

ArcA and AppY Antagonize IscR Repression of Hydrogenase-1 Expression under Anaerobic Conditions, Revealing a Novel Mode of O₂ Regulation of Gene Expression in *Escherichia coli*

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Transcription of the *Escherichia coli* hydrogenase-1 operon (*hyaABCDE*) is increased by the transcription factors ArcA and AppY under anaerobic growth conditions. However, IscR, which represses transcription of the *hyaA* promoter (P_{hyaA}) under aerobic conditions, was not known to repress transcription of this promoter under anaerobic conditions. Here, we report that ArcA and AppY increase P_{hyaA} expression under anaerobic conditions by antagonizing IscR binding at P_{hyaA} , since IscR repression is observed when either ArcA or AppY is eliminated. The ability of ArcA and AppY to act as antirepressors of IscR repression of P_{hyaA} depended on IscR levels, suggesting that IscR competes with ArcA and/or AppY for binding. In support of this competition model, electrophoretic mobility shift assays and DNase I footprinting showed that the ArcA and IscR binding sites overlap and that binding of ArcA and IscR is mutually exclusive. Unexpectedly, IscR with a C92A mutation (IscR-C92A), which mimics the clusterless form of the protein that is present predominantly under aerobic conditions, was a better repressor under anaerobic conditions of both P_{hyaA} and a constitutive promoter containing the IscR binding site from P_{hyaA} than wild-type IscR, which is predominantly in the [2Fe-2S] form under anaerobic conditions. This observation could not be explained by differences in DNA binding affinities or IscR levels, so we conclude that [2Fe-2S]-IscR is a weaker repressor of P_{hyaA} than clusterless IscR. In sum, a combination of ArcA and AppY antirepression of IscR function, lower levels of IscR, and weak repression by [2Fe-2S]-IscR leads to increased P_{hyaA} expression under anaerobic conditions.

Hydrogenases, which catalyze the reversible oxidation of H₂, are widespread throughout the prokaryotic world (36). Despite a detailed biochemical and structural understanding of these enzymes (11, 15), the importance of individual isoforms of hydrogenases to cellular physiology is often not known. In particular, hydrogenase-1 (Hyd-1), encoded by *hyaAB* of the *hyaABCDE* operon, is one of two hydrogenases that function in H₂ oxidation under anaerobic conditions in *Escherichia coli* (30). Recent studies have demonstrated that, unlike most hydrogenases, Hyd-1 is O₂ tolerant (20), raising the question of whether it might also have a role under O₂-limiting conditions. Expression of *E. coli hya* is also subject to various physiological inputs (i.e., anaerobiosis, stationary phase, nitrate, and phosphate starvation) (2, 5, 31), suggesting that it could have specialized roles.

It is well established that expression of the *hya* operon is increased under anaerobic fermentative conditions and decreased under aerobic or anaerobic respiratory conditions with nitrate, consistent with the proposal that Hyd-1 functions in fermentation, not respiration (30). NarL and NarP appear to be responsible for repression of *hya* expression in response to nitrate (5, 31), whereas the anaerobic transcription factors ArcA and AppY are required for the increased expression of the *hya* operon in response to anaerobiosis (5, 31). Under anaerobic conditions, the activity of the response regulator ArcA is increased through phosphorylation by the sensor kinase ArcB (1), while the activity of AppY is presumably increased by changes in *appY* transcription (6). More recently, another transcription factor, IscR, has been shown to directly repress the *hyaA* promoter (P_{hyaA}) but only under aerobic conditions when ArcA and AppY are not active (14, 28). The finding that P_{hyaA} was repressed by IscR under aerobic conditions was surprising since activation of P_{hyaA} under anaero-

bic conditions by ArcA and AppY would make an aerobic repressor unnecessary.

DNase I footprinting showed that IscR binds to a site from -55 to -29 relative to the more-downstream of two *hyaA* transcription start sites (-2 and +1), both of which are repressed by IscR in *in vitro* transcription assays with RNA polymerase σ^{70} (14). The sequence of the protected site indicated that it belongs to the type 2 class of IscR binding sites, which binds with similar affinities to either the [2Fe-2S] cluster or the apo (clusterless) form of IscR (28). Although recent data indicate that the [2Fe-2S] cluster occupancy of IscR is decreased under aerobic conditions (13), we did not expect any differences in IscR function between aerobic and anaerobic conditions for promoters containing type 2 sites since the affinities of the two forms of IscR for the *hyaA* promoter region were similar. Despite this, we found that under anaerobic growth conditions when IscR is predominantly in the [2Fe-2S] form, IscR did not regulate most promoters with type 2 sites, including P_{hyaA} (14).

In this study, we examined the roles of IscR, ArcA, and AppY in regulating the expression of the *hyaA* promoter under aerobic and anaerobic conditions. Using various combinations of mutant alleles, we investigated the role of ArcA and AppY in antagonizing

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IscR repression of P_{hyaA} expression under anaerobic growth conditions. DNA binding assays were used to determine whether IscR and ArcA bind simultaneously to the *hya* promoter region. To test whether increasing the concentration of IscR could overcome antirepression by ArcA and AppY, we varied the levels of both the wild-type and a clusterless mutant of IscR and monitored the expression of the *hya* promoter under anaerobic conditions. Our studies revealed that IscR repressor function is diminished under anaerobic conditions because of antirepression by ArcA and AppY, decreased IscR levels, and decreased repressor function of [2Fe-2S]-IscR.

