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Two Distinct Pathways Supply Anthranilate as a Precursor of the *Pseudomonas* Quinolone Signal[▽]

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Pseudomonas aeruginosa **is an opportunistic pathogen that causes serious infections in immunocompromised patients and those with cystic fibrosis (CF). This gram-negative bacterium uses multiple cell-to-cell signals to control numerous cellular functions and virulence. One of these signals is 2-heptyl-3-hydroxy-4-quinolone, which is referred to as the *Pseudomonas* quinolone signal (PQS). This signal functions as a coinducer for a transcriptional regulator (PqsR) to positively control multiple virulence genes and its own synthesis. PQS production is required for virulence in multiple models of infection, and it has been shown to be produced in the lungs of CF patients infected by *P. aeruginosa*. One of the precursor compounds from which PQS is synthesized is the metabolite anthranilate. This compound can be derived from the conversion of chorismate to anthranilate by an anthranilate synthase or through the degradation of tryptophan via the anthranilate branch of the kynurenine pathway. In this study, we present data which help to define the kynurenine pathway in *P. aeruginosa* and show that the kynurenine pathway serves as a critical source of anthranilate for PQS synthesis. We also show that the *kyn* pathway genes are induced during growth with tryptophan and that they are autoregulated by kynurenine. This study provides solid foundations for the understanding of how *P. aeruginosa* produces the anthranilate that serves as a precursor to PQS and other 4-quinolones.**

Pseudomonas aeruginosa is an opportunistic pathogen that can infect insects, plants, animals, and humans. This bacterium is one of the leading nosocomial pathogens in the United States and also causes chronic lung infections in most cystic fibrosis (CF) patients (21, 45, 48, 50). *P. aeruginosa* is ubiquitous in our environment and is notoriously resistant to antibiotics, making infections especially difficult to control and treat. When causing an infection, *P. aeruginosa* utilizes numerous virulence factors, many of which are controlled by cell-to-cell signaling. The *las* and *rhl* quorum-sensing systems function through the acyl-homoserine lactone intercellular signals *N*-(3-oxododecanoyl) homoserine lactone and *N*-butyryl homoserine lactone, respectively (36, 37). These signals control 6 to 11% of the *P. aeruginosa* genome (43, 47) and are required for virulence (see reference 8 for a review). *P. aeruginosa* also produces a third signal, which was identified as 2-heptyl-3-hydroxy-4-quinolone and is referred to as the *Pseudomonas* quinolone signal (PQS) (38).

PQS has been shown to control the expression of multiple virulence factors (9, 11, 31, 38), and it is produced by *P. aeruginosa* in the lungs of infected CF patients (5). In addition, quinolone signaling is required for virulence in nematodes, plants, and mice (9, 15, 16, 24, 28, 41, 52). We have shown that PQS acts as a coinducer for a LysR-type transcriptional activator called PqsR (also referred to as MvfR) (4, 46). In the presence of PQS, PqsR interacts with the promoter region of the *pqsABCDE* operon, which is part of the PQS synthetic gene cluster (7, 16), thereby creating a positive feedback loop for PQS production (46). It has also been found that 2-heptyl-4-quinolone, which is proposed to be the direct precursor of PQS, is capable of activating PqsR but with a potency that is 100-fold lower than that of PQS (52).

Along with PQS, *P. aeruginosa* produces at least 55 other quinolone compounds, many of which were identified because of their antibiotic activities (25, 26) (Note that PQS exhibited no antibiotic activity [38].) Studies of the synthesis of these compounds have shown that 4-quinolones are derived via the condensation of anthranilate and a fatty acid (6, 42). More specifically, we showed indirectly that anthranilate is a precursor for PQS and that the addition of an anthranilate analog to a *P. aeruginosa* culture would disrupt PQS production (3). In addition, Bredenbruch et al. (2) clearly showed that carbon 4 of PQS derives from anthranilic acid and carbon 2 derives from acetate, thereby proving that anthranilate and a fatty acid combine to produce 2-heptyl-4-quinolone, which can then be converted to PQS by a monooxygenase. With such compelling evidence to support the importance of anthranilate for PQS synthesis, it was clear that *P. aeruginosa* must have mechanisms in place to ensure that enough anthranilate is available to support both basic cellular functions and 4-quinolone synthesis. This is supported by the fact that *P. aeruginosa* appears to have multiple routes through which it can synthesize anthranilate. The genome of this organism encodes multiple proteins that are similar to an anthranilate synthase (www.pseudomonas.com), but only two of these (TrpEG and PhnAB) appear to supply anthranilate that is available for general cellular functions (13, 14). Most interestingly, one of these anthranilate synthases happens to be encoded by genes within the PQS synthetic region. Adjacent to the *pqsABCDE* operon is the *phnAB* operon, which is positively controlled by PqsR (4) and encodes the large and small subunits, respectively, of an anthranilate

