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RESEARCH ARTICLE

Ethanol Stimulates Trehalose Production through a SpoT-DksA-AlgU-Dependent Pathway in *Pseudomonas aeruginosa*

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ABSTRACT *Pseudomonas aeruginosa* frequently resides among ethanol-producing microbes, making its response to the microbially produced concentrations of ethanol relevant to understanding its biology. Our transcriptome analysis found that genes involved in trehalose metabolism were induced by low concentrations of ethanol, and biochemical assays showed that levels of intracellular trehalose increased significantly upon growth with ethanol. The increase in trehalose was dependent on the TreYZ pathway but not other trehalose-metabolic enzymes (TreS or TreA). The sigma factor AlgU (AlgT), a homolog of RpoE in other species, was required for increased expression of the *treZ* gene and trehalose levels, but induction was not controlled by the well-characterized proteolysis of its anti-sigma factor, MucA. Growth with ethanol led to increased SpoT-dependent (p)ppGpp accumulation, which stimulates AlgU-dependent transcription of *treZ* and other AlgU-regulated genes through DksA, a (p)ppGpp and RNA polymerase binding protein. Ethanol stimulation of trehalose also required acylhomoserine lactone (AHL)-mediated quorum sensing (QS), as induction was not observed in a Δ*lasR* Δ*rhlR* strain. A network analysis using a model, eADAGE, built from publicly available *P. aeruginosa* transcriptome data sets (J. Tan, G. Doing, K. A. Lewis, C. E. Price, et al., Cell Syst 5:63–71, 2017, https://doi.org/10.1016/j.cels.2017.06.003) provided strong support for our model in which *treZ* and coregulated genes are controlled by both AlgU- and AHL-mediated QS. Consistent with (p)ppGpp- and AHL-mediated quorum-sensing regulation, ethanol, even when added at the time of culture inoculation, stimulated *treZ* transcript levels and trehalose production in cells from post-exponential-phase cultures but not in cells from exponential-phase cultures. These data highlight the integration of growth and cell density cues in the *P. aeruginosa* transcriptional response to ethanol.

IMPORTANCE *Pseudomonas aeruginosa* is often found with bacteria and fungi that produce fermentation products, including ethanol. At concentrations similar to those produced by environmental microbes, we found that ethanol stimulated expression of trehalose-biosynthetic genes and cellular levels of trehalose, a disaccharide that protects against environmental stresses. The induction of trehalose by ethanol required the alternative sigma factor AlgU through DksA- and SpoT-dependent (p)ppGpp. Trehalose accumulation also required AHL quorum sensing and occurred only in post-exponential-phase cultures. This work highlights how cells integrate cell density and growth cues in their responses to products made by other microbes and reveals a new role for (p)ppGpp in the regulation of AlgU activity.

KEYWORDS (p)ppGpp, AlgU, DksA, *Pseudomonas aeruginosa*, SpoT, ethanol, microbe-microbe interaction, quorum sensing, trehalose

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Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium that can cause acute and chronic infections in a broad range of hosts. *P. aeruginosa* frequently causes chronic infections in individuals with the genetic disorder cystic fibrosis (CF). Diverse bacterial and fungal taxa often coinfect with *P. aeruginosa* in CF airways (1–5), and many of these taxa are robust fermenters capable of ethanol (EtOH) production (6, 7). Ethanol has also been identified as a volatile biomarker in exhaled breath condensates that discriminates between healthy individuals and those with CF (8).

Ethanol has a range of biological activities that vary based on its concentration. At concentrations in the 3% to 5% range and higher, ethanol can inhibit growth or kill *P. aeruginosa* (9–11). The effects of biologically produced concentrations of ethanol within the range experienced by organisms in polymicrobial communities (0.1 to 1.1%) (12–20) have been less well studied. Several studies have shown that 1% ethanol can alter pathogenesis and interspecies interactions (12, 14, 16, 18). In *Acinetobacter baumannii*, ethanol enhances virulence toward *Caenorhabditis elegans* (16) and *Galleria mellonella* (18). This may be due to enhanced production of cytotoxic phospholipase C and increased expression of nutrient uptake pathways (17). In *P. aeruginosa*, ethanol produced by *Candida albicans* influences the expression of the antifungal phenazine 5-methyl phenazine-1-carboxylic acid (5MPCA), and 1% ethanol was sufficient to modulate phenazine production and stimulate exopolysaccharide Pel- and Psl-mediated biofilm and pellicle formation (12, 15).

The *P. aeruginosa* alternative sigma factor AlgU, also named AlgT, has been well studied for its positive regulation of alginate, an exopolysaccharide (21, 22). AlgU is an extracytoplasmic sigma factor that is homologous to RpoE (σ^E or σ^{22}) in other Gram-negative bacteria (23). *P. aeruginosa* mutants lacking *algU* have increased resistance to hydrogen peroxide compared to their alginate-overproducing mucoid counterparts due to transcriptional derepression of the catalase gene *katA* but are more susceptible to host antimicrobial peptides (24, 25). In other species, σ^E is necessary for fitness in response to high concentrations of ethanol (3% to 10%) and inhibitory concentrations of salt (NaCl) (26–29). A well-known mechanism of σ^E activation in response to stresses that perturb the cell envelope is by proteolytic degradation of its anti-sigma factor by specific proteases (30, 31). In *P. aeruginosa*, the AlgU anti-sigma factor is MucA, and *mucA* mutations lead to high AlgU activity. Naturally occurring *mucA* mutants are frequently observed in populations from chronic *P. aeruginosa* lung infections, and these strains overproduce alginate (32, 33).

In *E. coli* and *Salmonella enterica*, σ^E activity can also be modulated by the alarmone (p)ppGpp (34, 35). (p)ppGpp is an intracellular molecular signal that is synthesized by either the synthase RelA or a hybrid synthase/hydrolase, SpoT (36), in response to nutrient limitation and various environmental stressors (37). (p)ppGpp can complex with the RNA polymerase binding protein DksA to promote transcription initiation and elongation and alter the effects of RNA polymerase-associated sigma factors, including RpoE (38, 39).

In this work, we show that a subinhibitory concentration of ethanol (1%) induces the expression of genes involved in trehalose metabolism and that biochemical assays detect significant increases in intracellular trehalose, a disaccharide that serves as both a compatible solute and a carbon source. Increased trehalose in response to ethanol required the TreYZ trehalose-biosynthetic enzymes but not the TreS trehalose synthase. Ethanol induction of *treZ* gene expression and trehalose requires the sigma factor AlgU. AlgU was activated not by release from MucA but rather in a manner dependent on (p)ppGpp. Ethanol caused a 2.5-fold increase in (p)ppGpp levels, which was specifically dependent on the SpoT (p)ppGpp synthase. The (p)ppGpp-binding protein DksA was required for ethanol-induced stimulation of *treZ* gene expression and trehalose levels. Consistent with previous reports (40, 41), acylhomoserine lactone (AHL)-mediated quorum sensing (QS) was also required for transcriptional induction of the *treZ* gene by ethanol, as a Δ*lasR* Δ*rhlR* mutant defective in AHL-mediated quorum sensing did not show increased trehalose levels in response to ethanol. The stimulation of trehalose levels by NaCl required AlgU, and trehalose stimulation in response to NaCl was lower,

but still occurred, in mutants lacking the factors necessary for the response to ethanol. Analysis of genes differentially expressed when ethanol is in the growth medium, performed using the gene expression model eADAGE (ensemble analysis using denoising autoencoders of gene expression), constructed with data from over 1,056 different samples (42), placed the *treYZ* genes among a cluster of coregulated genes within the AHL-controlled QS and AlgU regulons. Ethanol, even when added at the time of culture inoculation, stimulated AlgU-regulated genes and trehalose production in cells only during the post-exponential phase, which is consistent with our model, in which regulators that monitor growth and cell density cues are integrated into the *P. aeruginosa* response to ethanol.

RESULTS

Analysis of the transcriptome upon growth in the presence of ethanol. To examine the transcriptional response of *P. aeruginosa* to 1% ethanol, RNA from *P. aeruginosa* grown as colony biofilms for 16 h on tryptone agar with or without 1% ethanol was analyzed using *P. aeruginosa* Affymetrix GeneChips, as we have previously shown that ethanol increases Pel exopolysaccharide production under similar conditions (12). Similar to published results (12), the presence of 1% ethanol in the medium did not affect the number of CFU in colony biofilms (see Fig. S1A in the supplemental material).

Levels of 54 transcripts were higher by 2-fold or more in cells grown with ethanol, with a false-discovery rate (FDR)-corrected *P* value of less than 0.05, and the levels of 20 genes were found to be lower by 2-fold or more in the presence of ethanol (see Table S3A and B in the supplemental material). Among the most differentially expressed genes were those involved in trehalose metabolism: *treZ* (3-fold), *treA* (2.1-fold), and *treS* (2.5-fold) (43). Other genes that were changed upon growth with ethanol are discussed in more detail below. To determine if ethanol also led to increased levels of trehalose, intracellular trehalose concentrations were measured in cells grown with or without ethanol in lysogeny broth (LB), a nutrient-rich medium, and M63, a defined medium. The LB was pH buffered with HEPES because we observed that ethanol led to a lower final pH in *P. aeruginosa* cultures grown with ethanol (final pH 8.3 in the control and pH 6.5 in ethanol from an initial pH 7.1), despite similar growth kinetics (see Fig. S1B), as has been described for *Escherichia coli* (44) and *A. baumannii* (18). Ethanol led to significant increases in trehalose in both buffered LB (>2-fold higher with ethanol) and M63 (20-fold higher with ethanol).