MATERIALS AND METHODS

Strain construction. In-frame deletions of *arcA* and *lacY* were constructed by replacing the coding region with the *cat* cassette flanked by FLP recognition target (FRT) sites from pKD32 as described previously (8). Transduction with P1vir was used to move *arcA::cat*, *lacY::cat*, or *appY::kan* (from the Keio JW0553-1) (3) to the appropriate strain background(s) with selection for either 20 µg/ml chloramphenicol or 40 µg/ml kanamycin. In some instances, the antibiotic cassette was removed by transforming strains with pCP20, which encodes FLP recombinase (8), and screening for antibiotic sensitivity. In addition, transduction with P1vir was used to introduce *kan*-promoter-*lacZ* constructs into the appropriate strain background(s). Genetic manipulations were confirmed by colony PCR and, in some cases, DNA sequencing.

The chromosomal construct containing *iscR* under the control of P_{tac} and the *isc* operon under the control of P_{BAD} (*bla*-P_{tac}-*iscR*-FRT-*cat*-FRT-*araC*-P_{BAD}-*iscS*) (13) was the starting point to construct a variant with a C-to-A mutation at position 92 encoded by *iscR* [*iscR*(C92A)]. The region encompassing *bla*-P_{tac}-*iscR* was amplified by colony PCR and subsequently cloned into the HindIII and BamHI sites of pACYClacI⁹-*tet* (4) to form pPK9156 (Table 1). Quikchange (Agilent) was subsequently used to create pPK9157 containing *bla*-P_{tac}-*iscR*(C92A). The *bla*-P_{tac}-*iscR*(C92A) region was amplified and electroporated into strain PK9133 as described previously (13) to create strain PK9175. Both *bla*-P_{tac}-*iscR*-FRT-*cat*-FRT-*araC*-P_{BAD}-*iscS* from strain PK9520 and *bla*-P_{tac}-*iscR*(C92A)-FRT-*cat*-FRT-*araC*-P_{BAD}-*iscS* from strain PK9175 were moved to strains PK8153 (P_{hyaA}-*lacZ* Δ*lacY*::FRT) and PK9176 (P_{hyaA}-*lacZ* Δ*lacY*::FRT Δ*arcA*::FRT Δ*appY*::FRT) by transduction with P1vir by selecting for Ap^r and Cm^r with 10 mM arabinose to derepress P_{BAD}-*iscS*. After verification by DNA sequencing, the strains were transformed with pACYClacI⁹-*tet*.

A hybrid P_{syn(hyaA)} promoter, a synthetic promoter in which the IscR binding site from P_{hyaA} was incorporated into a constitutive variant of P_{tac} was constructed. P_{tac} from pPK9003 was first cloned into the BamHI and XhoI sites of pPK7035 to generate pPK9054. The P_{syn(hyaA)} promoter was constructed by Quikchange (Agilent) of pPK9054, in which the region from -52 to -28 relative to the transcription start site of P_{tac} in pPK9054 was replaced with the IscR binding site from P_{hyaA} (-55 to -31 relative to the downstream-most transcription start site [14]). The DNA fragment containing *kan*-P_{syn(hyaA)}-*lacZ* was electroporated and recombined onto the chromosome of BW25993/pKD46 using a previously described method (8) with selection for Km^r. Subsequently, an in-frame deletion of *lacY* was constructed by replacing the coding region with the *cat* cassette flanked by FLP recognition target (FRT) sites from pKD32, as described previously, and the antibiotic cassette was removed by transforming the strain with pCP20, which encodes FLP recombinase (8). Transduction with P1vir was used to introduce *kan*-P_{syn(hyaA)}-*lacZ* with Δ*lacY* into PK4854 and MG1655, selecting for Km^r; the strains were then transformed with pACYClacI⁹-*tet*. Finally, *bla*-P_{tac}-*iscR*-FRT-*cat*-FRT-*araC*-P_{BAD}-*iscS* and *bla*-P_{tac}-*iscR*(C92A)-FRT-*cat*-FRT-*araC*-P_{BAD}-*iscS* were introduced into PK10519 using P1vir transduction, selecting for Ap^r. The resulting strains were confirmed using colony PCR and sequencing.

β-Galactosidase assays. Three independent isolates of each strain were grown to saturation in morpholinepropanesulfonic acid (MOPS)

minimal medium with either 0.2% glucose (27) or, for strains containing *bla*-P_{tac}-*iscR*-FRT-*cat*-FRT-*araC*-P_{BAD}-*iscS* or *bla*-P_{tac}-*iscR*(C92A)-FRT-*cat*-FRT-*araC*-P_{BAD}-*iscS*, 20 mM arabinose (35) with the indicated concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG), 0.2% Casamino Acids (in Fig. 4) and tetracycline (10 µg/liter). The cultures were diluted 1:250 into the same medium containing the same additives and grown at 37°C under aerobic conditions to an optical density at 600 nm (OD₆₀₀) of ~0.2 or under anaerobic conditions to an OD₆₀₀ of ~0.1. The cultures were subsequently assayed for β-galactosidase activity (26). For comparison of aerobically and anaerobically grown cultures in Fig. 1, differences in cell numbers per OD₆₀₀ unit were accounted for by multiplying the Miller units from aerobic cultures by 1.55, as described previously (25). The average of three isolates repeated on at least three independent occasions is presented. In Fig. 3 and 4, fold repression was calculated by dividing the promoter activity in a strain lacking IscR by the promoter activity in the indicated strain. Standard error of the fold repression was calculated as described previously (18).

Purification of IscR and ArcA. Wild-type IscR was purified from PK7599 as described previously (14). N-terminal His₆-tagged ArcA was expressed from a pET21d (Novagen) derivative in BL21 (λDE3). The protein was isolated by passage over a Ni-nitrilotriacetic acid (NTA) matrix column (GE Healthcare) at 4°C and elution with 50 mM Tris-Cl, pH 7.0, 0.4 M NaCl, 1 mM EDTA, and 500 mM imidazole, followed by dialysis against 50 mM Tris-Cl, pH 7.0, 0.1 M NaCl, and 1 mM EDTA. The His₆ tag was removed from ArcA by overnight incubation with tobacco etch virus (TEV) protease at 4°C and passage over a Ni²⁺-agarose column (Qiagen). The protein concentrations of ArcA and IscR and the iron and sulfide content of IscR were determined colorimetrically, as described previously (33).

