

Ethanolamine Catabolism in *Pseudomonas aeruginosa* PAO1 Is Regulated by the Enhancer-Binding Protein EatR (PA4021) and the Alternative Sigma Factor RpoN

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ABSTRACT

Although genes encoding enzymes and proteins related to ethanolamine catabolism are widely distributed in the genomes of *Pseudomonas* spp., ethanolamine catabolism has received little attention among this metabolically versatile group of bacteria. In an attempt to shed light on this subject, this study focused on defining the key regulatory factors that govern the expression of the central ethanolamine catabolic pathway in *Pseudomonas aeruginosa* PAO1. This pathway is encoded by the *PA4022-eat-eutBC* operon and consists of a transport protein (Eat), an ethanolamine-ammonia lyase (EutBC), and an acetaldehyde dehydrogenase (PA4022). EutBC is an essential enzyme in ethanolamine catabolism because it hydrolyzes this amino alcohol into ammonia and acetaldehyde. The acetaldehyde intermediate is then converted into acetate in a reaction catalyzed by acetaldehyde dehydrogenase. Using a combination of growth analyses and β -galactosidase fusions, the enhancer-binding protein PA4021 and the sigma factor RpoN were shown to be positive regulators of the *PA4022-eat-eutBC* operon in *P. aeruginosa* PAO1. PA4021 and RpoN were required for growth on ethanolamine, and both of these regulatory proteins were essential for induction of the *PA4022-eat-eutBC* operon. Unexpectedly, the results indicate that acetaldehyde (and not ethanolamine) serves as the inducer molecule that is sensed by PA4021 and leads to the transcriptional activation of the *PA4022-eat-eutBC* operon. Due to its regulatory role in ethanolamine catabolism, PA4021 was given the name EatR. Both EatR and its target genes are conserved in several other *Pseudomonas* spp., suggesting that these bacteria share a mechanism for regulating ethanolamine catabolism.

IMPORTANCE

The results of this study provide a basis for understanding ethanolamine catabolism and its regulation in *Pseudomonas aeruginosa* PAO1. Interestingly, expression of the ethanolamine-catabolic genes in this bacterium was found to be under the control of a positive-feedback regulatory loop in a manner dependent on the transcriptional regulator PA4021, the sigma factor RpoN, and the metabolite acetaldehyde. Previously characterized regulators of ethanolamine catabolism are known to sense and respond directly to ethanolamine. In contrast, PA4021 (EatR) appears to monitor the intracellular levels of free acetaldehyde and responds through transcriptional activation of the ethanolamine-catabolic genes. This regulatory mechanism is unique and represents an alternative strategy used by bacteria to govern the acquisition of ethanolamine from their surroundings.

Ethanolamine serves as a source of carbon and nitrogen for a variety of bacteria, including members of the *Enterobacteriaceae*, *Pseudomonadaceae*, and *Firmicutes* (1–5). From studies mostly centered on *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Escherichia coli*, there is now a basic understanding of the catabolic steps involved in ethanolamine utilization. Extracellular ethanolamine enters the bacterial cell through simple diffusion or carrier-mediated transport (6). Upon entry, ethanolamine is cleaved into acetaldehyde and ammonia through the actions of an ethanolamine-ammonia lyase (EutBC), which uses adenosylcobalamin (AdoCbl) as a cofactor (3, 7, 8). An acetaldehyde dehydrogenase then converts acetaldehyde into acetate (9, 10). In addition to this set of core enzymes, some bacteria possess several other auxiliary proteins that aid in the catabolism of ethanolamine (11). For example, members of the *Enterobacteriaceae* often utilize an alcohol dehydrogenase (EutG) that reduces acetaldehyde to ethanol and shell proteins (EutSMNLK), which form a microcompartment to encapsulate the ethanolamine-degrading enzymes (10, 12–14).

Two types of regulatory systems have been described regarding ethanolamine catabolism. EutR is an AraC-like transcriptional

regulator that positively regulates expression of the *eut* genes in *S. Typhimurium* and *E. coli* (15–17). Induction of the *eut* genes by EutR involves ethanolamine and AdoCbl (15, 16). EutR is thought to be the regulator of ethanolamine catabolism for most *Enterobacteriaceae* (11). The other known regulatory system is found among *Firmicutes* and consists of the sensor kinase EutW and its cognate response regulator EutV (4). The presence of ethanol-

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amine leads to the autophosphorylation of EutW, which subsequently phosphorylates EutV (4). Phosphorylated EutV positively regulates transcription of the *eut* genes through an antitermination mechanism (18).

In the current study, we describe a third regulatory system for ethanolamine catabolism that was found to function in the opportunistic pathogen *Pseudomonas aeruginosa* PAO1. The *eutBC* genes are in an operon with genes encoding an acetaldehyde dehydrogenase (*PA4022*) and an ethanolamine transporter (*PA4023* or *eat*). The *PA4022-eat-eutBC* operon is preceded by a conserved $-24/-12$ promoter recognized by the alternative sigma factor σ^{54} or RpoN (19–21). Unlike sigma factors of the σ^{70} class, the RpoN RNA polymerase (RNAP) holoenzyme cannot spontaneously isomerize from a closed configuration to an open complex (19, 22). Instead, this transition requires a unique group of transcriptional regulators known as enhancer-binding proteins (EBPs) (21). EBPs interact specifically with RpoN and couple the energy of nucleotide hydrolysis to the opening of the RpoN-RNAP complex (21).

The presence of an RpoN promoter upstream of *PA4022-eat-eutBC* suggested that transcription of this operon is most likely regulated by an EBP. Accordingly, we searched for and identified the adjacent *PA4021* gene encoding the EBP that positively regulates expression of the *PA4022-eat-eutBC* genes in response to acetaldehyde, an intermediate of ethanolamine catabolism. Growth on ethanolamine and acetaldehyde-induced expression of the *PA4022-eat-eutBC* operon were dependent on both *PA4021* and RpoN. This is the first description of an acetaldehyde-responsive EBP, and based on conservation, many other *Pseudomonas* spp. are expected to regulate the catabolism of ethanolamine through a *PA4021*-RpoN mechanism.

MATERIALS AND METHODS

Bacteria and media. The *P. aeruginosa* and *Escherichia coli* strains used in this study are given in Table S1 in the supplemental material. The $\Delta PA4021$, $\Delta PA4022$, Δeat , and $\Delta eutB$ deletion mutants of *P. aeruginosa* PAO1 were constructed using established methods that have been described (23–25). Bacteria were grown in Lennox broth (LB) or minimal medium (22 mM KH_2PO_4 , 42 mM Na_2HPO_4 , 8.6 mM NaCl, 1.0 mM $MgSO_4$, 5.0 μM $FeSO_4$, and 2 mg liter⁻¹ cyanocobalamin [pH 7.0]). Minimal media were supplemented with carbon and nitrogen sources to final concentrations of 20 mM, unless otherwise stated. Solid bacteriological medium was prepared with the addition of Difco Bacto agar at 15 g liter⁻¹. For plasmid maintenance in *E. coli*, the medium was supplemented with carbenicillin (100 μg ml⁻¹), kanamycin (50 μg ml⁻¹), and/or gentamicin (20 μg ml⁻¹). For plasmid and marker selection in *P. aeruginosa*, carbenicillin (200 μg ml⁻¹) or gentamicin (30 μg ml⁻¹) was used as needed.

Plasmids and general molecular biology methods. Plasmids and primers (oligonucleotides) used in this study are given in Tables S2 and S3 in the supplemental material, respectively. Restriction endonucleases, T4 ligase, and Phusion polymerase used for cloning purposes were purchased from New England BioLabs. PCR was performed according to the recommended conditions for the Phusion polymerase. DNA was isolated using Promega nucleic acid purification kits.

Cloning of the *exaC*, *PA4021*, *PA4022*, *eat*, and *eutBC* genes. The *exaC*, *PA4021*, *PA4022*, *eat*, *eat-eutBC*, and *PA4022-eat-eutBC* genes were PCR amplified from genomic DNA of *P. aeruginosa* PAO1. The desired PCR products were gel purified, cloned into pCR-Blunt (Invitrogen), and verified through DNA sequencing (Genewiz). The *exaC*, *eat-eutBC*, and *PA4022-eat-eutBC* genes were subcloned into the XbaI/SacI sites of pBBR1MCS-5 (26) to yield the plasmids pBRL644, pBRL669, and pBRL668, respectively. The *PA4022* gene was subcloned into the KpnI/

XhoI sites of pBBR1MCS-5 to give plasmid pBRL579. The *eat* gene was subcloned into the HindIII/EcoRI sites of pBBR1MCS-5 to give plasmid pBRL581. The *PA4021* gene was subcloned into two different plasmids. In the first cloning event, *PA4021* was subcloned into the XbaI/SacI sites of $\Delta Plac$ -pBBR1MCS-5 (the pBBR1MCS-5 plasmid lacking the *Plac* promoter) (24) to give pBRL667. For the second cloning event, the *PA4021* gene was subcloned into the XbaI sites of pTrc99a with a forward orientation relative to the *trc* promoter to yield pBRL602.

Cloning of *exaC::lacZ*, *PA4022::lacZ*, *eat::lacZ*, and truncated *eat::lacZ* fusions. PCR was used to amplify the 5'-regulatory regions adjacent to the open reading frames (ORFs) for *exaC*, *PA4022*, and *eat*. The amplified 5'-regulatory regions (850 bp for *exaC*, 986 bp for *PA4022*, 2,716 bp for *eat*, and 928 bp for truncated *eat*) were then fused to the β -galactosidase (*lacZ*) ORF of *E. coli* through PCR (24, 27). The *PA4022::lacZ*, *eat::lacZ*, and truncated *eat::lacZ* fusions were subcloned into the XbaI site of $\Delta Plac$ -pBBR1MCS-5 (24) to yield the plasmids pBRL601, pBRL678, and pBRL597, respectively. The *exaC::lacZ* fusion was subcloned into the BamHI site of $\Delta Plac$ -pBBR1MCS-5 to give plasmid pBRL647. The GG dinucleotide (underlined) of -24 element of the putative RpoN promoter (TGGCCCCGGCCCTTGCT) upstream of the *PA4022* gene was changed to AA in plasmids pBRL601 and pBRL678 using the Q5 site-directed mutagenesis kit from New England BioLabs. The resulting plasmids pBRL679 and pBRL681 were sequenced to verify the presence of the desired mutations.

