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KynR, a Lrp/AsnC-Type Transcriptional Regulator, Directly Controls the Kynurenine Pathway in *Pseudomonas aeruginosa*▽

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The opportunistic pathogen *Pseudomonas aeruginosa* can utilize a variety of carbon sources and produces many secondary metabolites to help survive harsh environments. *P. aeruginosa* is part of a small group of bacteria that use the kynurenine pathway to catabolize tryptophan. Through the kynurenine pathway, tryptophan is broken down into anthranilate, which is further degraded into tricarboxylic acid cycle intermediates or utilized to make numerous aromatic compounds, including the *Pseudomonas* quinolone signal (PQS). We have previously shown that the kynurenine pathway is a critical source of anthranilate for PQS synthesis and that the kynurenine pathway genes (*kynA* and *kynBU*) are upregulated in the presence of kynurenine. A putative Lrp/AsnC-type transcriptional regulator (gene PA2082, here called *kynR*), is divergently transcribed from the *kynBU* operon and is highly conserved in Gram-negative bacteria that harbor the kynurenine pathway. We show that a mutation in *kynR* renders *P. aeruginosa* unable to utilize L-tryptophan as a sole carbon source and decreases PQS production. In addition, we found that the increase of *kynA* and *kynB* transcriptional activity in response to kynurenine was completely abolished in a *kynR* mutant, further indicating that KynR mediates the kynurenine-dependent expression of the kynurenine pathway genes. Finally, we found that purified KynR specifically bound the *kynA* promoter in the presence of kynurenine and bound the *kynB* promoter in the absence or presence of kynurenine. Taken together, our data show that KynR directly regulates the kynurenine pathway genes.

Pseudomonas aeruginosa is a Gram-negative bacillus that is ubiquitous throughout nature and infects a variety of hosts. It is a common nosocomial pathogen known to cause serious opportunistic infections in immunocompromised individuals (25). *P. aeruginosa* also causes a chronic infection in cystic fibrosis (CF) patients that is difficult, if not impossible, to eradicate and ultimately leads to increased morbidity and mortality in this population (4, 37). In order to survive during such infections and in many other harsh environments, *P. aeruginosa* utilizes numerous different carbon sources and produces a wide range of secondary metabolites (21, 24, 28, 35, 38). One potential nutrient, tryptophan, can be used by some bacteria to provide building blocks for many secondary metabolites, some of which function as siderophores, signaling molecules, and protective compounds (1, 11, 28, 44). Similar to eukaryotes, *P. aeruginosa* catabolizes tryptophan through the kynurenine pathway (11, 22). Kurnasov et al. utilized comparative genetics to identify several bacteria with putative kynurenine pathways, including *Bacillus anthracis*, *P. aeruginosa*, and *Bordetella pertussis* (22). The kynurenine pathway contrasts significantly from the major tryptophan catabolic pathway of *Escherichia coli* (and many other species of bacteria), which catabolizes L-tryptophan anaerobically into indole, pyruvate, and ammonia via a pyridoxal phosphate-dependent tryptophanase (41, 50). Nevertheless, with either catabolic pathway the ability to utilize both tryptophan and tryptophan breakdown products as a carbon and nitrogen source, and as precursors for many secondary metabolites, provides a unique advantage for survival within nutrient-limited and harsh environments.

The conversion of L-tryptophan into quinolinate via the kynurenine pathway has been demonstrated in multiple bacterial species (22). However, *P. aeruginosa* only encodes the genes for the anthranilate branch of the kynurenine pathway (23). This branch catabolizes L-tryptophan into anthranilate via a three-step enzymatic pathway (22). The three enzymes are encoded by *kynA* (which encodes a tryptophan-2,3-dioxygenase), *kynB* (kynurenine formamidase), and *kynU* (kynureninase) (see Fig. 7 for pathways) (11, 23). The *kynA* gene is located separately on the *P. aeruginosa* chromosome, while *kynB* and *kynU* are encoded in a putative operon. The kynurenine pathway was linked to *P. aeruginosa* virulence when it was shown that radiolabeled tryptophan was incorporated into the *Pseudomonas* quinolone signal (PQS) (11), which is important for virulence in multiple models of infection (10, 13, 27, 34, 49). These data also showed that despite the presence of two alternative pathways for anthranilate production, the kynurenine pathway is the main source of anthranilate for PQS production when *P. aeruginosa* is grown in the presence of tryptophan or tryptophan breakdown metabolites (11, 31).

Due to both the importance of the kynurenine pathway in the production of PQS and because tryptophan is a costly amino acid to synthesize (11, 52), it would be advantageous for *P. aeruginosa* to strictly regulate the catabolism of tryptophan. Multiple transcriptome arrays have shown that quorum sensing may regulate the kynurenine pathway (36, 43), and the quorum-sensing regulator LasR was predicted through chromatin

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype[a]	Reference
E. coli DH5α	F′ *endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* (*lacZYA-argF*)*U169 deoR* [ϕ80d*lac*Δ(*lacZ*)M15*recA1*]	48
P. aeruginosa strains		
PAO1	Wild type	19
PAO-R1	*lasR*::Tc deletion mutant derived from PAO1	14
PJF-KA1	*kynA* deletion mutant derived from PAO1	11
PJF-KB1	*kynB* deletion mutant derived from PAO1	11
PJF-KU1	*kynU* deletion mutant derived from PAO1	11
PAΔKynR	*kynR* deletion mutant derived from PAO1	This study
PAO-R1ΔKynR	*kynR lasR* deletion mutant derived from PAO-R1	This study
Plasmids		
pHERD30T	P_{BAD}-based shuttle vector, Gm^r	33
pKynRex	KynR expression vector, Gm^r	This study
pACYC184	General purpose cloning vector, Tet^r Chl^r	5
pKynRsubex	KynR expression vector derived from pKynRex, Chl^r	This study
pEX18Ap	Suicide vector, Amp^r	17
p*kynR*del	*kynR* deletion suicide vector, Amp^r	This study
pLP170	*lacZ* transcriptional fusion vector, Amp^r	32
pJF01	*kynB′-lacZ* transcriptional fusion, Amp^r	11
pJF03	*kynA′-lacZ* transcriptional fusion, Amp^r	11

[a] Abbreviations: Gm, gentamicin; Tet, tetracycline; Chl, chloramphenicol; Amp, ampicillin.

