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Mutational Analysis of the *Escherichia coli melR* Gene Suggests a Two-State Concerted Model To Explain Transcriptional Activation and Repression in the Melibiose Operon

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Transcription of the *Escherichia coli melAB* operon is regulated by the MelR protein, an AraC family member whose activity is modulated by the binding of melibiose. In the absence of melibiose, MelR is unable to activate the *melAB* promoter but autoregulates its own expression by repressing the *melR* promoter. Melibiose triggers MelR-dependent activation of the *melAB* promoter and relieves MelR-dependent repression of the *melR* promoter. Twenty-nine single amino acid substitutions in MelR that result in partial melibiose-independent activation of the *melAB* promoter have been identified. Combinations of different substitutions result in almost complete melibiose-independent activation of the *melAB* promoter. MelR carrying each of the single substitutions is less able to repress the *melR* promoter, while MelR carrying some combinations of substitutions is completely unable to repress the *melR* promoter. These results argue that different conformational states of MelR are responsible for activation of the *melAB* promoter and repression of the *melR* promoter. Supporting evidence for this is provided by the isolation of substitutions in MelR that block melibiose-dependent activation of the *melAB* promoter while not changing melibiose-independent repression of the *melR* promoter. Additional experiments with a bacterial two-hybrid system suggest that interactions between MelR subunits differ according to the two conformational states.

The AraC family of bacterial transcription factors contains a large number of activators that regulate transcription initiation at promoters controlling genes important for virulence, stress, and metabolism (reviewed in references 7, 8, 16, and 29). Members of the AraC family are defined by an ~110-amino-acid domain, containing two helix-turn-helix motifs, that recognizes ~20-bp operator sequences at target promoters. Many members of the AraC family also contain an ~170-amino-acid ligand-binding domain which regulates their activity. The *Escherichia coli* MelR protein appears to be a typical member of this family (33). Its function is to activate expression of the *E. coli* melibiose operon, *melAB*, in response to the availability of melibiose. MelR consists of an ~170-amino-acid melibiose-binding N-terminal regulatory domain joined to an ~110-amino-acid DNA-binding domain via an ~20-amino-acid linker (17). The aim of this work was to exploit mutational analysis to understand how melibiose binding modulates the activity of MelR.

The *E. coli melAB* and *melR* genes are expressed from divergent promoters, p*melAB* and p*melR*, whose transcription start sites are separated by 237 bp (32). The regulatory region between the two promoters is complex and contains five 18-bp DNA sites for MelR (known as sites 1, 1′, 2, 2′, and R) and two 22-bp DNA sites for the cyclic AMP receptor protein (CRP) (2, 30, 31) (Fig. 1). Transcription initiation at p*melAB* is totally dependent on MelR and melibiose. This requires the binding of MelR to operator site 2′, centered at position −42.5 upstream of the *melAB* transcript start site. Site 2′ overlaps the −35 element of p*melAB*, and MelR bound at site 2′ activates transcription by making a direct contact with the RNA polymerase σ subunit (10). MelR binds to site 2′ only in the presence of melibiose (2). In the absence of melibiose, MelR occupies the other four sites, and this results in repression of p*melR* (30). Repression of p*melR* requires MelR binding to site R, which overlaps the *melR* promoter, but also MelR binding to site 2, located 176 bp upstream. It has been proposed (30) that repression requires the formation of a DNA loop that is stabilized by MelR binding at site R and site 2 and that the presence of melibiose breaks this loop, resulting in derepression of p*melR*, occupation of site 2′, and induction of p*melAB* (Fig. 1). Thus, melibiose toggles MelR between a state where it represses p*melR* and is unable to activate p*melAB* to a state where p*melR* is derepressed and p*melAB* is activated. Our aim was to understand this transition. MelR, like many AraC family members, is insoluble at higher concentrations, and structural studies have proven impossible. Hence, here we have tackled the problem using genetic approaches.

MATERIALS AND METHODS

Bacterial strains, plasmids, and oligonucleotide primers. Bacterial strains and plasmids used in this work are described in Table 1, and oligonucleotide primers are listed in Table 2.

WAM1321 is a derivative of WAM132 carrying an in-frame deletion of the *melA* gene, constructed by the Datsenko and Wanner (4) method. This construction was made by using PCR primers D42648 and D42649 to amplify a DNA fragment carrying the *cat* gene from pKD3 flanked by upstream and downstream *melA* sequences. The fragment was electroporated into WAM132 carrying pKD46 encoding phage λ *red* functions, and crossovers were selected as chloramphenicol-resistant colonies. After checking the insertion of the *cat* gene using

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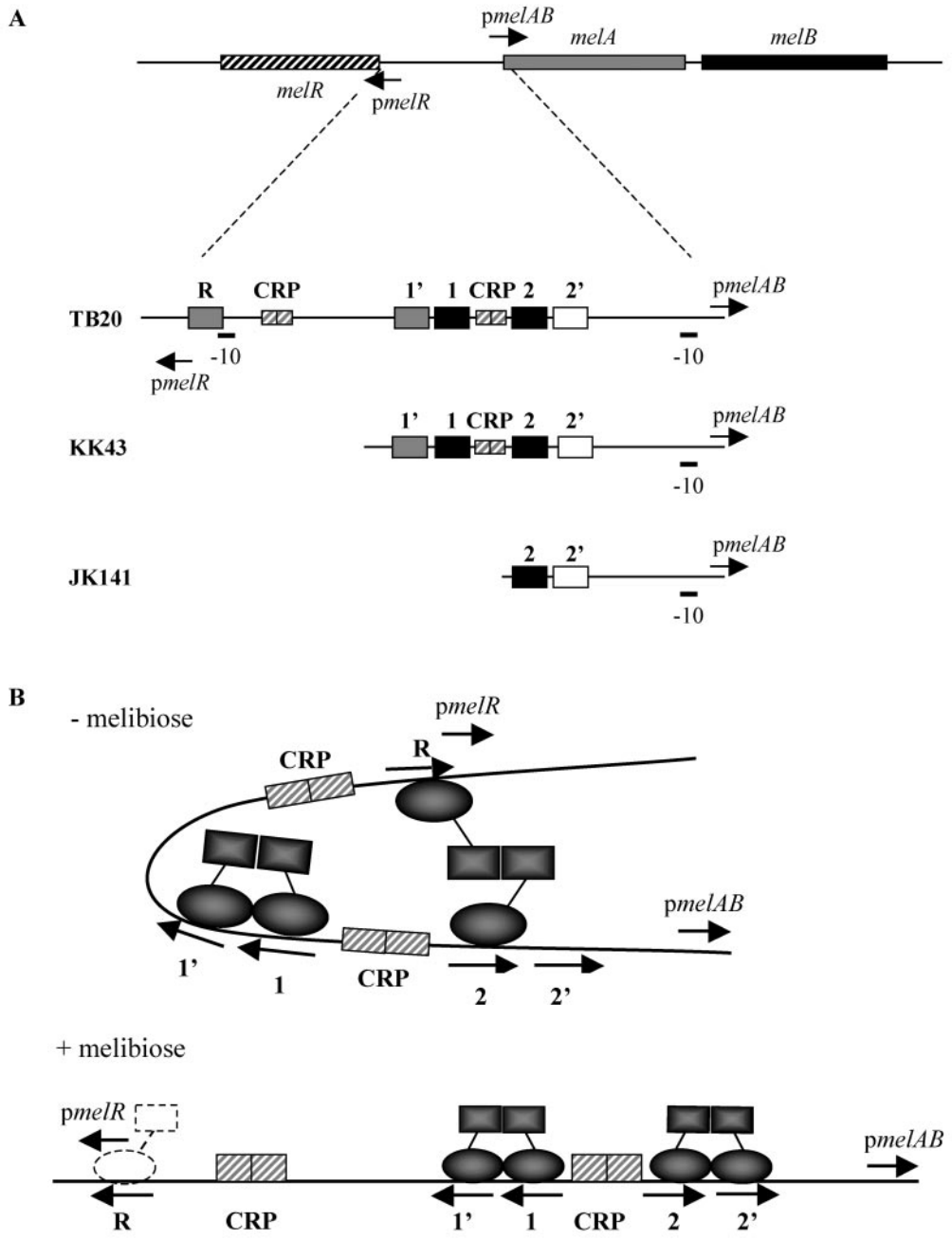

