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Finely Tuned Regulation of the Aromatic Amine Degradation Pathway in *Escherichia coli*

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FeaR is an AraC family regulator that activates transcription of the *tynA* and *feaB* genes in *Escherichia coli*. TynA is a periplasmic topaquinone- and copper-containing amine oxidase, and FeaB is a cytosolic NAD-linked aldehyde dehydrogenase. Phenylethylamine, tyramine, and dopamine are oxidized by TynA to the corresponding aldehydes, releasing one equivalent of H_2O_2 and NH_3. The aldehydes can be oxidized to carboxylic acids by FeaB, and (in the case of phenylacetate) can be further degraded to enter central metabolism. Thus, phenylethylamine can be used as a carbon and nitrogen source, while tyramine and dopamine can be used only as sources of nitrogen. Using genetic, biochemical and computational approaches, we show that the FeaR binding site is a TGNCA-N_8-AAA motif that occurs in 2 copies in the *tynA* and *feaB* promoters. We show that the coactivator for FeaR is the product rather than the substrate of the TynA reaction. The *feaR* gene is upregulated by carbon or nitrogen limitation, which we propose reflects regulation of *feaR* by the cyclic AMP receptor protein (CRP) and the nitrogen assimilation control protein (NAC), respectively. In carbon-limited cells grown in the presence of a TynA substrate, *tynA* and *feaB* are induced, whereas in nitrogen-limited cells, only the *tynA* promoter is induced. We propose that *tynA* and *feaB* expression is finely tuned to provide the FeaB activity that is required for carbon source utilization and the TynA activity required for nitrogen and carbon source utilization.

In *Escherichia coli*, TynA is a periplasmic amine oxidase containing copper and topaquinone cofactors (1). Aromatic amines, including phenylethylamine (PEA), tyramine, and dopamine, are oxidized by TynA to the corresponding aldehydes, in a reaction that releases one equivalent of H_2O_2 and NH_3 (Fig. 1A). Therefore, these monoamines can be used as the sole nitrogen source for growth. The aldehydes are further oxidized to the corresponding carboxylic acids by FeaB, a cytosolic NAD-linked aldehyde dehydrogenase (2). Phenylacetate (PA) can be further degraded to acetyl coenzyme A (acetyl-CoA) and succinyl-CoA, and therefore, PEA can be utilized as the sole carbon and energy source (3–6). In K-12 strains of *E. coli*, the carboxylic acids derived from tyramine and dopamine cannot be further catabolized, so these compounds can act only as nitrogen sources. Therefore, while TynA activity may allow for both carbon and nitrogen assimilation, FeaB activity is related solely to carbon and energy metabolism (3). Despite the potentially different physiological roles of TynA and FeaB, currently available information suggests that their genes are coordinately regulated by the product of the linked *feaR* gene, which is a transcriptional regulator from the AraC family (2, 5, 7, 8).

The AraC family includes over 800 members, most of which are thought to be transcriptional activators that function to regulate genes related to carbon metabolism, stress responses, or pathogenesis (9–11). With some exceptions, AraC family members are characterized by a conserved C-terminal DNA binding domain (CTD) and a nonconserved N-terminal domain (NTD). The nonconserved NTD contains the ligand binding site and, usually, the dimerization interface (9, 10). AraC family regulators that have been well characterized include AraC, MelR, XylS, RhaR, and RhaS (9, 12–18). FeaR is known to be required for the expression of *tynA* and *feaB* (7, 8), but its role and mechanism have not otherwise been characterized.

Besides FeaR, there is some evidence that *tynA* and *feaB* expression may also be modulated by other transcriptional regulators. We have previously shown that the nitric oxide (NO)-sensitive repressor NsrR binds to sites in the *tynA* and *feaB* promoters and has a small effect on the transcription of these genes (8, 19). In addition, there is evidence that *feaR* expression may be regulated by PhoB (20) and ArcA (P. J. Kiley, personal communication).

In this study, we used computational, genetic, and biochemical approaches to identify the FeaR binding site in the *tynA* and *feaB* promoter regions. We showed that the FeaR CTD can bind to DNA *in vitro* and can activate the *tynA* promoter *in vivo*. In full-length FeaR, the NTD appears to act to inhibit the CTD in the absence of the coactivator. We show that the expression of *feaR* is regulated by carbon or nitrogen limitation and is not subject to autoregulation by the FeaR protein. Overall, we find that *tynA* expression is activated in both carbon- and nitrogen-limited cells in the presence of a FeaR coactivator, while *feaB* can be activated only during carbon limitation. We also show that the coactivator for FeaR is probably an aldehyde (the substrate for FeaB) rather than an amine (the substrate for TynA).

MATERIALS AND METHODS

Bacterial strains, growth media, and culture conditions. The strains and plasmids used in this work are listed in Table S2 in the supplemental material. The methods used to make gene knockouts and to construct chromosomal promoter-*lacZ* fusions were described previously (8, 21–23). The *glnG*::*kan* and *nac*::*kan* mutations (in strain BW25113) were obtained from the Keio collection and then were transferred to the reporter strain by P1 transduction (21, 24). DNA sequences encoding the

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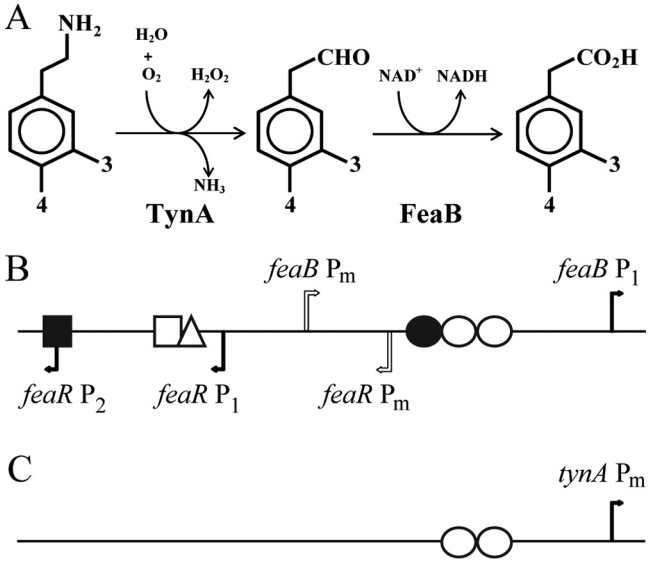

FIG 1 (A) Pathways for the catabolism of phenylethylamine, tyramine, and dopamine. The first reaction is catalyzed by the periplasmic amine oxidase (TynA) and the second reaction by an NAD-linked dehydrogenase (FeaB). Where substituents at the 3 and 4 positions are hydrogen, the three compounds are phenylethylamine, phenylacetaldehyde, and phenylacetate. With a hydroxyl group at the 4 position, they are tyramine, 4-hydroxyphenylacetaldehyde, and 4-hydroxyphenylacetate. With hydroxyl groups at both the 3 and 4 positions, they are dopamine, 3,4-dihydroxyphenylacetaldehyde, and 3,4-dihydroxyphenylacetate. (B and C) Schematics of the organization of the *feaR-feaB* intergenic region (B) and the *tynA* regulatory region (C). Transcription start sites are indicated by bent arrows. Verified and predicted binding sites for regulatory proteins are shown: FeaR (open circles), CRP (filled circle), NAC (open triangle), PhoB (open square), and NsrR (filled square). For additional details and DNA sequences, see Fig. 2.