DNase I footprinting and electrophoretic mobility shift assays. A DNA fragment containing the *hyaA* region from -200 to +40 relative to the transcription start site was excised by BamHI and HindIII digestion of pPK6842, as described previously (14). The fragment was radiolabeled using Klenow fragment (New England BioLabs) with [α-³²P]dGTP (GE Healthcare), and the DNA containing the *hyaA* region was isolated from a 5% nondenaturing gel, followed by purification via an Elutip-d column (Schleicher and Schuell). ArcA was phosphorylated by incubating ArcA with 50 mM disodium carbamyl phosphate in a buffer composed of 50 mM Tris, pH 7.9, 150 mM NaCl, and 10 mM MgCl₂ for 1 h at 30°C (21) and immediately used in the binding assays.

DNase I footprinting was performed at 37°C by incubating protein with 4 nM *hyaA* DNA, 40 mM Tris, pH 7.9, 70 mM KCl, and 0.1 mg/ml bovine serum albumin (BSA) for 10 min before the addition of 2 µg/ml DNase I and 10 mM MgCl₂. Assays were terminated after 30 s by addition of 300 mM sodium acetate (NaOAc) and 20 mM EDTA, pH 7.9; DNA was ethanol precipitated and resuspended in stop solution (USB Scientific) before being heated to 95°C for 30 s and loaded onto an 8% polyacrylamide gel with 7 M urea. An A+G ladder was made by formic acid modification of the radiolabeled DNA, followed by piperidine cleavage (23). The products were visualized using a Storm PhosphorImager (Molecular Dynamics).

Electrophoretic mobility shift assays were performed by incubating either 60% [2Fe-2S]-occupied as-purified wild-type IscR and/or phosphorylated ArcA with 5 nM radiolabeled DNA fragment containing the region from -200 to +40 of the *hyaA* promoter in 5% glycerol, 100 mM potassium glutamate (pH 7.5), 10 mM potassium phosphate (pH 7.5), 50 µg/ml BSA, 3 mM Tris-Cl (pH 7.9), and 10 mM MgCl₂. After a 10-min incubation at 37°C, samples were loaded onto a 10% nondenaturing acrylamide (38:2, acrylamide/bis-acrylamide) gel and run at 150 V for 2 h. The gels were visualized using a Storm PhosphorImager (Molecular Dynamics).

RESULTS

AppY and ArcA prevent IscR repression of the *hyaA* promoter under anaerobic conditions. In agreement with previous find-

