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GENETICS AND MOLECULAR BIOLOGY

YbeY Controls the Type III and Type VI Secretion Systems and Biofilm Formation through RetS in *Pseudomonas aeruginosa*

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ABSTRACT YbeY is a highly conserved RNase in bacteria and plays essential roles in the maturation of 16S rRNA, regulation of small RNAs (sRNAs), and bacterial responses to environmental stresses. Previously, we verified the role of YbeY in rRNA processing and ribosome maturation in *Pseudomonas aeruginosa* and demonstrated YbeY-mediated regulation of *rpoS* through an sRNA, ReaL. In this study, we demonstrate that mutation of the *ybeY* gene results in upregulation of the type III secretion system (T3SS) genes as well as downregulation of the type VI secretion system (T6SS) genes and reduction of biofilm formation. By examining the expression of the known sRNAs in *P. aeruginosa*, we found that mutation of the *ybeY* gene leads to downregulation of the small RNAs RsmY/Z, which control the T3SS, T6SS, and biofilm formation. Further studies revealed that the reduced levels of RsmY/Z are due to upregulation of *retS*. Taken together, our results reveal the pleiotropic functions of YbeY and provide detailed mechanisms of YbeY-mediated regulation in *P. aeruginosa*.

IMPORTANCE *Pseudomonas aeruginosa* causes a variety of acute and chronic infections in humans. The type III secretion system (T3SS) plays an important role in acute infection, and the type VI secretion system (T6SS) and biofilm formation are associated with chronic infections. Understanding of the mechanisms that control the virulence determinants involved in acute and chronic infections will provide clues for the development of effective treatment strategies. Our results reveal a novel RNase-mediated regulation of T3SS, T6SS, and biofilm formation in *P. aeruginosa*.

KEYWORDS biofilm, *Pseudomonas aeruginosa*, RetS, type III secretion system, YbeY, sRNA

YbeY is a highly conserved bacterial RNase that is involved in the maturation of 16S rRNA, ribosome quality control, regulation of sRNAs, and stress responses (1–6). Previous studies in *Escherichia coli* identified YbeY as a UPF0054 family metal-dependent hydrolase, and the three-dimensional crystal structure of YbeY revealed a conserved metal ion-binding region (7). The YbeY protein purified from *Sinorhizobium meliloti* displays metal-dependent endoribonuclease activity that cleaves both single-stranded (ssRNA) and double-stranded (dsRNA) RNA substrates (6). Deletion of *ybeY* in *E. coli* reduces protein translation efficiency by affecting the 30S ribosome subunits (8). Jacob et al. demonstrated that YbeY is a single-strand-specific endoribonuclease that plays key roles in ribosome quality control and 16S rRNA maturation together with RNase R in *E. coli* (1). The structural model of YbeY revealed a positively charged cavity similar to the middle domain of Argonaute (AGO) proteins involved in RNA silencing in eukaryotes (9). Recent studies in *Vibrio cholerae*, *S. meliloti*, and *E. coli* demonstrated that a defect in YbeY results in aberrant expression of small RNAs (sRNAs) and the corresponding target mRNAs (2, 9, 10).

Citation Xia Y, Xu C, Wang D, Weng Y, Jin Y, Bai F, Cheng Z, Kuipers OP, Wu W. 2021. YbeY controls the type III and type VI secretion systems and biofilm formation through RetS in *Pseudomonas aeruginosa*. Appl Environ Microbiol 87:e02171-20. https://doi.org/10.1128/AEM.02171-20.

Editor Knut Rudi, Norwegian University of Life Sciences

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Received 3 September 2020
Accepted 3 December 2020
Accepted manuscript posted online 11 December 2020
Published 12 February 2021

In pathogenic bacteria, YbeY has been found to play important roles in bacterial virulence. In *V. cholerae*, the absence of YbeY reduces the production of the cholera toxin and intestinal colonization in mice (2). In *Yersinia enterocolitica*, YbeY is required for intestinal adhesion and bacterial virulence (11). A defect in *ybeY* severely impairs the ability of *Brucella* to infect macrophages (12). However, the mechanisms by which YbeY affects bacterial virulence and stress response remain largely unknown.

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen that causes acute and chronic infections in humans (13). The bacterium possesses a variety of virulence determinants that contribute to pathogenesis. The type III secretion system (T3SS) is one of the major virulence factors that play critical roles in acute infections (14). It is a syringe-like machinery that directly injects effector proteins into mammalian cells, interfering with cell physiological functions or leading to cell death (14). The chronic infection caused by *P. aeruginosa* is usually accompanied by the formation of biofilm in which bacteria are protected by an extracellular matrix against host immune cells and antibacterial substances (15).

The type VI secretion system (T6SS) is a weapon for bacterial warfare and interfering with the functions of host cells (16). A number of T6SSs have been demonstrated to target competing bacteria and efficiently kill the competitors (17–20), which may play a key role in the survival and proliferation of the producer cells in a multimicrobial environment (21). *P. aeruginosa* harbors three T6SS clusters, namely, H1-, H2-, and H3-T6SS. The H1-T6SS is related to the adaptability of this bacterium to chronic infection (22, 23). A recent study in reference strain PA14 revealed that all the three T6SSs are under the control of the RetS-GacS/GacA-RsmA pathway and the transcriptional regulator AmrZ (24).

The RetS/LadS-GacS/GacA-RsmY/RsmZ-RsmA regulatory pathway plays a key role in the transition between acute and chronic infections. RetS inhibits the GacS-mediated phosphorylation of GacA through directly binding to GacS, whereas LadS promotes the phosphorylation of GacA. The two-component system GacS/GacA directly activates the expression of RsmY/RsmZ sRNAs that antagonize the function of RsmA through direct interaction. RsmA is an RNA binding protein that represses expression of T6SS genes and biofilm formation and activates the expression of T3SS genes (25–29). AmrZ is a DNA binding protein that controls gene expression at the transcriptional level. Unlike RsmA, which represses the expression of all three T6SS genes, AmrZ represses the expression of the H2-T6SS genes but activates the expression of the H1- and H3-T6SS genes (24).

Previously, we demonstrated that the *P. aeruginosa* endoribonuclease YbeY is involved in 16S rRNA maturation and ribosome assembly. In addition, we found that YbeY controls bacterial resistance to oxidative stresses through an sRNA, ReaL (30). In this study, we demonstrate that YbeY regulates the expression of T3SS and T6SS genes and biofilm formation through the RetS-GacS/GacA-RsmY/RsmZ-RsmA pathway, further revealing the pleiotropic function of YbeY in *P. aeruginosa*.

RESULTS

Mutation of *ybeY* enhances the expression of the T3SS genes and bacterial cytotoxicity. Our previous transcriptomic analyses revealed an upregulation of the T3SS genes in a PA14 Δ*ybeY* mutant (30). To understand the relationship between YbeY and the T3SS genes, we utilized reverse transcription-quantitative PCR (RT-qPCR) to verify the expression levels of the T3SS regulatory genes *exsA*, *exsC*, and *exsD*, the structural gene *pcrV*, and the effector gene *exoU*. All of the tested genes were upregulated about 9- to 13-fold in the Δ*ybeY* mutant and returned to wild-type levels by the complementation of the *ybeY* gene (Fig. 1A). Since T3SS plays a major role in the bacterial cytotoxicity, we performed an LDH release assay with the A549 human lung carcinoma cell line. Compared to the wild-type strain, the Δ*ybeY* mutant displayed enhanced cytotoxicity (Fig. 1B).

