**Pseudomonas aeruginosa** Ethanol Oxidation by AdhA in Low-Oxygen Environments

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**ABSTRACT** *Pseudomonas aeruginosa* has a broad metabolic repertoire that facilitates its coexistence with different microbes. Many microbes secrete products that *P. aeruginosa* can then catabolize, including ethanol, a common fermentation product. Here, we show that under oxygen-limiting conditions *P. aeruginosa* utilizes AdhA, an NAD-linked alcohol dehydrogenase, as a previously undescribed means for ethanol catabolism. In a rich medium containing ethanol, AdhA, but not the previously described PQQ-linked alcohol dehydrogenase, ExaA, oxidizes ethanol and leads to the accumulation of acetate in culture supernatants. AdhA-dependent acetate accumulation and the accompanying decrease in pH promote *P. aeruginosa* survival in LB-grown stationary-phase cultures. The transcription of adhA is elevated by hypoxia and under anoxic conditions, and we show that it is regulated by the Anr transcription factor. We have shown that lasR mutants, which lack an important quorum sensing regulator, have higher levels of Anr-regulated transcripts under low-oxygen conditions than their wild-type counterparts. Here, we show that a lasR mutant, when grown with ethanol, has an even larger decrease in pH than the wild type (WT) that is dependent on both anr and adhA. The large increase in AdhA activity is similar to that of a strain expressing a hyperactive Anr-D149A variant. Ethanol catabolism in *P. aeruginosa* by AdhA supports growth on ethanol as a sole carbon source and electron donor in oxygen-limited settings and in cells growing by denitrification under anoxic conditions. This is the first demonstration of a physiological role for AdhA in ethanol oxidation in *P. aeruginosa*.

**IMPORTANCE** Ethanol is a common product of microbial fermentation, and the *Pseudomonas aeruginosa* response to and utilization of ethanol are relevant to our understanding of its role in microbial communities. Here, we report that the putative alcohol dehydrogenase AdhA is responsible for ethanol catabolism and acetate accumulation under low-oxygen conditions and that it is regulated by Anr.

**KEYWORDS** AdhA, ExaA, *Pseudomonas aeruginosa*, ethanol, lasR

*Pseudomonas aeruginosa* has broad catabolic potential that contributes to its ability to thrive in diverse environments that often include other microbes. One environment in which *P. aeruginosa* metabolism has been well studied is in the mucus that accumulates during chronic infections in the lungs of individuals with cystic fibrosis. In this low-oxygen setting, *P. aeruginosa* encounters other pathogens, many of which are known to produce ethanol by fermentation (1). Ethanol has been detected in the volatiles expired from the lungs of cystic fibrosis (CF) patients infected with *P. aeruginosa* (2, 3), and several previous studies have shown that the addition of ethanol to the growth medium can alter *P. aeruginosa* metabolism, motility, and virulence factor production (4–7).

Ethanol metabolism in *P. aeruginosa* has mainly been studied with ethanol supplied as a sole carbon source under aerobic conditions, and that focus has been on the...
pyrroloquinoline quinone (PQQ)-dependent ethanol-oxidizing enzyme ExaA, which donates electrons directly to the cytochrome 550 protein, ExaB (8–11) (Fig. 1). Görisch and colleagues (8–10) described aerobic ethanol catabolism in detail, including a complex regulatory cascade initiated by ErbR/AgmR. The disruption of exaA, exaB, or genes involved in PQQ biosynthesis eliminates the ability to grow aerobically on ethanol as a sole carbon source on agar medium (11).

The *P. aeruginosa* genome encodes putative alcohol dehydrogenases other than ExaA, but the roles of these others in the oxidation of ethanol have not been clearly established. The protein encoded by *P. aeruginosa* PAO1 gene PA5427 was named AdhA based on the high structural similarity of its crystal structure to those of diverse characterized alcohol dehydrogenase enzymes that use NAD$^+$ as a cofactor (12). AdhA contains a catalytic domain (amino acids 1 to 157 and 292 to 342) with a Zn$^2+$ active site and a coenzyme-binding domain (residues 158 to 291) with a Rossmann fold motif that is characteristic of NAD$^+$ coenzyme-binding proteins (12, 13). The biological activity of this enzyme as an ethanol dehydrogenase in *P. aeruginosa*, however, was not described.