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Reference or source
Strains		
E. coli		
DH5α	F′/*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* [ϕ80d*lac*Δ(*lacZ*)*M15recA1*]	51
One Shot	*ccdB* survival T1 phage-resistant cells	Invitrogen
P. aeruginosa		
PAO1	Wild type	18
PA14	Wild type	40
PJF-KA1	*kynA* deletion mutant derived from strain PAO1	This study
PJF-KB1	*kynB* deletion mutant derived from strain PAO1	This study
PJF-KU1	*kynU* deletion mutant derived from strain PAO1	This study
PJF-PHNA1	*phnA* deletion mutant derived from strain PAO1	This study
S. aureus ATCC 25923	Clinical isolate; laboratory test strain	ATCC
Plasmids		
pEXGWAp	Gateway cloning vector adapted for *P. aeruginosa*	M. Wolfgang
pΔkynAsuc	*kynA* deletion suicide vector	This study
pΔkynBsuc2	*kynB* deletion suicide vector	This study
pΔkynUsuc	*kynU* deletion suicide vector	This study
pΔphnAsuc2	*phnA* deletion suicide vector	This study
pLP170	*lacZ* transcriptional fusion vector	39
pMTP121	Minimum tiling pathway cosmid 121 which contains *kynB*; Tet^r	20
pMTP153	Minimum tiling pathway cosmid 153 which contains *kynA*; Tet^r	20
pJF01	*kynB′-lacZ* transcriptional fusion	This study
pJF03	*kynA′-lacZ* transcriptional fusion	This study
pUCP22	*E. coli*/*P. aeruginosa* cloning vector	49
pJF100	*phnAB* complementation plasmid	This study

synthase (13). In addition, the three genes (*kynA*, *kynB*, and *kynU*) of the anthranilate branch of the kynurenine (*kyn*) pathway, which converts tryptophan to anthranilate, are present in *P. aeruginosa* (see www.pseudomonas.com). The importance of this catabolic pathway for PQS synthesis has been suggested by the data of D'Argenio et al. (7). We speculate that, as a key factor in the 4-quinolone synthesis pathway, the cellular supply of anthranilate could provide a viable drug target that if disrupted would lessen the virulence of *P. aeruginosa*. With this in mind, this study begins to sort out the sources of anthranilate for PQS production and demonstrates the importance of the kynurenine pathway for PQS biosynthesis.

MATERIALS AND METHODS

Strains and culture conditions. Bacterial strains and plasmids used in this study are described in Table 1. *P. aeruginosa* strains were maintained at −70°C in 10% skim milk and were freshly plated to begin each experiment. Bacteria were cultured at 37°C in peptone tryptic soy broth (PTSB) (33) or Luria-Bertani medium (LB), as noted. For experiments in minimal medium, bacteria were cultured in low-phosphate succinate medium (LPSM) (16), which was supplemented with the indicated kynurenine pathway metabolites as indicated below. Cultures were supplemented with 200 μg/ml carbenicillin when necessary to maintain plasmids.

Generation of mutant strains. Plasmid constructions were completed using *Escherichia coli* cloning strains listed in Table 1. Mutant alleles were generated using the PCR technique of splicing by overlap extension (19). Alleles were constructed to contain in-frame deletions in the coding DNA sequence corresponding to amino acids 11 to 246 for *kynA* (85% of protein sequence), 31 to 172 for *kynB* (66% of protein sequence), 36 to 377 for *kynU* (82% of protein sequence), and 45 to 499 for *phnA* (86% of protein sequence). Oligonucleotide primers used for mutant construction are listed in Table 2 and were designed so that PCR products would contain approximately 1 kb of DNA upstream or downstream from the splice junction. Primers were synthesized with *attB1* or *attB2* sequences to allow for the use of Gateway Cloning Technology (Invitrogen), and strain PAO1 chromosomal DNA was used as a PCR template. Once constructed, alleles were transferred into pEXGWAp using either BP or LR Clonase II enzyme mix (Invitrogen). Plasmids pΔkynAsuc, pΔkynBsuc2, pΔkynUsuc, and pΔphnAsuc2 (Table 1), which carry deletion mutant alleles for *kynA*, *kynB*, *kynU*, and *phnA*, respectively, were transformed into strain PAO1 by electroporation (12). Mutants were selected as described by Hoang et al. (17) by plating transformants first on medium containing carbenicillin and then on medium containing 6% sucrose to remove the vector sequence. Colonies of potential mutants were screened by PCR using appropriate flanking primers, and mutants were further confirmed by determining the DNA sequence of the PCR products.

Feeding and complementation experiments. For experiments with radioactive tryptophan, washed cells from overnight cultures were used to inoculate 10-ml cultures of LB supplemented with 5 μCi L-[5-^{3}H]tryptophan (specific activity, 32 Ci/mmol; Amersham Biosciences) to a turbidity at 660 nm (optical density at 660 nm [OD_{660}]) of 0.05. After 24 h of growth, 300-μl samples of each culture were extracted with 900 μl acidified ethyl acetate as described elsewhere (5). One-half of the resulting organic phase was evaporated to dryness at 37°C and reconstituted in 50 μl 1:1 acidified ethyl acetate-acetonitrile. Samples were analyzed by thin-layer chromatography (TLC) and photographed under long-wave UV light as described previously (5), and the position of PQS on the TLC plate was marked. The plate was air dried and then treated with En^{3}Hance spray (Perkin-Elmer) as recommended by the manufacturer. Kodak Biomax XAR film was exposed to the TLC plate for 4 days before development.

For complementation experiments, washed cells from overnight cultures grown in PTSB were used to inoculate 10-ml cultures of PTSB or LB supplemented with 1 mM L-tryptophan (Sigma), L-kynurenine (Sigma), or anthranilic acid (Acros) to an OD_{660} of 0.05. After 24 h of growth, 300-μl samples of each culture were extracted with 900 μl acidified ethyl acetate. Samples were prepared as described above and analyzed by TLC. The data presented are from cells grown in PTSB, and there was no difference in the data when cells were grown in LB. Experiments in which bacteria were cultured in minimal medium were performed in the same manner, except that bacteria were grown for 30 h before samples were collected for ethyl acetate extraction.

