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Involvement of *rppH* in Thermoregulation in *Pseudomonas syringae*

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Temperature, among other environmental factors, influences the incidence and severity of many plant diseases. Likewise, numerous traits, including the expression of virulence factors, are regulated by temperature. Little is known about the underlying genetic determinants of thermoregulation in plant-pathogenic bacteria. Previously, we showed that the expression of both *fliC* (encoding flagellin) and *syfA* (encoding a nonribosomal polypeptide synthetase) was suppressed at high temperatures in *Pseudomonas syringae*. In this work, we used a high-throughput screen to identify mutations that conferred overexpression of *syfA* at elevated temperatures (28°C compared to 20°C). Two genes, Psyr_2474, encoding an acyl-coenzyme A (CoA) dehydrogenase, and Psyr_4843, encoding an ortholog of RppH, which in *Escherichia coli* mediates RNA turnover, contribute to thermoregulation of *syfA*. To assess the global role of *rppH* in thermoregulation in *P. syringae*, RNA sequencing was used to compare the transcriptomes of an *rppH* deletion mutant and the wild-type strain incubated at 20°C and 30°C. The disruption of *rppH* had a large effect on the temperature-dependent transcriptome of *P. syringae*, affecting the expression of 569 genes at either 20°C or 30°C but not at both temperatures. Intriguingly, RppH is involved in the thermoregulation of ribosome-associated proteins, as well as of RNase E, suggesting a prominent role of *rppH* on the proteome in addition to its effect on the transcriptome.

Temperature is an important environmental factor that influences many aspects of microbial physiology and profoundly affects an organism's ability to survive and reproduce (1). Since microorganisms must appropriately both perceive and respond to changes in temperature, they possess some form of thermoregulated gene expression. Certain temperature responses appear to be conserved across diverse bacterial species, such as the cold shock and heat shock responses (2, 3). The stimulatory signals and regulatory mechanisms of these shock responses are also largely conserved. However, in addition to the shock responses, which maintain cellular functions after large and rapid temperature shifts, most bacterial species also possess more specialized forms of thermoregulation whose role is not necessarily to return the cell to homeostasis but, rather, to reprogram cells for fitness in the altered environment. For example, animal pathogens often use host body temperature as a cue to express virulence factors (4–6). In this setting, members of many taxa suppress the production of flagellar genes since they encode immune-eliciting antigens (i.e., flagellin) (7). While the suppression of flagellin at host temperatures is common in such animal pathogens, the stages within the flagellar hierarchy and the mechanisms by which such regulation is achieved differ between organisms (cf. *E. coli* [8], *Yersinia* [9–11], and *Listeria* [12]). Common thermoregulated traits, such as motility, therefore do not necessarily have conserved mechanisms of regulation. This may be a consequence of the myriad mechanisms by which thermoregulation can be achieved (reviewed in references 1 and 13). While the central components of thermoregulated gene expression and their interactions have been described in model organisms, such as *Escherichia coli* (14, 15), much less is known of these processes in most taxa.

Thermoregulation of certain traits in plant-pathogenic bacteria has been observed (16); however, our understanding of the mechanisms operative in these organisms remains limited. One of the most thoroughly studied thermoregulation systems in plant pathogens is the CorRS two-component signaling system that regulates coronatine biosynthesis in *Pseudomonas syringae* pv. glycinea (17–19). While this system is necessary for temperature regulation of coronatine biosynthesis, there appear to be additional components necessary for thermoregulation of this toxin (20). Furthermore, this two-component system appears to regulate only coronatine biosynthesis. Likewise, phaseolotoxin production is thermoregulated in *P. syringae* pv. phaseolicola by a process involving a small, noncoding RNA and, potentially, a metabolic intermediate, although the details of this process are currently unknown (21–23). More generally, it is unknown whether there are any conserved global thermoregulators in *P. syringae* similar to those described in *E. coli*. A better understanding of the molecular basis of thermoregulation in *P. syringae* will advance our understanding of common versus lineage-specific thermoregulation across diverse taxa. Additionally, knowledge of how a temperature signal is integrated into the regulatory networks of *P. syringae* will help elucidate the phenotypes that are thermoregulated and, thus, further our understanding of the genetic regulation that contributes to this organism's ability to be a successful epiphyte and pathogen in an environment where the temperature fluctuates regularly.

Previously, we demonstrated that the production of both the flagellum and the lipopeptide surfactant syringafactin were thermoregulated in *P. syringae* and that this regulation was due, at least in part, to reduced transcription of *fliC* and *syfA* at 30°C compared to their transcription at cooler incubation temperatures (24). While we found that *flgM* was necessary for the thermoregulation

Received 16 January 2014 Accepted 8 April 2014
Published ahead of print 11 April 2014
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.00057-14.

doi:10.1128/JB.00057-14

of *fliC*, it did not play any role in the thermoregulation of *syfA*, suggesting that additional means of transcriptional regulation must be functioning in *P. syringae*. In this study, we characterized thermoregulators in *P. syringae* by assessing *syfA* expression in a large collection of random transposon mutants and showed that one mutation greatly influenced the transcriptome in a temperature-dependent manner.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture media, and growth conditions. *Pseudomonas syringae* pv. syringae B728a (25) and mutant derivatives were routinely cultured in King's medium B (KB) broth or on KB plates supplemented with 1.5% (wt/vol) Difco agar technical (BD, Sparks, MD) at 28°C (26). *Escherichia coli* strains TOP10 (Life Technologies, Carlsbad, CA), S17-1 (27), and SM10(λpir) (28) were cultured in Luria-Bertani (LB) medium broth or on LB plates supplemented with 1.5% (wt/vol) Difco agar technical at 37°C (29). Antibiotics were used at the following final concentrations: 100 μg/ml rifampin, 50 μg/ml kanamycin, 15 μg/ml gentamicin, 20 μg/ml spectinomycin, 15 μg/ml tetracycline, and 30 μg/ml nitrofurantoin (NFT). The strains and plasmids used in this work are listed in Table S1 in the supplemental material. The primers used in this work are listed in Table S2.

The plate temperatures were routinely monitored using a CZ-IR thermometer (ThermoWorks, Lindon, UT). Incubator temperatures and relative humidity were routinely monitored using HOBO data loggers (Onset, Bourne, MA).