In *P. aeruginosa*, adhA transcripts are markedly elevated during hypoxic growth and under anoxic conditions in the presence of nitrate (14–17). Furthermore, adhA expression was greatly reduced upon deletion of anr, which encodes the oxygen-responsive transcription factor that controls the expression of genes involved in anaerobic and microoxic metabolism (14, 15). Eschbach and colleagues (18) examined *P. aeruginosa* AdhA for the potential to participate in the conversion of pyruvate to produce ethanol when oxygen is limiting. Although lactic acid and acetic acid were detected, no evidence for ethanol formation was found.

In this work, we show that in rich medium amended with ethanol, ethanol was oxidized with the accumulation of acetate under oxygen-limiting conditions and this was not dependent on ExaA but did require AdhA (see Fig. 1 for pathways). We also found that acetate accumulation and the concomitant reduction in pH increased survival during stationary phase. The ΔadhA mutant was also incapable of growth with ethanol as the sole carbon source and electron donor under microoxic or anoxic conditions with nitrate as an electron acceptor. We provide evidence that the transcription of adhA is regulated by Anr both in LB medium and during growth on ethanol as a sole carbon source in low-oxygen or anoxic environments. Together, these data suggest that AdhA is capable of ethanol oxidation for growth and for conversion to acetate and that it can work in conjunction with ExaAB to enable *P. aeruginosa* to catabolize ethanol in diverse environments.

RESULTS

**AdhA is responsible for accumulation of acetate from ethanol in LB.** We previously reported that *P. aeruginosa* strain PA14 grown in LB medium with 1% ethanol yields a lower final pH in stationary-phase culture supernatants than cultures grown in LB alone (4). Due to the production of ammonia from amino acid catabolism, the culture pH from early stationary-phase *P. aeruginosa* strain PA14 cultures (12 h, optical density [OD] of ~4) was 1.5 pH units higher than that in uninoculated medium (pH 8.7 versus pH 7.2 for uninoculated medium) (Fig. 2A, dashed line). When ethanol

Figure 1: Ethanol catabolism in *P. aeruginosa*. ExaA is a PQQ-linked enzyme that, with a soluble cytochrome c (ExaB), catalyzes the oxidation of ethanol to acetaldehyde. ExaC then oxidizes acetaldehyde to acetate.

**Anr** (Low O2 or anoxia with nitrate)
was present in the medium, the final culture pH was lower than when cultures were grown in LB alone (pH 8.1 versus pH 8.7) (Fig. 2A). High-pressure liquid chromatography (HPLC) analysis of P. aeruginosa wild-type (WT) culture supernatants at this time point (12 h) revealed that 36.3 mM ethanol had been consumed (Fig. 2B) and 11.1 mM acetate had accumulated in the medium (Fig. 2C). No acetate was detected in LB cultures without added ethanol, and no lactate, pyruvate, or succinate was detected in either the LB or LB plus ethanol culture supernatants by the HPLC method used (data not shown). The final ODs of cultures grown in LB plus 1% ethanol were similar for cultures grown with and without ethanol. All strains yielded a slightly higher OD at 600 nm (OD$_{600}$) value in the presence of ethanol, but the difference did not reach statistical significance for the wild type (Fig. 2D).