Increased trehalose in response to ethanol requires *treYZ* genes. In pseudomonads, such as *P. aeruginosa* and *Pseudomonas syringae*, trehalose can be synthesized by the TreYZ pathway, which converts glycogen to trehalose via a maltooligosyl trehalose synthase and glycosyl hydrolase (45), and by TreS, a trehalose synthetase that uses maltose as a substrate (Fig. 1A shows a schematic) (43, 46). Trehalose is degraded by the trehalase TreA; in other species, TreS also has trehalose-catabolic activities (47, 48).

To determine which metabolic pathway was responsible for increased trehalose in cells grown with ethanol, we used mutants lacking the 6-gene operon (PA14_36570 to PA14_36630) that contains *treY* and *treZ* genes (referred to here as a *treYZ* mutant [43]), the 3-gene operon that contains *treS* (referred to here as a *treS* mutant [43]), and an insertional mutant of *treA* (43, 49). While the *treS* mutant and *treA*::Tn*M* strain both showed a marked increase in trehalose in the presence of ethanol in comparison to controls, the *treYZ* mutant strain did not, suggesting the increase in trehalose was by the TreYZ pathway (Fig. 1B). To confirm that this was due to loss of the TreYZ pathway functionality, we confirmed these results in a *treZ*::Tn*M* mutant (see Fig. S2 in the supplemental material). The significantly higher levels of trehalose in the *treS* mutant relative to the wild type under both control and ethanol conditions suggested a role for TreS in trehalose catabolism in these cultures (50).

Ethanol catabolism occurs by the pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase ExaAB, and the resultant acetaldehyde is catabolized through a pathway that includes acetyl coenzyme A (acetyl-CoA) synthetase (AcsA). We have shown previously that ethanol-catabolic (*exaA*, *pqqB*, and *acsA*) mutants are defective

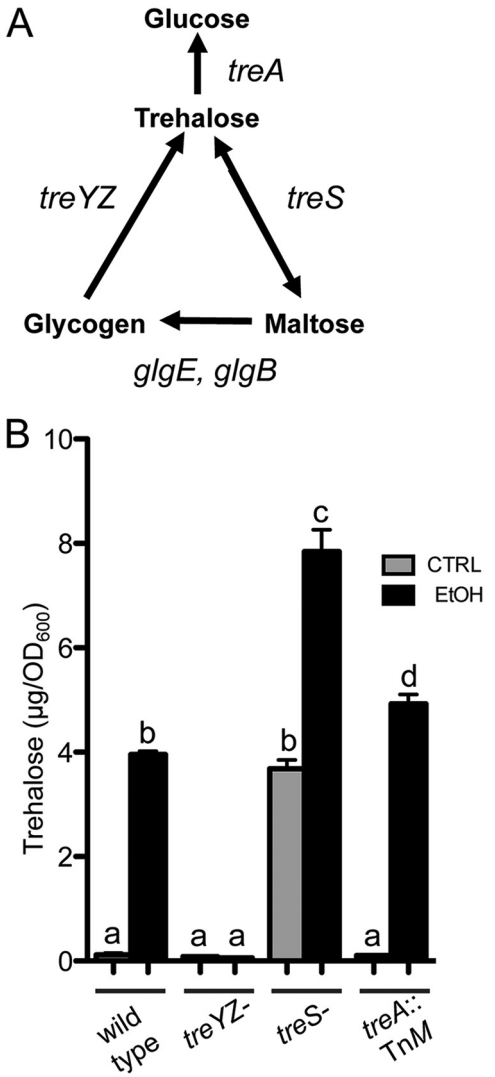

FIG 1 Trehalose accumulation in response to ethanol requires *treYZ*. (A) Schematic of trehalose-biosynthetic pathways in *P. aeruginosa*. (B) Trehalose levels in trehalose-metabolic mutants grown in M63 medium with and without 1% ethanol for 16 h. The data are representative of the results of at least 3 independent experiments with 3 biological replicates each. a-b, a-c, a-d, b-c, and c-d, $P < 0.0001$; b-d, $P < 0.02$ (based on two-way ANOVA and Tukey's multiple-comparison test). The error bars indicate standard deviations.

in growth on ethanol as a carbon source (12). Ethanol-catabolic mutants still showed stimulation of trehalose production with ethanol in the growth medium (see Fig. S3A in the supplemental material).

Because we previously showed that ethanol (1%) led to increased production of the Pel exopolysaccharide through the diguanylate cyclase WspR (12), we determined if changes in trehalose occurred in response to changes in Pel production. We found that both the *pelA* and *wspR* mutants still had higher levels of trehalose in cells grown with ethanol (see Fig. S3B), suggesting that changes in exopolysaccharide biosynthesis did not cause the increase in trehalose.

Ethanol induction of *treZ* gene expression and trehalose levels is dependent on AlgU. Several lines of evidence led us to hypothesize that the alternative sigma factor AlgU controlled the induction of trehalose-metabolic genes in response to ethanol. First, *treZ*, *treS*, and *treA* have been reported to be differentially expressed in *algU* mutant strains compared to a wild-type strain in transcriptomics studies (51, 52). Second, *osmC* and *pfpI*, two well-characterized members of the AlgU regulon (52–54),

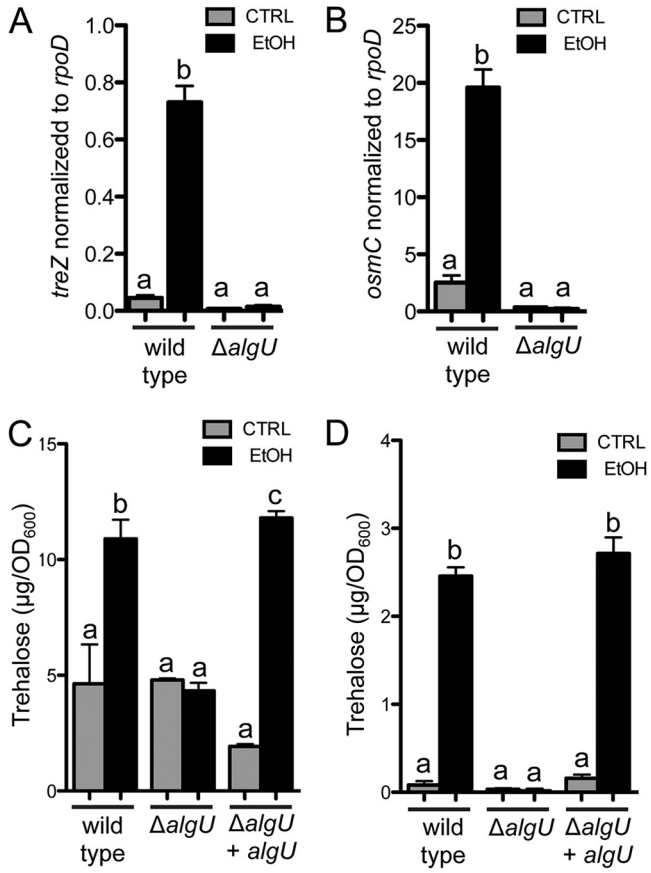

FIG 2 Analysis of the effects of ethanol on *treZ* and *osmC* transcript levels and intracellular trehalose in *P. aeruginosa* wild-type and Δ*algU* strains. (A and B) *treZ* (A) and *osmC* (B) transcript levels relative to *rpoD* after growth in the absence and presence of 1% ethanol in buffered LB for 16 h. a-b, $P < 0.0001$ (two-way ANOVA and Tukey's multiple-comparison test). (C and D) Trehalose levels in wild type, Δ*algU*, and Δ*algU* plus *algU* strains at the native locus in buffered LB (C) and M63 medium (D) with and without 1% ethanol for 16 h. (C) a-b, $P < 0.002$; a-c, $P < 0.0009$; b-c, not significant (NS). (D) a-b, $P < 0.0001$. (*P* values based on two-way ANOVA and Tukey's multiple-comparison test.) The data are representative of the results of at least three independent experiments, each with at least three biological replicates. The error bars indicate standard deviations.

were differentially expressed in our transcriptomics analysis of cells grown with or without 1% ethanol (see Table S3A).