Synthesis of hydrazone. The synthesis of valeric acid ethylidene hydrazone, referred simply to as hydrazone, was done as described previously, with minor modifications (28, 29). Briefly, in a round-bottom flask, valeric acid hydrazide (250 mg, 2.2 mmol) was dissolved in tetrahydrofuran and cooled in an ice bath. Acetaldehyde (5.4 ml, 96.1 mmol, 44 eq) was added dropwise, and the solution was stirred for 2 h. The reaction mixture was concentrated *in vacuo*. The hydrazone was then purified by flash column chromatography and concentrated *in vacuo* to yield a mixture composed of 84 mol% hydrazone (as a 1:1 mixture of diastereomers) and 16 mol% hydrazide as a white solid (275 mg). Nuclear magnetic resonance (NMR) data for the purified hydrazone are provided in Fig. S1 in the supplemental material.

Growth on ethanolamine. Analysis was done in triplicate for each strain and condition. Strains were grown in LB for 18 h at 200 rpm at 37°C. Minimal medium supplemented with the desired carbon and nitrogen source was inoculated with 1% (vol/vol) of the LB-grown culture. The inoculated cultures were grown for 4.5 h (nitrogen source testing) or 24 h (carbon source testing) at 200 rpm and 37°C. The absorbance at 600 nm (OD_{600}) was measured for each culture. For carbon source testing, minimal media were supplemented with 20 mM NH_4Cl and 20 mM carbon source (ethanolamine, acetate, acetoin or ethanol) or 10 mM carbon source (hydrazide or hydrazone). For nitrogen source testing, minimal media were supplemented with 20 mM succinate and 20 mM nitrogen source (ethanolamine, acetamide, glycine, NH_4Cl , KNO_3^- , or urea). Growth complementation experiments were done in a similar manner, except that medium was supplemented with 30 μg ml⁻¹ gentamicin.

Mapping the *PA4022-eat-eutBC* operon. The operon arrangement of the *PA4022-eat-eutBC* genes was examined using reverse transcriptase PCR (RT-PCR) (25). *P. aeruginosa* PAO1 was grown in minimal medium supplemented with 20 mM ethanolamine and 20 mM NH_4Cl . At an OD_{600} of 0.3, 0.5 ml of culture was treated with 1.0 ml of RNAProtect bacterial reagent (Qiagen), and RNA was then purified from the treated cells using the RNeasy kit (Qiagen) (25). Prior to cDNA synthesis, the purified RNA was checked for genomic DNA contamination by PCR designed to amplify the *rplU* gene (24, 30, 31). Following this quality check, reverse transcriptase reactions were conducted using 500 ng of purified RNA and the iScript cDNA synthesis kit (Bio-Rad). The resulting cDNA served as a template in PCRs aimed to amplify ~500-bp regions between the *PA4022-eat*, *eat-eutB*, and *eutB-eutC* genes with primers ZS466.f/ZS466.r, ZS467.f/ZS467.r, and ZS468.f/ZS468.r, respectively. PCR was also conducted using the purified RNA (negative control) and isolated

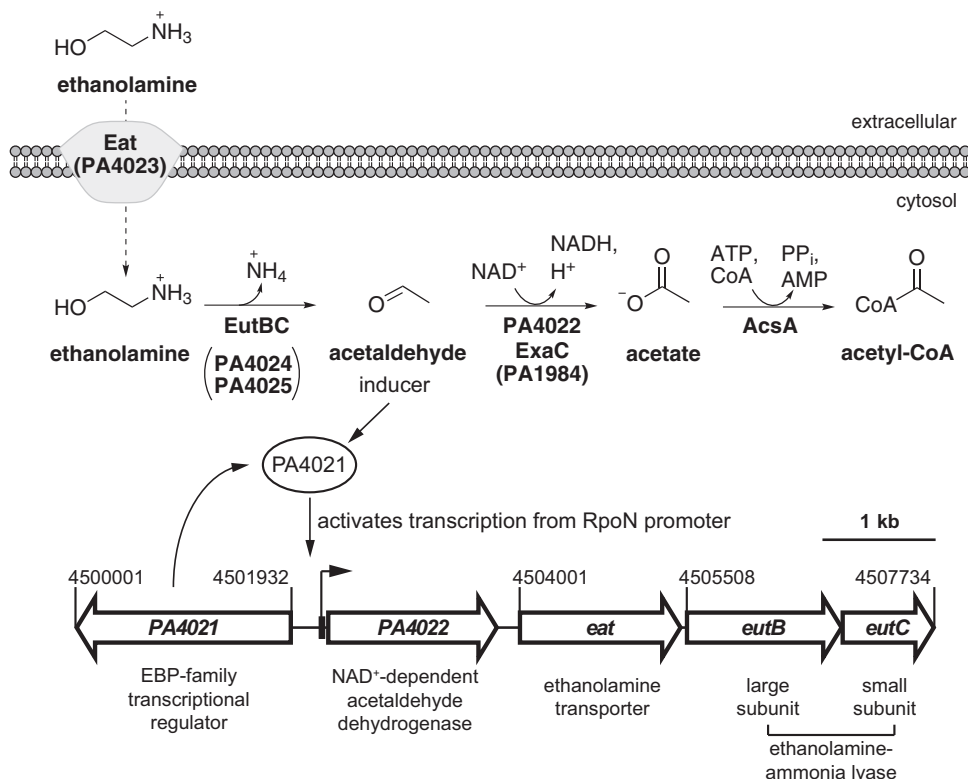


FIG 1 Proposed catabolic pathway for ethanolamine in *P. aeruginosa* PAO1. The *PA4022-eat-eutBC* operon encodes a central catabolic pathway for ethanolamine. Ethanolamine enters the bacterium through simple diffusion and/or transport mediated by Eat (PA4023). Intracellular ethanolamine is hydrolyzed into ammonia and acetaldehyde by ethanolamine-ammonia lyase, which is composed of large EutB (PA4024) and small EutC (PA4025) subunits. The acetaldehyde intermediate is converted into acetate via an NAD⁺-dependent acetaldehyde dehydrogenase, PA4022 and/or ExaC (PA1984). Acetyl coenzyme A (acetyl-CoA) synthetase (AcsA) is required for acetate utilization in *P. aeruginosa* (58) and therefore is predicted to be responsible for the activation of acetate into acetyl-CoA during ethanolamine catabolism. The results of the current study suggest that the EBP PA4021 activates transcription of the *PA4022-eat-eutBC* operon from an RpoN promoter in response to acetaldehyde; a mechanism that is essential for ethanolamine catabolism.

genomic DNA of *P. aeruginosa* PAO1 (positive control). PCRs were analyzed by agarose gel electrophoresis.

β-Galactosidase (LacZ) assays. Analysis was done in triplicate for each strain and condition. LacZ activity was measured using the Miller assay and is reported in Miller units (32). *P. aeruginosa* strains harboring *PA4022::lacZ* (pBRL601), *eat::lacZ* (pBRL678), *exaC::lacZ* (pBRL647), truncated *eat::lacZ* (pBRL597), mutated *PA4022::lacZ* (pBRL679), and mutated *eat::lacZ* (pBRL681) were grown in minimal medium supplemented with 20 mM acetate, 20 mM NH₄Cl, and 30 μg liter⁻¹ gentamicin to an OD₆₀₀ of 0.3. Substrates, which were prepared as 2.0 M solutions in dimethyl sulfoxide (DMSO), were added to a final concentration of 2.0 mM, and LacZ activity was measured at 90 min postaddition of substrate. For experiments involving *E. coli*, the Δ*lacZ* mutant strain BW25113 (33) was cotransformed with (i) *PA4022::lacZ* (pBRL601), *eat::lacZ* (pBRL678), or Δ*Plac*-pBBR1MCS-5 and (ii) plasmid-borne *PA4021* (pBRL602) or pTrc99a. The recombinant *E. coli* strains were grown at 200 rpm and 37°C in minimal medium supplemented with 40 mM glycerol, 20 mM NH₄Cl, 100 μg ml⁻¹ carbenicillin, and 20 μg ml⁻¹ gentamicin. At an OD₆₀₀ of 0.3, ethanolamine or acetaldehyde was added to a final concentration of 10 mM, and LacZ activity was measured at 2 h postaddition of substrate.

RESULTS

PA4021 gene is required for growth on ethanolamine in *P. aeruginosa* PAO1. The *PA4021* gene encodes an EBP that shares 60% homology (46% identity) with AcoR (PA4147), an EBP that regulates acetoin catabolism (34). Adjacent to *PA4021* is a gene

cluster encoding enzymes and proteins related to ethanolamine catabolism (Fig. 1). *PA4022* encodes a NAD⁺-dependent acetaldehyde dehydrogenase that has no significant homology to the EutE acetaldehyde dehydrogenases found in *Enterobacteriaceae* (29). *PA4023* encodes a homolog of the ethanolamine transporter Eat, a member of the amino acid-polyamine-organocation family (35). The large and small subunits of ethanolamine-ammonia lyase are putatively encoded by the *PA4024* (*eutB*) and *PA4025* (*eutC*) genes, respectively. The chromosomal clustering or arrangement of the *eat-eutBC* genes in *P. aeruginosa* PAO1 is similar to that of many other *Proteobacteria* (11). Located 68 bp upstream of the *PA4022* ORF is a putative -24/-12 promoter that is recognized by the sigma factor RpoN. EBPs are regulatory proteins that activate transcription from RpoN promoters. Therefore, based on proximity, *PA4021* was considered to be a potential activator of the *PA4022-eat-eutBC* genes in response to ethanolamine.