TABLE 1 Strains and plasmids used in this work

Strain or plasmid	Relevant genotype	Reference or source
Strains		
MG1655	$\lambda^- F^- rph-1$	Laboratory stock
PK4854	MG1655 $\Delta iscR$	33
BW25993/pKD46	<i>lacI^q hsdR514 $\Delta araBAD_{AH33} \Delta rhaBAD_{LD78}$</i> pKD46	8
PK7599	BL21(λ)DE $\Delta himA::tet$ pPK6161	33
PK7510	BW25993 $\Delta arcA::cat$	This study
PK7514	MG1655 $\Delta arcA::cat$	This study
PK7521	MG1655 $\Delta arcA::FRT$	This study
JW0553-1	$\Delta(araD-araB)567 \Delta lacZ4787(::rrnB-3) \Delta appY773::kan rph-1 \Delta(rhaD-rhaB)568 hsdR514$	Keio Collection
PK8713	MG1655 $\Delta appY::FRT$	This study
PK8714	PK4854 $\Delta appY::FRT$	This study
PK6881	BW25993 $P_{hyaA}-lacZ$	14
PK7573	MG1655 $P_{hyaA}-lacZ$	14
PK6886	PK4854 $P_{hyaA}-lacZ$	14
PK8716	PK8713 $P_{hyaA}-lacZ$	This study
PK7525	PK7521 $P_{hyaA}-lacZ$	This study
PK8717	PK8714 $P_{hyaA}-lacZ$	This study
PK8776	PK6886 $\Delta arcA::cat$	This study
PK8767	PK8716 $\Delta arcA::cat$	This study
PK8768	PK8717 $\Delta arcA::cat$	This study
PK8758	MG1655 $P_{hyaA(-50G)}-lacZ$	28
PK8759	PK4854 $P_{hyaA(-50G)}-lacZ$	28
PK9282	PK8713 $P_{hyaA(-50G)}-lacZ$	This study
PK9283	PK8714 $P_{hyaA(-50G)}-lacZ$	This study
PK9285	PK7514 $P_{hyaA(-50G)}-lacZ$	This study
PK9517	PK8759 $\Delta arcA::cat$	This study
PK9518	PK9283 $\Delta arcA::cat$	This study
PK9519	PK9282 $\Delta arcA::cat$	This study
PK9520	<i>bla-P_{tac}-iscR-FRT-cat-FRT-araC-P_{BAD}-iscS</i>	13
PK8137	PK6881 pKD46	This study
PK8139	PK8137 $\Delta lacY::cat$	This study
PK8148	MG1655 $P_{hyaA}-lacZ \Delta lacY::cat$	This study
PK8153	MG1655 $P_{hyaA}-lacZ \Delta lacY::FRT$	This study
PK8170	PK4854 $P_{hyaA}-lacZ \Delta lacY::FRT$	This study
PK9522	PK8153 <i>bla-P_{tac}-iscR-FRT-cat-FRT-araC-P_{BAD}-iscS</i>	This study
PK9524	PK9522 pACYC <i>lacI^q-tet</i>	This study
PK9133	FRT- <i>cat</i> -FRT- <i>araC</i> -P _{BAD}	13
PK9175	PK9133 <i>bla-P_{tac}-iscR(C92A)</i>	This study
PK9529	PK8153 <i>bla-P_{tac}-iscR(C92A)-FRT-cat-FRT-araC-P_{BAD}-iscS</i> pACYC <i>lacI^q-tet</i>	This study
PK9527	PK8713 $\Delta appY \Delta arcA::FRT$	This study
PK9176	PK9527 $P_{hyaA}-lacZ \Delta lacY::FRT$	This study
PK9178	PK9176 <i>bla-P_{tac}-iscR-FRT-cat-FRT-araC-P_{BAD}-iscS</i>	This study
PK9179	PK9178 pACYC <i>lacI^q-tet</i>	This study
PK9184	PK9176 <i>bla-P_{tac}-iscR(C92A)-FRT-cat-FRT-araC-P_{BAD}-iscS</i>	This study
PK9188	PK9184 pACYC <i>lacI^q-tet</i>	This study
PK10519	MG1655 $P_{syn(hyaA)}-lacZ \Delta lacY$	This study
PK10529	PK4854 $P_{syn(hyaA)}-lacZ \Delta lacY$	This study
PK10554	PK10519 <i>bla-P_{tac}-iscR-araC-P_{BAD}-iscS</i> pACYC <i>lacI^q-tet</i>	This study
PK10556	PK10519 <i>bla-P_{tac}-iscR(C92A)-araC-P_{BAD}-iscS</i> pACYC <i>lacI^q-tet</i>	This study
Plasmids		
pKD46	Phage λ <i>gam-bet-exo</i> genes under P _{araB} control	8
pKD32	FRT- <i>cat</i> -FRT	8
pCP20	Ap ^r	8
pACYC <i>lacI^q-tet</i>	<i>lacI^q</i> in pACYC184, Tet ^r	4
pPK9156	<i>bla-P_{tac}-iscR</i> cloned into HindIII/BamHI sites of pACYC <i>lacI^q-tet</i>	This study
pPK9157	pPK9156 <i>bla-P_{tac}-iscR(C92A)</i>	This study
pPK6842	P_{hyaA} cloned into pUC19- <i>spf</i> with XhoI site replacing SalI site	14
pPK9003	pRZ7411 based vector with <i>bla</i> from pBR322 at AflII site and P_{tac} from pPK7332 at AflII and NdeI sites	13
pPK7035	pBR322 with <i>kan</i> from pHP45 Ω and BamHI-NdeI fragment from pRS1553	17
pPK9054	pPK7035 with P_{tac} from pPK9003 cloned into BamHI and XhoI sites	This study
pPK6161	<i>iscR</i> in pET11a	33

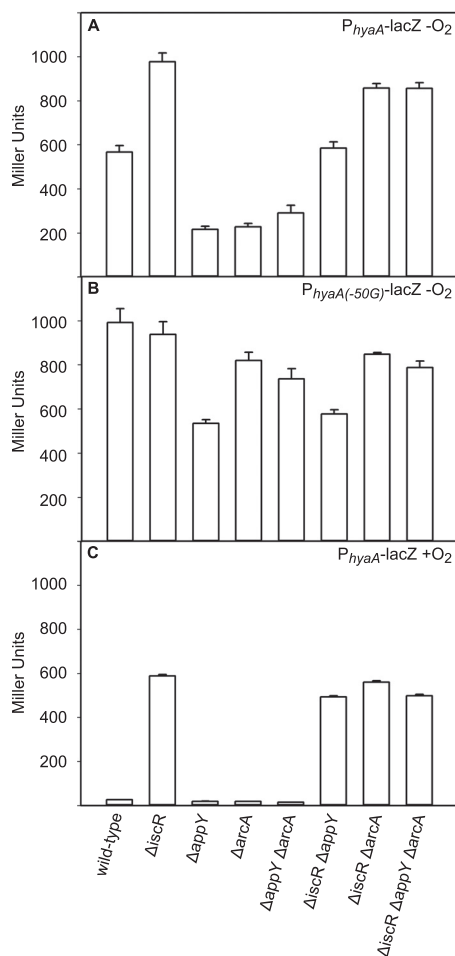


FIG 1 Expression levels of P_{hyaA} (A and C) or $P_{hyaA(-50G)}$ (B) fused to $lacZ$ in wild-type, $\Delta iscR$, $\Delta appY$, $\Delta arcA$, $\Delta appY \Delta arcA$, $\Delta iscR \Delta appY$, $\Delta iscR \Delta arcA$, or $\Delta iscR \Delta appY \Delta arcA$ strain backgrounds grown under anaerobic (A and B) or aerobic (C) conditions in MOPS minimal medium containing 0.2% glucose. Strains were assayed for β -galactosidase activity and normalized for cell number, as explained in Materials and Methods. Error bars are for the activity from three strain isolates repeated on at least three independent occasions.

ings, deletion of *arcA* or *appY* or both *arcA* and *appY* resulted in a 3- to 4-fold decrease in expression of P_{hyaA} - $lacZ$, whereas deletion of *iscR* alone showed only a slight increase in expression (5, 14, 31) (Fig. 1A). However, elimination of all three transcription factors (IscR, AppY, and ArcA) resulted in an unexpectedly high level of expression under anaerobic conditions, which was slightly greater than the level of expression in the parent strain (Fig. 1A). Therefore, the decreased expression in strains lacking ArcA and/or AppY under anaerobic conditions does not appear to result from a defect in activation by ArcA or AppY but, rather, from unmasking IscR repression in the absence of these other transcription factors. The repression by IscR in the absence of either ArcA or AppY appears to be a direct result of IscR binding, since the increase in expression observed from a mutation that is known to disrupt the IscR binding site (C-to-G mutation at position -50 in P_{hyaA} [$P_{hyaA(-50G)}$]) (28) mimicked the increase in expression of the $\Delta iscR$ strains (Fig. 1B).