In wild-type cells, quantitative real-time PCR (qRT-PCR) analysis found *treZ* to be 16-fold higher in cultures containing ethanol. These results are in line with our microarray analysis, indicating that ethanol stimulates trehalose gene expression in both planktonic cultures and colony biofilms, as others have shown similarities in gene expression patterns in the two growth states. In contrast, an in-frame Δ*algU* mutant had no significant difference in *treZ* expression between cells grown with and without ethanol (Fig. 2A). Like that of *treZ*, the expression of another AlgU-regulated gene, *osmC*, was higher (8-fold) upon growth with ethanol, and as expected, the differential expression was dependent on AlgU (Fig. 2B). The Δ*algU* mutant also did not show an increase in trehalose upon growth with ethanol (Fig. 2C), and its defect could be complemented by restoring *algU* to the native locus (Fig. 2C). While the sigma factor AlgU was required for the induction of *treZ* transcripts and trehalose production, a mutant lacking another sigma factor, RpoS, which has been shown to regulate trehalose levels in *E. coli* (55), did not differ from the wild type in its response to ethanol (see Fig. S4 in the supplemental material).

AlgU-dependent induction of trehalose in response to ethanol is independent of MucA cleavage and KinB regulation. AlgU activity is repressed by the anti-sigma

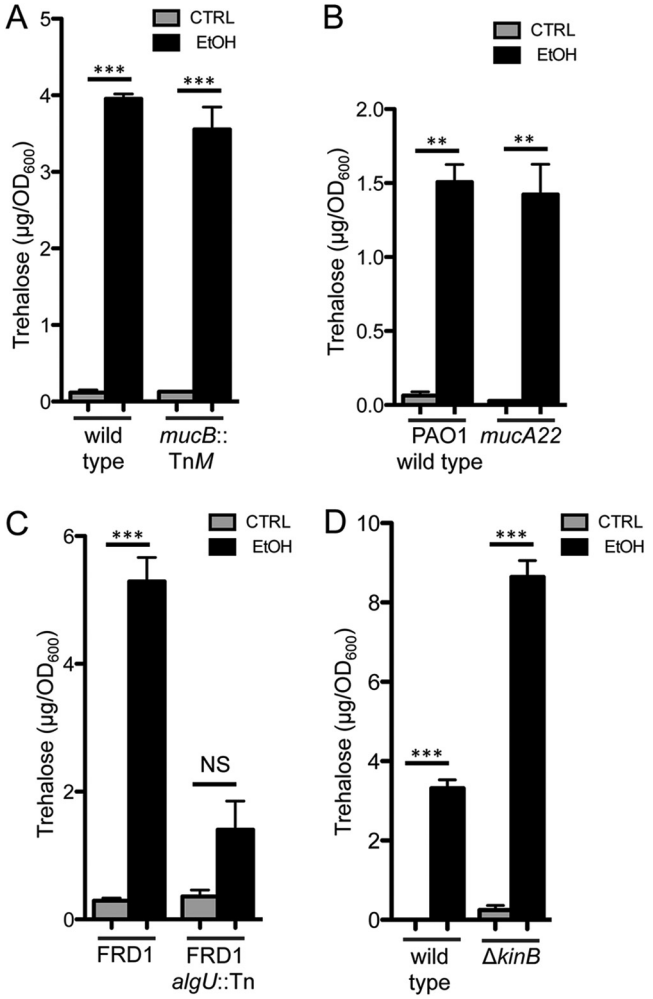

FIG 3 Trehalose levels in response to ethanol are independent of MucB, MucA cleavage, and KinB. Shown are trehalose levels of *P. aeruginosa* strain PA14 wild type and the validated *mucB* transposon mutant (A), *P. aeruginosa* strain PAO1 wild type and a *mucA* (MucA22) mutant (B), FRD1 and its isogenic *algU*::Tn derivative (C), and *P. aeruginosa* strain PA14 wild type and the Δ*kinB* mutant (D). Cultures were grown in M63 medium with and without 1% ethanol for 16 h. The data are representative of the results of at least 2 experiments, each with 3 biological replicates. The statistics are based on two-way ANOVA and Tukey's multiple-comparison test. ***, $P < 0.0001$; **, $P \leq 0.0002$. The error bars indicate standard deviations.

factor MucA, and proteolysis of MucA is a well-characterized means by which AlgU and its orthologs are activated (24, 53, 54, 56–60). MucA is bound by the periplasmic protein MucB, which inhibits MucA cleavage (61–63). Several stimuli, including high concentrations of ethanol (>3%) (26, 27), can lead to periplasmic stress and RpoE activation by anti-sigma factor degradation in other species. Several lines of evidence suggest that MucA cleavage was not the mechanism by which ethanol stimulated levels of *treZ* mRNA and trehalose. First, we found that ethanol increased trehalose to similar extents in wild-type and *mucB* mutant cells in a strain PA14 background (Fig. 3A); the *mucB* mutant has decreased stability of MucA, and the strain is mucoid, which indicates higher AlgU activation of genes involved in alginate biosynthesis. Second, ethanol stimulated trehalose levels in *P. aeruginosa* strain PAO1 in which *mucA* contained the *mucA22* mutation (Fig. 3B). The truncated MucA22 mutant, a variant frequently found among *P. aeruginosa* clinical isolates, is no longer regulated by proteolysis and no longer represses AlgU (64, 65). Lastly, ethanol also stimulated trehalose in the alginate-overproducing mucoid cystic fibrosis isolate FRD1 (66), which also has the *mucA22* allele (62, 67) (Fig. 3C). In FRD1, the stimulation of trehalose levels by ethanol required

the presence of AlgU, as the isogenic nonmucoid *algT/U*::Tn*501* (FRD440) derivative showed a large reduction in ethanol-stimulated trehalose (21) (Fig. 3C). These analyses showed that (i) ethanol induced responses similar to those in strain PA14 in the genetically distinct strains PAO1 and FRD1 and (ii) the increase in trehalose in response to ethanol does not require MucB or full-length MucA.

kinB loss of function has been associated with increased AlgU activity (53, 54) due to increased activity of the AlgB transcription factor, which stimulates AlgU expression; KinB phosphatase activity normally represses AlgB (68). Thus, we determined if KinB was required for the difference in trehalose levels in cells grown with or without ethanol. While the *kinB* mutant consistently had higher levels of trehalose under control and ethanol conditions than the wild type, KinB was not required for the effects of ethanol on trehalose levels (Fig. 3D). Together, these data support our model in which AlgU regulates trehalose through a mechanism that is not dependent on KinB.

Ethanol stimulation of trehalose requires SpoT-generated ppGpp. In *E. coli*, the activity of RpoE, an AlgU homolog, is influenced by (p)ppGpp (34, 38, 39, 69, 70); (p)ppGpp effects on *P. aeruginosa* AlgU have not yet been reported. (p)ppGpp can be synthesized by either of two enzymes, RelA or SpoT (71–73). SpoT also has (p)ppGpp-degrading activity, and because very high levels of (p)ppGpp are toxic, mutants lacking *spoT* are viable only in the absence of RelA activity (74, 75). Thus, we determined if ethanol stimulation of trehalose levels was altered in either the Δ*relA* or Δ*relA* Δ*spoT* strain. We found that while the Δ*relA* strain was like the wild type, ethanol did not influence trehalose levels in the Δ*relA* Δ*spoT* double mutant, suggesting that SpoT was required for trehalose accumulation in ethanol-grown cells (Fig. 4A). Complementation of the Δ*relA* Δ*spoT* double mutant with *spoT* restored trehalose levels to those in the wild type in the presence of ethanol (see Fig. S6 in the supplemental material). The induction of *treZ* and *osmC* was also dependent on SpoT (Fig. 4C and D).

To determine if 1% ethanol altered (p)ppGpp levels in the PA14 wild type, its levels were measured. We found that cells grown with ethanol had 3.45-fold higher levels of (p)ppGpp. The increase in (p)ppGpp was similar to that of the wild type in the *relA* mutant, but the Δ*relA* Δ*spoT* strain did not show an increase in (p)ppGpp in response to ethanol, indicating that ethanol stimulates (p)ppGpp production via SpoT (Fig. 4E).

Ethanol stimulation of *treZ* and trehalose levels requires DksA. The (p)ppGpp signal influences the activity of RNA polymerase-sigma factor complexes through DksA, an RNA polymerase binding protein. We found that a Δ*dksA* mutant no longer showed ethanol-induced trehalose accumulation and that the phenotype of the Δ*dksA* mutant was complemented by restoring *dksA* at the native locus (Fig. 4B). Consistent with the trehalose measurements, ethanol-induced increases in *treZ* and *osmC* expression were greatly reduced in the Δ*relA* Δ*spoT* and Δ*dksA* strains (Fig. 4C and D). Like those of the wild type, the growth kinetics of the Δ*dksA* mutant and the Δ*relA* Δ*spoT* mutant were not reduced by 1% ethanol, though the Δ*dksA* mutant grew more slowly than the wild type under control conditions, consistent with published reports (76, 77) (see Fig. S5 in the supplemental material).