FIG. 1. Organization of the *E. coli* melibiose operon regulatory region. (A) A not-to-scale illustration of the organization of the *melR*, *melA*, and *melB* genes, with the locations and orientation of p*melR* and p*melAB*. In the lower part of the figure, expanded views of the TB20, KK43, and JK141 fragments are shown, with the locations of the p*melAB* and p*melR* −10 elements and the different DNA sites for CRP (small hatched boxes) and MelR (larger boxes shaded according to binding hierarchy in the absence of melibiose). In this work, the TB20 fragment was cloned with EcoRI and HindIII linkers upstream and downstream of p*melR*, respectively, into pRW50 to give a p*melR*::*lac* fusion. The KK43 and JK141 fragments were cloned with EcoRI and HindIII linkers upstream and downstream of p*melAB*, respectively, into pRW50 to give a p*melAB*::*lac* fusion. (B) Interactions of MelR with the different sites in the absence and presence of melibiose as proposed by Wade et al. (30). In the absence of melibiose, MelR is unable to occupy site 2′, and an interaction between MelR bound at site 2 and site R causes strong repression of p*melR*. In the presence of melibiose, MelR occupies site 2′, the interaction between site 2 and site R is broken, and the strong repression of p*melR* is relieved. Weaker repression is due to residual binding of MelR to site R (dotted outline).

PCR, pKD46 was cured. The FLP recombinase, carried by pCP20, was then used to remove the *cat* insert to generate an in-phase deletion in the *melA* gene.

JK141-pRW50 was derived from KK43-pRW50 using PCR with primers D49091 and D10527 to construct a shorter p*melAB* fragment (Fig. 1). The resulting PCR product was restricted with EcoRI and HindIII and cloned into pRW50.

Plasmids pK-T25-MelR and pU-T18C-MelR and derivatives were constructed by cloning an XbaI-KpnI fragment encoding full-length or mutant MelR into

TABLE 1. Bacterial strains and plasmids used in this work

Bacterial strain or plasmid	Characteristics or description[a]	Origin
E. coli strains		
WAM131	*E. coli* K-12 *ara thi pro* Δ*lac*	Belyaeva et al. (2)
WAM132	Δ*melR* derivative of WAM131	Belyaeva et al. (2)
WAM1321	Δ*melA* derivative of WAM132	This work
BTH101	*E. coli* K-12 *cya-99*	Ladant (14)
Plasmids		
pKD3	Carries Cm^r flanked by *flp* sites	Datsenko and Wanner (4)
pKD46	Encodes λ *red* functions	Datsenko and Wanner (4)
pCP20	Encodes FLP recombinase	Datsenko and Wanner (4)
pJW15	Carries *melR* and Amp^r	Wade et al. (30)
pJW15 derivatives	Carrying different mutant *melR* alleles	This work
pJW15Δ*melR*	pJW15 derivative with deletion of *melR*	Wade et al. (30)
pRW50	Lac expression vector, carries Tet^r	Lodge et al. (15)
KK43-pRW50	pRW50 carrying KK43 p*melAB* fragment	Belyaeva et al. (2)
JK141-pRW50	pRW50 carrying JK141 p*melAB* fragment	This work
TB20-pRW50	pRW50 carrying TB20 p*melR* fragment	Wade et al. (30)
pK-T25	Carries T25 adenylate cyclase fragment; Kan^r	Karimova et al. (13)
pK-T25-zip	Carries T25:leucine zipper fusion	Karimova et al. (13)
pK-T25-MelR	Carries T25::*melR* fusion	This work
pK-T25-MelR derivatives	Carries T25::*melR* derivative fusions	This work
pU-T18C	Carries T18 adenylate cyclase fragment; Amp^r	Karimova et al. (14)
pU-T18C-zip	Carries T18::leucine zipper fusion	Karimova et al. (14)
pU-T18C-MelR	Carries T18::*melR* fusion	This work
pU-T18C-MelR derivatives	Carries T18::*melR* derivative fusions	This work

[a] Cm, chloramphenicol; Amp, ampicillin; Tet, tetracycline; Kan, kanamycin.

pK-T25 or pU-T18C plasmid that had been digested with XbaI and KpnI. The fragments were generated by PCR, using primers D37452 and D37453 with pJW15 encoding wild-type or mutant MelR as template, followed by digestion of the product with XbaI and KpnI.

