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O_2 as the Regulatory Signal for FNR-Dependent Gene Regulation in *Escherichia coli*

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With an oxystat, changes in the pattern of expression of FNR-dependent genes from *Escherichia coli* were studied as a function of the O_2 tension (pO_2) in the medium. Expression of all four tested genes was decreased by increasing O_2. However, the pO_2 values that gave rise to half-maximal repression ($pO_{0.5}$) were dependent on the particular promoter and varied between 1 and 5 millibars (1 bar = 10^5 Pa). The $pO_{0.5}$ value for the ArcA-regulated succinate dehydrogenase genes was in the same range ($pO_{0.5}$ = 4.6 millibars). At these pO_2 values, the cytoplasm can be calculated to be well supplied with O_2 by diffusion. Therefore, intracellular O_2 could provide the signal to FNR, suggesting that there is no need for a signal transfer chain. Genetic inactivation of the enzymes and coenzymes of aerobic respiration had no or limited effects on the $pO_{0.5}$ of FNR-regulated genes. Thus, neither the components of aerobic respiration nor their redox state are the primary sites for O_2 sensing, supporting the significance of intracellular O_2. Non-redox-active, structural O_2 analogs like CO, CN^-, and N_3^- could not mimic the effect of O_2 on FNR-regulated genes under anaerobic conditions and did not decrease the inhibitory effect of O_2 under aerobic conditions.

In response to O_2 availability, the transcriptional regulator FNR of *Escherichia coli* controls the expression of genes required for anaerobic metabolism, such as structural genes of anaerobic respiration, substrate transport, and biosyntheses of coenzymes for anaerobic metabolism (18, 37, 42, 43). The Arc system on the other hand controls the expression of many genes of aerobic metabolism in response to O_2 (20, 22). The Arc system belongs to the two-component regulatory family, with ArcB as the membrane sensor protein and ArcA as the response regulator. FNR is in the regulatory competent state only under anaerobic conditions (12, 15, 25), although it is present in rather constant amounts in *E. coli* grown under either aerobic or anaerobic conditions (18, 36, 44). The O_2-sensing mechanism has been attributed to an essential Fe cofactor (15, 17, 29, 35, 39), and according to recent experiments, this cofactor is an FeS cluster (1, 25). In vivo, FNR can switch reversibly from the inactive (aerobic) to the active (anaerobic) state (12). Apart from O_2, FNR can also be inactivated in vivo by applying positive redox potential to the medium, e.g., by the addition of ferricyanide (45). In vitro, DNA binding of FNR and transcriptional activation were stimulated by applying reducing conditions (15, 25). Therefore, a redox reaction at the FeS cofactor may trigger the functional switch of FNR.

How O_2 is sensed by FNR is not well understood. It is not known whether O_2 itself or a product of aerobic metabolism reacts with FNR and whether other mediators are required. The failure to isolate mutations in other loci which cause defective FNR function suggests that there are no specific protein components required for signal transfer or reaction with O_2. To further analyze the pathway by which O_2 affects FNR function, here the role of O_2 as the signal and effector was analyzed and quantified. The transition point of oxygen regulation ($pO_{0.5}$) was determined to obtain a quantitative measure for the effect of O_2 on FNR.

By using this same approach, the aerobic respiratory chain was studied as a potential site for O_2 sensing or for providing a signal. Mutants with defective components of the respiratory chain from NADH to O_2 were analyzed for effects on the $pO_{0.5}$ value of FNR regulation. The mutants used had defective NADH dehydrogenases (*ndh* and *nuo* genes) and oxidases (*cyo*, *cyd*, and *appBC* genes) and lacked ubiquinone biosynthesis (*ubiA*) to determine whether the components of aerobic respiration, their redox state, or the electron flux were essential for FNR function.

The reaction of O_2 with target proteins can be either by mere binding via a metal cofactor or by binding followed by a redox reaction. Examples for the former type of interaction are O_2 carrier proteins like hemoglobin, hemerythrin, or the O_2 sensor FixL from *Rhizobium meliloti*, which appears to sense oxic conditions by binding of molecular O_2 (14). Examples for the latter type are oxidases or oxygenases with cofactors like heme, binuclear iron, or FeS clusters (4, 38, 41). Direct interaction with O_2 can often be demonstrated by structural O_2 analogs like CO, NO, CN^-, and N_3^- which apparently are able to interact with most types of these cofactors or proteins (24, 26, 38, 41). Therefore, to test whether there are indications for a direct interaction between O_2 and FNR, here the structural (and non-redox-active) O_2 analogs CO, CN^-, and N_3^- were analyzed for their ability to mimic the effect of O_2 on FNR under anaerobic conditions or to compete with O_2 under aerobic conditions.

