Requirement for Terminal Cytochromes in Generation of the Aerobic Signal for the *arc* Regulatory System in *Escherichia coli*: Study Utilizing Deletions and *lac* Fusions of *cyo* and *cyd*

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Escherichia coli has two terminal oxidases for its respiratory chain: cytochrome *o* (low O₂ affinity) and cytochrome *d* (high O₂ affinity). Expression of the *cyo* operon, encoding cytochrome *o*, is decreased by anaerobic growth, whereas expression of the *cyd* operon, encoding cytochrome *d*, is increased by anaerobic growth. We show by the use of *lac* gene fusion that the expressions of *cyo* and *cyd* are under the control of the two-component *arc* system. In a *cyo*⁺ *cyd*⁺ background, expression of $\Phi(\text{cyo-lac})$ is higher when the organism is grown aerobically than when it is grown anaerobically. A mutation in either the sensor gene *arcB* or the pleiotropic regulator gene *arcA* almost abolishes the anaerobic repression. In the same background, expression of $\Phi(\text{cyd-lac})$ is higher under anaerobic growth conditions than under aerobic growth conditions. A mutation in *arcA* or *arcB* lowers both the aerobic and anaerobic expressions, suggesting that ArcA plays an activating role instead of the typical repressing role. Under aerobic growth conditions, double deletions of *cyo* and *cyd* lower $\Phi(\text{cyo-lac})$ expression but enhance $\Phi(\text{cyd-lac})$ expression. The double deletions also prevent elevated aerobic induction of the *lct* operon (encoding L-lactate dehydrogenase), another target operon of the *arc* system. In contrast, these deletions do not circumvent aerobic repression of the *nar* operon (encoding the anaerobic respiratory enzyme nitrate reductase) under the control of the pleiotropic *fnr* gene product. It thus appears that ArcB senses the presence of O₂ by the level of an electron transport component in reduced form or that of a nonautoxidizable compound linked to the process by a redox reaction, whereas Fnr senses O₂ by a different mechanism.

Escherichia coli can utilize a number of carbon and energy sources oxidatively but not fermentatively. Such sources include acetate, lactate, *sn*-glycerol-3-phosphate, succinate, fatty acids, and certain amino acids. To catabolize these compounds, the cell uses two oxidases for the terminal transfer of electrons from the substrate: cytochrome *o*, which has low O₂ affinity but a high $V_{\max}$, and cytochrome *d*, which has high O₂ affinity but a low $V_{\max}$ (25; for reviews, see references 1 and 23). The synthesis of many enzymes in these catabolic pathways is controlled by respiratory conditions of growth, since the enzyme levels are higher in aerobically grown than anaerobically grown cells (11, 23). We recently found that both *arcA* (min 0) and *arcB* (min 69.5) gene products are responsible for pleiotropic anaerobic repression (8, 11). These genes are members of the two-component regulatory family with conserved regions of amino acid sequence (21, 26). The sequence data indicate that ArcB is a membrane sensor and ArcA is a transcriptional regulator (4, 11a). The regulatory role of ArcA is also supported by a predicted helix-turn-helix structure on the C-terminal side of the polypeptide with a sequence fitting the consensus of the DNA-binding domain of certain regulator proteins (22). Furthermore, mutations in this domain destroy *arc* control (S. Iuchi, unpublished data).

Among the enzyme activity levels increased anaerobically by *arcA* or *arcB* mutations is that of ubiquinol oxidation. This activity reflects the composite electron flow rate from ubiquinol through cytochrome *o* (encoded by *cyo*) and cytochrome *d* (encoded by *cyd*). As a way of assessing the

effect of *arc* mutations on the level of each of the cytochromes, we exploited the fact that the inhibitor KCN has about 200 times higher affinity for cytochrome *o* than for cytochrome *d* (13, 24). By assaying ubiquinol oxidase activity in the presence or absence of KCN, we came to the conclusion that at least the synthesis of cytochrome *o* was under *arc* control (8, 11). In this study, we examined the regulation of *cyo* and *cyd* expression by the use of $\Phi(\text{cyo-lac})$ and $\Phi(\text{cyd-lac})$ protein fusions (Table 1). In addition, we explored the signal transduction pathway.

MATERIALS AND METHODS

Bacteria, phages, and plasmids. All strains used were derivatives of *E. coli* K-12. The origins and genotypes of the *E. coli* strains, bacteriophages, and plasmids are given in Table 1.