Transposon mutagenesis. To facilitate a mutagenesis screen using a spectinomycin resistance-conferring transposon, the *syfA* promoter was digested from $pP_{syfA\text{-}gfp}$ (spectinomycin resistance) using HindIII and EcoRI restriction endonucleases (New England BioLabs, Ipswich, MA) and cloned into pPROBE-gfp[tagless], where the green fluorescent protein (GFP) has a long half-life (kanamycin resistance) (30). This construct was electroporated into *P. syringae* B728a. Transposon mutants were generated using an approach similar to that described in reference 31, where mini-Tn*5* (mTn*5*) Sm/Sp was introduced into *P. syringae* B728a [$pP_{syfA\text{-}gfp(K)}$]. Briefly, *E. coli* cells harboring pUT mini-Tn*5* Sm/Sp (32) and *P. syringae* cells harboring $pP_{syfA\text{-}gfp(K)}$ were grown overnight in either LB or KB broth with proper antibiotic amendment at 37°C or 28°C, respectively, with shaking at 200 rpm. Cells were mixed in an approximate ratio of 1:3 (*E. coli*-*P. syringae*) and incubated overnight on KB medium without antibiotic amendment. Culture lawns were resuspended in 10 mM KPO_4 buffer, spread onto KB plates amended with spectinomycin and kanamycin, and incubated at 28 to 29°C. After 2 to 3 days of incubation, plates were observed under UV illumination and colonies displaying an observable increase in fluorescence compared to that of the majority of colonies were streaked to isolation and rescreened at 28 to 29°C to ensure phenotype reproducibility. A plate containing colonies of wild-type (WT) *P. syringae* harboring $pP_{syfA\text{-}gfp(K)}$ was routinely included for comparison at all stages of screening.

Regions flanking transposon insertions were obtained using arbitrarily primed PCR similar to the method described in references 31 and 33. Genomic DNA was isolated from transposon mutants using a DNeasy blood and tissue kit (Qiagen, Valencia, CA), which served as the template in two sequential PCRs using tn5sm-ext (first round of amplification) and tn5sm-int (second round of amplification) (see reference 31 for primer sequences). The sequenced PCR products were compared to the *P. syringae* B728a genome using BLAST on the Integrated Microbial Genomes website (http://img.jgi.doe.gov).

Targeted deletion of *rppH* and ACDH and complementation. *rppH* and acyl-coenzyme A (CoA) dehydrogenase (ACDH) deletion mutants were constructed in a fashion similar to that previously described for *flgM* (24). Briefly, approximately one kilobase of upstream and downstream genomic DNA flanking either gene was amplified using Phusion DNA polymerase (Thermo Scientific [previously Finnzymes], Lafayette, CO) with the knockout (KO) primers listed in Table S2 in the supplemental material. Amplified genomic fragments were combined with a kanamycin-FLP recombination target (*kan*-FRT) PCR fragment amplified from pKD13 (ACDH) or pKD4 (*rppH*) (34) for three-fragment overlap-extension PCR. The combined fragments were cloned into pTOK2T (35) to create p*rppH*-KO and pACDH-KO. p*rppH*-KO and pACDH-KO were electroporated into S17-1, which was mated with *P. syringae* B728a. Kanamycin-resistant and tetracycline-sensitive colonies derived from these matings (indicative of double-crossover homologous recombination) were verified using PCR. pFLP2Ω was electroporated into each individual mutant, and single colonies isolated on KB amended with spectinomycin. Five kanamycin-sensitive colonies (indicative of Flp-mediated *kan* cassette excision) from each mutant were grown overnight in KB broth without antibiotic amendment, and 10 spectinomycin-sensitive colonies from each deletion were screened by PCR for loss of pFLP2Ω. *rppH* and ACDH deletion mutants (referred to here as the Δ*rppH* strain and the ΔACDH strain, respectively) were confirmed by sequencing. The pACDH-KO construct was mated into the Δ*rppH* mutant and processed as described above to construct a double-deletion mutant.

The *rppH* and ACDH complementation vectors, p*rppH* and pACDH, respectively, were constructed by amplifying *rppH* and ACDH coding sequences, along with their upstream promoter regions, using Phusion DNA polymerase (Thermo Scientific, Lafayette, CO) and native_comp primers (see Table S2 in the supplemental material), followed by blunt-end cloning into pVSP61 (36), which was digested with EcoRI and blunted with T4 DNA polymerase (New England BioLabs). The complementation constructs were sequenced using the same primers to verify insertion.

Detection of biosurfactants. Biosurfactants were detected using an atomized oil assay (31). Briefly, bacterial cells were grown overnight in KB broth with appropriate antibiotics and resuspended to a final concentration of 2×10^8 CFU/ml. Five microliters of resuspended cells was spot inoculated onto solid KB medium amended with appropriate antibiotics. The plates were incubated for ca. 24 h at the temperature indicated below, and the surfactant area detected by the formation of bright oil drops after a mist of mineral oil was sprayed onto the plate.

Transcriptional reporter assays. Transcriptional reporter assays were performed similarly to a previously described method (37). Briefly, cultures were resuspended in 10 mM KPO_4 buffer and diluted to a final optical density at 600 nm (OD_{600}) of 0.1 to 0.2. GFP fluorescence intensity was determined using a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) with a 486-nm band-pass excitation filter and a 510- to 700-nm combination emission filter. Relative GFP fluorescence was determined by normalizing the fluorescence intensity (arbitrary units) by the optical density.

RNA isolation and qRT-PCR. RNA isolation and quantitative reverse transcription-PCR (qRT-PCR) were performed as described in other studies (24). Briefly, cultures were harvested with RNA*later* (Life Technologies, Carlsbad, CA) and stored for no longer than 1 week at 4°C prior to RNA isolation. Total RNA was isolated using TRIzol reagent (Life Technologies). Total RNA was DNase treated with Turbo DNA-*free* (Life Technologies), followed by column purification using an RNeasy minikit (Qiagen). RNA was reverse transcribed using SuperScript II reverse transcriptase (Life Technologies) with random primers (Life Technologies). Quantitative PCR (qPCR) was performed using a 7300 real-time PCR system (Life Technologies) with QuantiTect SYBR green I (Qiagen) on diluted cDNA. Samples not treated with reverse transcriptase were routinely included and exhibited no amplification following 35 cycles. *rpoD* and Psyr_3981 (encodes pseudouridine synthase, a gene that was found to be stably expressed in multiple regulatory mutant backgrounds, as well as under several different culture conditions [K. L. Hockett, R. A. Scott, and S. E. Lindow, unpublished data]), were routinely used as endogenous controls.

