



Role of RNase Y in *Clostridium perfringens* mRNA Decay and Processing

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ABSTRACT RNase Y is a major endoribonuclease that plays a crucial role in mRNA degradation and processing. We study the role of RNase Y in the Gram-positive anaerobic pathogen *Clostridium perfringens*, which until now has not been well understood. Our study implies an important role for RNase Y-mediated RNA degradation and processing in virulence gene expression and the physiological development of the organism. We began by constructing an RNase Y conditional knockdown strain in order to observe the importance of RNase Y on growth and virulence. Our resulting transcriptome analysis shows that RNase Y affects the expression of many genes, including toxin-producing genes. We provide data to show that RNase Y depletion repressed several toxin genes in *C. perfringens* and involved the *virR-virS* two-component system. We also observe evidence that RNase Y is indispensable for processing and stabilizing the transcripts of *colA* (encoding a major toxin collagenase) and *pilA2* (encoding a major pilin component of the type IV pili). Posttranscriptional regulation of *colA* is known to be mediated by cleavage in the 5' untranslated region (5'UTR), and we observe that RNase Y depletion diminishes *colA* 5'UTR processing. We show that RNase Y is also involved in the posttranscriptional stabilization of *pilA2* mRNA, which is thought to be important for host cell adherence and biofilm formation.

IMPORTANCE RNases have important roles in RNA degradation and turnover in all organisms. *C. perfringens* is a Gram-positive anaerobic spore-forming bacterial pathogen that produces numerous extracellular enzymes and toxins, and it is linked to digestive disorders and disease. A highly conserved endoribonuclease, RNase Y, affects the expression of hundreds of genes, including toxin genes, and studying these effects is useful for understanding *C. perfringens* specifically and RNases generally. Moreover, RNase Y is involved in processing specific transcripts, and we observed that this processing in *C. perfringens* results in the stabilization of mRNAs encoding a toxin and bacterial extracellular apparatus pili. Our study shows that RNase activity is associated with gene expression, helping to determine the growth, proliferation, and virulence of *C. perfringens*.

KEYWORDS *Clostridium perfringens*, RNase Y, posttranscriptional regulation, toxin regulation, type IV pili, ribonuclease, toxin gene

RNA degradation and turnover are essential processes in all organisms, and bacterial mRNAs are rapidly degraded to facilitate quick adaptation to changing environments (1). In addition to transcriptional control, posttranscriptional control is also important for gene regulation. RNA regulation without translation requires smaller amounts of energy and permits more rapid control of expression than regulation exerted through protein factors (2). Ribonucleases (RNases) are critical factors that determine transcript instability and function in posttranscriptional gene regulation. RNases are broadly divided into endo- or exoribonuclease classes. The former class of enzymes plays an important role in initiating degradation, while the latter mediates further transcript degradation (1).

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RNases have been well studied in *Bacillus subtilis*, and roles for RNases in the principle mRNA decay pathway of this bacterium have been predicted (3). RNase Y is the major endoribonuclease that cleaves transcripts; the cleaved fragments are then degraded by the 5' to 3' exoribonuclease activity of RNase J1 and/or the 3' exoribonuclease activity of polynucleotide phosphorylase (PNPase), RNase PH, RNase R, and YhaM (4–6). RNase Y affects mRNA turnover and normal proliferation in *B. subtilis* (7). The important role of RNase Y in bacterial pathogenesis is highlighted by the fact that RNase Y is conserved in the majority of *Firmicutes* and also affects mRNA degradation and gene expression in the Gram-positive pathogens *Staphylococcus aureus* and *Streptococcus pyogenes* (8–10).

C. perfringens is a Gram-positive spore-forming anaerobic bacterium that produces numerous extracellular enzymes and toxins (11). This organism is a causative agent of gas gangrene, food poisoning, and antibiotic-associated diarrhea (AAD). Rapid gene activation and repression are important for regulating virulence in *C. perfringens*, since virulence-associated gene expression is observed to peak during the exponential phase (12). The two-component system (TCS) VirR/VirS regulates a number of toxin genes (13, 14). The response regulator, VirR, is phosphorylated and activated by a sensor kinase, VirS, inducing target gene expression (15, 16). The transcription of a small RNA (sRNA), VR-RNA, is activated by VirR/VirS; this sRNA is an effector molecule and directly or indirectly regulates >100 genes (17). Taken together, the VirR/VirS-VR-RNA regulatory cascade is important for the regulation of virulence gene expression in *C. perfringens*. Type IV pili (TFP) are virulence factors for host-microbe interactions and are also involved in host cell adherence, motility, and biofilm formation (18–20). Bacterial pathogens quickly respond to environmental stimuli and possess multiple strategies in order to rapidly regulate genes to achieve effective infection (21). The rapid degradation of mRNA and RNA regulation affect the regulation of virulence factors in *C. perfringens*. It is not clearly known, however, whether mRNA decay and processing are involved in gene expression and pathogenesis of this organism.

A putative major RNase, RNase Y, is highly conserved in *Clostridia* and is predicted to be encoded by CPE1672 (here called the *rny* gene) in *C. perfringens*, based on sequence similarity. In this study, we constructed an RNase Y-depleted strain of *C. perfringens* to understand its importance for growth and virulence factor expression in *C. perfringens*. In the RNase Y-depleted strain, we observed a drastic attenuation of expression of toxin genes regulated by the VirR/VirS TCS cascade and a decrease in the expression of a major secreted toxin. Notably, transcripts of the collagenase gene *colA* were processed and accumulated in the presence of RNase Y, which suggests the involvement of RNase Y-dependent cleavage of *colA* transcripts in *colA* regulation. Additionally, RNase Y was involved in the processing and accumulation of *pilA2* mRNA (encoding a TFP pilin in the TFP biosynthesis operon, *pilD-pilB2-pilC2-pilA2*). These results show that RNase Y affects TFP component expression and host cell attachment. Transcriptome analysis of the RNase Y-depleted strain revealed the effects of RNase Y on the expression of a number of mRNAs. Thus, RNase Y plays a critical role in RNA degradation and gene expression in *C. perfringens*.