Growth conditions. For routine cultures, we used LB medium (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl). For β -galactosidase assays, cells were grown at 37°C in a mineral medium at pH 7.6 buffered by 0.1 M 3-(*N*-morpholino)propanesulfonate (MOPS) and supplemented with 0.03% casein acid hydrolysate (to prime growth) (10). Xylose was added as the principal carbon and energy source. Aerobic cultures were grown with vigorous rotatory agitation and harvested in mid-exponential phase. Anaerobic cultures were grown overnight in screw-capped test tubes filled to the top with medium and left undisturbed overnight. Agar plates were incubated anaerobically in sealed jars containing an atmosphere of H₂ and CO₂ (GasPak Anaerobic System; BBL Microbiology Systems, Cockeysville, Md). When used, fumarate was added to a concentration of 20 mM, xylose and potassium nitrate were added to 10 mM, 5-bromo-4-chloro-

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TABLE 1. *E. coli* K-12 strains, phages, and plasmids

Bacterium, phage, or plasmid	Genotype ^a	Derivation	Source or reference
Bacteria			
CC118	<i>araD139</i> Δ (<i>ara leu</i>)7697 <i>lacX74 phoA20 galE galK thi rpsE rpoB argE(Am) recA1</i>		16
GV102	F ⁻ Δ <i>cyo::kan galK2 galT22 hsdR514</i> ($r_K^- m_K^+$) <i>lacY1</i> or Δ (<i>lacIZY</i>)6 <i>met-31 supE44 supF58 trpR55</i>		K. Oden
GO103	F ⁻ Δ <i>cyd-1::kan gal rpsL Str^r thi</i>		K. Oden
GK101	F ⁻ Δ <i>cyd::cam recB21 recC22 del sbcA sbcB15 sbcC201 thr-1 ara-14 levB6</i> Δ (<i>gpt-proA</i>)62 <i>lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1</i>		C. Vibat
MC4100	F ⁻ <i>araD139</i> Δ (<i>argF-lac</i>)U169 <i>rpsL150 relA1 deoC1 flb-5301 ptsF25</i>		M. J. Casadaban
ECL525	F ⁻ Δ <i>frd-101 araD139</i> Δ (<i>argF-lac</i>)U169 <i>rpsL150 relA1 deoC1 flb-5301 ptsF25</i>		11
ECL561	Φ (<i>narC218-lac</i>)		9
ECL585	<i>arcA1 zji::Tn10</i>		11
ECL594	<i>arcB1 zgi::Tn10</i>		8
ECL933	Φ (<i>cyo-lac</i>) <i>bla⁺ cyo⁺</i>	λ VLH114 $\times$ ECL525	This study
ECL934	Φ (<i>cyo-lac</i>) <i>bla⁺ cyo⁺ arcA1 zji::Tn10</i>	P1(ECL585) $\times$ ECL933	This study
ECL935	Φ (<i>cyo-lac</i>) <i>bla⁺ cyo⁺ arcB1 zgi::Tn10</i>	P1(ECL594) $\times$ ECL933	This study
ECL936	Φ (<i>cyo-lac</i>) <i>bla⁺ Δcyo::kan</i>	P1(GV102) $\times$ ECL933	This study
ECL937	Φ (<i>cyo-lac</i>) <i>bla⁺ Δcyd::kan</i>	P1(GO103) $\times$ ECL933	This study
ECL938	Φ (<i>cyo-lac</i>) <i>bla⁺ Δcyo::kan arcA1 zji::Tn10</i>	P1(GV102) $\times$ ECL934	This study
ECL939	Φ (<i>cyo-lac</i>) <i>bla⁺ Δcyd::kan arcA1 zji::Tn10</i>	P1(GO103) $\times$ ECL934	This study
ECL940	Φ (<i>cyo-lac</i>) <i>bla⁺ Δcyo::kan arcB1 zgi::Tn10</i>	P1(GV102) $\times$ ECL935	This study
ECL941	Φ (<i>cyo-lac</i>) <i>bla⁺ Δcyd::kan arcB1 zgi::Tn10</i>	P1(GO103) $\times$ ECL935	This study
ECL942	Φ (<i>cyd-lac</i>) <i>bla⁺ cyd⁺</i>	λ GC101 $\times$ ECL525	This study
ECL943	Φ (<i>cyd-lac</i>) <i>bla⁺ cyd⁺ arcA1 zji::Tn10</i>	P1(ECL585) $\times$ ECL942	This study
ECL944	Φ (<i>cyd-lac</i>) <i>bla⁺ cyd⁺ arcB1 zgi::Tn10</i>	P1(ECL594) $\times$ ECL942	This study
ECL945	Φ (<i>cyo-lac</i>) <i>bla⁺ Δcyo::kan Δcyd::cam</i>	P1(GK101) $\times$ ECL936	This study
ECL946	Φ (<i>cyo-lac</i>) <i>bla⁺ Δcyo::kan Δcyd::cam arcA1 zji::Tn10</i>	P1(GK101) $\times$ ECL938	This study
ECL947	Φ (<i>cyo-lac</i>) <i>bla⁺ Δcyo::kan Δcyd::cam arcB1 zgi::Tn10</i>	P1(GK101) $\times$ ECL940	This study
ECL948	Φ (<i>cyd-lac</i>) <i>bla⁺ Δcyo::kan cyd⁺</i>	P1(GV102) $\times$ ECL942	This study
ECL949	Φ (<i>cyd-lac</i>) <i>bla⁺ Δcyo::kan cyd⁺ arcA1 zji::Tn10</i>	P1(GV102) $\times$ ECL943	This study
ECL950	Φ (<i>cyd-lac</i>) <i>bla⁺ Δcyo::kan cyd⁺ arcB1 zgi::Tn10</i>	P1(GV102) $\times$ ECL944	This study
ECL951	Φ (<i>cyd-lac</i>) <i>bla⁺ Δcyo::kan Δcyd::cam</i>	P1(GK101) $\times$ ECL948	This study
ECL952	Φ (<i>cyd-lac</i>) <i>bla⁺ Δcyo::kan Δcyd::cam arcA1 zji::Tn10</i>	P1(GK101) $\times$ ECL949	This study
ECL953	Φ (<i>cyd-lac</i>) <i>bla⁺ Δcyo::kan Δcyd::cam arcB1 zgi::Tn10</i>	P1(GK101) $\times$ ECL950	This study
ECL954	Φ (<i>narC218-lac</i>) Δ <i>cyo::kan</i>	P1(GV102) $\times$ ECL561	This study
ECL955	Φ (<i>narC218-lac</i>) Δ <i>cyo::kan Δcyd::cam</i>	P1(GK101) $\times$ ECL954	This study
Phages			
P1 <i>vir</i>			
λ RZ5	' <i>bla lacZ lacY</i> ' ⁺		20
λ VLH114	Φ (<i>cyo-lac</i>) <i>bla⁺</i>	See text	This study
λ GC101	Φ (<i>cyd-lac</i>) <i>bla⁺</i>		5
Plasmids			
pMLB1034	' <i>lacZ lacY</i> ' in pBR322		27
pRG110	<i>cyo⁺</i> in pBR322		2
pVLH114	Φ (<i>cyo-lac</i>) <i>bla⁺</i>	See text	This study

