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Regulatory Mechanisms of Exoribonuclease PNPase and Regulatory Small RNA on T3SS of *Dickeya dadantii*

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The type III secretion system (T3SS) is an essential virulence factor for many bacterial pathogens. Polynucleotide phosphorylase (PNPase) is one of the major exoribonucleases in bacteria and plays important roles in mRNA degradation, tRNA processing, and small RNA (sRNA) turnover. In this study, we showed that PNPase downregulates the transcription of T3SS structural and effector genes of the phytopathogenic bacterium *Dickeya dadantii*. This negative regulation of T3SS by PNPase occurs by repressing the expression of *hrpL*, encoding a master regulator of T3SS in *D. dadantii*. By reducing *rpoN* mRNA stability, PNPase downregulates the transcription of *hrpL*, which leads to a reduction in T3SS gene expression. Moreover, we have found that PNPase downregulates T3SS by decreasing *hrpL* mRNA stability. RsmB, a regulatory sRNA, enhances *hrpL* mRNA stability in *D. dadantii*. Our results suggest that PNPase decreases the amount of functional RsmB transcripts that could result in reduction of *hrpL* mRNA stability. In addition, bistable gene expression (differential expression of a single gene that creates two distinct subpopulations) of *hrpA*, *hrpN*, and *dspE* was observed in *D. dadantii* under in vitro conditions. Although PNPase regulates the proportion of cells in the high state and the low state of T3SS gene expression, it appears that PNPase is not the key switch that triggers the bistable expression patterns of T3SS genes.

Polynucleotide phosphorylase (PNPase), encoded by *pnp,* is widespread in prokaryotes as well as in the chloroplasts and mitochondria of eukaryotes. It is one of the most important exoribonucleases in the cell (Kinscherf and Apirion 1975; Kudla et al. 1996; Leszczyniecka et al. 2002) and has both 3′ to 5′ exoribonuclease activity and 3′-terminal oligonucleotide polymerase activity (Mohanty and Kushner 2000). Based on sequence homology, PNPase in most prokaryotes contains four conserved functional domains. An S1 domain and a KH domain are located at the C-terminus of PNPase, both of which are critical for the RNA binding and substrate recognition activity of PNPase (Stickney et al. 2005; Amblar et al. 2007). Two RNase PH-like domains are located at the N terminus, which are responsible for PNPase's phosphate-dependent exonuclease activity and its RNA polyadenylation activity (Zuo and Deutscher 2001).

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PNPase is involved in mRNA degradation, tRNA processing and degradation, small RNA (sRNA) turnover, and the addition of heteropolymeric tails to some RNAs (Kinscherf and Apirion 1975; Li and Deutscher 1994; Mohanty and Kushner 2000; Oussenko et al. 2005; Viegas et al. 2007). In bacteria, RNA degradation usually occurs in two stages. During the first stage, full-length RNA is cleaved into short fragments of RNA decay intermediates by endoribonucleases such as RNase E, RNase G, RNase III, and RNase P (Mackie 1998; Kushner 2002; Deutscher 2006). Following the initial endonucleolytic attack, these decay intermediates undergo complete breakdown into mononucleotides by exoribonucleases and oligoribonucleases. PNPase, along with other exoribonucleases such as RNase II and RNase R, contribute to the second stage of the RNA degradation process through its 3′ to 5′ exonuclease activity (Donovan and Kushner 1986; Deutscher 2006). In addition to exonuclease activity, PNPase can function as a poly(A) polymerase by adding nucleotides to an existing poly(A) tail of mRNA transcribed by PAP1 (Kinscherf and Apirion 1975; Li and Deutscher 1994; Mohanty and Kushner 2000; Oussenko et al. 2005; Viegas et al. 2007). The heteropolymeric tails synthesized by PNPase may further enhance the degradation of mRNA intermediates by disrupting the secondary structure at the 3′ termini of the RNA.

Several cellular metabolic processes are regulated by PNPase through its post-transcriptional control of mRNA. Yamanaka and Inouye (2001) have demonstrated that PNPase-mediated RNA degradation is required for the regulation of the cold-shock response in *Escherichia coli*. They discovered that a number of specific mRNAs induced by cold shock are degraded by PNPase at the end of the acclimation phase (Yamanaka and Inouye 2001; Polissi et al. 2003). In addition, PNPase regulates cellular responses to environmental changes by controlling regulatory sRNA turnover. Viegas and associates (2007) showed that PNPase is important for the decay of four regulatory sRNAs: CsrB, CsrC, MicA, and SraL. Andrade and Arraiano (2008) also illustrated that PNPase is important in controlling the amount of regulatory sRNA required for the expression of some outer-membrane proteins. Together, these reports demonstrate that, in addition to its role in the general degradation of RNA, PNPase-controlled RNA degradation can also be specific.

Dickeya dadantii 3937 (formerly *Erwinia chrysanthemi* 3937) is a gram-negative phytopathogenic bacterium that causes soft rot, wilt, and blight diseases on a wide range of plants. *D. dadantii* possesses a type III secretion system (T3SS), a major virulence factor in many gram-negative plant and animal pathogens (Alfano and Collmer 1997, 2004; Galan and Collmer 1999; Hueck 1998). The T3SS of *D. dadantii* is encoded by several structural and effector genes, such as *hrpA*, *hrpN*, and *dspE*,

which encode a T3SS pilus, harpin, and effector, respectively. The transcription of T3SS structural and effector genes is controlled by the master regulator and alternative σ factor, HrpL (Tang et al. 2006). *hrpL* expression is activated by RpoN (σ^{54}) in conjunction with HrpS, an NtrC family enhancer protein (Yap et al. 2005; Tang et al. 2006). The transcription of *hrpS* is upregulated by a two-component signal transduction system (TCSS), HrpX/HrpY (Tang et al. 2006). In addition to regulation at the transcriptional level, the expression of *hrpL* is also controlled at the post-transcriptional level by the RsmA/RsmB pair (Yang et al. 2008a,b). RsmA (repressor of stationary phase metabolites) is a sRNA-binding protein that binds to and promotes the degradation of *hrpL* mRNA. RsmB is a sRNA molecule that binds to RsmA and neutralizes its degradation effect on *hrpL* mRNA (Liu et al. 1998). The expression of RsmB is upregulated by another TCSS, GacS/GacA (Yang et al. 2008b).

Although the regulatory mechanism is unclear, microarray analysis of *Salmonella enterica* showed that PNPase affects mRNA levels of virulence genes located in *Salmonella* pathogenicity islands 1 and 2 that encode a syringe-like organelle of the T3SS (Clements et al. 2002). In *Yersinia pseudotuberculosis* and *Y. pestis*, PNPase is required for the optimal function of T3SS (Rosenzweig et al. 2005, 2007; Rosenzweig and Schesser 2007). In this report, we demonstrate that the PNPase of *D. dadantii* 3937 reduces mRNA stability of a key T3SS regulator, RpoN. RpoN instability results in a repressive effect on *hrpL* which ultimately downregulates the transcription of T3SS genes. Our results also suggest a role for PNPase in the turnover of an untranslated regulatory sRNA, RsmB, which may further affect the expression of T3SS genes in *D. dadantii.* Finally, bistable expression patterns of T3SS structural and effector genes were observed in *D. dadantii* 3937 and the regulatory effect of PNPase on the bistable expression of T3SS genes was examined.

RESULTS

PNPase downregulates the transcription of T3SS structural and effector genes.