RESULTS

RNase Y is important for the growth of *Clostridium perfringens* cells. We compared the sequence of the *rny* gene encoding RNase Y of *B. subtilis* with clostridial genome sequences and found that the *rny* gene is highly conserved in *Clostridia* (see Fig. S1 in the supplemental material). This suggests that the protein product of the *rny* gene in *Clostridia* also exhibits endoribonuclease activity and is involved in RNA degradation and processing. We attempted to construct a null mutant of the *rny* gene via double crossover to analyze RNase Y function in *C. perfringens*. However, construction was not successful. Despite the potential for low efficiency in electroporation with our method for homologous recombination with linear DNA fragments, we assumed that RNase Y was important for cell proliferation in *C. perfringens*. Due to this assumption, we then constructed a conditional knockdown *rny* strain by replacing the native

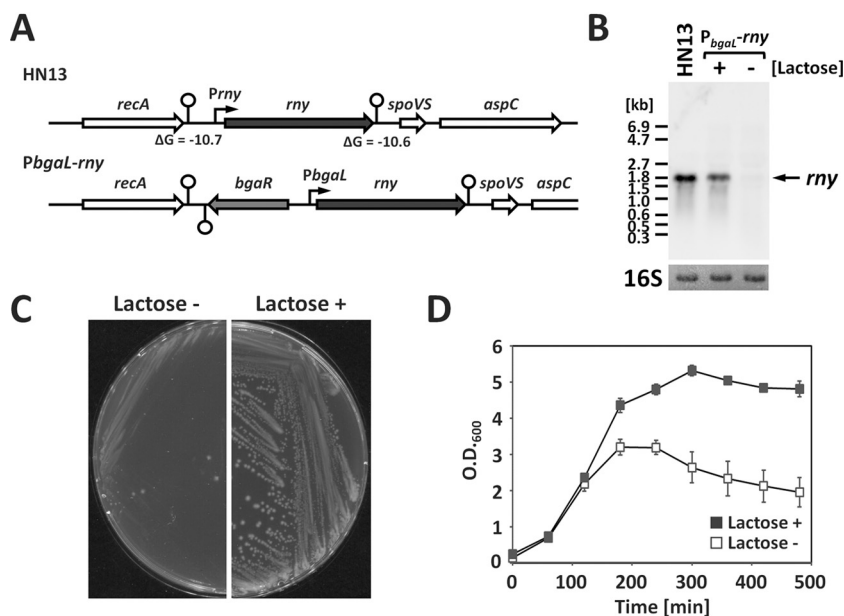


FIG 1 RNase Y is important for the growth of *C. perfringens*. (A) Schematics of the *rny* locus in HN13 and the *rny* conditional knockdown strain. The transcriptional start sites and terminators are represented by bent arrows and stem-loop structures, respectively. (B) Northern blot analysis of the *rny* gene. Each lane contained 2 μ g of total RNA isolated from the culture at the mid-exponential phase. The *rny* conditional knockdown strain was cultured with or without 1 mM lactose to induce *rny* gene expression under the *bgaL* promoter. 16S rRNA stained with methylene blue on the blots is indicated at the bottom as a loading control. (C) Lactose addition is required for growth of the *rny* conditional knockdown strain on GAM/2 plates. The *rny* conditional knockdown strain was anaerobically grown for 12 h on GAM/2 plates with or without 1 mM lactose under anaerobic conditions. (D) Effects of the inducer on cell growth in liquid culture. *rny* conditional knockdown strains were grown overnight in PGY medium containing 1 mM lactose, and the cells were washed twice with GAM broth. The cells were inoculated with and cultured in GAM broth with or without 1 mM lactose at 37°C.

rny promoter region with the lactose-inducible *bgaL* promoter (22, 23) (Fig. 1A). In the resultant strain, the *rny* promoter was replaced with the *bgaR* gene and the *bgaL* promoter, and we induced transcription of the *rny* gene by adding lactose to the culture medium (Fig. 1B). *rny* expression in response to lactose was stable and reproducible in different experiments (Fig. S2). We observed that for the *rny* conditional knockdown strain, cell growth on agar plates was drastically inhibited in the absence of lactose compared to that in the presence of lactose, which suggested the importance of RNase Y for *C. perfringens* cell growth (Fig. 1C).

RNase Y affects global gene expression. To determine the role of RNase Y in gene expression, we compared the transcriptome profiles of the *rny* conditional knockdown strain in the presence or absence of the inducer. The *rny* conditional knockdown strain was precultured in PGY medium containing 1 mM lactose, and the cells were washed twice with GAM broth. The washed cells were inoculated in GAM broth with or without lactose, and the optical densities of the culture were plotted (Fig. 1D). The growth rate of the strain both with and without lactose was comparable at mid-exponential phase; however, the absence of the inducer inhibited growth during the late-exponential or stationary phase (Fig. 1D). To account for the effects of these different growth rates exerted by the two culture conditions, we isolated total RNA from the cultures at mid-exponential phase (120 min; optical density at 600 nm [OD₆₀₀] of 1.0 to 2.0). Microarray analysis revealed the effects of RNase Y depletion on the mRNA levels of over 400 genes. The expression of 235 and 188 genes significantly decreased and increased, respectively (the log₂ ratio of the expression levels in the *rny* conditional knockdown strain with or without the inducer was less than -1 or greater than 1, and the *P* value was less than 0.05) (Fig. S3 and Tables S2 and S3). This indicates that RNase Y is involved in mRNA turnover and affects global gene expression in *C. perfringens*.