MATERIALS AND METHODS

Bacterial strains and strain constructions. The bacteria, phages, and plasmid used in this study are shown in Table 1. Transductions with P1 *kc* were performed by the method of Miller (28). Transductants were selected by growth on L broth (LB) agar in the presence of the appropriate antibiotics, chloramphenicol, kanamycin sulfate, ampicillin, and tetracycline (20, 30, 50, and 15 μg/ml, respectively). The relevant phenotypes were also verified. The *cyd* null phenotype was demonstrated by decreased aerobic growth on LB agar supplemented with 0.2 mM NaN_3 and 0.2 mM $ZnSO_4$ (19). The *nuo* and *ubi* phenotypes were recognized by the loss of aerobic growth on M9 minimal medium or agar supplemented with 20 mM acetate. The transcriptional fusion to *lacZ*, FFp*melR-lacZ* was transferred from the plasmid FF/182 (2) to the transducing phage λRZ5 (23) by the method of Ostrow et al. (30). Bacteria lysogenized with the recombinant phage were screened for the *lac*$^+$ Ap^r phenotype. Monolysogenic strains were identified by measurement of the β-galactosidase activity from at least six isolates of bacteria that had been made permeable (28). For infection of IMW91 deriv-

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TABLE 1. Strains of *E. coli* K-12, plasmid, and phages used

Strain, plasmid, or phage	Genotype	Reference or derivation
Strains		
MC4100	F$^-$ *araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 flb5301*	34
AN387	Wild type	46
AN385	AN387 but *ubiA*	46
SH205	*zah-735*::Tn*10* Δ(*argF-lac*)U169	33
NKSO2	*ndh*::Kan^r F$^-$ Man$^+$ Lac$^-$	8
SBS1201	HfrC Δ(*appBA*)*1198*::Kan^r *relA pit-10 tonA22*	7
MC021	MC4100 but *nuoB*::Kan^r	
ECL547	MC4100 but λ[Φ(*sdh′-′lacZ*)Hyb] Δ*frd101 sdh*$^+$	22
ECL947	MC4100 but λ[Φ(*cyo′-′lacZ*)Hyb] Δ*frd101 cyo*::Kan^r *cyd*::Cam^r *arcB1 zgi*::Tn*10*	20
ECL937	MC4100 but λ[Φ(*cyo′-′lacZ*)Hyb] Δ*frd101 cyd*::Kan^r	20
RM123	MC4100 but λ[Φ(*pfl′-′lacZ*)Hyb]	31
MC4100λPC25	MC4100 but λ[Φ(*dmsA′-′lacZ*)Hyb]	9
MC4100λJ100	MC4100 but λ[Φ(*frdA′-′lacZ*)Hyb]	23
IMW46	MC4100 but *nuoB*::Kan^r λ[Φ(*frdA′-′lacZ*)Hyb]	MC021(P1) × MC4100λJ100
IMW50	MC4100 but *cyo*::Kan^r λ[Φ(*frdA′-′lacZ*)Hyb]	ECL947(P1) × MC4100λJ100
IMW51	MC4100 but *cyd*::Kan^r λ[Φ(*frdA′-′lacZ*)Hyb]	ECL937(P1) × MC4100λJ100
IMW91	AN387 but *zah-735*::Tn*10* Δ(*argF-lac*)U169	SH205(P1) × AN387
IMW93	AN387 but *ubiA zah-735*::Tn*10* Δ(*argF-lac*)U169	SH205(P1) × AN385
IMW94	AN387 but λ[Φ(*frdA′-′lacZ*)Hyb] *zah-735*::Tn*10* Δ(*argF-lac*)U169	IMW91(λJ100)
IMW96	AN387 but *ubiA* λ[Φ(*frdA′-′lacZ*)Hyb]*zah-735*::Tn*10* Δ(*argF-lac*)U169	IMW93(λJ100)
IMW156	MC4100 but λ[Φ(FFp*melR-lacZ*)Hyb]	MC4100(λFFp*melR*)
IMW167	MC4100 but *nuoB*::Kan^r λ[Φ(FFp*melR-lacZ*)Hyb]	MC021(P1) × IMW156
IMW168	MC4100 but *cyd*::Cam^r λ[Φ(FFp*melR-lacZ*)Hyb]	ECL947(P1) × IMW156
IMW172	MC4100 but *ndh*::Kan^r λ[Φ(FFp*melR-lacZ*)Hyb]	NKS02(P1) × IMW156
IMW177	MC4100 but Δ(*appBA*)*1198*::Kan^r λ[Φ(FFp*melR-lacZ*)Hyb]	SBS1201(P1) × IMW156
Plasmid		
FF/182	Φ(FFp*melR-lacZ*)Hyb *bla*$^+$	3
Phages		
λRZ5	λ(*′bla ′lacZ lacY*$^+$)	30
λJ100	λ[Φ(*frdA′-′lacZ*)Hyb *bla*$^+$]	MC4100λJ100
λFFp*melR*	λ[Φ(FFp*melR-lacZ*)Hyb *bla*$^+$]	This work

atives, λJ100 was recovered from MC4100λJ100 by UV induction (34) for 15 s by a UV lamp (254-nm wavelength, 4 W, 15-cm distance). The lysate was tested for homogeneity by plating with top agar containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and a new lysate was made of a single plaque for further use.