Consistent with *PA4021* having a role in ethanolamine catabolism, a Δ*PA4021* mutant of *P. aeruginosa* PAO1 did not grow on ethanolamine as either a sole source of carbon (Fig. 2A) or nitrogen (Fig. 2B). The growth deficiency of the Δ*PA4021* mutant was similar to that of the Δ*eutB* mutant (Fig. 2), indicating the essentiality of *PA4021* for ethanolamine utilization. The growth of the Δ*eat* mutant on ethanolamine was identical to that of wild-type (WT) *P. aeruginosa* PAO1. This finding is in agreement with earlier studies suggesting that carrier-mediated transport is not es-

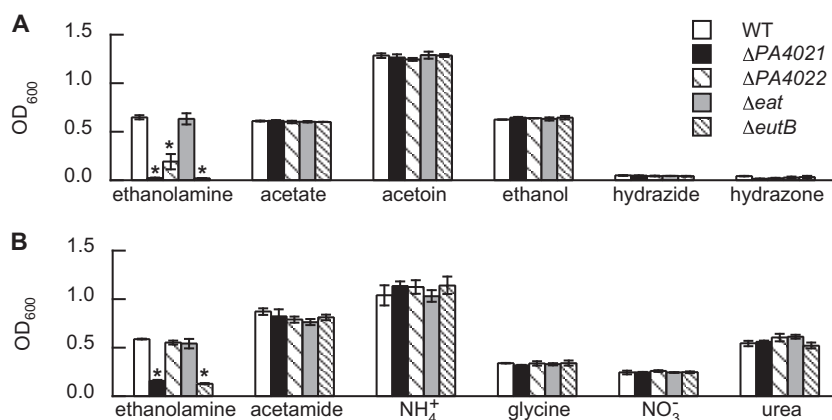


FIG 2 Growth on ethanolamine requires the *PA4021* gene in *P. aeruginosa* PAO1. Ethanolamine was provided as the sole source of either carbon (A) or nitrogen (B) for growing WT *P. aeruginosa* PAO1 and its isogenic mutants: the $\Delta PA4021$, $\Delta PA4022$, Δeat , and $\Delta eutB$ mutants. (A) As a carbon source, the $\Delta PA4021$ and $\Delta eutB$ mutants exhibited no growth on ethanolamine, whereas the $\Delta PA4022$ mutant displayed reduced growth. In comparison, the $\Delta PA4021$ and $\Delta PA4022$ mutants generated cell densities identical to that of the WT on carbon sources that intercept with ethanolamine at key catabolic intermediates, such as acetaldehyde and acetate. These results indicate that the *PA4021* and *PA4022* genes are necessary for catabolism of ethanolamine and not acetaldehyde in general. (B) As a nitrogen source in the presence of succinate, the $\Delta PA4021$ and $\Delta eutB$ mutants exhibited no growth on ethanolamine. In contrast, the growth of the $\Delta PA4022$ mutant was identical to that of the WT, indicating that *PA4022* is not required for the assimilation of ethanolamine as a nitrogen source. Strains were grown for 24 and 4.5 h on ethanolamine as a source of carbon and nitrogen, respectively. Significant differences in OD₆₀₀ values were determined using an analysis of variance (ANOVA) with Dunnett's *post hoc* test (α -value, 0.05) and are indicated with asterisks. Data points represent mean values ($n = 3$) \pm standard deviations (SD).

sential for ethanolamine utilization (6, 12). Deletion of the *PA4022* gene reduced the growth of *P. aeruginosa* PAO1 on ethanolamine as a sole source of carbon (Fig. 2A) but not nitrogen (Fig. 2B). In addition to *PA4022*, the *PA1984* (*exaC*) gene also encodes an acetaldehyde dehydrogenase that was originally identified as a possible component of the ethanol oxidation system in *P. aeruginosa* (29, 36). *PA4022* and *ExaC* are nearly identical proteins (98% identity), and both have been biochemically characterized as acetaldehyde dehydrogenases (29). This genetic redundancy would explain why *PA4022* was nonessential for growth on ethanolamine, i.e., *ExaC* was expressed or present under the conditions used. Interestingly, in addition to accepting acetaldehyde as a substrate, *PA4022* was shown to catalyze oxidative hydrolysis of the carbon-nitrogen double bond of hydrazones to generate hydrazides and acetate as products (29). Consumption of the acetate product was believed to be responsible for the growth of *P. aeruginosa* PAO1 on hydrazones (29). As shown in Fig. 2A, we did not observe growth on hydrazone for any of the *P. aeruginosa* strains used in this study. The discrepancy between our result and that of the earlier study is unclear.

Plasmid-based expression of the *PA4022-eat-eutBC* genes rescues the growth of the $\Delta PA4021$ mutant on ethanolamine. It was reasoned that the inability of the $\Delta PA4021$ mutant to grow on ethanolamine was due to insufficient expression of the *PA4022-eat-eutBC* genes. Indeed, expression of the entire *PA4022-eat-eutBC* operon from the *lac* promoter on the broad-host-range plasmid pBBR1MCS-5 rescued the growth of the $\Delta PA4021$ mutant on ethanolamine as a carbon source (Fig. 3A). Growth of the $\Delta PA4021$ mutant reached $\sim 25\%$ of WT levels when only the *eat-eutBC* genes were expressed. In contrast, plasmid-based expression of only an acetaldehyde dehydrogenase (*PA4022* or *exaC*) or the *Eat* transporter did not restore the growth of the $\Delta PA4021$ mutant on ethanolamine.

Growth on ethanolamine was measured for plasmid-carrying strains of the $\Delta PA4022$ mutant (Fig. 3B), Δeat mutant (Fig. 3C),

$\Delta eutB$ mutant (Fig. 3D), and WT (Fig. 3E). As expected, plasmid-based expression of *PA4022* or *exaC* was sufficient to genetically complement the $\Delta PA4022$ mutant (Fig. 3B). The cell densities observed for the plasmid-carrying strains of the Δeat mutant (Fig. 3D) and WT (Fig. 3E) were similar in value. Plasmid-based expression of the *PA4022-eat-eutBC* genes fully restored the growth of the $\Delta eutB$ mutant, whereas the expression of *eat-eutBC* resulted in partial recovery, at $\sim 25\%$ of the WT levels (Fig. 3D). It is worth noting that while plasmid-based expression of the *eat-eutBC* genes partially compensated for the growth deficiencies of the $\Delta PA4021$ and $\Delta eutB$ mutants, the presence of this plasmid actually reduced the overall growth of the $\Delta PA4022$ mutant, Δeat mutant, and WT strains on ethanolamine. It is feasible that overexpression of the *eat-eutBC* genes generated high levels of *EutBC* activity, which led to abnormally large amounts of acetaldehyde, resulting in toxicity and growth inhibition of the strains.

***PA4022-eat-eutBC* genes are synthesized as a single transcript in the presence of ethanolamine.** Although the *PA4022-eat-eutBC* genes are clustered on the chromosome, it was not experimentally known whether these genes formed a single transcription unit. To address this, *P. aeruginosa* PAO1 was grown on ethanolamine as a carbon source to an OD₆₀₀ of 0.3. RNA was purified and subsequently used for cDNA synthesis. PCR was performed on the synthesized cDNA using primers designed to specifically amplify regions between the *PA4022-eat*, *eat-eutB*, and *eutB-eutC* genes. Electrophoresis of the PCRs revealed the presence of only DNA fragments having the sizes of the predicted *PA4022-eat*, *eat-eutB*, and *eutB-eutC* amplicons (Fig. 4). This result indicates that the *PA4022-eat-eutBC* genes are arranged as an operon in *P. aeruginosa* PAO1.

Expression of *PA4022::lacZ* and *eat::lacZ* is induced by acetaldehyde. Plasmid-based *LacZ* fusions were constructed to assess expression of the *PA4022-eat-eutBC* locus. Additionally, because acetaldehyde is an intermediate of ethanolamine catabolism, it was considered necessary to measure the expres-

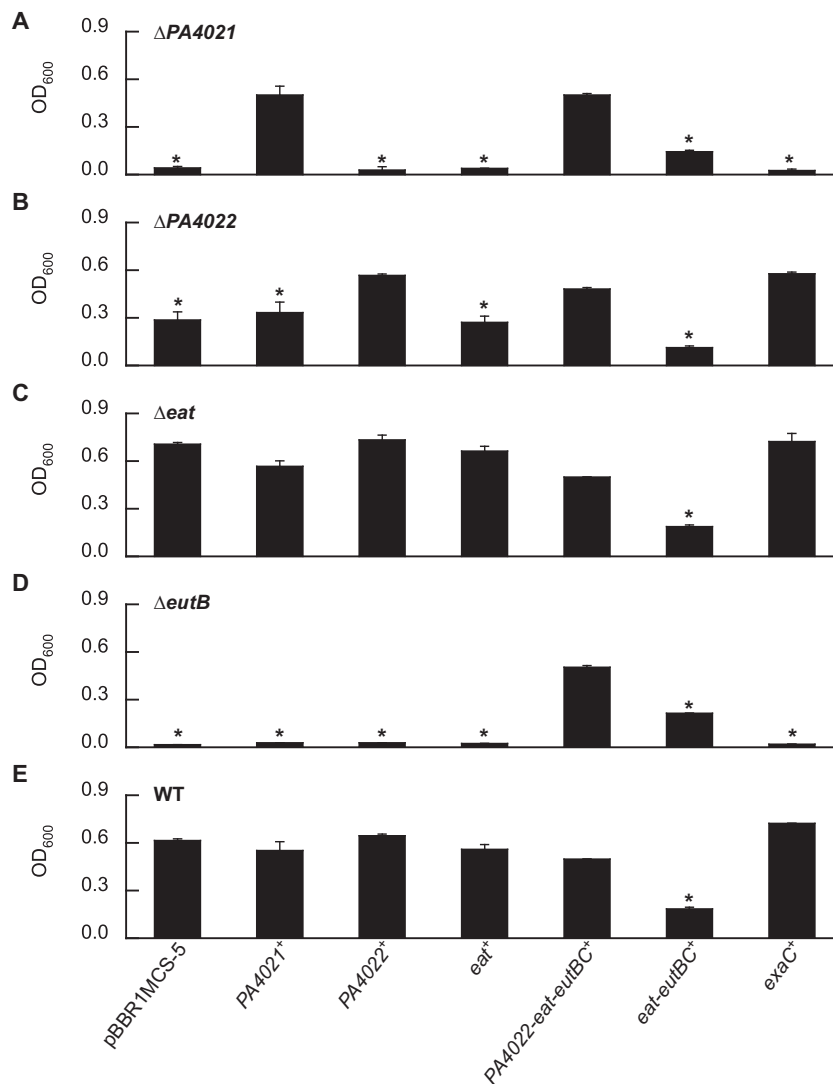


FIG 3 Expression of the *PA4022-eat-eutBC* genes from the *lac* promoter on the plasmid pBBR1MCS-5 restores the growth of the $\Delta PA4021$ mutant on ethanolamine. The $\Delta PA4021$ (A), $\Delta PA4022$ (B), Δeat (C), and $\Delta eutB$ (D) mutants, in addition to the WT (E), were transformed with derivatives of pBBR1MCS-5 that carried either *PA4021*, *PA4022*, *eat*, *PA4022-eat-eutBC*, *eat-eutBC*, or *exaC*. Genetic complementation of the mutants was then determined by growing the recombinant strains on ethanolamine as the sole carbon source for 24 h. Significant differences in OD₆₀₀ values were determined using an ANOVA with Dunnett's *post hoc* test (α -value, 0.05) and are indicated with asterisks. Data points represent mean values ($n = 3$) \pm SD.

sion of *exaC*. The *lacZ* ORF of *E. coli* was fused to the 5'-regulatory region of either *PA4022*, *eat*, or *exaC* to generate the *PA4022::lacZ*, *eat::lacZ*, and *exaC::lacZ*, respectively (Fig. 5A). For the *PA4022::lacZ* and *exaC::lacZ* fusions, the 5'-regulatory region consisted of the $\sim 1,000$ -bp sequence upstream of the *PA4022* or *exaC* ORF, respectively. For the *eat::lacZ* fusion, the 5'-regulatory region ($\sim 2,700$ bp) consisted of three key features: (i) the $\sim 1,000$ -bp 5'-regulatory region upstream of *PA4022* ORF, (ii) the *PA4022* ORF, and (iii) the 209-bp intergenic region between the *PA4022* and *eat* ORFs.