mRNA sequencing. All protocols and analyses (including statistical tests for differentially expressed transcripts and assessment of gene functional enrichment) related to mRNA sequencing were performed exactly

as described previously (24). Briefly, total RNA was isolated as described above, with 16S and 23S rRNA removed using a Ribo-Zero rRNA removal kit (Gram-negative bacteria) (Epicentre, Madison, WI) according to the manufacturer's protocol. mRNA samples were assayed with a 2100 Bioanalyzer (Agilent, Santa Clara, CA) to confirm the removal of rRNA. mRNA and double-stranded DNA (dsDNA) were routinely quantified using the qBit RNA and dsDNA HS assay (Life Technologies, Carlsbad, CA), respectively. See Hockett et al. (24) for details regarding the construction of libraries to be sequenced using the Illumina HiSeq 2000. Three biological repeats were sequenced per temperature treatment per strain on three separate flow cells. Reads were aligned to the *P. syringae* pv. syringae B728a genome using Burrows-Wheeler Aligner (38), allowing for a maximum of three mismatches between a given read and the reference genome. The number of reads that overlapped with a given gene were counted using a custom script. Differential expression of genes and statistical significance were assessed using edgeR (39). Briefly, significance was established by comparing the gene expression levels, normalized by the trimmed mean of M values (TMM; where M is the log expression ratio per gene between treatments) (40), of three biological replicates incubated at either 20°C or 30°C (six samples total), using the empirical Bayes estimation and exact tests based on the negative binomial distribution. Alternatively, significance was established by comparing the gene expression levels of three biological replicates of the two strains (WT and Δ*rppH* strains) at either 20°C or 30°C. Genes were considered significantly differentially expressed if the *P* value (after adjustment for multiple comparisons) for a difference in expression between the two treatments was less than or equal to 0.05. Expression data are available at the Integrated Microbial Genome website (http://img.jgi.doe.gov). Thermoregulated genes in the WT strain are reported in reference 24. All genes thermoregulated in the Δ*rppH* strain are presented in Table S3 in the supplemental material. Genes differentially regulated between the Δ*rppH* strain and the wild-type strain at 20°C are presented in Table S4. Genes differentially regulated between the Δ*rppH* and the wild-type strain at 30°C are presented in Table S5.

RESULTS

A transposon mutagenesis screen uncovers mutations in genes encoding an ACDH and a Nudix hydrolase, causing overexpression of *syfA* at 28°C. Since the expression of *syfA* is suppressed at 28 to 30°C compared to its expression at cooler growth temperatures, we hypothesized that one or more negative regulators are necessary to mediate such thermorepression (24). To identify such regulators, we assessed the GFP fluorescence of a large collection of insertional mutants (mini-Tn*5*) of a *P. syringae* strain that harbored a plasmid containing a fusion of the *syfA* promoter (P_{syfA}) with a *gfp* reporter gene. A total of 13 mutants that exhibited a visible increase in GFP fluorescence compared to that of the wild-type strain were found in a screen of approximately 30,000 colonies grown at 28°C (Table 1). Six of the mutants were considerably more fluorescent than the other seven. Characterization of the sites of transposon insertion in the six highly fluorescent mutants revealed that two unique genes, Psyr_2474, encoding an acyl-CoA dehydrogenase (ACDH), and Psyr_4843, encoding a Nudix (nucleoside diphosphate linked to moiety X) hydrolase, were each disrupted independently in three mutants (Fig. 1). Because disruption of these two genes always conferred a pronounced hyperexpression of P_{syfA} at high temperatures and since such mutants were found multiple times, they were further investigated.

Psyr_2474 encodes an acyl-CoA dehydrogenase. Psyr_2474 is annotated as an acyl-CoA dehydrogenase, which is predicted to be involved in multiple metabolic pathways by the KEGG database (41). Generally, acyl-CoA dehydrogenases catalyze the desaturation at positions α,β of CoA-conjugated fatty acids derived from β-oxidation or amino acid metabolism (42). Both Psyr_2474 and Psyr_2473 (encoding a putative LysR-type transcriptional regulator) are highly conserved across diverse pseudomonads (see Table S6 in the supplemental material).

TABLE 1 Genes disrupted in transposon mutants of *Pseudomonas syringae* that overexpress *syfA* at 28 to 29°C

Locus tag	Annotation	Fluorescence	No. of times disrupted
Psyr_1083	Nucleoid-associated protein	+	1
Psyr_2474	Acyl-CoA dehydrogenase	++	3
Psyr_2759	Eukaryotic-like DNA topoisomerase I	+	1
Psyr_3575	Phenylalanine-4-hydroxylase, monomeric form	+	1
Psyr_3700	Protein of unknown function, DUF306	+	1
Psyr_4005	Hypothetical protein	+	1
Psyr_4202	Sodium:neurotransmitter symporter	+	1
Psyr_4493	PAS-GGDEF domains	+	1
Psyr_4843	Nudix hydrolase	++	3

Psyr_4843 encodes a putative Nudix hydrolase probably involved in mRNA turnover. Psyr_4843 is annotated as a Nudix hydrolase, an enzyme family widespread throughout the tree of life that catalyzes the general reaction in which a nucleoside diphosphate linked to moiety X is converted to a nucleoside monophosphate plus phosphate linked to moiety X (43). Psyr_4843 is closely related to RppH of *E. coli* based on both amino acid conservation (65% identity/80% similarity) and syntenic conservation (see Fig. S1 in the supplemental material). Additionally, since the KEGG database predicts that Psyr_4843 is the *P. syringae* B728a ortholog of *rppH*, we will refer to Psyr_4843 as *rppH*. RppH has recently been shown to stimulate mRNA decay through its action as a decapping enzyme, whereby it cleaves the 5′ pyrophosphate from an mRNA molecule, generating a 5′-monophosphorylated transcript (44). The resultant transcript, having a 5′-monophosphate, subsequently becomes a more favorable substrate for RNase E and RNase G, resulting in its degradation (45, 46).

ACDH and *rppH* knockout mutants require a higher incubation temperature than the WT strain for full thermorepression of *syfA* but are unaffected in thermorepression of *fliC*. The con-

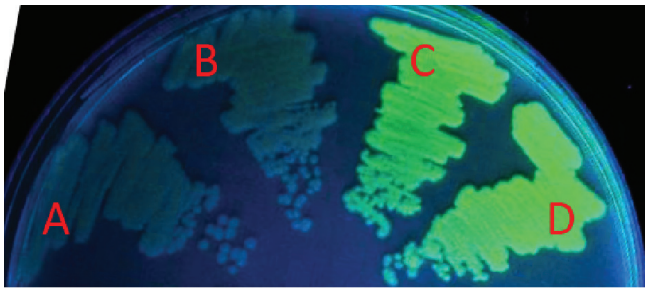

FIG 1 *syfA* promoter activity of selected transposon mutants. GFP fluorescence exhibited by wild-type *Pseudomonas syringae* B728a harboring a plasmid containing a fusion of the *syfA* promoter with a *gfp* reporter gene (A and B) and this strain having a Tn*5* insertion in Psyr_2474 (acyl-CoA dehydrogenase) (C) or Psyr_4843 (Nudix hydrolase) (D).