Generation of random mutations in *melR*. Error-prone PCR was used to amplify an EcoRI-HindIII fragment encoding *melR* using pJW15 as a template and primers D5431 and D4600. In these experiments, we used *Taq* DNA polymerase and buffer conditions as described by Barne et al. (1). Fragments from different reactions were digested with EcoRI and HindIII, purified, and recloned into pJW15 to generate independent libraries of mutations. DNA from the libraries was electroporated into tester strains carrying p*melAB*::*lac* fusions as described below in Results. Transformants were screened either on minimal medium plates containing 20 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) or MacConkey lactose plates, with 80 μg/ml ampicillin and 35 μg/ml tetracycline. For minimal medium, we used M9 containing 0.3% fructose and 0.1% Casamino Acids, as previously described (32). After selection of each pJW15 derivative, encoding mutant MelR, the entire EcoRI-HindIII fragment encoding *melR* was sequenced in the University of Birmingham Functional Genomics laboratory (http://www.genomics.bham.ac.uk/) using primer D5431.

Combination of different *melR* mutations. Most derivatives of pJW15 encoding MelR with two or more substitutions were made by exploiting the unique NsiI site corresponding to codon 100. The YD25 FY53, KE123 DG256, and NI183 FS191 double mutants were made by megaprimer PCR using the D38456, D38392, and D37665 primers, respectively, together with the flanking D5431 or D4600 primers. The QR238 TA277 derivative was isolated as a spontaneous pJW15 mutant during screening for MelR mutants competent for melibiose-independent activation of p*melAB*.

TABLE 2. Oligonucleotide primers

Name	Sequence
D42648	5′-CTGCCATGATGAAGTTATTCAAGCAAGCCAGGAGATCTGCGTGTAGGCTGGAGCTGCTTC-3′
D42649	5′-CCGGAATAAATCCCCCGGCGCAGTAGCGTTTAGTCGCGTTCATATGAATATCCTCCTTAG-3′
D49091	5′-GCAGAATTCGATCTGAGTTTAT-3′
D10527	5′-GCAGGTCGTTGAACTGAGCCTGAAATTCAGG-3′
D37452	5′-GCTAGTCTAGAGATGAATACAGATACGTTTATG-3′
D37453	5′-GCGGGGTACCCGTTAGCCGGGAAACGTCTGGCG-3′
D5431	5′-ACCTGACGTCTAAGAAACC-3′
D4600	5′-GTAGTCGGTGTGTTCAC-3′
D38456	5′-GATATGCGGTGCGCGAAACTCAAT-3′
D38392	5′-GCGAATTTGCTCGTTCGGACTGTTT-3′
D37665	5′-CGCCATGCGCAATCTTATGTTAGCC-3′

Assays for activation and repression by mutant MelR derivatives. WAM132 or WAM1321 cells carrying pRW50 with either p*melAB*::*lac* or p*melR*::*lac* fusions and pJW15 encoding *melR* were grown aerobically overnight at 30°C in M9 medium containing 0.3% fructose, 0.1% Casamino Acids, 80 μg/ml ampicillin, and 35 μg/ml tetracycline as previously described (32). The next day, 100-μl aliquots were inoculated into 5 ml of fresh culture either without or with added melibiose (10 mM). These cultures were grown aerobically at 30°C for several hours until the A_{600} reached 0.3 to 0.4. At this point, cultures were lysed with toluene, and β-galactosidase activities were measured as described by Miller (18). Activities were used to measure MelR-dependent activation of p*melAB* and MelR-dependent repression of p*melR*. Note that these assays were performed at 30°C to avoid complications due to the thermosensitivity of the MelB melibiose permease (28).

Bacterial two-hybrid assays. The bacterial adenylate cyclase two-hybrid (BACTH) assay, as described by Karimova et al. (13), was used to monitor interactions between *melR* fused to the T18 and T25 segments of the *Bordetella pertussis* adenylate cyclase. The *E. coli cya* strain BTH101 was transformed by derivatives of pU-T18C and pK-T25, and transformants were plated on MacConkey lactose or MacConkey maltose plates containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. For β-galactosidase assays, transformants were grown aerobically at 30°C in LB medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin to an A_{600} of 0.3 to 0.4. Cultures were lysed with toluene, and activities were measured as described by Miller (18).

RESULTS

Melibiose-independent activation of p*melAB* by MelR. We previously showed that MelR-dependent activation of p*melAB* could be readily monitored using the low-copy-number, broad-host-range *lac* fusion plasmid pRW50, carrying the KK43 p*melAB* promoter fragment (KK43-pRW50) (2). The KK43 p*melAB* fragment carries two pairs of DNA sites for MelR

(sites 1 and 1′ and sites 2 and 2′) separated by a DNA site for CRP (Fig. 1). In the absence of melibiose, MelR and CRP bind to form a complex, but site 2′ is not occupied. The occupation of site 2′, and concomitant transcription activation, is triggered by melibiose (2). To screen for MelR mutants with altered regulatory properties, we used the *E. coli* Δ*melR* Δ*lac* strain, WAM132, transformed with KK43-pRW50. Plasmid pJW15 was used to supply MelR to activate expression of the p*melAB*::*lac* fusion carried by KK43-pRW50. Mutant *melR* libraries were made by using error-prone PCR to amplify a DNA fragment encoding the *melR* gene and recloning this DNA into pJW15.

WAM132 cells carrying KK43-pRW50 and pJW15 encoding wild-type *melR* score as Lac^+ on X-Gal indicator plates containing melibiose and score as Lac^- in the absence of melibiose. Table 3 lists β-galactosidase activity measurements, which showed that p*melAB* activity is very low in the absence of melibiose and that melibiose triggers a >50-fold increase in p*melAB* activity that is MelR dependent. DNA from eight independent preparations of pJW15 carrying randomly mutated *melR* was electroporated into WAM132 cells containing KK43-pRW50. Transformants were plated onto X-Gal indicator plates in the absence of melibiose. We reasoned that colonies exhibiting an enhanced Lac^+ phenotype must carry pJW15 plasmids encoding MelR mutants (designated MelR*) able to activate p*melAB* in the absence of melibiose. After screening and purification of such colonies, extraction of pJW15 DNA, and back-transformation into the test strain, we isolated 107 mutant pJW15 derivative candidates. DNA sequencing revealed that, among these, 29 carry single base changes that give rise to single amino acid substitutions in MelR. These changes are spread throughout the entire length of MelR and are listed in Table 3.

To quantify the effects of the different substitutions, the β-galactosidase activities in cultures of WAM132 cells containing KK43-pRW50 and each mutant pJW15 derivative, grown in the absence of melibiose, were measured. The data in Table 3 show that the changes increase p*melAB* activity by factors ranging from 1.5-fold to 14-fold. None of the substitutions resulted in full melibiose-independent activation, and many caused only marginal increases in expression. The biggest effect, seen with the GD71 substitution, corresponds to only ~25% of the full activation. Results in Table 3 show that, with each mutant MelR, the addition of melibiose increases the activity of p*melAB*. In most cases, the observed expression is similar to that observed with wild-type MelR in the presence of melibiose.