The *LacZ* fusions were initially used to identify the inducers of the *PA4022-eat-eutBC* genes in *P. aeruginosa* PAO1. *P. aeruginosa* PAO1 harboring the *PA4022::lacZ*, *eat::lacZ*, or *exaC::lacZ* was grown in acetate-minimal medium to an OD₆₀₀ of 0.3 and then challenged with an array of substrates provided at a final concentration of 2.0 mM. As anticipated, ethanolamine induced *LacZ*

activity 7-fold for *PA4022::lacZ* (Fig. 5B) and 2-fold for *eat::lacZ* (Fig. 5C). Interestingly, the addition of acetaldehyde also induced *LacZ* activity, i.e., 7-fold for *PA4022::lacZ* and 5-fold for *eat::lacZ*. The addition of acetoin and ethanol caused a 2-fold increase in the expression of *PA4022::lacZ*. Acetaldehyde is an intermediate of acetoin and ethanol catabolism (37, 38), which might account for the inducing affects these substrates have on *PA4022::lacZ*. The expression of *eat::lacZ*, however, was not significantly affected by acetoin or ethanol. In the presence of hydrazine, *LacZ* activity increased 6-fold for *PA4022::lacZ* and 4-fold for *eat::lacZ* (Fig. 5B and C). As mentioned earlier, *PA4022* was reported to be involved in the breakdown of hydrazones (29), and it was interesting to find that a hydrazine did positively regulate expression of the *PA4022* gene. Hydrazones are compounds having a basic structure of $R_1R_2C=N-NR_3R_4$ (39). The similarities in electronic properties shared between acetaldehyde and acyl hydrazones (40) might be

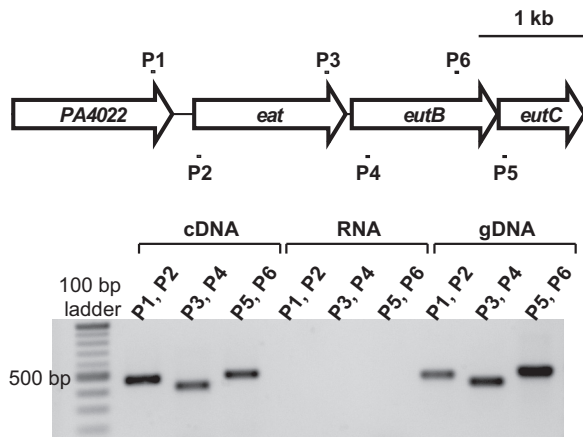


FIG 4 The *PA4022-eat-eutBC* genes are arranged in an operon in *P. aeruginosa* PAO1. RT-PCR was used to detect the *PA4022-eat-eutBC* transcript in *P. aeruginosa* PAO1 grown on ethanolamine. RNA was purified from ethanolamine-grown cells and subsequently used for cDNA synthesis. Regions (~500 bp) between *PA4022-eat*, *eat-eutB*, and *eutB-eutC* were PCR amplified from synthesized cDNA using primers P1 and P2, P3 and P4, and P5 and P6, respectively. As expected, PCR products corresponding to *PA4022-eat*, *eat-eutB*, and *eutB-eutC* were observed in reactions that used cDNA or genomic DNA (gDNA) as the template. These products were not observed from PCRs using the purified RNA as the template.

the reason why the hydrazine used in this study induced the expression of the *PA4022::lacZ* and *eat::lacZ*.

Last, expression of *exaC::lacZ* did not significantly change with the addition of any of the tested substrates (Fig. 5D). The LacZ activity of *exaC::lacZ* (~900 MU) was >3-fold higher than the non-induced (background) LacZ activity measured for *PA4022::lacZ* (~250 MU) and *eat::lacZ* (~250 MU). Little is known on the expression of *exaC* in *P. aeruginosa* PAO1 (36, 41), but these results clearly show that the expression of *exaC* is not regulated in the same acetaldehyde-dependent manner as *PA4022*.

***PA4021* is essential for induction of *PA4022::lacZ* and *eat::lacZ*.** Ethanolamine and acetaldehyde were identified as potential inducers of the *PA4022-eat-eutBC* genes. To investigate this further, expression of the *PA4022::lacZ* and *eat::lacZ* was measured in the $\Delta PA4021$, $\Delta PA4022$, Δeat , and $\Delta eutB$ mutants. As shown in Fig. 6, the addition of ethanolamine or acetaldehyde did not induce expression of *PA4022::lacZ* (Fig. 6A) or *eat::lacZ* (Fig. 6B) in the $\Delta PA4021$ mutant. This result is consistent with *PA4021* being a positive regulator of the *PA4022-eat-eutBC* genes.

In the case of the $\Delta PA4022$ mutant, ethanolamine and acetaldehyde induced the expression of *PA4022::lacZ* by 2-fold (Fig. 6A). Furthermore, the noninduced (background) expression levels of *PA4022::lacZ* were 5-fold higher in the $\Delta PA4022$ mutant than those observed in the WT. Acetaldehyde is an intermediate in both the biosynthesis and catabolism of ethanol, which is a by-product of *P. aeruginosa* PAO1 metabolism (38). An inability to catabolize or remove acetaldehyde generated from cellular metabolism might account for the elevated background expression of *PA4022::lacZ* in the $\Delta PA4022$ mutant. In comparison, the expression of *eat::lacZ* in the $\Delta PA4022$ mutant increased 2- and 5-fold with the addition of ethanolamine and acetaldehyde, respectively (Fig. 6B), but the background levels of *eat::lacZ* in the $\Delta PA4022$ mutant were similar in value to those measured in WT. Unlike *PA4022::lacZ*, the *eat::lacZ* fusion possesses an intact *PA4022* gene

(see Fig. 5). The expression of the *eat::lacZ* fusion, which is carried on a low-copy-number plasmid, could generate sufficient *PA4022* protein to counter the $\Delta PA4022$ mutation. This would explain why *eat::lacZ* behaved in the same way for both the $\Delta PA4022$ mutant and WT strains.

The fold changes observed for *PA4022::lacZ* and *eat::lacZ* in the Δeat mutant were similar in magnitude to the fold changes measured in the WT (Fig. 6). This result held true for the $\Delta eutB$ mutant as well but only regarding acetaldehyde, which induced the expression of *PA4022::lacZ* by 7-fold and *eat::lacZ* by 4-fold. In contrast, ethanolamine did not induce expression of *PA4022::lacZ* or *eat::lacZ* in the $\Delta eutB$ mutant. These results are consistent with the idea that acetaldehyde (the product of the *EutBC* reaction) is the inducer of the *PA4022-eat-eutBC* genes in *P. aeruginosa* PAO1.

***PA4022::lacZ* and *eat::lacZ* are induced by acetaldehyde in *E. coli* expressing the *PA4021* gene.** The *PA4021* gene was cloned under the *trc* promoter of the expression plasmid pTrc99a. The resulting plasmid was then introduced into the *E. coli* $\Delta lacZ$ mutant that harbored either *PA4022::lacZ* or *eat::lacZ*. The recombinant *E. coli* strains were grown in glycerol-minimal medium to an OD₆₀₀ of 0.3 and then challenged with either ethanolamine or acetaldehyde. While the addition of ethanolamine did not induce expression of either LacZ fusion, acetaldehyde caused LacZ activity to increase 8-fold for *PA4022::lacZ* and 4-fold for *eat::lacZ* (Fig. 7). *PA4021* was essential for this increase in expression, because replacement of the plasmid-borne *PA4021* with empty pTrc99a resulted in background levels of induction of *PA4022::lacZ* and *eat::lacZ*.

Growth on acetaldehyde does not require *PA4021* or *PA4022*. It was clear that expression of *PA4022::lacZ* was induced by acetaldehyde, but it was uncertain as to whether *PA4022* and its regulator *PA4021* are essential in the utilization of acetaldehyde. Due to the toxicity and volatile nature of this compound, *P. aeruginosa* strains were grown in sealed tubes completely filled with minimal medium containing 10 mM acetaldehyde and 20 mM KNO₃. After an incubation period of 48 h at 37°C, the $\Delta PA4021$ and $\Delta PA4022$ mutants were found to have cell densities identical to that of the WT in the presence of acetaldehyde (see Fig. S2 in the supplemental material). However, under these oxygen-limiting conditions, growth on ethanolamine as a carbon source was absent for the $\Delta PA4021$ mutant and reduced by ~50% for the $\Delta PA4022$ mutant. Even though these results indicate that the *PA4021* and *PA4022* genes are not needed for growth on acetaldehyde, they do reaffirm that these genes are crucial in the catabolism of ethanolamine in *P. aeruginosa* PAO1.

Growth on ethanolamine and induction of *PA4022::lacZ* and *eat::lacZ* is dependent on RpoN. The results suggested that *PA4021* was necessary for the activation of the *PA4022-eat-eutBC* genes in response to acetaldehyde. Because EBPs activate transcription from RpoN promoters, we next sought to verify that RpoN and its cognate promoter upstream of *PA4022* were key factors in the regulation of ethanolamine catabolism in *P. aeruginosa* PAO1. As shown in Fig. 8, ethanolamine did not support the growth of an *rpoN* mutant (42) unless the *PA4022-eat-eutBC* genes were expressed from the *lac* promoter on the plasmid pBBR1MCS-5 (Fig. 8A). In addition, neither ethanolamine nor acetaldehyde induced expression of *PA4022::lacZ* and *eat::lacZ* in an *rpoN* mutant (Fig. 8B).

RpoN recognizes a distinct and highly conserved -24/-12 promoter with the consensus sequence TGGCAC-N₆-TGCT (20).