immunoprecipitation-chip analysis to bind to the promoter region of *kynB* (15). Our laboratory has also shown that the transcription of both *kynA* and *kynB* was significantly increased in the presence of kynurenine (11). With these data in mind, we began to search for a transcriptional regulator that could specifically regulate *kynA* and *kynB* in the presence of kynurenine. We identified a putative transcriptional regulator encoded by gene PA2082, which is divergently transcribed from *kynB* and is homologous to the Lrp/AsnC family of transcriptional regulators. Lrp/AsnC-type regulators are known to control amino acid metabolism, and though Lrp has a global regulatory role in *E. coli*, many other regulators within the family have more specific regulons (3, 51). We demonstrate here that the protein encoded by gene PA2082 directly binds to and regulates the kynurenine pathway genes, and we therefore propose that this protein be named the kynurenine pathway regulator (KynR).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *E. coli* and *P. aeruginosa* strains were maintained in 30% glycerol and 10% skim milk (Difco), respectively, at −80°C and were freshly plated to begin each experiment. All strains and plasmids used are listed in Table 1. Bacteria were cultured at 37°C in Luria-Bertani medium (LB), Vogel Bonner minimal medium supplemented with 0.5% glycerol (VBG) (42), or sole carbon source medium (SCM); containing 73.4 mM K_2HPO_4, 16.76 mM $NaNH_5PO_4 \cdot 4H_2O$, 0.8 mM $MgSO_4$, and with either 10 mM L-tryptophan (TSCM) or 10 mM L-alanine (ASCM) (pH 7.0). Cultures were supplemented with the kynurenine pathway metabolites as indicated below. Growth was monitored spectrophotometrically based on the optical density at 600 nm (OD_{600}) for *E. coli* or OD_{660} for *P. aeruginosa.* To maintain plasmids, 30 μg/ml chloramphenicol, 100 μg/ml ampicillin, 200 μg/ml carbenicillin, or 15 μg/ml (*E. coli*) or 30 μg/ml (*P. aeruginosa*) gentamicin was added when appropriate.

In order to generate an expression plasmid for KynR, a 568-bp DNA fragment, which began at the *kynR* start codon (ATG) and ended 46 bp downstream from the stop codon, was amplified by PCR using strain PAO1 chromosomal DNA as the template. The oligonucleotide primers used for this amplification were engineered to contain a single HindIII site downstream from the stop codon (Table 2). Vector plasmid pHERD30T, which contains an *araBAD* (P_{BAD}) promoter to control gene expression, was digested sequentially with SmaI and HindIII. The digested plasmid DNA was ligated with the *kynR* PCR fragment, which had also been digested with HindIII, to create the plasmid pKynRex. This plasmid was used to amplify *kynR* and regulatory elements for use in a two-plasmid system in *E. coli.* PCR was used to amplify both the *kynR* and divergently transcribed *araC* to ensure all elements necessary for controlled expression of KynR were included in the subcloned plasmid. Primers were located 52 bp downstream from *araC* and 46 bp downstream from *kynR*. The resulting PCR fragment, as well as pACYC184, were sequentially digested with SalI and HindIII and ligated together, generating plasmid pKynRsubex.

TABLE 2. Primers used in this study

Primer use and name	Sequence (5′–3′)[a]
Primers for mutagenesis	
2082-F1-remake	AAAAAA**GAATTC**GAGGTAGATCACGCCGTC
PA2082Rev1	CCGGCCTTGATCTCCTGCAGTCCTGGTTGCTCATCCGCCC
PA2082Fwd2	GGGCGGATGAGCAACCAGGACTGCAGGAGATCAAGGCCGG
PA2082Rev2	AAAAAA**GAATTC**GAACTTGGCGGCGATGG
Primers for KynR expression	
2082atgfwd	ATGTCCCTGGACGCCATCGA
2082 Rev Herd	AAAA**AAGCTT**CGTCCCTCTCATTGCACT
Herd2082exsubRev	AAAA**GTCGAC**CGAAGCAGGGTTATGCAGC
EMSA	
kynApromoterfwd	GAGTGAGGGCAAGGACACAT
kynApromoterrev	CGCGAGTGATCCGAAATTCG
kynBpromoterfwd	GACTGATGTCCCAGTAGCGG
kynBpromoterrev	GACGGAGAATGCGCAGATCG
FwdKynU-EMSA	GGTACTTGTAGGTGCAGCCG
RevKynU-EMSA	CAAGACCGGCTACCTGCACG

[a] Restriction sites are indicated by bold type.

Generation of *kynR* mutants. A splicing-by-overlap extension protocol was used to generate mutant alleles (47). Alleles were constructed to contain in-frame deletions in the coding DNA sequences corresponding to amino acids 25 to 140 for KynR (73% of the protein sequence). Primers were designed to contain approximately 1 kb of DNA both upstream and downstream from the splice junction, and each primer added an EcoRI restriction site to both ends of the PCR product. Both the *kynR*del PCR product and pEX18Ap (suicide vector) were digested with EcoRI and ligated together. The resulting plasmid, p*kynR*del, was transformed into strains PAO1 and PAO-R1 by electroporation (6). Mutants were selected by plating transformants on medium containing carbenicillin and then on medium containing 6% sucrose to remove the vector sequence (17). PCR was used to screen colonies, and DNA sequencing of PCR products was used to confirm mutants.

PQS production. Washed cells from overnight cultures were used to inoculate 10-ml cultures of LB or VBG supplemented with either water, 1 mM L-tryptophan, 1 mM L-kynurenine, or 1 mM anthranilate (final concentrations) to an OD_{660} of 0.05. After 6 and 24 h of growth, 300-μl samples of each culture were extracted with 900 μl of acidified ethyl acetate as previously described (8). One-half of the resulting organic phase was evaporated to dryness at 37°C, and 50 μl of 1:1 acidified ethyl acetate-acetonitrile was used to reconstitute the extract. Samples were analyzed by thin-layer chromatography (TLC), visualized by long-wave UV light, and photographed (8).

β-Gal assays for *P. aeruginosa*. Cells from overnight cultures of *P. aeruginosa* grown in VBG were washed and resuspended in fresh medium to an OD_{660} of 0.05 and incubated at 37°C with shaking at ≥180 rpm for 6 h. At that time, either water or 1 mM L-kynurenine was added, and cultures continued to grow at 37°C with shaking at ≥180 rpm for an additional 18 h. The cells were collected by centrifugation at 12,000 × *g* for 2 min, resuspended in fresh VBG, and assayed for β-Galactosidase (β-Gal) activity in duplicate. Data are presented in Miller units as the mean ± standard deviation [$\sigma^{(n-1)}$] of at least 3 separate experiments.