For some of the substitutions, we measured the concentration of melibiose required to increase expression of the p*melAB*::*lac* fusion. To do this, the assays were made in strain WAM1321, a derivative of WAM132 carrying an in-phase deletion of the *melA* gene. Recall that *melA* encodes the α-galactosidase that hydrolyzes melibiose to glucose and galactose. Data in Table 3 show that, with pJW15 encoding wild-type MelR, 4 μM melibiose is required for 50% of the melibiose-dependent induction of p*melAB* activity. With pJW15 encoding the YD25, FY53, KE123, SF167, KR182, or NI183 MelR* substitutions, only 0.2 to 0.5 μM melibiose is required.

In the next series of experiments, we investigated whether combining different substitutions located in different parts of MelR would result in a mutant protein better able to activate p*melAB* in the absence of melibiose. Thus, we constructed pJW15 derivatives that encode the following double substitutions: YD25 FY53, FY53 SF167, FY53 KR182, FY53 DG256,

TABLE 3. β-Galactosidase activity in WAM132 Δ*melR* Δ*lac* cells[a]

pJW15 derivative encoding *melR*	β-Galactosidase activity p*melAB*::*lac* (KK43)		Melibiose concn (μM) required for 50% induction
	No melibiose	10 mM melibiose	
No *melR*	11	11	
Wild-type *melR*	17	980	4 ± 2
Single mutants			
QE14	51	997	
EV24	40	1,225	
YD25	232	1,070	0.2 ± 0.1
YH25	38	1,048	
YN25	42	1,078	
RP27	190	598	
IT37	33	960	
SN41	32	1,254	
FY53	124	1,080	0.4 ± 0.1
IV68	34	1,000	
GD71	241	1,098	
IT73	33	1,080	
GS119	85	923	
KE123	77	941	0.5 ± 0.1
QR128	131	980	
QR140	31	1,080	
EG156	133	1,421	
ST167	105	1,077	
SF167	133	970	0.5 ± 0.1
KR182	68	1,068	0.5 ± 0.1
NI183	140	990	0.5 ± 0.1
NS183	70	1,400	
QR190	163	774	
FL191	32	902	
FS191	34	931	
SG194	35	1,049	
QR238	30	995	
DG256	190	1,215	
TA277	30	1,030	
Double mutants			
YD25 FY53	310	1,209	
FY53 SF167	210	1,113	
FY53 KR182	440	1,150	
FY53 DG256	594	1,261	
KE123 DG256	742	1,279	
NI183 FS191	170	1,090	
QR238 TA277	318	1,035	
Quadruple mutants			
YD25 FY53 QR238 TA277	870	1,023	
YD25 FY53 NI183 FS191	920	1,034	

[a] The first column of the table lists the substitutions in different MelR derivatives selected as competent for melibiose-independent activation of p*melAB*. Activities are expressed in the units described by Miller (18) and were determined using the Miller protocol. The data in columns 2 and 3 are averages of at least four independent determinations that differed by no more than 10%. Cells were grown aerobically in defined medium with fructose as a carbon source either with or without 10 mM melibiose and were harvested in exponential phase at an optical density at 600 nm of ~0.3. Column 4 lists the melibiose concentration required for 50% of the melibiose-dependent activation of p*melAB*. These estimates are derived from assays of p*melAB*::*lac* expression in the WAM1321 Δ*melA* strain grown in media with different concentrations of melibiose (0.1, 1.0, and 10 μM and 0.1, 1.0, and 10 mM).

KE123 DG256, NI183 FS191, and QR238 TA277. Results in Table 3 show that, in combination, the effects of the different substitutions were additive, and in some cases synergy was found. Hence, with pJW15 carrying the double substitutions, p*melAB* activity in the absence of melibiose ranges from 17 to 75% of the activity with wild-type MelR in the presence of melibiose. Finally, pJW15 derivatives were constructed encoding MelR YD25 FY53 together with the NI183 FS191 or QR238 TA277 changes. Further data listed in Table 3 show that, with pJW15 carrying these quadruple substitutions, p*melAB* activity in the absence of melibiose rises to >90% of the activity seen with wild-type MelR in the presence of melibiose.

Repression of p*melR* by MelR∗ mutants. The simplest explanation of our data is that melibiose switches MelR from a conformation that is unable to activate p*melAB* to a conformation that is able to activate p*melAB* and that the different MelR∗ substitutions favor adoption of the latter conformation to different extents. In our previous work (30), we showed that, in the absence of melibiose, the expression of a p*melR*::*lac* fusion could be repressed >10-fold by MelR and that this repression required MelR binding to site R, overlapping p*melR*, and to site 2, located 176 bp upstream. Since repression is greatly reduced by melibiose, we investigated whether it is reduced by the different MelR∗ substitutions. To do this we used the Δ*melR* Δ*lac* strain, WAM132, transformed with pRW50 carrying the TB20 p*melR* promoter fragment (TB20-pRW50). The TB20 p*melR* fragment carries the *melR* promoter and upstream sequences, including MelR site 2 (Fig. 1). Plasmid pJW15 was used to supply wild-type or mutant MelR to repress expression of the p*melR*::*lac* fusion carried by TB20-pRW50.

WAM132 cells containing TB20-pRW50 and pJW15 encoding wild-type MelR score as Lac$^-$ on MacConkey or X-Gal indicator plates. A Lac$^+$ phenotype is observed if pJW15 is replaced by empty vector plasmid. Table 4 lists β-galactosidase activity measurements, which confirmed that, in the absence of melibiose, p*melR* activity is repressed >10-fold by wild-type MelR. With one exception, each of the different single MelR∗ substitutions results in a small reduction in the MelR-dependent repression of p*melR*. The exception is the GS119 MelR∗ substitution, which results in a much greater reduction in repression. Table 4 also shows measurements of the repression of p*melR* by MelR carrying different combinations of substitutions. Double-substituted MelR represses p*melR* less efficiently, and repression by MelR carrying YD25 FY53 together with the NI183 FS191 or QR238 TA277 changes is minimal.