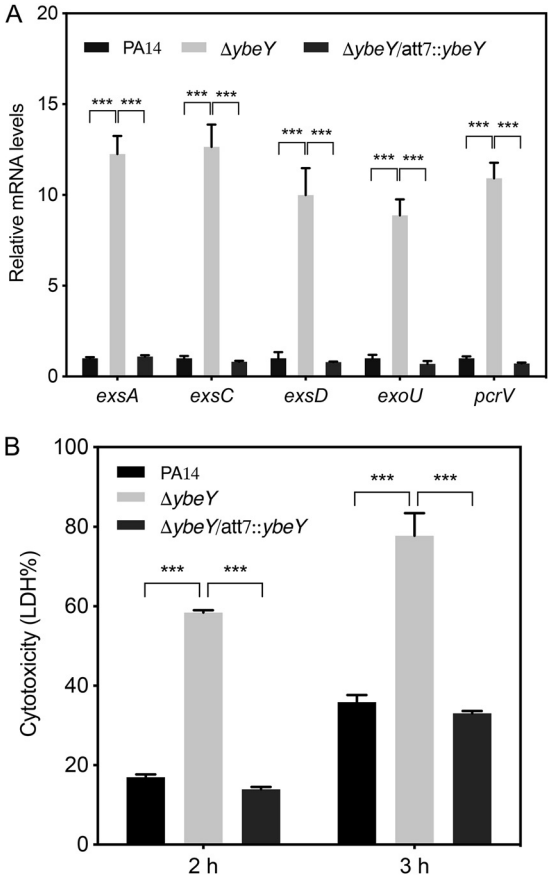

FIG 1 YbeY is involved in the regulation of the T3SS. (A) Wild-type PA14, the Δ*ybeY* mutant, and the complemented strain were grown in LB to an OD_{600} of 1. The relative mRNA levels of the T3SS genes were determined by RT-qPCR. Results represent means ± standard deviations (SD). (B) A549 cells were infected with the indicated strains at an MOI of 50 for 2 or 3 h. The relative cytotoxicity was determined by the LDH release assay. Results represent means ± SD. ***, $P < 0.001$ by Student's *t* test.

YbeY influences the expression of the T3SS and T6SS genes and biofilm formation through the RsmY/RsmZ-RsmA pathway. YbeY is an endoribonuclease that has been shown to control the expression of *rpoS* through the sRNA ReaL (30). We hypothesized that YbeY affects the expression of the T3SS genes through sRNAs. Thus, we examined the levels of 36 known *P. aeruginosa* sRNAs by RT-qPCR. Previously, we found that mutation of *ybeY* reduces the bacterial growth rate (30). Therefore, we increased the inoculum of the Δ*ybeY* mutant to achieve an optical density at 600 nm (OD_{600}) of 1, the same as the wild-type strain at the same time before RNA isolation. However, it took longer for the Δ*ybeY* mutant to achieve an OD_{600} of 3.0. The growth curves and sample collection points are shown in Fig. 2A. The expression of 21 and 18 sRNAs was altered (fold change, >2) by the mutation of *ybeY* in exponential and stationary growth phases, respectively (Fig. 2B and C). Of note, the sRNAs RsmY and RsmZ were two of the most downregulated sRNAs in the exponential and stationary growth phases in the Δ*ybeY* mutant. Complementation of the *ybeY* gene in the Δ*ybeY* mutant restored the levels of RsmY and RsmZ (Fig. 2D). The mRNA level of *rsmA* was not affected by the mutation of *ybeY* (Fig. 2D).

Previous studies revealed that upregulation of RsmY/Z leads to downregulation of the T3SS genes (31, 32). To investigate whether RsmY/Z is involved in the regulation of the T3SS genes by YbeY, we overexpressed RsmY or RsmZ in the Δ*ybeY* mutant, which reduced the expression levels of the T3SS genes and the bacterial cytotoxicity (Fig. 3A and B). Deletion of the *rsmA* gene in the Δ*ybeY* mutant reduced the expression of the T3SS genes and the cytotoxicity (Fig. 3C and D).

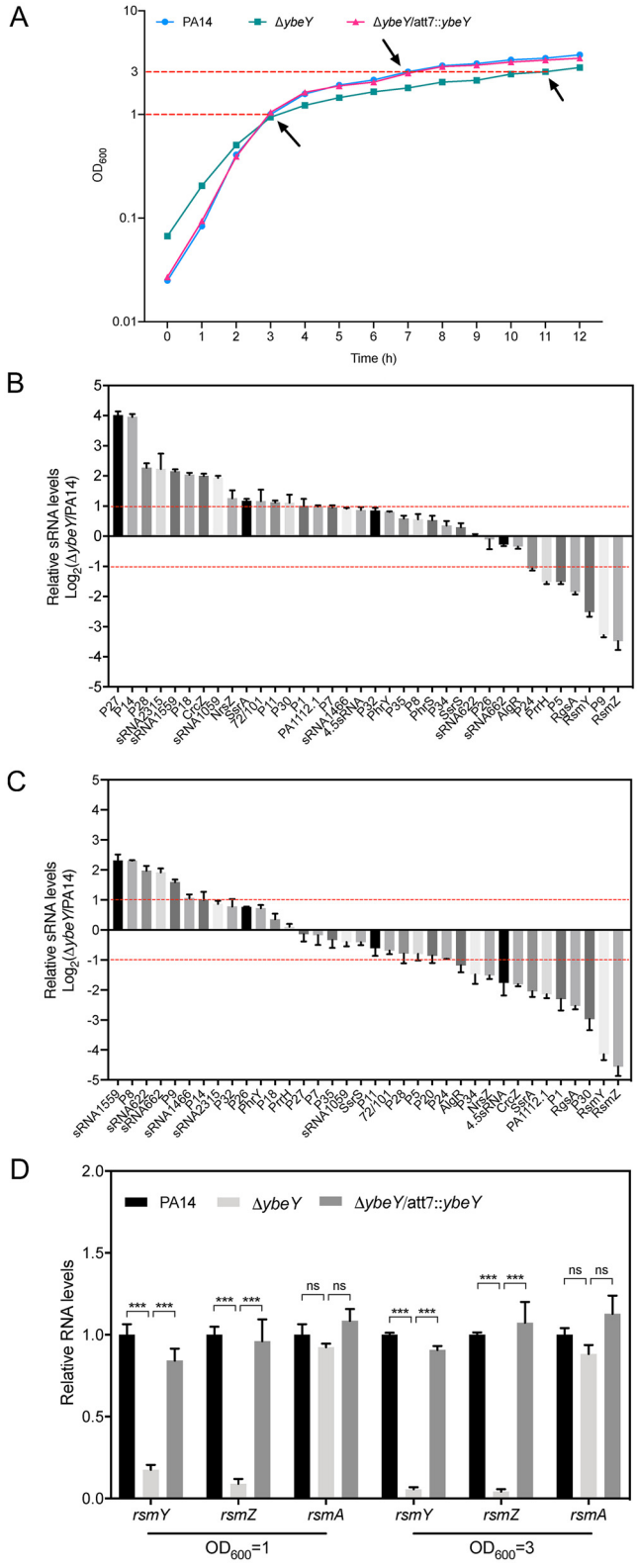

FIG 2 YbeY controls the expression of sRNAs but not the expression of *rsmA*. (A) The growth curves of wild-type PA14, the Δ*ybeY* mutant, and the complemented strain. The bacteria were grown in LB overnight. Aliquots of 0.3 ml of the cultures of the wild-type PA14 and the complemented strain or 0.9 ml of the culture of the Δ*ybeY* mutant were subcultured into 30 ml fresh LB medium and grown at 37°C with agitation at 200 rpm. The OD_{600} was monitored every hour for 12 h. The sample collection points are indicated by arrows. Bacteria were grown to an OD_{600} of 1 (B) or 3 (C). Total