β-Gal assays for *E. coli*. Overnight cultures of *E. coli* carrying the appropriate plasmids were grown in LB and used to inoculate 10-ml cultures of fresh medium to an OD_{600} of 0.08. The subcultures were incubated at 37°C with shaking at ≥180 rpm until an OD_{600} of 0.3 was reached. Then, 2-ml aliquots were transferred to tubes that contained 0.1% L-arabinose to induce *kynR* expression, and either water, 1 mM L-kynurenine, 1 mM L-tryptophan, or 1 mM anthranilic acid (final concentrations) was added. The cells were incubated for 2 h at 37°C with shaking at ≥180 rpm. After incubation, the β-Gal activity produced by each culture was assayed, and data are reported as the mean ± $\sigma^{(n-1)}$ of at least 3 independent experiments.

Purification of KynR. The method used for purification of KynR is a modification of those described by Madhusudhan et al. for the purification of BkdR from *Pseudomonas putida* (26). A 10-ml overnight culture of *E. coli* strain DH5α(pKynRex) was used to inoculate 250 ml of LB and was incubated at 37°C with shaking at ≥180 rpm until an OD_{600} of 0.7 was reached. At this point, 1% L-arabinose (Sigma-Aldrich) was added to induce *kynR* expression, and the culture was grown for an additional 3 h at 37°C with shaking at ≥180 rpm. Cells were harvested by centrifugation at 6,000 × *g* for 10 min and then resuspended in 20 mM Tris-HCl (pH 7.4) and 1 mM dithiothreitol (DTT) (buffer A). The cells were lysed by sonication, and the lysate was cleared by centrifugation at 37,000 × *g* for 1 h at 4°C (Beckman Coulter Optima L-100 XP ultracentrifuge). KynR purification was performed at room temperature, and all samples were kept on ice or at 4°C until purification was complete. The soluble fraction (90 mg of total protein) was applied to a HiTrap DEAE Sepharose Fast Flow column (1 ml; GE Healthcare) in buffer A at a flow rate of 0.5 ml/min. The column was washed with buffer A until the A_{280} returned to 0 (~50 ml). Bound proteins were eluted with a 30-ml linear gradient of 0 to 0.3 M NaCl at a flow rate of 1 ml/min, and 3-ml fractions were collected. Samples from the collected fractions were precipitated using deoxycholic acid and tricholoracetate acid (DCA/TCA) and then separated by 15% SDS-PAGE to identify fractions containing KynR. These fractions were collected and dialyzed against a buffer containing 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT (buffer B) overnight at 4°C. The dialyzed fractions were then applied to a HiTrap heparin column (5-ml; GE Healthcare) equilibrated with buffer B. The column was washed with buffer B until the A_{280} returned to 0 (~24 ml). Bound proteins were eluted with a 50-ml linear gradient of 0.1 to 0.4 M NaCl in buffer B at a flow rate of 1 ml/min, and 3-ml fractions were collected. Again, proteins from the collected fractions were precipitated with DCA/TCA and were separated on a 15% SDS-polyacrylamide gel to identify the fractions containing KynR. All fractions containing KynR were pooled and dialyzed against 20 mM Tris-HCl (pH 7.8) and 1 mM DTT (buffer C) overnight at 4°C. The dialyzed fraction was then added to a HiTrap DEAE Sepharose Fast Flow column (1 ml; GE Healthcare) equilibrated with buffer C. The column was washed with buffer C until the A_{280} returned to 0 (~12 ml), bound proteins were eluted with a 30-ml linear gradient of 0 to 0.3 M NaCl at a flow rate of 1 ml/min, and 3-ml fractions were collected. Samples from collected fractions were precipitated with DCA/TCA and separated on a 15% SDS-PAGE to identify the fractions that contained KynR. The fractions containing KynR resulted in 95% purity of KynR as judged by SDS-PAGE (data not shown). Glycerol was added to the pooled KynR fractions to a final concentration of 15%, and the protein was stored at −80°C.

Column fractions during purification were monitored by absorbance measurements at 280 and 260 nm with a NanoDrop ND-1000 spectrophotometer. After purification, the final protein concentration was 4.34 mg/ml (244 mol) of protein, as determined by a Bradford assay using Bio-Rad reagents. In addition, protein concentrations for electrophoretic mobility shift assays (EMSA) were determined by the Bradford assay using Bio-Rad reagents.

EMSA. PCR was used to generate DNA fragments containing the *kynA* (228 bp, +208 bp to −20 bp relative to ATG) and *kynB* (200 bp, +169 bp to −31 bp relative to the ATG) promoter regions. An internal fragment of *kynU* (157 bp, +527 bp to +685 bp relative to the ATG) was also generated by PCR as a negative control. DNA fragments were labeled with [γ-^{32}P]ATP (Perkin-Elmer, Wellesley, MA) by using T4 polynucleotide kinase (Invitrogen, Carlsbad, CA). The binding assays were carried out in buffer containing 20 mM Tris-HCl (pH 7.4), 50 mM $NaCl_2$, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 10% glycerol. Each reaction mixture contained 0.3 μg of salmon sperm DNA, approximately 1.5 × 10^4 cpm radiolabeled DNA, and 0 to 488 pmol of protein. Binding reactions were performed in both the presence and absence of 0.1 mM L-kynurenine or 0.1 mM L-tryptophan. Reaction mixtures were incubated at room temperature for 20 min and separated by electrophoresis at 4°C on a native 10% polyacrylamide gel (29:1 acrylamide–bis-acrylamide, 1× Tris-borate buffer [89 mM Tris and 89 mM borate, pH 8.0], and 2.5% glycerol) for 5 min at 200 V and then for 4 h at 100 V. After electrophoresis, the gels were dried and visualized by autoradiography.