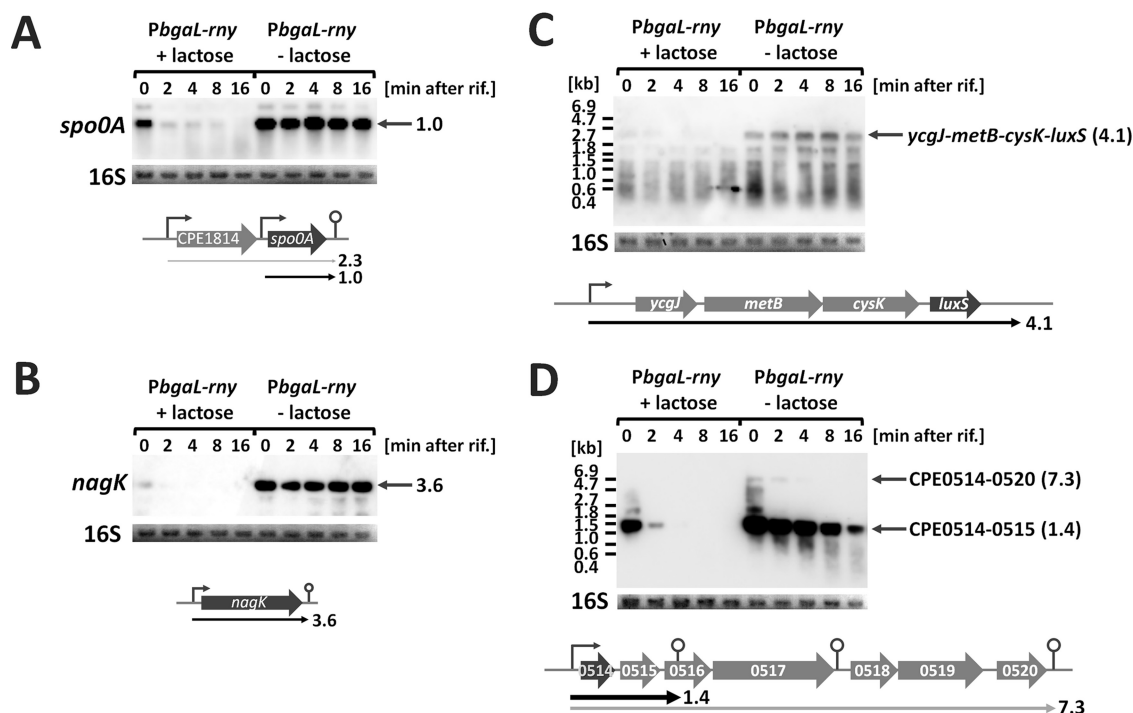


FIG 2 RNase Y affects the stability of specific transcripts *in vivo*. Half-lives of *spo0A* (A), *nagK* (B), *ycgJ-metB-cysK-luxS* (C), and CPE0514-CPE0520 (D) mRNAs. *rny* conditional knockdown strains were cultured with or without 1 mM lactose to mid-exponential phase at 37°C. Total RNA was isolated prior to or after the addition of rifampin (rif; 200 $\mu\text{g ml}^{-1}$). In each lane, 2 μg of total RNA was loaded and hybridized with *spo0A*-, *nagK*-, *luxS*-, or CPE0514-specific probes. The lengths of the transcripts detected are indicated in kilobases. 16S rRNA stained with methylene blue on the blots is indicated at the bottom as a loading control. Schematics of genetic maps are shown below. The predicted transcriptional start sites and terminators are indicated by bent arrows and stem-loop structures, respectively. Arrows represent the mRNAs detected by Northern blotting.

RNase Y is involved in destabilizing specific mRNAs. According to our transcriptome analysis, a number of mRNAs accumulated under RNase Y-depleted conditions, suggesting these mRNAs are direct targets for degradation by RNase Y (Table S2). To analyze the effects of RNase Y on mRNA half-life, we selected the following genes that accumulated when RNase Y was depleted: *spo0A*, encoding a sporulation master regulator (24); monocistronic *nagK*, encoding the μ -toxin hyaluronidase (25); the tetracistronic operon *ycgJ-metB-cysK-luxS*, encoding the AI-2-producing enzyme LuxS (26); and the long operon CPE0514-CPE0520, which encodes putative extracellular proteins and the two-component system (27). The monocistronic *spo0A* and *nagK* mRNAs were highly stable under RNase Y-depleted conditions, although they were rapidly degraded after the addition of rifampin in the presence of the inducer lactose (Fig. 2A and B). In addition, the 4.1-kb *ycgJ-metB-cysK-luxS* mRNA was unstable and undetectable 2 min after rifampin addition in the presence of the inducer but was stable and detectable at 16 min when RNase Y was depleted (Fig. 2C). We detected the 1.4-kb CPE0514-CPE0515 bicistronic mRNA and the 7.3-kb CPE0514-CPE0520 polycistronic mRNA and observed stabilization of the bicistronic mRNA upon RNase Y depletion (Fig. 2D). This shows that in *C. perfringens* RNase Y is involved in the degradation of at least four and possibly more transcripts.

RNase Y impacts toxin gene expression through the VirR/VirS two-component system. The expression of several major toxin genes, specifically *plc*, *pfoA*, *colA*, *ccp*, and *cpb2*, was decreased under RNase Y-depleted conditions (Fig. 3A and Table 1; Table S3). RNase Y depletion also decreased the amount of extracellular secreted alpha-toxin, a phospholipase C (Fig. 3B). Furthermore, expression of the *vrr* gene, which encodes a small regulatory RNA, VR-RNA, decreased under RNase Y-depleted conditions (Fig. 3A). We postulated that RNase Y affects toxin production via the VirR/VirS-VR-RNA cascade because toxin genes were either directly or indirectly regulated by the VirR/VirS-VR-

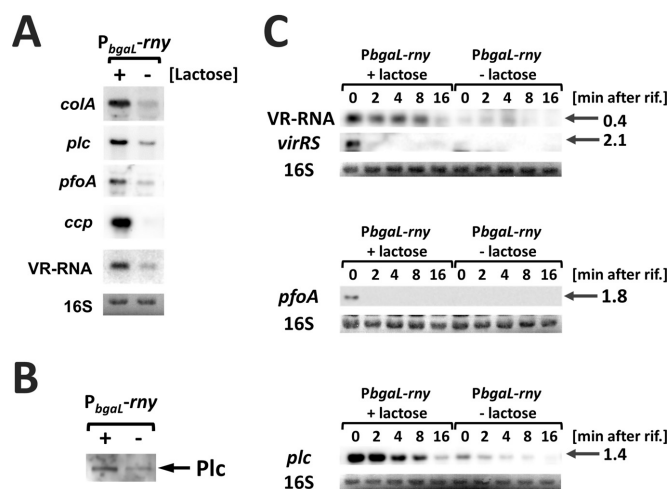


FIG 3 RNase Y affects the expression of toxin genes through the VirR/VirS–VR-RNA cascade. (A) The expression of virulence-associated genes was affected by RNase Y. Total RNAs (2 μ g) isolated from cells cultured for 2 h at 37°C were used for Northern blotting of *colA*, *plc*, *pfoA*, *ccp*, and VR-RNA. 16S rRNA stained with methylene blue on the blots is indicated at the bottom as a loading control. (B) Western blotting of Plc proteins in the culture supernatant. Extracellular proteins equivalent to 0.01 OD₆₀₀ units were separated by SDS-PAGE and were probed with anti-Plc antisera. (C) Half-lives of *virRS* mRNA and VirR/VirS target gene mRNAs in *rny* conditional knockdown strains. *rny* conditional knockdown strains were cultured with or without 1 mM lactose to mid-exponential phase at 37°C. Total RNA was isolated prior to or after the addition of rifampin (200 μ g ml⁻¹). In each lane, 2 μ g of total RNA was loaded and hybridized with VR-RNA-, *plc*-, *vir*-, or *pfoA*-specific probes. The lengths of the detected transcripts are indicated in kilonucleotides on the right. 16S rRNA stained with methylene blue on the blots is indicated at the bottom as a loading control.

RNA cascade (17). We examined the mRNA half-life of the *plc* gene, which encodes the alpha-toxin phospholipase C, the *virRS* gene, which encodes the response regulator VirR, and the sensor kinases VirS and VR-RNA. RNase Y depletion negatively affected the amount of each transcript but demonstrated little impact on the stability of *plc*, *virRS* mRNA, and VR-RNA (Fig. 3B). Northern blots and transcriptome analysis (the log₂ ratio with/without inducer was -2.1) indicated a decrease in *virRS* mRNA under RNase Y-depleted conditions (Fig. 3B). The *virRS* transcript was highly unstable; we were unable to detect it 2 min after the addition of rifampin with or without the inducer lactose. Thus, RNase Y affects *virRS* gene expression at the transcriptional level, not at the posttranscriptional level. The expression of the VirR direct target gene *pfoA*, whose promoter sequence contains the VirR protein-binding box (15), also decreased. However, transcript stability was not affected by RNase Y depletion (Fig. 3C).