The effects of PNPase were analyzed on three representative T3SS structural and effector genes: *hrpA*, *hrpN*, and *dspE*. A *pnp* deletion mutant (Ech152) (Table 1) was constructed and the mRNA levels and promoter activities of *hrpA*, *hrpN*, and *dspE* were examined in Ech152 and the wild-type strain, *D. dadantii* 3937. Northern blot analysis revealed an increase in *hrpA* mRNA in Ech152 (Fig. 1A). The *hrpA* mRNA was restored to the wild-type level upon complementation of Ech152 with pML1231 (Fig. 1A). The mRNA levels of *hrpN*, and *dspE*, along with *hrpA*, were also measured by real-time polymerase chain reaction (PCR) in *D. dadantii* 3937 and Ech152. Compared with the wild type, significantly higher amounts of *hrpA* (relative expression ratio 16.44, $P = 0.002$), *hrpN* (relative expression ratio 48.39, $P \leq 0.001$), and *dspE* (relative expression ratio 34.51, $P \leq 0.001$) mRNA were observed in Ech152 (Fig. 1B). Promoter activities of *hrpA, hrpN*, and *dspE* were also examined in *D. dadantii* 3937 and Ech152 carrying plasmids with promoter green fluorescence protein (GFP) transcriptional fusions phrpA, phrpN, and pdspE, respectively. Higher promoter activities of *hrpA, hrpN*, and *dspE* were observed in Ech152 by a fluorescence-activated cell sorter (FACS) (Fig. 1C). These results strongly suggest that PNPase downregulates the transcription of *hrpA*, *hrpN,* and *dspE*.

PNPase downregulates *hrpL* transcription by affecting *rpoN* mRNA stability.

In *D. dadantii* 3937, HrpL is a master regulator of genes encoding T3SS structural and effector proteins. Therefore, downregulation of *hrpL* transcription would lead to a reduction in the expression of *hrpA*, *hrpN*, and *dspE*. To investigate the regulatory effect of PNPase on *hrpL*, both the mRNA level and promoter activity of *hrpL* were examined in the wild type and Ech152. Compared with the wild-type strain, an increase in both *hrpL* promoter activity and mRNA level was observed in Ech152 (Fig. 2A and B). This indicates that the transcription of *hrpL* is downregulated by PNPase, which explains the decrease in *hrpA, hrpN*, and *dspE* transcription. Because *hrpL* is upregulated by RpoN and HrpS at the transcriptional level (Chatterjee et al. 2002; Yap et al. 2005), the mRNA levels of *rpoN* and *hrpS* in *D. dadantii* 3937 and Ech152 were also examined. Compared with *D. dadantii* 3937, an 11-fold increase of *rpoN* mRNA levels ($P \leq 0.001$) was observed in the *pnp* mutant (Fig. 2B). No significant difference in *hrpS* mRNA level ($P > 0.05$) was observed between the wild-type strain and Ech152 (Fig. 2B). This indicates that the increase in *hrpL* promoter activity observed in Ech152 is due to the increased amount of *rpoN* mRNA. The *rpoN* mRNA stability was then tested in *D. dadantii* 3937 and Ech152 (Fig. 3A). Compared with the wild-type strain, the *rpoN* mRNA decay rate was much slower in Ech152 (*rpoN* mRNA half-life: wild type, 8.64 min; Ech152, 241.38 min). Together, these results demonstrate that PNPase functions to reduce *rpoN* mRNA stability which, in turn, has negative effects on *hrpL* transcription and, consequently, on the transcription of the T3SS structural and effector genes.

PNPase affects *hrpL* mRNA stability and RsmB sRNA transcript pattern.

In *D. dadantii* 3937, in addition to the transcriptional regulation by RpoN and HrpS, the *hrpL* mRNA level is also controlled through the GacS-GacA-RsmB-RsmA-HrpL post-transcriptional regulatory pathway (Yang et al. 2008a,b). To determine whether PNPase controls *hrpL* at the post-transcriptional level, an RNA decay assay was performed to examine the *hrpL* mRNA stability in *D. dadantii* 3937 and Ech152 (Fig. 3B). A reduction in the *hrpL* mRNA decay rate (*hrpL* mRNA half-life: wild type, 9.16 min; Ech152, 14.46 min) was observed in Ech152 compared with *D. dadantii* 3937, suggesting that PNPase is involved in the post-transcriptional control of *hrpL* mRNA levels.

Previous reports have shown that PNPase plays an important role in sRNA turnover (Viegas et al. 2007; Andrade and Arraiano 2008). RsmB is an abundant regulatory sRNA in *D. dadantii* that has an essential role in the post-transcriptional regulation of *hrpL* by antagonizing RsmA and alleviating the degradation effect of RsmA on *hrpL* mRNA. We hypothesize that PNPase may regulate *hrpL* through RsmA-RsmB. Northern blot analysis was performed to compare the RsmB RNA levels in *D. dadantii* 3937 and Ech152. A major RsmB transcript of approximately 250 nucleotides (nt) was observed in both the wild type and the *pnp* mutant (Fig. 4A). Interestingly, in Ech152, in addition to the 250-nt transcript, shorter RsmB transcripts of approximately 155, 170, and 210 nt were observed in abundance (Fig. 4A). The RsmB transcript pattern was restored to that of the wild type when Ech152 was complemented with pML1231. Similar levels of *rsmA* mRNA were observed in *D. dadantii* 3937 and Ech152, suggesting that PNPase does not have a regulatory effect on *rsmA* (Fig. 4B). The total amount of RsmB transcripts (comprising full-length transcripts plus truncated transcripts) in Ech152 is much higher than the total amount of RsmB RNA transcripts (comprising full-length transcripts only) in *D. dadantii* 3937.

RsmB truncated transcripts in *pnp* mutant have intact 5′ ends and incomplete 3′ ends.

After deletion of *pnp*, an abundance of truncated RsmB transcripts were observed in Ech152. For this reason, we sought to

determine which parts were missing from the truncated RsmB transcripts in Ech152. Therefore, a primer extension assay was performed to determine whether the truncated RsmB transcripts have the same 5′ ends as the full-length RsmB transcripts in the wild type. Two primers were designed for use in the primer extension analysis: primer 1 and primer 2 that anneal to the 5′ and 3′ ends of RsmB, respectively (Fig. 5B). Initial reactions with primer 1 yielded a single primer extension product (band I) in both the wild type and Ech152, which indicates that the 5′ ends of the RsmB transcripts in both strains are intact and identical