These data also show that both AppY and ArcA must be present to prevent IscR repression of P_{hyaA} under anaerobic conditions,

since loss of either transcription factor is sufficient to permit repression by IscR. For example, the decreased expression of P_{hyaA} that arises when strains lack ArcA ($AppY^+$ and $IscR^+$) or AppY ($ArcA^+$ and $IscR^+$) is reversed by the further elimination of IscR from either strain (Fig. 1A). ArcA alone appears to slightly repress P_{hyaA} under anaerobic conditions, since a strain containing deletions of *appY* and *iscR* ($ArcA^+$) showed an intermediate expression level (Fig. 1A). However, ArcA repression is not observed when all three transcription factors are present, suggesting that the major effect of ArcA under anaerobic conditions is to increase expression of P_{hyaA} by preventing IscR repression.

ArcA and AppY had no effect on P_{hyaA} expression under aerobic conditions, as expected (Fig. 1C), since ArcA is not active aerobically and since AppY is poorly expressed under these growth conditions (1, 6). In contrast, IscR was able to repress P_{hyaA} - $lacZ$ expression under aerobic conditions, as previously reported (14, 28). Consistent with the notion that P_{hyaA} expression is regulated by IscR repression aerobically and by ArcA and AppY antirepression of IscR anaerobically, P_{hyaA} - $lacZ$ expression levels in the absence of any transcription factors were similar under anaerobic and aerobic conditions (Fig. 1A and C).

ArcA competes for binding with IscR at the *hyaA* promoter.

To examine how ArcA interferes with IscR repression of P_{hyaA} , *in vitro* DNA binding experiments were carried out with isolated ArcA and IscR (Fig. 2A and B). The location of the ArcA binding site within the *hyaA* promoter region was determined by DNase I footprinting, which showed that phosphorylated ArcA bound *hyaA* DNA from position -32 to +3 relative to the transcription start site (Fig. 2A). As expected, no footprint was detected with similar concentrations of unphosphorylated ArcA (data not shown). Comparison of the DNA sequence within the bound region to the predicted ArcA consensus (5'-GTTAATTAATGTTA-3') (10, 24) shows a match from position -17 to +1 on the bottom strand (Fig. 2C). The location of the ArcA footprint overlapped the predicted -10 promoter hexamer for binding RNA polymerase σ^{70} .

Notably, the region protected by ArcA (-32 to +3) was also found to overlap the region protected by IscR (-55 to -29) (Fig. 2A and C) (14), suggesting that binding of ArcA and IscR to *hyaA* promoter DNA may be mutually exclusive. To test whether IscR and ArcA can bind simultaneously, electrophoretic mobility shift assays were performed with radiolabeled *hyaA* DNA, IscR, and/or phosphorylated ArcA. The protein-DNA complex observed with phosphorylated ArcA was larger than the complex with [2Fe-2S]-IscR. However, no complex with a size larger than the ArcA-DNA complex was observed when both proteins were present in the binding assay, indicating that binding of ArcA and IscR to *hyaA* promoter DNA is mutually exclusive (Fig. 2B). Furthermore, preincubation of *hyaA* DNA with phosphorylated ArcA followed by addition of IscR or vice versa did not produce an additional complex, suggesting that the order of addition does not affect binding (data not shown). Thus, ArcA and IscR appear to compete for binding to the *hyaA* promoter.

No binding site information is available for AppY, and attempts to carry out binding studies with AppY were unsuccessful due to an inability to express amounts sufficient for its isolation. Therefore, while the role of AppY in preventing repression by IscR is unclear, the simplest model from the combined results is that both AppY and ArcA act as antirepressors, competing with the repressor IscR for binding to P_{hyaA} .