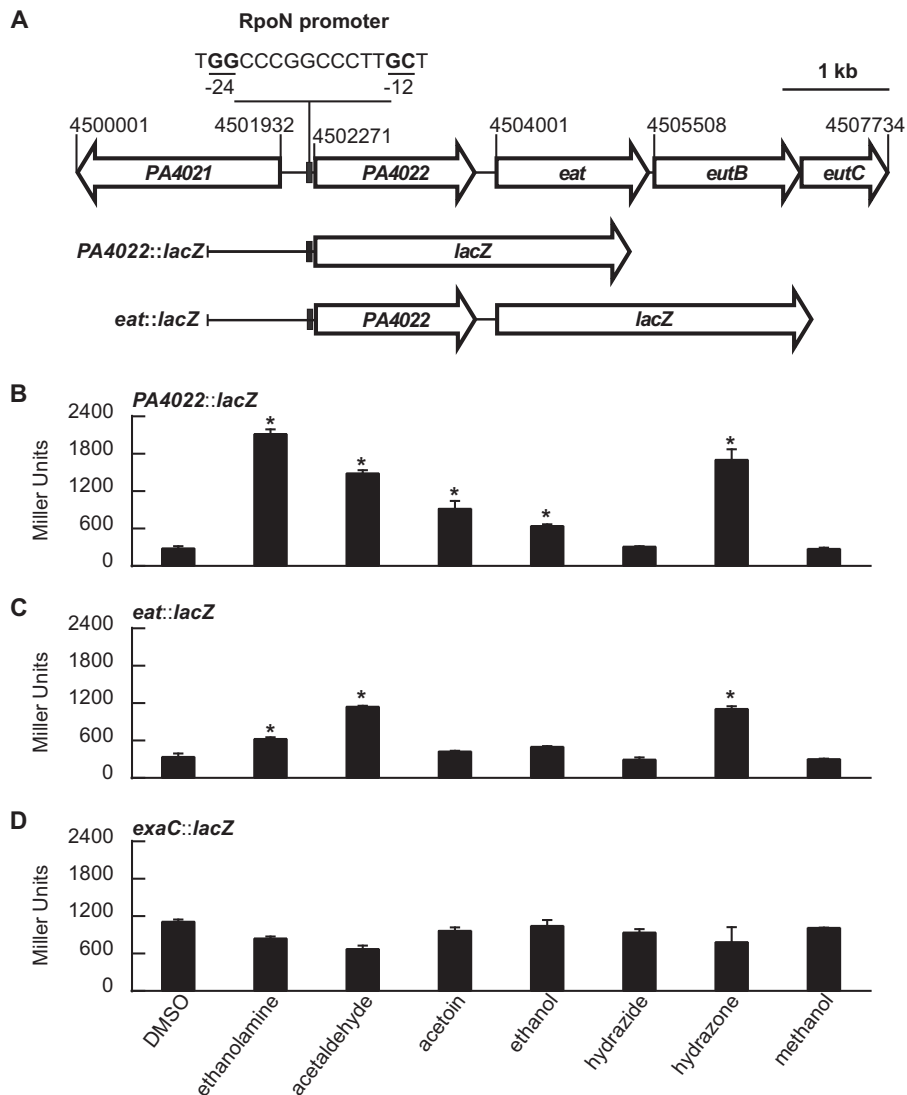


FIG 5 Acetaldehyde is an inducer of *PA4022::lacZ* and *eat::lacZ* in *P. aeruginosa* PAO1. (A) *PA4022::lacZ* and *eat::lacZ* were constructed by fusing the 5'-regulatory regions of each of these genes with *lacZ*. Fusions were individually cloned into a promoter-less plasmid Δ Plac-pBBR1MCS-5. (B) Expression of *PA4022::lacZ* increased with the addition of ethanolamine, acetaldehyde, acetoin, ethanol, or hydrazone. (C) Expression of *eat::lacZ* increased with the addition of ethanolamine, acetaldehyde or hydrazone. (D) Expression of the *exaC::lacZ* fusion did not significantly change with the addition of any substrate. *P. aeruginosa* PAO1 carrying the LacZ fusions was grown in acetate-minimal medium to an OD_{600} of 0.3 and then challenged with a 2.0 mM concentration of the indicated substrate. LacZ activities were measured 90 min postaddition of substrate. Significant differences in LacZ activities were determined using an ANOVA with Dunnett's *post hoc* test (α -value, 0.05) and are indicated with asterisks. Data points represent mean values ($n = 3$) \pm SD.

The GG and GC dinucleotides of the -24 and -12 elements (underlined), respectively, are critical for promoter activity. Positioned 68 bp upstream of the *PA4022* ORF is a putative RpoN promoter with the sequence TGGCCCGGCCCTTGCT (Sigma 54 Promoter Database [<http://www.sigma54.ca>]). To validate the importance of this promoter, the conserved GG dinucleotide of the -24 element was changed to AA in *PA4022::lacZ* and *eat::lacZ*, which afterwards were assayed for LacZ activity in *P. aeruginosa* PAO1. As shown in Fig. 9, expression of the mutated *PA4022::lacZ* and *eat::lacZ* did not increase with the addition of ethanolamine or acetaldehyde. In parallel to these experiments, a truncated version of *eat::lacZ* was constructed by fusing the \sim 1,000-bp upstream region of *eat* with the *E. coli lacZ* ORF. Notably, this truncated *eat::lacZ* fusion does not possess the 5'-regulatory region up-

stream of *PA4022* and therefore is not under the control of the RpoN promoter in question. The background expression of the truncated *eat::lacZ* fusion was similar in value to that of the full-length or untruncated *eat::lacZ* (Fig. 9). However, expression of the truncated *eat::lacZ* fusion did not change with the addition of ethanolamine or acetaldehyde. This highlights the necessity of the RpoN promoter for the induction of the *PA4022-eat-eutBC* operon in response to acetaldehyde.

DISCUSSION

Prior to this study, there was little information regarding ethanolamine catabolism in *Pseudomonas*. In fact, the only things known about this subject were that (i) the *eutBC* genes are found in the genome sequences of several *Pseudomonas* spp. and (ii) EutBC

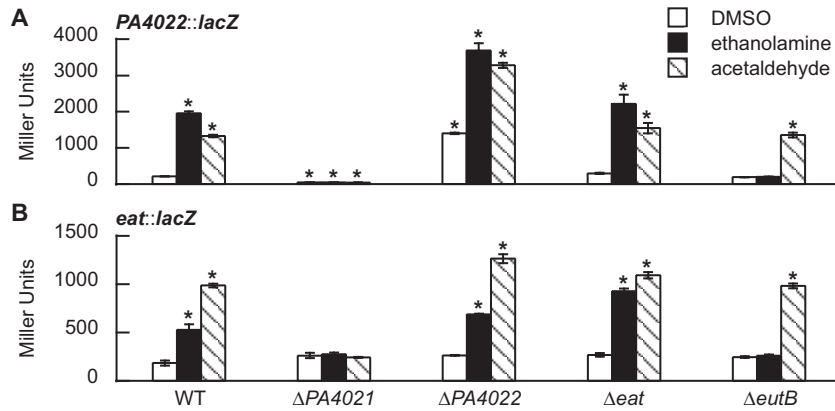


FIG 6 Induction of *PA4022::lacZ* and *eat::lacZ* are dependent on *PA4021*. Expression of *PA4022::lacZ* (A) and *eat::lacZ* (B) were measured in the $\Delta PA4021$, $\Delta PA4022$, Δeat , and $\Delta eutB$ mutants. Strains carrying the LacZ fusions were grown in acetate-minimal medium to an OD_{600} of 0.3 and then challenged with a 2.0 mM concentration of the indicated substrate. LacZ activities were measured 90 min postaddition of substrate. Significant differences in LacZ activities were determined using an ANOVA with Dunnett's *post hoc* test (α -value, 0.05) and are indicated with asterisks. Data points represent mean values ($n = 3$) \pm SD.

activity had been reported for a few species, such as *P. denitrificans* and *P. putida* (1, 11). This limited information does indicate that under certain conditions, ethanolamine might be a necessary or desirable nutrient for a variety of *Pseudomonas* species. For enteric bacteria, the human intestine has been suggested to be a rich source of ethanolamine, which is primarily available in the form of the phospholipid phosphatidylethanolamine (43–45). In addition to phosphatidylethanolamine, *Pseudomonas* spp. could potentially have access to free or non-lipid-bound ethanolamine, because plants biosynthesize ethanolamine through direct decarboxylation of serine (46). It is interesting to note that ethanolamine utilization has been connected with virulence in pathogenic strains of *S. Typhimurium* and *E. coli* (17, 44, 45, 47). This raises questions about the physiological relevance or biolog-

ical importance of ethanolamine catabolism among *Pseudomonas* species. As a first step in addressing this complex problem, this study identified and evaluated several key elements, e.g., genes and small molecules, surrounding ethanolamine catabolism in *P. aeruginosa* PAO1.

The primary focus of this study was the *PA4022-eat-eutBC*

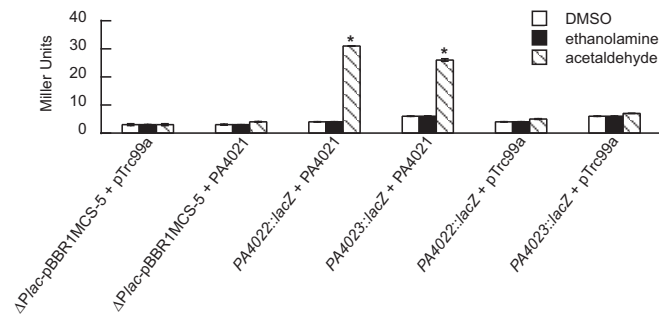


FIG 7 Heterologous expression of *PA4021* allows for acetaldehyde induction of *PA4022::lacZ* and *eat::lacZ* in *E. coli*. The *PA4021* gene was cloned into pTrc99a, and the resulting plasmid was transformed into an *E. coli* $\Delta lacZ$ mutant, which harbored *PA4022::lacZ*, *eat::lacZ*, or empty plasmid control ($\Delta Plac$ -pBBR1MCS-5). The recombinant *E. coli* strains were assayed for LacZ activity in the presence of acetaldehyde, ethanolamine, and no substrate. The addition of acetaldehyde caused a >4-fold increase in LacZ activity for the *PA4022::lacZ* and *eat::lacZ*. The addition of ethanolamine had no effect. Substitution of the plasmid-borne *PA4021* with empty plasmid pTrc99a resulted in no induction of either LacZ fusion in the presence of acetaldehyde. (Strains carrying the LacZ fusions were grown in glycerol-minimal medium to an OD_{600} of 0.3 and then challenged with a 2.0 mM concentration of the indicated substrate. LacZ activities were measured 2 h postaddition of substrate. Significant differences in LacZ activities were determined using an ANOVA with Dunnett's *post hoc* test (α -value, 0.05) and are indicated with asterisks. Data points represent mean values ($n = 3$) \pm SD.