Table 1. Strains, plasmids, and primers used in this study

Strains, plasmids, and primers[a]	Characteristics or sequences (5′ to 3′)[b]	Reference or source
Strains		
Dickeya dadantii 3937	Wild-type strain of *D. dadantii* isolated from *Saintpaulia ionantha*	Hugouvieux-Cotte-Pattat, N., UMR-CNRS, Villeneuve, France
Ech152	*D. dadantii* 3937 Δ*pnp*::kan, Km^r	This work
Ech153	*D. dadantii* 3937 with transposon mini*Himar RB1* insertion in *rsmB*, Km^r	Yang et al. 2008a
Plasmids		
pWM91	Sucrose-based counter-selectable plasmid, Ap^r	Metcalf et al. 1996
pML123	RSF1010-derived expression and *lac*-fusion broad-host-range vector, Gm^r	Labes et al. 1990
pML1231	pML123 derivative with PCR fragment containing *pnp*, Gm^r	This work
pML1233	pML123 derivative with PCR fragment containing full length *rsmB*, Gm^r	This work
pML1234	pML123 derivative with PCR fragment containing *rsmB* with an intact 5′ end and an incomplete 3′ end lacking 41 bp, Gm^r	This work
pML1235	pML123 derivative with PCR fragment containing *rsmB* fragment with an intact 5′ end and an incomplete 3′ end lacking 88 bp, Gm^r	This work
pPROBE-AT	Promoter-probe vector, Ap^r	Miller et al. 2000
phrpA	pPROBE-AT derivative with PCR fragment containing *hrpA* promoter region, Ap^r	Yang et al. 2008B
phrpN	pPROBE-AT derivative with PCR fragment containing *hrpN* promoter region, Ap^r	YangB et al. 2008
pdspE	pPROBE-AT derivative with PCR fragment containing *dspE* promoter region, Ap^r	Peng, Yang et al. 2006
phrpL	pPROBE-AT derivative with PCR fragment containing *hrpL* promoter region, Ap^r	Yang et al. 2007
phrpS	pPROBE-AT derivative with PCR fragment containing *hrpS* promoter region, Ap^r	Yang et al. 2008A
prsmA	pPROBE-AT derivative with PCR fragment containing *rsmA* promoter region, Ap^r	This work
Primers		
PNP_A	TCGGCGCCTGTTCTATCTAT	This work
PNP_B	GGGACTCTGGGGTTCGAAATCTAGAATCCATGCTGACCATTAC	This work
PNP_C	CCAGTAGCTGACATTCATCCCTCGAGATTTCAAAGTGGCCGGTA	This work
PNP_D	CATCGTAAGGAGCTGCCAATC	This work
PNP_F	GACCTAGGCGAGAAGCTG	This work
PNP_R	CTATTGCTGGTCCGATCC	This work
rsmB_F	GTCGCCACGCAGGATAGC	This work
rsmB_R	CATGGTATCGATGCAGAACG	This work
rsmB1234_F	CAAGAGCTCACCTTGTTGCTTCCCTGCTC	This work
rsmB1234_R	GCATCTAGAGTCGCCACGCAGGATAGC	This work
rsmB1235_F	CAAGAGCTCGTCCCTGATGGTCCTCCTA	This work
rsmB1235_R	GCATCTAGAGTCGCCACGCAGGATAGC	This work
Terminator_F	GCATCTAGAATCGGTTCCCTCTTTTCGTT	This work
Terminator_R	CATGGATCCCTCCAGCATCCTCACTCCTC	This work
rplU forward-qRTPCR	GCGGCAAAATCAAGGCTGAAGTCG	This work
rplU reverse-qRTPCR	CGGTGGCCAGCCTGCTTACGGTAG	This work
16S forward-qRTPCR	GTCATCATGGCCCTTACGAG	This work
16S reverse-qRTPCR	CCGGACTACGACGCACTTTA	This work
hrpA forward-qRTPCR	CAGCAATGGCAGGCATGCAG	This work
hrpA reverse-qRTPCR	CTGGCCGTCGGTGATTGAGC	This work
hrpN forward-qRTPCR	TCGGCAGCGGTCTGAACGAC	This work
hrpN reverse-qRTPCR	CCAGCGACAACGGCGAGAA	This work
dspE forward-qRTPCR	GATGGCGGAGCTGAAATCGTTC	This work
dspE reverse-qRTPCR	CCTTGCCGGACCGCTTATCATT	This work
rpoN forward-qRTPCR	ACTGGCGCTGGAAAGCAACC	This work
rpoN reverse-qRTPCR	GGCAGCTCGTCGGGCATATC	This work
hrpL forward-qRTPCR	GATGATGCTGCTGGATGCCGATGT	This work
hrpL reverse-qRTPCR	TGCATCAACAGCCTGGCGGAGATA	This work
hrpS forward-qRTPCR	TGGAAGGCGAAACCGGCACC	This work
hrpS reverse-qRTPCR	GCACGGCGGCGCAGTTCAC	This work
hrpA forward-Northern	GGGACTTTCTAACGCAGCAG	This work
hrpA reverse-Northern	AATAGCTTTGGCCGCTTTCT	This work
rsmA forward-Northern	CGAGTTGGCGAAACCCTCAT	This work
rsmA reverse-Northern	AATAGGAGGTAGGCTGAGAC	This work
rsmB forward-Northern	CGCGATTTTTGTACGGCTAT	This work
rsmB reverse-Northern	CGATTTCTCGGTTCCCTCTT	This work
RsmB primer 1	TTTAGGCTCCTGCCCCACC	This work
RsmB primer 2	CTCTTAGTTCGTTTGCAGCAGTCC	This work
RsmB probe I	CCCCACCGGCATTCCCAGGCCGGCTCTCATTCTCCATCCTGGAGGTGTCCCTAA TTTCATCCTGAAA	This work
RsmB probe II	AGGGAACCGATTTCTCGGTTCCCTCTTAGTTCGTTTGCAGCAGTCCCGCTACCT TGTTGCTTCCCTGCTCGTCCTT	This work

[a] qRTPCR = quantitative reverse-transcription polymerase chain reaction.
[b] Km^r Ap^r and Gm^r indicate kanamycin, gentamycin, and ampicillin resistance, respectively. PCR = polymerase chain reaction.

(Fig. 5A and B). In addition, the primer extension signal produced by primer 1 was considerably stronger in Ech152 compared with the wild type (signal intensity ratio of Ech152/*D. dadantii* 3937 = 4.38 using primer 1) (Fig. 5A). Based on the signal intensity, this result also suggests that the abundant truncated RsmB transcripts observed in Ech152 have intact 5′ ends that could be detected in abundance by primer 1 in the primer extension assay.

Subsequent primer extension analysis using primer 2 (anneals to 3′ end of RsmB) was also performed to compare the 5′ ends of RsmB transcripts in the wild type and Ech152. Reactions with primer 2 also yielded a single primer extension product (band II), indicating that the 5′ ends of RsmB are identical in Ech152 and wild-type strains. Interestingly, the Ech152 primer extension signal produced by primer 2 was only slightly more intense than the signal in the wild-type strain (signal intensity ratio of Ech152/*D. dadantii* 3937 = 1.57 using primer 2) (Fig. 5A). Because primer 2 anneals to the 3′ end of RsmB, only those transcripts with intact 3′ ends will produce a primer extension signal (Fig. 5B, scheme of primer extension analysis

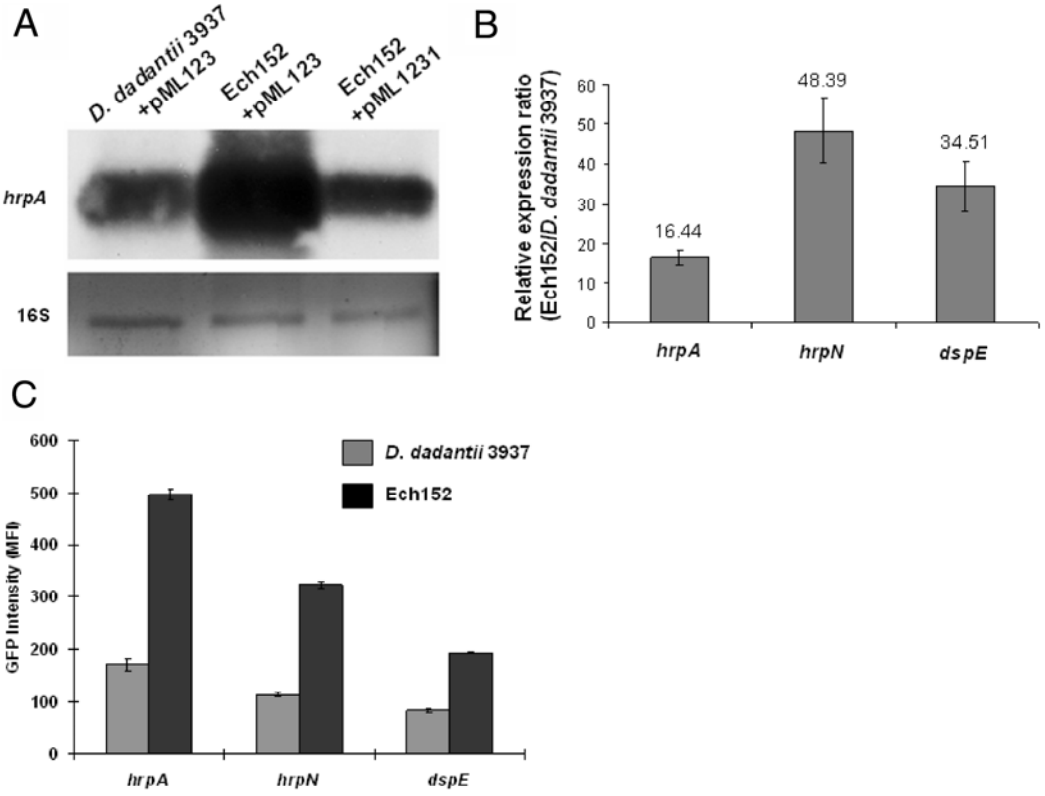

Fig. 1. A, Northern blot analysis of *Dickeya dadantii* 3937 with plasmid pML123, *pnp* mutant Ech152 with pML123, and pML1231 using *hrpA* as a hybridization probe. 16S rRNA was used as RNA loading control. **B,** Relative mRNA levels of *hrpA, hrpN*, and *dspE* in Ech152 compared with *D. dadantii* 3937, measured by real-time polymerase chain reaction. Similar results have been observed in two independent experiments. **C,** Promoter activities of *hrpA, hrpN*, and *dspE* in *D. dadantii* 3937 (gray bar) and Ech152 (black bar). *D. dadantii* 3937 and Ech152 carrying reporter plasmids phrpA, phrpN, and pdspE were cultured in minimal medium at 28°C for 12 h and the promoter activities were measured by flow cytometry. Values of mean fluorescence intensity are average green fluorescent protein fluorescence intensities of total bacterial population and are representative of two experiments. Three replicates were used in this experiment.