To determine if ethanol catabolism in LB was due to activity of the characterized ethanol dehydrogenase ExaAB, we examined ethanol catabolism in a mutant with an in-frame deletion of exaA and a validated exaA::TnM insertion mutant (5, 19), both of which were generated in the strain PA14 background. Both mutants acidified the culture medium, consumed ethanol, and accumulated acetate in cultures with ethanol present, suggesting that ExaA was not required and that another ethanol catabolic enzyme was present (Fig. 2A to C). In light of this result, we examined whether AdhA, a putative NAD$^+$-linked alcohol dehydrogenase encoded by PA14_71630 (PA5427 in strain PAO1) contributed to ethanol catabolism and acetate accumulation. We con-
constructed and analyzed an in-frame mutant lacking adhA and found that it did not have a lower pH when grown in LB plus 1% ethanol than when grown in LB alone (Fig. 2A). The ΔadhA mutant showed only a minor decrease in the ethanol concentration of the medium (4.7 mM), and no acetate was detected (Fig. 2B and C). The defect in the adhA mutant was complemented by replacement of the adhA gene at the native locus (Fig. 2A to C), while a ΔadhA ΔexaA double mutant phenocopied the ΔadhA mutant. Together, these data indicate that ethanol was required for the accumulation of acetate in P. aeruginosa cultures grown in LB and that this phenomenon was dependent on adhA, but not exaA, in P. aeruginosa strain PA14. A similar requirement for adhA for acidification of LB medium in cultures with ethanol was observed in P. aeruginosa strain PAO1, a genetically distinct laboratory strain (Fig. 2E).

Consistent with our observation that AdhA activity was responsible for the lower culture pH in LB with ethanol at 12 h (Fig. 2), culture pH in LB with ethanol at 24 h showed even greater differences between the wild type and the adhA or adhA exaA mutants (pH 6.5 for wild type and 8.7 for mutants).

**Ethanol catabolism through AdhA increases stationary-phase survival.** On the basis of our initial observations that cultures grown with ethanol remained turbid for longer in stationary phase, and in light of previous studies in *Escherichia coli* that show ethanol can promote stationary-phase survival in rich media such as LB (20), we tested the hypothesis that ethanol catabolism by AdhA promoted stationary-phase survival. In LB cultures with no ethanol, strain PA14 wild type, and ΔadhA and ΔadhA ΔexaA mutants all showed a significant decrease in viability over time (8 h, 24 h, and 48 h), with no significant differences between strains (Fig. 3, LB). In LB with 1% ethanol, the wild-type cultures showed the absence of or a reduction in viability loss compared to that of LB cultures without ethanol, indicating that ethanol was protective. The ΔadhA and ΔadhA ΔexaA mutants did not receive the same benefit from the presence of ethanol in the medium (Fig. 3, LB + EtOH). To determine if ethanol was protective in late stationary phase because of its effects on culture pH or because of other factors, the experiment also included cultures grown in LB that was buffered to pH 7. We found that maintaining a pH of 7 was sufficient to mitigate the loss of viability in late stationary phase relative to that in unbuffered medium in the wild-type and all mutant strains (Fig. 3, LB pH 7 versus LB), similarly to what has been observed for *E. coli* (21). We also performed a kinetic analysis of the contribution of AdhA to *P. aeruginosa* culture pH and growth in LB in the absence and presence of ethanol by measuring
culture density and culture pH over the course of 30 h for both the WT and the adhA deletion mutant (see Fig. S1 in the supplemental material). We observed a reduction in pH in the presence of added ethanol only in the WT beginning at ~8 h postinoculation. The decrease in the high stationary-phase OD exhibited by the WT without ethanol and the ΔadhA mutant with and without ethanol was not observed until 16 h, by which time a decrease in pH by almost 1 pH unit was observed only in the WT with ethanol (Fig. S1A and B). Analysis of the culture densities and pH values at 12 h and 26 h revealed some small level of protection by ethanol even in the ΔadhA mutant in the absence of a pH change, suggesting that the effects of ethanol are multifactorial (Fig. S1C and D). Together, these data suggest that the ability of AdhA to convert ethanol to acetate in the late stationary phase of growth even in rich media can mitigate the toxic alkaline pH condition and its effects on cells.