RNase Y is responsible for *colA* primary transcript processing. We previously observed base pairing between VR-RNA and the *colA* primary transcript, resulting in a partial melting of the stem-loop structure in the 5' untranslated region (5'UTR) of the transcript (28). The primary *colA* transcript is unstable because the stem-loop structure masks the ribosome binding site (RBS), thereby inhibiting translation. Partial melting of the *colA* 5'UTR stem-loop structure via VR-RNA–*colA* base pairing induces cleavage of the *colA* mRNA 5'UTR; this induced cleavage activates translation and stabilizes the transcript (28) (Fig. 4A). The RNase responsible for this cleavage was not previously identified. As described above, expression of *colA* mRNA decreased after RNase Y depletion (Fig. 4B). Thus, RNase Y is potentially involved in *colA* primary mRNA cleavage.

RNase Y depletion represses *colA* expression, which potentially is a secondary effect that results from decreased amounts of VR-RNA because the amount of VR-RNA decreased under RNase Y-depleted conditions. However, to exclude this possibility, we analyzed *colA* expression at 45°C. At higher temperatures, the stem-loop structure is partially melted, which can induce *colA* 5'UTR processing without VR-RNA (29) (Fig. 4A). If *colA* repression under RNase Y-depleted conditions is a secondary effect of VR-RNA

TABLE 1 Effect of RNase Y on virulence-associated genes^a

Locus	Gene	Product name	Expression level log ₂ ratio ^b	P value
Loci with increased expression under RNase Y-depleted condition				
CPE0030	<i>hlyA</i>	Hemolysin-related protein	1.99	0.065
CPE0452	<i>entC</i>	Enterotoxin	1.88	0.019
CPE1234	<i>nagJ</i>	Hyaluronidase	3.44	0.007
CPE1279	<i>nagK</i>	Hyaluronidase	2.61	0.001
Loci with decreased expression under RNase Y-depleted condition				
CPE0036	<i>plc</i>	Phospholipase C	-2.88	0.101
CPE0163	<i>pfoA</i>	Perfringolysin O	-3.38	0.049
CPE0173	<i>colA</i>	Collagenase	-3.10	0.087
CPE0846	<i>ccp</i>	Alpha-clostripain	-5.75	0.097
CPE0957	<i>vrr</i>	Hypothetical protein CPE0957	-2.49	0.044
CPE1231		Surface protein	-1.07	0.008
CPE1258	<i>entA</i>	Putative enterotoxin	-2.81	0.052
CPE1500	<i>virS</i>	Sensor histidine kinase VirS	-1.57	0.046
CPE1818	<i>hlyD</i>	Hemolysin A	-1.04	0.004
PCP17	<i>cpb2</i>	Beta2-toxin	-2.30	0.356
PCP57	<i>cna</i>	Collagen adhesin	-1.27	0.163
Unaffected loci				
CPE0191	<i>nagH</i>	Hyaluronidase	-0.18	0.083
CPE0378		Myosin cross-reactive antigen family protein	-0.02	0.838
CPE0437	<i>hlyB</i>	Hemolysin	-0.38	0.131
CPE0553	<i>nanJ</i>	Exo-alpha-sialidase	-0.41	0.285
CPE0606	<i>entD</i>	Enterotoxin	-0.66	0.050
CPE0725	<i>nanI</i>	Exo-alpha-sialidase	-0.59	0.048
CPE0737		Putative fibronectin-binding protein	-0.70	0.004
CPE0881	<i>nagI</i>	Hyaluronidase	0.58	0.001
CPE1354	<i>entB</i>	Enterotoxin	0.43	0.034
CPE1474	<i>hlyC</i>	Hemolysin III	-0.85	0.072
CPE1501	<i>virR</i>	DNA-binding response regulator, LytTR family	-0.85	0.048
CPE1523	<i>nagL</i>	Hyaluronidase	0.04	0.647
CPE1847		Fibronectin-binding protein	0.52	0.046
CPE1915	<i>hlyE</i>	Hemolysin III	-0.76	0.218

^aVirulence-associated genes were predicted by Shimizu et al. (45).

^bLog₂ ratio of expression in the *my* conditional knockdown strain without the inducer to expression with the inducer.

repression, RNase Y depletion should have no effect on *colA* expression at 45°C. However, because RNase Y depletion reduced *colA* expression even at 45°C, we can infer that the repression of *colA* due to RNase Y depletion is independent of VR-RNA (Fig. 4B). Moreover, we observed stabilization of *colA* mRNA through the induction of RNase Y expression (Fig. 4C).

We employed 5' RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) to discriminate between primary and processed *colA* transcripts, which enabled us to determine whether RNase Y affects *colA* primary transcript processing. Treatment with 5'-dependent exoribonuclease (TEX) and pyrophosphatase (PPH) eliminates processed transcripts and makes the primary transcripts detectable by PCR. The PCR products were detected in both the presence and absence of the inducer (Fig. 4D, +TEX +PPH). However, to detect the processed transcripts we used the total RNA that was not treated with PPH and TEX for PCR. In this case, the processed transcripts were amplified when we induced RNase Y expression (Fig. 4D, NT). This PCR product was not amplified when the RNAs were treated with TEX, indicating that the PCR product corresponds to the processed transcripts (Fig. 4D, TEX). Based on these results, RNase Y is responsible for the cleavage and stabilization of *colA* mRNA.

Effect of RNase Y on the expression of a type IV pilus component, *pilA2*. In *C. perfringens*, the type IV pili (TFP) are involved in motility, attachment to eukaryotic cells,