Growth and media. For growth experiments, strains were grown in M9 minimal medium supplemented with acid-hydrolyzed casein (1 g liter^{-1}) and L-tryptophan (0.5 g liter^{-1}) unless otherwise stated. The carbon sources, glucose (20 or 50 mM), succinate (20 mM), and acetate (20 mM), were added as stated for individual experiments. The medium was inoculated from cultures grown overnight under the same conditions to an A_{578} of 0.05. Strain RM123 was grown in sodium phosphate (0.1 M, pH 7.0)-buffered LB medium with 50 mM glucose. Primary cultures of *ubiA* strains were grown under anoxic conditions to limit reversion, and after every growth experiment, the cultures were assayed for the lack of revertants. Batch cultures were grown aerobically in flasks containing medium filled to 5 to 10% of the maximal volume with vigorous shaking, and cultures were grown anaerobically in sealed bottles under an O_2-free atmosphere (6). For strain constructions with *ubiA* derivatives, the medium was supplemented with 1 mM hydroxybenzoate to prevent revertants (49). Cell densities were measured as the A_{578}. An A_{578} of 1 corresponds to 1.5×10^9 cells ml^{-1}.

Growth in the presence of CO, KCN, or NaN_3. The bacteria were grown in sealed flasks (1 liter) containing 10 ml of M9 medium with glucose plus fumarate and 990 ml of gas phase. In addition to N_2, the gas phase contained 212 (aerobic growth), 21 (microaerobic growth) or 0 (anaerobic growth) millibars (1 bar = 10^5 Pa) of O_2. KCN and NaN_3 were included in the medium, CO in the gas phase as indicated for individual experiments. The medium was inoculated with 2 ml of a preculture (A_{578} of 0.5) grown under identical conditions. Samples were drawn at an A_{578} of 0.3 (logarithmic growth phase) and analyzed for β-galactosidase.

Growth in the oxystat. Growth of *E. coli* at defined oxygen tensions (pO_2) was performed in an oxystat (Biostat MD; Braun, Melsungen, Germany) in batch cultures. The pO_2 of the medium was measured continuously with an O_2 electrode. The pO_2 was held constant by an alternating supply of air (valve I) and N_2 (valve II). The switch between the valves occurred in response to the actual pO_2 in the medium. When the actual pO_2 fell below 98% of the set value, valve I opened and valve II closed. After the set value was reached, valve I closed, and valve II opened. The flow of N_2 was 0.8 liter/min. The flow of air was increased manually from 0.2 to 1.0 liter/min during growth to compensate for the increasing oxygen consumption. Additionally, 0.2 liter of N_2 per min was added continuously through a third valve which decreased the fluctuations of pO_2 in the medium. In cultures of bacteria with low O_2 consumption like *ubiA* mutants, a constant pO_2 was maintained by using only the regulated air valve and the permanent N_2 supply. O_2 tensions from 0.3 to 212 millibars could be obtained by this method with fluctuations within 5% of the set value.

Enzyme activities. Cell extracts were prepared from aerobically grown bacteria (M9 medium with glucose) harvested at mid-exponential growth phase (A_{578} of 0.5). After the cells were washed in 50 mM potassium phosphate buffer, the cells were resuspended in buffer at an A_{578} of 5 and disrupted in a French press at maximal pressure. Cell debris was removed by centrifugation for 15 min at $10{,}000 \times g$. Oxygen uptake (NADH → O_2) by cell extracts was measured with a Clark-type oxygen electrode at 37°C in 50 mM potassium phosphate buffer (pH 7.0). All samples were equilibrated with air before substrate (1.5 mM NADH) was added. Protein concentrations were determined by the biuret method with KCN (5). The β-galactosidase activity was measured by the method of Miller in bacteria that had been made permeable (28).

RESULTS

Growth of the bacteria in the oxystat at defined oxygen tension (pO_2). Growth of the bacteria at defined pO_2 was performed in an oxystat. Values of pO_2 ranging from 0.3 to 212 millibars (i.e., air saturation) could be maintained accurately over a broad range of cell densities during exponential growth (Fig. 1). The fluctuation of the actual pO_2 in the medium was significantly less than ±0.5 millibar at microaerobic conditions (≤10 millibars) and about 0.6 millibar at higher O_2 concentrations. Because of the short duration of the growth experiments (3 to 6 h), the electrode showed no significant drift even at the

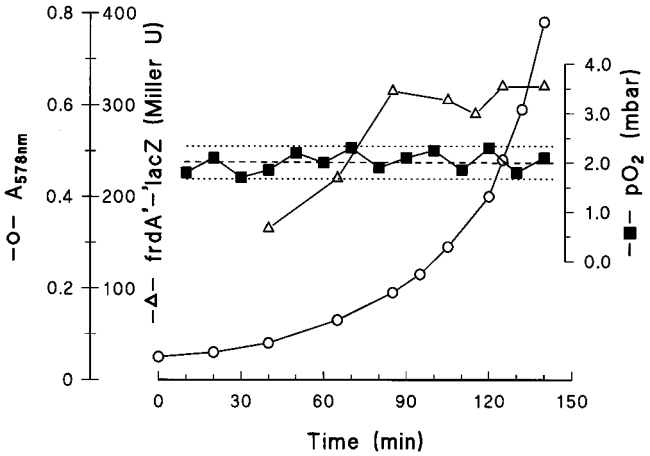

FIG. 1. Growth of *E. coli* MC4100λJ100 in the oxystat at defined pO_2 (2 millibars) and expression of *frdA'-'lacZ*. Growth (A_{578}) was performed in M9 medium supplemented with 20 mM glucose and 50 mM fumarate. The actual pO_2 was recorded by the oxystat. The dashed line shows the set point (2 millibars of O_2), and the dotted lines show the upper and lower limits of the measured values (± 0.3 millibars of O_2). Expression of *frdA'-'lacZ* was measured as the β-galactosidase activity. mbar, millibar (1 bar = 10^5 Pa).