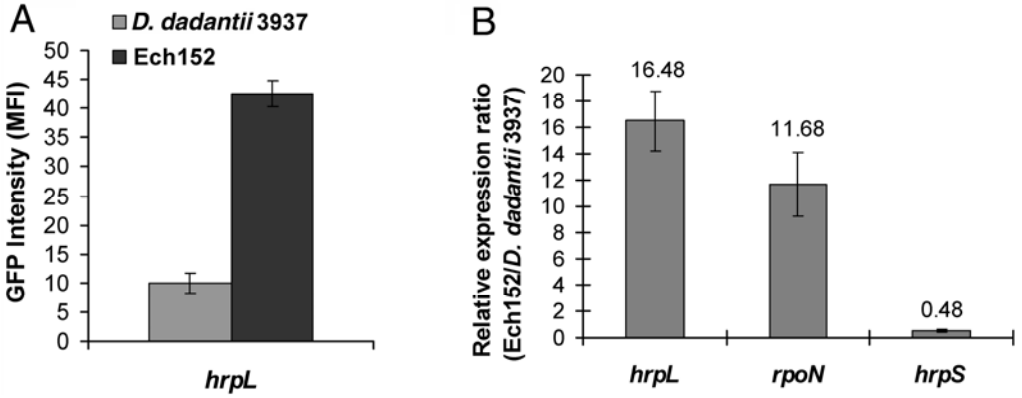

Fig. 2. A, Promoter activities of *hrpL* in *Dickeya dadantii* 3937 (gray bar) and in Ech152 (black bar). **B,** Relative mRNA levels of *hrpL, rpoN*, and *hrpS* of Ech152 compared with *D. dadantii* 3937, measured by real-time polymerase chain reaction. *D. dadantii* 3937 and Ech152 carrying reporter plasmids phrpA, phrpN, and pdspE were cultured in minimal medium at 28°C for 12 h and the promoter activities were measured by flow cytometry. Values of mean fluorescence intensity are average green fluorescent protein fluorescence intensities of total bacterial population and are representative of two experiments. Three replicates were used in this experiment.

with primer 1 and primer 2). Primer extension signals of similar intensity were produced in both Ech152 and the wild type, which suggests that the population of truncated RsmB transcripts that are most abundant in Ech152 lack different portions from the 3′ ends and are unable to generate a signal with primer 2 (Fig. 5B).

Finally, a Northern blot was performed to compare the 3′ and 5′ ends of RsmB transcripts in Ech152 with those of the wild-type strain. In this assay, two hybridization probes were used, probe I (anneals to the 5′ end of RsmB) and probe II (anneals to the 3′ end of RsmB). Similar to the product generated by the full-length RsmB probe (Fig. 4A), an RsmB transcript pattern consisting of full-length and multiple truncated RsmB transcripts was observed in Ech152 using probe I (Fig. 5C). However, when probe II was used in the Northern blot, only two major bands were detected in Ech152 (Fig. 5C). Again, these results confirm that the truncated RsmB transcripts present in Ech152 have similar intact 5′ ends detectable by probe I but incomplete 3′ ends that are undetectable by probe II (Fig. 5D).

Sequestering activity of artificially designed truncated RsmB transcripts.

In addition to the full-length 250-nt transcript, abundant 3′-end truncated RsmB transcripts (sizes of 155, 170, and 210 nt) were present in Ech152 (Fig. 4A). Given that RsmB is an untranslated sRNA which functions by directly binding RsmA to sequester its degradation effect on T3SS, we speculate that these truncated RsmB transcripts are still able to bind and sequester RsmA. Two artificially designed *rsmB* DNA fragments encoding transcripts with intact 5′ ends and truncated 3′ ends were cloned into pML123, generating the complementation plasmids pML1234 and pML1235. pML1234 contains an *rsmB* fragment that includes the *rsmB* promoter region, an intact 5′ end of the *rsmB* gene, and a truncated 3′ end lacking the last 41 bp of *rsmB*. pML1235 contains an *rsmB* fragment that includes the *rsmB* promoter region, an intact 5′ end of the *rsmB* gene, and a truncated 3′ end lacking the last 88 bp of *rsmB*. Another plasmid containing full-length *rsmB* was also constructed and designated pML1233 (Table 1). The plasmids pML1233, pML1234, and pML1235 were each electroporated into an *rsmB* mutant (Ech153), and the *hrpA* expression in the wild type and Ech153 carrying the different complementation plasmids was examined by Northern blot. In *D. dadantii*, the expression of *hrpA* is regulated by the RsmA-RsmB pair through HrpL (Yang et al. 2008a). Thus, the level of *hrpA* expression reflects the ability of RsmB sRNA to sequester the degradation effect of RsmA on *hrpL* mRNA. The Northern blot analysis showed that the *hrpA* expression was completely abolished in Ech153 (Fig. 6). However, the level of *hrpA* mRNA in Ech153 was restored by pML1233. Interestingly, the plasmids pML1234 and pML1235 carrying artificially designed 3′ end truncated *rsmB* fragments could partially restore *hrpA* expression in Ech153 (Fig. 6). The sequestration effect of these 3′-end truncated RsmB fragments on RsmA was also confirmed by measuring the promoter activity of *hrpA* in the wild type and Ech153 carrying pML1233, pML1234, or pML1235. The complementation plasmid pML1233 was able to restore *hrpA* promoter activity in Ech153 (Table 2). The *hrpA* promoter activity was partially restored upon introduction of pML1234 and pML1235 into Ech153 (Table 2). These results suggest that RsmB transcripts with short truncations at the 3′ end may retain the ability to sequester RsmA, and as a consequence, enhance *hrpA* expression, albeit with reduced activity.

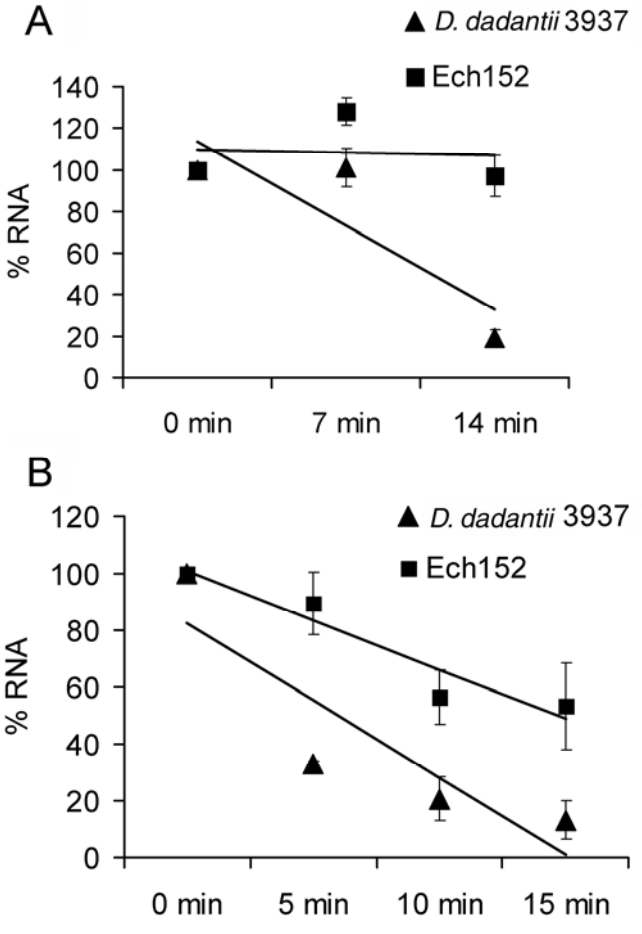

Fig. 3. A, *rpoN* RNA stability in *Dickeya dadantii* 3937 (triangles) and in Ech152 (squares). Slopes of *rpoN* RNA decay curves of *D. dadantii* 3937 and Ech152 are –40.5 and –1.45, respectively. **B,** *hrpL* RNA stability in *D. dadantii* 3937 (triangles) and in Ech152 (squares). Slopes of *hrpL* RNA decay curves of *D. dadantii* 3937 and Ech152 are –28.28 and –17.29, respectively. Similar results were observed in two independent experiments and the result of one is shown here.