RESULTS

Identification of a putative regulator of the kynurenine pathway. To begin our search for potential regulators of the kynurenine pathway, we first analyzed the predicted function of annotated genes located near the kynurenine pathway operons in *P. aeruginosa* (The Pseudomonas Genome Database). The kynurenine pathway genes are located separately on the *P. aeruginosa* chromosome, with *kynB* and *kynU* located in a putative polycistronic operon and *kynA* located on a distant putative monocistronic operon (Fig. 1A). A potential transcriptional regulator encoded by gene PA2082 was divergently transcribed from *kynB* in *P. aeruginosa* and appeared to be a member of the Lrp/AsnC family of transcriptional regulators (Fig. 1B). These regulators are known to be important for controlling amino acid metabolism and are often encoded adjacent to pathway genes that they regulate (3). We used a comparative genomics approach and began to examine other bacteria that harbor the kynurenine pathway genes to determine if they also possessed similar regulator proteins. Both the *Burkholderiaceae* and *Pseudomonadaceae* families encode a PA2082 homolog divergently oriented from either *kynB* or *kynU* in the *Burkholderia*, *Ralstonia*, *Cupavarius*, *Bordetella*, and *Pseudomonas* genera (Fig. 1B). *P. aeruginosa* only contains the anthranilate branch of the kynurenine pathway, while some of the *Burkholderia* species and *Pseudomonas fluorescens* apparently contain the pathway to catabolize tryptophan into quinolinate or the siderophore quinolobactin (22, 28). Also, *Bacillus cereus* and *Bacillus anthracis* contain the kynurenine pathway genes (23), but these species have a TetR-like regulator divergently oriented from *kynU* that may be responsible for an alternative regulatory scheme. Overall, these findings suggest that the putative transcriptional regulator encoded by PA2082, which we hereby designate KynR (kynurenine path-

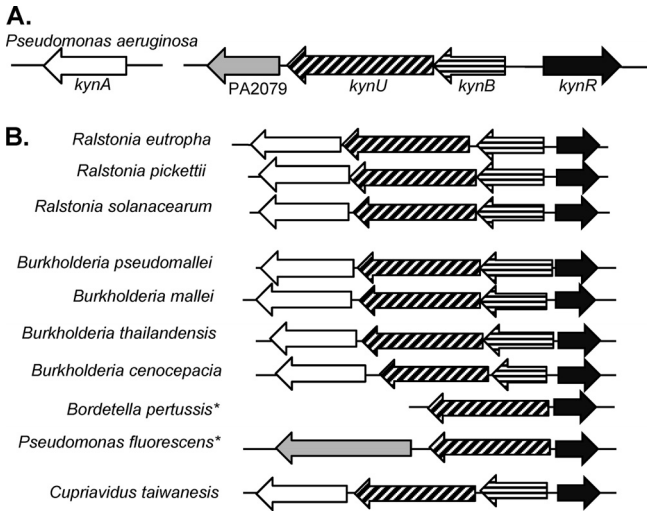

FIG. 1. The *kynR* gene is conserved in other Gram-negative bacteria that have the kynurenine pathway genes. (A) The two loci of the kynurenine pathway genes in *P. aeruginosa.* (B) A schematic to show the conservation of *kynR.* The shading on each gene corresponds to the homologous genes in panel A. *, *kynB* and *kynA* are located separately on the chromosome in a putative operon.

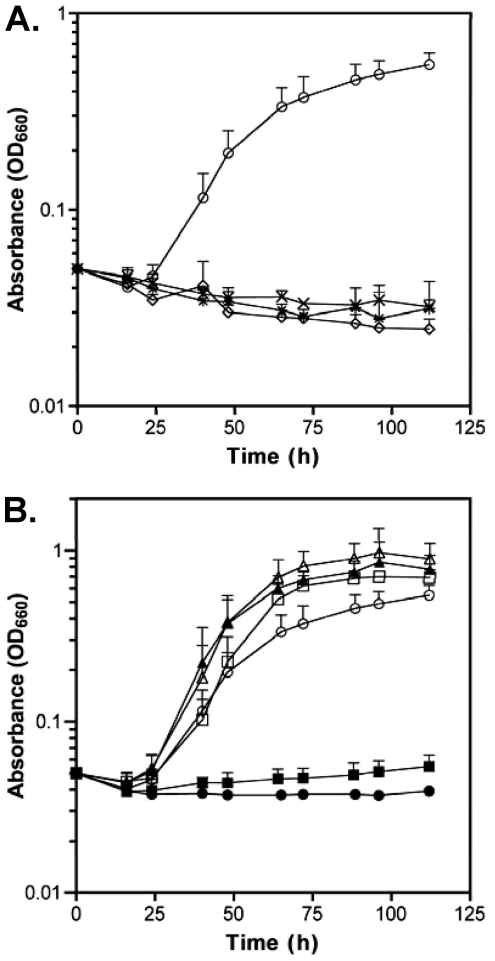

FIG. 2. Utilization of L-tryptophan as a sole carbon source. (A) Bacterial strains PAO1 (circles), PJF-KA1 (X), PJF-KB1 (diamonds), and PJF-KU1 (asterisks) were grown in TSCM, and the OD_{660} was measured. (B) Bacterial strains PAO1 (open circles), PAO1 with 0.1% arabinose (open squares), PAO1(pKynRex) with 0.1% arabinose (open triangles), PAΔKynR (closed circles), PAΔKynR with 0.1% arabinose (closed squares), and PAΔKynR(pKynRex) with 0.1% arabinose (closed triangles) were grown in TSCM, and the OD_{660} was measured. Values depict the mean $\pm \sigma^{(n-1)}$ from at least three separate experiments.

way regulator), might play a role in regulating tryptophan catabolism in *P. aeruginosa.*

In order to examine the role of KynR in tryptophan catabolism, we assessed the impact of KynR on kynurenine pathway-dependent phenotypes. The kynurenine pathway is the only enzymatic pathway identified in *P. aeruginosa* capable of degrading tryptophan (22). To confirm this, we grew mutants containing deletions of the kynurenine pathway genes *kynA* (PJF-KA1), *kynB* (PJF-KB1), and *kynU* (PJF-KU1) in a minimal salts medium with tryptophan as the only carbon source (TSCM) (Fig. 2A). The wild-type strain PAO1 utilized tryptophan as a sole carbon source and grew to an OD_{660} of over 0.5 (Fig. 2A). However, the kynurenine pathway mutants were unable to grow with only tryptophan as a carbon source (Fig. 2A). As a control, the strains were also grown on alanine as a sole carbon source (ASCM), and the kynurenine pathways mutants were able to grow to wild-type levels (data not shown). These results confirmed that the kynurenine pathway is required for tryptophan catabolism in *P. aeruginosa.* Next, we tested whether the *kynR* mutant strain PAΔKynR was able to grow on tryptophan as a sole carbon source (Fig. 2B). The results indicated that, like the kynurenine pathway mutants, PAΔKynR was unable to grow on tryptophan alone. In order to determine if the inhibition of growth was due to the loss of *kynR*, strains PAΔKynR and PAO1 were transformed with a plasmid harboring wild-type *kynR* under an arabinose-inducible promoter (pKynRex). Expression of KynR in strain PAΔKynR caused growth on tryptophan to be similar to that of the parent strain PAO1 (Fig. 2B). Since *P. aeruginosa* can utilize arabinose as a carbon source, we also included control cultures to ensure that the arabinose that was added for KynR expression was not affecting the growth phenotypes, and we observed that the effect was negligible (Fig. 2B). Overall, these data indicated that a *kynR* mutant was unable to grow on tryptophan as a sole carbon source and further supported the notion that KynR has a role in the regulation of the kynurenine pathway.