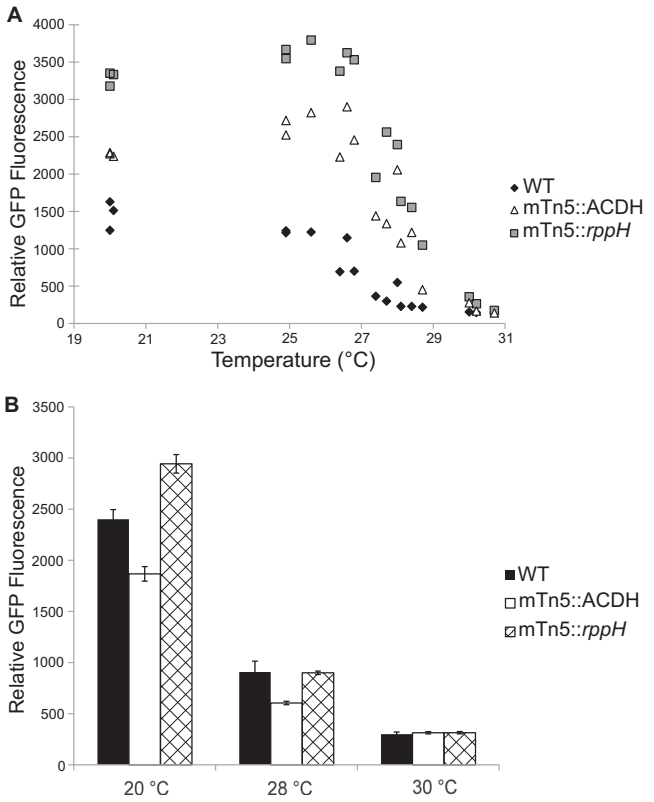

FIG 2 Effect of temperature on *syfA* and *fliC* promoter activities in transposon mutants. Cell-normalized GFP fluorescence obtained from growth at various temperatures of wild-type *Pseudomonas syringae* B728a (WT) and mutant strains containing the indicated Tn*5* insertions in the ACDH gene or *rppH* and harboring either *syfA*::*gfp* (A) or *fliC*::*gfp* (B) reporter gene fusions. Error bars in panel B represent the standard deviations of the means.

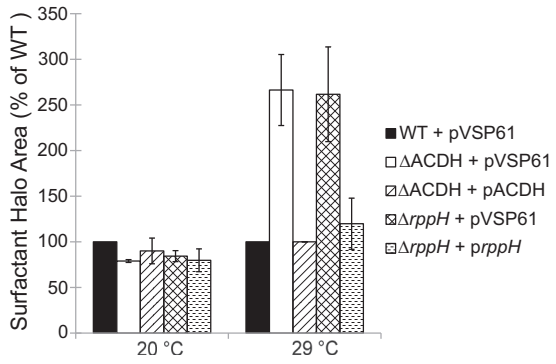

FIG 3 Complementation of ΔACDH and Δ*rppH* strains. Areas of surfactant spread on plates with colonies of wild-type *Pseudomonas syringae* B728a or mutant strains with knockout of a gene encoding ACDH or *rppH* and harboring either pVSP61 alone or pVSP61 containing Psyr_2474 (ΔACDH + pACDH) or Psyr_4843 (Δ*rppH* + p*rppH*), expressed as the percentage of the surfactant area produced by the wild-type strain. Error bars represent the standard deviations of the means.

tribution of *rppH* and ACDH to thermoregulation in *P. syringae* was examined in mutants in which these loci were disrupted (mTn*5*::*rppH* and mTn*5*::ACDH, respectively). Compared to the *syfA* expression in the wild-type strain, both mutants overexpressed *syfA* at 20°C, as well as at higher temperatures up to ~30°C, although both mutants expressed *syfA* at levels comparable to the level in the wild-type strain at the highest temperatures assayed (Fig. 2A). Neither mutant appeared to differ substantially from the wild-type strain in the expression of *fliC*, a gene previously shown to be thermorepressed (24), at any temperature, indicating that neither gene contributed to the thermoregulation of *fliC* (Fig. 2B).

To confirm the genetic basis of the mutant phenotypes described above, we constructed deletion mutants with a targeted deletion at each locus (ΔACDH and Δ*rppH* strains), as well as complementing them with the full-length genes. The deletion mutants exhibited phenotypes similar to those of the corresponding insertional mutants (the mTn*5*::*rppH* strain exhibited slightly greater *syfA* expression than the Δ*rppH* strain at both temperatures); both overexpressed *syfA* at 20°C and 29°C (see Fig. S2 in the supplemental material). Both mutants also produced more surfactant at 29°C, evident as a larger halo of modified oil drops surrounding colonies when assessed with an atomized oil assay (Fig. 3). In contrast, at 29°C, the surfactant production of the mutants harboring the corresponding gene expressed in *trans* from the native promoter in pVSP61 was much lower than that of the corresponding mutant itself and did not differ from that of the wild-type strain (Fig. 3).

***rppH* likely regulates *syfA* via *syfR*.** *syfA* is regulated by *syfR*, a LuxR-type regulator (31). Using qRT-PCR, we determined that *syfR* is thermoregulated similarly to *syfA*, with greater transcript abundance at cooler temperatures (see Fig. S3 in the supplemental material). Similar thermoregulation was observed using the *syfR* promoter fused to a *gfp* reporter gene (data not shown). The Δ*rppH* strain also exhibited a loss of *syfR* thermoregulation, similar to the results for *syfA* (see Fig. S3). Thermoregulation of both *syfR* and *syfA* are restored when *rppH* is complemented in *trans* (Fig. 4). These experiments were performed at warm temperatures between 28°C and 29°C, a temperature range where there is a significant difference between the *syfA* expression levels in the WT and Δ*rppH* strains. At higher temperatures (≥30°C), the transcript abundance of both *syfR* and *syfA* is strongly suppressed in both the WT and Δ*rppH* strain (see Fig. S4), consistent with the results of studies using *gfp* reporter gene fusions (Fig. 2A).