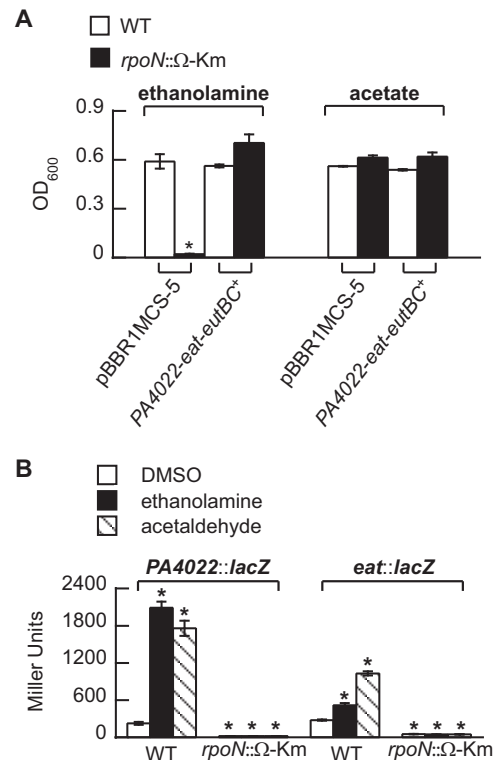


FIG 8 Ethanolamine catabolism is dependent on RpoN. (A) No growth on ethanolamine was observed for an *rpoN* mutant (*rpoN::Ω-Km*) (42). This growth deficiency was alleviated via plasmid-based expression of the *PA4022-eat-eutBC* genes. (B) There was no induction of *PA4022::lacZ* and *eat::lacZ* in the *rpoN* mutant. Significant differences in measured values were determined using an ANOVA with Dunnett's *post hoc* test (α -value, 0.05) and are indicated with asterisks. Data points represent mean values ($n = 3$) \pm SD.

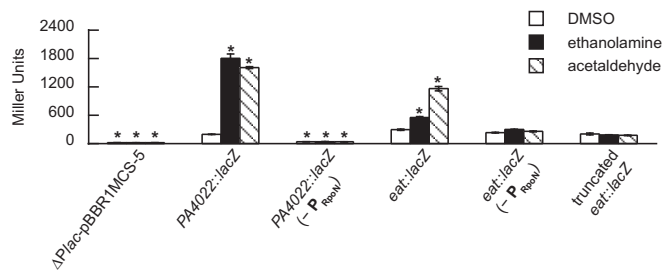


FIG 9 Induction of *PA4022::lacZ* and *eat::lacZ* is controlled by an RpoN promoter. Transcription of the *PA4022-eat-eutBC* operon in response to acetaldehyde was thought to be driven by an RpoN promoter located 68 bp upstream of *PA4022*. To address the functionality of this RpoN promoter, the conserved GG dinucleotide of the -24 element was changed to AA in both *PA4022::lacZ* and *eat::lacZ*. This mutation ($-P_{RpoN}$) resulted in no induction of either *lacZ* fusion in the presence of ethanolamine or acetaldehyde. Additionally, when the 5'-regulatory region of *eat::lacZ* was truncated so as to include only the $\sim 1,000$ -bp sequence immediately upstream of *eat* (truncated *eat::lacZ*), *LacZ* activity did not increase with the addition of ethanolamine or acetaldehyde. (*P. aeruginosa* PAO1 carrying the *LacZ* fusions was grown in acetate-minimal medium to an OD₆₀₀) of 0.3 and then challenged with a 2.0 mM concentration of the indicated substrate. *LacZ* activities were measured 90 min postaddition of substrate. Significant differences in *LacZ* activities were determined using an ANOVA with Dunnett's *post hoc* test (α -value, 0.05) and are indicated with asterisks. Data points represent mean values ($n = 3$) \pm SD.

operon, because it encodes proteins at the core of ethanolamine catabolism, including an acetaldehyde dehydrogenase (*PA4022*), the ethanolamine transporter *Eat* (*PA4023*), and ethanolamine-ammonia lyase *EutBC* (*PA4024* and *PA4025*). Taken together, these proteins comprise an entire catabolic pathway that hypothetically enables *P. aeruginosa* PAO1 to transport and degrade ethanolamine into the metabolite acetate. However, growth analysis of the $\Delta PA4022$, Δeat , and $\Delta eutB$ mutants revealed that these genes were not equally important with regard to *P. aeruginosa* PAO1 using ethanolamine as a source of carbon and/or nitrogen. *EutBC* is known as an essential enzyme in ethanolamine catabolism, and as expected, deletion of the *eutB* gene completely abolished the growth of *P. aeruginosa* PAO1 on ethanolamine. *Eat* is a predicted transport protein thought to be involved in the uptake of extracellular ethanolamine (5, 11). Deletion of the *eat* gene in *P. aeruginosa* PAO1 did not impact the growth of this bacterium on ethanolamine. Published data indicate that carrier-mediated transport has an observable effect only when ethanolamine is present at micromolar concentrations or in an acidic environment (6). Overall, the observed phenotypes for the Δeat and $\Delta eutB$ mutants of *P. aeruginosa* PAO1 are consistent with previous findings describing the roles of these genes in other ethanolamine-degrading bacteria (5, 12).

Deletion of the *PA4022* gene affected the growth of *P. aeruginosa* PAO1 differently, depending on whether ethanolamine was a carbon or nitrogen source. When ethanolamine served as the sole carbon source, deletion of the *PA4022* gene reduced the growth of *P. aeruginosa* PAO1 by $\sim 50\%$. The catabolic breakdown of acetaldehyde into acetate is expected to be hindered in the $\Delta PA4022$ mutant, which would account for its reduced growth on ethanolamine as a carbon source. In contrast, the $\Delta PA4022$ mutant displayed wild-type growth on a preferred carbon source, with ethanolamine as the sole nitrogen source. This is understandable, because *EutBC* catalyzes the hydrolysis of ethanolamine to release ammonia, thereby fulfilling the nitrogen demands of the cell.

These results suggest that *PA4022* participates in the catabolism of ethanolamine, but its presence is not absolutely essential. As stated earlier, *P. aeruginosa* PAO1 possesses two genes, *exaC* (*PA1984*) and *PA4022*, encoding acetaldehyde dehydrogenases that are 98% identical in amino acid sequence and have similar enzymatic properties (29). The genetic redundancy or dual function of the *exaC* and *PA4022* genes has been previously demonstrated for *P. aeruginosa* PAO1 when grown on ethanol, which is also catabolized through an acetaldehyde intermediate (29). Therefore, activity from *ExaC* most likely compensates for the $\Delta PA4022$ mutation regarding ethanolamine catabolism as well.

The results of this study suggest a role for *PA4022* in the catabolism of ethanolamine. However, a search of the literature revealed that *PA4022* was previously characterized as a hydrazone dehydrogenase, an enzyme that hydrolyzes the carbon-nitrogen double bond of hydrazones to yield acetate and hydrazides as products. Hydrazones are not known or considered to be abundant in nature (39). Some examples of naturally occurring hydrazones include gyromitrin (48) of the mushroom *Gyromitra esculenta* and yoropyrazone (49), an alkaloid isolated from *Streptomyces*. As it pertains to *P. aeruginosa*, growth on a relatively simple hydrazone (valeric acid ethylidene hydrazide) was dependent on *PA4022* and *ExaC* (29). It was thought that the acetate product liberated from the hydrolysis of hydrazone is a source of carbon and energy for bacterial growth. Contrary to this earlier finding, we did not observe any significant growth of *P. aeruginosa* PAO1 on hydrazone. Various types of minimal media, conditions (shaking versus static), and different strains (*P. aeruginosa* PAO1, PA14, and PAK) were used, but none proved successful for growing *P. aeruginosa* on hydrazone. Nonetheless, *PA4022* was shown to catalyze the hydrolysis of hydrazones *in vitro* (29), and we found that the expression of a *PA4022::lacZ* fusion increased 6-fold in response to hydrazone. Both results implicate *PA4022* in the degradation of hydrazones even though the growth data presented in this study question the validity of hydrazone as a viable carbon source for all strains of *P. aeruginosa*. More studies are needed to understand hydrazone utilization among various strains of *P. aeruginosa*.

Analysis of the *PA4022::lacZ* and *eat::lacZ* fusions revealed that expression of the *PA4022-eat-eutBC* operon is under the control of positive-feedback regulation exerted through the EBP *PA4021*, the sigma factor RpoN, and the metabolite acetaldehyde. The identification of acetaldehyde as the inducer of the *PA4022-eat-eutBC* operon was an unanticipated result, because ethanolamine has been shown to directly regulate expression of the *eut* genes in bacteria of the *Enterobacteriaceae* and *Firmicutes* (4, 15, 17). However, several lines of evidence clearly point to acetaldehyde as being the inducer of the *PA4022-eat-eutBC* operon and not ethanolamine as expected. First, the expression of *PA4022::lacZ* and *eat::lacZ* was repeatedly found to be induced with the addition of acetaldehyde. Second, the addition of ethanolamine failed to induce the expression of *PA4022::lacZ* and *eat::lacZ* in the $\Delta eutB$ mutant, suggesting that *EutBC* is required for induction. Third, background expression of *PA4022::lacZ* was elevated 5-fold in the absence of the acetaldehyde dehydrogenase *PA4022*. Last, acetaldehyde (but not ethanolamine) induced the expression of *PA4022::lacZ* and *eat::lacZ* in *E. coli* cells that heterologously expressed the *PA4021* gene.

One possible explanation for acetaldehyde serving as the inducer of the *PA4022-eat-eutBC* operon instead of ethanolamine is

the fact that *P. aeruginosa* PAO1 does not have a microcompartment dedicated to ethanolamine catabolism. For *Enterobacteriaceae*, ethanolamine catabolism occurs within an intracellular microcompartment consisting of the shell proteins EutSMNLK (12). The microcompartment is believed to contain or capture the toxic and volatile acetaldehyde, which allows for a more efficient utilization of this compound (50, 51). Homologs of the EutSMNLK shell proteins are not encoded in the genome of *P. aeruginosa* PAO1. To counter the absence of a microcompartment and maximize its growth potential on ethanolamine, *P. aeruginosa* PAO1 uses an acetaldehyde-responsive mechanism to dictate transcription of the *PA4022-eat-eutBC* operon. The outcome of this positive-feedback regulation is the production of adequate and balanced enzymatic activity that readily degrades ethanolamine into acetate while keeping acetaldehyde levels at a minimum to avoid toxicity and loss of carbon due to volatility. The results from the genetic complementation experiments illustrate the importance of balance in the Eat-EutBC-PA4022 pathway. When only the *eat-eutBC* genes were overexpressed in *P. aeruginosa* PAO1, its growth was significantly reduced on ethanolamine. This growth inhibition was likely a direct result of toxic concentrations of intracellular acetaldehyde generated by the excess activities of Eat-EutBC.