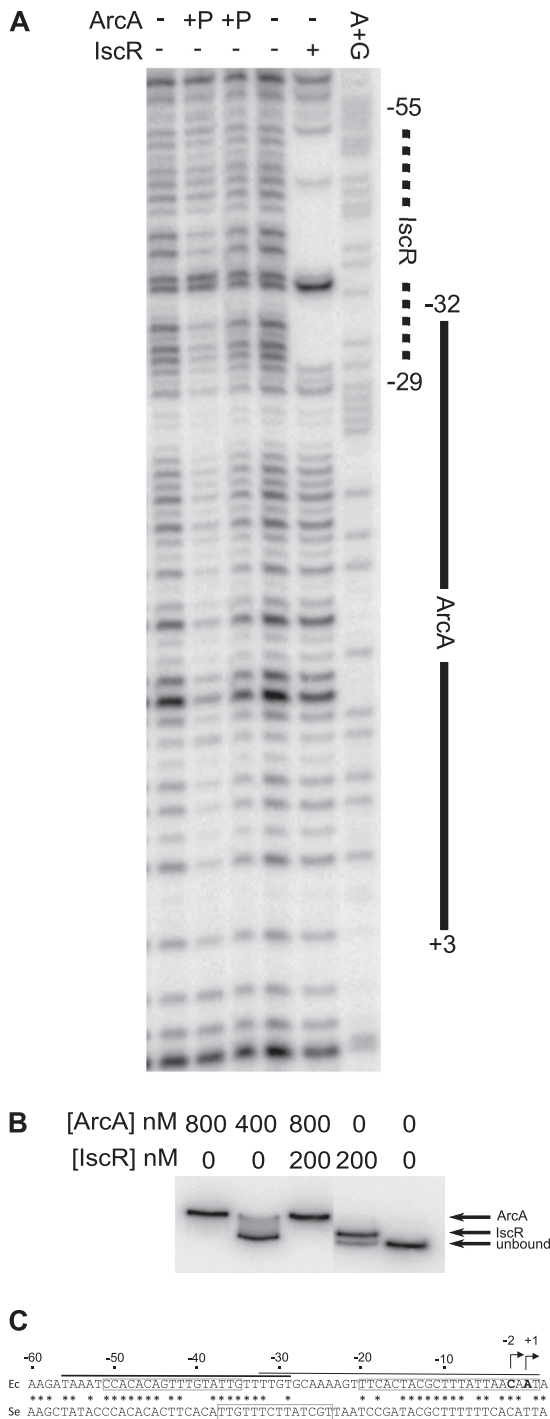


FIG 2 (A) DNase I footprinting of ArcA and IscR binding to the *hyaA* promoter region. The labeled DNA is the top strand from -200 to $+40$ relative to the *hyaA* transcription start site. Regions protected by IscR (dashed line) or phosphorylated ArcA (solid line) are marked, and numbers are relative to the downstream transcription start site. Lanes 1 and 4 lack IscR or ArcA; lanes 2 and 3 contain 800 nM and 400 nM phosphorylated ArcA, respectively; lane 5 contains 800 nM as-purified IscR; lane 6 is a Maxam and Gilbert A+G ladder made from the same DNA. (B) Electrophoretic mobility shift assay using the same radiolabeled *hyaA* DNA as the DNase I footprinting assay. Lanes 1 and 2 contain 800 nM and 400 nM phosphorylated ArcA, respectively; lane 3 contains 200 nM as-purified IscR and 800 nM phosphorylated ArcA; lane 4 contains 200 nM as-purified IscR; lane 5 contains no protein. (C) The regulatory region of the *hyaA* promoter from *E. coli* (Ec) and *S. enterica* (Se). The upstream

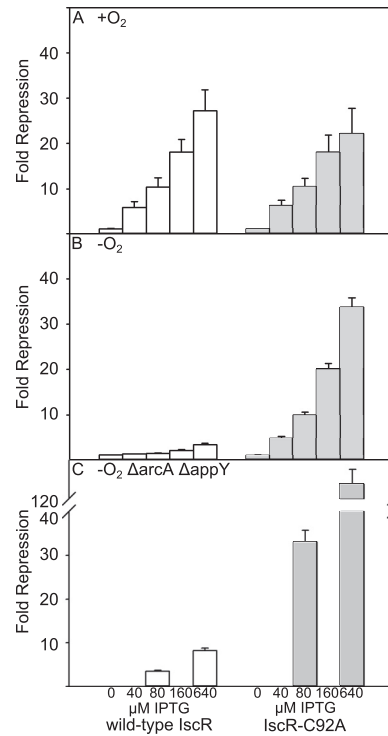


FIG 3 Expression levels of P_{hyaA} fused to *lacZ* in strains containing either *bla-P_{tac}-iscR-FRT-cat-FRT-araC-P_{BAD}-iscS* (white bars) or *bla-P_{tac}-iscR(C92A)-FRT-cat-FRT-araC-P_{BAD}-iscS* (shaded bars) in either a wild-type strain (A and B) or in a strain lacking AppY and ArcA (C). Cells were grown under aerobic (+O₂) or anaerobic (−O₂) conditions in MOPS minimal medium containing 20 mM arabinose, tetracycline (10 μg/liter), and the indicated concentration of IPTG and were assayed for β-galactosidase activity. Error bars represent data from three independent isolates repeated on at least three independent occasions.

Lower IscR levels also contribute to decreased IscR regulation under anaerobic conditions. Since ArcA competes with IscR for binding to P_{hyaA} , we examined whether the proposed antirepression mechanism is dependent on the low IscR protein levels observed under anaerobic conditions compared to levels observed under aerobic conditions (13). Therefore, a strain containing both *iscR* under the control of P_{tac} and *iscSUA hscBA fdx* under the control of P_{BAD} was grown aerobically (Fig. 3A) or anaerobically (Fig. 3B) in the presence of different IPTG concentrations to determine the effect of various IscR levels within the range normally produced under either condition. In addition, the levels of the Isc pathway were maintained at levels similar to aerobic conditions by the addition of 20 mM arabinose (data not shown). We found that as the levels of IscR were increased (by increasing the IPTG concentration) under both conditions, there was a corresponding increase in repression by IscR (Fig. 3). However, much greater repression was observed anaerobically than aerobically at the same concentrations of IPTG, consistent with direct IscR repression at P_{hyaA} without competition from ArcA and AppY aerobically. Fur-

IscR and downstream ArcA sites are boxed in the *E. coli* sequence (top). A predicted FNR site is boxed in the *S. enterica* sequence (bottom). Asterisks indicate conserved residues. The thick line is the IscR footprinted region, and the thin line is the ArcA footprinted region. Arrows indicate the positions of the two transcription initiation sites (31).

thermore, by similarly increasing the levels of IscR in a strain lacking ArcA and AppY, we found that less IscR was required for repression anaerobically (Fig. 3C) than in strains containing AppY and ArcA (Fig. 3B). Therefore, downregulation of IscR levels under anaerobic conditions appears to be important for proper regulation of P_{hyaA} , such that IscR competition with ArcA and AppY for P_{hyaA} binding is maintained at a low level, keeping P_{hyaA} from being repressed anaerobically.