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Since the GacS/GacA-RsmY/Z-RsmA pathway reciprocally regulates the T3SS, T6SS, and biofilm formation (24, 25), we suspected that YbeY is involved in the regulation of the T6SS and biofilm formation. We then examined the expression levels of H1- and H3-T6SS genes that are regulated in the same patterns by GacS/GacA and AmrZ. Indeed, RT-qPCR results revealed downregulation of *hcp-1*, *vgrG1a*, *hcp3*, and *hsiB3* in the Δ*ybeY* mutant (Fig. 3E). In addition, the Δ*ybeY* mutant displayed reduced biofilm formation (Fig. 3F). Deletion of *rsmA* in the Δ*ybeY* mutant restored the expression of the T6SS genes and biofilm formation (Fig. 3E and F). These results demonstrate that YbeY plays an important role in the transition between acute and chronic infections through the RsmY/RsmZ-RsmA regulatory pathway.

YbeY regulates the expression of RsmY/Z through RetS. The expression of *rsmY* and *rsmZ* is directly activated by the GacS/GacA two-component system. RetS inhibits the GacS-mediated phosphorylation of GacA through directly binding to GacS, whereas LadS promotes the phosphorylation of GacA (25–29). To understand the mechanism of the downregulation of *rsmY* and *rsmZ* in the Δ*ybeY* mutant, we monitored the promoter activities of the two genes by *lacZ* transcriptional fusions (P_{rsmY}-*lacZ* and P_{rsmZ}-*lacZ*). The LacZ levels of both of the constructs were lower in the Δ*ybeY* mutant and returned to wild-type levels by the complementation of the *ybeY* gene (Fig. 4A), indicating a reduction at the transcriptional level. The transcription of *rsmY* and *rsmZ* is directly activated by the GacS/GacA two-component regulatory system (28). However, the mRNA levels of *gacS* and *gacA* were not affected by the mutation of *ybeY* (Fig. 4B). We then examined the genes regulating the activity of the GacS/GacA system. Mutation of *ybeY* resulted in upregulation of *retS*, whereas the expression of *ladS* and *hptB* was not affected (Fig. 4B). By utilizing a transcriptional fusion between the *retS* promoter and a *lacZ* gene (P_{retS}-*lacZ*), we found the promoter activity of *retS* was increased in the *ybeY* mutant and returned to wild-type levels by the complementation of the *ybeY* gene (Fig. 4C). These results led us to speculate that the upregulation of *retS* represses the expression of *rsmY* and *rsmZ* and subsequently leads to the activation of the T3SS genes and suppression of the T6SS genes and biofilm formation. To test our hypothesis, we knocked out *retS* in the Δ*ybeY* mutant, which resulted in increased levels of RsmY/Z (Fig. 4D). In addition, deletion of *retS* in the Δ*ybeY* mutant reduced expression of the T3SS genes and cytotoxicity (Fig. 5A and B) and increased the expression of the H1- and H3-T6SS genes as well as biofilm formation (Fig. 5C and D). These results demonstrate that YbeY plays an important role in the regulation of T3SS, T6SS, and biofilm formation through RetS.

Mutation of *ybeZ* results in phenotypes similar to those of the Δ*ybeY* mutant. In our previous research, we found that YbeZ binds to YbeY and is involved in the maturation of 16S rRNA and the response to oxidative stress (30). Therefore, we speculated that YbeZ plays a role in the regulation of the T3SS, T6SS, and biofilm formation. Indeed, mutation of *ybeZ* resulted in upregulation of T3SS genes and enhanced cytotoxicity (Fig. 6A and B). In addition, the Δ*ybeZ* mutant displayed downregulation of T6SS genes and reduced biofilm formation (Fig. 6C and D). Consistent with this, the Δ*ybeZ* mutant displayed similar expression levels of the genes encoding RsmY, RsmZ, and RetS (Fig. 6E). In combination, these results demonstrate that YbeZ is involved in the regulation of transition between acute and chronic infections through RetS.

DISCUSSION

Ribonucleases play important roles in bacterial stress responses and regulation of virulence factors. YbeY is a conserved endoribonuclease that plays pleiotropic roles in

FIG 2 Legend (Continued)
RNA was purified, and the relative sRNA levels were determined by RT-qPCR. The relative levels of the small RNAs in the Δ*ybeY* mutant compared to those in wild-type PA14 are shown. Results represent means ± SD. The red lines represent a fold change of 2. (D) The bacteria were grown in LB to an OD_{600} of 1 or 3. The relative RNA levels of *rsmY-rsmZ* and *rsmA* were determined by RT-qPCR. Results represent means ± SD. ***, $P < 0.001$ by Student's *t* test. ns, not significant.