Bistable expression of T3SS genes.

A subpopulation of *D. dadantii* 3937 cells grown in a homogenous medium was shown to have elevated expression of *dspE* (Peng et al. 2006). The expression level of *dspE* in the total cell population can be divided into low-state (basal level

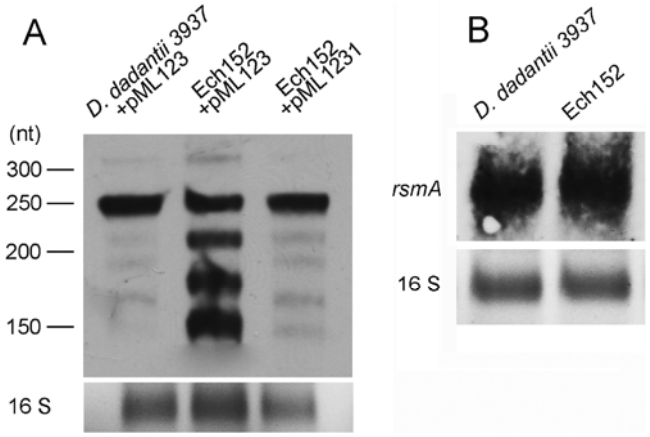

Fig. 4. A, Northern blot analysis of *Dickeya dadantii* 3937 with plasmid pML123, *pnp* mutant Ech152 with pML123, and Ech152 with plasmid pML1231 using a full-length *rsmB* hybridization probe. A biotin-labeled RNA marker was used to determine the size of the RsmB RNA fragments. **B,** Northern blot analysis of *D. dadantii* 3937 and Ech152 using an *rsmA* probe.

of T3SS gene expression) and high-state (high level of T3SS gene expression). In this study, single-cell gene expression patterns of *hrpA*, *hrpN*, *dspE*, *hrpL*, *hrpS*, and *rsmA* were further examined in *D. dadantii* 3937 carrying the promoter-*gfp* transcriptional fusion constructs phrpA, phrpN, pdspE, phrpL, phrpS, and prsmA, respectively, by FACS. Interestingly, only the T3SS structural and effector genes *hrpA*, *hrpN*, and *dspE* showed a bistable gene expression pattern (Fig. 7A, gray shade). The T3SS regulatory genes *hrpS, hrpL,* and *rsmA* showed a monostable gene expression pattern (Supplementary Fig. 1).

Because an increase in expression of the T3SS structural and effector genes was observed in Ech152, we examined the role of PNPase in regulating the bistable gene expression of *hrpA*, *hrpN*, and *dspE*. Gene expression patterns of the T3SS genes were analyzed in Ech152 by FACS (Fig. 7A, black line). Compared with the wild-type strain, an increased and monophasic-like expression of *hrpA*, *hrpN*, and *dspE* was observed in Ech152 cells after 12 h (Fig. 7A, black line). Although a majority of Ech152 cells expressed *hrpA* at the high-state level at 12 h (Fig. 7A, black line), a subpopulation of cells expressed *hrpA* at the low-state level in Ech152 in the early growth stages (Fig. 7B, 7, 9, and 11 h, black line). Similarly, a bistable gene expression pattern of *hrpN* and *dspE* was observed in the *pnp* mutant in the early growth stages but a monomodal-like

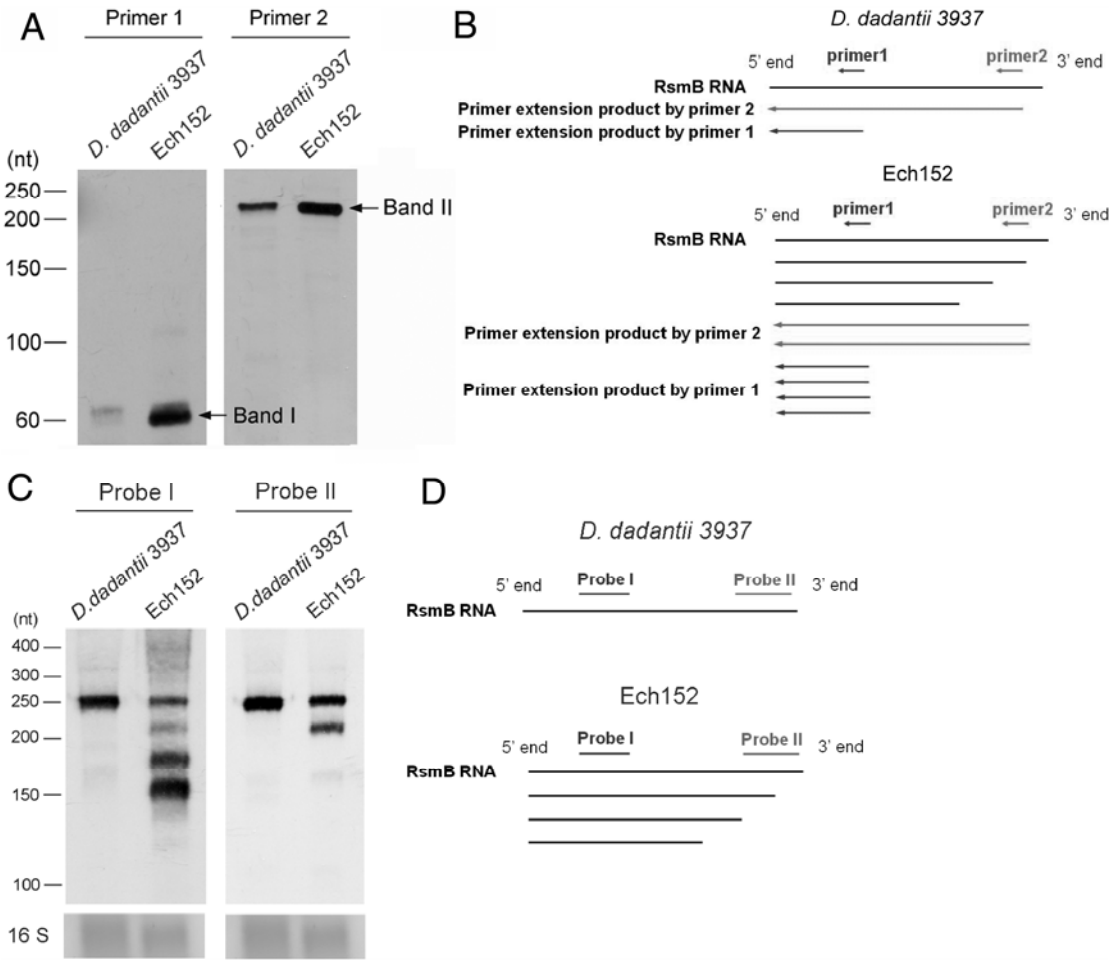

Fig. 5. A, Primer extension assay of RsmB in *Dickeya dadantii* 3937 and Ech152 using primer 1 (anneals to the 5′ end of RsmB RNA) and primer 2 (anneals to the 3′ end of RsmB RNA). Bacterial strains were grown in minimal medium at 28°C for 12 h. Similar results were observed among three individual experiments. **B,** Scheme of primer extension analysis with primer 1 and primer 2. **C,** Northern blot analysis of *Dickeya dadantii* 3937 and Ech152 using *rsmB* probe I and probe II. **D,** Scheme of Northern blot analysis with probe I and probe II.