^a Δ *cyo::kan*, Kanamycin resistance gene inserted into a truncated *cyo*; Δ *cyd-1::kan*, kanamycin resistance gene inserted into a truncated *cyd*; Δ *cyd::cam*, chloramphenicol resistance gene inserted into a truncated *cyd*.

3-indolyl- β -D-galactopyranoside (X-Gal) was added to 40 μ g/ml, ampicillin was added to 100 μ g/ml, chloramphenicol was added to 15 μ g/ml, kanamycin was added to 25 μ g/ml, and tetracycline was added to 10 μ g/ml.

β -Galactosidase assay. The enzyme activity in whole cells was assayed (the cells were rendered permeable by the addition of 1 drop of 0.1% sodium dodecyl sulfate and 2 drops of chloroform to the cell suspension) at 30°C by measuring the hydrolysis of *o*-nitrophenyl- β -D-galactoside at 420 nm. The specific activity was expressed in units by the method of Miller (17).

Construction of pVLH114 containing Φ (*cyo-lac*). Plasmid pRG110 contained the entire *cyo* operon. There were two

*Hind*III sites, both located within the *cyoB* gene (2). The plasmid was digested with *Hind*III to remove a part of *cyoB*. The remaining DNA was shortened from both ends by digestion with BAL 31 exonuclease. Samples of the digestion mixture were removed at various time intervals, and the reaction was stopped with 0.2 M ice-cold ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid. The BAL 31-digested DNAs were blunt-ended and ligated to *Bam*HI linkers. The mixture was then digested with *Bam*HI and *Pvu*II to liberate, from the appropriately processed plasmid, fragments containing the *cyo* operon truncated from downstream. After isolation from the mixture by electrophoresis in a 0.7% agarose gel and purification by using a

FIG. 1. Construction of λ VLH114 containing Φ (*cyo-lac*). For details of the construction process, see Materials and Methods. Recombination 1 occurred within the homologous regions of *lacZY*, and recombination 2 occurred within the homologous regions of *bla*. The phenotypes exhibited by host cells bearing the particular phage or plasmid are indicated on the right. A, J, cI, and S are marker λ genes.

GeneClean kit, fragments containing the desired truncated *cyo*, with a blunt end on the 5' side (the side cut by *Pvu*II) and a sticky end on the 3' side (the side cut by *Bam*HI), were then ligated into the vector pMLB1034 digested with *Sma*I (blunt end) and *Bam*HI (sticky end). The digested vector pMLB1034 retained *lacZ*, lacking its promoter, ribosome-binding site, and the first eight codons of the coding sequence (28). The ligation mixture was transformed into *E. coli* CC118 (16), and transformants were isolated that were blue on ampicillin-X-Gal-LB agar. Restriction analysis confirmed the presence of a *cyo* insert in the plasmid DNA, pVLH114, from one of the blue colonies.