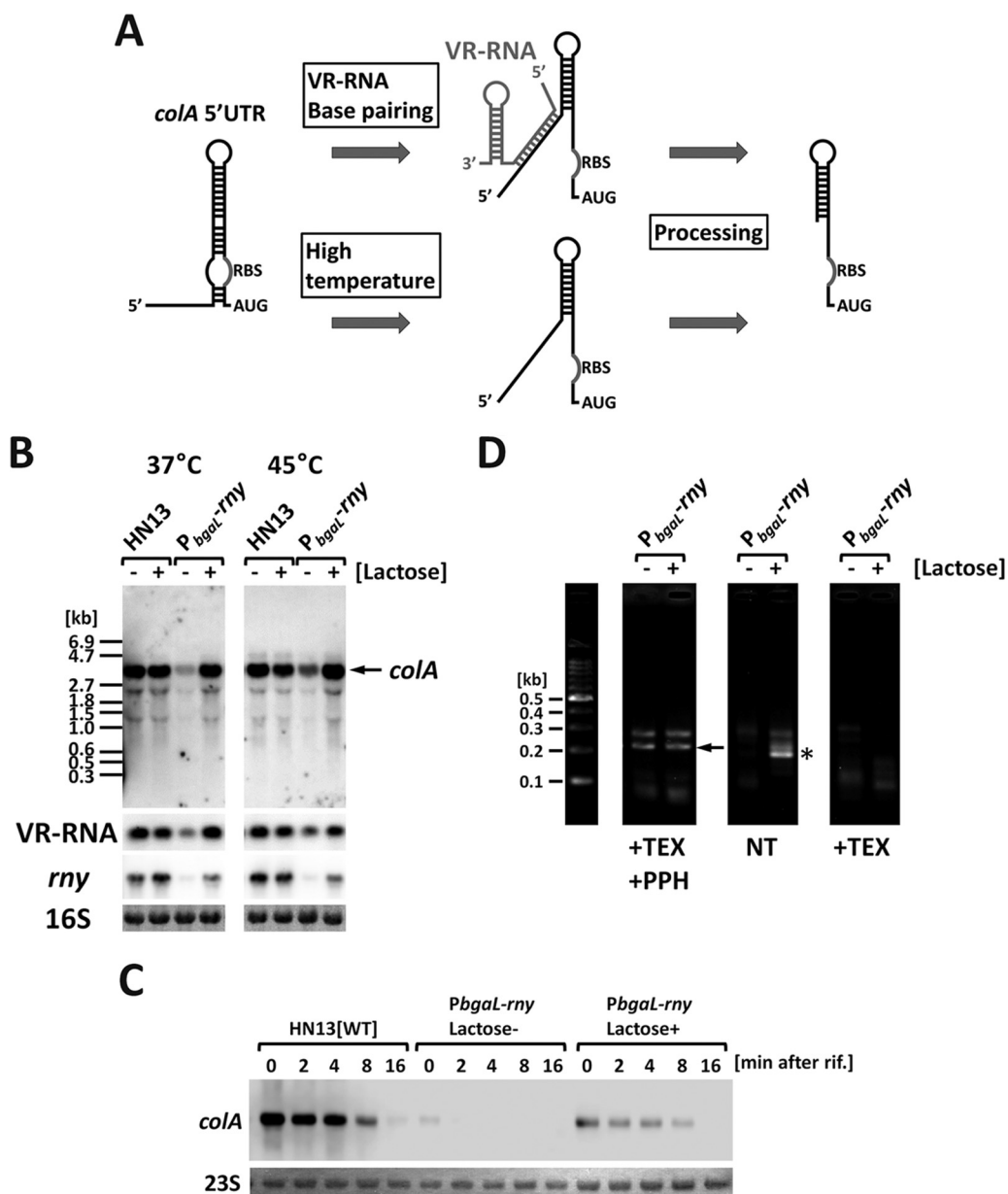


FIG 4 *colA* 5'UTR cleavage requires RNase Y. (A) A model of posttranscriptional regulation in the *colA* mRNA 5'UTR. The long stem-loop structure in the *colA* 5'UTR is partially melted by VR-RNA base pairing or high temperatures, and this structural change induces cleavage. The *colA* RBS in the processed transcripts is free from the RNA secondary structure, and translation was stimulated. (B) Northern blotting of *colA* mRNA in the *rny* conditional knockdown strain. Total RNA was isolated from cultures grown to mid-exponential phase at 37 or 45°C with or without lactose, and 2 μg of RNA was dissolved in denaturing agarose. VR-RNA and *colA* and *rny* mRNA were detected using DIG-labeled specific DNA probes. 16S rRNA stained with methylene blue on the blots is indicated at the bottom as a loading control. (C) The half-life of *colA* mRNA was affected by RNase Y. *rny* conditional knockdown strains or HN13 was cultured with or without 1 mM lactose to mid-exponential phase at 45°C. Total RNA was isolated prior to or after the addition of rifampin (200 μg ml⁻¹). In each lane, 2 μg of total RNA was loaded and hybridized with *colA*-specific probes. 16S rRNA stained with methylene blue on the blots is indicated at the bottom as a loading control. (D) 5'RLM-RACE analysis of *colA* transcripts. Total RNA isolated from *rny* conditional knockdown strains cultured at 45°C with or without lactose was treated with or without terminal-dependent exonuclease (TEX) and pyrophosphatase (PPH) to distinguish primary transcripts from processed transcripts. The PCR products were resolved on a 2% agarose gel and stained with ethidium bromide. Asterisks and arrows show PCR products derived from processed and primary transcripts (estimated product sizes are 182 and 244 bp), respectively.

and biofilm formation, suggesting an important role for TFP in pathogenesis (18, 19, 30). A TFP biosynthesis operon, *pilD-pilB2-pilC2-pilA2*, encodes the PilD peptidase and PilA2 pilin, a major component of TFP (Fig. 5A). The expression of *pilA2* was drastically repressed under RNase Y-depleted conditions (the log₂ ratio of the expression levels

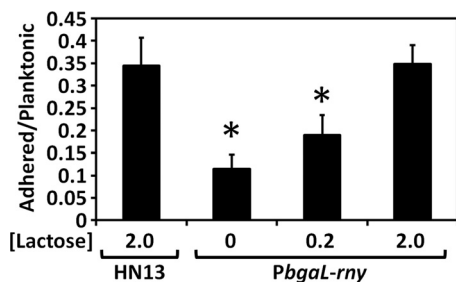


FIG 6 RNase Y depletion decreases attachment to polystyrene surface and biofilm formation. *C. perfringens* was grown with or without lactose for 24 h. Cell adhesion activity was calculated by measuring the OD₆₀₀ of adherent or planktonic cells. Asterisks show statistically significant differences with respect to HN13 ($P < 0.05$). P values were calculated using a two-tailed unpaired t test with Welch's correction.

under RNase Y-depleted conditions was -2.65) (Table S3). Although we have predicted that there is only one promoter sequence upstream of *pilD*, we detected both a monocistronic *pilA2* mRNA and polycistronic *pilD-pilB2-pilC2-pilA2* mRNA by Northern blotting (20) (Fig. 5B). The amount of monocistronic *pilA2* mRNA was reduced under RNase Y-depleted conditions (Fig. 5B), and therefore we hypothesized that RNase Y-dependent posttranscriptional cleavage occurs in polycistronic mRNA.

To determine whether monocistronic *pilA2* mRNA is a processed product, the 5' termini of the polycistronic *pilD-pilB2-pilC2-pilA2* mRNA and the monocistronic *pilA2* mRNA were detected by 5'RLM-RACE (Fig. 5C and D). Prior to RNA adaptor ligation, we treated the total RNA with pyrophosphatase (PPH). This allowed us to detect the PCR products amplified from the 5' terminus of *pilD* mRNA, which indicates that the 5' terminus of *pilD* mRNA is a transcription start site (Fig. 5C). Additionally, a typical σ^A consensus sequence was found in the region upstream of the predicted *pilD* transcriptional start site (Fig. 5E). However, the 250-bp PCR products amplified from the 5' terminus of *pilA2* mRNA were completely eliminated in the presence of TEX; this indicates that monocistronic *pilA2* mRNA is a processed transcript (Fig. 5D and F). Although we detected a 100-bp band in the TEX- and PPH-treated lanes (Fig. 5D), we can justifiably assume that this is the result of nonspecific amplification; PCR products of less than 100 bp are too small to correspond to the monocistronic *pilA2* transcripts, because we detected only monocistronic *pilA2* transcripts of greater than 500 nucleotides by Northern blot analysis (Fig. S4).

To examine the effect of processing on mRNA, we compared the stability of the primary and processed mRNA in wild-type cells. We observed increased stability of the processed monocistronic *pilA2* mRNA over the primary polycistronic transcripts (Fig. 5G). Moreover, the amount of PilA2 protein decreased under RNase Y-depleted conditions (Fig. 5H). Therefore, we reason that RNase Y is involved in the processing and stabilization of *pilA2* mRNA, which is required for maximal PilA2 protein expression.