CTD and full-length FeaR were amplified by PCR (primers are listed in Table S1 in the supplemental material) and ligated into pBAD24 (25). For β-galactosidase assays, cultures were grown in rich medium (LB) or in defined medium (26) with the indicated carbon and nitrogen sources. For growth with nonpreferred nitrogen sources, ammonium sulfate was replaced with sodium sulfate. For defined medium with PEA as the carbon and nitrogen source (PEA medium), Casamino Acids (0.05% [wt/vol]) were also added. Growth on PEA is temperature sensitive (6) and is significantly improved by the addition of Casamino Acids to growth media. PEA has limited solubility in water, so it was added directly to the bulk medium, which was then sterilized by filtration. Phenylacetaldehyde (PAL) was solubilized in dimethyl sulfoxide (DMSO) prior to addition to growth media. Because PAL is toxic (and insoluble in aqueous buffers), it was added in 0.1 mM aliquots at 2-h intervals during the growth of cultures.

Promoter analysis. The 250-bp, 150-bp, 142-bp, 140-bp, 133-bp, 132-bp, 129-bp, and 126-bp DNA fragments upstream of the *tynA* start codon (*tynA5-1* to *tynA5-8*), and the 612-bp, 250-bp, 109-bp, 96-bp, 89-bp, 75-bp, and 63-bp DNA fragments upstream of the *feaB* start codon (*feaB5-1* to *feaB5-7*) were amplified by PCR. The promoter fragments were cloned into pSTBlue-1 as described previously (22). Promoter fusions to *lacZ* were constructed in pRS415, transferred to λRS45, and integrated into the chromosome as described previously (22, 23). Mutations were introduced into the *tynA5-1* clone using the Invitrogen QuikChange site-directed mutagenesis kit and appropriate mutagenic primers (see Table S1 in the supplemental material). Mutant *tynA* promoters were fused to *lacZ* in pRS415 and then transferred to the chromosome (22, 23). 5′ transcription start sites were determined by rapid amplification of 5′ cDNA ends (RACE), using the TaKaRa 5′-full RACE core set according to the manufacturer's directions. The primers used for RACE are listed in Table S1 in the supplemental material.

Purification of the FeaR CTD. The C-terminal domain (CTD) and the linker region of FeaR were identified by sequence alignment of five AraC family proteins (FeaR, AraC, MelR, RhaR, and RhaS) using T-coffee (27). The DNA sequence corresponding to the CTD and linker region was amplified by PCR and ligated into pET-21a(+) (Novagen) in frame with sequences encoding a C-terminal hexahistidine tag. The recombinant plasmid was transformed into *E. coli* strain BL21(λDE3) for overexpression of the His-tagged CTD. CTDhis was purified using the His GraviTrap kit (GE Healthcare). Protein concentrations were determined using the 660-nm protein assay reagent (Pierce).

DNA binding assay. 5′ biotin-labeled *tynA* and control (*ytfE*) promoters were amplified by PCR and gel purified. DNA binding buffer [10 mM Tris (pH 7.5), 100 mM KCl, 1 mM dithiothreitol (DTT), 50 ng/μl poly(dI · dC), 100 ng/μl salmon sperm DNA, 5% glycerol, 0.05% NP-40, 0.5 mM EDTA, 200 μg/ml bovine serum albumin (BSA)] was incubated at the room temperature with or without CTDhis for 1 min (Pierce LightShift chemiluminescent electrophoretic mobility shift assay [EMSA] kit).

TABLE 1 Activities of the *feaR*, *tynA*, and *feaB* promoters in cultures grown in different media

Growth condition[a]	β-Galactosidase activity (Miller units)[b]								
	feaR-lacZ	Δ*feaR* *feaR-lacZ*	*feaR-lacZ* (anaerobic)	*tynA-lacZ*	Δ*feaR* *tynA-lacZ*	*tynA-lacZ* (anaerobic)	*feaB-lacZ*	Δ*feaR* *feaB-lacZ*	*feaB-lacZ* (anaerobic)
Glucose + $(NH_4)_2SO_4$ (preferred medium)	476 (43)	322 (8)	160 (11)	6 (0.9)	3 (0.3)	ND	219 (19)	229 (15)	ND
Glycerol + $(NH_4)_2SO_4$ (glycerol medium)	951 (85)	890 (22)	322 (17)	5 (0.7)	3 (0.7)	ND	225 (18)	230 (17)	ND
Glucose + glutamine (glutamine medium)	1,857 (123)	2,123 (130)	450 (45)	3 (0.3)	3 (0.6)	ND	142 (8)	170 (12)	ND
Glucose + alanine (alanine medium)	2,106 (115)	2,048 (186)	NG	5 (0.4)	2 (0.2)	ND	115 (5)	154 (14)	ND
Glycerol + tyramine (tyramine medium)	2,803 (321)	NG	561 (46)	1,665 (156)	NG	5 (0.6)	545 (16)	NG	ND
Preferred medium + tyramine	389 (35)	ND	ND	5 (0.1)	ND	11 (0.6)	206 (9)	ND	34 (3)
Glycerol medium + tyramine	953 (70)	ND	ND	400 (50)	ND	26 (2)	464 (41)	ND	94 (10)
Glycerol medium + phenylethylamine	1,056 (121)	ND	ND	549 (31)	ND	3 (0.4)	577 (25)	ND	113 (9)
Glutamine medium + tyramine	2,658 (98)	ND	ND	312 (40)	ND	7 (0.6)	174 (13)	ND	20 (2)

[a] Carbon and nitrogen source in the defined minimum medium for cell growth.
[b] Values are means of duplicate measurements from each of three independent cultures. Numbers in parentheses are standard deviations. NG, no growth; ND, not done.