Anr regulates AdhA-dependent ethanol oxidation in wild-type and ΔlasR strains. We and others showed previously that adhA transcript levels are as much as 40-fold lower in a Δannr mutant than in the wild type during growth under microoxic or anoxic conditions (14, 15). Thus, we speculated that Anr was regulating adhA in dense LB cultures when oxygen levels decreased due to cell respiration. Indeed, like the ΔadhA strain, the Δanr mutant culture had a higher pH after growth in LB plus 1% ethanol after 16 h than the wild type or the ΔadhA strain (Fig. 4). We previously reported the creation of a strain in the PAO1 background in which native anr is replaced with an allele that encodes Anr-D149A, a variant that is active even in the presence of oxygen, and demonstrated that the activity is 2 to 4 times higher than that of native Anr in microoxic environments (22). For the present study, we constructed a PA14 strain in which anr was replaced with anr-D149A and saw greater acidification of LB plus 1% ethanol medium than with the otherwise isogenic wild-type PA14, indicating that increased Anr activity leads to even more ethanol catabolism and acetate accumulation in LB medium (Fig. 4A). To determine if AdhA was sufficient to restore ethanol conversion of acetate in the Δannr strain, we tested whether expression of adhA under the control of an arabinose-inducible promoter supported medium acidification in LB amended with ethanol. Indeed, this was the case. The Δannr mutant with adhA, but not with the empty vector, restored medium acidification in LB plus 1% ethanol (Fig. 4B).
LasR is a global transcriptional regulator of the acyl-homoserine lactone quorum sensing system (23), and *P. aeruginosa* isolates with loss-of-function mutations in *lasR* are commonly recovered from both the clinic and in the environment (24–27). LasR loss-of-function mutants isolated from CF airways have been shown to confer growth advantages on certain carbon and nitrogen sources (25). Previous studies have shown that under microoxic conditions, Anr activity is higher in both engineered and naturally occurring *lasR*-defective strains than in their counterparts with functional LasR (14, 24).

In the study by Hammond et al. (14), *adhA* was found to be 5-fold higher (*P* < 0.005) in *lasR* mutants than in their genetically similar counterparts with *lasR* intact, across four pairs of strains, including strains PA14 and PAO1. Thus, we tested whether ethanol-dependent acidification of the medium was greater in Δ*lasR* mutant cultures. Like the strain bearing the Anr-D149A variant, Δ*lasR* cultures showed greater acidification at 16 h than the wild type or the *lasR*-complemented strain (Fig. 4A). Deletion of either *adhA* or *anr* in the Δ*lasR* background eliminated the acidification phenotype (Fig. 4A). Together, these data strongly suggest that Anr induction of AdhA promotes ethanol oxidation to acetate, resulting in reduced medium pH, and that conditions or backgrounds that increase Anr activity will enhance the rate of ethanol conversion to acetate in LB.

**Anr-regulated AdhA can support growth of *P. aeruginosa* strains PA14 and PAO1 on ethanol as a sole carbon source under low-oxygen and anoxic conditions with nitrate.** In light of *adhA* regulation by Anr (Fig. 4A) and its ability to oxidize ethanol to acetate in LB (Fig. 2B), we sought to determine if AdhA would be required to support growth on ethanol as a sole carbon source in low-oxygen environments. The Δ*adhA* mutant was grown under microoxic conditions (a 0.2% oxygen atmosphere) in M63 liquid medium with 1% ethanol as the sole carbon source. Under these low-oxygen conditions, the Δ*adhA* strain showed a marked defect in growth that was fully complemented by reconstitution of the mutant with the native *adhA* gene (Fig. 5A). The Δ*exaA* mutant did not have a defect in microoxic growth and in fact showed a slight significant increase in growth that was complemented by the reconstitution of the *exaA* gene at the native locus. The Δ*exaA* Δ*adhA* mutant was similar in growth to the *adhA* single mutant (Fig. 5A). All strains grew equally well in M63 medium with glucose as the sole carbon source in 0.2% oxygen, with an average OD$_{600}$ of 0.71 ± 0.09 across strains. The low final OD$_{600}$ of ~0.07 in the *adhA* mutant strains was likely an inoculation remnant, since we added inoculum to an OD of 0.05 (Fig. 5A and 5B).