Construction of *lacZ* reporter plasmids and β-galactosidase (β-Gal) assays. A *kynA′-lacZ* reporter plasmid was constructed by using PCR to amplify a 496-bp fragment of the *kynA* upstream region (−412 to +84 relative to the *kynA* translational start site). For this amplification, the oligonucleotide primers contained XhoI and XbaI restriction sites and cosmid clone pMTP153 was used as

TABLE 2. Primers used to construct *P. aeruginosa* mutant strains

Primer name	Sequence[a]
kynA upstream 1	5′-TACAAGAAAGCTGGGTGCAGGCATATCCGCTGCGGTTCCA-3′
kynA upstream 2	5′-GATGATGCGCTCCACCGTGGTCACCTGGGCCTGGGAGTGAGGGCAAGG-3′
kynA downstream 1	5′-GGCGTGATCTACCTCGACGGCAACTTCACCCCGCTTTACACCCGCTTC-3′
kynA downstream 2	5′-TACAAAAAAGCAGGCTTCGACCCCCGCTGTTTCGGCTACC-3′
kynB upstream 1	5′-TACAAGAAAGCTGGGTGCGACGCTGGATGATCAGGTTGCG-3′
kynB upstream 2	5′-CACGTCGTCGAGGACCACGCCTTCCCATTCCTGCTGGAACGGCGTGTC-3′
kynB downstream 1	5′-GACACGCCGTTCCAGCAGGAATGGGAAGGCGTGGTCCTCGACGACGTG-3′
kynB downstream 2	5′-TACAAAAAAGCAGGCTTTCGATGAACAGATCGGCGAGGGC-3′
kynU upstream 1	5′-TACAAGAAAGCTGGGTTGCAGGTGACGGAGAATGCGCAGA-3′
kynU upstream 2	5′-GAAGCGGGTGTAAAGCGGGGTGAAGTTGCCGTCGAGGTAGATCACGCC-3′
kynU downstream 1	5′-GGCGTGATCTACCTCGACGGCAACTTCACCCCGCTTTACACCCGCTTC-3′
kynU downstream 2	5′-TACAAAAAAGCAGGCTGCCAGGGTCAGCAGGTAGAACACC-3′
phnA upstream 1	5′-TACAAAAAAGCAGGCTCGCTGGTTGAAGGAGGGATCAGCC-3′
phnA upstream 2	5′-CAGCGCCTTGTTGCGGGTTTCCTCGCAGTCGAACAGCATCCGGTTGGC-3′
phnA downstream 1	5′-GCCAACCGGATGCTGTTCGACTGCGAGGAAACCCGCAACAAGGCGCTG-3′
phnA downstream 2	5′-TACAAGAAAGCTGGGTGACATGCTGCGTCTGGTGGAAGCC-3′

[a] *att* sequences are underlined.

a source of template DNA. The amplified fragment was digested with XhoI and XbaI, purified from an agarose gel, and ligated into pLP170 digested with the same enzymes, to produce reporter plasmid pJF03. Similarly, a *kynB′-lacZ* reporter plasmid was constructed by using PCR to amplify a 378-bp fragment of the *kynB* upstream region (−289 to +89 relative to the *kynB* translational start site). For this amplification, the oligonucleotide primers contained XhoI and XbaI restriction sites and cosmid clone pMTP121 was used as a source of template DNA. The amplified fragment was digested, purified from an agarose gel, and ligated into pLP170 digested with the same enzymes, to produce reporter plasmid pJF01. Both gene fusions were sequenced to ensure that mutations were not introduced during DNA manipulations. Plasmids were transformed into *P. aeruginosa* strains by electroporation (12).

For experiments to test the effects of kynurenine pathway metabolites on *kynA′* and *kynB′-lacZ* expression, washed cells from overnight cultures grown in LPSM were used to inoculate 10-ml subcultures of LPSM to an OD_{660} of 0.05. Subcultures were grown for 6 hours at 37°C, and then 1-ml aliquots were transferred to tubes and supplemented with either water, 1 mM L-tryptophan, 1 mM L-kynurenine, or 1 mM anthranilic acid. Cultures were incubated in tubes at 37°C for 18 h, and then cells were collected by centrifugation, resuspended in fresh LPSM, and assayed for β-Gal activity in duplicate. Data are presented in Miller units as the mean ± the standard deviation (σ^{n-1}) of four separate experiments.

***Staphylococcus aureus* plate killing assay.** The ability of *P. aeruginosa* strains to lyse *S. aureus* was tested as described by Mashburn et al. (29). An overnight culture of *S. aureus* strain ATCC 25923 was grown in LB, diluted to an OD_{600} of 0.1 in fresh medium, and used to thoroughly swab an LB plate. Overnight cultures of *P. aeruginosa* strains grown in LB were diluted to an OD_{660} of 3.0, and 2 μl of each was spotted onto the freshly swabbed plate of *S. aureus*. The plate was incubated for 24 h at 37°C and then photographed.