Table 2. *hrpA* promoter activity in *Dickeya dadantii* 3937 carrying plasmid pML123 and *rsmB* mutant Ech153 carrying plasmids pML123, pML1233, pML1234, and pML1235[a]

	Mean fluorescence intensity[b]		
Gene Promoter	**6 h**	**12 h**	**24 h**
D. dadantii 3937 (phrpA, pML123)	46.0 ± 5.6	110.4 ± 3.7	140.3 ± 8.4
Ech153 (phrpA, pML123)	4.2 ± 2.8	4.4 ± 0.8	7.8 ± 2.1
Ech153 (phrpA, pML1233)	90.1 ± 4.4	210.1 ± 12.9	252.7 ± 13.6
Ech153 (phrpA, pML1234)	23.2 ± 3.4	61.7 ± 6.9	42.2 ± 5.5
Ech153 (phrpA, pML1235)	19.1 ± 1.9	44.1 ± 3.2	36.2 ± 5.3
D. dadantii 3937 (pPROBE-AT)	2.3 ± 0.1	9.8 ± 1.4	8.2 ± 3.7

[a] *D. dadantii* 3937 carrying pPROBE-AT was used as a control for basal level of green fluorescent protein (GFP) expression.
[b] Promoter activities were measured at 6, 12, and 24 h of bacterial growth in minimal medium at 28°C by flow cytometry. Values of mean fluorescence intensity are average GFP fluorescence intensities of total bacterial population and are representative of two experiments. Three replicates were used in this experiment.

expression pattern was observed after 12 h (data not shown). Because PNPase regulates the transcription of *hrpL* but does not have a regulatory effect on HrpS and RsmA (Figs. 2B and 4B), an increase in *hrpL* promoter activity but similar gene expression patterns of *hrpS* and *rsmA* were observed in Ech152 compared with *D. dadantii* 3937.

DISCUSSION

PNPase is a well-known exoribonuclease that is ubiquitous in bacteria and eukaryotes. In this work, we presented evidence that PNPase plays an important role in the regulation of T3SS in *D. dadantii* (Fig. 8). We revealed that, by reducing the *rpoN* mRNA stability, PNPase downregulates the transcription of *hrpL* and, consequently, the transcription of T3SS structural and effector genes. To our knowledge, this is the first report describing the regulation of PNPase on the T3SS of a phytopathogenic bacterium. In addition, we also provided evidence that PNPase affects the RsmB turnover by controlling the levels of fragmented RsmB transcripts in *D. dadantii*. Although we do not have direct evidence that these fragmented RsmB transcripts are functional in vivo or that their presence in the cell contributes to the control of T3SS expression, the information we present here provides innovative insights toward understanding the relationship between the structural integrity of the regulatory sRNAs and their function during the regulation of T3SS.

In *Yersinia* spp., PNPase plays an important role in controlling the expression of T3SS genes. PNPase deficiency results in increased expression of T3SS genes under in vitro growth conditions (Rosenzweig et al. 2007). However, because the transcription of those T3SS genes showed no difference among the wild type and *pnp* mutant, the author proposed that PNPase might regulate the T3SS genes at the post-transcriptional level. In contrast, our results showed that PNPase regulates the transcription of the T3SS structural and effector genes through the T3SS master regulator HrpL in *D. dadantii*. In addition, similar *hrpA* RNA degradation rates have been observed in *D. dadantii* and Ech152 (Supplementary Fig. 2). This result further demonstrates that, apart from *Yersinia* spp., PNPase does not directly regulate the T3SS structural and effector genes in *D. dadantii*. In *S. enterica*, PNPase downregulates the expression of *Salmonella* plasmid virulence genes (*spv* genes) through SpvR (Ygberg et al. 2006). PNPase controls the T3SS through a master regulator in both *Dickeya* and *Salmonella* spp. However, unlike HrpL, which activates the expression of T3SS genes as an ECF family alternative σ factor, SpvR regulates *spv* genes as a transcriptional regulator and its activation requires the participation of σ factor RpoS. It is interesting to realize that, although *Yersinia*, *Salmonella*, and *Dickeya* spp. infect different hosts, regulation of their T3SS genes all involve PNPase. This gives us some indication that PNPase might serve as a general

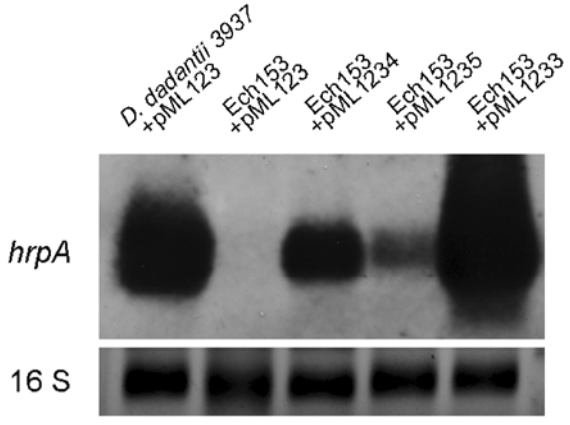

Fig. 6. Northern blot analysis of *Dickeya dadantii* 3937 with plasmid pML123 and the *rsmB* mutant (Ech153) with plasmids pML123, pML1234, pML1235, and pML1233, using *hrpA* as a hybridization probe. pML1233 contains the full-length *rsmB* fragment. pML1234 contains an *rsmB* fragment lacking 41 bp from the 3′ end and pML1235 contains an *rsmB* fragment lacking 88 bp from the 3′ end. pML123 is the empty vector control.

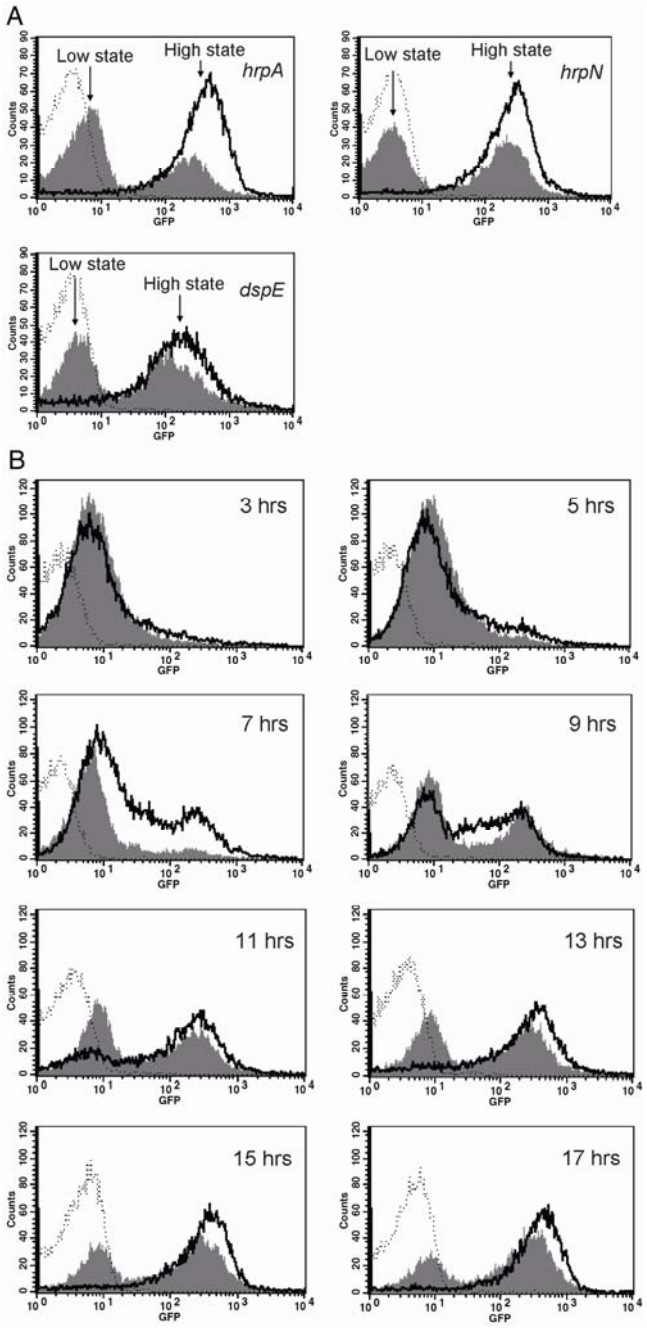

Fig. 7. A, Expression patterns of *hrpA, hrpN,* and *dspE* in *Dickeya dadantii* 3937 and Ech152. *D. dadantii* 3937 and Ech152 carrying reporter plasmids phrpA, phrpN, and pdspE were cultured in minimal medium at 28°C for 12 h and the promoter activities were measured by flow cytometry. **B,** Expression pattern of *hrpA* in *Dickeya dadantii* 3937 and Ech152 at different time points of growth in minimal medium at 28°C. In both A and B, the y-axis represents the cell counts and the x-axis represents green fluorescent protein (GFP) intensity (mean fluorescence intensity). The dotted line depicts the basal level of GFP expression in *D. dadantii* 3937 containing pPROBE-AT vector control. The gray shade represents expression patterns of target genes in *D. dadantii* 3937 and the black line represents expression patterns of target genes in Ech152.

mechanism to fine tune the control of virulence gene expression in different bacterial pathogens.