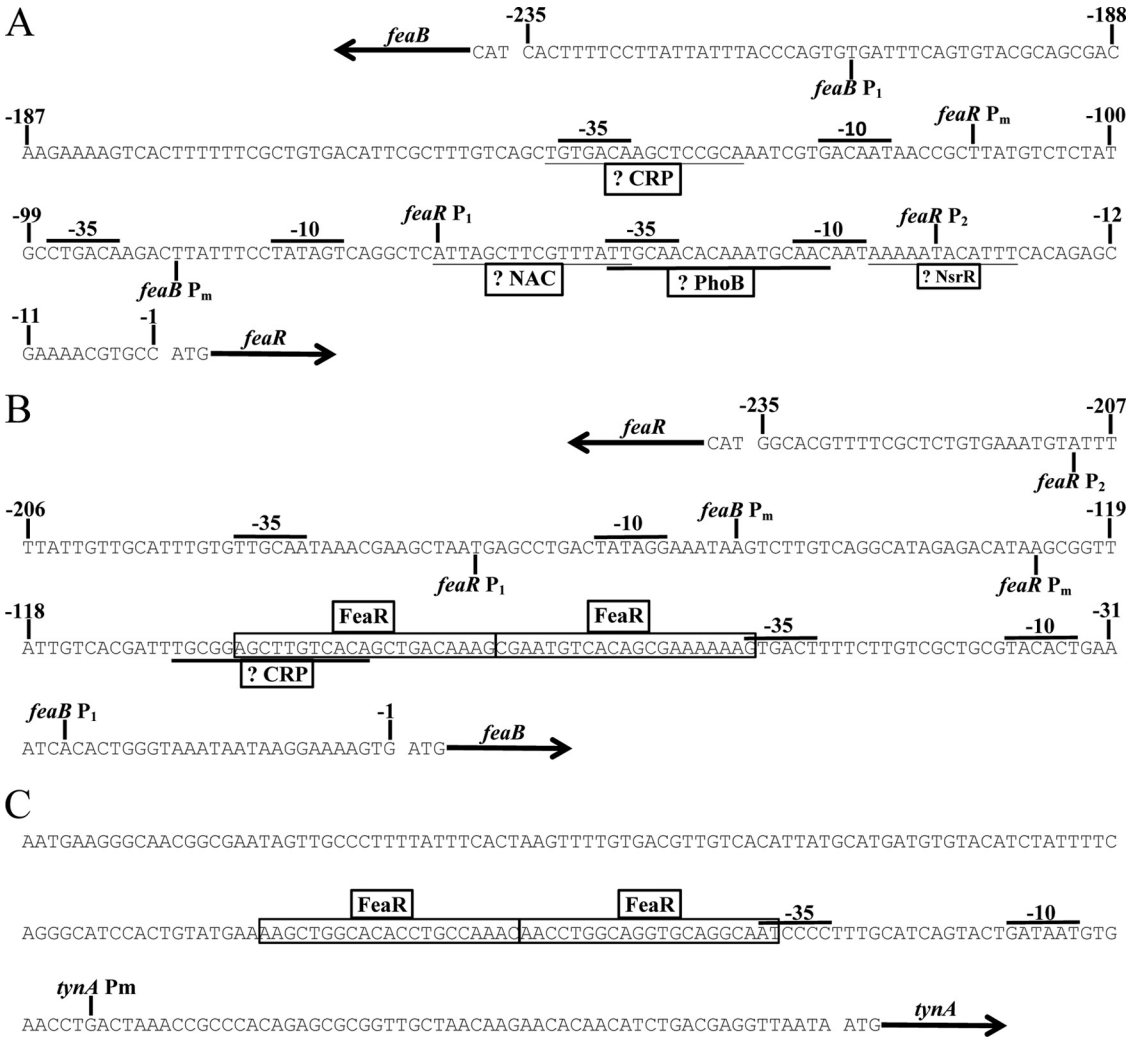

FIG 2 Transcription start sites of *feaR* (A), *feaB* (B), and *tynA* (C) as determined by 5′ RACE. The binding sites for FeaR as defined in this study are boxed. Suggested binding sites for CRP, NAC, PhoB, and NsrR are underlined. Promoter elements (−35 and −10) associated with the mapped transcription start sites are also indicated.

Biotin-labeled DNA (1 nM) was added to the solution and incubated for 20 min. Protein-DNA complexes were then resolved on 8% polyacrylamide gels. The biotin-labeled DNA was transferred to a Biodyne B membrane (Pall Corporation) and then detected using the chemiluminescent nucleic acid detection module (Pierce).

The DNA binding activity of the FeaR CTD was also assayed by fluorescence anisotropy (28, 29). The rhodamine-X (ROX)-labeled 31-nucleotide (nt) site 1 and site 2 contain 21 nt of the first and second repeats of the FeaR binding site, respectively, flanked by 5 nt upstream and downstream of the full-length FeaR binding site. cDNA strands were annealed by heating at 95°C for 2 min in Tris-EDTA (TE) buffer and then cooling to 25°C (at a rate of 1°C per min). ROX-labeled DNA fragments (5 nM) were incubated with 3 ml FA buffer (10 mM Tris [pH 7.4], 200 mM KCl, 1 mM EDTA, 5% glycerol, 25 μg/ml BSA, 75 μg/ml salmon sperm DNA) for 10 min, and then CTD (5 nM to 1,300 nM) was added and the reaction mixture incubated for 2 min. The anisotropy change was measured in a Varian Cary Eclipse fluorimeter. The binding isotherm was fit to equation 1 (28) using Kaleidagraph (Synergy Software):

$$\Delta A = \Delta A_T\,(\mathrm{CTD}^{\mathrm{nH}}/K_d^{\mathrm{nH}})/(1 + \mathrm{CTD}^{\mathrm{nH}}/K_d^{\mathrm{nH}}) \quad (1)$$

where ΔA is the change in fluorescence anisotropy, ΔA_T is the total change in anisotropy, CTD is the total protein concentration at each point in the titration, K_d is the dissociation constant, and nH is the Hill coefficient.

For competition assays, 345 nM CTD was incubated in 3 ml FA buffer for 5 min, and then 5 nM ROX-labeled site 1 or site 2 was added and the reaction mixture incubated for a further 10 min. Unlabeled competitor DNAs (16 to 1,500 nM competitors [see Table 2]) were added, and the anisotropy change was measured after equilibration for 4 min. The data were fit to equation 2 (29):

$$\text{fraction bound} = \mathrm{FB}_{\max}[1 - ([\text{competitor}]/(\mathrm{IC}_{50} + [\text{competitor}]))] \quad (2)$$

where $\mathrm{FB}_{\max}$ is the fraction bound in the absence of competitor, IC_{50} is the concentration of competitor required for half-maximal inhibition of binding, and the fraction bound is defined according to equation 3 (29):

$$\text{fraction bound} = (\Delta A - A_{\mathrm{free}})/(\Delta A_T - A_{\mathrm{free}}) \quad (3)$$

where A_{free} is the anisotropy in the absence of protein.

RESULTS

Regulation of the *feaR* promoter. In order to study the regulation of *feaR*, *feaB*, and *tynA*, the promoters of the three genes were fused to *lacZ*, and the fusions were transferred to the *E. coli*

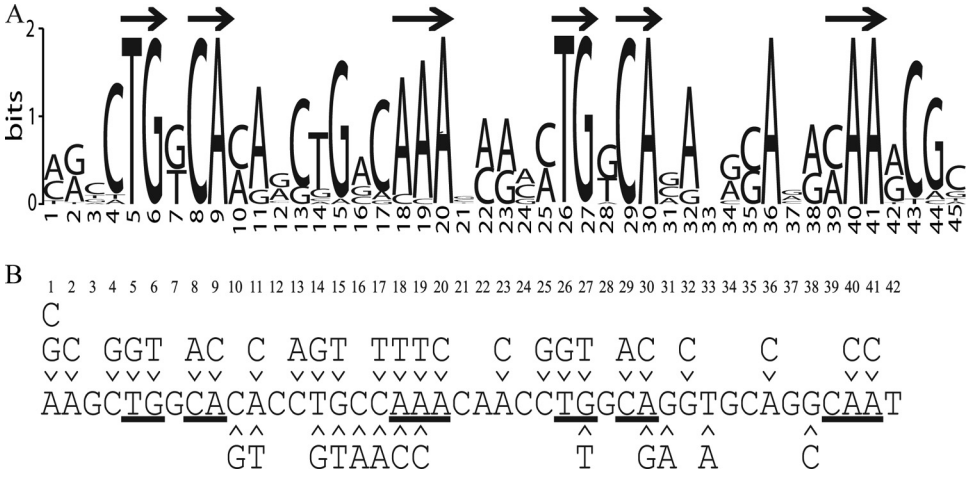

FIG 3 (A) The FeaR binding site. Computational prediction of the FeaR binding site is shown. The *tynA* and *feaB* promoter regions from different organisms were used in a search for enriched sequence motifs using the MEME algorithm. Arrows indicate nucleotides that constitute the directly repeated TGNCA-N_8-AAA, which is the proposed FeaR consensus binding site. (B) Mutations that were introduced into the FeaR binding site. Nucleotides are numbered according to the sequence logo. Those nucleotides that are within the TGNCA-N_8-AAA motif are underlined. Mutations above the sequence were introduced for *in vitro* DNA binding assays. Mutations below the sequence were used for *in vivo* reporter fusion assays.