PQS production is affected in a *kynR* mutant. Previous studies showed that the kynurenine pathway mutants produced little to no detectable PQS and suggested that the kynurenine pathway is the main source of anthranilate for PQS production when tryptophan or its breakdown metabolites are present (11). Therefore, we were interested in testing whether a mutation in *kynR* would also have an effect on PQS production. Strains PAO1 and PAΔKynR were grown in LB and VBG supplemented with tryptophan, kynurenine, anthranilic acid, or water (as a control). The cultures were grown for either 6 or 24 h and then were extracted with acidified ethyl acetate. The extracts were analyzed by TLC to assay both PQS and anthranilate production. After 6 and 24 h of growth in LB or LB supplemented with tryptophan, strain PAΔKynR produced less

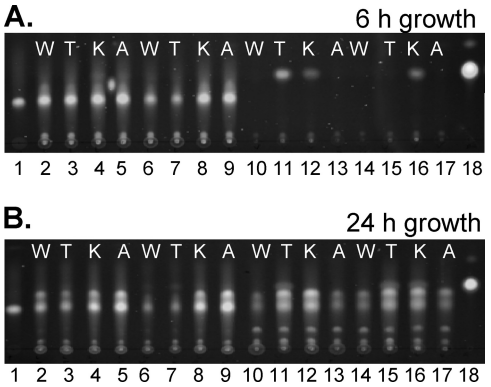

FIG. 3. The production of anthranilate and PQS is decreased in the *kynR* mutant. Ethyl acetate extracts from cultures grown for 6 (A) and 24 h (B) were analyzed by TLC. Equal volumes of extracts were resolved in each lane, and the addition of water (W), 1 mM L-tryptophan (T), 1 mM L-kynurenine (K), or 1 mM anthranilic acid (A) to the respective culture is indicated at the top of each lane. Lane 1 contains 75 ng of PQS, and lane 18 contains 1.25 ng of anthranilic acid (controls). Lanes 2 to 5 contain extracts from strain PAO1 grown in LB; lanes 6 to 9 contain extracts from strain PAΔKynR grown in LB; lanes 10 to 13 contain extracts from strain PAO1 grown in VBG; lanes 14 to 17 contain extracts from strain PAΔKynR grown in VBG.

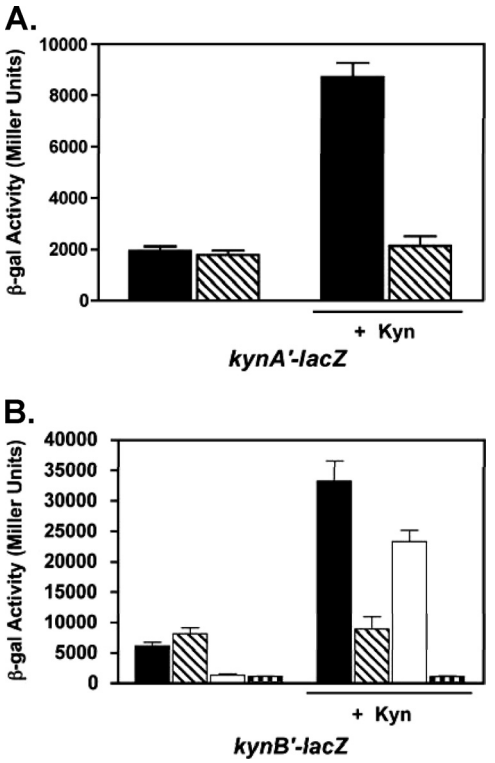

FIG. 4. The kynurenine pathway genes are not induced by kynurenine in a *kynR* mutant. β-Gal activity of *kynA′-lacZ* fusion (pJF03) (A) and (B) *kynB′-lacZ* fusion (pJF01) (B) in strains PAO1 (solid bars), PAΔKynR (striped bars), PAO-R1 (open bars), and PAO-R1ΔKynR (stippled bars) were assayed in cultures grown in VBG for 6 h and then supplemented with either water (as a control) or 1 mM L-kynurenine for 18 h. β-Gal activity is presented in Miller units as the mean ± $\sigma^{(n-1)}$ of results from duplicate assays from at least three separate experiments.

PQS (Fig. 3A and B, lanes 6 and 7) than the wild-type strain PAO1 (Fig. 3A and B, lanes 2 and 3). This suggested that *kynR* affects the supply of anthranilate for PQS production by positively controlling an element of the kynurenine pathway.

We then saw something unexpected, when the addition of kynurenine to LB resulted in wild-type amounts of PQS being produced by strain PAΔKynR after both 6 and 24 h (Fig. 3A and B, lane 8). This suggested that KynR was not the only factor that controls *kynBU*, which must be induced for the conversion of kynurenine to anthranilate to be used for PQS synthesis (see Fig. 7 for pathway). PQS was also extracted from both strains grown in a minimal medium to assess the effects of supplementing with individual kynurenine pathway metabolites. The production of PQS by strains PAO1 and PAΔKynR grown in VBG differed somewhat from those in LB but followed a similar trend. PQS was not detectable from either strain after 6 h of growth in VBG, but the addition of tryptophan or kynurenine to the medium induced the production of anthranilate by the wild-type strain PAO1 (Fig. 3A, lanes 11 and 12). Unlike the wild-type strain PAO1, strain PAΔKynR only produced anthranilate with the addition of kynurenine and not when tryptophan was added (Fig. 3A, lanes 15 and 16). After 24 h of growth in VBG, PQS was detectable from both strains under all conditions, and anthranilate was no longer detectable after presumably being used by the cells (Fig. 3B, lanes 10 to 17). Taken together, the data suggest that KynR indirectly regulates PQS production, presumably through the kynurenine pathway, but that it is not absolutely required for PQS to be produced.