In this study, it appears that PNPase plays an important role in RsmB turnover in *D. dadantii* 3937, and the absence of PNPase activity results in the production of abundant truncated RsmB transcripts in Ech152. At this stage, it is uncertain whether these truncated RsmB transcripts are still biologically functional and maintain the ability to sequester RsmA. Artificially designed truncated RsmB transcripts lacking either 41 or 88 nt from the 3′ end could partially restore the complete lack of *hrpA* expression in the *rsmB* mutant (Fig. 6; Table 2). This suggests that the 3′ truncated RsmB transcripts in the *pnp* mutant may remain biologically functional and retain partial RsmA sequestering activity, which would result in a reduction of *hrpL* mRNA degradation in Ech152. Indeed, 14 putative RsmA-binding sites were predicted in the full-length 250-nt RsmB RNA of *D. dadantii* 3937 (Supplementary Fig. 3). These RsmA-binding sites are primarily located within the loops of predicted stem-loops of the RsmB RNA secondary structure. The truncated RsmB transcripts in Ech152 still contain many of the predicted RsmA binding sites at the 5′ end, which suggests that these truncated transcripts may still be able to bind to RsmA.

In *Escherichia coli*, both CsrB (RsmB homologue) and CsrC sRNAs contain multiple binding sites to bind CsrA (RsmA homologue) and sequester its regulatory function (Liu et al. 1997). Dubey and associates (2005) demonstrated that randomly synthesized RNA ligands containing single CsrA-binding sites with an appropriate stem loop structure can bind to the CsrA protein in vitro. In comparison, our results showed that, although the fragmented RsmB RNAs retain the majority of the RsmA-binding sites after truncation at the 3′ end, their RsmA sequestering ability has been significantly compromised (Fig. 6). This implies that a single RsmA-binding site with an appropriate secondary structure may be sufficient to bind RsmA but structural integrity is crucial for proper regulatory function of RsmB.

In *Bacillus subtilis*, *comK*, a gene encoding a master transcriptional activator, displays a bistable gene expression pattern during the late exponential growth phase. This leads to the bistable expression of the genes responsible for competence development and causes the separation of competent and noncompetent populations in the bacterial cells (Hahn et al. 1994). Bistable expression patterns of *hrpA*, *hrpN*, and *dspE* were observed in *D. dadantii* 3937 (Fig. 7A.). However, the T3SS master regulator gene *hrpL* does not show a bistable expression pattern in *D. dadantii* 3937. This observation is different than that reported on the bistable expression of competence development genes in *B. subtilis*. Compared with the wild-type strain, a high level of *hrpL* expression and a constant bistable expression pattern of *hrpA, hrpN*, and *dspE* with a large proportion of high state cells were observed in Ech152 (Fig. 7A). This result suggests that PNPase controls the ratio of cells that express high-state and low-state levels of *hrpA*, *hrpN*, and *dspE*. By repressing *hrpL* expression, PNPase prevents the shift of *hrpA* expression from the low-state toward the high-state level. In addition, because bistable expression patterns of *hrpA*, *hrpN*, and *dspE* were observed in Ech152 at the early stage of bacterial growth, it is unlikely that PNPase is the key regulator involved in the switch from

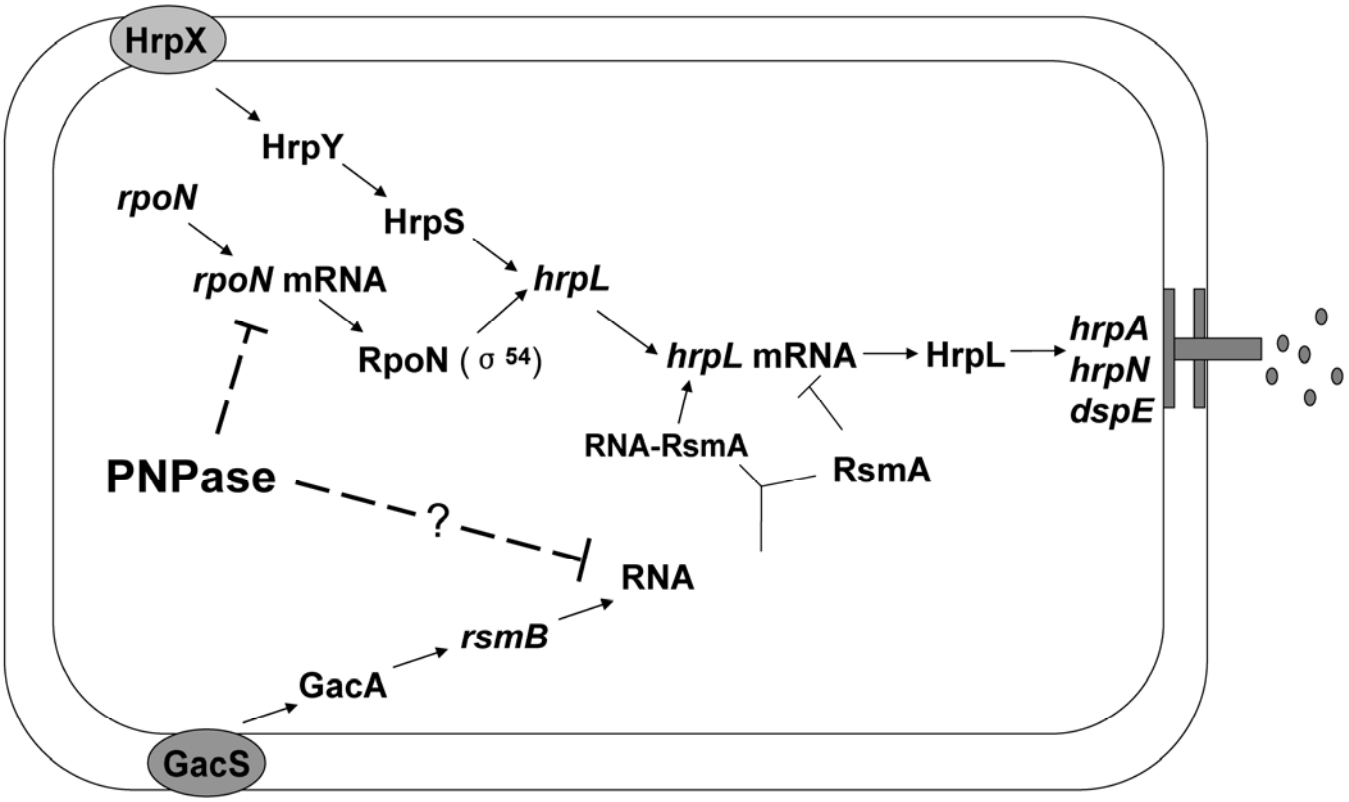

Fig. 8. Model of type III secretion system (T3SS) gene regulation by polynucleotide phosphorylase (PNPase) in *Dickeya dadantii*. HrpL, the master regulator of the T3SS structural and effector genes, activates the transcription of *hrpA*, *hrpN*, and *dspE*. The transcription of *hrpL* is upregulated by HrpS, an enhancer protein, in conjunction with RpoN, a σ^{54} factor. *hrpL* mRNA stability is controlled by RsmA, a small-RNA-binding protein which binds to *hrpL* mRNA and promotes *hrpL* mRNA degradation. GacS-GacA activates RsmB, which encodes a regulatory small RNA that binds to and neutralizes the degradation effect of RsmA on *hrpL* mRNA. PNPase has a negative regulatory effect on the expression of T3SS genes *hrpA, hrpN*, and *dspE*. PNPase downregulates *hrpL* transcription by reducing *rpoN* mRNA stability. PNPase also reduces *hrpL* mRNA stability. In this report, it is speculated that PNPase has a negative effect on RsmB RNA levels and structural integrity and, thereby, is involved in the post-transcriptional modulation of *hrpL*.