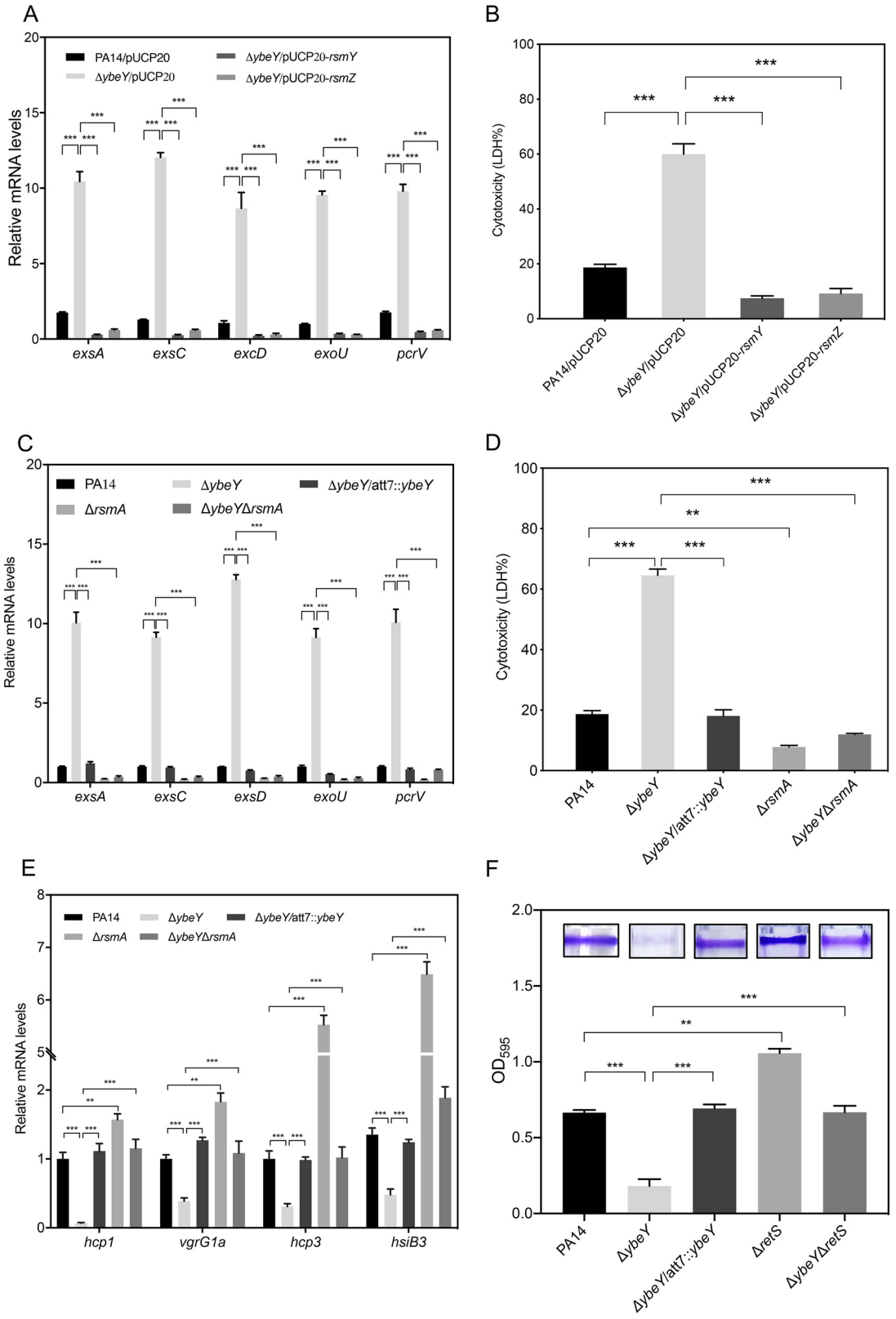

FIG 3 YbeY controls the expression of the T3SS and T6SS genes and biofilm formation through RsmY/Z-RsmA. (A) The indicated strains were grown in LB to an OD_{600} of 1. The relative mRNA levels of the T3SS genes were determined by RT-qPCR. Results represent means ± SD. (B) A549 cells were infected with the indicated strains at an MOI of 50 for 2 h. The relative cytotoxicity was determined by the LDH release assay. (C) The relative mRNA levels of the T3SS genes were determined by RT-qPCR. Results represent means ± SD. (D) The relative bacterial cytotoxicity was determined by the LDH

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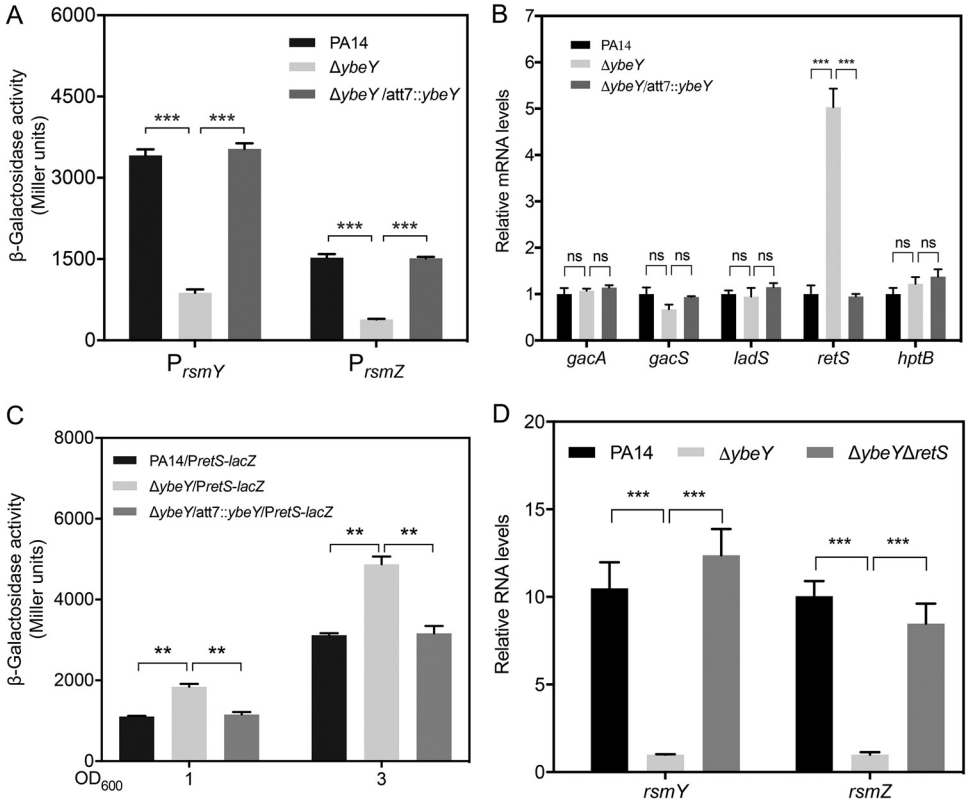

FIG 4 YbeY controls the expression of *rsmY* and *rsmZ* through RetS. (A) PA14, the Δ*ybeY* mutant, and the complemented strain containing the P_{rsmY}-*lacZ* or P_{rsmZ}-*lacZ* transcriptional fusion were cultured in LB to an OD_{600} of 1. The bacteria were collected and subjected to the β-galactosidase activity assay. (B) Wild-type PA14, the Δ*ybeY* mutant, and the complemented strain were grown in LB to an OD_{600} of 1. The relative mRNA levels of *gacA*, *gacS*, *ladS*, *retS*, and *hptB* were determined by RT-qPCR. Results represent means ± SD. (C) PA14, the Δ*ybeY* mutant, and the complemented strain containing the P_{retS}-*lacZ* transcriptional fusion were cultured in LB to an OD_{600} of 1 or 3. The bacteria were collected and subjected to the β-galactosidase activity assay. (D) Wild-type PA14, the Δ*ybeY* mutant, and the Δ*ybeY* Δ*retS* mutant were grown in LB to an OD_{600} of 1. The relative RNA levels of *rsmY* and *rsmZ* were determined by RT-qPCR. Results represent means ± SD. ns, not significant; **, $P < 0.01$; ***, $P < 0.001$; by Student's *t* test.

bacterial physiology and virulence (1–6). In *V. cholera*, mutation in the *ybeY* gene resulted in complete loss of mouse colonization and biofilm formation (2). In *E. coli*, YbeY has been shown to play important roles in bacterial resistance to heat shock, oxidative stresses, and a variety of antibiotics (33). Deletion of the *ybeY* gene in the plant pathogen *Agrobacterium tumefaciens* reduced the bacterial growth rate, motility, and stress tolerance (34). In *Yersinia enterocolitica* serotype O:3, YbeY is involved in the regulation of the genes of the *Yersinia* virulence plasmid (pYV) and multiple regulatory small RNAs (11). In enterohemorrhagic *E. coli* (EHEC), YbeY is required for the expression of the T3SS genes. Further studies revealed that mutation of *ybeY* reduces the amount of initiating ribosomes, leading to destabilization of the T3SS gene mRNA (35). Previously, we found that YbeY controls bacterial resistance to oxidative stresses through a small RNA (sRNA), ReaL, and participates in the maturation of 16S rRNA in *P. aeruginosa*. Here, we demonstrated that YbeY is involved in the regulation of serval sRNAs in *P. aeruginosa*. In addition, we found that mutation of *ybeY* results in the up-

FIG 3 Legend (Continued)
release assay. (E) The indicated strains were grown in LB to an OD_{600} of 1. The relative mRNA levels of the T6SS genes were determined by RT-qPCR. (F) The indicated strains were grown in 96-well plates for 20 h. The wells were washed with PBS and stained with 1% crystal violet. The crystal violet was dissolved in ethanol and measured at a wavelength of 595 nm. Results represent means ± SD. **, $P < 0.01$; ***, $P < 0.001$; both by Student's *t* test.