MelR mutants that are less able to activate p*melAB*. The substitutions in the MelR∗ mutants described above appear to bias MelR towards a conformation that can activate p*melAB* but is unable to repress p*melR*. To find complementary mutants, stabilized in the conformation that represses p*melR* but unable to activate p*melAB*, we screened the eight libraries of mutated pJW15 using two steps. In the first step, pJW15 DNA was electroporated into the WAM131 Δ*lac* strain containing plasmid KK43-pRW50 carrying a p*melAB*::*lac* fusion. Since strain WAM131 is *melR*$^+$, colonies score as Lac$^+$ on MacConkey lactose indicator plates either with or without pJW15 encoding MelR. However, some *melR* alleles unable to activate p*melAB* result in Lac$^-$ colonies, and 50 such colonies were selected. In the second step, pJW15 DNA was purified from each colony and retransformed into WAM132 cells containing TB20-pRW50. Most of the 50 pJW15 mutant DNAs gave rise to Lac$^+$ colonies. These DNAs, which encode mutant MelR that is unable to repress p*melR*, presumably due to a defect in

TABLE 4. β-Galactosidase activity in WAM132 Δ*melR* Δ*lac* cells grown in the absence of melibiose[a]

pJW15 derivative encoding *melR*	β-Galactosidase activity	
	p*melR*::*lac* (TB20)	p*melAB*::*lac* (KK43)
No *melR*	274	11
Wild-type *melR*	25	17
Single mutants		
QE14	37	51
EV24	35	40
YD25	65	232
YH25	45	38
YN25	45	42
RP27	50	190
IT37	27	33
SN41	38	32
FY53	60	124
IV68	38	34
GD71	60	241
IT73	37	33
GS119	154	85
KE123	50	77
QR128	42	131
QR140	36	31
EG156	49	133
ST167	39	105
SF167	40	133
KR182	37	68
NI183	51	140
NS183	40	70
QR190	60	163
FL191	29	32
FS191	31	34
SG194	25	35
QR238	65	30
DG256	52	190
TA277	37	30
Double mutants		
YD25 FY53	205	310
FY53 SF167	140	210
FY53 KR182	135	440
FY53 DG256	231	594
KE123 DG256	143	742
NI183 FS191	55	170
QR238 TA277	103	318
Quadruple mutants		
YD25 FY53 QR238 TA277	263	870
YD25 FY53 NI183 FS191	269	920

[a] The first column of the table lists the substitutions in different MelR derivatives selected as competent for melibiose-independent activation of p*melAB*. The second column lists the measured β-galactosidase activities in WAM132Δ*melR* Δ*lac* cells carrying a p*melR*::*lac* fusion and the different MelR derivatives. The third column lists the measured β-galactosidase activities in cells carrying a p*melAB*::*lac* fusion and different MelR derivatives (as in Table 3). Activities, expressed in the units described by Miller (18), are the averages of at least four independent determinations that differed by no more than 10%. Cells were grown aerobically in defined medium without melibiose, with fructose as a carbon source, and were harvested in exponential phase at an optical density at 600 nm of ~0.3.

TABLE 5. β-Galactosidase activity in WAM132 Δ*melR* Δ*lac* cells carrying MelR mutants defective in activation of p*melAB*[a]

pJW15 derivative encoding *melR*	β-Galactosidase activity		
	p*melAB*::*lac* (KK43)		p*melR*::*lac* (TB20), no melibiose
	No melibiose	10 mM melibiose	
No *melR*	11	12	274
Wild-type *melR*	16	1,021	25
Single mutants			
NS50	12	10	25
FL53	16	220	52
PS81	17	263	64
IT95	11	11	22
TA117	10	187	21
TI117	6	12	62
ND183	7	137	44
AT201	6	6	30
MT243	5	4	27
LS251	6	13	61
SR271	5	9	26

[a] The first column of the table lists the substitutions in different MelR derivatives selected as defective for melibiose-dependent activation of p*melAB*. The second and third columns list the measured β-galactosidase activities in WAM132 Δ*melR* Δ*lac* cells carrying a p*melAB*::*lac* fusion and the different MelR derivatives. Cells were grown aerobically in defined medium without (column 2) or with (column 3) 10 mM melibiose, with fructose as a carbon source, and were harvested in exponential phase at an optical density at 600 nm of ~0.3. The fourth column lists the measured β-galactosidase activities in WAM132 Δ*melR* Δ*lac* cells carrying a p*melR*::*lac* fusion and the different MelR derivatives. Activities, expressed in the units described by Miller (18), are the averages of at least four independent determinations that differed by no more than 10%.

DNA binding, were discarded. However, this second screening step identified 11 pJW15 derivatives encoding MelR that were still able to repress p*melR*, despite being defective in the activation of p*melAB*. Each of these mutant MelR derivatives carried a single substitution. The different changes, listed in Table 5, are distributed throughout MelR.

To quantify the effects of the different changes, the β-galactosidase activities in cultures of WAM132 cells containing KK43-pRW50 and each mutant pJW15 derivative were measured without and with melibiose. Data in Table 5 show that, for seven of the mutants (NS50, IT95, TI117, AT201, MT243, LS251, and SR271), 10 mM melibiose is unable to induce p*melAB* activity. In contrast, with four of the mutants (FL53, PS81, TA117, and ND183), melibiose induces p*melAB* activity, but to a lesser level than with wild-type MelR. Titrations with melibiose revealed that the concentration of melibiose required for 50% induction is increased by five- to eightfold compared to wild-type MelR (C. L. Webster, unpublished data). Interestingly, other substitutions of F53 or N183 create MelR* derivatives that are triggered at lower melibiose concentrations (Table 3).

To quantify the effects of the 11 changes on repression of p*melR*, the β-galactosidase activities in cultures of WAM132 cells containing TB20-pRW50 and each mutant pJW15 derivative were measured in the absence of melibiose. These assays showed that each of the different mutant MelR derivatives was able to repress p*melR* (Table 5). Maximum repression was found with the NS50, IT95, TA117, MT243, and SR271 mutants. Since the NS50, IT95, MT243, and SR271 substitutions result in noninducible (NI) MelR, we conclude that these changes lock MelR in the minus-melibiose conformation.