MG1655 chromosome (8). β-Galactosidase activities were measured in cultures grown in defined media with different carbon and nitrogen sources (8, 26). In some cases, TynA substrates (PEA or tyramine) were used as the sole source of nitrogen, and/or these were added as inducers to media also containing other nitrogen sources (Table 1). The *feaR* promoter showed a basal level of activity in defined medium with glucose as the sole carbon source and ammonia as the sole nitrogen source (preferred medium). The promoter activity increased about 2-fold when the carbon source was replaced by glycerol (glycerol medium) and 4- to 6-fold when the nitrogen source was glutamine, alanine, or tyramine (glutamine, alanine, or tyramine medium). Addition of tyramine to the preferred medium or deletion of *feaR* had no effect on *feaR* promoter activity (Table 1), indicating that there is no autoregulation of *feaR* expression. Growth with glucose as the carbon source and tyramine as the nitrogen source was not possible, perhaps reflecting glucose repression of *feaR* expression (see below).

The transcription start site of *feaR* was determined by rapid amplification of 5′ cDNA ends (RACE). According to the RACE results, *feaR* transcription initiates from three sites, P_m (m for minor), P_1, and P_2, which are located 111, 66, and 26 bp upstream of the translation initiation codon, respectively (Fig. 1B and 2A). Based on the frequency of different clones recovered from the RACE procedure, all three sites are used in the preferred medium (6% of clones started at P_m, 38% at P_1, and 56% at P_2), while P_1 is the only promoter used in cells grown on defined medium with PEA as the sole carbon source (PEA medium) and is the dominant promoter used in glycerol medium (78% P_1 and 22% P_2), and P_2 is the only promoter used in glutamine medium (Fig. 2A). A sequence that is a good match to the cyclic AMP (cAMP) receptor protein (CRP) binding site is centered at 71.5 bp upstream of the P_1 promoter, which is suggestive of a class I type activation mechanism by CRP-cAMP (30–32). Regulation of P_1 by CRP-cAMP would be consistent with the preferential utilization of this promoter in glycerol medium. In contrast, the P_2 promoter is used preferentially in cells grown on a nonpreferred nitrogen source, which is suggestive of regulation by NtrC or the nitrogen assimilation control protein (NAC) (33–36). Upregulation of *feaR* in glutamine medium was abolished in *ntrC* (*glnG*) and *nac* mutants (see Fig. S1 in the supplemental material), which is consistent with NAC acting as a direct regulator of *feaR*, since *nac* expression is NtrC dependent (33–37). Accordingly, there is a predicted NAC binding site associated with the P_2 promoter (Fig. 1B and 2A) and no predicted binding sites for NtrC or σ^{54}.

Regulation of the *feaB* and *tynA* promoters. The *feaB* promoter showed a relatively low activity unless a substrate for the TynA/FeaB pathway was present in the growth medium (Table 1). Thus, there is not a simple correlation between *feaR* expression and the activity of its target promoter. The likely explanation is that a pathway substrate or intermediate is required to act as the coactivator for FeaR. Also, activation of the *feaB* promoter above its basal level required growth on a nonglucose carbon and energy source (for example, compare activities in preferred medium plus tyramine and in glycerol medium plus tyramine [Table 1]). In medium with a nonpreferred nitrogen source (glutamine medium plus tyramine), *feaB* activity remained low. Two transcription start sites were mapped 149 and 27 bp upstream of the *feaB* translation initiation codon and named P_m and P_1, respectively. The P_m promoter was used in the preferred medium; while only P_1 was used in cells growing on PEA medium. This pattern of promoter utilization is consistent with the presence of predicted FeaR and CRP binding sites upstream of the P_1 promoter (Fig. 1B and 2B).

Unlike the *feaB* promoter, the *tynA* promoter was almost silent under noninducing conditions. Activation of *tynA* required the presence of either tyramine or PEA in the medium (tyramine medium, PEA medium, or glycerol medium with tyramine or PEA). The requirement for an inducer for *tynA* promoter activity is consistent with our detection of only a single transcription start site, which is associated with FeaR binding sites (Fig. 1C; Fig. 2C). Unlike *feaB* activity, *tynA* activity could be elevated above its basal level by addition of tyramine to the glycerol medium or glutamine medium (Table 1). Thus, in the presence of an inducer, *tynA* ex-

pression is elevated in cells growing on nonpreferred carbon and nitrogen sources.

Activity of the *feaR* promoter was at basal levels under anaerobic conditions in all growth media tested (Table 1). Accordingly, *tynA* could not be induced by pathway substrates in anaerobic cultures, and *feaB* promoter activity was consistently lower than that observed in aerobic cultures. We conclude that expression of the PEA pathway is shut down during anaerobic growth, which can be explained by the recent identification of *feaR* as a target for ArcA regulation (P. J. Kiley, personal communication).

Overall, our data show that the *feaR* gene is upregulated during growth on nonpreferred carbon and nitrogen sources. This increase in *feaR* expression is not sufficient to activate expression of *tynA* and *feaB* unless a pathway inducer is also present (although a nonphysiological increase in FeaR abundance may lead to induction of its targets in the absence of inducer [see Fig. S3 in the supplemental material]). Elevated levels of *feaB* expression require growth on glycerol and either PEA or tyramine, while *tynA* can be induced by PEA or tyramine in media containing either glucose (with glutamine as the nitrogen source) or glycerol. Thus, *tynA* can be induced by PEA or tyramine in either carbon- or nitrogen-limited cultures, while *feaB* is induced only in carbon-limited cultures.

The coactivator for FeaR. The molecule that functions as the coactivator for FeaR is not known, though previous data and results reported above indicate that it is a substrate or intermediate of the TynA/FeaB pathway (3, 5, 7). We have been unable to test ligand binding to FeaR directly, since purified soluble protein is not available in sufficient yields (see below). We suspected that the coactivator for FeaR might be a FeaB substrate rather than a TynA substrate because (i) the TynA substrate is oxidized in the periplasm and it is the reaction product that is (presumably) transported into the cell and (ii) in multiple genomes, the *feaR* and *feaB* genes very frequently cooccur, whereas *tynA* is also present in only a subset of those genomes. The pattern of gene distribution suggests that FeaR more often functions as an activator of *feaB*, thus making it likely that the FeaR coactivator is an aldehyde rather than an amine (Fig. 1A). We measured *tynA* promoter activity in a wild-type strain and a *tynA* mutant in glycerol medium supplemented with PEA, PAL, or PA (see Fig. S2 in the supplemental material). The activation of *tynA* in glycerol medium plus PEA is dependent on TynA activity, consistent with the suggestion that the inducer is the product of the TynA reaction. Further, *tynA* is upregulated by addition of PAL to growth media, and this effect does not require TynA activity (see Fig. S2 in the supplemental material). In addition, PA is not able to activate *tynA* in either strain (see Fig. S2 in the supplemental material). These results suggest that the aldehyde is the direct inducer for the PEA catabolic pathway and is the likely coeffector of FeaR.