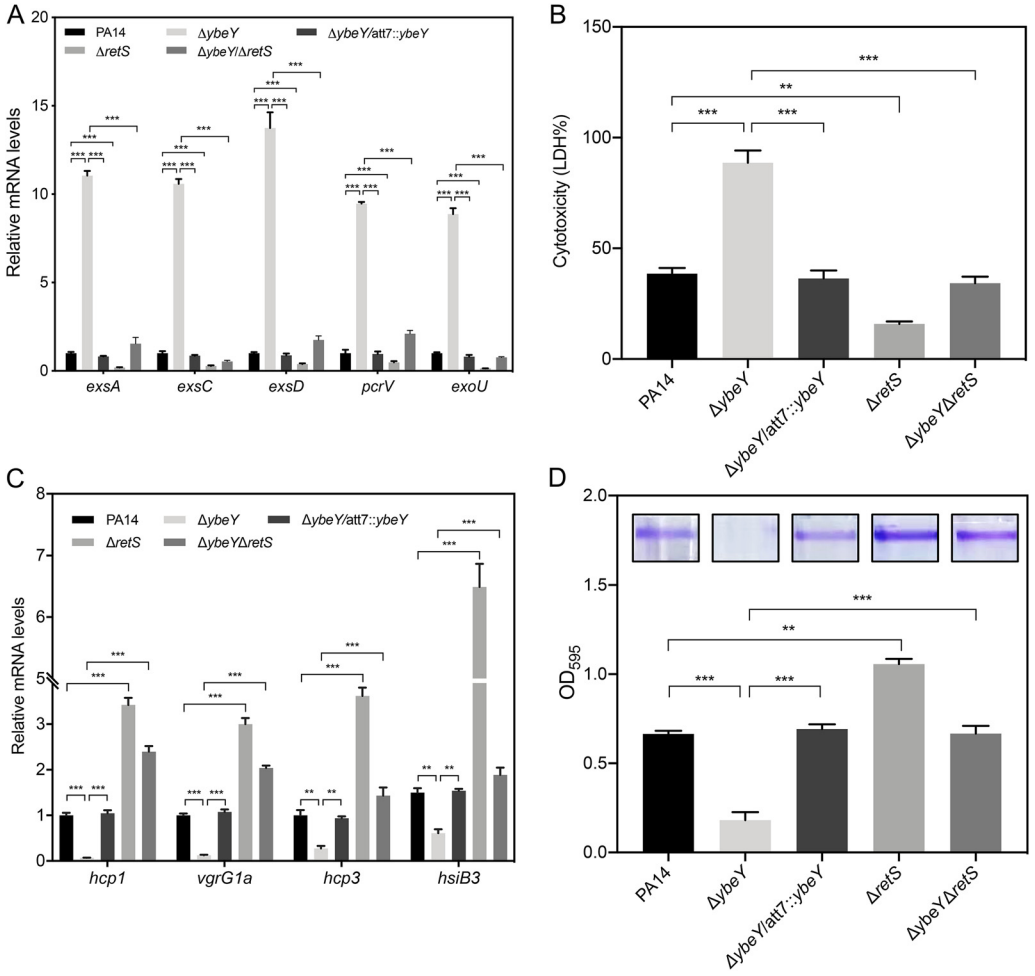

FIG 5 YbeY controls biofilm formation and the expression of T3SS and T6SS genes through RetS. (A) The indicated strains were grown in LB to an OD_{600} of 1. The relative mRNA levels of the T3SS genes were determined by RT-qPCR. Results represent means ± SD. (B) A549 cells were infected with the indicated strains at an MOI of 50 for 3 h. The relative cytotoxicity was determined by the LDH release assay. (C) The indicated strains were grown in LB to an OD_{600} of 1. The relative mRNA levels of the T6SS genes were determined by RT-qPCR. Results represent means ± SD. (D) The indicated strains were grown in 96-well plates for 20 h. The wells were washed with PBS and stained with 1% crystal violet. The crystal violet was dissolved in ethanol and measured at a wavelength of 595 nm. Results represent means ± SD. **, $P < 0.01$; ***, $P < 0.001$; both by Student's *t* test.

regulation of the T3SS genes. Further studies revealed that YbeY regulates the expression of the T3SS genes through the GacA/S-RsmY/Z-RsmA pathway by regulating the expression of *retS* (Fig. 7).

Previous studies revealed that the expression of *retS* is repressed by the two-component system PhoP/PhoQ and activated by the transcriptional regulator CysB (36, 37). Our results revealed the downregulation of *phoP* and upregulation of *cysB* in the Δ*ybeY* mutant (data not shown). However, overexpression of *phoP* or a knockout of *cysB* in the Δ*ybeY* mutant did not reduce the expression of *retS* and the T3SS genes (data not shown). Thus, the mechanism of the upregulation of *retS* in the *ybeY* mutant remains elusive and requires further studies.

The T6SS is a weapon that targets competing bacteria and efficiently kills the competitors (17–20). We found that the *ybeY* mutation resulted in downregulation of all three T6SS genes and a reduction of the ability to kill other bacteria (data not shown). A recent study revealed that all the three T6SSs are under the control of the RetS-GacS/GacA-RsmA pathway, and the H2-T6SS plays a major role in bacterial killing in the reference strain PA14 (24). In our study, we found that knocking out *rsmA* or *retS* in