The induction of the *PA4022-eat-eutBC* operon by acetaldehyde is achieved through an EBP-RpoN mechanism. Previously, PA4021 was one of several uncharacterized EBPs in *P. aeruginosa* PAO1 (52). However, the proximity of the *PA4021* gene to the *PA4022-eat-eutBC* operon in addition to the presence of an RpoN promoter upstream of the *PA4022* gene were strong indicators that PA4021 might be an EBP that regulates transcription of the *PA4022-eat-eutBC* operon in relation to ethanolamine catabolism. This prediction appears to be correct because (i) deletion of the *PA4021* gene eliminated the growth of *P. aeruginosa* PAO1 on ethanolamine, (ii) there was no induction of *PA4022::lacZ* or *eat::lacZ* in the Δ *PA4021* mutant, and (iii) expression of only the *PA4021* gene was essential and sufficient for induction of *PA4022::lacZ* and *eat::lacZ* in nonnative *E. coli*. Similar results were observed regarding the regulatory role of RpoN: the *rpoN* gene was required for growth on ethanolamine, there was no induction of *PA4022::lacZ* and *eat::lacZ* in an *rpoN* mutant, and mutation of the RpoN promoter in the 5'-regulatory regions of *PA4022::lacZ* and *eat::lacZ* rendered them unresponsive to induction by acetaldehyde.

Importantly, these findings provide a working model for the regulation of the *PA4022-eat-eutBC* operon in *P. aeruginosa* PAO1. In this model, PA4021 is expected to sense and bind to acetaldehyde. The binding of acetaldehyde would then stimulate PA4021 to adopt an active conformation that leads to the transcriptional activation of the *PA4022-eat-eutBC* operon in an RpoN-dependent manner. The transcript levels of *PA4022-eat-eutBC* would ultimately vary depending on the concentration of intracellular acetaldehyde. When grown in acetate-minimal medium, the LacZ activity of *PA4022::lacZ* was 5-fold higher in the Δ *PA4022* mutant than in the wild-type *P. aeruginosa* PAO1. This result not only indicates that acetaldehyde catabolism is inhibited in the Δ *PA4022* mutant, but more significantly, transcriptional activation of the *PA4022* locus by PA4021 is not strictly limited to ethanolamine catabolism but also occurs during basic cellular metabolism to aid in the breakdown of intracellular acetaldehyde. However, the *PA4021* and *PA4022* genes were not required for

growth on acetaldehyde, ethanol, or acetoin (with ethanol and acetoin being catabolized through an acetaldehyde intermediate). It would appear that even though PA4021 and its target gene *PA4022* comprise a system involved in the sensing and breakdown of acetaldehyde, this system is not required for growth on all compounds catabolized through an acetaldehyde intermediate.

The regulatory function of PA4021 is reminiscent of the transcriptional regulator AlcR of *Aspergillus nidulans* (53). In this microorganism, AlcR is a regulator of ethanol catabolism that responds directly to acetaldehyde and upregulates the transcription of genes encoding alcohol (*alca*) and aldehyde (*alda*) dehydrogenases (53, 54). Similar to the situation described herein for *P. aeruginosa* PAO1, growth on compounds that are degraded into acetaldehyde induce expression of the *alca* and *alda* genes in *Aspergillus nidulans* (55, 56). Additionally, the aldehyde intermediates generated from the catabolism of other compounds, such as D-galacturonic acid and putrescine, also induce expression of the *alca* and *alda* genes (56). It would be interesting to determine what other aldehydes are sensed by PA4021 and induce expression of the *PA4022* locus in *P. aeruginosa* PAO1.

PA4021 is a founding member of a class of EBPs in which transcriptional activity is directly controlled by acetaldehyde. Like other transcriptional regulators, PA4021 has a modular structure (663 amino acid residues) consisting of an N-terminal region possessing both GAF (residues 68 to 199) and PAS (residues 235 to 311) domains, a central RpoN-interaction domain (residues 334 to 500), and a C-terminal DNA-binding region possessing an FIS-type helix-turn-helix motif (residues 595 to 636). GAF and PAS domains serve as sensory input sites for transcriptional regulators (52) and are probably involved in relaying the presence of acetaldehyde to the transcriptional activity of PA4021. The FIS-type helix-turn-helix motif in the C-terminal region does suggest that PA4021 binds to specific DNA sequences upstream of the RpoN promoter in the 5'-regulatory region of the *PA4022-eat-eutBC* operon. A more in-depth study on the biochemical characteristics of the PA4021 protein is needed to identify both its DNA binding sequence and other genes that are regulated by this EBP.

Based on the results of the current study, we recommend PA4021 be given the name EatR to signify its regulatory function in ethanolamine catabolism in *P. aeruginosa* PAO1. Homologs of the *eatR* (*PA4021*) gene and the *PA4022-eat-eutBC* operon are clustered in the genomes for many *Pseudomonas* spp., including common strains of *P. putida*, *P. denitrificans*, and *P. fluorescens* (57). This suggests that a variety of *Pseudomonas* strains use an EatR-RpoN mechanism for regulating ethanolamine catabolism. However, regulation of ethanolamine catabolism through EatR-RpoN does not appear to be universal among *Pseudomonas* species. For example, the regulatory *eutR* gene is adjacent to the *eat-eutBC* operon in the genomes of some strains of *P. denitrificans*, *P. oleovorans*, and *P. stutzeri*, indicating that these specific strains use EutR to regulate ethanolamine catabolism. Homologs of the *PA4022-eat-eutBC* genes are distributed in the genomes of *P. syringae*, but the presence of a regulatory gene (*eutR* or *eatR*) is inconsistent or variable among strains. Hopefully, this study will create interest in the microbiology community to explore ethanolamine catabolism further in *Pseudomonas*, with the ultimate goal being a clarification of this catabolic process and its biological importance for this group of bacteria.

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REFERENCES

- Blackwell CM, Scarlett FA, Turner JM. 1976. Ethanolamine catabolism by bacteria, including *Escherichia coli*. *Biochem Soc Trans* 4:495–497. <http://dx.doi.org/10.1042/bst0040495>.
- Blackwell CM, Turner JM. 1978. Microbial metabolism of amino alcohols. Formation of coenzyme B₁₂-dependent ethanolamine ammonia-lyase and its concerted induction in *Escherichia coli*. *Biochem J* 176:751–757.
- Roof DM, Roth JR. 1988. Ethanolamine utilization in *Salmonella* Typhimurium. *J Bacteriol* 170:3855–3863.
- Del Papa MF, Perego M. 2008. Ethanolamine activates a sensor histidine kinase regulating its utilization in *Enterococcus faecalis*. *J Bacteriol* 190:7147–7156. <http://dx.doi.org/10.1128/JB.00952-08>.
- Garsin DA. 2010. Ethanolamine utilization in bacterial pathogens: roles and regulation. *Nat Rev Microbiol* 8:290–295. <http://dx.doi.org/10.1038/nrmicro2334>.
- Penrod JT, Mace CC, Roth JR. 2004. A pH-sensitive function and phenotype: evidence that EutH facilitates diffusion of uncharged ethanolamine in *Salmonella enterica*. *J Bacteriol* 186:6885–6890. <http://dx.doi.org/10.1128/JB.186.20.6885-6890.2004>.
- Chang GW, Chang JT. 1975. Evidence for the B₁₂-dependent enzyme ethanolamine deaminase in *Salmonella*. *Nature* 254:150–151. <http://dx.doi.org/10.1038/254150a0>.
- Scarlett FA, Turner JM. 1976. Microbial metabolism of amino alcohols. Ethanolamine catabolism mediated by coenzyme B₁₂-dependent ethanolamine ammonia-lyase in *Escherichia coli* and *Klebsiella aerogenes*. *J Gen Microbiol* 95:173–176.
- Jones PW, Turner JM. 1984. Interrelationships between the enzymes of ethanolamine metabolism in *Escherichia coli*. *J Gen Microbiol* 130:299–308.
- Stojiljkovic I, Bäumlner AJ, Heffron F. 1995. Ethanolamine utilization in *Salmonella* Typhimurium: nucleotide sequence, protein expression, and mutational analysis of the *chtA chtB eutE eutJ eutG eutH* gene cluster. *J Bacteriol* 177:1357–1366.
- Tsoy O, Ravcheev D, Mushegian A. 2009. Comparative genomics of ethanolamine utilization. *J Bacteriol* 191:7157–7164. <http://dx.doi.org/10.1128/JB.00838-09>.
- Kofoed E, Rappleye C, Stojiljkovic I, Roth J. 1999. The 17-gene ethanolamine (*eut*) operon of *Salmonella* Typhimurium encodes five homologues of carboxysome shell proteins. *J Bacteriol* 181:5317–5329.
- Tanaka S, Sawaya MR, Yeates TO. 2010. Structure and mechanisms of a protein-based organelle in *Escherichia coli*. *Science* 327:81–84. <http://dx.doi.org/10.1126/science.1179513>.
- Chowdhury C, Sinha S, Chun S, Yeates TO, Bobik TA. 2014. Diverse bacterial microcompartment organelles. *Microbiol Mol Biol Rev* 78:438–468. <http://dx.doi.org/10.1128/MMBR.00009-14>.
- Roof DM, Roth JR. 1992. Autogenous regulation of ethanolamine utilization by a transcriptional activator of the *eut* operon in *Salmonella* Typhimurium. *J Bacteriol* 174:6634–6643.
- Sheppard DE, Roth JR. 1994. A rationale for autoinduction of a transcriptional activator: ethanolamine ammonia-lyase (EutBC) and the operon activator (EutR) compete for adenosyl-cobalamin in *Salmonella* Typhimurium. *J Bacteriol* 176:1287–1296.
- Luzader DH, Clark DE, Gonyar LA, Kendall MM. 2013. EutR is a direct regulator of genes that contribute to metabolism and virulence in enterohemorrhagic *Escherichia coli* O157:H7. *J Bacteriol* 195:4947–4953. <http://dx.doi.org/10.1128/JB.00937-13>.
- Baker KA, Perego M. 2011. Transcription antitermination by a phosphorylated response regulator and cobalamin-dependent termination at a B₁₂ riboswitch contribute to ethanolamine utilization in *Enterococcus faecalis*. *J Bacteriol* 193:2575–2586. <http://dx.doi.org/10.1128/JB.00217-11>.
- Buck M, Cannon W. 1992. Specific binding of the transcription factor sigma-54 to promoter DNA. *Nature* 358:422–424. <http://dx.doi.org/10.1038/358422a0>.
- Barrios H, Valderrama B, Morett E. 1999. Compilation and analysis of sigma(54)-dependent promoter sequences. *Nucleic Acids Res* 27:4305–4313. <http://dx.doi.org/10.1093/nar/27.22.4305>.
- Buck M, Gallegos MT, Studholme DJ, Guo Y, Gralla JD. 2000. The bacterial enhancer-dependent sigma(54) (sigma(N)) transcription factor. *J Bacteriol* 182:4129–4136. <http://dx.doi.org/10.1128/JB.182.15.4129-4136.2000>.
- Merrick MJ. 1993. In a class of its own—the RNA polymerase sigma factor sigma 54 (sigma N). *Mol Microbiol* 10:903–909. <http://dx.doi.org/10.1111/j.1365-2958.1993.tb00961.x>.
- Choi KH, Schweizer HP. 2005. An improved method for rapid generation of unmarked *Pseudomonas aeruginosa* deletion mutants. *BMC Microbiol* 5:30. <http://dx.doi.org/10.1186/1471-2180-5-30>.
- Lundgren BR, Villegas-Peñaranda LR, Harris JR, Mottern AM, Dunn DM, Boddy CN, Nomura CT. 2014. Genetic analysis of the assimilation of C₅-dicarboxylic acids in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 196:2543–2551. <http://dx.doi.org/10.1128/JB.01615-14>.
- Sarwar Z, Lundgren BR, Grassa MT, Wang MX, Gribble M, Moffat JF, Nomura CT. 2016. GcsR, a TyrR-like enhancer-binding protein, regulates expression of the glycine cleavage system in *Pseudomonas aeruginosa* PAO1. *mSphere* 1:e00020-16. <http://dx.doi.org/10.1128/mSphere.00020-16>.
- Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, Jr, Peterson KM. 1995. Four new derivatives of the broad-host-range cloning vector pBRR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166:175–176. [http://dx.doi.org/10.1016/0378-1119\(95\)00584-1](http://dx.doi.org/10.1016/0378-1119(95)00584-1).
- Lundgren BR, Harris JR, Sarwar Z, Scheel LR, Nomura CT. 2015. The metabolism of (R)-3-hydroxybutyrate is regulated by the enhancer-binding protein PA2005 and the alternative sigma factor RpoN in *Pseudomonas aeruginosa* PAO1. *Microbiology* 161:2232–2242. <http://dx.doi.org/10.1099/mic.0.000163>.
- Itoh H, Suzuta T, Hoshino T, Takaya N. 2008. Novel dehydrogenase catalyzes oxidative hydrolysis of carbon-nitrogen double bonds for hydrazine degradation. *J Biol Chem* 283:5790–5800.
- Taniyama K, Itoh H, Takuwa A, Sasaki Y, Yajima S, Toyofuku M, Nomura N, Takaya N. 2012. Group X aldehyde dehydrogenases of *Pseudomonas aeruginosa* PAO1 degrade hydrazones. *J Bacteriol* 194:1447–1456. <http://dx.doi.org/10.1128/JB.06590-11>.
- Lundgren BR, Thornton W, Dornan MH, Villegas-Peñaranda LR, Boddy CN, Nomura CT. 2013. Gene *PA2449* is essential for glycine metabolism and pyocyanin biosynthesis in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 195:2087–2100. <http://dx.doi.org/10.1128/JB.02205-12>.
- MacEachran DP, Ye S, Bomberger JM, Hogan DA, Swiatecka-Urban A, Stanton BA, O'Toole GA. 2007. The *Pseudomonas aeruginosa* secreted protein PA2934 decreases apical membrane expression of the cystic fibrosis transmembrane conductance regulator. *Infect Immun* 75:3902–3912. <http://dx.doi.org/10.1128/IAI.00338-07>.
- Zhang X, Bremer H. 1995. Control of the *Escherichia coli* *rrnB* P1 promoter strength by ppGpp. *J Biol Chem* 270:11181–11189. <http://dx.doi.org/10.1074/jbc.270.19.11181>.
- Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* 97:6640–6645. <http://dx.doi.org/10.1073/pnas.120163297>.
- Krüger N, Steinbüchel A. 1992. Identification of *acoR*, a regulatory gene for the expression of genes essential for acetoin catabolism in *Alcaligenes eutrophus* H16. *J Bacteriol* 174:4391–4400.
- Jack DL, Paulsen IT, Saier MH. 2000. The amino acid/polyamine/organocation (APC) superfamily of transporters specific for amino acids, polyamines and organocations. *Microbiology* 146:1797–1814.
- Schober M, Görisch H. 1999. Cytochrome *c*₅₅₀ is an essential component of the quinoprotein ethanol oxidation system in *Pseudomonas aeruginosa*: cloning and sequencing of the genes encoding cytochrome *c*₅₅₀ and an adjacent acetaldehyde dehydrogenase. *Microbiology* 145:471–481.
- Huang M, Oppermann FB, Steinbüchel A. 1994. Molecular characterization of the *Pseudomonas putida* 2,3-butanediol catabolic pathway. *FEMS Microbiol Lett* 124:141–150. <http://dx.doi.org/10.1111/j.1574-6968.1994.tb02726.x>.
- Filipiak W, Sponring A, Baur MM, Filipiak A, Ager C, Wiesenhofer H, Nagl M, Troppmair J, Amann A. 2012. Molecular analysis of volatile