AlgU is required for, and SpoT and DksA contribute to, the induction of trehalose by high NaCl. In *P. aeruginosa* and other species, trehalose is induced by high NaCl levels (46, 78). Ausubel and colleagues (43) found that elevated trehalose levels under high-NaCl conditions required TreYZ (43), and we confirmed these results (Fig. 5A and B). Furthermore, we found that trehalose induction in response to high NaCl was absent in the Δ*algU* mutant (Fig. 5C), as it was for ethanol (Fig. 1C and D). While (p)ppGpp and DksA were necessary for induction of trehalose in response to ethanol, the Δ*dksA* and Δ*relA* Δ*spoT* strains still showed significant induction of trehalose in response to NaCl (Fig. 5C). The level of induction, however, was significantly lower in the Δ*dksA* and Δ*relA* Δ*spoT* strains than in the wild type and the Δ*relA* strain, suggesting that SpoT-dependent (p)ppGpp and the (p)ppGpp-responsive RNA polymerase binding protein DksA contributed to the response in cells from post-exponential-phase cultures (Fig. 5C and D).

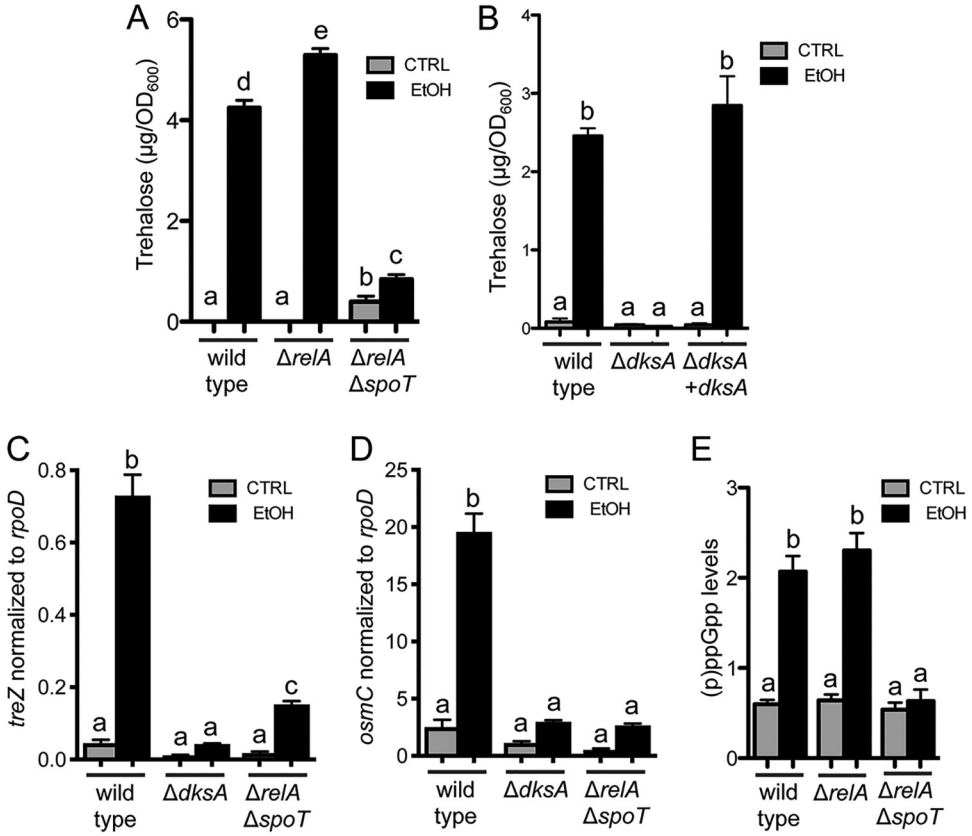

FIG 4 *dksA* and *spoT* are required for trehalose accumulation and *treZ* and *osmC* expression, and *spoT* is necessary for increased (p)ppGpp in response to ethanol. (A and B) Trehalose levels of PA14 wild type, Δ*relA*, and Δ*relA* Δ*spoT* (A) and PA14 wild-type, Δ*dksA*, and Δ*dksA* plus *dksA* (B) strains in M63 medium with and without 1% ethanol for 16 h. (C and D) *treZ* (C) and *osmC* (D) transcripts normalized to *rpoD* in PA14 wild-type, Δ*dksA*, and Δ*relA* Δ*spoT* strains in buffered LB at 16 h with and without 1% ethanol. The data are representative of the results of at least 3 independent experiments with at least 3 biological replicates each. (E) (p)ppGpp quantification (relative fluorescence units [RFU], 470/380 nm/OD_{600} unit) of PA14 wild-type, Δ*relA,* and Δ*relA* Δ*spoT* strains in M63 medium with and without 1% ethanol for 16 h. The data are representative of the results of at least 2 independent experiments. The statistics are based on two-way ANOVA and Tukey's multiple-comparison test. (A) a-b, $P = 0.0179$; a-c and d-e, $P = 0.0001$; a-d, a-e, b-d, b-e, c-d, and c-e, $P < 0.0001$; b-c, NS. (B) a-b, $P < 0.0001$. (C) a-b and b-c, $P < 0.0001$; a-c, $P \leq 0.05$. (D) a-b, $P < 0.0001$. (E) a-b, $P < 0.0001$. The error bars indicate standard deviations.

Quorum-sensing master regulators are necessary for the ethanol induction of trehalose. Schuster et al. (40, 41) found that *treZ* and other genes involved in trehalose biosynthesis and *osmC* were at lower levels in a PAO1 Δ*lasR* Δ*rhlR* mutant than in the wild type. They reported that *treZ* and *osmC* fell within a subset of QS-controlled genes that were induced later in growth in batch culture than other QS-controlled genes (40, 41). We found that the increase in levels of *treZ* and *osmC* (Fig. 6A and B) or trehalose (Fig. 6C) in ethanol-grown cells did not occur in the Δ*lasR* Δ*rhlR* strain and that the lack of response did not appear to be due to ethanol effects on growth in the strain (see Fig. S5). Analysis of trehalose levels in *lasR* and *rhlR* single mutants was also performed. We found that *lasR* was necessary for the accumulation of trehalose in response to ethanol and that the defect in the Δ*lasR* mutant was complemented by providing *lasR* back at the native locus (see Fig. S7 in the supplemental material). The Δ*rhlR* mutant phenotype was consistent within an experiment but variable across experiments (discussed further below) (see Fig. S7). Since *lasR* or both AHL-responsive transcription factors were necessary for the stimulation of trehalose in cells grown with ethanol, we determined if ethanol enhanced AHL-mediated quorum sensing, thereby inducing trehalose levels. To do so, we monitored the expression of *rhlI*, a quorum-sensing-controlled gene regulated by LasR and RhlR, using an *rhlI-lacZ* promoter fusion (79). PA14 wild type and the Δ*lasR* Δ*rhlR* strains were grown with or without ethanol. As expected, levels of