RNase Y may also play an important role in TFP biosynthesis and function. RNase Y likely affects cell-substrate attachment and biofilm formation in *C. perfringens*, because TFP are involved in host cell attachment and biofilm formation (19, 30). We cultured the *C. perfringens* strains in polystyrene microtiter plates and measured the amount of adhered cells and biofilm biomass. The attachment and biofilm formation by the RNase Y conditional knockdown strain to polystyrene surfaces were significantly decreased compared to those of the wild-type HN13 strain (Fig. 6). In the presence of lactose, these amounts increased to the same levels observed for the wild-type strain (Fig. 6). This shows that RNase Y activity has an important role in attachment and biofilm formation in *C. perfringens*.

RNase Y is involved in the degradation of mRNAs of the protein secretion system. It is believed that *C. perfringens* secretes extracellular enzymes and toxins through a Sec-dependent pathway because these protein sequences are predicted to contain a signal peptide (31) (Table S4). Under RNase Y-depleted conditions, the

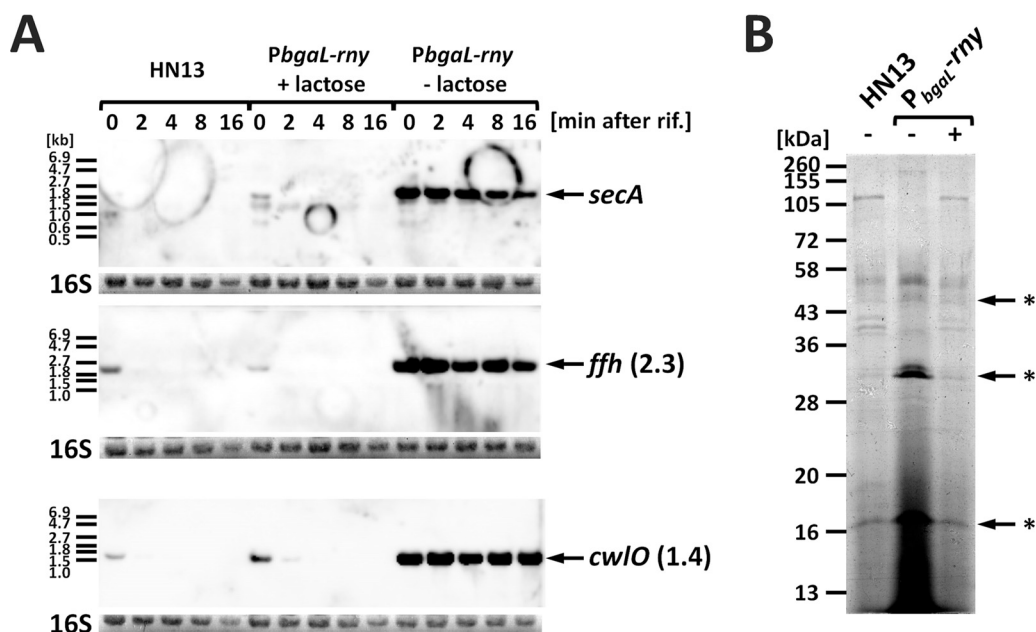


FIG 7 RNase Y affects the Sec secretion system. (A) *rny* conditional knockdown strains or HN13 were cultured with or without 1 mM lactose to mid-exponential phase at 37°C. Total RNA was isolated prior to or after the addition of rifampin (200 $\mu\text{g ml}^{-1}$). In each lane, 2 μg of total RNA was loaded and hybridized with *secA*-, *ffh*-, and *cwIO*-specific probes. The lengths of the detected transcripts are indicated in kilobases on the right. 16S rRNA stained with methylene blue on the blots is indicated at the bottom as a loading control. (B) Extracellular protein expression in HN13 or *rny* conditional knockdown strains. Extracellular proteins equivalent to 0.04 OD₆₀₀ units were prepared from the supernatant of the culture grown for 6 h at 37°C and were loaded in each lane. Arrows indicate CwIO proteins estimated based on molecular size.

expression of the *ffh* gene, which encodes a signal recognition particle protein, was highly upregulated according to our microarray analysis (Table S2). This observation led us to analyze Sec-dependent protein secretion in the *rny* conditional knockdown strain. We found that the *secA* and *ffh* mRNAs were stable when RNase Y was depleted but were unstable and thus undetectable 2 min after the addition of rifampin in the presence of RNase Y (Fig. 7A). This result is consistent with the transcriptome analysis and suggests the involvement of RNase Y in *secA* and *ffh* transcript turnover. The *cwIO* gene encodes a cell wall lytic endopeptidase, and in *B. subtilis* transcript stability is regulated by RNase Y (32). Northern blot analysis showed the *cwIO* transcript stability was increased under RNase Y-depleted conditions in *C. perfringens* (Fig. 7A). The 45-, 30-, and 15-kDa proteins have been predicted to be CwIO fragments and are the major extracellular proteins in this organism (22). We investigated the amounts of CwIO protein present in the culture supernatant of the *rny* conditional knockdown strain. The 45-, 30-, and 15-kDa proteins accumulated in the *rny* conditional knockdown strain cultured without lactose, suggesting the effects of RNase Y depletion on the Sec secretion system in *C. perfringens* (Fig. 7B).

DISCUSSION

RNase Y is a major endoribonuclease and plays an important role in mRNA turnover in *C. perfringens*. Cells are unable to grow normally under RNase Y-depleted conditions (Fig. 1), indicating the importance of RNase Y for the proliferation and physiological activity of *C. perfringens*. RNase Y depletion impacts a large number of genes in various functional classes, which suggests that combinatorial effects cause cell growth defects (see Fig. S3 in the supplemental material). In this study, we measured lactose-inducible RNase Y expression at the transcript level by Northern blotting. We cannot exclude the possibility that the amount of RNase Y protein was not accurately controlled by lactose. However, the stability of several transcripts was drastically affected as a function of the lactose content (Fig. 2 and 7). Thus, lactose plays a significant role in the regulation of RNase Y protein levels in the *rny* conditional knockdown strain. We attempted to

inactivate *rny* using a gene replacement system but were unsuccessful, so it is still unclear whether RNase Y is essential for *C. perfringens* growth. The *B. subtilis* RNase Y deletion strain exhibits major defects in cell morphology and growth but is viable (33); however, to inactivate RNase Y in *B. subtilis*, high recombination efficiency appears to be required. In *C. perfringens*, we suspect that low transformation and recombination efficiency is the cause of our failure to generate an RNase Y deletion strain.