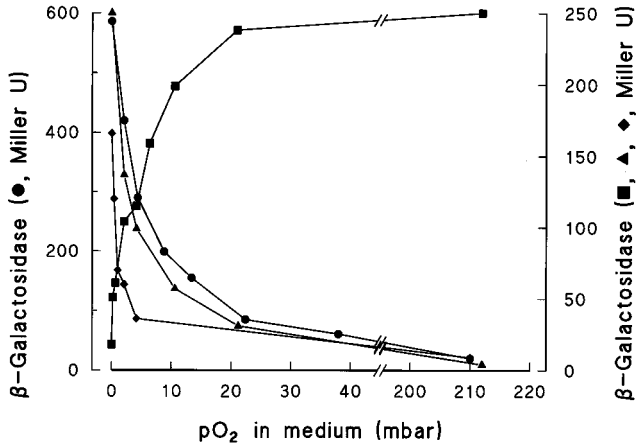

FIG. 2. Expression of β-galactosidase from *frdA'-'lacZ*, FFp*melR-lacZ*, *dmsA'-'lacZ*, and *sdh'-'lacZ* as a function of pO_2 in the medium. For each of the datum points (maximal standard deviation, 11%), at least three independent growth experiments were performed in the oxystat. Samples were drawn at A_{578}s between 0.4 to 0.8 and analyzed for β-galactosidase activity. Symbols: ●, *E. coli* MC4100λJ100 (*frdA'-'lacZ*); ▲, *E. coli* IMW156 (FFp*melR-lacZ*); ◆, *E. coli* MC4100λPC25 (*dmsA'-'lacZ*); ■, *E. coli* ECL547 (*sdh'-'lacZ*).

low pO_2 values. Therefore, this method was suitable to grow the bacteria at constant pO_2 values under aerobic and microaerobic conditions.

The expression of FNR-regulated genes under defined pO_2 values in the medium was studied initially in a strain containing a *lacZ* gene fusion to *frd*, which encodes fumarate reductase. In the experiment shown in Fig. 1, bacteria carrying a *frdA'-'lacZ* fusion were grown under a uniform O_2 tension (2 millibars) and the amount of *frdA'-'lacZ* expressed was observed during growth. The specific activities of β-galactosidase were constant during the logarithmic growth phase after adaptation of the bacteria. This was true for anaerobic, microaerobic, and aerobic growth conditions (data not shown). Thus, the slight fluctuations in the pO_2 of the medium at a given O_2 concentration did not significantly alter gene expression. Nevertheless, to account for any variation in pO_2, for each pO_2 value the data were collected from at least three independent growth experiments and at mid-exponential growth (A_{578} of 0.4 to 0.7). By this method, the variation in β-galactosidase was less than 11%, and reproducible correlations between activity and pO_2 were obtained.

Expression of FNR-dependent genes as a function of pO_2. To determine the effect of O_2 concentration on FNR-dependent gene regulation, the expression of *frdA'-'lacZ* (*frd*, which encodes fumarate reductase), *dmsA'-'lacZ* (*dms*, which encodes dimethylsulfoxide reductase) and FFp*melR-lacZ* (which carries a synthetic FNR consensus site in front of the *melR* promoter) was measured by growing the bacteria at various pO_2 values in the oxystat (Fig. 2). Glucose was included in the medium to enable growth under conditions of aerobic respiration, fermentation, and anaerobic respiration at comparable growth rates. The expression of *frdA'-'lacZ* and the other tested FNR-dependent genes decreased in a similar way by hyperbolic functions in response to increasing pO_2. The FFp*melR-lacZ* fusion with the synthetic FNR consensus site showed a response to the pO_2 very similar to those of the natural promoters. However, the sensitivities to O_2 were different, as indicated by the shift of the curves to higher or lower values for different fusions. The response of *pfl'-'lacZ*, which is positively regulated by ArcA in addition to FNR under anaerobic conditions (31, 32), was also monophasic and very similar to that of *frdA'-'lacZ* (not shown). The monophasic curve could be due to a similar pO_2-dependent regulation of *pfl* by both regulators (see the following paragraph) or to the predominant regulation by one regulator (FNR) (32).

From the graphs in Fig. 2, pO_2 values which correspond to 50% repression or induction ($pO_{0.5}$ value) compared with the maximal value can be derived. The $pO_{0.5}$ values ranged from

TABLE 2. $pO_{0.5}$ values for half-maximal expression of FNR-dependent genes and of *sdh* (ArcA regulated)

Gene fusion	Strain	Condition for growth[a]	β-Galactosidase activity (Miller units)[b]	$pO_{0.5}$ (millibars)[c]
frdA'-'lacZ	MC4100λJ100	Glucose	245	4.7 ± 0.6
		Glucose + fumarate	410	4.7 ± 0.8
		Glucose (28°C)	207	4.5
		Glucose, LB	400	4.5 ± 0.8
pfl'-'lacZ	RM123	Glucose (pH 6 and 7)	3,530	5.0 ± 1.0
dmsA'-'lacZ	MC4100λPC25	Glucose	166	1.0 ± 0.2
FFp*melR-lacZ*	IMW156	Glucose	260	2.9 ± 0.4
sdh'-'lacZ	ECL547	Glucose	275	4.6 ± 0.5

[a] Grown in an oxystat and at 37°C unless stated otherwise.
[b] Measured after growth under anaerobic conditions.
[c] The $pO_{0.5}$ values and standard deviations were determined from curves like those in Fig. 2. 1 bar = 10^5 Pa.