[2Fe-2S]-IscR exhibits decreased repressor function at the $hyaA$ promoter. Despite the fact that we observed more repression of the $hyaA$ promoter with increasing levels of IscR, the extent of P_{hyaA} repression under anaerobic conditions was never as great as that measured under aerobic conditions, even in the absence of AppY and ArcA (Fig. 1 and 3). The observed difference in repression between anaerobic and aerobic growth conditions was not due to insufficient IscR concentrations since the highest IPTG concentrations resulted in IscR protein levels well above those observed in aerobic cells (determined by Western blotting) (data not shown). Since we have previously observed a difference in IscR cluster occupancy between anaerobic and aerobic conditions (13), we examined the possibility that [2Fe-2S]- and apo-IscR might function differently in their abilities to repress P_{hyaA} . To determine whether cluster occupancy affects the ability of IscR to repress under anaerobic conditions, we measured P_{hyaA} - $lacZ$ expression while increasing the levels of IscR with the C92A mutation (IscR-C92A), which lacks the [2Fe-2S] cluster. We found that under anaerobic conditions, more repression of P_{hyaA} was observed with IscR-C92A than with wild-type IscR at the same IPTG concentrations (Fig. 3B), despite the fact that protein levels were similar for wild-type IscR and IscR-C92A (data not shown). A further, but small, increase in repression of P_{hyaA} was observed with IscR-C92A when ArcA and AppY were eliminated (Fig. 3C), indicating that the ability of apo-IscR to repress P_{hyaA} under anaerobic conditions is largely indifferent to the presence of ArcA and AppY. Hence, [2Fe-2S]-IscR appears to be a weaker repressor of P_{hyaA} than apo-IscR.

In order to test IscR repression of the $hyaA$ site in the absence of other factors that might unknowingly affect P_{hyaA} regulation anaerobically, we incorporated the IscR binding site from P_{hyaA} into a constitutively active promoter [$P_{syn(hyaA)}$]. In this synthetic promoter construct, the 25-bp IscR binding site from P_{hyaA} was aligned in the same register relative to the $-35 \sigma^{70}$ promoter recognition element of P_{tac} . When we tested the ability of different levels of wild-type IscR (containing the [2Fe-2S] cluster) and IscR-C92A (apo mutant) to repress the synthetic promoter under anaerobic conditions, we found that the apo mutant repressed more efficiently than the wild-type IscR (Fig. 4). Since removing the IscR binding site from the context of P_{hyaA} results in a similar preference for promoter repression by the apo form of IscR, it is unlikely that there is another factor dictating the cluster dependence of P_{hyaA} repression by IscR. Thus, in addition to decreased IscR protein levels under anaerobic conditions, the poor repressing function of the [2Fe-2S] form of IscR, which predominates under anaerobic conditions, contributes to overall regulation of P_{hyaA} under anaerobic conditions.

DISCUSSION

By examining the expression of the hya operon encoding Hyd-1 in *E. coli* K-12, we have uncovered a new antirepression mechanism

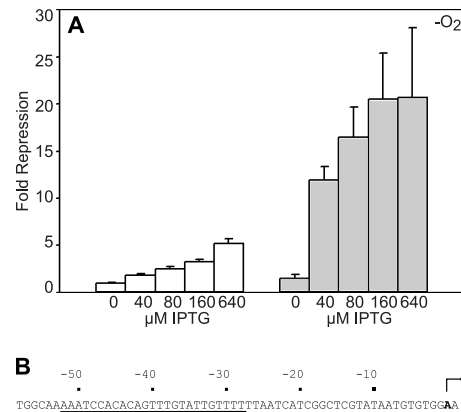


FIG 4 (A) Expression levels of $P_{syn(hyaA)}$ fused to $lacZ$ in strains containing either bla - P_{tac} - $iscR$ -FRT- cat -FRT- $araC$ - P_{BAD} - $iscS$ (white bars) or bla - P_{tac} - $iscR(C92A)$ -FRT- cat -FRT- $araC$ - P_{BAD} - $iscS$ (shaded bars). Cells were grown under anaerobic conditions in MOPS minimal medium containing 20 mM arabinose, tetracycline (10 μ g/liter), 0.2% Casamino Acids, and the indicated concentration of IPTG and were assayed for β -galactosidase activity. Error bars for fold repression are given for the data from three strain isolates repeated on at least three independent occasions. (B) Sequence of $P_{syn(hyaA)}$ promoter fused to $lacZ$. The IscR binding site from P_{hyaA} (underlined) was incorporated into a construct containing a constitutively active variant of P_{tac} fused to $lacZ$. An arrow represents the position of the transcription initiation site.

that results in O₂-regulated gene expression. We found that under anaerobic conditions two transcription factors, ArcA and AppY, work together to overcome the repression of P_{hyaA} by IscR that occurs under aerobic conditions. Antirepression by the anaerobic transcription factors ArcA and AppY, in addition to a combination of decreased levels of wild-type IscR and the weak repressing function of [2Fe-2S]-IscR, resulted in the lack of repression by IscR under anaerobic conditions. Thus, these data suggest a new mechanism by which promoters can be regulated by O₂ availability.

ArcA, AppY, and IscR act combinatorially to control $hyaA$ promoter expression levels under anaerobic conditions. Previous studies (5, 31) showed that deletion of ArcA or AppY results in decreased expression of P_{hyaA} under anaerobic conditions, which led to the hypothesis that ArcA and AppY activated P_{hyaA} . However, the mechanism by which activation occurred was unknown. Our data indicate that the decrease in expression upon elimination of ArcA or AppY is not due to activation by either transcription factor, but, rather, to repression by IscR in the absence of ArcA and/or AppY. Based on the DNase I footprint and electrophoretic mobility shift assays presented in this study, we propose that ArcA antagonizes IscR repression by partially occluding IscR binding. Although isolation of AppY will be required before we can elucidate how this protein inhibits IscR repression at P_{hyaA} , the simplest explanation is that both AppY and ArcA block IscR binding. Taken together, these data show how multiple transcription factors can act combinatorially to achieve an effect not possible by a single transcription factor.