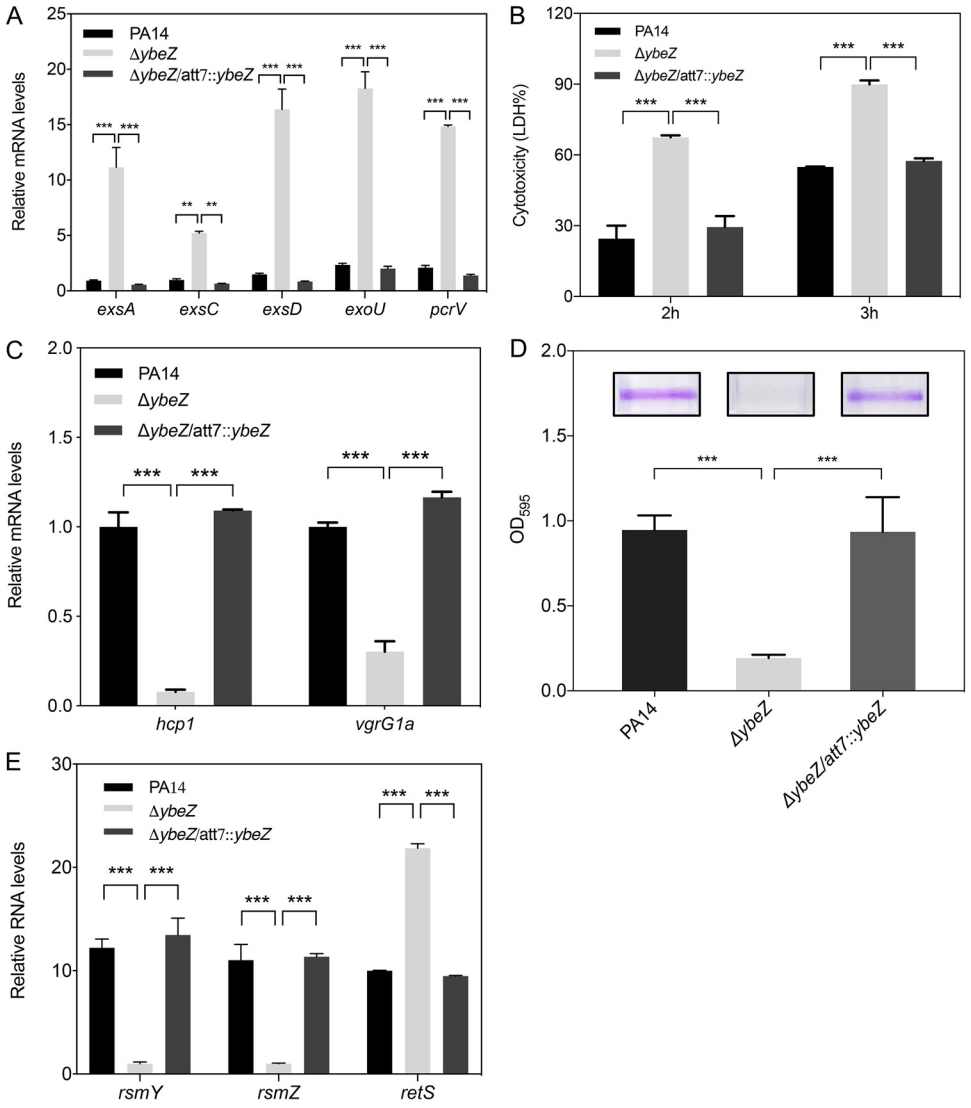

FIG 6 YbeZ influences the expression of the T3SS and T6SS genes and biofilm formation. (A) Wild-type PA14, the Δ*ybeZ* mutant, and the complemented strain were grown in LB to an OD_{600} of 1. The relative mRNA levels of the T3SS genes were determined by RT-qPCR. Results represent means ± SD. (B) Cytotoxicity of wild-type PA14, the Δ*ybeZ* mutant, and the complemented strain. A549 cells were infected with the indicated strains at an MOI of 50 for 2 or 3 h. The relative cytotoxicity was determined by the LDH release assay. (C) The indicated strains were grown in LB to an OD_{600} of 1. The relative mRNA levels of the T6SS genes were determined by RT-qPCR. Results represent means ± SD. (D) The indicated strains were grown in 96-well plates for 20 h. The wells were washed with PBS and stained with 1% crystal violet. The crystal violet was dissolved in ethanol and measured at a wavelength of 595 nm. (E) The bacteria were grown in LB to an OD_{600} of 1. The relative RNA levels of *rsmY*, *rsmZ*, and *retS* were determined by RT-qPCR. Results represent means ± SD. **, $P < 0.01$; ***, $P < 0.001$; both by Student's *t* test.

the context of *ybeY* mutation could not restore the expression of the H2-T6SS genes and the ability to kill other bacteria (data not shown), indicating that additional factors control the expression of the H2-T6SS genes. Previous study has shown that the transcriptional regulator AmrZ directly represses the expression of the H2-T6SS genes but activates the expression of the H1- and H3-T6SS genes (24). Our preliminary results demonstrated an upregulation of *amrZ* in the *ybeY* mutant (data not shown). Currently, we are making efforts to understand the mechanism of YbeY-mediated regulation on *amrZ*.

sRNAs affect the stabilities and translation efficiencies of mRNAs through complementary base pairing, which is a key regulatory mechanism of bacterial gene

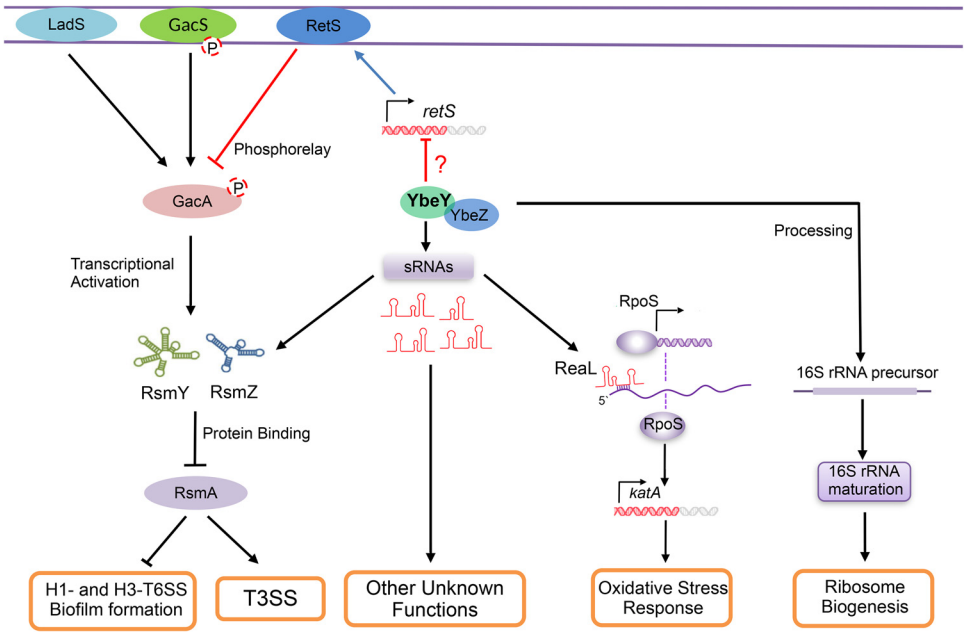

FIG 7 Roles of YbeY/YbeZ in *P. aeruginosa*. YbeY/YbeZ are involved in the maturation of 16S rRNA and ribosome assembly (30). Meanwhile, YbeY influences the levels of multiple sRNAs. One of the direct regulatory targets of YbeY is the sRNA ReaL. ReaL binds to the 5′-untranslated region of the *rpoS* mRNA, inhibiting its translation (58). RpoS is an alternative sigma factor that has been demonstrated to contribute to bacterial responses to oxidative stresses by activating the expression of the major catalase KatA in *P. aeruginosa* (59). YbeY directly degrades ReaL, thereby positively regulating the expression of RpoS (30). In addition, YbeY and YbeZ control the expression of the T3SS and T6SS genes as well as biofilm formation through the RetS-GacS/GacA-RsmY/Z-RsmA pathway. YbeY and YbeZ repress the transcription of *retS* through an unknown mechanism. RetS directly binds to GacS, which inhibits the phosphorelay-dependent activation of GacA. GacA activates the expression of two sRNAs, RsmY and RsmZ, that antagonize the function of RsmA. RsmA is a posttranscriptional regulator that activates the expression of the T3SS genes and represses the expression of the T6SS genes as well as the extracellular polysaccharide biosynthesis *pel* and *psl* genes, which are important for biofilm formation (25–29).

expression (31, 38, 39). Although bacterial sRNAs affect a wide range of biological processes, including energy utilization and metabolism, pathogenicity, and antibiotic resistance, our understanding of the regulation of sRNAs is still limited (40–43). Ribonucleases play an important role in cellular RNA metabolism processes, such as mRNA degradation and rRNA/tRNA maturation, and have emerged as the main posttranscriptional regulators of sRNAs (44–46). RNase E and PNPase have been shown to be involved in the degradation of the free pool of sRNAs (47, 48). In addition, RNases are also involved in the maturation process of sRNAs (45). Recent studies in *V. cholerae*, *S. meliloti*, and *E. coli* have shown that YbeY is involved in the regulation of sRNAs (2, 9, 10).