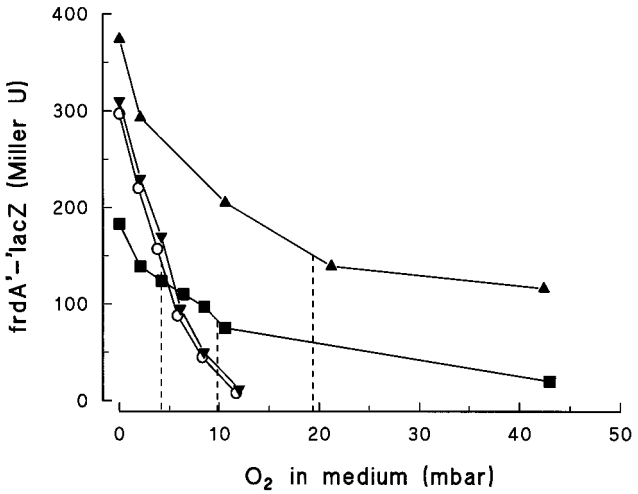

FIG. 3. Expression of *frdA'-'lacZ* in the parental strain of *E. coli* and *cyo*, *cyd*, and *ubiA* mutant strains at different pO_2 values in the medium. Growth was performed in the oxystat as described in the legend to Fig. 2. Symbols: ○, *E. coli* MC4100λJ100 (parental strain); ▼, *E. coli* IMW50 (*cyo* mutant); ■, *E. coli* IMW51 (*cyd* mutant); ▲, *E. coli* IMW96 (*ubiA* mutant).

1.0 to 5 millibars (Table 2) and provide a measure for the O_2 sensitivity of expression for the tested genes. Since the strains carrying the FNR-dependent gene fusions were isogenic derivatives of strain MC4100, the different values reflect differences in the target promoters. For *frdA'-'lacZ* (Table 2) and FFp*melR-lacZ*, the effects of medium composition, growth substrates, and temperature on $pO_{0.5}$ were also tested. Neither the presence of fumarate, which stimulates *frd* expression, nor temperature and medium composition, which can affect the growth rate, distinctly affected the $pO_{0.5}$. The $pO_{0.5}$ values for *frdA* and FFp*melR* promoters therefore are rather independent of other variables like medium composition and growth substrates under the test conditions.

Expression of ArcA-ArcB-regulated *sdh* as a function of pO_2. A second system important for the shift from aerobic to anaerobic pathway in the catabolism of *E. coli* are the two-component ArcA-ArcB regulators (22). The response regulator ArcA controls mainly the expression of aerobic metabolism in response to O_2 availability. The *sdh'-'lacZ* fusion (*sdh*, which encodes succinate dehydrogenase) is known to be repressed by ArcA under anoxic conditions (22). In the experiment shown in Fig. 2, the expression increased at elevated pO_2 and the curve was almost a mirror image to that of *frdA'-'lacZ*. The $pO_{0.5}$ was in the same range as that of *frdA'-'lacZ* (Table 2).

Effects of respiratory mutations on $pO_{0.5}$. Since O_2 is used as the terminal electron acceptor in aerobic respiration, mutants lacking various components of the major aerobic respiratory chain (NADH → O_2) were analyzed to test if the effect of O_2 on FNR required function of this chain. The transition points ($pO_{0.5}$) for *frdA'-'lacZ* and FFp*melR-'lacZ* expression were determined (Fig. 3 and Table 3) in strains with defective NADH dehydrogenase I (*nuo* genes) or II (*ndh* gene) or with defective quinoloxidase cytochrome *bo* (*cyo* genes) or cytochrome *bd* (*cyd* genes) or lacking ubiquinone (*ubiA*). A mutant with a defective putative third oxidase (*appBC* genes) (10) was also used, since the gene products could operate either as an additional quinoloxidase or as an O_2 sensor. Apart from the *ubiA* mutant, all derivatives were from the same parental strain.

The effects of these mutations on expression of *frdA'-'lacZ* were studied by measuring the β-galactosidase activity after growth in the oxystat at different pO_2 values (Fig. 3). None of the electron transport components was essential for FNR function, since in any of the mutants with these mutations, anaerobic expression and FNR-dependent regulation was maintained. In the *nuo* (not shown) and *cyo* null mutants, no changes in either parameter were observed for the expression of *frdA'-'lacZ* compared with the wild type (Fig. 3). In the *cyd* and *ubiA* mutants, however, differences are seen. The anaerobic expression of the fusion in the *cyd* mutant was only about half that of the parental strain. With increasing pO_2, the expression of the *frdA'-'lacZ* fusion decreased as in the parental strain. However, the slope of the curves was more gentle, causing increased $pO_{0.5}$ values for the *ubiA* and *cyd* mutants. The same changes were obtained with independent isolates of these same mutant strains (data not shown). Similar experiments were also performed with respiratory mutations carrying *lacZ* fused to the synthetic FNR-regulated promoter FFp*melR* which should be less susceptible to regulation by other factors. The titration curves of the strains and their responses to the mutations were similar to those of the *frdA'-'lacZ* fusions, but the effects of the mutations were less distinct.