The nucleotide sequence of the junction of Φ (*cyo-lac*) in pVLH114 was determined by the dideoxy method of Sanger et al. (27) using the double-stranded plasmid DNA (7) and an 18-mer primer (5'-GAAAGGGGGATGTGCTGC-3'). This primer (synthesized in the Genetic Engineering Facility of the University of Illinois) complements the *lacZ* DNA encoding amino acid residues 28 to 33. The data indicate that the in-frame gene fusion occurred at amino acid 75 of the *cyoA* subunit (the most promoter-proximal cistron in the *cyo* operon).

Construction of a lambda phage bearing Φ (*cyo-lacZ*). Plasmid pVLH114 containing Φ (*cyo-lac*) was transformed into *E. coli* MC4100 by selecting for ampicillin resistance (Amp^r) and scoring for blue colonies on X-Gal-LB agar. A clone of MC4100(pVLH114) was then infected with a λ RZ5 lysate prepared as described by Silhavy et al. (28). Figure 1 illustrates the structure of λ RZ5, which contains *bla* and *lacZ* both truncated from the 5' end but an intact *lacY* (12). A double recombination between the λ RZ5 and the plasmid pVLH114 within the existing homologous regions of *lacZY* and *bla* should result in a λ phage with a complete *bla* gene and the Φ (*cyo-lac*) fusion. Such a recombinant phage (λ VLH114) was selected from the parental phage (λ RZ5) by the abilities to confer Amp^r to the host and to form blue colonies on X-Gal-LB agar.

Construction of Φ (*cyo-lac*) and Φ (*cyd-lac*) λ lysogens. To analyze the expression of a single copy of the *lac* fusion, the appropriate carrier λ phage was integrated into the *att* λ site of the chromosome of strain ECL525. To transduce Φ (*cyo-lac*), cells grown on LB broth containing 5 mM maltose and 0.1 M MOPS at pH 7.6 were infected with λ VLH114. The

TABLE 2. Effects of *arcA* and *arcB* mutations on Φ (*cyo-lac*) and Φ (*cyd-lac*) expression^a

Strain	Relevant genotype	β -Galactosidase (U)		β -Galactosidase ratio (+O ₂ /-O ₂)
		-O ₂	+O ₂	
ECL933	Φ (<i>cyo-lac</i>) <i>cyo</i> ⁺	8	130	16
ECL934	Φ (<i>cyo-lac</i>) <i>cyo</i> ⁺ <i>arcA</i> 1	63	110	1.7
ECL935	Φ (<i>cyo-lac</i>) <i>cyo</i> ⁺ <i>arcB</i> 1	66	90	1.4
ECL942	Φ (<i>cyd-lac</i>) <i>cyd</i> ⁺	830	160	0.19
ECL943	Φ (<i>cyd-lac</i>) <i>cyd</i> ⁺ <i>arcA</i> 1	280	73	0.26
ECL944	Φ (<i>cyd-lac</i>) <i>cyd</i> ⁺ <i>arcB</i> 1	260	34	0.13

^a Cells were grown aerobically or anaerobically at 37°C in a mineral medium containing xylose as the principal carbon and energy source and casein acid hydrolysate as a growth stimulant.

transductants were selected for Amp^r, and β -galactosidase activity in permeabilized cells was measured. The Φ (*cyd-lac*) fusion in λ GC101, constructed by an approach similar to that of the Φ (*cyo-lac*) fusion (5), was transduced into strain ECL525 in a similar manner.

Construction of strains by generalized transduction. For transduction of the *arcA*1 or *arcB*1 mutation, P1 particles were prepared from strain ECL585 or strain ECL594. The transductants were selected for tetracycline resistance (Tc^r) and scored for dye susceptibility on agar containing 0.2 mg of toluidine blue o per ml, 1.0% tryptone, and 0.8% NaCl. For transduction of Δ *cyo*::*kan*, P1 particles were prepared from strain GV102. The transductants were selected for kanamycin resistance (Kan^r). For transduction of Δ *cyd*::*kan*, P1 particles were prepared from strain GO103. The transductants were selected for Kan^r and Amp^r. In this case it was also necessary to select for Amp^r in order to obtain clones that retained the prophage λ VLH114 [bearing Φ (*cyo-lac*) *bla*] or the prophage λ GC101 [bearing Φ (*cyd-lac*) *bla*] integrated at the *att* λ site close to *cyd*. For transduction of Δ *cyd*::*cam* into Δ *cyo*::*kan* strains, P1 particles were prepared from strain GK101. The transductants were selected on chloramphenicol-ampicillin-glucose agar. Glucose supported good growth despite the absence of the terminal oxidases.