Transcripts associated with translation, amino acid import and metabolism, and other processes are not suppressed at high temperatures in a Δ*rppH* mutant. As *rppH* is involved in mediating mRNA turnover, we wanted to assess whether it may have a general role in the thermoregulation of transcript abundance in *P. syringae*. Our hypothesis was that there would be fewer transcripts whose abundances were decreased by incubation at warmer temperatures. To assess the role of *rppH* in global thermoregulation, we compared the transcriptome of the Δ*rppH* strain incubated at 20°C or 30°C to that of the wild-type strain incubated at the same temperatures. While this high temperature precluded us from observing an effect of the *rppH* mutation on *syfA* and *syfR* transcript abundances, it was necessary to enable a direct comparison with the temperature-dependent transcriptome in the wild-type strain, where 1,445 transcripts were thermoregulated (24).

In the Δ*rppH* strain, the expression of 1,150 transcripts was significantly different at the two temperatures (see Table S3 in the supplemental material); 636 were induced and 514 were repressed at 30°C compared to their expression at 20°C (Fig. 5A). Of the 1,150 temperature-regulated transcripts, 726 were also influenced

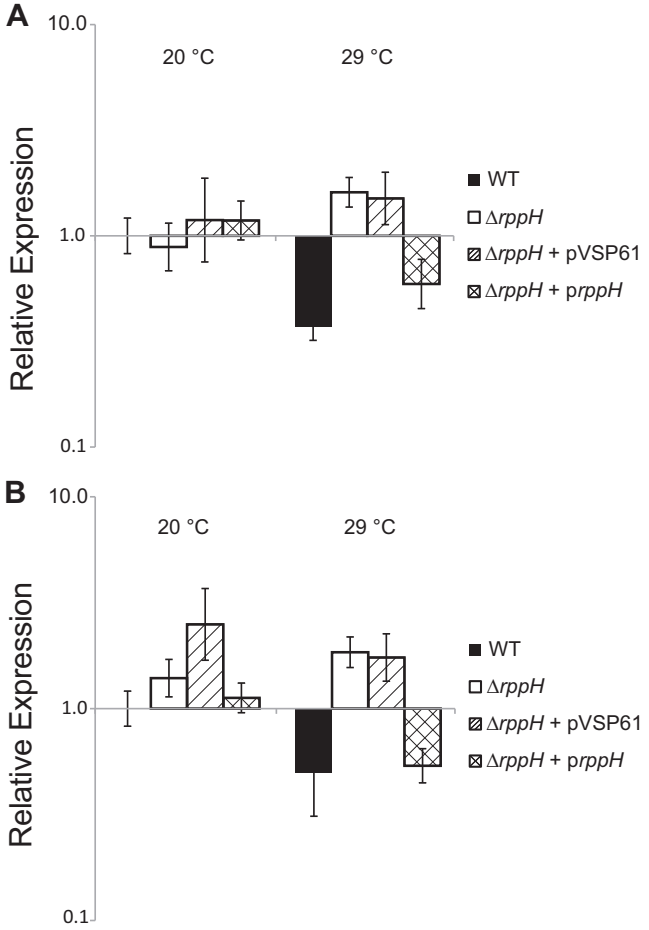

FIG 4 Effect of temperature on *syfA* and *syfR* transcript abundances in the Δ*rppH* strain with complementation. The transcript abundance of *syfR* (A) and *syfA* (B) was determined by qRT-PCR analysis of wild-type *Pseudomonas syringae* B728a and the Δ*rppH* strain harboring either no vector, pVSP61, or pVSP61 containing *rppH* and grown at 20°C or 29°C. All abundance values are calculated relative to the result for the wild-type strain grown at 20°C. Error bars represent the 95% confidence interval.

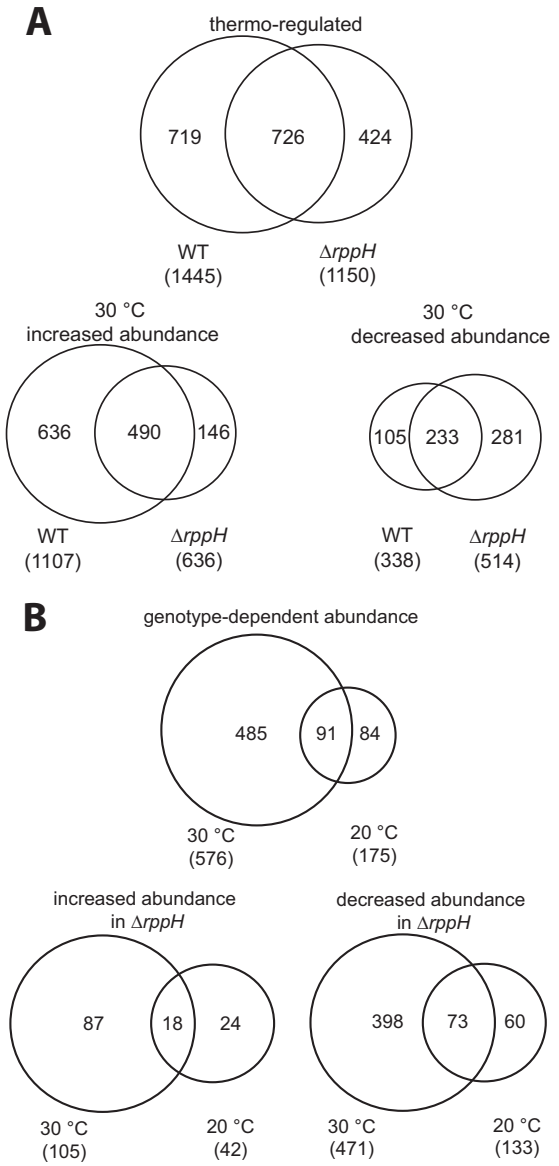

FIG 5 Effects of temperature and *rppH* deletion on *P. syringae* transcriptome. (A) Comparison of the numbers of thermoregulated transcripts in a wild-type strain and a Δ*rppH* mutant of *Pseudomonas syringae* B728a. (B) Comparison of the numbers of transcripts differentially regulated in the wild type and the Δ*rppH* mutant of *Pseudomonas syringae* at different growth temperatures.

by temperature in the wild-type strain (Fig. 5A). When grown at 30°C, a total of 576 transcripts differed significantly in expression in the Δ*rppH* strain compared to their expression in the wild-type strain (see Table S4); 105 transcripts were more abundant in the mutant, while 471 were less abundant in the mutant (Fig. 5B). When grown at 20°C, a total of 175 transcripts differed in abundance in the Δ*rppH* strain compared to their levels in the wild-type strain (see Table S5); 42 transcripts were more abundant in the mutant, while 133 were less abundant in the mutant (Fig. 5B). Taken together, these results demonstrated that deletion of *rppH* significantly affected the transcriptome of *P. syringae* and that its effect was largely but not entirely temperature dependent. For example, more than three times as many transcripts were misregulated in the Δ*rppH* strain compared to their regulation in the wild-type strain at 30°C than at 20°C.

