Received: 29 January 2020 | Revised: 22 March 2020 | Accepted: 24 March 2020

DOI: 10.1111/1348-0421.12791

NOTE

Microbiology and Immunology WILEY

Expression of small RNAs of *Bordetella pertussis* colonizing murine tracheas

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Funding information
Japan Society for the Promotion of Science, Grant/Award Numbers: 17H04075, 19K16638, 19K23845; JSPS KAKENHI

Abstract

We performed RNA sequencing on *Bordetella pertussis*, the causative agent of whooping cough, and identified nine novel small RNAs (sRNAs) that were transcribed during the bacterial colonization of murine tracheas. Among them, four sRNAs were more strongly expressed *in vivo* than *in vitro*. Moreover, the expression of eight sRNAs was not regulated by the BvgAS two-component system, which is the master regulator for the expression of genes contributing to the bacterial infection. The present results suggest a BvgAS-independent gene regulatory system involving the sRNAs that is active during *B. pertussis* infection.

KEYWORDS

Bordetella pertussis, BvgAS, gene regulatory system, *in vivo* RNA sequencing, small RNA

Bordetella pertussis causes whooping cough, a contagious respiratory disease that has been resurging recently despite high vaccination coverage.[1,2] This organism produces multiple virulence factors, including toxins and adhesins, the expression of which is largely regulated by the BvgAS two-component system, consisting of the sensor kinase BvgS and response regulator BvgA.[3] At 37°C in standard *Bordetella* media, the BvgAS system activates the transcription of a set of genes (Bvg-activated genes) including various virulence genes. Conversely, this system is inactivated at temperatures lower than 26°C or in the presence of $MgSO_4$ (40–50 mM) or nicotinic acid (10–20 mM), and *B. pertussis* eventually does not express the Bvg-activated genes. The former bacterial state is called the Bvg^+ phase, and the latter is the Bvg^- phase. The BvgAS system is considered to play a major role in the expression of genes involved in the pathogenesis of *B. pertussis*; however, recent *in vivo* studies

Abbreviations: BG, Bordet–Gengou; Bpr, *B. pertussis* sRNA; DIG, digoxigenin; IGR, intergenic region; RACE, rapid amplification of cDNA end; RgtA, repressor of glutamate transport; RNA-seq, RNA sequencing; sRNA, small RNA; SS, Stainer–Scholte; WT, wild type.

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found that several Bvg-activated genes were repressed in *B. pertussis* colonizing the respiratory tracts of mice.[4,5] van Beek *et al.* also reported that approximately 30% of all genes were differentially expressed between *in vitro* and *in vivo* conditions.[4] Furthermore, a *B. pertussis* clinical strain, the BvgAS system of which was dysfunctional due to a spontaneous mutation in the *bvgS* gene, was isolated from a pertussis patient.[6] These findings suggest that a complex mechanism, besides the BvgAS system, is involved in the regulation of the bacterial gene expression during the course of infection.

Bacterial small RNAs (sRNAs) are functional noncoding RNA molecules that range between 50 and 500 nucleotides in length.[7] Previous studies identified numerous sRNAs in various pathogenic and commensal bacteria using a computational analysis and laboratory-based techniques, such as microarrays, Northern blotting, and RNA sequencing (RNA-seq).[8–11] Most sRNAs post-transcriptionally upregulate or downregulate downstream gene expression by affecting the stability and translational efficiency of target messenger RNAs (mRNAs) through base pairing with them.[12,13] A wide variety of physiological processes, including metabolism, stress responses, and the expression of virulence genes, are regulated by sRNAs.[14–18] In *B. pertussis*, many types of sRNAs have been identified or predicted by an *in silico* analysis and RNA-seq on the bacteria grown *in vitro*.[9,11,14] However, it currently remains unclear whether *B. pertussis* sRNAs are involved in the regulation of *in vivo* gene expression, which is associated with the establishment of bacterial infection. In the present study, we performed *in vivo* RNA-seq on *B. pertussis* colonizing the murine tracheas and identified novel sRNAs that were strongly expressed during colonization.