RESULTS AND DISCUSSION

Effects of *arcA* and *arcB* mutations on Φ (*cyo-lac*) and Φ (*cyd-lac*) expression. In a wild-type (*cyo*⁺ *cyd*⁺) background, the expression of Φ (*cyo-lacZ*) was repressed by a factor of 16 by anaerobic growth (Table 2). The mutation in either *arcA*1 or *arcB*1 raised the anaerobic activity level about eightfold. Since Arc shows properties of a transcriptional regulator, we conclude that the regulation of *cyo* is mainly at the level of mRNA synthesis. This conclusion is consistent with the observation of Minagawa et al., who showed that expression of a Φ (*cyo-lac*) operon fusion was regulated by the presence of O₂ (18). It might be noted, however, that the *arc* mutations did not fully abolish anaerobic repression (Table 2). The residual repression might be attributed either to leakiness of the *arc* mutations or to control by Fnr (P. A. Cotter and R. P. Gunsalus, Abstr. Annu. Meet. Am. Soc. Microbiol. 1990, H-66, p. 165), which is known as a pleiotropic regulator for anaerobic respiration (3, 15, 19, 31).

A different regulation pattern was observed with the Φ (*cyd-lacZ*) fusion. This difference was not surprising, since the level of cytochrome *d*, an O₂-scavenging enzyme, was reported to be elevated when O₂ became rate limiting for

growth (5, 14). In a wild-type (*cyo*⁺ *cyd*⁺) background, the level of $\Phi(\text{cyd-lac})$ expression was higher in anaerobically grown than in aerobically grown cells (Table 2). The *arcA1* and the *arcB1* mutation diminished $\Phi(\text{cyd-lac})$ expression under both growth conditions. It thus seems that ArcA positively regulates the *cyd* operon, in contrast to the negative regulation of the other known target operons involved in aerobic metabolism (8, 11).

Effects of deletion of *cyo*, *cyd*, or both on aerobic expression of $\Phi(\text{cyo-lac})$ and *lct*. In a previous attempt to define the stimulus for the Arc system, we excluded a direct effect of O₂. In that experiment the target operon used was $\Phi(\text{sdh-lacZ})$, in which an *sdh* gene encoding a subunit of succinate dehydrogenase was joined to *lacZ*. Comparison of $\Phi(\text{sdh-lacZ})$ cells grown on xylose fermentatively or on xylose with fumarate, nitrate, or O₂ as an exogenous electron acceptor showed that the level of β -galactosidase activity increased when an external oxidant was provided. Moreover, the degree of increase depended on the midpotential of the terminal electron acceptor; fumarate ($E^{\circ} = +30$ mV) raised the activity level by about 45% over that of the fermentative level, nitrate ($E^{\circ} = +420$ mV) raised the activity level by about 150%, and O₂ ($E^{\circ} = +820$ mV) raised the activity level by about 850% (11). It therefore appears that, as the flow of electrons through the transport system becomes more effective, the repression signal is progressively dampened. If this view is correct, then restricting the flow of electrons should enhance the repression signal.

To test this hypothesis, we asked whether depriving the cell of one or both terminal cytochromes might lower the aerobic expression of $\Phi(\text{cyo-lac})$. It should be noted first, however, that a previous study showed that the V_{max} of cytochrome *o* in aerobically grown cells was in the range of 1.1 to 1.5 μmol of O₂ per nmol of the heme protein per min. The corresponding value for cytochrome *d* was 0.7 μmol of O₂ per nmol of heme protein per min (25). Thus, cytochrome *d* might contribute significantly to the total O₂ consumption rate, even when O₂ was not severely depleted. This possibility was confirmed by the increased cyanide sensitivity (greater dependence on cytochrome *o*) of both the oxygen uptake and growth rates of a mutant lacking cytochrome *d* (6). Moreover, we found that deletion of either *cyo* or *cyd* slightly reduced the aerobic growth rate of the cells in LB medium, whereas double deletions severely reduced both the growth rate and the growth yield (data not shown). Aerobic expression of $\Phi(\text{cyo-lac})$ was lowered about 60% by the *cyo* deletion (Table 3). Deletion of *cyd* had a similar effect. Double deletion of *cyo* and *cyd* decreased aerobic $\Phi(\text{cyo-lac})$ expression to a level less than half of that observed in mutants with a single deletion. These results indicate that a cellular redox component in the electron transport chain or a compound connected to the chain by a redox reaction serves as the direct signal for the Arc system and that the signal compound is not readily autoxidizable. As expected, both the *arcA1* and the *arcB1* mutations prevented signal sensing by mutants lacking cytochrome *o*, cytochrome *d*, or both (Table 3).

To test whether only $\Phi(\text{cyo-lac})$ is regulated in this manner, we determined the aerobic activity level of L-lactate dehydrogenase, which is encoded by another arc target operon, *lct* (11; J. H. Scott, S. Iuchi, and E. C. C. Lin, submitted for publication). The dehydrogenase activity level of the *cyo cyd* double mutant was 1/10 that of the *cyo*⁺ *cyd*⁺ parent strain. The low level of the enzyme was restored to normal by either the *arcA1* or the *arcB1* mutation (data not shown).