Transcriptome analysis revealed the global effects of RNase Y on gene expression, including toxin genes. We conducted a transcriptome analysis in the mid-exponential phase when the growth rate of the strain under both lactose-positive and -negative conditions were comparable. However, the downregulation of the toxin genes under the RNase Y-depleted condition might correlate with a decrease in growth rate and may not be specific to RNase Y. Meanwhile, we found that RNase Y depletion affected the expression of virulence factors under VirR/VirS TCS control. VirR/VirS–VR-RNA is the major regulatory cascade involved in toxin gene regulation; over 100 genes are controlled by the VirR/VirS–VR-RNA cascade, suggesting indirect effects of RNase Y on the expression of many genes through the VirR/VirS TCS (34). RNase Y depletion did not affect *virR-virS* mRNA stability, suggesting RNase Y affects *virR-virS* expression at the transcriptional level. The mRNA encoding the *virR-virS* gene regulator may be processed or degraded by RNase Y.

RNase Y is an endoribonuclease responsible for mRNA turnover in *B. subtilis*, *S. aureus*, and *S. pyogenes* (7–10, 35, 36). We suggest that this enzyme also plays an important role in RNA degradation in *C. perfringens*, because the expression of 188 genes was upregulated in our microarray experiment under RNase Y-depleted conditions. A Northern blot analysis of *spo0A*, *nagK*, *ycgl-metB-cysK-luxS*, CPE0514–CPE0515, *secA*, *ffh*, and *cwI* also supported this hypothesis. The transcript of the *cwI* homolog in *B. subtilis* is unstable and is cleaved by RNase Y in its 5'UTR, which initiates degradation of the transcript (32). In *C. perfringens*, *cwI* transcripts are also highly unstable and are undetectable 2 min after the addition of rifampin. *cwI* mRNA may be cleaved by RNase Y, triggering transcript degradation. In addition, several target genes directly regulated by RNase Y have been identified in *B. subtilis*. The glycolytic *gapA* operon is composed of the *cggR-gapA-pgk-tpi-pgm-eno* genes and is cleaved at a position upstream of the *gapA* open reading frame (ORF) by RNase Y in *B. subtilis* (37). In *C. perfringens*, the operon is conserved and composed of the *cggR-gapC-pgk-tpiA-pgm* genes. The longer *gapC* operon mRNAs were detected under RNase Y-depleted conditions, which suggests that the effects of RNase Y on gene expression are realized through mRNA cleavage in *C. perfringens* (Fig. S5).

The kappa-toxin collagenase is a major virulence factor and is encoded by the *colA* gene. The *colA* mRNA 5'UTR forms a long stem-loop structure that masks the RBS, thereby inhibiting translation and rendering the transcripts unstable (28). There are two ways to partially melt the secondary structure of the *colA* mRNA and induce processing in the 5'UTR: (i) base pairing of the sRNA VR-RNA or (ii) elevating temperatures (29). The processed transcripts are stable and translatable because the RBS is free from the secondary structure. However, the enzyme that cleaves *colA* transcripts is unknown. Under RNase Y-depleted conditions, we saw that *colA* transcripts became unstable even at higher temperatures. Additionally, we could not detect the processed transcript. From this we can induce that RNase Y is responsible for *colA* processing and stabilization and, furthermore, is coupled to sRNA- or temperature-dependent *colA* regulation. Endoribonuclease E (RNase E) is a major enzyme involved in RNA turnover in gamma proteobacteria such as *Escherichia coli*. RNase E is involved not only in maintaining steady-state mRNA levels but also in the sRNA-dependent regulation of specific mRNAs (38). In *B. subtilis*, RNase Y is thought to be the functional equivalent of RNase E and functions in concert with the RoxS sRNA in regulation of *ppnKB* and *sucCD* (39, 40). *colA* regulation by VR-RNA and RNase Y is the first example of *trans*-acting sRNAs and endoribonucleases functioning in concert in clostridia. RNase Y may be an important factor for sRNA-dependent posttranscriptional regulation in *Clostridium* and *Bacillus* (41, 42).

In this study, we also revealed the effects of RNase Y on the expression of the TFP gene *pilA2*, which encodes a pilin that is the primary component of TFP. The TFP biosynthesis operon consists of the *pilD*, *pilB2*, *pilC2*, and *pilA2* genes, and these genes are cotranscribed from the promoter located upstream of the *pilD* gene. Northern blotting detected *pilA2* monocistronic mRNA in addition to *pilD-pilB2-pilC2-pilA2* polycistronic mRNA. Promoter prediction, the reporter assay, and 5'RLM-RACE indicated the absence of any promoter immediately upstream of the *pilA2* gene (20) (Fig. 5). This indicates that *pilA2* monocistronic mRNA is the processed product of primary polycistronic transcripts and consequently that RNase Y is required for the efficient processing of *pilA2* mRNA.

The PilA2 pilin, a major component of TFP, polymerizes and forms a pilus structure (43). TFP activity and ability to rapidly respond to the environment is tightly controlled by the amount of *pilA2* transcripts. We know that RNase Y-mediated *pilA2* transcript processing actually controls *pilA2* expression because *pilD-pilB2-pilC2-pilA2* polycistronic mRNA is unstable, while processed *pilA2* monocistronic mRNA is stable. The 5'-proximal RNA structure of the *pilA2* monocistronic mRNA was predicted to form a stem-loop structure (data not shown), which might contribute to the stability of the transcript (44). We recently observed the accumulation of monocistronic *pilA2* mRNA in cells grown at 37°C compared to 25°C, which highly suggests that PilA2 protein amounts and processing depends on temperature (20). In fact, it is known that TFP-mediated cell surface adhesion decreased at 25°C (20). Western blotting for glutathione S-transferase (GST) expressed from the *pilD* promoter revealed minimal effects of temperature on promoter activity (Fig. S6). RNase Y depletion decreased *C. perfringens* attachment to polystyrene surfaces and biofilm formation (Fig. 6). Therefore, the RNase Y-dependent cleavage of *pilA2* is important for TFP response to environmental change and its relative functions in cell adhesion and biofilm formation.

RNase Y depletion also leads to the accumulation of mRNAs encoding two components of the Sec-dependent secretion system, SecA and Ffh. *C. perfringens* has no secretion system other than the Sec system (45). Therefore, the Sec secretion system is important for the physiology and proliferation of this organism. In *B. subtilis*, transcriptome analysis of three ribonucleases, RNase Y, RNase J1, and RNase III, has been performed, but the ribonucleases involved in regulating the Sec secretion components have not been identified (36). The results shown in Fig. 7 reveal the posttranscriptional effects of RNase Y on *secA* and *ffh* expression. The Sec system in *C. perfringens* must be rapidly regulated in response to the environment and requires RNase Y-dependent posttranscriptional degradation. The smaller *secA* mRNA was detected in the presence of RNase Y, as shown in Fig. 7. Thus, *secA* mRNA may be cleaved by RNase Y.

The major RNase RNase Y is involved in mRNA turnover and cleaves specific mRNAs. This RNase also affects toxin production and secretion, cell adhesion, and cell proliferation in *C. perfringens*. Furthermore, a number of sRNAs have been predicted in the genus *Clostridium*, and these sRNAs are potentially involved in physiological functions (42). RNase Y is more likely to have an important role in sRNA function, because this enzyme is involved in posttranscriptional *colA* regulation by VR-RNA, an sRNA in *C. perfringens*. Moreover, RNase Y activity affects the amounts of specific transcripts related to toxin, adherence, and protein secretion. Therefore, RNase Y is potentially a good target to control pathogenic, solventogenic, and cellulose-degrading clostridia.