Computational prediction of the FeaR binding site. To identify the FeaR binding site, *tynA* promoter regions and *feaR-feaB* intergenic regions from different bacteria were collected and used in a search for enriched sequence motifs using the MEME algorithm (38). Sequences were collected only from species that have all three genes, and identical or very similar (<15-bp changes) sequences were discarded. In all, 15 *tynA* promoters and 10 *feaB* promoters were used for the motif search. The sequence logo generated by MEME is about 45 bp (Fig. 3A), and a number of patterns that might represent protein binding sites can be discerned in this sequence logo. Experimental results presented elsewhere in

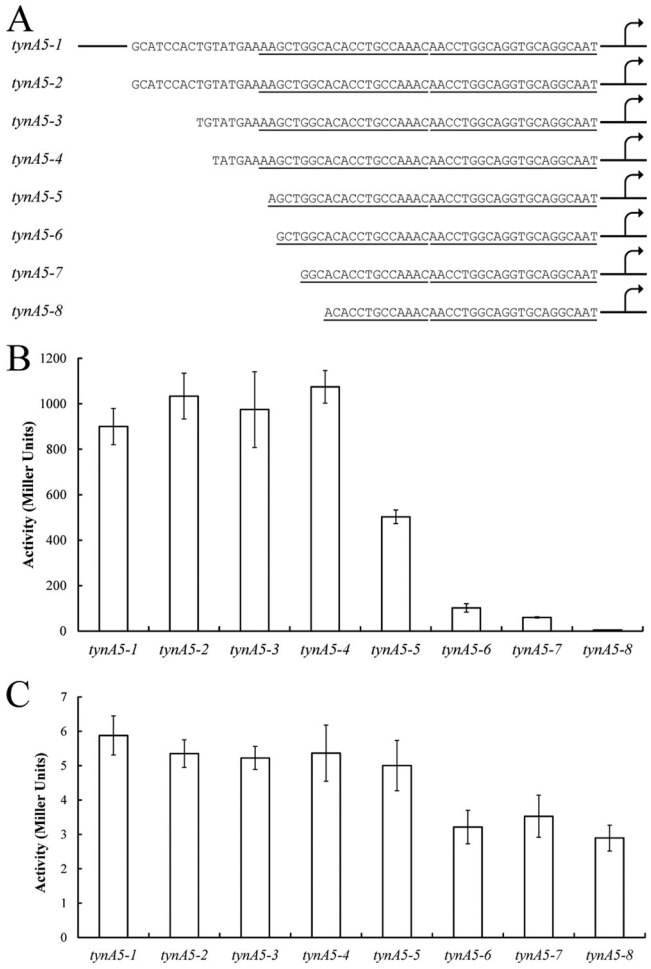

FIG 4 Deletion analysis of the *tynA* promoters. (A) The *tynA* promoter was truncated as indicated and fused to *lacZ* for measurements of β-galactosidase activity. The predicted FeaR binding sites are underlined. (B and C) Promoter activity was measured in cultures grown in PEA medium (B) and in glycerol medium (C). Activities are the means of duplicate measurements from each of three independent cultures, and error bars are standard deviations. The 5′ end of *tynA5-1* is shown schematically; this fusion contains 250 bp upstream of the *tynA* translational start site.

this paper suggest that the sequence is best interpreted as a direct repeat of two 16-bp motifs with the core consensus TGKCA-N_8-MAA (where K is G or T and M is C or A). The 3′ end of the promoter-proximal FeaR binding site is 32 bp upstream of the *tynA* P_1 and *feaB* P_1 start sites (Fig. 2); in other words, the spacing between these sequence elements and the downstream transcription start sites is precisely conserved.

Deletion analysis of the *tynA* and *feaB* promoters. With some information about the locations of transcription start sites and potential FeaR binding sites, we next designed 5′ truncations of the *tynA* and *feaB* promoters, which were fused to *lacZ*. Promoter activities were measured in defined medium with PEA as the sole carbon source (PEA medium) and in glycerol medium. In PEA medium, *tynA* promoter activity remained high until the first nucleotide of the promoter-distal potential FeaR binding site was deleted. Further deletions completely abolished *tynA* promoter activity (Fig. 4). In the case of *feaB*, there was an ~50% drop in activity for the deletion that extends into the predicted CRP bind-

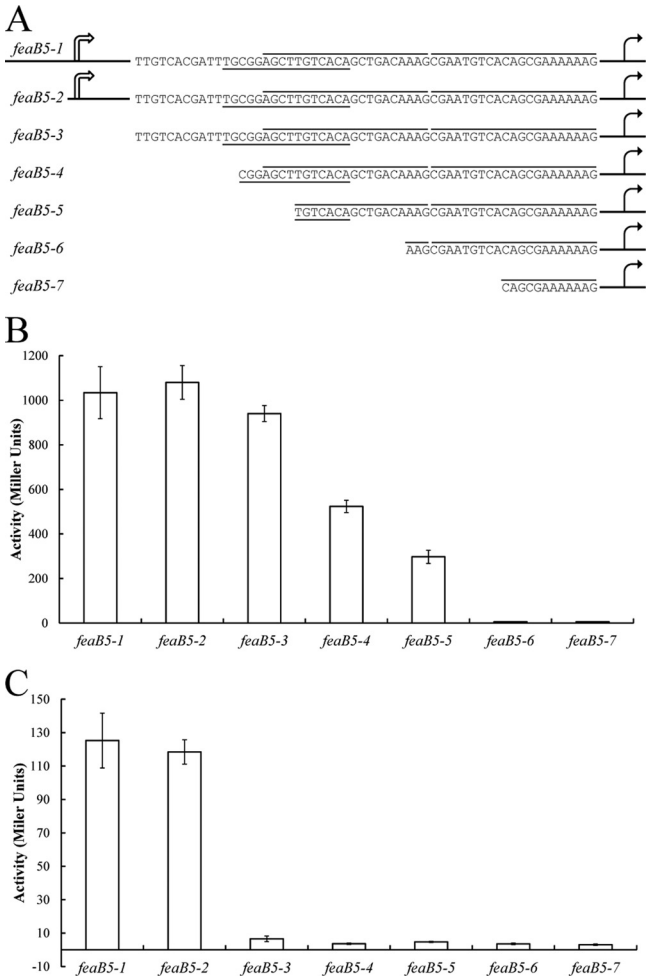

FIG 5 Deletion analysis of the *feaB* promoters. (A) The *feaB* promoter was truncated as indicated and fused to *lacZ* for measurements of β-galactosidase activity. The FeaR binding sites are highlighted by lines above the sequence, and the predicted CRP binding site is underlined. (B and C) Promoter activity was measured in cultures grown in PEA medium (B) and in glycerol medium (C). Activities are the means of duplicate measurements from each of three independent cultures, and error bars are standard deviations. The 5′ ends of *feaB5-1* and *feaB5-2* are shown schematically; these fusions contain 612 and 250 bp, respectively, upstream of the *feaB* translational start site.

ing site (Fig. 5). This is consistent with the previous observation that the utilization of tyramine as nitrogen source probably requires transcription activation by CRP-cAMP (Table 1). Interestingly, the *feaB5-3* deletion, which had full promoter activity in PEA medium, showed no activity in glycerol medium (Fig. 5C). This deletion removes the P_m promoter, which is therefore probably active during growth in glycerol medium. The results of the deletion analysis are consistent with the proposed locations of FeaR and CRP binding sites (Fig. 2).