Interactions between MelR subunits measured by BACTH. We reasoned that the difference between the form of MelR unable to activate p*melAB* but competent for repression of p*melR* and the alternative form that can activate p*melAB* but is unable to repress p*melR* might be, in part, due to subunit-subunit interactions. Since, to date, purified MelR has been refractory to biophysical investigation, we sought to study these interactions using the well-characterized BACTH assay (13). This relies on the observation that *Bordetella pertussis* adenylate cyclase consists of two independently folding domains and that this adenylate cyclase can become active when the two domains are brought together in the cell. Thus, when the T18 and T25 fragments are expressed as separate entities, host cells score as negative for adenylate cyclase activity, but if T18 and T25 are fused to interacting partners, hosts can score as a positive. Recall that adenylate cyclase catalyzes the synthesis of cyclic AMP, whose levels in *E. coli* can be monitored by plate assays of maltose phenotypes or enzyme assays of β-galactosidase activity. Thus, *E. coli* strain BTH101, which is defective for adenylate cyclase (*cya*), was transformed with plasmids pU-T18C and pK-T25 that express, respectively, the *Bordetella pertussis* adenylate cyclase T18 and T25 fragments. Transformants score as Mal^- and contain low levels of β-galactosidase. However, results summarized in Table 6 show that cells transformed with pU-T18C and pK-T25 derivatives encoding fusions of the T18 and T25 fragments to wild-type MelR score as Mal^+ and contain significantly increased levels of β-galactosidase. The explanation for this is that MelR self-associates and brings together the T18 and T25 fragments to generate adenylate cyclase activity. This association is unchanged by the

TABLE 6. β-Galactosidase activity in BTH101 *cya* cells containing pK-T25 and pU-T18C derivatives[a]

pK-T25 derivative	pU-T18C derivative	β-Galactosidase activity	Phenotype on MacConkey maltose plates
pK-T25	pU-T18C	50	White (Mal^-)
pK-T25-zip	pU-T18C-zip	690	Red (Mal^+)
pK-T25-MelR	pU-T18C-MelR	271	Red
pK-T25-MelR YD25 FY53 NI183 FS191	pU-T18C-MelR YD25 FY53 NI183 FS191	73	White
pK-T25-MelR MT243	pU-T18C-MelR MT243	248	Red

[a] Activities are expressed in the units described by Miller and were determined using the Miller protocol (18). The data shown are the averages of at least four independent measurements that differed by no more than 10%. Cells were grown aerobically at 30°C in LB medium containing 50 μg/ml kanamycin and 100 μg/ml ampicillin and were harvested in exponential phase at an optical density at 600 nm of ~0.4. Column 4 lists the observed phenotype of the starting colony on MacConkey maltose plates after overnight growth at 30°C.

TABLE 7. β-Galactosidase activity in WAM132 Δ*melR* Δ*lac* cells carrying KK43 or JK141 p*melAB*::*lac* fusions and pJW15[a]

MelR encoded by pJW15 derivative	β-Galactosidase activity			
	KK43-pRW50		JK141-pRW50	
	No melibiose	With melibiose	No melibiose	With melibiose
Wild type	23	1043	3	28
YD25 FY53 NI183 FS191	875	929	234	703

[a] Activities are expressed in the units described by Miller and were determined using the Miller protocol (18). The data shown are the averages of at least four independent values that differed by no more than 10%. Cells were grown aerobically in defined medium with fructose as a carbon source either with or without 10 mM melibiose and were harvested in exponential phase at an optical density at 600 nm of ~0.3.

MT243 substitution, which appears to freeze MelR in its melibiose-free conformation. In contrast, when MelR carrying the YD25, FY53, NI183, and FS191 substitutions is fused to the T18 and T25 fragments, cells score as Mal$^-$ and contain low levels of β-galactosidase. Since the combination of the YD25, FY53, NI183, and FS191 substitutions converts MelR to its melibiose-triggered state, we conclude that the association between MelR subunits must differ according to their conformation.

Mutant MelR that is active in the absence of melibiose has less need for CRP. When triggered by melibiose, wild-type MelR requires the assistance of CRP to activate transcription at p*melAB* (31). The likely explanation for this is that a nucleoprotein complex of DNA-MelR and CRP is needed for melibiose-activated MelR to occupy site 2′. To examine whether MelR that is frozen in the activating conformation has the same requirement for CRP, we constructed the JK141 promoter, which lacks MelR-binding sites 1′ and 1 and the p*melAB* DNA site for CRP (Fig. 1), and cloned the resulting fragment in pRW50 to give plasmid JK141-pRW50. Results in Table 7 show that wild-type MelR is unable to activate expression from the JK141 promoter. This was expected, since the CRP requirement for MelR-dependent activation of p*melAB* is well established (31). However, MelR carrying the YD25, FY53, NI183, and FS191 substitutions, as well as being able to activate p*melAB* carried by the KK43 fragment, is also able to activate the JK141 promoter. This shows that MelR carrying these substitutions has a reduced requirement for CRP to activate p*melAB*.

DISCUSSION

Melibiose is needed for wild-type MelR to activate transcription initiation at p*melAB*. This is because activation requires MelR binding to site 2′ that overlaps the p*melAB* −35 hexamer element, and melibiose is required for wild-type MelR to occupy this site (2). However, in the absence of melibiose, MelR still binds at site 2 and at site R, and this results in strong repression of p*melR* (30). This strong repression is relieved by melibiose and, thus, melibiose toggles MelR between two alternative states, one that activates p*melAB* and one that represses p*melR* (Fig. 1B). Our goal was to use genetic analysis to investigate these two states.

The best-understood AraC family member is the *Escherichia coli* AraC protein itself, which is also toggled between two states by its ligand, arabinose (reviewed in references 23 and 24). AraC-dependent transcription regulation has been most studied at the *araBAD* and *araC* genes, which are expressed from divergent promoters, p*araBAD* and p*araC*, whose transcription start sites are separated by 166 bp. Activation of p*araBAD* requires AraC to bind at adjacent 20-bp operator sites, *I1* and *I2*, centered at positions −63.5 and −43.5 upstream of the transcription start site. The *I2* site overlaps the −35 element of p*araBAD*, and AraC normally occupies this site only in the presence of arabinose. In the absence of arabinose, AraC binds to site *I1* and an upstream site *O2*, and this results in repression of p*araC*. Thus, arabinose converts AraC from a form that binds to distal targets (*O2* and *I1*) to a form that binds to adjacent targets (*I1* and *I2*). To explain this, Schleif and his colleagues proposed the light switch model (23, 24), which was derived from X-ray structural analyses of the AraC N-terminal arabinose-binding domain. These studies (26, 27) showed that the AraC N-terminal domain contains a cupin fold that carries the binding site for arabinose and that the extreme N-terminal arm (AraC residues 1 to 20) folds over bound arabinose. Schleif and coworkers have found that, in the absence of arabinose, this N-terminal arm switches to interacting with the AraC DNA-binding domain (9, 25). Hence, in the X-ray structure of the AraC N-terminal domain without arabinose, this arm is unstructured and cannot be seen. The light switch model proposes that, in the absence of arabinose, the interaction of the AraC N-terminal arm with the C-terminal DNA-binding domain constrains the subunits of the AraC dimer in an orientation that makes it energetically favorable to bind to distal (*O2* and *I1*) rather than adjacent (*I1* and *I2*) targets (11, 25). This model is supported by genetic analyses, notably, mutations that alter amino acids in the AraC N-terminal arm that result in AraC-dependent activation of p*araBAD* in the absence of arabinose (19, 21, 22, 34, 35).