While these results confirmed that *rppH* affects the transcriptome in a temperature-dependent manner, our hypothesis predicted that, in general, we would observe less thermosuppression in the Δ*rppH* strain than in the wild-type strain, a pattern that we did not observe. More transcripts were repressed at 30°C than at 20°C in the Δ*rppH* strain than in the wild-type strain (514 and 338 genes for the Δ*rppH* strain and the WT strain, respectively). Furthermore, more transcripts had lower rather than higher abundance in the Δ*rppH* strain compared to the wild-type strain at 30°C (471 and 105 transcripts suppressed and enhanced, respectively). That is, in the Δ*rppH* strain, there were more thermosuppressed transcripts than in the wild-type strain and the deletion of *rppH* appeared to exert a generally negative effect on transcript abundance at 30°C. Based on the predicted function of RppH, it seemed unlikely that this enzyme would directly increase the abundance of the transcripts of its target genes. A more likely possibility was that RppH negatively regulates an intermediate regulator(s) that is itself a negative regulator, potentially in a tem-

TABLE 2 Thermorepressed transcripts in wild-type *Pseudomonas syringae* B728a that are more abundant in the Δ*rppH* strain at 30°C

Category, locus tag	Gene	Ratio of thermorepressed transcripts for indicated strain(s) and temp(s)[a]			
		30°C/20°C		Δ*rppH* strain/WT strain	
		WT strain	Δ*rppH* strain	30°C	20°C
Translation					
Psyr_4525	*rpsD*	**0.5**	1.2	**2.6**	1.1
Psyr_4526	*rpsK*	**0.5**	1.1	**2.4**	1.1
Psyr_4538	*rplN*	**0.5**	1.1	**2.5**	1.0
Psyr_4539	*rpsQ*	**0.5**	1.1	**2.6**	1.1
Psyr_4540	*rpmC*	**0.4**	1.1	**2.6**	1.0
Psyr_4541	*rplP*	**0.5**	1.1	**2.4**	1.0
Psyr_4542	*rpsC*	**0.5**	1.2	**2.3**	1.0
Psyr_4543	*rplV*	**0.5**	1.1	**2.2**	1.1
Psyr_4544	*rpsS*	**0.5**	1.1	**2.2**	1.1
Psyr_4545	*rplB*	**0.5**	1.1	**2.3**	1.0
Psyr_4546	*rplW*	**0.5**	1.1	**2.7**	1.1
Psyr_4547	*rplD*	**0.4**	1.1	**2.7**	1.1
Psyr_4548	*rplC*	**0.5**	1.1	**2.4**	1.0
Psyr_4550	*tuf*	**0.5**	1.2	**2.3**	1.0
Psyr_4551	*fusA*	**0.5**	1.1	**2.3**	1.0
Psyr_4552	*rpsG*	**0.6**	1.1	**2.0**	1.0
Psyr_4557	*rplJ*	**0.6**	1.0	**1.9**	1.1
Amino acid metabolism and transport					
Psyr_1072	*aapJ*	**0.6**	1.6	**1.7**	0.6
Psyr_1073	*aapQ*	**0.4**	1.5	**2.2**	0.6
Psyr_1096	*gcvP*	**0.5**	0.9	**2.0**	1.2
Psyr_1097	*gcvH*-2	**0.5**	0.9	**2.5**	1.4
Psyr_2470	*liuA*	**0.4**	**0.5**	**1.9**	1.5
Psyr_3908	*gltI*	**0.4**	0.9	**2.6**	1.4
Psyr_3909	*gltJ*	**0.3**	0.9	**3.8**	1.3
Psyr_3910	*gltK*	**0.4**	0.9	**3.0**	1.3
Psyr_3911	*gltL*	**0.5**	0.8	**1.8**	1.2
Secretion/efflux/export					
Psyr_5134		**0.5**	0.9	**1.9**	1.1
Transport					
Psyr_3999		**0.2**	0.9	**5.5**	1.4
Psyr_4175		**0.2**	0.6	**10.7**	**3.9**
RNA degradation					
Psyr_1638	*rne*	**0.4**	0.8	**2.0**	1.0
Nitrogen metabolism					
Psyr_4817	*glnA*-1	**0.3**	0.8	**2.0**	0.8
Carbohydrate metabolism and transport					
Psyr_0944	*prsA*	**0.5**	1.1	**1.9**	0.9
Psyr_2440	*mltE*	**0.4**	**0.6**	**2.0**	1.4
Fatty acid metabolism					
Psyr_0749	*fadD*	**0.3**	**0.3**	**1.9**	**2.1**
Quaternary ammonium compound metabolism and transport					
Psyr_3238	*dhcB*	**0.5**	0.7	**1.9**	1.2
Transcription					
Psyr_4524	*rpoA*	**0.5**	1.2	**2.4**	1
Phytotoxin synthesis and transport					
Psyr_2601	*salA*	**0.5**	0.9	**3**	**1.9**
Transport (peptides)					
Psyr_4235		**0.5**	1.6	**2.7**	0.8
Psyr_4238	*dppA*-1	**0.5**	1.3	**2.1**	0.8
Hypothetical					
Psyr_0750		**0.1**	**4.1**	**3.5**	**2.1**
Psyr_5135		**0.5**	0.9	**1.9**	1.2
Unannotated					
Psyr_2239		**0.4**	1.5	**2.2**	1.4
Psyr_5136		**0.5**	0.9	**1.9**	1.2

[a] Values in boldface are significant at a *P* value of ≤0.05.

perature-dependent manner. To address this possibility, we determined those transcripts that were simultaneously (i) lower in abundance at 30°C than at 20°C in the wild-type strain and (ii) greater in abundance in the Δ*rppH* strain than in the WT strain at 30°C. Such transcripts were thermosuppressed in the wild-type strain, and this thermosuppression was dependent on *rppH*. There were 43 transcripts that fulfilled these criteria. This group was enriched in genes related to the functional categories of translation and amino acid transport and metabolism (Table 2). To discount those transcripts that might be overexpressed in the Δ*rppH* strain at both temperatures (indicative of temperature-independent overexpression in the Δ*rppH* strain), we determined whether any of these 43 transcripts were more abundant in the Δ*rppH* strain than in the wild-type strain at 20°C. Only four transcripts (Psyr_0749, Psyr_0750, Psyr_2601 [*salA*], and Psyr_4175) exhibited such temperature-independent increased abundance, whereas the other 39 transcripts were more abundant in the Δ*rppH* strain only at 30°C. Intriguingly, RNase E (Psyr_1638) was more abundant in the Δ*rppH* strain than in the wild-type strain at 30°C but not at 20°C. RNase E has recently been shown to stimulate 5′-monophosphate-dependent (i.e., RppH-dependent) transcript degradation, as well as 5′-monophosphate-independent (i.e., RppH-independent) transcript degradation. This suggested that the Δ*rppH* strain may exhibit increased turnover of RNase E-dependent but RppH-independent transcripts. Since RNase E was only overexpressed in the Δ*rppH* strain at 30°C and not at 20°C, this effect would be temperature dependent.

