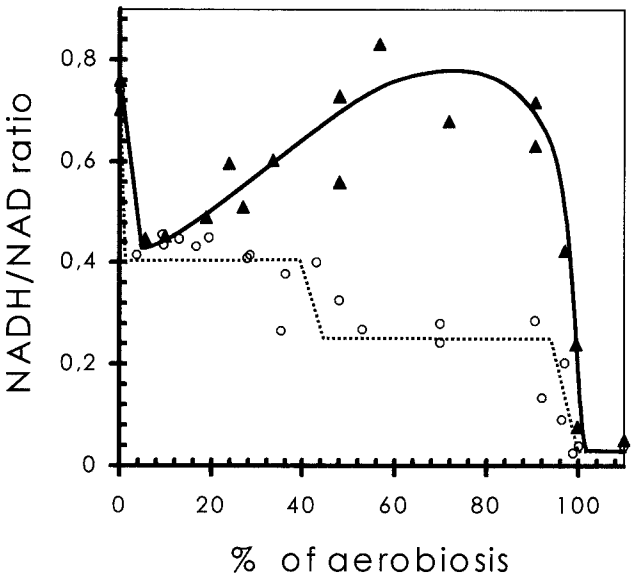

FIG. 6. Effect of the oxygen supply rate on the NADH/NAD ratio in the wild-type (open symbols) and Δ*arcA* (filled symbols) strains. Data for the wild-type strain are derived from reference 1.

a very low oxygen supply (5 to 10% aerobiosis) in the Δ*arcA* cultures was indistinguishable from that of the wild-type cells: it dropped to a value of approximately 60% of the anaerobic NADH/NAD ratio. An oxygen supply above 25 to 30% aerobiosis, in contrast, resulted in strikingly different responses by the wild type and the Δ*arcA* cells. Whereas in the wild type the NADH/NAD ratio decreased in response to an increasing oxygen availability, in the mutant cells, addition of oxygen above this range of 5 to 10% aerobiosis resulted in a continuous increase of the ratio up to anaerobic levels. This high ratio was observed at up to 90% aerobiosis. Above this range, the NADH/NAD ratio in the Δ*arcA* cultures, much as in the wild type, dropped dramatically to the level observed in aerobic cells.

DISCUSSION

Although the ArcAB two-component system has been the subject of many studies at the molecular level, the question of its role in the cell's physiology has never been addressed quantitatively. Here, for the first time the contribution to catabolism by the transcriptional regulation mediated by the Arc system is analyzed quantitatively in steady-state chemostat cultures.

Several conclusions may be drawn based on the data presented here. Firstly, the effect of an *arcA* deletion is most profound under oxygen-restricted growth conditions. These effects concern the catabolic efficiency (Y_{glc}), the intracellular redox state, the in vivo TCA cycle activity, the respiration rate, and the electron distribution over the alternative terminal oxidases. Only minor effects of the lack of this transcriptional regulator are seen under fully aerobic and fully anaerobic conditions. The above alterations may be explained by a lack of repression of NADH-producing enzymes, specifically those of the TCA cycle, under conditions of oxygen deprivation in the Δ*arcA* cultures: the increased TCA cycle activity results in an increased production rate of reduced electron carriers (NADH). Their elevated steady-state pools subsequently may cause increased respiration rates. Thus, it seems that ArcAB is primarily involved in maintaining the microaerobic redox state of the cell.

It is well documented that synthesis of PFL is under positive control of the ArcAB system, whereas the opposite is true for PDHc (6, 10, 15, 18, 22). However, deletion of *arcA* did not have a significant effect on the in vivo fluxes through these enzymes. With respect to PDHc, it may well be that the lack of repression of its synthesis is compensated for by the increased steady-state pool of NADH, which is known to inhibit its specific activity in vitro (20). For PFL, it was shown previously (1) that its expression level under extreme microaerobic conditions exceeded that under anaerobic conditions in a wild-type strain and that this pattern was lost in a Δ*arcA* strain. Yet, the flux through PFL is always highest under fully anaerobic conditions. Apparently, the microaerobic increase in expression results in overcapacity of the system, which explains why no effects on flux are observed in the mutant strain at the relatively low growth rate studied here. In addition, the observed lower maximal growth rate of the mutant when grown under oxygen restriction may be related to its decreased PFL expression level.

The pattern of electron flux distribution through the respiratory chain was found to be dramatically changed in cells lacking ArcA. Almost throughout the full range of oxygen-restricted growth, a shift is observed towards the use of cytochrome *bo* oxidase. This is consistent with the fact that ArcA represses cytochrome *bo* oxidase synthesis under microaerobic conditions (9, 23).

Previously (1, 2) we proposed a physiological function for the Arc system in ensuring rapid consumption of oxygen in order to maintain low intracellular oxygen concentrations and at the same time allowing the oxygen-sensitive, active PFL enzyme to function in maintaining a high catabolic flux. This suggestion was supported by the homeostatic effects on the steady-state dissolved oxygen tension by changed expression of the alternative terminal oxidases upon changes in oxygen supply. The analysis of the physiology of a Δ*arcA* strain presented here is not contradictory to the proposed function, although the observation that in vivo PFL activity was not altered in the deletion mutant under our experimental conditions may seem so. It seems that the lack of ArcA has no adverse effect on the physiology (although its growth efficiency is slightly lowered at the high end of microaerobiosis). However, it should be realized that our data concern steady-state conditions only. In nature, such conditions will not prevail, and it is expected that cells are optimized to a large extent to cope with changes in the supply of nutrients, i.e., oxygen. In this respect, the loss of a homeostatic response with respect to oxygen is an important observation. It is tempting to hypothesize that the mutant strain will be less able to respond adequately to sudden changes in oxygen availability, as indeed is exemplified by the erratic behavior of the mutant strain in establishing a new steady state upon changes in oxygen supply under high microaerobic conditions. A quantitative analysis of the response of this strain to oxygen pulses is being carried out currently.

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