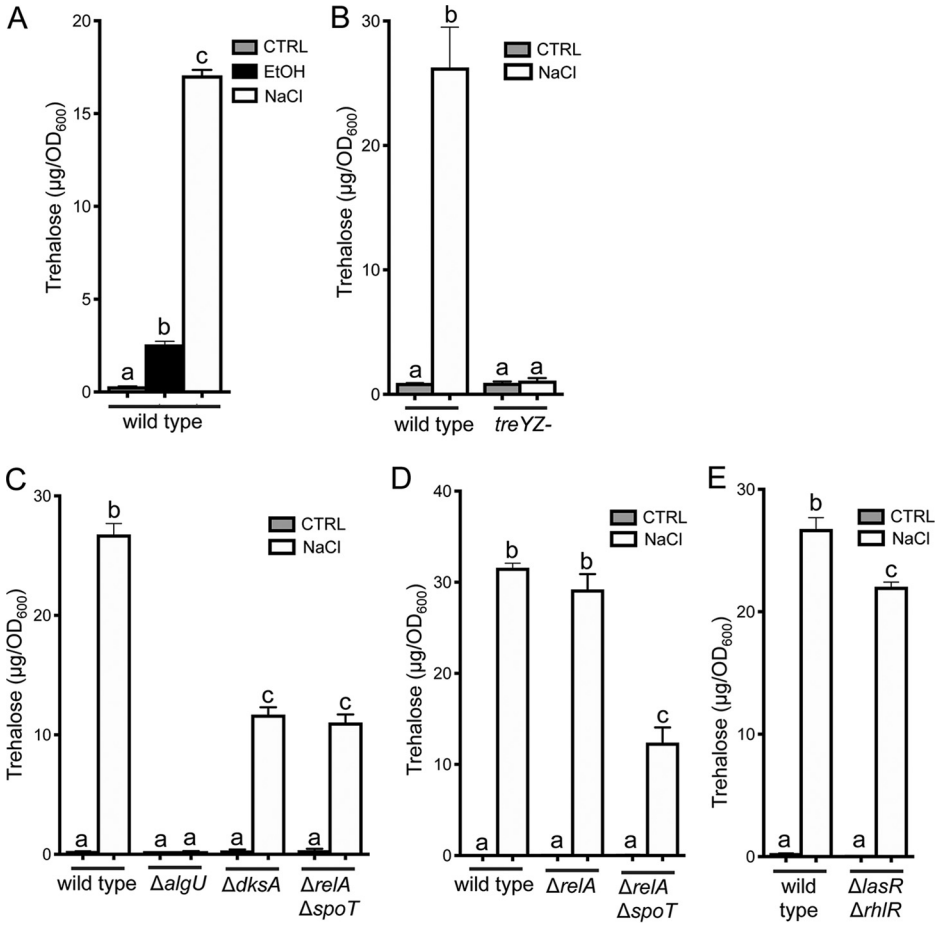

FIG 5 Trehalose accumulation in response to NaCl requires TreYZ and AlgU. (A) NaCl (500 mM) stimulates trehalose accumulation. (B) NaCl-stimulated accumulation of trehalose is dependent on the TreYZ pathway. (C) NaCl-stimulated trehalose accumulation requires *algU*, and *dksA* and *spoT* contribute. (D) NaCl-stimulated trehalose accumulation is independent of *relA*. (E) Trehalose accumulation in NaCl in the Δ*lasR* Δ*rhlR* strain is similar to that in the wild type. Cultures were grown for 16 h in M63 medium with 1% ethanol or 500 mM NaCl as indicated. The data are representative of the results of two or more experiments, each with at least 3 biological replicates. The statistics are based on one-way ANOVA (A) or two-way ANOVA (B, C, and D) and Tukey's multiple-comparison test. (A) a-b, $P = 0.0005$; a-c and b-c, $P < 0.0001$. (B) a-b, $P < 0.0001$. (C) a-b, a-c, a-d, and b-c, $P < 0.0001$. (D) a-b, a-c, a-d, and b-c, $P < 0.0001$. (E). a-b and a-c, $P < 0.0001$; b-c, $P = 0.0007$. The error bars indicate standard deviations.

β-galactosidase (β-Gal) activity were much lower in the Δ*lasR* Δ*rhlR* mutant than in the wild type, and we found no significant difference in *rhlI* promoter activity in ethanol-grown cells compared to cells from control conditions (Fig. 6D). The transcriptomics analysis of *P. aeruginosa* grown with or without ethanol as described above did not find evidence for ethanol affecting AHL-mediated quorum sensing broadly (see Table S3A and B). Additionally, while AHL quorum sensing was required for trehalose accumulation in response to ethanol, it was not necessary for trehalose accumulation in response to high NaCl (Fig. 5E); there was, however, a significant reduction in trehalose levels in the Δ*lasR* Δ*rhlR* strain compared to the wild type in NaCl, suggesting that this mechanism played a role.

Ethanol-responsive genes comprise a distinct cluster within a structured network of QS-controlled genes also regulated by AlgU. Together, our data present a complex scheme in which global regulators, AlgU and transcription factors involved in AHL-mediated quorum sensing, control the trehalose-biosynthetic genes *treYZ* and *osmC* and levels of trehalose in cells grown with ethanol. Our findings support previous reports that separately found *treZ* and *osmC* genes to be among the genes controlled by AlgU (51, 52) and AHL-mediated quorum sensing (40, 41). To test the hypothesis that

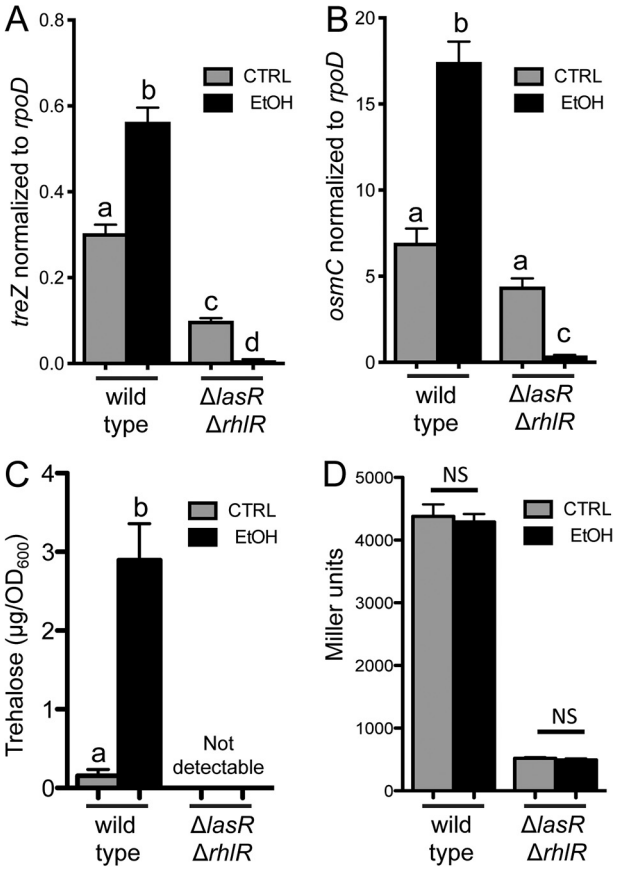

FIG 6 AHL quorum-sensing regulation is required for trehalose accumulation and increased *treZ* and *osmC* transcripts in response to ethanol. (A and B) Expression of *treZ* (A) and *osmC* (B) genes normalized to *rpoD* in PA14 wild-type and Δ*lasR* Δ*rhlR* strains in buffered LB with and without 1% ethanol for 16 h. (C) Trehalose quantification of PA14 wild-type and Δ*lasR* Δ*rhlR* cells grown planktonically in M63 medium with and without 1% ethanol for 16 h. (D) β-Galactosidase assay of *rhlI-lacZ* promoter activity in PA14 wild-type and Δ*lasR* Δ*rhlR* cells grown planktonically in M63 medium with and without 1% ethanol for 16 h. The data are representative of the results of at least 2 independent experiments, each with 3 or more biological replicates. The statistics are based on two-way ANOVA and Tukey's multiple-comparison test. (A) a-b, a-d, b-c, and b-d, $P < 0.0001$; a-c, $P = 0.0004$; c-d, $P = 0.0395$. (B) a-b and b-c, $P < 0.0001$; a-c, $P < 0.02$. (C) a-b, $P = 0.0002$. The error bars indicate standard deviations.

a subset of genes are members of both the AlgU and QS regulons and that ethanol specifically altered the expression of this subset of genes, we used eADAGE, a machine learning algorithm used to generate a model for *P. aeruginosa* expression patterns from 1,051 publicly available transcriptome samples (42, 80, 81). The eADAGE model learned 600 expression signatures, and within each signature, genes had different weights. Similarities in weights across signatures for genes indicate a correlation in expression levels across the large *P. aeruginosa* transcriptomics data set. Pairwise Pearson correlation coefficients of the genes in the eADAGE model can be visualized as edge weights in a network where nodes are genes (eADAGE [80]).

In the network shown in Fig. 7, we present the relationships in expression patterns for (i) genes that were found to be differentially expressed in response to ethanol (nodes with orange borders) and (ii) the set of genes differentially expressed by more than 5-fold in a Δ*lasR* Δ*rhlR* strain compared to the wild type, which included *treZ* and *osmC*, as reported by Schuster et al. (40) (blue nodes) (a gene list is shown in Table S3D). Using a data set that characterized the AlgU regulon by comparing a Δ*algU* mutant to the wild type under AlgU-inducing heat shock conditions (51), we identified genes in the two data sets mentioned above that were regulated by AlgU (yellow nodes) (see Table S3C for a gene list) or that were altered in both the Δ*lasR* Δ*rhlR* and Δ*algU* strains (green nodes).