- metabolites released specifically by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *BMC Microbiol* 12:113. <http://dx.doi.org/10.1186/1471-2180-12-113>.
39. Blair LM, Sperry J. 2013. Natural products containing a nitrogen-nitrogen bond. *J Nat Prod* 76:794–812. <http://dx.doi.org/10.1021/np400124n>.
 40. Sugiura M, Kobayashi S. 2005. *N*-acylhydrazones as versatile electrophiles for the synthesis of nitrogen-containing compounds. *Angew Chem Int Ed Engl* 44:5176–5186. <http://dx.doi.org/10.1002/anie.200500691>.
 41. Görisch H. 2003. The ethanol oxidation system and its regulation in *Pseudomonas aeruginosa*. *Biochim Biophys Acta* 1647:98–102. [http://dx.doi.org/10.1016/S1570-9639\(03\)00066-9](http://dx.doi.org/10.1016/S1570-9639(03)00066-9).
 42. Heurlier K, Dénervaud V, Pessi G, Reimann C, Haas D. 2003. Negative control of quorum sensing by RpoN (σ 54) in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 185:2227–2235. <http://dx.doi.org/10.1128/JB.185.7.2227-2235.2003>.
 43. Krivan HC, Franklin DP, Wang W, Laux DC, Cohen PS. 1992. Phosphatidylserine found in intestinal mucus serves as a sole source of carbon and nitrogen for *Salmonellae* and *Escherichia coli*. *Infect Immun* 60:3943–3946.
 44. Thiennimitr P, Winter SE, Winter MG, Xavier MN, Tolstikov V, Huseby DL, Sterzenbach T, Tsois RM, Roth JR, Bäuml AJ. 2011. Intestinal inflammation allows *Salmonella* to use ethanolamine to compete with the microbiota. *Proc Natl Acad Sci U S A* 108:17480–17485. <http://dx.doi.org/10.1073/pnas.1107857108>.
 45. Bertin Y, Girardeau JP, Chaucheyras-Durand F, Lyan B, Pujos-Guillot E, Harel J, Martin C. 2011. Enterohaemorrhagic *Escherichia coli* gains a competitive advantage by using ethanolamine as a nitrogen source in the bovine intestinal content. *Environ Microbiol* 13:365–377. <http://dx.doi.org/10.1111/j.1462-2920.2010.02334.x>.
 46. Rontein D, Nishida I, Tashiro G, Yoshioka K, Wu WI, Voelker DR, Basset G, Hanson AD. 2001. Plants synthesize ethanolamine by direct decarboxylation of serine using a pyridoxal phosphate enzyme. *J Biol Chem* 276:35523–35529. <http://dx.doi.org/10.1074/jbc.M106038200>.
 47. Kendall MM, Gruber CC, Parker CT, Sperandio V. 2012. Ethanolamine controls expression of genes encoding components involved in interkingdom signaling and virulence in enterohemorrhagic *Escherichia coli* O157:H7. *mBio* 3(3):e00050-12. <http://dx.doi.org/10.1128/mBio.00050-12>.
 48. Michelot D, Toth B. 1991. Poisoning by *Gyromitra esculenta*—a review. *J Appl Toxicol* 11:235–243. <http://dx.doi.org/10.1002/jat.2550110403>.
 49. Abdelfattah MS, Toume K, Ishibashi M. 2012. Yoropyrazone, a new naphthopyridazone alkaloid isolated from *Streptomyces* sp. IFM 11307 and evaluation of its TRAIL resistance-overcoming activity. *J Antibiot (Tokyo)* 65:245–248.
 50. Brinsmade SR, Paldon T, Escalante-Semerena JC. 2005. Minimal functions and physiological conditions required for growth of *Salmonella enterica* on ethanolamine in the absence of the metabolosome. *J Bacteriol* 187:8039–8046. <http://dx.doi.org/10.1128/JB.187.23.8039-8046.2005>.
 51. Penrod JT, Roth JR. 2006. Conserving a volatile metabolite: a role for carboxysome-like organelles in *Salmonella enterica*. *J Bacteriol* 188:2865–2874. <http://dx.doi.org/10.1128/JB.188.8.2865-2874.2006>.
 52. Studholme DJ, Dixon R. 2003. Domain architectures of sigma54-dependent transcriptional activators. *J Bacteriol* 185:1757–1767. <http://dx.doi.org/10.1128/JB.185.6.1757-1767.2003>.
 53. Felenbok B, Flippin M, Nikolaev I. 2001. Ethanol catabolism in *Aspergillus nidulans*: a model system for studying gene regulation. *Prog Nucleic Acid Res Mol Biol* 69:149–204. [http://dx.doi.org/10.1016/S0079-6603\(01\)69047-0](http://dx.doi.org/10.1016/S0079-6603(01)69047-0).
 54. Flippin M, Mathieu M, Cirpus I, Panozzo C, Felenbok B. 2001. Regulation of the aldehyde dehydrogenase gene (*aldA*) and its role in the control of the coinducer level necessary for induction of the ethanol utilization pathway in *Aspergillus nidulans*. *J Biol Chem* 276:6950–6958. <http://dx.doi.org/10.1074/jbc.M005769200>.
 55. Flippin M, Kocalkowska J, Felenbok B. 2002. Characteristics of physiological inducers of the ethanol utilization (*alc*) pathway in *Aspergillus nidulans*. *Biochem J* 364:25–31. <http://dx.doi.org/10.1042/bj3640025>.
 56. Flippin M, Kocalkowska J, Felenbok B. 2003. Relationships between the ethanol utilization (*alc*) pathway and unrelated catabolic pathways in *Aspergillus nidulans*. *Eur J Biochem* 270:3555–3564. <http://dx.doi.org/10.1046/j.1432-1033.2003.03738.x>.
 57. Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FSL. 2016. Enhanced annotations and features for comparing thousands of *Pseudomonas* genomes in the *Pseudomonas* genome database. *Nucleic Acids Res* 44:D646–D653. <http://dx.doi.org/10.1093/nar/gkv1227>.
 58. Kretschmar U, Khodaverdi V, Adrian L. 2010. Transcriptional regulation of the acetyl-CoA synthetase gene *acsA* in *Pseudomonas aeruginosa*. *Arch Microbiol* 192:685–690. <http://dx.doi.org/10.1007/s00203-010-0593-5>.