AdhA also supported ethanol utilization as the sole carbon source in *P. aeruginosa* when growing anoxically using nitrate respiration for energy generation (Fig. 5B).

**FIG 5** Growth of *P. aeruginosa* PA14 strains on ethanol as a sole carbon source. (A) Growth in M63 liquid medium with ethanol as the sole carbon source in 0.2% oxygen. (B) *P. aeruginosa* strains grown in M63 liquid medium with ethanol as a sole carbon source in anoxia with 50 mM KNO$_3$. Data are representative of at least three independent experiments, each with three or more biological replicates. Statistics are based on one-way ANOVA and Tukey’s multiple-comparison test; ***,* P < 0.01; ****, *P < 0.001; ns, not significant. Statistics presented indicate differences from the WT unless otherwise indicated.
wild type, the ΔadhA mutant, and the ΔadhA-complemented strain were grown in M63 liquid medium with 1% ethanol as the sole carbon source and 50 mM nitrate. This experiment showed that adhA significantly contributes to growth on ethanol under anoxic conditions. Although the exaA deletion mutant grew quite well, the slightly reduced growth of the ΔexaA strain relative to that of the wild type and the reduced growth of the ΔexaA ΔadhA double mutant relative to that of the ΔadhA single mutant suggested that exaA contributed to a small amount of the mostly AdhA-dependent ethanol utilization as the sole carbon source under anoxic denitrifying conditions (Fig. 5B). Further study would be needed to evaluate this interesting possibility. Under anoxic conditions in medium with glucose as the sole carbon source and with nitrate as the terminal electron acceptor, all strains grew similarly to the wild type, with an OD600 of 0.39 ± 0.05, except for the Δanr mutant which did not grow. Our finding that AdhA is needed for growth on ethanol as the sole carbon source under anoxic denitrifying conditions is consistent with observations that ethanol can be utilized as an electron donor under these denitrifying conditions, but no data or details about the exact nature of this ethanol dehydrogenase were reported in their paper.

Like the ΔadhA mutant, the Δanr mutant was unable to grow on ethanol as a sole carbon source at 0.2% oxygen, and that defect was complemented by restoring anr to the strain (Fig. 6). To determine if the defect in growth in the Δanr mutant on ethanol in low oxygen was due to the inability to induce adhA, the Δanr strain expressing adhA was again employed. In this background, overexpression of adhA was sufficient to partially rescue growth of a Δanr mutant relative to that of a Δanr strain bearing the empty vector (Fig. 6). The inability to completely restore growth to the Δanr mutant upon overexpression of adhA suggests that other Anr-regulated factors such as the high-affinity cytochrome c oxidases are required for fitness in low-oxygen environments during growth on ethanol.

To complement our analysis of the role of AdhA in ethanol catabolism in liquid M63 with ethanol as the sole carbon source under microoxic and anoxic conditions, we also analyzed the role of AdhA in growth on ethanol under normoxic culture conditions. Consistent with previously published results (5, 9, 10), exaA was necessary and sufficient for growth on ethanol on agar medium at 21% oxygen in both strains PA14 and PAO1 (see Fig. S2A). The adhA mutants in both PA14 and PAO1 showed no defect in aerobic...
growth on ethanol on an agar medium and the phenotype of the ΔadhA ΔexaA mutant was similar to that of the ΔexaA single mutant (Fig. S2A). When ethanol was replaced with 0.2% glucose as sole C source, all strains grew equally well on the M63 agar medium (Fig. S2A). However, in liquid medium (M63 with ethanol as the sole carbon source in 5-ml cultures in test tubes on a roller drum), both exaA and adhA were required for growth equal to that in the WT PA14 and WT PAO1 backgrounds (Fig. S2B and C, respectively). These data suggest that even under normoxic atmospheric conditions, metabolic activity in cells within liquid media in culture tubes created sufficient oxygen restriction to induce adhA and require its participation, along with exaA, to achieve maximal growth under these conditions of limited aeration. All strains grew equally well in M63 medium with glucose as the sole carbon source, with an average OD600 of 0.94 ± 0.2. Together, these data suggest that AdhA, under the control of Anr, can contribute to ethanol catabolism under diverse culture conditions and, in some cases, can work in parallel with the ExaAB ethanol catabolic complex.