Linking of expression of Hyd-1 with the cytochrome bd -II oxidase. Immediately downstream of the hya operon are the genes $cbdAB$ (also known as $appCB$) encoding subunits of the cytochrome bd -II oxidase. Previous *in vivo* studies indicated that $cbdAB$ expression is also decreased in strains lacking AppY and ArcA (6). Furthermore, transcription of both $cbdAB$ and hya oper-

ons is regulated by similar growth conditions, including stationary phase, phosphate starvation, and anaerobiosis (2). The similar regulation of these two operons suggests that they may function in a common pathway. Since cytochrome *bd*-II oxidase functions to reduce O₂ to H₂O (34), it is reasonable to speculate that under low-O₂ conditions, H₂ oxidation via Hyd-1 might provide reducing equivalents for the cytochrome *bd*-II oxidase and explain the O₂ tolerance of Hyd-1. If this is indeed the case, then the combinatorial control that we have observed of P_{hyaA} may also facilitate expression under low-O₂ conditions.

Novel features of IscR regulation. The lack of repression of P_{hyaA} under anaerobic conditions appears to be linked to the lower anaerobic levels of IscR (10-fold less than aerobic protein levels [13]) since artificially increasing the levels of wild-type IscR to those found under aerobic conditions resulted in increased repression of P_{hyaA} by IscR, even in the presence of ArcA and AppY. Hence, the concentration of IscR, in addition to regulation by ArcA and AppY, is important for determining the anaerobic expression levels of P_{hyaA}.

Surprisingly, we also found that even in the absence of AppY and ArcA, wild-type IscR could not repress P_{hyaA} anaerobically as well as an IscR mutant that lacked the Fe-S cluster or as well as wild-type IscR aerobically at the same protein concentrations. The only reported difference regarding IscR between aerobic and anaerobic conditions is that under anaerobic conditions IscR appears to be more highly occupied with the [2Fe-2S] cluster than its aerobic counterpart (13). Since *in vitro* DNA binding experiments have shown that [2Fe-2S]-IscR and apo-IscR bind the *hyaA* DNA site with the same affinities (16, 28), our data imply that [2Fe-2S]-IscR is a weaker repressor than the apo form at P_{hyaA}. The differential repressor activities of [2Fe-2S]- and apo-IscR are reminiscent of [2Fe-2S]-SoxR, which binds DNA in both an active and inactive state but regulates transcription only in the active state (12, 16). However, additional experiments will be required to explain this observation.

Interestingly, the mechanism described here for the regulation of the *hyaA* promoter may be relevant to the IscR-dependent regulation of other promoters that contain type 2 IscR binding sites and are regulated by multiple transcription factors, including the *sufA*, *hybO*, and *sodA* promoters (7, 14, 31, 37). However, the combination of transcription factors active at each promoter varies, suggesting that the mechanism of regulation of these promoters is different. How IscR interacts with other transcription factors to regulate this subset of promoters is therefore in need of further study.

ArcA- and IscR-dependent regulation of the *hyaA* promoter is not conserved in a close relative. Surprisingly, the mechanism of O₂ regulation of P_{hyaA} described here for *E. coli* K-12 is not conserved in the close relative *Salmonella enterica* serovar Typhimurium. Rather, *hya* expression in *S. enterica* is regulated not by IscR or ArcA but, rather, by FNR under anaerobic conditions (38). However, comparison of the *hyaA* upstream promoter regions from the two organisms suggests that while the IscR binding site is conserved, a single base pair mismatch to the ArcA site may decrease DNA binding, thus preventing ArcA regulation (Fig. 2C). Further work is necessary to understand the significance of this finding, including mapping the transcription start site for the *hya* operon from *S. enterica*, since it is not known whether the positions of the promoter elements are conserved. However, the sequence similarities between the *S. enterica* and *E. coli* promoter

regions may suggest a recent loss in the regulatory control by ArcA and IscR, with a gain of FNR regulation in this closely related enterobacterium.

Role of ArcA as an antirepressor. Phosphorylated ArcA directly regulates many genes, including those encoding aerobic or anaerobic respiratory enzymes, and typically functions as a repressor (19, 32). Therefore, the role of ArcA in antirepression of IscR at P_{hyaA} described in this study is more consistent with its typical function as a repressor than the previously suggested role as an activator at this promoter. However, the role of ArcA as an anaerobic antirepressor of IscR at P_{hyaA} raises the question of whether the decreased expression of other promoters in strains lacking ArcA is due to the loss of ArcA activation or to the unmasking of repression by another transcription factor. Several other promoters that show decreased expression in strains lacking ArcA (*erpA*, *cydA*, *ndh*, *caiT*, and *focA* promoters) (9, 19, 32) are also regulated by other transcription factors, so antirepression by ArcA might be possible at other targets. Alternatively, these transcripts may be targets of the small RNA ArcZ (22) although transcript analysis of an *S. enterica* *arcZ* mutant did not show regulation of these genes by ArcZ (29). Because ArcA has not yet been shown to activate transcription *in vitro*, it remains to be seen whether ArcA functions as an activator or as an antirepressor at other promoters, whose expression decreases upon deletion of *arcA*.

In sum, we have found that a combinatorial strategy leads to ArcA and AppY overcoming IscR repression at P_{hyaA}. Thus, we have uncovered a new antirepression mechanism to increase gene expression under anaerobic conditions that does not require direct activation of RNA polymerase. Additional studies will be needed to determine the extent to which an antirepression mechanism is used to control the expression of other O₂-regulated genes in *E. coli* K-12 and other facultative bacteria.

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