The effects of the mutations on the transition points are summarized in Table 3. Inactivation of *nuo*, *cyo*, and *appBC* had no significant effects on the $pO_{0.5}$ values of the fusions. In the *cyd* mutant, the $pO_{0.5}$ values for both *lacZ* fusion-contain-

TABLE 3. Effects of mutations in structural genes involved in aerobic respiration or in ubiquinone biosynthesis on the $pO_{0.5}$ of *frdA'-'lacZ* and FFp*melR-lacZ* expression

Mutation	Growth rate (h^{-1})[a,b]	NADH → O_2 (U/g)[b]	*frdA'-'lacZ*[b]		FFp*melR-lacZ*[c]	
			Strain	$pO_{0.5}$[d]	Strain	$pO_{0.5}$
None (parental)	1.26	180	MC4100λJ100	4.7 ± 0.8	IMW156	2.8 ± 0.6
ndh	1.39	60		ND[e]	IMW172	1.2 ± 0.2
nuo	1.26	180	IMW46	5.1 ± 0.5	IMW167	2.8 ± 0.6
cyo	1.19	145	IMW50	4.7 ± 0.6		ND
cyd	1.39	165	IMW51	10.0 ± 0.8	IMW168	3.8 ± 0.4
appBC	1.39	ND		ND	IMW177	2.5 ± 0.5
ubiA	0.52	15	IMW96[f]	19.7 ± 0.3		ND

[a] Growth with glucose in M9 medium (air saturated).
[b] Derivatives of MC4100λJ100 (*frdA'-'lacZ*) with the respective mutations.
[c] Derivatives of IMW156 (FFp*melR-lacZ*) with the respective mutations.
[d] $pO_{0.5}$ values and standard deviations determined as described in the legend to Fig. 2 (M9 medium).
[e] ND, not determined.
[f] Derivative of IMW94.

ing strains were increased to different extents. The transition point in the *ubiA* mutant was determined only for the *frdA'-'lacZ* fusion because of the poor growth of the *ubiA* mutants. Introduction of a *ndh* mutation caused a slight decrease of the $pO_{0.5}$ value. In summary, mutations which inactivate single steps of aerobic respiration or the overall reaction had either none or only moderate effects on FNR function, depending on the mutation. None of the mutations, however, was essential for FNR-dependent regulation. Thus, none of the components is specifically required, but there might be some interference.

Growth rate and rates of aerobic respiration were determined for the various mutant strains (Table 3). The rates for aerobic growth on glucose were similar for most of the mutant and parental strains. Only the *ubiA* mutation caused a strong decrease in the growth rate. The aerobic respiration rate (NADH $\rightarrow$ O_2) was not remarkably changed in the *nuo*, *cyo*, and *cyd* mutants. However, in the *ndh* and *ubiA* mutant strains, the respiration rate was significantly decreased. The diminished respiration rate in the *ndh* mutant supports earlier findings that the corresponding NADH dehydrogenase II is important for NADH oxidation in aerobically grown bacteria (8, 16). In the *ubiA* mutant, aerobic respiration was less than 1/10 of the parental strain. This mutant strain converted glucose mostly to lactate and some acetate during aerobic growth, and only a small portion was oxidized to CO_2 (40). This strain is completely devoid of ubiquinone (47), and the residual O_2 consumption might be due to respiration via demethylmenaquinone, which is present to some extent in aerobically grown *E. coli* (46, 47). Thus, the lack of ubiquinone in the *ubiA* mutant severely inhibited aerobic respiration which has to affect the steady-state rate of electron transport and the redox state of the components. The experiments therefore indicate that neither the individual components of respiration nor electron flux or the redox state of the chain provides essential signals for FNR regulation.

Effects of O_2 analogs on *frdA'-'lacZ* expression. The results of the previous sections had shown that neither distinct components and presumably also not the operation of aerobic respiration are essential for FNR function. One possibility is that O_2 reacts directly with FNR, although there is no evidence for this type of interaction. The direct interaction could consist in a mere binding of O_2 to FNR or binding followed by oxidation of the cofactor. To test whether there are indications for a direct interaction, here the effects of the structural O_2 analogs CO, CN^-, and N_3^- on FNR function were analyzed under aerobic and anaerobic conditions. A strain carrying a *frdA'-'lacZ* fusion was grown anaerobically in the presence of high concentrations of the analogs and assayed for expression of the fusion (Fig. 4). None of the analogs was able to repress *frd'-'lacZ* significantly in contrast to O_2. Thus, either there was no specific binding of the analogs, or binding without oxidation is not sufficient for inhibition of FNR function.