DISCUSSION

Pseudomonas aeruginosa is remarkable in its ability to grow in diverse environments. Although, for years, only one pathway for ethanol oxidation and utilization in this organism has been known (10), it is unsurprising that P. aeruginosa possesses other enzymes that carry out the same metabolic process, regulated by entirely different signals. Through this work, we show that AdhA contributes to the catabolism of ethanol in a rich medium (LB plus 1% ethanol) and in a minimal medium with ethanol as the sole carbon source under oxygen-limiting conditions. In the absence of oxygen, ethanol also supported growth with the electron acceptor nitrate, a phenomenon also observed in the complex microbial communities of wastewater treatment systems (29). AdhA-dependent ethanol catabolism in LB was concomitant with acetate accumulation in culture supernatants, and the resulting acidification contributed to increased P. aeruginosa survival by counteracting the alkalinization that develops due to deamination of amino acids.

We suggest that AdhA-dependent accumulation of acetate from ethanol had not been previously observed in P. aeruginosa because previous studies largely investigated the aerobic oxidation and utilization of ethanol as a sole carbon source under oxic conditions. In the present study, we observed acetate accumulation when we added ethanol to LB, a rich medium, and incubated it with Pseudomonas in a test tube on a roller drum apparatus. We hypothesize that the rapid respiratory activity of cells after reaching late log phase had depleted much of the dissolved oxygen in the broth on the roller tube apparatus. This would be expected to increase the level of AdhA activity, which is markedly elevated by hypoxia as explained above. The obvious question that arises is why the acetate formed was not further metabolized under the LB medium conditions. We hypothesize that this is due to the presence of amino acids in the medium activating the previously characterized catabolite repression of acetate utilization in P. aeruginosa (9, 30).

It is interesting to consider why ethanol requires AdhA for catabolism under microoxic conditions. We and others found that microoxic growth of P. aeruginosa is sufficient to markedly elevate levels of transcripts that encode the NAD⁺-linked alcohol dehydrogenase adhA (PA5427) while not elevating the levels of transcripts associated with the PQQ-dependent ethanol oxidation pathway (14–17). This difference may explain why AdhA is active under these conditions but does not explain why the PQQ-dependent ExaA is not. We do not yet know if ExaA is expressed but not active or if it is not produced in cells grown under low oxygen. ExaA is localized to the periplasm and donates electrons to a soluble cytochrome c₅₅₀. ExaB, which mediates electron transfer between ExaA and an unknown cytochrome c oxidase (10). One might speculate that this activity may not function under microoxic conditions. In contrast, AdhA is predicted to be cytosolic with only NAD⁺ as the electron acceptor. The reoxidation of NADH may be more favorable than the reoxidation of reduced factors in the ExaAB complex when oxygen is at very low levels or under denitrifying conditions.
Published findings suggest that an additional factor, catabolite repression, may repress elements of the Exa pathway, since the amino acids present in LB have been shown capable of exerting catabolite repression on several P. aeruginosa enzymatic activities (31). There is direct evidence for catabolite repression of some of the enzymes of the POQ ethanol oxidation pathway (9, 28, 30). However, there is no evidence for the catabolite repression of AdhA, and there is indirect evidence suggesting that growth on amino acids does not cause repression of the marked elevation of adhA transcript levels induced by microoxic growth conditions (14, 16). Therefore, we suggest that future studies may find that many nutrient compounds exert catabolite repression on exaA and other genes encoding enzymes in the POQ ethanol oxidation pathway but that the oxidation of ethanol to acetate by AdhA is not similarly subject to catabolite repression. Thus, in environments like the cystic fibrosis lung environment, where oxygen gradients are steep, nutrients are abundant, and ethanol-producing microbes are often present, Anc and AdhA may be particularly important factors to consider in determining if locally produced ethanol is catabolized by P. aeruginosa.