RESULTS

Tryptophan provides a PQS precursor. We have previously shown that anthranilate is a precursor for PQS (3). In addition, Bredenbruch et al. (2) nicely demonstrated that acylated 4-quinolone compounds are produced through the condensation of anthranilate and a β-keto-decanoic acid. With the knowledge that anthranilate is a precursor for PQS, it made sense that the genes (*phnAB*) for the large and small subunits of an anthranilate synthase are contained within the PQS synthetic gene cluster. However, we previously reported the curious finding that some of the genes of the kynurenine pathway were required for PQS production (7). This led us to believe that the supply of anthranilate for PQS synthesis was derived from at least two cellular sources. With this in mind, we began to explore the role of the kynurenine pathway in PQS synthesis. The kynurenine pathway is well characterized in eukaryotic organisms, where it is responsible for the breakdown of tryptophan into other compounds (see reference 44). Recently, it was shown that a relatively small group of bacteria (including *P. aeruginosa*) possess genes which encode proteins that are homologous to enzymes of the kynurenine pathway (22). In *P. aeruginosa*, three of these genes encode homologs of tryptophan 2,3-dioxygenase (KynA), *N*-formyl-kynurenine formamidase (KynB), and kynureninase (KynU), which are proposed to convert tryptophan into anthranilate (23) (Fig. 1). Most interestingly, Matthijs et al. (30) also presented data which suggested that *Pseudomonas fluorescens* probably utilizes the kynurenine pathway to produce quinolobactin, a quinoline compound that acts as a siderophore. Therefore, we hypothesized that tryptophan degraded via the kynurenine pathway could serve as a precursor for PQS. To learn more about the importance of tryptophan degradation for PQS synthesis, we began our studies by growing *P. aeruginosa* strain PAO1 in the presence of radiolabeled tryptophan in rich medium. (Note that results did not differ between when LB was used as a growth medium and when PTSB was used as a growth medium.) After 24 h of growth with ring-labeled [^{3}H]tryptophan, the culture was extracted with acidified ethyl acetate and extracts were resolved by TLC to visualize PQS (Fig. 2A). The location of PQS was marked, and the TLC plate was then dried and exposed to X-ray film (Fig. 2B). The result of this experiment showed that the growth of *P. aeruginosa* in the presence of radioactive tryptophan resulted in the production of radioactive PQS (Fig. 2A and B, lanes 2). Under our solvent conditions, which have been used to purify PQS (38), tryptophan did not migrate from the origin (Fig. 2B, lane 1). However, a radioactive compound that corresponded to the exact location of PQS appeared on the autoradiograph (Fig. 2B, lane 2), indicating that PQS was radioactive and that tryptophan could serve as a precursor for PQS. To expand on these studies, we created *P. aeruginosa* isogenic, in-frame deletion mutants for *kynA*, *kynB*, and *kynU* (see Materials and Methods). Each mutant was grown in rich medium in the presence of radioactive tryptophan and analyzed as described above, in order to determine whether tryptophan conversion to PQS continued to occur. We found that PQS production was not detected in

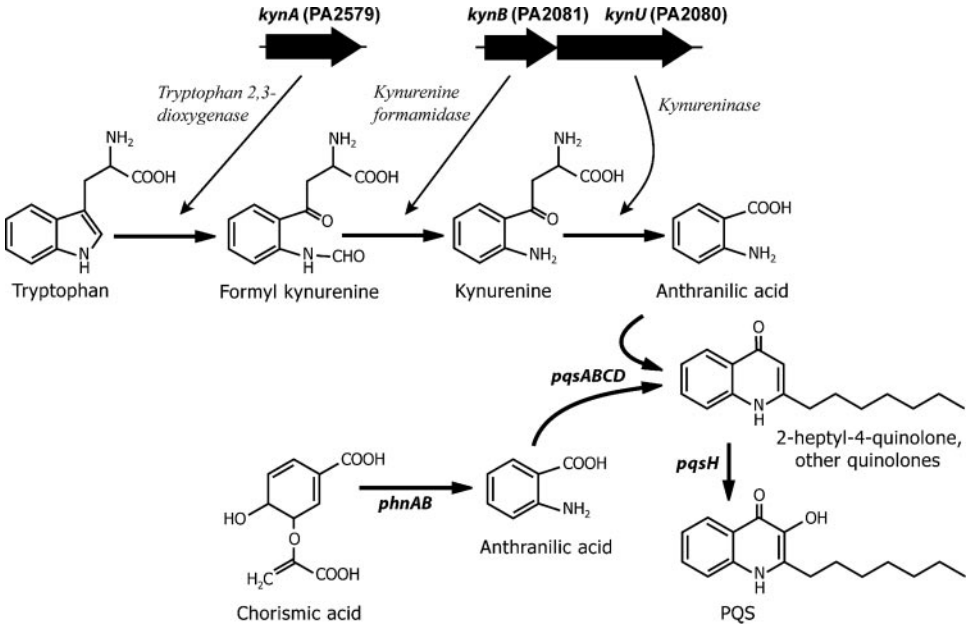

FIG. 1. Predicted model of the *P. aeruginosa* kynurenine pathway and its relationship to PQS synthesis. In this model, anthranilate for PQS synthesis is provided by either tryptophan degradation or the PhnAB anthranilate synthase depending on environmental conditions. The PA numbers indicate the position of genes in the strain PAO1 genome sequence provided at www.pseudomonas.com.

the *kynA* and *kynU* mutants and that the *kynB* mutant produced a greatly reduced amount of PQS compared to the wild-type strain PAO1 (Fig. 2A, lanes 2 to 5). No radioactive PQS was detected from any of the mutant strains (Fig. 2B). Overall, these data implied that the kynurenine pathway is an important source of anthranilate for the synthesis of PQS.