TABLE 3. Effects of deletion of *cyo* and *cyd* on aerobic expression of $\Phi(\text{cyo-lac})$ or $\Phi(\text{cyd-lac})$ ^a

Relevant genotype	Expression of fusion:			
	$\Phi(\text{cyo-lac})$		$\Phi(\text{cyd-lac})$	
	Strain	β -Gal ^b (U)	Strain	β -Gal (U)
<i>cyo</i> ⁺ <i>cyd</i> ⁺	ECL933	140	ECL942	200
<i>cyo</i> ⁺ <i>cyd</i> ⁺ <i>arcA1</i>	ECL934	120	ECL943	100
<i>cyo</i> ⁺ <i>cyd</i> ⁺ <i>arcB1</i>	ECL935	130	ECL944	82
Δcyo <i>cyd</i> ⁺	ECL936	52	ECL948	170
Δcyo <i>cyd</i> ⁺ <i>arcA1</i>	ECL938	150	ECL949	110
Δcyo <i>cyd</i> ⁺ <i>arcB1</i>	ECL940	140	ECL950	73
<i>cyo</i> ⁺ Δcyd	ECL937	53	ND ^c	ND
<i>cyo</i> ⁺ Δcyd <i>arcA1</i>	ECL939	120	ND	ND
<i>cyd</i> ⁺ Δcyd <i>arcB1</i>	ECL941	100	ND	ND
Δcyo Δcyd	ECL945	23	ECL951	610
Δcyo Δcyd <i>arcA1</i>	ECL946	150	ECL952	130
Δcyo Δcyd <i>arcB1</i>	ECL947	130	ECL953	110

^a Cells were grown aerobically as described in Table 2, footnote a.

^b β -Gal, β -Galactosidase.

^c ND, Not done.

Effects of *cyo* and *cyd* deletions on aerobic expression of $\Phi(\text{cyd-lac})$. Since ArcA appeared to serve as an activator of $\Phi(\text{cyd-lac})$ during O₂ deprivation (Table 2), loss of cytochromes *o* and *d* should increase aerobic expression of the fusion if the signaling processes for the regulation of both *cyd* and *cyo* are the same. Although the loss of cytochrome *o* alone did not significantly affect the aerobic expression of $\Phi(\text{cyd-lac})$, the loss of both cytochromes *o* and *d* increased the expression level by threefold (Table 3). As expected, in either the *cyo*⁺ *cyd*⁺ or the Δcyo Δcyd background, the *arcA1* or the *arcB1* mutation greatly lowered aerobic expression of $\Phi(\text{cyd-lac})$.

Different signals for Fnr and ArcA. Unlike the numerous genes that specify reactions linked to aerobic respiration and whose expression is increased by abundance of O₂, many genes that specify reactions in anaerobic respiration become highly inducible when O₂ is deficient. The increased anaerobic expression of these genes depends on transcriptional activation by the pleiotropic *fnr* gene product (in certain cases additional controlling elements are involved) (3, 15, 19, 31). Interestingly, Fnr was recently found in two laboratories to require Fe²⁺ for function (29, 32). This finding raises the possibility that the regulatory state of Fnr depends on the intracellular [Fe²⁺], which varies with the [Fe²⁺]/[Fe³⁺] ratio set by the intracellular redox potential (33).

Since both Fnr and ArcA are converted into the functional state when there is a lack of O₂, the question arises as to whether the two systems respond to the same signal. Two tests were carried out. First, we compared the effect of the Fe²⁺ chelator, *o*-phenanthroline (20 μM), on the anaerobic activation of the Fnr-controlled $\Phi(\text{frd-lac})$ (in which the promoter of the *frd* operon encoding the anaerobic respiratory enzyme fumarate reductase was fused to *lacZ*) with that on the anaerobic repression of the ArcA-controlled operon $\Phi(\text{sdh-lac})$. The chelator, which lowered the expression of $\Phi(\text{frd-lac})$ by a factor of five, did not significantly affect the expression of $\Phi(\text{sdh-lac})$ (data not shown). Hence, Fe²⁺ apparently is not involved in regulating the behavior of ArcA.

Second, we extended the experiments on the double deletion Δcyo Δcyd to include the Fnr-controlled *nar* operon (encoding the anaerobic respiratory enzyme nitrate reductase) (30) as a target of regulation. The double deletion did not relieve aerobic repression of the $\Phi(\text{nar-lac})$ operon

TABLE 4. Effect of deletion of *cyo* and *cyd* on aerobic expression of $\Phi(nar-lac)^a$

Strain	Relevant genotype	β -Galactosidase (U)		β -Galactosidase ratio (-O ₂ /+O ₂)
		-O ₂	+O ₂	
ECL561	$\Phi(nar-lac) cyo^+ cyd^+$	440	13	34
ECL955	$\Phi(nar-lac) \Delta cyo \Delta cyd$	410	20	21

^a Cells were grown aerobically or anaerobically as described in Table 2, footnote a, but with nitrate added as an inducer.

fusion (Table 4). This finding is in contrast to the marked influence of the double deletion $\Delta cyo \Delta cyd$ on expression of $\Phi(cyo-lac)$ and $\Phi(cyd-lac)$ (Table 3).