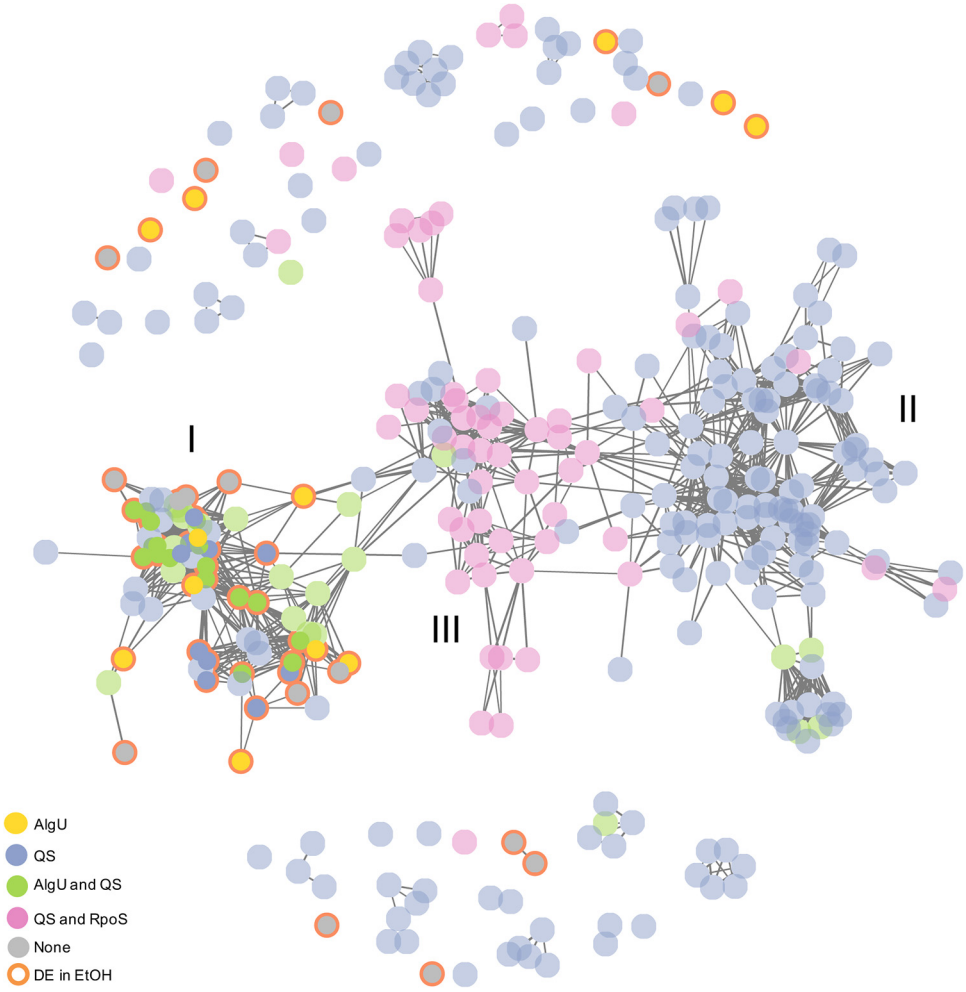

FIG 7 Ethanol-responsive genes are enriched for those with overlapping AlgU and QS control. eADAGE gene-gene network analysis shows ethanol-responsive genes and the QS regulon, with overlapping AlgU genes indicated. (I) Enrichment region for genes coordinately regulated by AlgU and QS (green) and ethanol-responsive genes (orange outline). (II) Enrichment region for "canonical" quorum-sensing genes, including *lasI*, *lasB*, *rhlR*, *rhlI*, and *rhlA*. (III) Enrichment region for QS genes within the RpoS regulon.

The majority of genes included in the network were connected by edges, revealing strongly correlated expression patterns across the large data compendium comprised of the results of experiments performed by different laboratories with different strains and under different conditions over more than a decade (42, 80, 81). The connected genes fell into three major clusters (I, II, and III). The majority (80%) of the QS-controlled genes that were also AlgU controlled (Fig. 7, green nodes) (52) were found in cluster I. Ethanol-responsive transcripts (orange borders), including *treZ* and *osmC*, were exclusively localized to cluster I. Statistical analysis found that genes differentially expressed in response to ethanol (see Table S3A) represented 12.2% of AlgU-controlled genes (51) and 8.6% of QS-controlled gene sets as defined above (see Table S3 for gene lists) but comprised 44% of the genes present in both regulons. The enrichment of the intersection of AlgU- and QS-controlled genes over the sets of either all AlgU- or all QS-regulated genes was significant ($P = 0.001$ and $P = 0.00002$, respectively). Visualization of a cluster of ethanol-responsive genes within the gene-gene network comprised of the complete AlgU regulon (51) is shown in Fig. S8 in the supplemental material.

Cluster II genes contained many genes known to be regulated mainly by LasR or RhlR and their cognate signals (40, 41). Examples of genes in cluster II are *lasI*, *lasB*, *rhlR*,

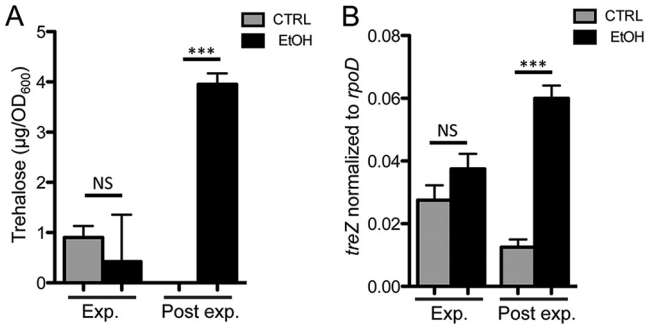

FIG 8 Ethanol stimulates *treZ* gene expression and trehalose levels in cells from post-exponential-phase (Post exp.) but not exponential-phase (Exp.) cultures. (A) Trehalose quantification of cells grown in M63 medium with and without 1% ethanol for 6 h to an OD_{600} of ~1.0 (Exp.) or for 16 h (Post exp.). (B) *treZ* gene expression normalized to *rpoD* in cultures grown in buffered LB with and without 1% ethanol to the same densities as for panel A. The data are representative of the results of 3 experiments, each with 2 to 4 biological replicates. The statistics are based on two-way ANOVA and Tukey's multiple-comparison test. ***, $P \leq 0.0008$. The error bars indicate standard deviations.

rhlI, and *rhlA*. The lack of any of the ethanol-responsive genes in cluster II is consistent with our finding that ethanol did not alter expression of *rhlI* (Fig. 6D) and supports our model in which ethanol did not broadly induce the entire QS regulon.

Cluster III contained genes from the QS-controlled gene set that were previously described by Schuster and Greenberg (41) as also being differentially expressed in an *rpoS* mutant (Fig. 7, pink nodes) (see Table S3E for a gene list). Of the 56 genes in cluster III, 47 (41) and 29 (51) genes were differentially expressed in separate published studies describing the RpoS regulon. Ethanol-responsive genes were not among the genes in cluster III, supporting the above-mentioned data showing that ethanol-induced trehalose levels were not dependent on *rpoS* (see Fig. S4). Specific enrichment in AlgU- and QS-coregulated genes among the genes upregulated in response to ethanol is consistent with ethanol activating only a subset of the AlgU- and AHL-controlled regulons.

Ethanol induces trehalose after entry into post-exponential phase. Increased trehalose levels and *osmC* and *treZ* transcripts in cultures with ethanol was dependent on both SpoT-synthesized (p)ppGpp and AHL-mediated quorum sensing; signals associated with growth restriction, often due to nutrient limitation; and high cell density, respectively. Analysis of trehalose levels in control and ethanol-containing cultures found that ethanol affected trehalose levels only in cells from post-exponential-phase cultures but not in cells from exponential-phase cultures. This relationship was observed in both M63 medium (Fig. 8A) and buffered LB medium (1.96 μg/optical density at 600 nm [OD_{600}] unit in control cultures versus 2.81 μg/OD_{600} unit in ethanol-containing cultures; $P = 0.1339$). Expression of *treZ* was also not affected by ethanol in cells from exponential-phase cultures but was affected in cells from the same cultures collected after entry into post-exponential phase (Fig. 8B). Together, these data support a model in which the induction of trehalose in response to ethanol by AlgU requires other signals from quorum-sensing- and growth-restriction-associated pathways.

DISCUSSION

The data presented above led us to propose a model, based largely on genetic analyses, in which ethanol activates AlgU through stimulation of (p)ppGpp, synthesized by SpoT, and activation of DksA-dependent transcription. Chromatin immunoprecipitation experiments have shown that AlgU binds to the promoter for the *treYZ*-containing operon (51). AHL-mediated quorum sensing through LasR and/or RhlR was required for AlgU-dependent activation of *treZ* and *osmC* and increased levels of trehalose, and thus ethanol induction of trehalose was observed in cultures only after AHL-mediated QS was induced. Our data do not indicate that ethanol led to a global increase in expression of the QS regulon. Based on these data, we propose that even

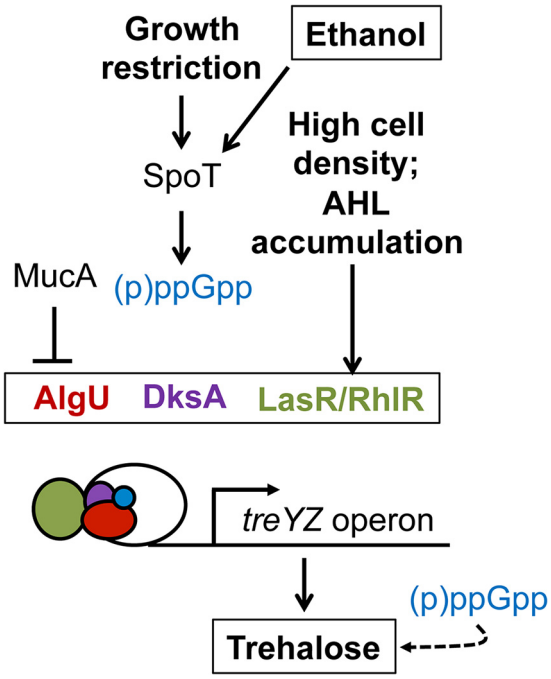

FIG 9 Model describing ethanol stimulation of the AlgU regulon and trehalose production. Our data support a model in which cell density and growth signals determine the cell's response to ethanol. The colors of the text are reflected in the schematic of the transcriptional-regulatory complex.

though ethanol was present over the course of growth, increased trehalose biosynthesis in response to ethanol occurs only when cells have sensed a quorum and when (p)ppGpp synthesis can be stimulated, perhaps because of concomitant nutrient limitation signals, which are known to activate SpoT (Fig. 9). These data highlight a nuanced response to a microbially produced molecule, ethanol, in *P. aeruginosa*, and this underscores how microbe-microbe interactions may change with shifts in physiological states and extracellular signal concentrations.