DNA binding assays. Attempts to purify FeaR using several different approaches yielded material that was highly insoluble, aggregation prone, and/or obtained in very low yields. In this respect, FeaR is similar to other AraC family members (9, 12, 39). For *in vitro* DNA binding assays, we therefore sought to take advantage of the fact that the isolated CTDs of AraC-type proteins often retain sequence-specific DNA binding activity (15, 16, 40–43). We purified a hexa-His-tagged derivative of the FeaR CTD,

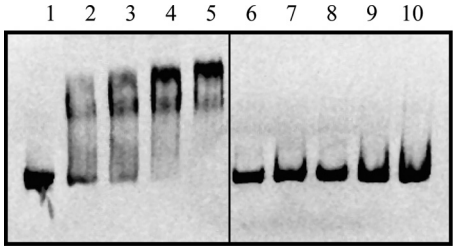

FIG 6 Gel retardation assay of FeaR CTD binding to the *tynA* promoter. A fragment from the *ytfE* promoter was used as a negative control. The labeled DNAs were *tynA* (lanes 1 to 5) and *ytfE* (lanes 6 to 10), and the protein concentrations were 0 (lanes 1 and 6), 250 nM (lanes 2 and 7), 500 nM (lanes 3 and 8), 750 nM (lanes 4 and 9), and 1,000 nM (lanes 5 and 10).

having first confirmed that this form of the protein is able to activate the *tynA* promoter *in vivo* when expressed at nonnative levels (data not shown). In a gel retardation DNA binding assay, the CTD bound specifically to a 250-bp fragment from the *tynA* promoter region, showing evidence of two retarded species (Fig. 6). This behavior is consistent with the presence of two FeaR binding sites in the *tynA* noncoding region.

To further analyze the FeaR binding site predicted by MEME, we used fluorescence anisotropy to measure binding of the FeaR CTD to DNA fragments from the *tynA* promoter region with mutations in the predicted binding sites. Two 31-bp DNA fragments containing the promoter-proximal (site 1) and -distal (site 2) binding sites were used. These DNA fragments were fluorescently labeled and used in measurements of fluorescence anisotropy in the presence of increasing concentrations of the FeaR CTD (28). The CTD bound to site 1 and site 2 with estimated dissociation constants of 301 ± 47 nM and 219 ± 18 nM, respectively. In each case, the Hill coefficient for binding was ~0.9, consistent with noncooperative binding to a single site (Fig. 7A).

To address the importance of specific residues within the FeaR binding sites, we performed competition binding assays with unlabeled DNAs containing single-site substitutions. In these assays, a 31-bp labeled DNA fragment containing either site 1 or site 2 was approximately half saturated with 345 nM CTD, and unlabeled DNA fragments were titrated into the DNA-protein complex (Fig. 7B; Table 2). For each sequence, we calculated the IC_{50}, i.e., the concentration that is required for half-maximal dissociation of the preexisting complex (29). The IC_{50}s for wild-type site 1 and site 2 were 660 and 580 nM, respectively, and almost all of the mutations increased these values. Four single-site mutations in each site abolished the ability of the sequence to compete for binding (Table 2), implicating these residues as especially important for CTD binding. All eight of these substitutions fall with the TGNCA-N_8-AAA element that is present in both fragments, and so the data are consistent with the proposal that this sequence is the FeaR binding site. With one exception (site 1-A19T), all other substitutions within the TGNCA-N_8-AAA motifs increased the IC_{50} by between 6- and 12-fold. In contrast, substitutions outside the proposed FeaR sites increased the IC_{50} by 4-fold at the most.

At an earlier stage of this analysis, we had made some point mutations in the region of the FeaR binding sites in the *tynA* promoter and fused the mutant promoters to *lacZ*. While this mutagenesis was not comprehensive, it is noticeable that of the four mutations within the TGNCA-N_8-AAA motifs, three (A18C, G27T, and A30G) reduced promoter activity by more than a factor