When the analogs were present during aerobic or microaerobic growth, the growth rate decreased by a factor of 1.4 to 2 depending on the analog, presumably a result of inhibition of respiration. The inhibitors are known to reach the cytoplasm at sufficient concentrations, as demonstrated by inhibition of the oxidases at the cytoplasmic aspect of the membranes in growing bacteria. However, the expression of *frdA'-'lacZ* did not increase significantly under aerobic conditions with the analogs present (Table 4). Even under microaerobic conditions when the analogs were present in about fivefold excess over the O_2 concentration, none of the reagents could relieve the O_2-dependent repression. Therefore, it appears that the analogs could neither transfer FNR to the aerobic state nor compete with O_2 for binding under the conditions tested. The lack of response confirms that the inhibition of respiration which occurs under these conditions also does not affect FNR function, in agreement with the results obtained with the respiratory mutants.

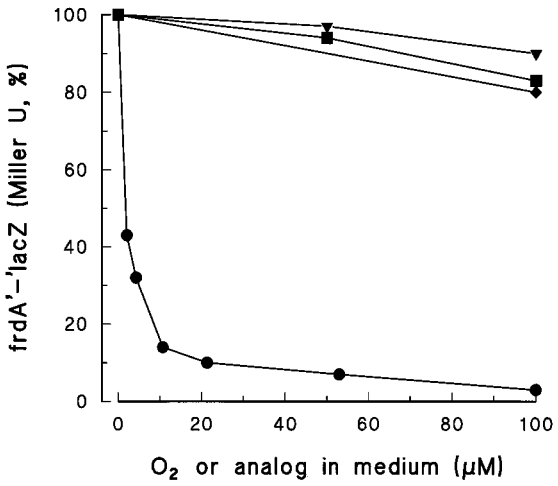

FIG. 4. Effects of the O_2 analogs CN^-, N_3^-, and CO on *frdA'-'lacZ* expression of *E. coli* MC4100λJ100 during anaerobic growth and comparison to the effect of O_2. The experiments with O_2 (●) were performed in the oxystat, and the experiments with KCN (■), NaN_3 (▼), and CO (◆) were performed under anaerobic conditions. CO was supplied to the gas phase, and growth was performed under vigorous shaking to ensure equilibration of CO with the medium. 100% activity corresponds to 480 Miller units.

DISCUSSION

The $pO_{0.5}$ values of O_2-regulated promoters of respiration. The oxystat cultures showed clear correlations between pO_2 and gene expression, and distinct values for the half-maximal expression ($pO_{0.5}$) of FNR-regulated genes could be determined. Comparison of the $pO_{0.5}$ values of FNR- and ArcA-regulated genes suggests that the range from 1 to 5 millibars of O_2 represents the transition point from aerobic to anaerobic metabolism. Because of the common transition point, a coordinated substitution of the aerobic pathways by the anaerobic pathways is achieved. Coordinated regulation makes sense for the bacteria because of the multiple metabolic changes involved in the transition from aerobic to anaerobic metabolism (43). The values are similar to earlier estimations of regulatory pO_2 values in the gas phase (13).

The FNR content in cells of *E. coli* is known to be rather constant during aerobic and anaerobic growth. However, the

TABLE 4. Effects of O_2 analogs on the expression of *frdA'-'lacZ* in *E. coli* MC4100λJ100 under aerobic and microaerobic growth conditions

O_2 analog (100 μM in medium)	Growth rate (h^{-1})[a]	β-Galactosidase activity (Miller units)[b]	
		Aerobic growth	Microaerobic growth[c]
None	1.47	28	55
KCN	0.42	33	82
NaN_3	1.31	38	46
CO	ND	43	74

[a] Aerobic growth conditions. ND, not determined.
[b] 245 Miller units for anaerobic growth.
[c] 21 millibars of O_2.

TABLE 5. Comparison of FNR sites to the regulatory O_2 tension ($pO_{0.5}$)

Promoter	Sequence of FNR site[a]	Additional regulators	No. of differences to consensus sequence[b]	$pO_{0.5}$ (millibars)[c]
FFp*melR*	TTGATN$_4$ATCAA		0	2.9
pfl (promoter P6)	ATGATN$_4$ATCAA	ArcA (positive)	1	5.0
dmsA	TTGATN$_4$AACAA	NarL (negative)	1	1.0
frdA	ATCGAN$_4$GTCAA	NarL (negative)	5	4.7

[a] FNR site sequences taken from references 11 and 37.
[b] FNR consensus site sequence, TTGATN$_4$ATCAA.
[c] $pO_{0.5}$ values from Table 2.

portion of the active form has to increase gradually with decreasing pO_2 (12). Therefore, the different $pO_{0.5}$ values indicate that the promoters require different amounts of active FNR for transcriptional activation. However, the $pO_{0.5}$ values are not related to the quality (as measured by the degree of identity to the consensus sequence) of the respective FNR site as shown in Table 5. Promoters with FNR sites differing in one position from the consensus FNR site sequence can have $pO_{0.5}$ values of 1.0 millibar (*dmsA*) or 5.0 millibars (*pfl*), and FNR sites with different sequences such as those of *pfl* and *frdA* can have very similar $pO_{0.5}$ values. Other factors, such as the location of the FNR site, the type of deviation from the consensus FNR site sequence, or the effect of additional regulators, must be important.