To assess the viability of the predicted kynurenine pathway shown in Fig. 1, we grew our *kyn* mutants in rich medium in the presence of either exogenous tryptophan, kynurenine, or anthranilic acid. After overnight growth, the cultures were extracted with acidified ethyl acetate and PQS was assayed by TLC. The results of this experiment showed that the *kynA* and *kynB* mutants were able to produce PQS when grown in the presence of kynurenine and anthranilate but not when supplemented with tryptophan (Fig. 3A and B). In addition, the *kynU* mutant was able to produce PQS when grown in the presence of anthranilate but not when grown with tryptophan or kynurenine (Fig. 3B). PQS production by the wild-type strain PAO1 was not affected by any of these metabolites (Fig. 3A). (Note that the second compound of the kynurenine pathway, *N*-formyl-kynurenine, is not commercially available and was not tested.) It should also be noted here that the organization of the *kynB* and *kynU* genes suggests that they form an operon (Fig. 1). Their open reading frames are separated by only three nucleotides and are on the same strand of DNA. If this is the case, we can be assured that our *kynB* mutation did not have a polar effect on *kynU*, because exogenous kynurenine restored PQS production to strain PJF-KB1 (*kynB*) (Fig. 3B). Taken together, the data presented above support the model of the *P. aeruginosa* kynurenine pathway shown in Fig. 1, where trypto-

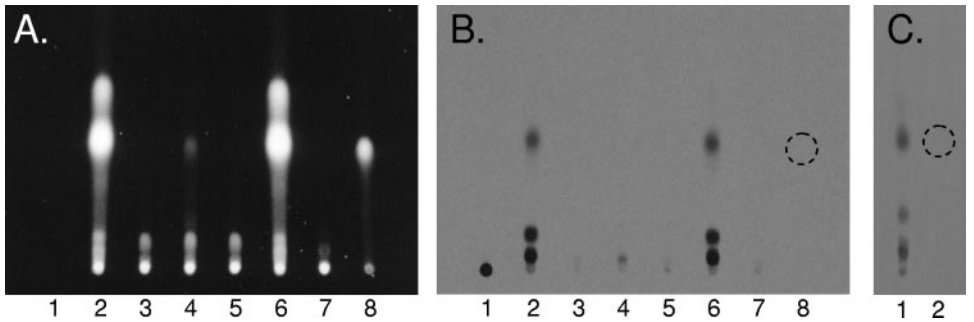

FIG. 2. *P. aeruginosa* incorporates radioactive tryptophan into PQS. *P. aeruginosa* strains were grown in rich medium (LB) in the presence of radioactive tryptophan for 24 h, and ethyl acetate extracts derived from 300 μl of culture (or uninoculated medium control) were analyzed by TLC. (A and B) Photograph (A) and autoradiograph (B) of resolved TLC plate. Lanes 1 contain 0.001 μCi L-[5-^{3}H]tryptophan, and lanes 8 contain 25 ng synthetic PQS. Lanes 2 to 6 contain culture extracts from strains PAO1, PJF-KA1, PJF-KB1, PJF-KU1, and PJF-PHNA1, respectively. Lanes 7 contain an extract of uninoculated culture medium. (C) Autoradiograph of a resolved TLC plate containing an extract from a strain PA14 culture grown in the presence of radioactive tryptophan. Lane 1 contains the strain PA14 culture extract, and lane 2 contains 25 ng of synthetic PQS. The dashed circle indicates the position of synthetic PQS as determined by alignment with the corresponding TLC plate.

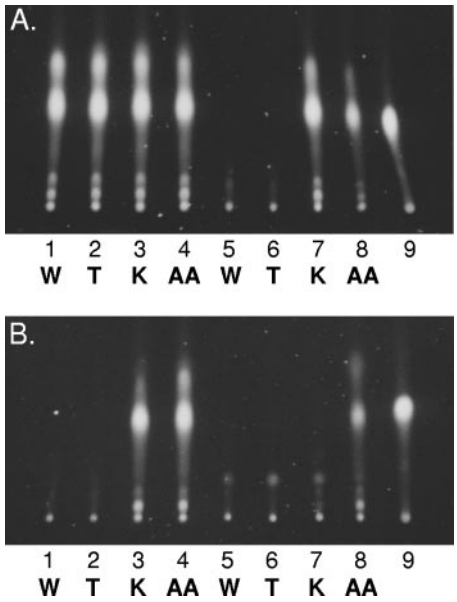

FIG. 3. Compounds from the kynurenine pathway are able to restore PQS production in *kyn* mutant strains. Ethyl acetate extracts from cultures grown in rich medium supplemented with water (W), L-tryptophan (T), L-kynurenine (K), or anthranilic acid (AA) were analyzed by TLC. Equal volumes of extracts (derived from 300 μl of culture) were resolved in each lane, and the compound added to each culture is indicated below the lane number. (A) Lanes 1 to 4 contain extracts from strain PAO1 cultures, and lanes 5 to 8 contain extracts from strain PJF-KA1 cultures. (B) Lanes 1 to 4 contain extracts from strain PJF-KB1 cultures, and lanes 5 to 8 contain extracts from strain PJF-KU1 cultures. Lane 9 of each thin-layer chromatograph contains 25 ng synthetic PQS.

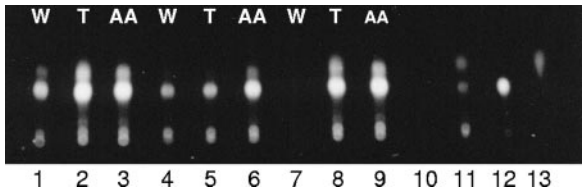

FIG. 4. The *phnAB* operon is important for PQS production in minimal medium. Ethyl acetate extracts from cultures grown in LPSM supplemented with water (W), 1 mM L-tryptophan (T), or 0.5 mM anthranilic acid (AA) were analyzed by TLC. Equal volumes of extracts were resolved in each lane, and the compound added to each culture is indicated at the top of each lane. Lanes 1 to 3 contain extracts from strain PAO1 cultures, lanes 4 to 6 contain extracts from strain PJF-KA1 cultures, and lanes 7 to 9 contain extracts from strain PJF-PHNA1 cultures. Lane 10 contains an extract from a culture of PJF-PHNA1 carrying vector plasmid pUCP22, and lane 11 contains an extract from a culture of PJF-PHNA1 carrying *phnAB* plasmid pJF100. Lane 12 contains 25 ng of synthetic PQS, and lane 13 contains 34 ng of anthranilic acid.

phan is converted to kynurenine by KynA and KynB and kynurenine is then converted to anthranilate by KynU. In addition, the fact that anthranilic acid restored PQS production to all three mutants also suggested that, in rich medium, the kynurenine pathway is the main source of anthranilate for PQS production.