KynR is required for kynurenine-dependent induction of kynurenine pathway genes. Thus far, the phenotypes of the *kynR* mutant have implied that KynR plays a role in the kynurenine pathway. In addition, our previous work had shown that the transcription of both *kynA* and *kynB* was induced in the presence of kynurenine (11). Therefore, we decided to determine if KynR affected the expression of *kynA* and *kynB* in a defined medium (VBG) where we could control the concentration of kynurenine. To study this, we performed β-Gal assays in strains PAO1 and PAΔKynR harboring the respective *kynA′-lacZ* and *kynB′-lacZ* transcriptional fusion plasmids pJF03 and pJF01. Our data showed that after 24 h of growth in VBG medium in the absence of kynurenine, the expression levels of both *kynA* and *kynB* were similar in both the parental and *kynR* mutant strains (Fig. 4A and B). However, the addition of 1 mM kynurenine resulted in a large induction of both *kynA* and *kynB* in the wild-type strain PAO1, but this induction did not occur in the *kynR* mutant (Fig. 4A and B). These data led us to conclude that in the presence of kynurenine, KynR positively regulates *kynA* and *kynB* transcription.

It has been suggested from microarray experiments that *kynB* is directly regulated by the quorum-sensing regulator LasR (15). Since our data in Fig. 3 also suggested that a factor in addition to KynR was regulating *kynBU*, it seemed logical that we should examine whether LasR affected *kynBU* transcription. The data of Fig. 4B showed that *kynB* transcription was greatly decreased in a *lasR* mutant when kynurenine was not present but that the addition of kynurenine would override the *lasR* mutation. In addition, a *lasR kynR* double mutant exhibited no transcription of *kynB* in the presence or absence

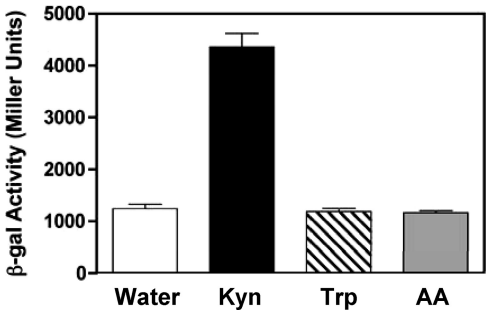

FIG. 5. KynR can induce expression of *kynB′-lacZ* in the presence of L-kynurenine in *E. coli. E. coli* strain DH5α(pJF01)(pKynRex) was grown in LB medium in the presence of water as a control water (white bar), 1 mM L-kynurenine (black bar), 1 mM L-tryptophan (striped bar), or 1 mM anthranilate (gray bar). After 3 h of growth the cultures were induced with 0.1% arabinose and grown for an additional 3 h. The cultures were then assayed for β-Gal activity, which is presented in Miller units as the mean ± $\sigma^{(n-1)}$ of results from at least three experiments.

of kynurenine (Fig. 4B). Taken together, the data of Fig. 4 lead us to conclude that *kynBU* is controlled by LasR when kynurenine is absent and by KynR when kynurenine is available.

KynR directly regulates the kynurenine pathway. Our data so far showed that KynR is required for the kynurenine-dependent increase of both *kynA* and *kynB* transcription (Fig. 4A and B), but whether this regulation was direct or indirect was unknown. To eliminate any possibility of endogenous *P. aeruginosa* factors altering the results of our *kynA* and *kynB* transcriptional fusion assays, we utilized an *E. coli* two-plasmid system to try to establish a more direct link between KynR and the induction of *kynA* and *kynB.* (It is important to point out here that *E. coli* does not contain the genes for the kynurenine pathway.) *E. coli* strain DH5α harboring either a *kynA′-lacZ* (pJF03) or a *kynB′-lacZ* (pJF01) transcriptional fusion and the KynR overexpression plasmid (pKynRsubex) was grown in LB in the presence of tryptophan, kynurenine, or anthranilate, and the expression of KynR was induced with the addition of 0.1% arabinose. The results presented in Fig. 5 show that the expression of *kynB′-lacZ* was only induced in the presence of KynR and kynurenine and not when tryptophan or anthranilate was present. Unfortunately, the similar assays performed with the *kynA′-lacZ* fusion showed that it was constitutively active in *E. coli*, and direct regulation by KynR could not be demonstrated in this experiment (data not shown). Nevertheless, the results of Fig. 5 suggest that KynR can directly activate the expression of *kynB* and that kynurenine acts as a coinducer for KynR.

To determine if KynR directly binds to the *kynA* and *kynB* promoter regions in response to kynurenine, we purified KynR and performed EMSA. KynR was purified from *E. coli* strain DH5α(pKynRex) and incubated with either the *kynA* or *kynB* promoter region in the presence or absence of kynurenine. We also added tryptophan in separate binding reaction mixtures to determine if it had an effect on the ability of KynR to bind DNA. As a nonspecific binding control, an internal *kynU* fragment was radiolabeled and utilized in binding reactions with KynR and kynurenine. The results of all EMSA are shown in Fig. 6. These data indicate that KynR only bound to the *kynA* promoter in the presence of kynurenine (Fig. 6A, right panel), and KynR was unable to bind *kynA* in the absence of a coinducer (Fig. 6A, left panel). Additionally, tryptophan had no effect on the ability of KynR to bind to the *kynA* promoter (Fig. 6C, left panel). In contrast to *kynA*, the *kynB* promoter was bound by KynR in both the presence and absence of kynurenine (Fig. 6B). This binding was not affected by tryptophan (Fig. 6C, right panel) and indicated that the *kynA* and *kynB* promoters are recognized by KynR in different manners. It should also be noted that in the presence of kynurenine, an observable shift with *kynB* occurred with 1.9 pmol of KynR (Fig. 6B, right panel), while 7.62 pmol of KynR was needed for a *kynB* shift to occur in the absence of kynurenine (Fig. 6B, left panel). This showed that KynR has a greater affinity for the *kynB* promoter in the presence of kynurenine. Furthermore, compared to the interaction with *kynA*, the binding of KynR to *kynB* exhibited a more complex pattern of DNA migration retardation as the protein concentration increased. These results suggest that the binding of KynR-kynurenine to the *kynA* promoter represented the binding of a single complex to the DNA and that multiple KynR complexes, with or without a kynurenine coinducer, most likely interact with the *kynB* promoter. Taken together, these data indicated that KynR binds to the *kynA* promoter in a kynurenine-dependent manner, while the interaction of KynR with the *kynB* promoter occurs in both the presence and absence of kynurenine in a less constricted manner.