The oxygen-responsive transcription factor Anc can be active at high cell densities or in biofilms under normoxic conditions as well as under microoxic or anoxic conditions, and several published transcriptomics analyses of the Anc regulon and gene expression comparisons between oxic and microoxic or anoxic environments showed that Anc is a key regulator of adhA (14–16, 32). It is perhaps curious that an NAD+/H+ linked ethanol oxidizing system is activated by Anc and hypoxia, since low-oxygen conditions limit the oxidation of NADH. We, like others (18), have considered the possibility that P. aeruginosa uses AdhA to ferment pyruvate to ethanol, regenerating NAD+/H+, but we never observed ethanol in supernatants from LB-grown cultures, even when extracellular acetate was provided in the medium. We noted that the ΔadhA mutant, compared to the WT, overproduced phenazines in both the PA14 and PAO1 backgrounds (see Fig. S2A in the supplemental material). This observation may indicate an imbalance in the NAD+/NADH ratio upon deletion of adhA (given the role of phenazines in redox homeostasis) (33). Furthermore, as ethanol addition to strain PA14 causes overproduction of some phenazine pigments apparent on mature colony growth on agar plates (5, 6), we suggest that ethanol concentrations are higher in the strain lacking AdhA and that this leads to higher levels of pyocyanin and perhaps other phenazines than in the wild type.

The finding that P. aeruginosa accumulates significant amounts of acetic acid from added ethanol has not been reported previously. Acetate accumulations in other species such as an Acetobacter sp. and Gluconobacter spp., which are used for the commercial production of vinegar, generate large amounts of extracellular acetic acid from ethanol oxidation using an ExaAB-like system, including a PQQ-linked alcohol dehydrogenase (28, 34). A very early study reported that many Pseudomonas fluorescens strains accumulated acetic acid from ethanol in a peptone medium; but in that study, the P. aeruginosa strain did not, although it did use ethanol as a sole carbon source (35).

Previous studies in E. coli may help to understand our finding of the effect of ethanol to increase the survival of P. aeruginosa in LB. In E. coli, a similar viability loss in late stationary phase occurs, and this is prevented by ethanol addition to the LB. In these studies, the addition of ethanol to LB also caused an acidification and decrease in the highly alkaline pH that develops in LB. Further work led to the conclusion that in E. coli, ethanol protection against viability loss is due to more than just an indirect effect of ethanol on pH through the enzymatic conversion of ethanol to acetic acid (20, 36) and that very low concentrations of ethanol are protective. Ferraro and Finkel (37) suggest that very low concentrations of ethanol may also increase long-term viability in LB of some other genera, including Pseudomonas, but not the viability of all genera tested. Together, these data suggest that ethanol is at times an important resource as an inducer, a carbon source, a compound that can be used to modulate environmental pH, and an agent that increases long-term bacterial persistence.
MATERIALS AND METHODS

Strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. Bacteria were maintained on 1.5% agar LB (lysogeny broth) plates (38). Where stated, ethanol (200 proof) was added to the medium (liquid or molten agar) to a final concentration of 1%. For LB medium buffered to pH 7, 100 mM HEPES acid was added to LB and the pH brought up to 7 by titration with NaOH. The exaA::TnM strain was maintained on LB with 60 μg/ml gentamicin (19).