In this study, we found that mutation of the *ybeY* gene influenced the expression of multiple sRNAs. For example, *crcZ*, which is related to carbon metabolism, is upregulated in the exponential phase but downregulated in the stationary phase, indicating that YbeY is involved in the growth phase-dependent metabolism regulation. The production of sRNA P27, PrrH, and NrsZ, involved in quorum sensing, was altered by the *ybeY* mutation (49–51). The RpoS-dependent sRNA RgsA, which regulates Fis and AcpP, is downregulated, which might be due to the defective expression of *rpoS* in the *ybeY* mutant (52). These results imply that YbeY plays a role in the regulation of quorum-sensing genes. SsrA is a critical component of the *trans*-translation system that is involved in the release of ribosomes stalled on mRNAs (53). In the *ybeY* mutant, SsrA is upregulated in the exponential phase but downregulated in the stationary phase, indicating that YbeY affects mRNA translation in a growth phase-dependent manner. However, the functions of the remaining sRNAs are not known. Nevertheless, these

TABLE 1 Bacterial strains, plasmids and primers used in this study[a]

Strain, plasmid, or primer	Description or sequence (5′–3′)	Source (reference) or function
E. coli		
DH5α	*F*$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15	57
S17-1	Δ(*lacZYA-argF*)*U169 hsdR17* ($r_K^-m_K^+$) λ-*thi pro hsdR recA traC*$^+$	57
P. aeruginosa		
PA14	Wild type	60
$\triangle$*ybeY*	PA14 deleted of *ybeY*	30
$\triangle$*ybeY*/att7::*ybeY*	$\triangle$*ybeY* with *ybeY* inserted on chromosome with mini-Tn*7*T insertion; GEN^r	30
$\triangle$*ybeZ*	PA14 deleted of *ybeZ*	30
$\triangle$*ybeZ*/att7::*ybeZ*	$\triangle$*ybeZ* with *ybeZ* inserted on chromosome with mini-Tn*7*T insertion; GEN^r	30
$\triangle$*rsmA*	PA14 deleted of *rsmA*	This study
$\triangle$*ybeY*$\triangle$*rsmA*	PA14 deleted of *ybeY* and *rsmA*	This study
$\triangle$*retS*	PA14 deleted of *retS*	This study
$\triangle$*ybeY*$\triangle$*retS*	PA14 deleted of *ybeY* and *retS*	This study
PA14/pUCP20	PA14 with empty plasmid pUCP20; CAR^r	This study
$\triangle$*ybeY*/pUCP20	$\triangle$*ybeY* with empty plasmid pUCP20; CAR^r	This study
$\triangle$*ybeY*/pUCP20-*rsmY*	$\triangle$*ybeY* with plasmid pUCP20-*rsmY*; CAR^r	This study
$\triangle$*ybeY*/pUCP20-*rsmZ*	$\triangle$*ybeY* with plasmid pUCP20-*rsmZ*; CAR^r	This study
Plasmids		
pUCP20	*Escherichia-Pseudomonas* shuttle vector without *lac* promoter; AMP^r	61
pEX18Tc	Gene replacement vector; TET^r, *oriT*$^+$, *sacB*$^+$	61
pUC18T-mini-Tn7T-Gm	Mini-Tn*7* base vector from insertion into chromosome attTn*7* site; GEN^r	61
pDN19lacΩ	Promoterless *lacZ* fusion vector; SPT^r, STR^r, TET^r	57
pEX18Tc-$\triangle$*rsmA*	*rsmA* gene of PA14 deletion on pEX18Tc; TET^r	This study
pEX18Tc-$\triangle$*retS*	*retS* gene of PA14 deletion on pEX18Tc; TET^r	This study
pUCP20-*rsmY*	Overpression of *rsmY* on pUCP20; CAR^r	This study
pUCP20-*rsmZ*	Overpression of *rsmZ* on pUCP20; CAR^r	This study
Primers		
RsmA-L-F	CCGGAATTCGCACATCGACGACACCCAC	*rsmA* deletion
RsmA-L-R	TGCTCTAGACCCGACGAGTCAGAATCAGC	*rsmA* deletion
RsmA-R-F	TGCTCTAGAAGAAAGATCAAGAGCCAAACCA	*rsmA* deletion
RsmA-R-R	CCCAAGCTTCTTAGTCTTGCCCCCTATGGA	*rsmA* deletion
RsmA-T-F	AGGGTGAGTGACGCTGGCA	$\triangle$*rsmA* screen
RsmA-T-R	GCCGCCTGAATCAACCTCTA	$\triangle$*rsmA* screen
RetS-L-F	CCCAAGCTTGAAGCCAAGTGCGAGAACGT	*retS* deletion
RetS-L-R	TGCTCTAGAGAGCAGAAGCAGCAGGAAGC	*retS* deletion
RetS-R-F	TGCTCTAGAGGTGCTGATGGACTGCGAGA	*retS* deletion
RetS-T-F	CGGCCACTTGGCTATAATCC	$\triangle$*retS* screen
RetS-T-R	CAGGACAGCACGAAGAAGGG	$\triangle$*retS* screen
PA1805-RT-F	ATCAGTCTCAATGAAGTC	RT-PCR
PA1805-RT-R	CATGGATGGATCGAAATC	RT-PCR
RpsL-RT-F	GTAAGGTATGCCGTGTACG	RT-PCR
RpsL-RT-R	CACTACGCTGTGCTCTTG	RT-PCR
ExsA-RT-F	GCTATGTCGTAAGTACCA	RT-PCR
ExsA-RT-R	GAAGCCTTGTAGAAACTG	RT-PCR
ExsC-RT-F	CAGCTTCAACCGCCATTG	RT-PCR
ExsC-RT-R	CGCATACAACTGGACCTTG	RT-PCR
ExsD-RT-F	AGAGGTGCGGCAGATTCTCC	RT-PCR
ExsD-RT-R	ATCATCGACTGCGGCACG	RT-PCR
ExoU-RT-F	AACACATTAGCAGCGAGAT	RT-PCR
ExoU-RT-R	AGCAGCAACTCAGAGAAG	RT-PCR
PcrV-RT-F	CACGCTCTATGGCTATGC	RT-PCR
PcrV-RT-R	AAGGTATCCAGATTGCTCAG	RT-PCR
RsmA-RT-F	GAAGGAAGTCGCCGTACA	RT-PCR
RsmA-RT-R	TAATGGTTTGGCTCTTGATCTTT	RT-PCR
RsmY-RT-F	CCAAAGACAATACGGAAA	RT-PCR
RsmY-RT-R	GTTTTGCAGACCTCTATC	RT-PCR
RsmZ-RT-F	CAACCCCGAAGGTTC	RT-PCR
RsmZ-RT-R	CAGTCCCTCGTCATC	RT-PCR
GacA-RT-F	CCTGATGATCGCCAACTG	RT-PCR
GacA-RT-R	ATAGGTATTCACGGTCTTCG	RT-PCR
GacS-RT-F	GAGGAAATGCAGCACAAC	RT-PCR