DISCUSSION

We previously showed that the transcription of both *kynA* and *kynB* was induced in the presence of kynurenine (11). This led us to believe that a transcriptional regulator was responsible for the induction of the kynurenine pathway genes in the presence of kynurenine. With this in mind, we identified KynR as the transcriptional regulator for the kynurenine pathway. The *kynR* gene is divergently transcribed from *kynB* and is found in the same genomic context in other Gram-negative bacteria with kynurenine pathway genes (Fig. 1). To begin to characterize the role of KynR in the expression of the kynurenine pathway genes, we first determined that *P. aeruginosa* required *kynA*, *kynB*, and *kynU* to grow on tryptophan as a sole carbon source (Fig. 2). This phenotype provided a testable method to determine if KynR was important for expression of the kynurenine pathway. When the *kynR* mutant was grown in TSCM, it was unable to utilize tryptophan as a sole carbon source, thereby indicating that KynR was involved in tryptophan degradation and leading us to explore its role as the regulator of the kynurenine pathway genes.

Due to the similar phenotypes of the kynurenine pathway mutants and *kynR* mutants when grown on tryptophan as a sole carbon source, we felt that this regulator would probably affect PQS production. We previously showed that the kynurenine pathway is the main source of anthranilate for PQS production in rich media and that *kynA* and *kynU* mutants produce no detectable PQS while *kynB* mutants produce trace amounts of PQS (11). To our surprise, the data indicated that a mutation in *kynR* caused only a partial decrease in PQS production (Fig. 3). This was probably due to the fact that we had mutated a pathway regulator as opposed to a structural gene (i.e., *kynA*,

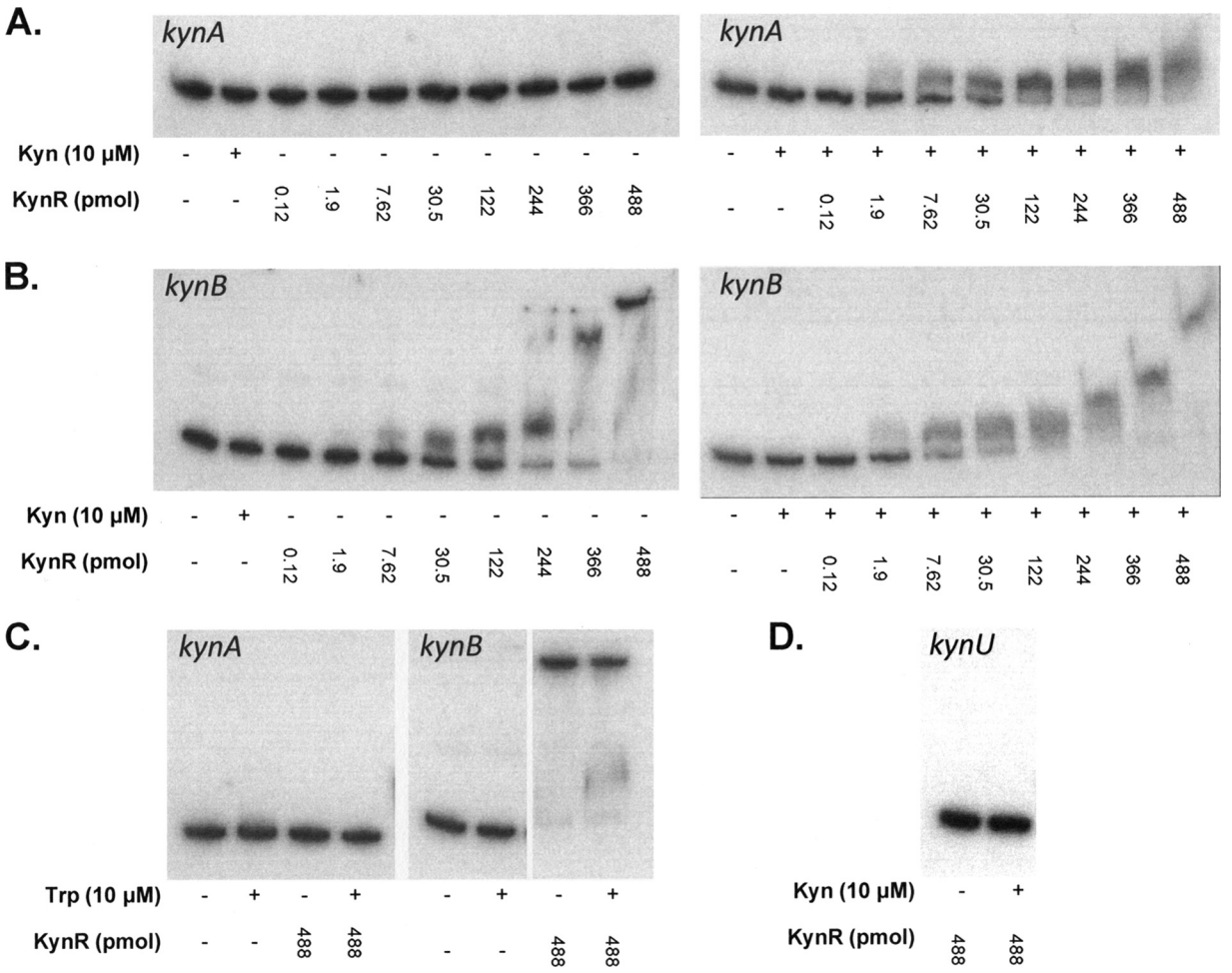

FIG. 6. KynR binds to the *kynA* and *kynB* promoter regions. (A) KynR was added to [γ-^{32}P]*kynA* in both the presence and absence of 10 μM kynurenine (kyn). (B) KynR was added to [γ-^{32}P]*kynB* in both the presence and absence of 10 μM kynurenine. (C) KynR was added to [γ-^{32}P]*kynA* and [γ-^{32}P]*kynB* in both the presence and absence of 10 μM tryptophan (Trp). (D) KynR was added to a [γ-^{32}P]*kynU* DNA fragment in both the presence and absence of 10 μM kynurenine. The total amounts of KynR are indicated below each lane. Total binding reaction mixtures were electrophoresed on nondenaturing 6% polyacrylamide gels. Gels were dried, and overlaid X-ray film was exposed for 2 days before being developed.

-B, or *-U*) that directly acts to break down tryptophan. We also found that the addition of kynurenine to the *kynR* mutant resulted in a wild-type level of PQS production, which means that *kynBU* was at least partly induced in the absence of KynR. This was explained by showing that *kynB* is positively regulated by LasR (Fig. 4), indicating that multiple regulators control this gene.