It is not surprising that we saw variation in the levels of trehalose induced by ethanol given that multiple dynamic systems are involved in its regulation. The trehalose levels in cultures with ethanol were statistically significantly higher than those in control cultures in 23 independent experiments performed on different days, with each containing three biological replicates inoculated from three different overnight cultures. If each experiment was treated as an independent data point, the average increase in trehalose levels in response to ethanol was 3.5 μg/OD$_{600}$ unit with a *P* value of $<$0.0001. In the absence of ethanol, trehalose levels ranged from undetectable to 0.5 μg/OD$_{600}$ unit, and in the presence of 1% ethanol, trehalose values ranged from 2 to 6.4 μg/OD$_{600}$ unit.

The model for AlgU activation by (p)ppGpp and DksA represents a new mechanism by which the important sigma factor AlgU may be activated in *P. aeruginosa*. (p)ppGpp-dependent activation of *E. coli* RpoE, an AlgU ortholog, has been reported previously (34) and has been associated with the growth phase (82), but activation by noninhibitory concentrations of ethanol has not been reported. Our data showed that ethanol, at a 1% concentration, did not activate AlgU through effects on the MucA or MucB protein (Fig. 3), which are targeted for cleavage in response to periplasmic or envelope stress. In the presence of 0.5 M NaCl, however, a stimulus that is expected to induce periplasmic stress, DksA- and SpoT-independent stimulation of trehalose was observed, presumably because some AlgU activation occurred through MucA cleavage, but DksA and SpoT did contribute to the strength of the response, suggesting that these mechanisms can work together.

The intersection of QS and AlgU regulation is interesting. Future experiments will

also determine if LasR and/or RhlR directly interacts with the promoter upstream of the *treYZ*-containing operon and if that interaction occurs only when AlgU and DksA are complexed with RNA polymerase. Evidence for direct activation of *osmC* and *treYZ* expression by LasR comes from the finding that overexpression of *lasR* but not *rhlR* was sufficient to induce their expression. Consistent with this, the Δ*lasR* strain was defective in the induction of trehalose. While we observed little variability between replicates within each experiment, we did observe variability in the importance of Δ*rhlR* across experiments, suggesting that an unidentified variable influenced the need for RhlR for *treYZ* induction. Thus, we propose that either transcription factor can influence the expression of *treYZ*, perhaps with different kinetics, as has been shown for other genes (83). In separate studies with Δ*dksA* and Δ*relA* Δ*spoT* mutants in strain PAO1, DksA and (p)ppGpp have independently been associated with both the positive and negative regulation of genes that are differentially expressed in a Δ*lasR* Δ*rhlR* mutant (77, 84–88) so there may be a complex relationship between these signaling pathways.

The biological role of AlgU-induced trehalose in *P. aeruginosa* in response to stresses or in microbial communities is not yet known. AlgU has been implicated in both the positive and negative regulation of genes involved in oxidative and osmotic stress responses in other pseudomonads (89–92). For example, in different pathovars of *P. syringae*, AlgU regulates oxidative- and osmotic-stress response genes transcriptionally in response to osmotic stress (91) and contributes to plant disease independently of alginate (92). In *Pseudomonas fluorescens*, an *algU* mutant was significantly more sensitive to osmotic stress than the wild type (89). *P. fluorescens* AlgU appears to be necessary for desiccation stress tolerance but dispensable for tolerance of 3% hydrogen peroxide, 1.9% paraquat, 5% sodium hypochlorite, heat shock, pH extremes, and the reducing agent dithiothreitol (89). In *P. aeruginosa*, AlgU has been described as having a negative role in oxidative-stress resistance and can be protective against host innate immune factors through its regulation of alginate (24, 25). We did not observe a protective benefit of growth with 1% ethanol in oxidative stress, osmotic stress, and dessication assays under the growth conditions used in these studies (data not shown). Trehalose has been reported to protect against osmotic, oxidative, heat, and cold stress by stabilizing proteins and reducing the formation of denatured protein aggregates (93–97) and to act as a carbon reserve (93, 98), and recent studies have found that trehalose can stabilize outer membrane vesicles (99). Trehalose can accumulate in the cytoplasm and periplasm and be secreted, making it an interesting molecule to consider in the context of microbe-microbe interactions. *P. syringae* survival as an epiphyte and *P. aeruginosa* plant pathogenesis both require the ability to make trehalose (43, 100). Interestingly, exogenous trehalose and wild-type-derived trehalose in coculture rescued the attenuated trehalose mutant phenotype in *P. aeruginosa Arabidopsis* pathogenicity *in planta*, with the requirement for trehalose being independent of osmoprotection (43). Pseudomonads can accumulate a variety of osmoprotectants in addition to trehalose, including betaine, ectoine, and *N*-acetylglutaminylglutamine amide (NAGGN) (100). This redundancy may contribute to the fact that trehalose mutants are not more sensitive to tested stresses in laboratory assays (43).

In addition to *treZ* and other trehalose metabolism genes, cluster I included 38 genes found within the genome island that spans from PA2134 to PA2192 (PA14_36980 to PA14_36345) (54, 101, 102). Sixteen of the 54 genes (~30%) differentially expressed in response to ethanol were in this chromosomal region. Many of the genes in this chromosomal region that are differentially expressed in ethanol could participate in survival of stresses likely to be present in mixed-species communities formed with ethanol-producing microbes. For example, *glgE* is involved in the metabolism of glycogen, a carbon and energy storage molecule that accumulates when carbon is in excess relative to other growth-limiting nutrients (103). Other genes within the genome island encode putative ion transporters, double-strand break repair enzymes, and two catalases. Other ethanol-induced genes within cluster I included *osmC*, *sprP*, and *pfpI*. OsmC can be protective against oxidative stress caused by exposure to elevated

osmolarity and hyperoxides through an unknown mechanism (104). SprP is a subtilase protease (105), and *pfpI* codes for a protease that plays a role in DNA protection under nonstress conditions and in the presence of hydrogen peroxide (106). Mutation of either *sprR* or *pfpI* in *P. aeruginosa* has pleiotropic effects (105, 106).

The next important question involves the mechanism by which ethanol stimulates (p)ppGpp. Others have shown that (p)ppGpp levels increase in response to higher concentrations of ethanol and that in *E. coli*, the addition of ethanol mimics amino acid starvation (107). They speculated that ethanol (and other short-chain alcohols) may interfere with amino acid uptake (107). Ethanol may also directly impact ribosome activity (108) or other pathways through effects on cell membranes, and the fact that responses vary based on the growth phase provides a useful tool to understand how ethanol and ethanol-producing microbes influence other bacteria.

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 plates (109). Where stated, ethanol (200 proof) was added to the medium (liquid or molten agar) to a final concentration of 1% unless otherwise stated. NaCl to a final concentration of 500 mM was added to liquid media as indicated. Mutants from the PA14 nonredundant (NR) library were grown on LB with 60 μg/ml gentamicin (49). When strains from the NR library were used, the location of the transposon insertion was confirmed using site-specific primers. The primers are listed in Table S2 in the supplemental material. Where stated, LB medium was buffered to pH 8 with 100 mM HEPES buffer (referred to as buffered LB). M63 medium contained 0.2% glucose and 2% Casamino Acids (110). When ethanol was supplied as a sole carbon source, glucose and amino acids were omitted. Planktonic cultures were grown at 37°C on a roller drum.

For the growth curves shown in Fig. S1, overnight cultures were diluted in 5 ml fresh buffered LB medium in 18- by 150-mm borosilicate glass tubes with or without 1% ethanol to an OD_{600} of ~0.05 and incubated at 37°C on a roller drum. Similar culture vessels and volumes were used in all assays unless otherwise specified. OD_{600} measurements were taken on a Genesys 6 spectrophotometer. For the data in Fig. S5, overnight cultures were diluted in fresh LB medium buffered to pH 8 with HEPES with or without 1% ethanol to an OD_{600} of ~0.05, and 150 μl of the cell suspension was pipetted into 96-well plates. The plates were grown at 37°C with continuous shaking at ~150 rpm. OD_{600} measurements were taken at 16 h of incubation using a microplate reader.