Construction of in-frame deletions, complementation, and plasmids. The construction of plasmids, including in-frame deletion and complementation constructs, was completed using yeast cloning techniques in Saccharomyces cerevisiae as previously described (39) unless otherwise stated. The primers used for plasmid construction are listed in Table S2. In-frame deletion and single-copy complementation constructs were made using the allelic replacement vector pMQ30 (39). Promoter fusion constructs were made using a modified pMQ30 vector with lacZ-GFP fusion integrating at the neutral att site on the chromosome. All plasmids were purified from yeast using Zymoprep Yeast Plasmid Miniprep II according to the manufacturer’s protocol and transformed into electrocompetent E. coli strain S17 by electroporation. Plasmids were introduced into P. aeruginosa by conjugation, and recombinants were obtained using sucrose counterselection and genotype screening by PCR. The primers are listed in Table S2. The exaA::TnM strain was confirmed with locus-specific primers as previously published (5).

Growth on ethanol as a sole carbon source. For the assessment of growth on ethanol as a sole carbon source, M63 medium with 1% ethanol was used (40). Microoxic cultures were grown in 1 ml medium in a single well of a 12-well plastic culture plate in a chamber with a controlled atmosphere (Coy Products) at 37°C set to maintain a 0.2% O2 atmosphere. M63 minimal medium with either 0.2% glucose or 1% ethanol as the sole carbon source was used. Cultures were inoculated to an initial OD600 of 0.05 in fresh medium from overnight liquid cultures grown in LB. OD600 was measured 48 h postinoculation using a Genesys 6 spectrophotometer. Normoxic cultures grown in 5 ml of M63 minimal medium with either 0.2% glucose or 1% ethanol as the sole carbon source were inoculated at an initial OD600 of 0.05 from overnight liquid cultures grown in LB and incubated in 18-mm by 150-mm borosilicate culture tubes at 37°C on a roller drum. The OD600 was measured 24 h postinoculation using a Genesys 6 spectrophotometer. Anoxic cultures were grown in 2 ml of M63 minimal medium with either 0.2% glucose or 1% ethanol as the sole carbon source and supplemented with 50 mM KNO3. Cultures were inoculated at an initial OD600 of 0.05 into fresh medium from overnight liquid cultures grown in LB in 18-mm borosilicate culture tubes in 15-ml conical screw-cap tubes (Sarstedt) at 37°C on a roller drum. Tubes were placed in 2.5-liter AnaeroPack system rectangular jars (Mitsubishi Gas Chemical Co., Inc.) with one GasPak EZ sachet (BD). These cultures were grown for 5 days at 37°C prior to measuring OD600 using a Genesys 6 spectrophotometer.

Ethanol catabolism experiments in LB medium. For experiments in which P. aeruginosa was grown in liquid LB, LB plus 1% ethanol, or LB buffered to pH 7, strains were first grown overnight in LB for 14 to 16 h, and ~100 μl of the overnight culture was used to inoculate 5 ml of the test medium to an initial OD of 0.05. For pH measurements at the designated time points, culture supernatant was applied to Millipore pH indicator strips (pH 6.5 to 10.0); colors were compared to the reference and values were recorded immediately.

HPLC analysis of culture supernatants. For HPLC, culture supernatants were collected after pelleting cells by centrifugation for 1 min at 13,000 rpm. Culture supernatant (400 μl) plus 40 μl of 10% sulfuric acid solution was added to Costar Spin-X 0.22-μm filter centrifuge tubes and spun for 5 min at 13,000 rpm. One hundred microliters of each sample was transferred to an HPLC autosampler vial (Chromglass CV-1042-1232), and one 40-μl injection of each sample was run for 30 min. Chemical peaks were detected by refractive index using an Aminex HPX-87H column (Bio-Rad, Hercules, CA) with a 2.5 mM sulfuric acid solution mobile phase. Chemical concentrations were determined using calibration curves based on three dilution standards of pyruvate, lactate, acetate, and ethanol.

Statistics. Unless otherwise stated, data are based on three biological replicates with the means and standard deviations calculated. Data are representative of at least three independent experiments containing multiple replicates. Unless stated otherwise, means and standard deviations were calculated in GraphPad Prism 8, and analyses were completed using a one-way analysis of variance (ANOVA) and Tukey’s multiple-comparison test, with P values indicated in figure legends.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00393-19.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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