In addition to its temperature-dependent effects on the transcriptome, RppH affected the abundance of 90 transcripts regardless of the incubation temperature. Two functional categories (cell division and phytotoxin synthesis and transport) were more common among the 18 transcripts that were more abundant in the Δ*rppH* strain than in the wild-type strain (Table 3). The 72 transcripts that were generally less abundant in the Δ*rppH* strain were enriched for those encoding phage and insertion elements (Table 3). These results indicate that RppH exerts a complex regulatory effect on the *P. syringae* transcriptome, altering the abundance of transcripts in both temperature-dependent and temperature-independent manners.

TABLE 3 Gene functional categories enriched for those transcripts that were either increased or decreased in abundance in the Δ*rppH* strain relative to their abundance in the wild-type strain of *Pseudomonas syringae* when grown at different temperatures

Category, locus tag	Gene	Annotation	Abundance ratio for Δ*rppH* strain/WT strain at[a]: 20°C	30°C
Cell division				
Psyr_1611	*minE*	Cell division topological specificity factor	1.9	2.5
Psyr_1612	*minD*	Septum site-determining protein	1.9	2.7
Phage and IS elements				
Psyr_1030	*nfrB*	Bacteriophage N4 adsorption protein B	0.4	0.6
Psyr_2764	NA[b]	Hypothetical protein	0.5	0.5
Psyr_2766	NA	Hypothetical protein	0.4	0.5
Psyr_2805	NA	Hypothetical protein	0.4	0.5
Psyr_2806	NA	Bacteriophage lambda NinG	0.4	0.3
Psyr_2807	NA	NinB	0.3	0.2
Psyr_2808	NA	Hypothetical protein	0.2	0.1
Psyr_2809	NA	Hypothetical protein	0.3	0.2
Psyr_2810	NA	Hypothetical protein	0.3	0.3
Psyr_2811	NA	Hypothetical protein	0.2	0.2
Psyr_2816	NA	Hypothetical protein	0.2	0.2
Psyr_2817	NA	Hypothetical protein	0.2	0.3
Psyr_2818	NA	Hypothetical protein	0.2	0.3
Psyr_2819	NA	Hypothetical protein	0.1	0.4
Psyr_2820	*recT*	Recombinase	0.2	0.3
Psyr_2821	*recE*	Promotes recombination by RecT	0.3	0.4
Psyr_2822	NA	Hypothetical protein	0.3	0.4
Psyr_2823	NA	Hypothetical protein	0.3	0.5
Psyr_2828	NA	C-5 cytosine-specific DNA methylase	0.3	0.5
Psyr_2831	NA	Hypothetical protein	0.2	0.5
Psyr_2832	NA	Phage integrase	0.3	0.4
Psyr_2845	NA	Hypothetical protein	0.2	0.5
Psyr_2846	NA	Phage integrase	0.3	0.5
Psyr_4586	NA	Hypothetical protein	0.2	0.2
Psyr_4587	NA	Baseplate J-like protein	0.2	0.2
Psyr_4588	NA	Phage GP46	0.2	0.2
Psyr_4589	NA	Phage baseplate assembly protein V	0.2	0.2
Psyr_4590	NA	Bacteriophage Mu P	0.2	0.2
Psyr_4591	NA	DNA circulation	0.2	0.2
Psyr_4592	NA	Phage tail tape measure protein TP901	0.2	0.2
Psyr_4595	NA	Bacteriophage Mu tail sheath	0.2	0.2

[a] All values listed are significant at a *P* value of ≤0.05.
[b] NA, not available.

DISCUSSION

In this study, we used a high-throughput screen to identify thermoregulators of *syfA* and gained thereby broader insight into the genetic components that mediate responses to temperature in *P. syringae*. Two genes, ACDH and *rppH*, both appear to contribute to the proper thermoregulation of *syfA*, while *rppH* is essential for the thermoregulation of many other transcripts. These two genes thus appear to play a role in the temperature-dependent behavior of *P. syringae*. To our knowledge, this is the first report of the involvement of either of these genes in thermoregulation. Whether ACDH and *rppH* have a role in direct thermosensing or are involved in the signal cascade that regulates temperature-sensitive genes but do not sense the temperature themselves remains to be determined.

Both the ACDH and *rppH* mutant required higher incubation temperatures than the WT strain for complete thermorepression of *syfA*. These mutations, uncovered in a mutagenesis screen and confirmed by site-directed deletion mutagenesis, as well as by complementation, were clearly causative for the overexpression of *syfA* and surfactant at 28 to 29°C. However, both the ΔACDH strain and the Δ*rppH* strain exhibited repression of *syfA* at temperatures of ≥30°C, indicating that additional thermoregulator(s) control temperature-dependent syringafactin expression. Preliminary studies indicated that stacked deletions of ACDH and *rppH* (ΔACDH/Δ*rppH*) resulted in loss of thermoregulation of *syfA* at 30°C but that this mutant still failed to produce syringafactin at this or higher temperatures (K. L. Hockett and S. E. Lindow, unpublished results). These results suggest that there may be layers of thermoregulation, where ACDH and *rppH* are important for thermoregulation at moderately warm temperatures while other factors ensure thermoregulation at warmer temperatures. The finely tuned sensitivity to incubation temperature, where there is a phenotypic difference between the Δ*rppH* and wild-type strains at 28 to 29°C with regard to *syfA* expression but no discernible difference between the strains at 30°C or warmer temperatures, is notable. Such precise regulation is not wholly surprising, given that bacterial RNA-based temperature sensors are able to respond to 1°C changes in temperature (47). While it is not clear why the transition from 29°C to 30°C results in the *rppH* deletion strain no longer allowing for increased expression of *syfA* above the level in the wild-type strain, we can speculate that at the warmer temperatures (≥30°C), the cells are likely starting to experience heat stress. The optimal growth temperature for *P. syringae* is 28°C, and it exhibits a marked decrease in growth rate at around 32°C or warmer (48). Indeed, we have observed that wild-type cells incubated at ca. 32°C are elongated compared to cells incubated at cooler temperatures, likely indicating arrest of cell division as a stress response. Whatever the cause of the transition in phenotype at 30°C, further studies of the interaction between ACDH and *rppH*, as well as the potential involvement of additional, yet-to-be-identified thermoregulators, will be fruitful.