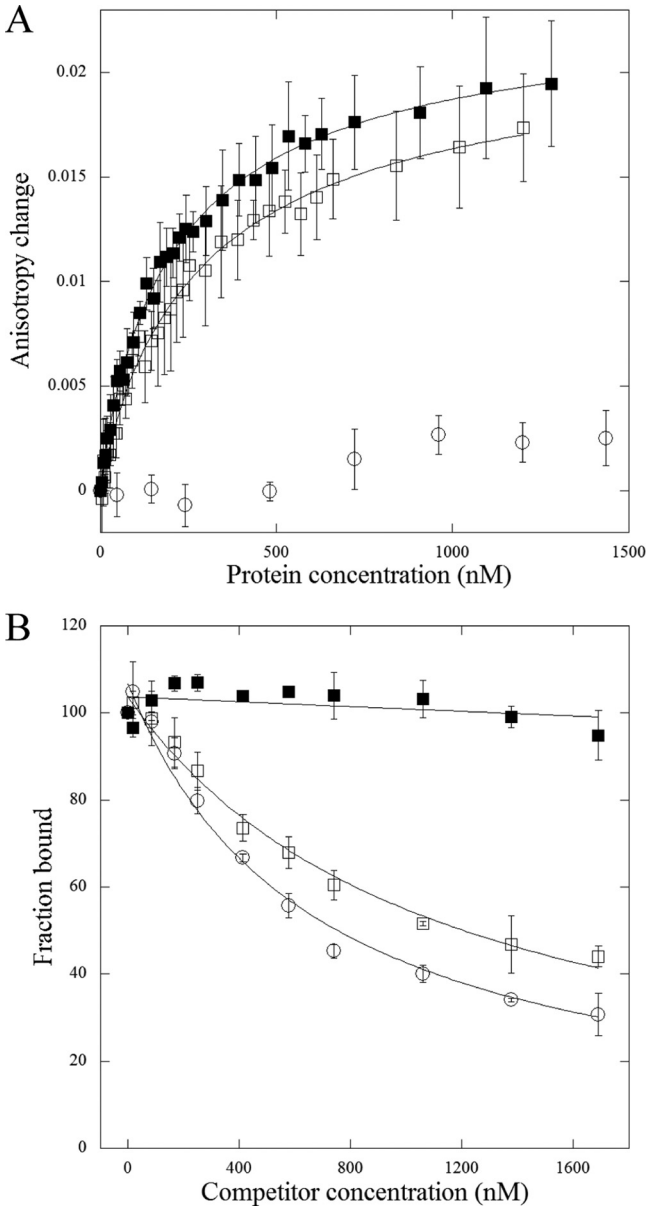

FIG 7 (A) Assay of DNA binding by the FeaR CTD by fluorescence anisotropy. The purified CTD was titrated into fluorescently labeled DNAs: site 1 (open squares), site 2 (filled squares), and a negative control, the *nrdH* promoter (open circles). Each data point is the mean of three measurements, and the plot lines show the fit to equation 1 (see Materials and Methods). The estimated dissociation constants are 301 nM ± 47 nM for site 1 and 219 nM ± 18 nM for site 2. (B) Competition assay using DNAs with mutations in the FeaR binding sites. Unlabeled DNAs were titrated into preformed complexes between the FeaR CTD and a labeled DNA. Each data point is the mean of three determinations, and data were fit to equation 2 (see Materials and Methods). Reactions are shown for DNAs that do not (T5G) (filled squares) and do (C17T) (open squares) compete with the wild-type sequence (competition with wild-type DNA is shown with open circles). Data for all mutations are shown in Table 2.

TABLE 2 DNA binding competition assay with mutant FeaR binding sites

Competitor[a]	DNA sequence[b]	IC_{50} (nM)[c]
Site 1	ATGAA<u>AAGCTGGCACACCTGCCAAAC</u>CCCCT	660 (50)
Site 1-A1G	ATGAA<u>gAGCTGGCACACCTGCCAAAC</u>CCCCT	910 (51)
Site 1-A1C	ATGAA<u>cAGCTGGCACACCTGCCAAAC</u>CCCCT	1,070 (46)
Site 1-A2C	ATGAA<u>AcGCTGGCACACCTGCCAAAC</u>CCCCT	1,330 (65)
Site 1-C4G	ATGAA<u>AAGgTGGCACACCTGCCAAAC</u>CCCCT	700 (40)
Site 1-T5G	ATGAA<u>AAGCgGGCACACCTGCCAAAC</u>CCCCT	No competition
Site 1-G6T	ATGAA<u>AAGCTtGCACACCTGCCAAAC</u>CCCCT	No competition
Site 1-C8A	ATGAA<u>AAGCTGGaACACCTGCCAAAC</u>CCCCT	No competition
Site 1-A9C	ATGAA<u>AAGCTGGCcCACCTGCCAAAC</u>CCCCT	No competition
Site 1-A11C	ATGAA<u>AAGCTGGCACcCCTGCCAAAC</u>CCCCT	1,570 (106)
Site 1-C13A	ATGAA<u>AAGCTGGCACACaTGCCAAAC</u>CCCCT	691 (47)
Site 1-T14G	ATGAA<u>AAGCTGGCACACCgGCCAAAC</u>CCCCT	2,000 (82)
Site 1-G15T	ATGAA<u>AAGCTGGCACACCTtCCAAAC</u>CCCCT	1,810 (85)
Site 1-C17T	ATGAA<u>AAGCTGGCACACCTGCtAAAC</u>CCCCT	1,120 (60)
Site 1-A18T	ATGAA<u>AAGCTGGCACACCTGCCtAAC</u>CCCCT	4,120 (362)
Site 1-A19T	ATGAA<u>AAGCTGGCACACCTGCCAtAC</u>CCCCT	1,370 (86)
Site 1-A20C	ATGAA<u>AAGCTGGCACACCTGCCAAcC</u>CCCCT	5,800 (517)
Site 2	ATGAA<u>AACCTGGCAGGTGCAGGCAAT</u>CCCCT	580 (43)
Site 2-A23C	ATGAA<u>AcCCTGGCAGGTGCAGGCAAT</u>CCCCT	950 (32)
Site 2-C25G	ATGAA<u>AACgTGGCAGGTGCAGGCAAT</u>CCCCT	650 (54)
Site 2-T26G	ATGAA<u>AACCgGGCAGGTGCAGGCAAT</u>CCCCT	No competition
Site 2-G27T	ATGAA<u>AACCTtGCAGGTGCAGGCAAT</u>CCCCT	No competition
Site 2-C29A	ATGAA<u>AACCTGGaAGGTGCAGGCAAT</u>CCCCT	No competition
Site 2-A30C	ATGAA<u>AACCTGGCcGGTGCAGGCAAT</u>CCCCT	5,010 (361)
Site 2-G32C	ATGAA<u>AACCTGGCAGcTGCAGGCAAT</u>CCCCT	2,650 (156)
Site 2-A36C	ATGAA<u>AACCTGGCAGGTGCcGGCAAT</u>CCCCT	1,840 (101)
Site 2-A40C	ATGAA<u>AACCTGGCAGGTGCAGGCcAT</u>CCCCT	7,846 (700)
Site 2-A41C	ATGAA<u>AACCTGGCAGGTGCAGGCAcT</u>CCCCT	No competition

[a] Promoter-proximal (site 1) and -distal (site 2) FeaR binding sites from the *tynA* promoter. Nucleotides are numbered according to the sequence logo (Fig. 3A).
[b] The site 1 and site 2 sequences are underlined, and mutations are in lowercase.
[c] Numbers in parentheses are errors estimated from the data fitting.

of 5 (Fig. 8). Of the remaining 11 mutations that were outside the motifs, two had no effect (C16A and C17A), and eight (C10G, A11T, T14G, G15T, C17T, G31A, T33A, and G38C) reduced the promoter activity to 80 to 40% of the wild-type level. Altogether, our data suggest that the FeaR binding site is a direct repeat of two 16-bp elements. Within each 16-bp sequence, nucleotides within the TGNCA-N_8-AAA motif make significant contributions to FeaR binding, while some nucleotides outside the motif also make minor contributions.

The function of the N-terminal domain. Sequences encoding FeaR and the CTD were ligated into pBAD24 for expression from the arabinose-inducible *araBAD* promoter. The plasmids were then transformed into a *tynA-lacZ* reporter strain to test the activity of FeaR and the CTD. Expression of the CTD led to high-level *tynA* promoter activity, in both the presence and absence of tyramine (see Fig. S3 in the supplemental material). The full-length

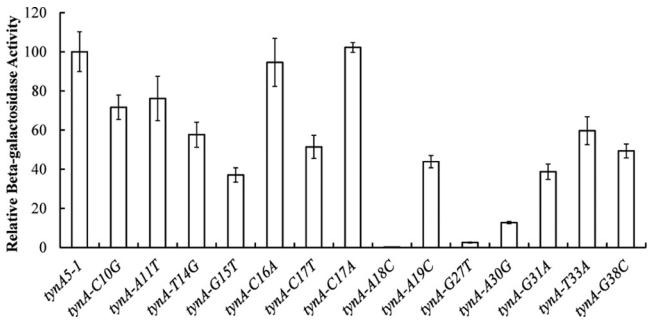

FIG 8 Site-directed mutagenesis of the FeaR binding site in the *tynA* promoter (Fig. 3). Mutations were introduced at positions in and near the FeaR binding sites, and mutant promoters were fused to *lacZ* for measurements of β-galactosidase activity. Activities are the means of duplicate measurements in each of three independent cultures, and error bars are standard deviations.