MATERIALS AND METHODS

Bacterial strains and culturing conditions. *Clostridium perfringens* strain 13 (45), HN13 (22), and derivative strains were cultured on Gifu anaerobic medium/2 (GAM/2) agar plates (Nissui Co., Japan) under anaerobic conditions using an Anaeropack system (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan) and PGY (3% proteose peptone no. 3, 2% glucose, 1% yeast extract, and 0.1% sodium thioglycolate) or GAM liquid broth supplemented with 1 mM lactose. *Escherichia coli* JM109 was cultured in LB medium supplemented with 50 $\mu\text{g ml}^{-1}$ ampicillin or 25 $\mu\text{g ml}^{-1}$ chloramphenicol.

Oligonucleotides. The oligonucleotides used in this study are listed in Table S1 in the supplemental material.

Conditional knockdown strain construction. We constructed the *my* conditional knockdown strain using a galactose counterselection system and lactose-inducible promoter (22, 23). The upstream and

downstream regions of the *rny* promoter region were amplified using primer pairs NOB-0642/NOB-0643 and NOB-0644/NOB-0645 and digested with Sall/SacI and BamHI/PstI, respectively. The *bgaR* ORF and *bgaL* promoter region were amplified using primers NOB-0488/NOB-0489 and digested with SacI/BamHI. These fragments were ligated and cloned into the Sall/PstI site of pCM-GALK (22), and the resulting plasmid was introduced into *C. perfringens* HN13 via electroporation. Agar plates containing 1 mM lactose with or without 20 μ g/ml chloramphenicol were used for isolation of the strains. We confirmed the generated strain by PCR and DNA sequencing.

Northern blot analysis. The *rny* conditional knockdown strains were cultured overnight in PGY medium containing 1 mM lactose. The cells were washed twice with GAM broth and inoculated in fresh GAM broth containing either 1 mM or no lactose to reach an OD₆₀₀ of 0.1. The cultures were incubated for 2 h at 37°C or 1.5 h at 45°C to reach a mid-exponential growth phase. Total RNA was extracted from *C. perfringens* using the SV total RNA isolation system according to the manufacturer's instructions (Promega, Tokyo, Japan). A Northern blot analysis was performed as previously described (20). Digoxigenin (DIG)-labeled DNA probes were generated using DIG-High Prime (Roche), and template DNAs were amplified using primers that are listed in Table S1 or that were previously described (12).

Microarray analysis. Transcriptome analysis of the RNase Y-depleted strain was performed using a custom *C. perfringens* DNA 15K array (TaKaRa Bio Inc., Japan), which contains the coding DNA sequences based on reference data for *C. perfringens* strain 13 from the National Center for Biotechnology Information (NCBI). We isolated the total RNA from the *rny* conditional knockdown strain grown with or without 1 mM lactose to mid-exponential phase and used the RNA for cDNA synthesis. Cy3-labeled cRNA was then synthesized via *in vitro* transcription using the Low Input Quick Amp WT labeling kit, one-color (Agilent Technologies, Tokyo, Japan). We hybridized the cRNAs to the custom DNA array and then scanned and analyzed the fluorescence using an Agilent DNA microarray scanner (G2565CA; Agilent Technologies). TaKaRa Bio Inc. supported probe labeling, hybridization, and partial data analysis. To determine fold ratios of gene expression, we compared the signal intensities of the *rny* conditional knockdown strains grown in the presence of lactose. The *P* values were determined using an unpaired Welch's *t* test.

5' RLM-RACE. The 5' ends of the transcripts were analyzed by 5' RLM-RACE as described previously, with some modifications (28). Total RNA (10 μ g) was left untreated or was treated with 1 U of Terminator exonuclease in 20 μ l of reaction buffer at 30°C for 1 h. We then purified the RNA with phenol-chloroform-isoamylalcohol (PCI) extraction and ethanol precipitation. RNA samples equivalent to 3 μ g were left untreated or were treated with 5 U of RNA 5' polyphosphatase in 10 μ l of reaction buffer at 37°C for 1 h. We then incubated 2 μ l of untreated or 5' polyphosphatase-treated RNA (equivalent to 0.6 μ g of RNA) with the 5' RACE adaptor and 5 U of T4 RNA ligase (Life Technologies, Tokyo, Japan) at 14°C for 16 h. The total RNA that ligated with the adaptor (equivalent to 0.12 μ g of RNA) was used as a template for reverse transcription, which was performed with PrimeScript reverse transcriptase (TaKaRa Bio). We amplified cDNAs via nested PCR using the primers listed in Table S1. The amplified DNA products were cloned into *E. coli* using the pUC18 vector. The resulting vector sequences were determined by DNA sequencing using M13 primers.

Attachment and biofilm formation assay. *C. perfringens* cells were inoculated from cultures grown in PGY into the wells of 24-well microtiter plates containing 1 ml of GAM broth with or without lactose. The cells were grown anaerobically for 24 h at 37°C. After removing culture supernatants and floating cells, we then washed the wells with 1 ml of phosphate-buffered saline (PBS). The residual cells adhering to the substratum were resuspended in 1 ml of PBS via thorough pipetting. OD₆₀₀ readings were obtained for floating and adherent cells.

Western blot analysis. Extracellular proteins equivalent to an OD₆₀₀ of 0.01 were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 2.5% skim milk in Tris-buffered saline containing 0.05% Tween 20. Plc proteins were probed using anti-CPa (*Clostridium perfringens* alpha-toxin) and anti-rabbit IgG (GE Healthcare) antibodies diluted 1:1,000 and 1:20,000, respectively, in Can Get signal solution (Toyobo, Osaka, Japan). The bound antibodies were detected using Immunostar LD (Wako, Osaka, Japan). For PilA2 detection, we used an anti-PilA2-specific peptide (QANSKDYLVPDGKPYDL)-directed antibody (Sigma-Aldrich) diluted to 1:5,000.

Extraction of extracellular proteins. *C. perfringens* HN13 and *rny* conditional knockdown strains were cultured overnight in TY-G1 medium (3% tryptone, 2% yeast extract, 1% glucose, and 0.1% sodium thioglycolate) containing 1 mM lactose. The cells were washed twice with TY-G1 medium and inoculated in fresh TY-G1 containing 1 mM or no lactose to reach an OD₆₀₀ of 0.1. The cultures were incubated for 6 h at 37°C, and then the culture supernatants were collected via centrifugation at 5,000 \times *g* for 10 min. The culture supernatants that passed through a 0.2- μ m-pore-size membrane filter were incubated with 20% trichloroacetic acid (TCA). After washing with ice-cold acetone, precipitated proteins were dissolved in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 2.5% mercaptoethanol, 0.1% bromophenol blue).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00703-16>.

TEXT S1, PDF file, 1.7 MB.

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