The striking parallels between AraC and MelR led us to consider whether the light switch model applies to MelR. Although we have no structural data for MelR, its domain organization appears to be similar to AraC (Fig. 2). In particular, amino acid sequence similarities argue that MelR residues 25 to 100 constitute a ligand-binding cupin fold (5, 6) and MelR residues 190 to 302 fold as an AraC family DNA-binding domain (29). In preliminary experiments, we targeted mutations to the segment of *melR* encoding the 20 N-terminal amino acids of MelR, but we were unable to find any changes that resulted in MelR capable of melibiose-independent activation of p*melAB* (T. A. Belyaeva, unpublished data). The subsequent random mutational analysis of the entire *melR* gene, presented in this paper, argues that the light switch model cannot apply to MelR.

Our analysis revealed that substitutions resulting in melibiose-independent activation of p*melAB* fall at loci throughout MelR and do not cluster in its N-terminal or its DNA-binding domain. Individual substitutions confer only incremental degrees of melibiose independence, though full melibiose independence is found when different substitutions are combined. In all the cases that were tested, the melibiose independence due to different substitutions was additive, suggesting that the conversion to the activating state of MelR requires changes in

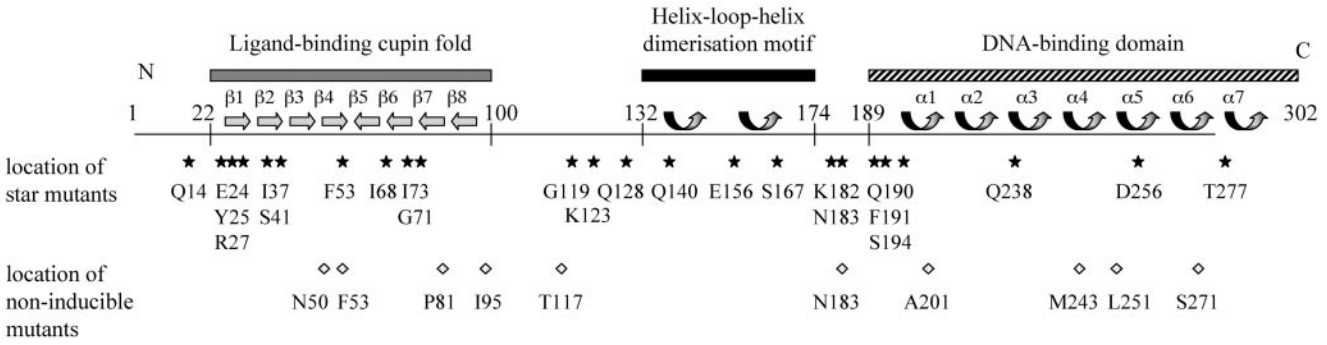

FIG. 2. Domain organization of the *E. coli* MelR protein. The 302 amino acids of MelR are illustrated as a horizontal line, annotated with different structural features. The locations of a ligand-binding cupin fold, consisting of eight β-sheet elements (5, 6) and a helix-loop-helix dimerization motif, deduced from similarities with AraC and published AraC structures (26, 27), are shown. The location of the DNA-binding domain, consisting of seven α-helix elements and deduced from similarities with MarA and the published MarA structure (20), is shown. The lower part of the figure shows the locations of different substitutions that confer the ability to partially activate p*melAB* in the absence of melibiose (stars) and the locations of substitutions that interfere with melibiose-dependent activation (diamonds).

all of the different segments of the protein (Fig. 2). Substitutions in different parts of MelR must be combined to create fully active melibiose-independent MelR. Thus, we suppose that melibiose binding to the MelR cupin fold triggers a concerted series of conformation changes that affect the N-terminal domain, the C-terminal domain, and the connecting linker. These changes appear to be mimicked by the different MelR* substitutions that we isolated.

Our results are most easily interpreted by a two-state model for MelR in which one state is fully competent for activation of p*melAB* but is unable to repress p*melR*, and vice versa for the other state. Thus, all the changes that confer melibiose independence for p*melAB* activation relieve repression of p*melR* in the absence of melibiose, and there is a clear correlation between the two functions (Table 4). In accord with this, we identified four NI mutants of MelR, NS50, IT95, MT243, and SR271, which appear to lock MelR in the minus-melibiose conformation and are fully competent for repression of p*melR* (Table 5). Due to the difficulty of working with purified MelR, we resorted to using an artificial bacterial two-hybrid system to investigate the two states of MelR. Since it is likely that MelR-MelR interactions are important for both activation of p*melAB* and repression of p*melR*, we suppose that that differences recorded in Table 5 are due to differences in these interactions. Presumably, in one state but not the other, the MelR-MelR interaction results in a productive interaction between the T18 and T25 adenylate cyclase fragments.

From our study, we can conclude that, though the ligand-free forms of both *E. coli* AraC and MelR proteins strongly repress expression from their own promoters, different mechanisms are used for ligand-dependent switching to a state that can activate transcription. For AraC in the absence of ligand, the N-terminal arm constrains the DNA-binding C-terminal domain (23, 24). Arabinose removes this constraint, and the C-terminal domain is then able to activate transcription at p*araBAD*. Consistent with this, Bustos and Schleif (3) showed that the isolated AraC C terminal is competent for this activation. In contrast, the corresponding C-terminal domain of MelR alone is unable to activate p*melAB* (12, 17). Thus, the binding of melibiose to the MelR N-terminal domain is required to transmit an activatory signal to the MelR C-terminal domain, and both the N- and C-terminal domains are required for activation of p*melAB* expression. The nature of this signal is not understood, but the scattering of substitutions that affect switching suggests that all segments of MelR are involved. Interestingly, some of the substitutions on our MelR* mutants fall in the linker that joins the N-terminal ligand-binding domain and the C-terminal DNA-binding domain. This suggests that the interdomain linker may play more than a neutral role as signals are passed between the two domains.