The results from the two different approaches suggest that although the O₂ effect on Fnr is likely to be exerted via [Fe²⁺], the O₂ effect on the Arc system is not. Although the exact nature of the direct signal for the Arc system remains to be identified, the receptor is most likely ArcB. We have recently sequenced the gene and shown its product to be membrane associated. The deduced sequence of the protein (778 residues) indicates that the N-terminal end of the molecule traverses the cytoplasmic membrane twice, with a small segment in the periplasmic space (7 amino acid residues). The rest of the polypeptide, the bulk, should be in the cytoplasm (11a). Because of the location of ArcB, we are inclined to believe that the signal comes from the cytoplasmic membrane and is generated by changing the ratio of the reduced form to the oxidized form of some compound, such as a flavin, a heme, or a quinone. In any case, it appears that when cells grow anaerobically, ArcB perceives the unfavorable respiratory state and transmits the signal by activating ArcA. The activated ArcA in turn represses the target operons such as *cyo* or activates the target operons such as *cyd*. When cells grow aerobically, the signal is discharged by effective conduction of electrons from the donor substrates to O₂ via the terminal cytochromes. This working model is illustrated in Fig. 2, which shows the reduced form of X (see legend to Fig. 2) as the direct signal, or effector, for ArcB. By analogy with other two-component regulatory systems, ArcB is expected to autophosphorylate at a histidyl residue

FIG. 2. A model for the *arc* regulatory system. Aerobic respiration is depicted by the sequential reactions in the plasma membrane. SH₂ is a substrate (e.g., succinate) of a primary dehydrogenase. X represents an unidentified intermediate of the respiration process (or a compound linked to the process by a redox reaction). R represents the reduced form, and O represents the oxidized form. The X(R) is shown acting as the direct signal for ArcB. The stimulated ArcB then undergoes autophosphorylation and communicates the signal to ArcA by transfer of the phosphoryl group. ArcA-P then becomes a transcriptional repressor (-) or activator (+) of promoters of the *arc* modulon (11; Iuchi et al., in press). The *cyo* and the *lct* operons are members of the family of repressible target operons, whereas *cyd* is a member of the family of activatable target operons.

in a conserved sequence. The phosphoryl group is then transferred to ArcA, thereby converting it to a transcriptional regulator.

ACKNOWLEDGMENTS

We thank T. Kaysser, K. Oden, and C. Vibat for supplying bacterial strains and J. A. Fee for discussion of the possible role of [Fe²⁺] as a redox signaling system. We thank Alison Ulrich for valuable assistance in the preparation of the manuscript.

This work was supported by Public Health Service grant 1-R01-GM40993 from the National Institutes of Health to E.C.C.L. and Public Health Service grant HL16101 from the National Institutes of Health and Department of Energy grant DEFGO2-78ER13716 to R.B.G.

LITERATURE CITED

1. Anraku, Y., and R. B. Gennis. 1987. The aerobic respiratory chain of *Escherichia coli*. Trends Biochem. Sci. 12:262-266.
2. Au, D. C.-T., and R. B. Gennis. 1987. Cloning of the *cyo* locus encoding the cytochrome *o* terminal oxidase complex of *Escherichia coli*. J. Bacteriol. 169:3237-3242.
3. Chippaux, M., D. Guidici, A. Abou-Jaoude, F. Casse, and M. Pascal. 1978. A mutation leading to the total lack of nitrite reductase activity in *Escherichia coli* K12. Mol. Gen. Genet. 160:225-229.
4. Drury, L. S., and R. S. Buxton. 1985. DNA sequence analysis of the *dye* gene of *Escherichia coli* reveals amino acid homology between the Dye and OmpR proteins. J. Biol. Chem. 260:4236-4242.
5. Georgiou, C. D., T. J. Dueweke, and R. B. Gennis. 1988. Regulation of expression of the cytochrome *d* terminal oxidase in *Escherichia coli* is transcriptional. J. Bacteriol. 170:961-966.
6. Green, G. N., and R. B. Gennis. 1983. Isolation and characterization of an *Escherichia coli* mutant lacking cytochrome *d* terminal oxidase. J. Bacteriol. 154:1269-1275.
7. Hattori, M., and Y. Sakaki. 1985. Dideoxy sequencing method using denatured plasmid templates. Anal. Biochem. 152:232-238.
8. Iuchi, S., D. C. Cameron, and E. C. C. Lin. 1989. A second global regulator gene (*arcB*) mediating repression of enzymes in aerobic pathways of *Escherichia coli*. J. Bacteriol. 171:868-873.
9. Iuchi, S., and E. C. C. Lin. 1987. Molybdenum effector of fumarate reductase repression and nitrate reductase induction in *Escherichia coli*. J. Bacteriol. 169:3720-3725.
10. Iuchi, S., and E. C. C. Lin. 1987. The *narL* gene product activates the nitrate reductase operon and represses the fumarate reductase and trimethylamine N-oxide reductase operons in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 84:3901-3905.
11. Iuchi, S., and E. C. C. Lin. 1988. *arcA* (*dye*), a global regulatory gene in *Escherichia coli* mediating repression of enzymes in aerobic pathways. Proc. Natl. Acad. Sci. USA 85:1888-1892.
- 11a. Iuchi, S., Z. Matsuda, T. Fujiwara, and E. C. C. Lin. 1990. The *arcB* gene of *Escherichia coli* encodes a sensor-regulator protein for anaerobic repression of the *arc* modulon. Mol. Microbiol. 4:715-727.
12. Jones, H. M., and R. P. Gunsalus. 1987. Regulation of *Escherichia coli* fumarate reductase (*frdABCD*) operon expression by respiratory electron acceptors and the *fnr* gene product. J. Bacteriol. 169:3340-3349.
13. Kita, K., K. Konishi, and Y. Anraku. 1984. Terminal oxidases of *Escherichia coli* aerobic respiratory chain. II. Purification and properties of cytochrome *b*_{558-d} complex from cells grown with limited oxygen and evidence of branched electron-carrying systems. J. Biol. Chem. 259:3375-3381.
14. Kranz, R. G., and R. B. Gennis. 1983. Immunological characterization of the cytochrome *o* terminal oxidase from *Escherichia coli*. J. Biol. Chem. 258:10614-10621.
15. Lambden, P. R., and J. R. Guest. 1976. Mutants of *Escherichia coli* unable to use fumarate as an anaerobic electron acceptor. J. Gen. Microbiol. 97:145-160.
16. Manoil, C., and J. Beckwith. 1985. *TnphoA*: a transposon probe