(Continued on next page)

TABLE 1 (Continued)

Strain, plasmid, or primer	Description or sequence (5′–3′)	Source (reference) or function
GacS-RT-R	GTTCTGGATCTCGATGGT	RT-PCR
RetS-RT-F	GACTACGTGCAGACCATC	RT-PCR
RetS-RT-R	CTTGGAGATGTCGAGGAT	RT-PCR
LadS-RT-F	GATGCTGATCTACAACCT	RT-PCR
LadS-RT-R	GAAGCGATATAGAGGATGT	RT-PCR
HptB-RT-F	CATCTCGATGATCGTGTTC	RT-PCR
HptB-RT-R	GAAGGTATCCAGCAGGAC	RT-PCR
Hcp1-RT-F	AGGACCTGTCGTTCACCAA	RT-PCR
Hcp1-RT-R	ATAGTGCTTGCCGCTGGA	RT-PCR
VgrG1a-RT-F	GAGACCAGCTTCGACTTCATC	RT-PCR
VgrG1a-RT-R	CTTCTGCTCATGGCGGAAC	RT-PCR
Hcp3-RT-F	ACATCAAAGGCGACAGCC	RT-PCR
Hcp3-RT-R	GTTGCTGACGTCGTTGGT	RT-PCR
HsiB3-RT-F	ATCACCTACGACGTCGAGAT	RT-PCR
HsiB3-RT-R	GTCGATGTCGACGAAACGC	RT-PCR

[a]GEN^r, gentamicin resistance; AMP^r, ampicillin resistance; TET^r, tetracycline resistance; CAR^r, carbenicilin resistance; STR^r, streptomycin resistance; SPT^r, spectinomycin resistance; KAN^r, kanamycin resistance. Enzyme cleavage sites are underlined.

results indicate that YbeY participates in multiple sRNA-mediated regulation processes in physiological functions in *P. aeruginosa*. Further studies are warranted to understand the functions of these sRNAs and the mechanisms of YbeY-mediated regulation of them.

In many bacterial species, including *P. aeruginosa*, *Staphylococcus aureus*, and *E. coli*, the *ybeZ* gene is in the same operon as the *ybeY* gene (30, 33, 54). We previously demonstrated the interaction between YbeY and YbeZ in *P. aeruginosa* and found that mutation of *ybeZ* resulted in a defective response to oxidative stresses similar to that of the *ybeY* mutant. In this study, we found that mutation of *ybeZ* resulted in the increased expression of T3SS and cytotoxicity, as seen in the *ybeY* mutant. These results suggest that YbeY and YbeZ function together in the transition between acute and chronic infections through RetS. YbeZ contains a nucleoside triphosphate hydrolase and an ATP binding domain. However, the exact function of YbeZ remains elusive and warrants further studies.

Overall, our results reveal pleiotropic roles of YbeY in the regulation of T3SS, T6SS, biofilm formation, and oxidative stress response in *P. aeruginosa*. Analyses of the global gene and sRNA expression profiles under various environmental stresses might reveal additional roles of YbeY and the regulatory pathways mediated by this endonuclease.

MATERIALS AND METHODS

Bacteria strains and plasmids. The bacterial strains, primers, and plasmids used in this study are listed in Table 1. Bacteria were cultured in L-broth medium (LB; 10 g/liter tryptone, 5 g/liter yeast, 5 g/liter NaCl) at 37°C with agitation at 200 rpm (27). Antibiotics were used at the following concentrations: for *E. coli*, 100 μg/ml ampicillin, 50 μg/ml kanamycin, 10 μg/ml gentamicin, and 10 μg/ml tetracycline; for *P. aeruginosa*, 50 μg/ml tetracycline, 50 μg/ml gentamicin, and 150 μg/ml carbenicillin. Chromosomal gene mutations were generated as described previously (55).

RNA isolation and RT-qPCR. Bacteria cultured overnight were diluted 1:100 into fresh LB and cultured at 37°C to the late log phase (OD_{600} of 1). Aliquots of 1.5 ml bacteria were collected by centrifugation and resuspended in 0.5 ml TRIzol reagent (Thermo Fisher Scientific, USA). Total RNA was extracted by chloroform extraction and isopropanol precipitation. Residual DNA was digested with RNase-free recombinant DNase I (TaKaRa, Dalian, China). RNA was dissolved in RNase-free water. cDNAs were synthesized using random primers and reverse transcriptase (TaKaRa, Dalian, China). RT-qPCR was performed with the SYBR II green supermix (TaKaRa, Dalian, China). The ribosomal gene *rpsL* or PA1805 was used as the internal control (56).

Cytotoxicity assays. Bacterial cytotoxicity was determined by the lactate dehydrogenase (LDH) release assay. The A549 cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% (vol/vol) thermally inactivated fetal bovine serum, streptomycin (100 mg/ml), and penicillin G (100 U/ml) at 37°C with 5% CO_2. A total of 2×10^5 cells were inoculated into each well of a 24-well plate and cultured overnight. Bacteria were cultured at 37°C in LB to the late log phase (OD_{600} of 1), and then the bacterial cells were washed twice in phosphate-buffered saline (PBS). Before infection, the cell culture medium was replaced by DMEM with 2.5% bovine serum albumin (BSA). The cells were

infected with the indicated strains of bacteria at a multiplicity of infection (MOI) of 50. After adding bacteria to the cells, the plate was centrifuged at 700 × *g* for 10 min to synchronize the infection. The LDH level in the medium was determined with the LDH cytotoxicity assay kit (Beyotime, Shanghai, China) at 2 or 3 h postinfection. Treatment with the LDH release reagent provided by the kit was used as a control for total LDH release. The percentage of cytotoxicity was calculated according to the manufacturer's instructions.

Biofilm formation assays. The bacteria were cultured at 37°C to an OD_{600} of 1 and then diluted 1:40 into fresh LB to an OD_{600} of 0.025. A volume of 150 μl of the bacterial suspension was added into each well of a 96-well plate and cultured at 37°C for 20 h. The culture medium was discarded, and the wells were washed three times with fresh PBS and dried at 65°C for 15 min. The wells were then stained with 1% crystal violet for 20 min, washed with PBS, and dried at 65°C. Aliquots of 200 μl ethanol were added into each well and incubated with gentle shaking at room temperature. The crystal violet solution was measured at a wavelength of 595 nm.

β-Galactosidase assay. The bacteria were cultured at 37°C to an OD_{600} of 1. A volume of 0.5 ml of the bacterial culture was collected by centrifuging and resuspended in 1.5 ml Z buffer (60 mM NaH_2PO_4, 60 mM Na_2HPO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0). The β-galactosidase activity was determined as previously described (57).

Data availability. The transcriptome data that support the findings of this study have been deposited in the NCBI Sequence Read Archive (SRA) with the accession code PRJNA574019. The plasmids constructed in this study are available from Weihui Wu (wuweihui@nankai.edu.cn).

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Project of China (2017YFE0125600), the National Science Foundation of China (31670130, 31970680, and 31870130), the Tianjin Municipal Science and Technology Commission (19JCYBJC24700), and the program of China Scholarships Council (201906200035). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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