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REFERENCES

1. **Barne, K. A., J. A. Bown, S. J. Busby, and S. D. Minchin.** 1997. Region 2.5 of the *Escherichia coli* RNA polymerase sigma70 subunit is responsible for recognition of the extended −10′ motif at promoters. EMBO J. **16:**4034–4040.
2. **Belyaeva, T. A., J. T. Wade, C. L. Webster, V. J. Howard, M. S. Thomas, E. I. Hyde, and S. J. Busby.** 2000. Transcription activation at the *Escherichia coli melAB* promoter: the role of MelR and the cyclic AMP receptor protein. Mol. Microbiol. **36:**211–222.
3. **Bustos, S. A., and R. F. Schleif.** 1993. Functional domains of the AraC protein. Proc. Natl. Acad. Sci. USA **90:**5638–5642.
4. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
5. **Dunwell, J. M., A. Culham, C. E. Carter, C. R. Sosa-Aguirre, and P. W. Goodenough.** 2001. Evolution of functional diversity in the cupin superfamily. Trends Biochem. Sci. **26:**740–746.
6. **Dunwell, J. M., A. Purvis, and S. Khuri.** 2004. Cupins: the most functionally diverse protein superfamily? Phytochemistry **65:**7–17.
7. **Egan, S. M.** 2002. Growing repertoire of AraC/XylS activators. J. Bacteriol. **184:**5529–5532.
8. **Gallegos, M.-T., R. Schleif, A. Bairoch, K. Hofmann, and J. L. Ramos.** 1997. AraC/XylS family of transcriptional regulators. Microbiol. Mol. Biol. Rev. **61:**393–410.
9. **Ghosh, M., and R. F. Schleif.** 2001. Biophysical evidence of arm-domain interactions in AraC. Anal. Biochem. **295:**107–112.
10. **Grainger, D. C., C. L. Webster, T. A. Belyaeva, E. I. Hyde, and S. J. Busby.** 2004. Transcription activation at the *Escherichia coli melAB* promoter: interactions of MelR with its DNA target site and with domain 4 of the RNA polymerase sigma subunit. Mol. Microbiol. **51:**1297–1309.

11. **Harmer, T., M. Wu, and R. Schleif.** 2001. The role of rigidity in DNA looping-unlooping by AraC. Proc. Natl. Acad. Sci. USA **98:**427–431.
12. **Howard, V. J., T. A. Belyaeva, S. J. Busby, and E. I. Hyde.** 2002. DNA binding of the transcription activator protein MelR from *Escherichia coli* and its C-terminal domain. Nucleic Acids Res. **30:**2692–2700.
13. **Karimova, G., J. Pidoux, A. Ullmann, and D. Ladant.** 1998. A bacterial two-hybrid system based on a reconstituted signal transduction pathway. Proc. Natl. Acad. Sci. USA **95:**5752–5756.
14. **Karimova, G., A. Ullmann, and D. Ladant.** 2001. Protein-protein interaction between *Bacillus stearothermophilus* tyrosyl-tRNA synthetase subdomains revealed by a bacterial two-hybrid system. J. Mol. Microbiol. Biotechnol. **3:**73–82.
15. **Lodge, J., J. Fear, S. Busby, P. Gunasekaran, and N.-R. Kamini.** 1992. Broad host range plasmids carrying the *Escherichia coli* lactose and galactose operons. FEMS Microbiol. Lett. **74:**271–276.
16. **Martin, R. G., and J. L. Rosner.** 2001. The AraC transcriptional activators. Curr. Opin. Microbiol. **4:**132–137.
17. **Michán, C. M., S. J. Busby, and E. I. Hyde.** 1995. The *Escherichia coli* MelR transcription activator: production of a stable fragment containing the DNA-binding domain. Nucleic Acids Res. **23:**1518–1523.
18. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Reed, W. L., and R. F. Schleif.** 1999. Hemiplegic mutations in AraC protein. J. Mol. Biol. **294:**417–425.
20. **Rhee, S., R. G. Martin, J. L. Rosner, and D. R. Davies.** 1998. A novel DNA-binding motif in MarA: the first structure for an AraC family transcription activator. Proc. Natl. Acad. Sci. USA **95:**10413–10418.
21. **Ross, J. J., U. Gryczynski, and R. Schleif.** 2003. Mutational analysis of residue roles in AraC function. J. Mol. Biol. **328:**85–93.
22. **Saviola, B., R. Seabold, and R. Schleif.** 1998. Arm-domain interactions in AraC. J. Mol. Biol. **278:**539–548.
23. **Schleif, R.** 2000. Regulation of the L-arabinose operon of *Escherichia coli*. Trends Genet. **16:**559–565.
24. **Schleif, R.** 2003. AraC protein: a love-hate relationship. Bioessays **25:**274–282.
25. **Seabold, R. R., and R. F. Schleif.** 1998. Apo-AraC actively seeks to loop. J. Mol. Biol. **278:**529–538.
26. **Soisson, S. M., B. Macdougall-Shackleton, R. Schleif, and C. Wolberger.** 1997. Structural basis for ligand-regulated oligomerization of AraC. Science **276:**421–425.
27. **Soisson, S. M., B. Macdougall-Shackleton, R. Schleif, and C. Wolberger.** 1997. The 1.6 Å crystal structure of the AraC sugar-binding and dimerization domain complexed with D-fucose. J. Mol. Biol. **273:**226–237.
28. **Tamai, E., T. Shimamoto, M. Tsuda, T. Mizushima, and T. Tsuchiya.** 1998. Conversion of temperature-sensitive to -resistant gene expression due to mutations in the promoter region of the melibiose operon in *Escherichia coli*. J. Biol. Chem. **273:**16860–16864.
29. **Tobes, R., and J. L. Ramos.** 2002. AraC-XylS database: a family of positive transcriptional regulators in bacteria. Nucleic Acids Res. **30:**318–321.
30. **Wade, J. T., T. A. Belyaeva, E. I. Hyde, and S. J. Busby.** 2000. Repression of the *Escherichia coli melR* promoter by MelR: evidence that efficient repression requires the formation of a repression loop. Mol. Microbiol. **36:**223–229.
31. **Wade, J. T., T. A. Belyaeva, E. I. Hyde, and S. J. Busby.** 2001. A simple mechanism for co-dependence on two activators at an *Escherichia coli* promoter. EMBO J. **20:**7160–7167.
32. **Webster, C., K. Kempsell, I. Booth, and S. Busby.** 1987. Organisation of the regulatory region of the *Escherichia coli* melibiose operon. Gene **59:**253–263.
33. **Webster, C., L. Gardner, and S. Busby.** 1989. The *Escherichia coli melR* gene encodes a DNA-binding protein with affinity for specific sequences located in the melibiose operon regulatory region. Gene **83:**207–213.
34. **Wu, M., and R. Schleif.** 2001. Mapping arm-DNA binding domain interactions in AraC. J. Mol. Biol. **307:**1001–1009.
35. **Wu, M., and R. Schleif.** 2001. Strengthened arm-dimerization domain interactions in AraC. J. Biol. Chem. **276:**2562–2564.