The PhnAB anthranilate synthase supplies anthranilate during growth in minimal medium. The finding that the kynurenine pathway was required for PQS synthesis was interesting because previous data showed that one *phnA* mutant (strain MP710) did not produce PQS in rich medium, while others did produce PQS (16). Since *phnA* is located between the *pqsABCDE* and *pqsR* operons in the PQS synthetic gene cluster, it would seem logical that *phnA*, which encodes an anthranilate synthase (13), would supply the anthranilate needed for PQS synthesis. To try to understand our data, we constructed an isogenic, in-frame *phnA* deletion mutant in the same PAO1 strain which was used previously (16). Interestingly, this mutant, strain PJF-PHNA1, produced PQS at a level similar to that of the wild-type strain PAO1 (Fig. 2A, lane 6), and it was able to convert radiolabeled tryptophan into PQS (Fig. 2B, lane 6). We confirmed this result by using a different suicide vector to construct a second strain PAO1 *phnA* deletion mutant, which had a tetracycline resistance gene inserted within *phnA* (data not shown). This mutant also produced PQS at a level comparable to that of the wild-type strain (data not shown). We then analyzed strain MP710 by PCR and Southern blot analysis to determine the location of the tetracycline resistance gene insertion. PCR amplification of chromosomal DNA and subsequent DNA sequencing showed that the *phnA* gene of MP710 was not disrupted, and Southern blot analysis indicated that this strain most likely resulted from a spurious recombination event that inserted a mutating plasmid within either the *pqsC* or the *pqsD* gene (data not shown). This discovery was in agreement with previous mutant complementation experiments which showed that PQS production was restored to strain MP710 when it contained a plasmid harboring *pqsABCD* (16). With this problem clarified, it was obvious from the data presented above and elsewhere (7) that the kynurenine pathway, and not *phnAB*, was important for PQS synthesis during growth in rich medium. To ensure that this effect was not unique to strain PAO1, we tested the ability of strain PA14, another *P. aeruginosa* clinical isolate that is commonly used for research purposes, to convert tryptophan into PQS. The data presented in Fig. 2C show that when grown in the presence of radiolabeled tryptophan, strain PA14 produced radioactive PQS. This indicated that the importance of the kynurenine pathway for PQS production was not exclusive to strain PAO1.

We next assayed the ability of *P. aeruginosa* to produce PQS in minimal medium in the presence or absence of tryptophan or anthranilate. In minimal medium with succinate as a carbon source, strain PAO1 produced PQS and this production was increased when the medium was supplemented with tryptophan or anthranilate (Fig. 4, lanes 1 to 3). Unlike the result from rich medium, the *kynA* mutant produced PQS in unsupplemented succinate minimal medium (Fig. 4, lane 4). This production did not change when the *kynA* mutant culture was supplemented with tryptophan, but PQS synthesis increased when anthranilate was added before growth (Fig. 4, lanes 5 and 6). These data implied that, in minimal medium, anthranilate is supplied from a source other than the kynurenine pathway. This conclusion was supported by data which showed that strain PJF-PHNA1 did not make PQS in minimal medium (Fig. 4, lane 7). However, PQS production returned to strain PJF-PHNA1 when either tryptophan or anthranilate was added to the medium (Fig. 4, lanes 8 and 9). It is also important to note that PQS production by strain PJF-PHNA1 was

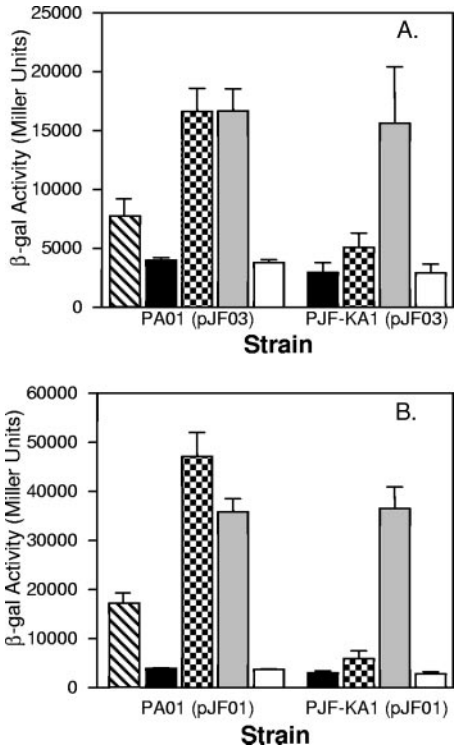

FIG. 5. The transcription of *kynA* and *kynB* is increased in the presence of tryptophan or kynurenine. β-Gal activity of a *kynA′-lacZ* fusion on pJF03 (A) or a *kynB′-lacZ* fusion on pJF01 (B) in strains PAO1 and PJF-KA1 was assayed in cultures grown in LPSM supplemented with either water (black bars), 1 mM L-tryptophan (checkered bars), 1 mM L-kynurenine (light gray bars), or 1 mM anthranilic acid (white bars) for 24 h. For reference, the striped bars represent data derived from unsupplemented cultures grown in PTSB medium. β-Gal activity is presented in Miller units as the mean $\pm \sigma^{n-1}$ of results from duplicate assays from at least three separate experiments. As a reference, parental vector plasmid pLP170 in strain PAO1 cultured in LPSM supplemented with water produced 814 ± 292 Miller units of β-Gal activity.