- for protein export signals. *Proc. Natl. Acad. Sci. USA* **82**:8129–8133.
17. Miller, J. H. 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. Minagawa, J., H. Nakamura, I. Yamato, T. Mogi, and Y. Anraku. 1990. Transcriptional regulation of the cytochrome *b₅₆₂-o* complex in *Escherichia coli*. Gene expression and molecular characterization of the promoter. *J. Biol. Chem.* **265**:11198–11203.
19. Newman, B. M., and J. A. Cole. 1978. The chromosomal location and pleiotropic effects of mutations of the *nirA*⁺ gene of *Escherichia coli*: the essential role of *nirA*⁺ in nitrite reduction and in other anaerobic redox reactions. *J. Gen. Microbiol.* **106**:1–12.
20. Nixon, B. T., C. W. Ronson, and F. M. Ausubel. 1986. Two-component regulatory systems responsive to environmental stimuli share strongly conserved domains with the nitrogen assimilation regulatory genes *ntrB* and *ntrC*. *Proc. Natl. Acad. Sci. USA* **83**:7850–7854.
21. Ostrow, K. S., T. J. Silhavy, and S. Garrett. 1986. *cis*-Acting sites required for osmoregulation of *ompF* expression in *Escherichia coli* K-12. *J. Bacteriol.* **168**:1165–1171.
22. Pabo, C. O., and R. T. Sauer. 1984. Protein-DNA recognition. *Annu. Rev. Biochem.* **53**:293–321.
23. Poole, R. K., and W. J. Ingledew. 1987. Pathways of electrons to oxygen, p. 170–200. In F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
24. Pudek, M. R., and P. D. Bragg. 1974. Inhibition by cyanide of the respiratory chain oxidases of *Escherichia coli*. *Arch. Biochem. Biophys.* **164**:682–693.
25. Rice, C. W., and W. P. Hempfling. 1978. Oxygen-limited continuous culture and respiratory energy conservation in *Escherichia coli*. *J. Bacteriol.* **134**:115–124.
26. Ronson, C. W., B. T. Nixon, and F. M. Ausubel. 1987. Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. *Cell* **49**:579–581.
27. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
28. Silhavy, T. J., M. L. Berman, and L. Enquist. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. Spiro, S., R. E. Roberts, and J. R. Guest. 1989. FNR-dependent repression of the *ndh* gene of *Escherichia coli* and metal ion requirement for FNR-regulated gene expression. *Mol. Microbiol.* **3**:601–608.
30. Stewart, V. 1982. Requirement of Fnr and NarL functions for nitrate reductase expression in *Escherichia coli* K-12. *J. Bacteriol.* **151**:1320–1325.
31. Stewart, V. 1988. Nitrate respiration in relation to facultative metabolism in enterobacteria. *Microbiol. Rev.* **52**:190–232.
32. Trageser, M., and G. Unden. 1989. Role of cysteine residues and of metal ions in the regulatory functioning of FNR, the transcriptional regulator of anaerobic respiration in *Escherichia coli*. *Mol. Microbiol.* **3**:593–599.
33. Unden, G., M. Trageser, and D. Duchene. 1990. Effect of positive redox potentials (<+400 mV) on the expression of anaerobic respiratory enzymes in *Escherichia coli*. *Mol. Microbiol.* **4**:315–319.