Intracellular O_2 is suggested to provide the regulatory signal to FNR. O_2 can diffuse readily into the cytoplasm of *E. coli*. Under aerobic and microaerobic conditions, the O_2 supply to the cytoplasm exceeds O_2 consumption by respiration by orders of magnitude (42, 43). At the $pO_{0.5}$ values corresponding to the transition from aerobic to anaerobic metabolism (1 to 5 millibars of O_2), the supply is predicted to amount to 3.4 to 17.1 mmol of O_2 min^{-1} g of $protein^{-1}$ and is thus much higher than the consumption (0.3 to 0.4 mmol of O_2 min^{-1} g of $protein^{-1}$). Therefore, the intracellular pO_2 should be similar to the extracellular pO_2 values if pO_2 is $\geq$1 millibar. Only at very low pO_2 (<0.1 millibar) does the consumption exceed the supply and the cytoplasm should become anoxic (42).

In summary, there is no indication and no requirement for a specific signal transfer chain from the membrane to FNR. Under all conditions where FNR is in the inactive (aerobic) state (pO_2 of $\geq$1 millibar), free O_2 can be assumed to be present within the bacteria. It can be assumed therefore that intracellular O_2 is responsible for FNR oxidation (see the below equation). The reaction could be mediated by cellular Fe ions or other (unspecific) redox mediators. Reversible interconversion of FNR by redox reactions also requires the presence of reducing agents for FNR reduction. Thiols (RS^- in the equation below) like reduced glutathione present in the bacteria (27) could be responsible for this part of the reaction.

$$\mathrm{FNR \cdot [FeS]^{reduced}} \underset{RS^-}{\overset{O_2 \text{ or } Fe(CN)_6^{3-}}{\rightleftharpoons}} \mathrm{FNR \cdot [FeS]^{oxidized}}$$

In earlier experiments it was shown that FNR can be converted to the inactive state not only by O_2 but also by oxidants like hexacyanoferrate ($Fe(CN)_6^{3-}$ in the above equation above) (45). This was taken as an indication that a redox reaction is involved in the functional transition of FNR. This view is in agreement with the recent demonstration of a redox-sensitive FeS cluster in FNR (25). The results shown here suggest that in vivo the physiological oxidant of FNR is intracellular O_2.

Non-redox-active O_2 analogs have no effect on FNR function in vivo. The non-redox-active O_2 analogs CO, CN^-, and N_3^- had no significant effects on FNR function and could neither substitute for O_2 nor outcompete the effects of O_2. Many enzymes or proteins with various types of prosthetic groups interacting with molecular oxygen are known to respond to O_2 analogs (24, 26, 38, 41). Absence of a response of FNR under any condition could indicate either that binding to the sensor without oxidation is not sufficient to provide the signal or that specific and tight binding of O_2 (and of the analog) is not required for reaction. Therefore, there is no indication for a direct interaction between FNR and molecular oxygen, and (unspecific) low-molecular-weight redox mediators like Fe ions could be required for reaction between FNR and O_2. For a direct proof of these assumptions, experiments with isolated FNR must be performed.

Components of aerobic electron transport are not essential for FNR function. None of the mutations causing lack of enzymes or coenzymes of aerobic respiration affected FNR function essentially. Therefore, aerobic electron transport and its components apparently are not the primary sites for signal recognition and transfer to FNR. These results confirm earlier suggestions by Iuchi et al. (20, 21). However, there was a shift of the $pO_{0.5}$ to higher or lower values in the *ndh*, *ubiA*, and *cyd* mutants. Various explanations for the shift are conceivable. The effects are smaller for the synthetic FFp*melR* promoter than for *frdA′*. This could indicate that the effects are in part independent of FNR and could be to some extent due to other factors acting on *frdA* but not on FFp*melR*. However, it is also possible that a component of aerobic respiration that is not directly involved in signal transfer influences the oxidation or reduction of FNR or forms a product of aerobic metabolism interacting with FNR.

"Respiratory protection" apparently plays no role in FNR-dependent regulation. "Respiratory protection" describes a phenomenon by which O_2-sensitive cytosolic enzymes are protected from inactivation by O_2 caused by respiration and decreased O_2 diffusion to the cytoplasm. This mechanism among others was suggested to protect nitrogenase in *Rhizobium* or *Klebsiella* species under microaerobic conditions (19, 48). Respiratory protection, however, does not affect O_2 sensing by FNR as shown here, and the effects of the respiratory mutations on FNR function do not support a role for respiratory protection. This can be concluded from the experiments with the *ubiA* mutant which shows severely inhibited respiration. In the mutant strain, the $pO_{0.5}$ for FNR function was not decreased as expected by involvement of respiratory protection. Rather, there was an increase in the $pO_{0.5}$ which contradicts the operation of respiratory protection. Moreover, at the transition points (1 to 5 millibars of O_2) the diffusion of O_2 (3.4 to 17 mmol of O_2 min^{-1} g of $protein^{-1}$) is predicted to exceed by far the respiration rate (0.4 mmol of O_2 min^{-1} g of $protein^{-1}$),

making the operation of respiratory protection also very unlikely (42, 43). Only at a pO_2 of $\ll$1 millibar should respiration be able to inhibit diffusion to cytoplasm.

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