Construction of in-frame deletions, complementation, and plasmids. Construction of plasmids, including in-frame deletion and complementation constructs, was completed using yeast cloning techniques in *Saccharomyces cerevisiae* as previously described (111) unless otherwise stated. The primers used for plasmid construction are listed in Table S2 in the supplemental material. In-frame deletion and single-copy complementation constructs were made using the allelic-replacement vector pMQ30 (46). Deletions of *relA* and *spoT* were introduced in the PA14 strain using the pEX18Gm suicide vector to create unmarked deletion mutants (112), as previously described (113). Promoter fusion constructs were made using a modified pMQ30 vector with a *lacZ-GFP* fusion integrating at the neutral *att* site on the chromosome. The *rhlI* promoter region was amplified from PA14 genomic DNA (gDNA) using Phusion high-fidelity DNA polymerase with primer tails homologous to the modified pMQ30 ATT KI vector containing the *lacZ-GFP* reporters. The 195-bp upstream promoter region includes an RhlR-binding site as annotated at the *Pseudomonas* Genome Database (121) at positions −192 to +3. All the plasmids assembled using yeast cloning 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. The *spoT* complementation construct was made by linearizing the pMQ30 vector using EcoRI. The linearized plasmid was treated with shrimp alkaline phosphatase purchased from New England Biolabs and cleaned using a Qiagen PCR purification kit (Qiagen, Hilden, Germany). *spoT* fragment 1 was amplified from *P. aeruginosa* PA14 genomic DNA for a 1,369-bp PCR product. *spoT* fragment 2 was amplified from PA14 genomic DNA for a 1,468-bp PCR product. *spoT* fragment 3 was amplified from PA14 genomic DNA for a 1,268-bp PCR product. All amplified DNA was treated with DpnI to remove any remaining template and then cleaned using a Qiagen PCR purification kit. The vector and *spoT* fragments were assembled using NEBuilder HiFi DNA assembly master mix and incubated at 60°C for 1 h. The DNA was then transformed into high-efficiency DH5α chemically competent cells. The plasmid was isolated using a Zyppy plasmid miniprep kit (Zymo Research, Irvine, CA) and then screened by digestion with NruI. The plasmid was confirmed by sequencing. Plasmids were introduced into *P. aeruginosa* by conjugation, and recombinants were obtained using sucrose counterselection and genotype screening by PCR.

Microarray analysis. Cultures of *P. aeruginosa* PA14 wild type were grown overnight in LB at 37°C on a roller drum, and 5 μl of the overnight culture was spotted onto tryptone plates (1% tryptone, 1.5% agar) (114) with or without 1% ethanol. The plates were incubated at 37°C for 16 h. Colonies were scraped up from the plates for RNA isolation using a Qiagen RNeasy Mini kit. The samples were DNase treated using an Invitrogen Turbo DNA-Free kit. As previously described (115), cDNAs labeled with biotin-ddUTP (Enzo Bio-Array terminal labeling kit; Affymetrix) were hybridized to *Pseudomonas* GeneChips using a GeneChip fluidics station 450 (Affymetrix) according to the manufacturer's instruc-

tions. The GeneChips were scanned in the Dartmouth Genomics and Microarray Laboratory using a GeneChip Scanner 3000 7G (Affymetrix), and the BioConductor Affy library was used to read CEL file data. The data were normalized with RMA in BioConductor (116).

eADAGE analysis. Genes upregulated 2-fold or more were analyzed within the context of their expression patterns in a compendium of 1,051 publicly available microarrays from the Gene Expression Omnibus as determined by a machine learning model, eADAGE (42). Pearson correlations of greater than 0.5 between the learned parameters corresponding to each gene were visualized by edge weights in the resultant network (80). Genes differentially expressed between the wild type and a Δ*lasR* Δ*rhlR* mutant strain sampled at different time points over the course of growth are referred to as the QS-controlled regulon (40) (see Table S3D). The QS-controlled genes are presented as a network, plotted using the Fruchterman-Reingold force-directed algorithm, in which correlations in gene expression are indicated with the presence of edges and genes with shorter edges are more strongly correlated in expression pattern. The network was generated in R using the "network" (117, 118), "GGally" (119), and "ggplot2" (120) packages. The genes within the QS-controlled gene set that were also differentially expressed upon deletion of *algU* (51) are indicated as green nodes, and genes within the QS-controlled gene set that were also differentially expressed upon deletion of *rpoS* (41) (see Table S3E) are indicated as pink nodes. QS-controlled genes that were not differentially expressed upon deletion of AlgU or RpoS are presented as blue nodes. The complete gene list for each data set and accompanying R code are available in the supplemental material. If necessary, PA14 gene numbers gene were converted to PAO1 ortholog gene numbers, and PAO1 gene numbers were converted to gene names, using *P. aeruginosa* PA14 109 orthologs and *P. aeruginosa* PAO1 107 annotations from the *Pseudomonas* Genome Database (121).

Quantitative-PCR analysis of transcripts. For quantitative real-time PCR experiments, cultures of the indicated strains of *P. aeruginosa* were grown for 16 h in 5 ml of buffered LB at 37°C on a roller drum. RNA was isolated from planktonic cultures using a Qiagen RNeasy Mini kit. The samples were DNase treated using an Invitrogen Turbo DNA-Free kit. cDNA was synthesized with a RevertAid H-minus first-strand synthesis kit using the GC-rich protocol at the following temperatures: 25°C for 5 min, 50°C for 60 min, and 70°C for 5 min. The synthesized cDNA was diluted 1:5 in molecular-grade water and stored at −20°C. Quantitative-PCR expression analysis was performed using an Applied Biosystems 7500 real-time PCR system with Bio-Rad SsoFast Evagreen Supermix and the primers listed in Table S2. A cycling regimen of 95°C for 30 s, 39 cycles of 95°C for 10 s and 60°C for 5 s, and a final 65°C for 3 s was used. Experimental transcripts were normalized to the housekeeping gene *rpoD*.

Measurement of trehalose in cells. Trehalose was quantified from whole-cell lysates as described previously (122, 123) with slight modifications. Briefly, bacterial cultures were grown for 16 h in LB or M63 medium with or without 1% ethanol as indicated in 18-mm borosilicate culture tubes at 37°C on a roller drum. The cultures were inoculated from strains grown on LB plates. A 250-μl volume of culture was concentrated to an OD_{600} of 8.0 in sterile water. Cell suspensions were boiled for 10 min to lyse the cells. The resulting lysate was centrifuged at 16,000 × *g*, and 100-μl aliquots of lysate were transferred to new tubes. One tube of lysate was treated with 1 μl of trehalase (Sigma-Aldrich) enzyme or vehicle control. Glucose in the lysate samples was quantified using a glucose oxidase kit (Sigma; catalog no. GAGO20). Trehalose concentrations were calculated based on a standard curve and subtraction of the basal glucose and are expressed relative to OD units.

(p)ppGpp measurements. Cultures inoculated at an OD_{600} of 0.05 were grown for 16 h to an OD_{600} of ~2.0 in 5 ml M63 medium with and without 1% ethanol in 18-mm culture tubes at 37°C with shaking at 250 rpm. About 2 ml of each cultures was pelleted at 10,000 × *g* for 5 min, and the (p)ppGpp was extracted as described previously (124). Briefly, the pellets were suspended in 200 μl of 10 mM Tris-HCl, pH 7.8, containing 1 mg/ml lysozyme and 15 mM magnesium acetate. The suspensions were vortexed for 3 s and subjected to two freeze-thaw cycles. Then, 15 μl of a 10% deoxycholate solution was added, and the suspensions were vortexed for 15 s. Finally, 200 μl of 10 mM Tris-HCl, pH 7.8, containing 15 mM magnesium acetate was added, and the samples were centrifuged at 12,000 × *g* for 10 min. The supernatants were used for (p)ppGpp quantification, which was performed using the chemosensor Bis-Zn^{2+}-dipicolylamine (PyDPA) as previously described (125). The measurements of (p)ppGpp-PyDPA complex were carried out immediately after probe addition using fluorescence spectroscopy (excitation wavelength [Ex]/emission wavelength [Em] = 344/480 nm) in a Tecan Infinite M1000 plate reader. To account for the interference of other nucleotides, Δ*relA* Δ*spoT* mutant extracts were used, and the absolute (p)ppGpp values were determined using a calibration curve with purified (p)ppGpp (TriLink Biotechnologies) spanning a linear range of 0.4 to 6 μM (p)ppGpp. For normalized (p)ppGpp measurements, fluorescence at Ex/Em values of 344/480 nm was normalized by the fluorescence at Ex/Em values of 344/380 nm (free nucleotides complex with PyDA) and by the OD_{600} of the washed cultures.

Measurement of β-galactosidase in reporter fusion strains. Cells with an *rhlI* promoter fusion to *lacZ-GFP* genes integrated at the *attB* locus were grown in 5 ml of LB at 37°C. About 1 ml of overnight culture was pelleted, washed twice, and resuspended in phosphate-buffered saline (PBS). The washed cells were diluted to a starting OD_{600} of 0.05 in 5 ml of M63 medium with and without 1% EtOH. After 16 h on a roller drum at 37°C, β-Gal activity was measured as described by Miller (126).

Statistics. Unless otherwise stated, data are based on three biological replicates with the mean and standard deviations calculated and are representative of the results of at least three independent experiments containing multiple replicates. Unless otherwise stated, means and standard deviations were calculated in Graph Pad Prism 8, and analyses were completed using a two-way analysis of variance (ANOVA) and Tukey's multiple-comparison test, with *P* values indicated in the figure legends. Regulon enrichments were determined by hypergeometric tests using the "phyper" function in the "stats" package in R.

Accession number(s). The data for our microarray analysis of the *P. aeruginosa* PA14 wild type in response to ethanol has been uploaded to the GEO repository (https://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE124852.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00794-18.

SUPPLEMENTAL FILE 1, PDF file, 1.7 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.

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