To determine how KynR affects the kynurenine pathway genes, we analyzed the transcriptional activity of *kynA* and *kynB* in our *kynR* mutant. Our data showed that both *kynA* and *kynB* were induced only in the presence of both KynR and kynurenine (Fig. 4), thereby implying that kynurenine was acting as a coinducer for KynR. These studies were then taken a step further when we showed that KynR and kynurenine were required and sufficient for the activation of *kynB* in *E. coli* (Fig. 5). This suggested that the regulation of *kynB* by KynR was direct and made it clear that the regulation of the kynurenine pathway in *P. aeruginosa* differs from tryptophan degradation pathways in other bacteria, in which the expression of the pyridoxal phosphate-dependent tryptophanase is posttranscriptionally regulated through attenuation (50). Taken together, the data we accumulated suggested that the kynurenine pathway is directly regulated in a positive way by KynR in a kynurenine-dependent manner.

To explore this potential direct regulation, we utilized purified KynR in DNA binding assays to study its interaction with the *kynA* and *kynB* promoters (Fig. 6). These studies showed that KynR bound to the *kynA* promoter only in the presence of kynurenine and that it would bind to the *kynB* promoter in the presence or absence of kynurenine. The addition of kynurenine did cause KynR to have a higher affinity for the *kynB* promoter, but the ability to bind *kynB* in the absence of kynurenine was interesting and not entirely unexpected. Ligand-independent binding by Lrp/AsnC-type regulators is typically observed when the regulator and target gene are divergently transcribed and adjacent to each other, as is the case with *kynB* and *kynR* (3). Such genetic organization often results in decreased transcription of either the regulator or both the

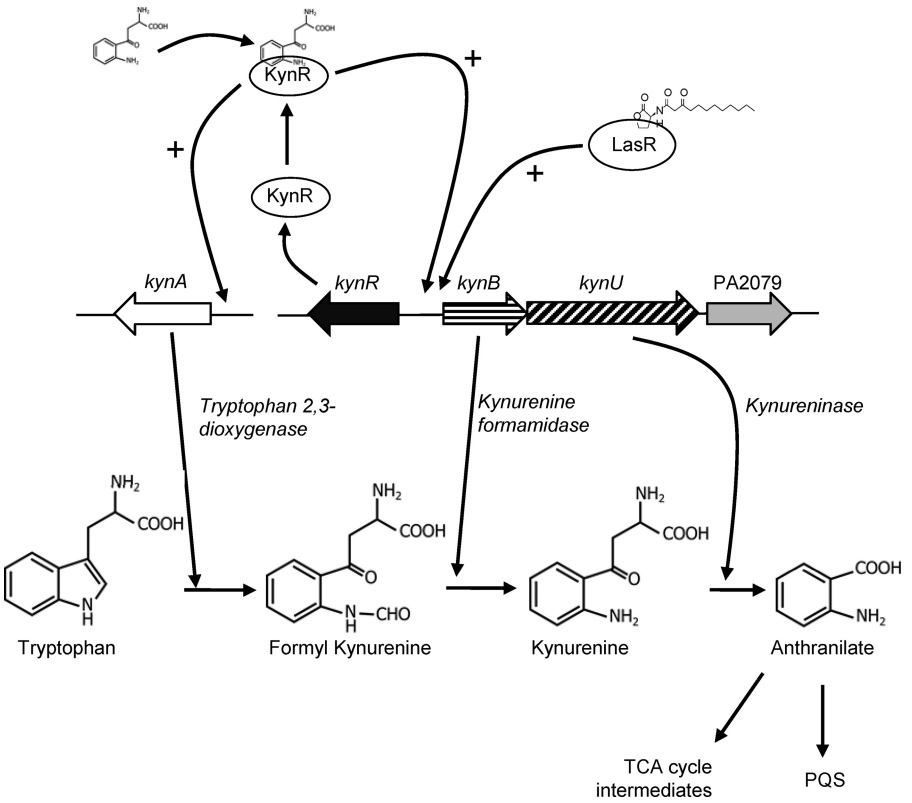

FIG. 7. Proposed model for KynR regulation and the kynurenine biosynthetic pathway. In this model, tryptophan is degraded via three separate enzymes and made into anthranilate. Anthranilate can either be used for the production of 4-quinolones, such as PQS, or is broken down into TCA cycle intermediates. KynR interacts with kynurenine to become a transcriptional activator for both *kynA* and *kynBU*.

regulator and target gene (3), but we have not demonstrated this for *kynB* or *kynR*.

Another interesting observation from the EMSA experiments was the differences that were observed between the binding complexes that KynR formed with the *kynA* and *kynB* promoter regions. The *kynA*-KynR-kynurenine complexes migrated the same regardless of the KynR concentration, while the *kynB*-KynR complexes had different migration rates with increasing concentrations of KynR (with and without kynurenine present). This type of migration pattern could be the result of KynR binding to multiple sites within the promoter region. Multiple binding sites, as well as DNA bending, have been reported in several Lrp/AsnC-type regulators in *E. coli* and other bacteria (9, 12, 20, 45, 46). In addition, Lrp/AsnC-type transcriptional regulators can bind as multimers both in the presence and absence of a ligand and are capable of acting as both activators and repressors (3, 51), so this seems to be the most likely explanation for the results seen with the *kynB* promoter binding assays.

Overall, our findings show that tryptophan degradation in *P. aeruginosa* is upregulated by a positive feedback mechanism and suggest that the pathway would be activated in tryptophan-rich environments. To help understand this, we have included a proposed model of tryptophan degradation by *P. aeruginosa* (Fig. 7). This model incorporates our previous results, which showed that the kynurenine pathway is a major source of anthranilate for PQS production in the presence of tryptophan and tryptophan-breakdown products (11). Similarly, Chugani and Greenberg (7) demonstrated that the kynurenine pathway was necessary for the expression of *catB*, a gene encoding an enzyme involved in anthranilate catabolism. These findings suggest that the kynurenine pathway could be active in amino acid-rich environments, such as in the CF lung (1, 30, 39). While one study found only 10 μM free tryptophan in CF sputum (30), it is well known that CF sputum also contains increased amounts of protein (2, 18, 29, 40). *P. aeruginosa* is well known to utilize proteases during infections within the CF lung (16, 39), and these enzymes could readily liberate tryptophan from host or bacterial proteins found in sputum. The ability of *P. aeruginosa* to catabolize tryptophan via the kynurenine pathway would provide a good source of both carbon for growth and anthranilate for the production of 4-quinolones (and many other secondary metabolites) during human infections (11). Whether the kynurenine pathway provides anthranilate for the production of PQS or for nutrient acquisition through anthranilate catabolism, this pathway provides a unique tool for *P. aeruginosa* to regulate virulence through PQS or survival through carbon and nitrogen source acquisition.

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