monostable to bistable expression of T3SS genes. The key regulator for the bistable expression of T3SS in *D. dadantii* 3937 is yet to be identified.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

Bacterial strains and plasmids used in this study are listed in Table 1. Wild-type *D. dadantii* 3937 and mutants were stored at –80°C in 15% glycerol. *D. dadantii* strains were grown in Luria-Bertani (LB) medium or minimal medium at 28°C with glucose as the carbon source (Yang et al. 2007). *E. coli* strains were grown in LB at 37°C. Antibiotics were added to the media at the following concentrations: kanamycin, 50 μg/ml; gentamycin, 25 μg/ml; and ampicillin, 100 μg/ml. The *pnp* deletion mutant (Ech152) was constructed by crossover PCR mutagenesis as previously described (Yang et al. 2002). Briefly, DNA fragments flanking *pnp* were amplified by PCR using primer sets PNP_A/PNP_B and PNP_C/PNP_D (Table 1). Crossover PCR was performed using the flanking regions and a kanamycin cassette as templates and primers PNP_A and PNP_D. The *pnp* deletion fragment was cloned into the *Spe*I/*Not*I sites of pWM91. The resulting plasmid was mobilized into *D. dadantii* 3937 by electroporation. To select strains with chromosomal deletions, transconjugants with kanamycin and ampicillin resistance were plated on mannitol glutamate containing 5% sucrose and kanamycin. Colonies having sucrose and kanamycin resistance and ampicillin sensitivity were isolated. The mutation was confirmed by PCR using primers PNP_F/PNP_R and by sequencing. The *rsmB* transposon mutant Ech153 was obtained from a previous transposon mutant library (Yang et al. 2008a).

The plasmids pML1231 and pML1233 carrying *pnp* and *rsmB*, respectively, were constructed as follows. DNA fragments containing *pnp* or *rsmB* with promoters and terminator regions were amplified by PCR from *D. dadantii* 3937 genomic DNA with primer sets PNP_F/PNP_R and rsmB_F/rsmB_R, respectively. The *pnp* and *rsmB* fragments were purified, digested with *Xba*I and *Sac*I, and cloned into the broad-host-range expression vector, pML123, digested with the same enzymes (Labes et al. 1990).

pML123 derivatives pML1234 and pML1235, containing DNA fragments encoding truncated RsmB transcripts with intact 5′ ends and incomplete 3′ ends, were constructed using the following method. DNA fragments containing the *rsmB* promoter and coding sequence lacking either 41 bp (*rsmB*41) or 88 bp (*rsmB*88) from the 3′ end were PCR amplified from *D. dadantii* 3937 genomic DNA with primer sets rsmB1234_F/rsmB1234_R and rsmB1235_F/rsmB1235_R, respectively. Another DNA fragment containing the terminator region of the *rsmB* gene (*rsmB*Term) was also PCR amplified with Term_F and Term_R primers. The 3′ ends of the *rsmB*41 and *rsmB*88 fragments and the 5′ end of the *rsmB*Term fragment were digested with *Xba*I. *rsmB*41 and *rsmB*88 fragments were individually ligated to *rsmB*Term and then amplified by PCR with primers rsmB1234/5_F and Term_R. The recombinant fragments were digested with *Sac*I and *Bam*HI and cloned into pML123 digested with the same enzymes to generate pML1234 (contains an *rsmB* fragment which lacks 41 bp from the 3′ end) and pML1235 (contains an *rsmB* fragment which lacks 88 bp from the 3′ end). All the constructs have been confirmed by DNA sequencing.

The *rsmA* promoter region was PCR amplified from *D. dadantii* 3937 genomic DNA, ligated into the pCR2.1-TOPO TA cloning vector, and then subcloned into the *Xba*I/*Sac*I sites of the promoter-*gfp* vector pPROBE-AT (Leveau and Lindow 2001), generating prsmA.

FACS assay.

The bacterial cells carrying promoter-*gfp* transcriptional fusion plasmids were subcultured in minimal medium at 28°C to induce T3SS gene expression. Cells were harvested at the time points indicated in the respective figures, washed, and diluted to approximately 10^6 CFU/ml with 1× phosphate-buffered saline (8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 per liter, pH 7.2 to 7.4) before analysis. The GFP fluorescence signals were measured using a four-color BD FACSCalibur (BD Biosciences, San Jose, CA, U.S.A.) equipped with 488- and 633-nm lasers. Bacteria were electronically gated based on forward and side light-scatter properties. All GFP fluorescence measurements were taken using the 488-nm laser and FL1 channel on the gated population. The results were analyzed using Cell Quest software (BD Biosciences).

RNA extraction and real-time PCR analysis.

Bacterial strains were cultured in minimal medium at 28°C for 12 h. Total RNA was isolated by using the TRI reagent method (Sigma-Aldrich, St. Louis) and treated with Turbo DNA-free DNase (Ambion, Austin, TX, U.S.A.). The iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, U.S.A.) was used to synthesize cDNA from 0.5 μg of DNase-treated total RNA. The Real Master Mix (Eppendorf, Westbury, NY, U.S.A.) was used for real-time PCR reactions to quantify the cDNA level of target genes. *rplU* mRNA stability appears to be unaffected by PNPase (Supplementary Fig. 4) and, therefore, was used as an endogenous control for data analysis (Mah et al. 2003; Kuchma et al. 2005). Data were collected by the Opticon 2 system (Bio-Rad) and analyzed using the Relative Expression Software Tool as described by Pfaffl and associates (2002). Primers used for the real-time PCR are listed in Table 1.

Northern blot analysis.

Bacterial strains were grown in minimal medium, and RNA was isolated and treated with DNase as mentioned above for real-time PCR analysis. Each RNA sample (10 μg) was analyzed by Northern blot analysis using a NorthernMax kit (Ambion) according to the manufacturer's instructions. Hybridization probes used to detect the mRNA of target genes were PCR amplified and labeled with biotin using a BrightStar Psoralen-Biotin kit (Ambion). Signals were developed using the BrightStar BioDetect kit (Ambion). 16S rRNA was visualized under UV transilluminator (Syngene, Frederick, MD, U.S.A.) and used as an internal control for normalization of RNA. For the Northern blot analysis of RsmB RNA, a 6 M urea/6% polyacrylamide gel was used to separate the sRNA fragments.

Primer extension analysis.

Primers used for reverse transcription were synthesized and labeled with biotin at the 5′ end by Fisher Scientific (Pittsburgh). The reaction mixtures for synthesis of cDNA contained a total of 2 pmol of primer, 10 μg of RNA, and 200 U of Superscript III RT (Invitrogen, Carlsbad, CA, U.S.A.). The primer extension products were separated in 6 M urea/6% polyacrylamide gels and transferred to nylon membranes (Immobilon-Ny+ Transfer Membrane; Millipore, Bedford, MA, U.S.A.). Signals were developed using a BrightStar BioDetect kit (Ambion). A biotin-labeled RNA marker (BrightStar RNA Century Markers; Ambion) was used to determine the sizes of the primer-extension products. The band intensities were quantified using a Bio Imaging system (Syngene).

RNA stability assay.

Bacterial strains were cultured in minimal medium at 28°C for 12 h. Rifampicin (500 μg/ml) and nalidixic acid (20 μg/ml) were added to bacterial cultures to stop nucleic acid synthesis.

Incubation was continued and culture aliquots were withdrawn at the times indicated in the respective figures. Bacterial cells were collected by centrifugation and were resuspended in RNA*later* Solution (Ambion) to stop RNA decay. Total RNA was isolated and real-time PCR was performed to quantify the RNA of target genes using the methods described above. 16S rRNA was used as the endogenous control for data analysis. The percentage of RNA remaining after termination of RNA synthesis was calculated by comparing the amounts of *hrpL* and *rpoN* mRNA measured at different time points with the quantity of mRNA present at time 0 min. The mRNA stability curves were plotted and the slopes of the stability curves were calculated.

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