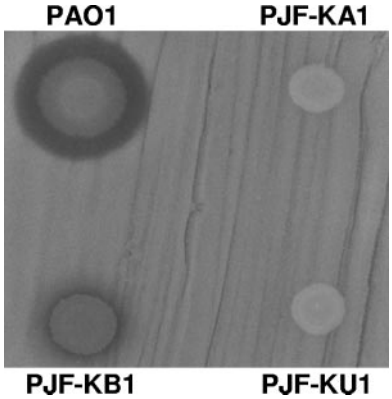

FIG. 6. Mutants in the kynurenine pathway have a reduced ability to lyse *S. aureus.* Bacteria from overnight cultures of the indicated *P. aeruginosa* strains were spotted onto a plate that had been swabbed with *S. aureus*. The plate was incubated for 24 h at 37°C and photographed. The figure presented is representative of data obtained from at least four separate experiments.

complemented by a plasmid which contained *phnAB* controlled by its natural promoter (Fig. 4, lane 11). It is not clear why the amount of PQS produced by the complemented strain was lower than that produced by the wild-type strain, but the simplest explanation is that this was due to a negative effect exerted by the high copy number of *phnAB*. Nevertheless, the data described above suggested that *phnAB* supplied anthranilate for PQS synthesis when tryptophan was not available and that the kynurenine pathway was the major supplier of anthranilate when tryptophan was present.

The expression of *kynA and kynB* is regulated by kynurenine. To further explore the role of the kynurenine pathway in PQS synthesis, we began studies of the expression of *kynA* and *kynB*. Reporter plasmids containing either a *kynA′-lacZ* or a *kynB′-lacZ* transcriptional fusion were constructed (see Materials and Methods) and transformed into the wild-type strain PAO1 or the *kynA* mutant PJF-KA1. Cultures were then grown in minimal medium in order to determine the effects of various supplemented metabolites. For comparison, the wild-type strain containing each plasmid was also grown in rich medium. The results of these experiments showed that *kynA* and *kynB* are regulated in a similar manner. Compared to their expression in rich medium, the expression of both *kynA′-lacZ* and *kynB′-lacZ* decreased in minimal medium where tryptophan and its breakdown products were absent (Fig. 5). When tryptophan or kynurenine was added to minimal medium in which the wild-type strain was grown, both the expression of *kynA* and that of *kynB* were greatly induced (Fig. 5). However, in a *kynA* mutant this large induction was not seen in the presence of tryptophan but was seen only in the presence of kynurenine (Fig. 5). This implied that the kynurenine pathway was positively autoregulated by kynurenine. In addition, supplementation of medium with anthranilate had no effect on *kynA* or *kynB* transcription (Fig. 5), indicating that the cellular pool of anthranilate does not affect the expression of kynurenine pathway genes. Taken together, the data presented in Fig. 4 and Fig. 5 indicate that the genes of the kynurenine pathway are induced in the presence of tryptophan in order to provide the anthranilate that is used in PQS synthesis.

Kynurenine pathway mutants have a reduced ability to kill *Staphylococcus aureus*. To determine whether the kynurenine pathway would affect 4-quinolone production at a level that is biologically significant, we assayed the ability of *kyn* mutants to compete with other species. *P. aeruginosa* possesses the ability to kill several gram-positive bacterial species, including *S. aureus*, and it has been shown elsewhere that this ability is dependent on genes necessary for the production of PQS and other 4-quinolone compounds (10, 29). We tested our kynurenine pathway mutants in order to determine whether they were also deficient in the ability to kill *S. aureus*. To assay this, *P. aeruginosa* strains were inoculated onto L agar plates that had been freshly swabbed with *S. aureus* and the bacteria were grown overnight. The results of this experiment showed that the *kynA* and *kynU* mutants were unable to kill *S. aureus* (Fig. 6), and the *kynB* mutant, which produced a small amount of PQS (Fig. 2), had a reduced ability to kill *S. aureus* compared to that of the wild-type strain (Fig. 6). These data agreed with those of Fig. 2 and demonstrated that, in strain PAO1, the ability to degrade tryptophan was necessary for PQS and other

4-quinolones to be synthesized at a level which produced a biological effect.