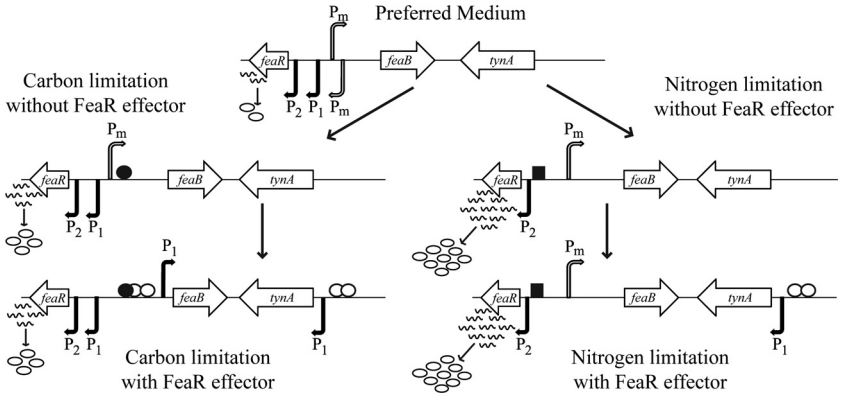

FIG 9 Summary of the known and proposed mechanisms that regulate transcription of *feaR*, *tynA*, and *feaB*. Active transcription start sites are represented by arrows, with filled arrows for relatively strong promoters and open arrows for weak promoters. The FeaR protein is represented by open circles, CRP-cAMP by filled circles, and NAC by squares. In glycerol-grown cells, *feaB* is transcribed at low level from P_m, and *tynA* is not expressed. Upon addition of a FeaR effector, the *feaB* P_1 promoter is activated by FeaR and CRP-cAMP and *tynA* is activated by FeaR. In cells using glutamine as the nitrogen source, *feaB* is expressed at a low level from P_m and *tynA* is not transcribed. In the presence of a FeaR effector, *feaB* is not expressed, most likely because CRP-cAMP is absent under these conditions. On the other hand, *tynA* transcription is activated by FeaR. Note that the figure is not drawn to scale.

FeaR expressed from pBAD24 activated the *tynA* promoter significantly above basal levels (to ~500 units) in the absence of a coactivator and to high levels in the presence of tyramine. Thus, activation by FeaR requires the NTD, as is the case for other AraC family members. Interestingly, the CTD activated *tynA* better than full-length FeaR in the absence of the coactivator, and this pattern was reversed in the presence of the ligand, suggesting that the NTD might function as an inhibitor of the CTD in the absence of the coactivator.

DISCUSSION

We have developed a model to describe the regulation of *tynA* and *feaB* expression that accounts for the data presented in this paper (Fig. 9). During growth on glucose and ammonia (preferred medium), *feaR* and *feaB* are expressed at basal levels, while *tynA* expression is almost silent. During growth on nonpreferred carbon or nitrogen sources (glycerol medium and glutamine medium), *feaR* expression is elevated 2- to 6-fold, but there is no activation of *tynA* or *feaB* in the absence of the FeaR coactivator. During carbon limitation in the presence of the FeaR coactivators (glycerol medium plus tyramine/PEA), both *tynA* and *feaB* are upregulated. However, during nitrogen limitation in the presence of the FeaR coactivators (glutamine medium plus tyramine), only *tynA* is upregulated, while *feaB* expression remains at a basal level. This regulatory pattern fits with the physiological roles of TynA and FeaB, in the sense that both are required for assimilation of monoamines as a source of carbon and energy, while only TynA is required when pathway substrates are serving only as a nitrogen source (Fig. 1). Thus, while *tynA* and *feaB* are coordinately regulated by FeaR, their expression is also fine-tuned by other regulators (CRP and NAC) and by the regulation of *feaR* expression in order to optimize enzyme activities according to the nutritional environment.

In addition to CRP and NAC, *feaR* may also be repressed by PhoB (20) and ArcA (P. J. Kiley, personal communication). The oxidation of PEA, tyramine, or dopamine to the corresponding aldehydes releases one equivalent of H_2O_2 and NH_3 at the expense of one equivalent of O_2. There is a physiological rationale for the repression of *feaR* by PhoB and ArcA: O_2 is required for the catabolism of monoamines, and H_2O_2 is a threat to membrane integrity during phosphate starvation (20). The H_2O_2 that is released by PEA catabolism is known to cause oxidative stress, activating the OxyR regulon and so stimulating the synthesis of enzymes that remove H_2O_2 (44). The *feaR* promoter is under the regulation of multiple global regulators, as is the case for about 50% of all *E. coli* genes (45). FeaR controls expression of a pathway for the degradation of potentially toxic aromatic compounds. The aldehydes that are the substrates for FeaB are particularly toxic, which may account for the fact that *feaB* is expressed at a significant level even in the absence of its substrates. A mutation in *tynA* causes constitutive expression of the SOS response (46), suggesting a role for TynA in removing a genotoxic compound, and we have suggested that TynA and FeaB may have a role in catabolizing toxic nitrated aromatic amines (8). Tyramine and PEA are found in food and in the gastrointestinal (GI) tract as products of the microbial decarboxylation of tyrosine and phenylalanine (47, 48). They may also play signaling roles, for example, influencing swarming motility and the adherence of pathogens to host cells (49–51). Tyramine-induced adherence may involve its binding to adrenergic receptors in intestinal tissue (49). It is also possible that tyramine interferes with signaling via the bacterial adrenergic sensor kinases QseC and QseE (52).

It is common for AraC family members to bind to two direct or inverted repeats upstream of the −35 region of the target promoters (39, 53, 54). Similarly, the FeaR binding site is two direct repeats upstream of the −35 region of *tynA* and *feaB*. However, unlike AraC, MelR, and XylS (15, 55, 56), the two FeaR binding sites have similar binding affinities, at least for binding to the FeaR CTD. Despite the fact that *feaR* and *feaB* are divergently transcribed with overlapping regulatory regions (Fig. 1), we find no evidence for autoregulation of *feaR*. The single predicted CRP site in the *feaR-feaB* intergenic region may be responsible for activation of both genes. This arrangement differs from that of (for example) the *melR-melAB* and *rhaS-rhaBAD* regulatory regions, in which the regulatory genes are subject to autoregulation and separate CRP dimers activate the divergently oriented promoters (14, 57).

The FeaR CTD can better activate the *tynA* promoter than

full-length FeaR in the absence of the coactivator; this pattern is reversed in the presence of coactivator. This suggests a dual role of for the NTD: inhibitory in the absence of ligand and stimulatory in the presence of ligand. This inhibitory function of the NTD is not found in MelR and RhaS/RhaR (18, 56, 58) but is found in AraC and XylS (15, 41, 59). The AraC NTD binds to the CTD and holds the CTD in a conformation favoring DNA looping in the absence of arabinose.

In conclusion, we have studied the regulation of the aromatic amine degradation pathway of *E. coli* and found that the genes encoding the first two enzymes of the pathway are subject to complex regulation involving FeaR and other proteins. The regulatory mechanisms appear to be coordinated to facilitate optimal expression of the two enzymes according to their different physiological roles and to the nutritional environment.

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