Our understanding of the role of ACDH in thermoregulation

remains limited to its effect on *syfA* expression. Based on its predicted function and the conservation of neighboring genes in different taxa, we speculate that it modifies the pool of a key metabolic intermediate(s), such as an amino acid(s) or fatty acid(s) that regulates the expression of *syfA*. As the syringafactins are a family of closely related lipopeptides, consisting of an acyl chain linked to a short peptide head group rich in leucine (49), ACDH could be involved in one or more pathways linked to the production of syringafactin precursors. The LysR-type regulator encoded by the adjacent, oppositely oriented gene Psyr_2473 is a likely regulator of such a metabolic intermediate, as this arrangement of genes encoding enzymes flanked by those encoding a LysR-type regulator is common (50).

We chose to perform temperature-dependent global transcriptome analysis on the Δ*rppH* strain because of a possibility that this gene is involved in controlling transcript stability, based on its characterized function in *E. coli* (44). Our results suggest that RppH regulates transcripts in both a temperature-dependent and -independent manner. A potential explanation for such a phenomenon would be that temperature-dependent regulation of a transcript is due to the 5′ untranslated region (UTR) of a particular transcript rather than being an inherent quality of RppH activity. Thermoregulation of translation by RNA thermometers (RNATs) is well described (51, 52). In RNATs, temperature-dependent conformational changes in the 5′ UTR of a temperature-sensitive transcript either allow or block access of the ribosome to the ribosomal binding site, thus allowing or inhibiting translation. Such conformational changes are the consequence of alternative, temperature-dependent stem-loop structures forming and can result from either *trans*-acting (intermolecular) or *cis*-acting (intramolecular) interactions. Temperature-dependent transcript stability has also been described, but the manner in which temperature regulates transcript stability remains poorly understood. Two well-described examples of temperature-dependent RNA stability include the *cspA* transcript, encoding a cold shock protein in *E. coli* (53), and the small RNA DsrA, which is important in establishing *rpoH* expression at cool but not warm temperatures (15). In both examples, the RNA molecules are stable at cool temperatures but are rapidly degraded when shifted to a warmer temperature. Importantly, in both cases, processing by RNase E is involved in processing the RNA molecules into inactive forms (53, 54). Despite the involvement of RNase E in processing these RNAs into inactive forms, a mechanistic understanding of how temperature alters their stability remains to be described. Our model of RppH-mediated thermoregulation is mechanistically similar to the function of RNATs in that thermoregulated transcripts might possess a 5′ UTR stem-loop structure that maintains transcript stability at cool temperatures but melts at warmer temperatures, thus facilitating processing by RppH and RNase E. Both RppH-mediated decapping and RNase E-mediated cleavage are inhibited by stem-loop structures at the 5′ terminus of transcripts (44, 55–57).

One of the most striking temperature-dependent phenotypes for which *rppH* is required is the expression of ribosomal proteins (Table 2). Previous work in *Pseudomonas* indicated that elevated expression of the ribosomal protein operon *infC-rpmI-rplT* (by increasing the copy number) partially restored toxin and protease production in *gacS* and *gacA* mutants of *P. syringae* and *Pseudomonas fluorescens* (58, 59), suggesting that the balance of ribosomal proteins in the cell can modulate certain phenotypes. We thus hypothesize that the ribosome itself might potentially play a role in mediating temperature-dependent phenotypes. There is evidence to support this hypothesis. Ribosomal function appears to be a regulatory input for both the cold shock and heat shock response, given that translation-inhibiting antibiotics can stimulate the cold shock response in both *E. coli* and *Bacillus subtilis* at sublethal doses (60, 61), while depletion of 4.5S rRNA or treatment with antibiotics that decrease translational fidelity induce the heat shock response (60, 62). This suggests that perturbation of ribosome function can mimic the effects of a large temperature shift. In addition, one of the cellular effects of heat shock is the downregulation of a subset of ribosomal proteins (63, 64). Conversely, the expression of ribosomal or ribosome-associated proteins of *B. subtilis* increases following cold shock, as well as during prolonged cool incubation (65–67). It has been hypothesized that the modification of ribosomes to function at cooler temperatures is one of the major acclimation processes that occurs when cells experience cool temperatures (68). More pertinently, in *E. coli*, RppH associates with ribosome precursor particles, and its abolishment leads to an increase of the ratio of 30S to 70S ribosomal complexes (69), suggesting that disruption of *rppH* may lead to altered ribosomal function in *P. syringae*. If ribosomal function is, in fact, altered in the Δ*rppH* strain, we might expect an even more pronounced change of the proteome than of the transcriptome.

The mRNA-decapping function of RppH also results in RNase E- and RNase G-mediated degradation of messages and is complicated by the fact that mRNA turnover can be catalyzed by several independent mechanisms. Most relevant to this work is the recognition that RNase E itself can cleave transcripts in both a 5′-monophosphate-dependent and -independent manner, these functions being genetically separable (70, 71). Our data indicated that the RNase E transcript, like the transcripts for ribosomal proteins, were more abundant at 20°C than at 30°C in the wild-type strain, while the Δ*rppH* strain expressed these transcripts equally at both temperatures. There are no previous reports of temperature-regulated RNase E transcript abundance. In *E. coli*, the *rne* transcript is autoregulated but in an RppH-independent manner, in that RppH does not cleave pyrophosphate from the *rne* transcript (72). Our results indicated that RppH is involved in the regulation of *rne* in *P. syringae* but only at warm temperatures. A similar effect was observed in *E. coli*, where deletion of *rppH* led to an apparent increase in RNase E activity (as measured by RNase E autoregulation) (71). This result suggests that while degradation of transcripts via an RppH-dependent mechanism may be abolished in an RppH mutant, it could stimulate the degradation of transcripts via an RppH-independent but RNase E-dependent mechanism, which would functionally resemble thermoregulation due to its preferential effect at higher temperatures.

ACKNOWLEDGMENTS

We are grateful to Gwyn Beattie for providing the gene functional category assignments for *P. syringae*. We thank Y. Mai for technical assistance with collection and analysis of transposon mutants. We thank Konstantinos Billis at the Joint Genome Institute for determining the gene read counts.

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