DISCUSSION

We have previously shown that anthranilate is a precursor of PQS and that an anthranilate analog would inhibit PQS production (3). This led us to propose that anthranilate and the enzymes which produce it may serve as targets for the development of novel therapeutic agents. With this in mind, we set out to investigate how *P. aeruginosa* produces the anthranilate that is condensed with a fatty acid to form the 4-quinolone compound that serves as a direct precursor of PQS and other 4-quinolone compounds. In this report, we demonstrated that the degradation of tryptophan through the kynurenine pathway is an important source of anthranilate for PQS production. Our data showed that supplementing a *P. aeruginosa* culture with radioactive tryptophan resulted in the production of radiolabeled PQS and that kynurenine pathway mutants could not produce radioactive PQS (Fig. 2). The kynurenine pathway is well characterized in mammals, but little is known about the aerobic degradation of tryptophan in bacteria (see reference 23). Studies by others have shown that the *P. aeruginosa* KynB homolog and the *Pseudomonas fluorescens* KynA homolog are important for tryptophan degradation (23, 30). In *P. aeruginosa*, the kynurenine pathway is proposed to function through three enzymes (Fig. 1). Tryptophan would enter the pathway and be converted to formyl-kynurenine by a tryptophan 2,3-dioxygenase (KynA), and formyl-kynurenine would then be converted to kynurenine by a kynurenine formamidase (KynB). The final conversion of kynurenine to anthranilate would be catalyzed by a kynureninase (KynU). Our data indicated that *kynA* and *kynU* mutants produced no PQS and that a *kynB* mutant produced a greatly reduced amount of PQS (Fig. 2). These results are similar to those reported by D'Argenio et al. (7), who showed that *kynA* and *kynB* were important for PQS production in an autolytic *P. aeruginosa* double mutant that overproduced PQS. We also presented data which imply that, as expected, KynU is responsible for the conversion of kynurenine to anthranilate in *P. aeruginosa* (Fig. 3). In addition, our data suggested that KynA and KynB were responsible for converting tryptophan to kynurenine in *P. aeruginosa* (Fig. 3), which agrees with the report by Kurnasov et al. (23), who showed that *kynB* from *P. aeruginosa* encodes a functional kynurenine formamidase. Overall, the data presented here indicated that the kynurenine pathway is a major source of the anthranilate used to synthesize PQS.

Previously it was assumed that the anthranilate synthase encoded by *phnAB* provided the majority of anthranilate for PQS synthesis due to the fact that the *phnAB* operon is adjacent to, and coregulated with, the genes necessary for PQS biosynthesis (10). However, evidence that an alternative pathway may supply anthranilate has been implied by data from multiple publications. In the first study which identified the PhnAB anthranilate synthase, a *phnA* mutant continued to produce pyocyanin (13), even though PQS is necessary for pyocyanin production (10, 11, 16). In addition, a strain PA14 *phnA* mutant was able to lyse *Bacillus subtilis* at a level that was approximately 70% of that seen with the parental strain, while a strain PA14 *pqsA* mutant was completely attenuated in the ability to lyse *B. subtilis* (35). This suggested that, unlike the *pqsA* mutant, the *phnA* mutant was still capable of producing the 4-quinolone compounds that were responsible for lysing gram-positive bacteria (10, 27). It has also been suggested that anthranilate for PQS production could be derived from another functional anthranilate synthase (i.e., TrpEG) encoded by *P. aeruginosa*. The TrpEG anthranilate synthase has been shown to be responsible for producing the anthranilate needed for tryptophan synthesis (14). While our current data do not rule out the possibility of a role for TrpEG in PQS synthesis, it seems that it is unlikely due to the fact that *trpG* is down-regulated during stationary-phase growth, when PQS is maximally produced, and the TrpEG anthranilate synthase is inhibited by tryptophan (13). Therefore, we speculate that the results seen in these earlier publications were probably due to tryptophan degradation through the kynurenine pathway rather than the result of anthranilate synthesis by tryptophan biosynthetic enzymes.

As for the role of the PhnAB anthranilate synthase in PQS production, our data showed that a kynurenine pathway mutant continued to make PQS in minimal medium (Fig. 4), suggesting that anthranilate was being produced by an alternative pathway. However, a *phnA* mutant failed to produce PQS when grown in minimal medium, and PQS production was restored by the addition of either exogenous anthranilic acid or a plasmid-borne copy of *phnAB* (Fig. 4). These data indicated that the PhnAB anthranilate synthase was able to provide sufficient anthranilate for PQS production in the absence of tryptophan or its breakdown products. It is unclear why the PhnAB anthranilate synthase was unable to support PQS production in our kynurenine pathway mutants when they were cultured in rich medium, and further studies of the regulation and activity of PhnAB are necessary to determine the specific environmental conditions which cause it to become active. Nevertheless, the presence of two independent pathways that produce anthranilate that is used by *P. aeruginosa* for the synthesis of 4-quinolones implies that anthranilate is an important metabolite for the pathogenesis of *P. aeruginosa*.

We also began preliminary studies of the regulation of the *kyn* genes in *P. aeruginosa* in order to gain a better understanding of the role of this pathway in PQS biosynthesis. Using *lacZ* reporter fusions, we found that the expression of *kynA* and *kynB* was strongly induced in the presence of tryptophan, and further investigation suggested that this induction was specifically caused by the tryptophan breakdown product L-kynurenine (Fig. 5). We also found that the expression of *kynA* and *kynB* was increased in rich medium (which contains tryptophan), compared to expression in minimal medium (Fig. 5), suggesting that the *kyn* pathway is up-regulated to provide anthranilate for PQS production when excess tryptophan is present. This finding is especially interesting in light of the fact that sputum from the lungs of CF patients has been found to be rich in amino acids and is an excellent carbon source to support the growth of *P. aeruginosa* (1, 32, 34). It is also interesting that Palmer et al. (34) found that *P. aeruginosa* strain PA14 grown in minimal medium supplemented with tryptophan produced approximately threefold-more PQS than when it was grown in minimal medium alone and approximately five times more PQS when grown with CF sputum as the sole carbon source than when grown with glucose. Our data

are in agreement with these findings and add more support to the theory that 4-quinolone production is increased in the amino acid-rich growth environment found in the CF patient lung. This leads us to speculate that the kynurenine pathway may be particularly important to support PQS production when tryptophan is readily available, as in the lungs of CF patients. This would make the kynurenine pathway especially attractive to those interested in exploring novel targets for the development of new therapeutic treatments for *P. aeruginosa* infections in CF patients.

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