In vivo expression of sRNAs were analyzed by RNA-seq using tracheas of three mice independently infected with *B. pertussis*-type strain 18323. This organism was grown at 37°C on Bordet–Gengou agar (Becton Dickinson, Franklin Lakes, NJ) containing 1% HIPOLY-PEPTON (Nihon Pharmaceutical, Tokyo, Japan), 1% glycerol, 15% defibrinated horse blood, and 10 μg/mL ceftibuten (BG plate). The bacteria recovered from the colonies on BG plates were suspended in Stainer–Scholte (SS) broth[19] to obtain an OD_{650} of 0.2, and cultured at 37°C for 14 hr with shaking. Bacterial CFUs were estimated from OD_{650} values according to the following equation: 1 $OD_{650} = 3.3 \times 10^9$ CFU/mL. Seven-week-old male C57BL/6J mice (CLEA Japan, Osaka, Japan) were anesthetized with a mixture of medetomidine (Kyoritsu Seiyaku, Tokyo, Japan), midazolam (Teva Takeda Pharma, Nagoya, Japan), and butorphanol (Meiji Seika Pharma, Yokohama, Japan) at final doses of 0.3, 2, and 5 mg/kg body weight, respectively, and intranasally inoculated with *B. pertussis* 18323 (1×10^7 CFU) in 50 μL of SS medium using a micropipette with a needle-like tip. On Day 4 after inoculation, mice were killed with pentobarbital, and the tracheas were excised and frozen in liquid nitrogen. Total RNA was extracted from the tracheas with TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA), treated with RNase-Free DNase (Takara Bio, Shiga, Japan), and then purified with the PureLink RNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Bacterial and murine ribosomal RNAs (rRNAs) were simultaneously depleted from the total RNA using the Ribo-Zero rRNA Removal Kit for Human/Mouse/Rat and Gram-Negative Bacteria (Illumina, San Diego, CA). The quality and quantity of RNA samples were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Reverse transcription was performed with the rRNA-depleted RNA, SuperScript III Reverse Transcriptase (Thermo Fisher Scientific), and Random Primer N9 (Takara Bio), and double-stranded DNA was then synthesized using DNA polymerase I (Klenow fragment [3′–5′ exo-]; New England Biolabs, Ipswich, MA). The resultant complementary DNA (cDNA) was sheared to approximately 600 bp fragments using Covaris S220 (Covaris, Woburn, MA) and purified with Agencourt AMPure XP beads (Beckman Coulter, Miami, FL). Libraries of the cDNA fragments were then prepared with the KAPA Library Preparation Kit (Kapa Biosystems, Wilmington, MA) and TruSeq adapters (Illumina), and sequenced with a HiSeq 2500 (Illumina) to obtain 101 bp single-end reads. The sequenced reads were mapped to the genomic DNA of *B. pertussis* 18323 (GenBank: NC_018518.1) using CLC Genomics Workbench, version 8.0.3 (CLC bio, Waltham, MA). All animal experiments were approved by the Animal Care and Use Committee of the Research Institute for Microbial Disease, Osaka University, and conducted according to the Regulations on Animal Experiments at Osaka University. The numbers of total sequenced reads were 54, 143, and 137 million, and 0.06%, 0.72%, and 0.04% of the reads in each sample were aligned to the genome sequence of *B. pertussis* 18323. A large portion of the reads aligned to the bacterial genome corresponded to protein-, rRNA-, and transfer RNA-coding sequences (95.5%, 99.9%, and 97.3%), whereas the residual reads were aligned to the intergenic regions: the numbers of reads were 1180, 1316, and 836, respectively. We predicted that these noncoding sequences located in the intergenic regions are potential sRNA sequences. Among these sRNA candidates, we selected nine novel sRNAs, for which the number of sequenced reads was more than 20 counts, and designated them *B. pertussis* sRNA (Bpr) 1–9 according to a previous study[9] (Table 1). Homologous sRNAs to Bpr1–9 were not found in the public databases

TABLE 1 Genetic localization of Bpr in *Bordetella pertussis* 18323

sRNA	Coordinate[a]		Predicted sRNA size (base)	IGR length[b]	Adjacent genes	Number of reads[c]			
	Start	Stop				1	2	3	Average
Bpr1[d]	615294	615326	33	253	*bn118_rs02945/purM*	536	369	485	463
Bpr2[d]	615326	615294	33	253	*bn118_rs02945/purM*	93	57	125	92
Bpr3	3280265	3280530	266	893	*rplN/gabD*	157	30	62	83
Bpr4	971049	971071	23	231	*bn118_rs04605/IS481*	69	26	92	62
Bpr5	3280631	3280424	208	893	*rplN/gabD*	113	22	9	48
Bpr6	1174521	1174937	417	727	*map/rpsB*	87	19	33	46
Bpr7	1175217	1174967	251	727	*map/rpsB*	76	21	8	35
Bpr8	410106	409944	163	595	*sphB1/ppc*	77	19	9	35
Bpr9	1432753	1432869	117	308	*bn118_rs06780/rplS*	43	18	8	23
recA	2148903	2147842	1062	–	*recX/ompR*	30	46	45	40

[a]Coordinate in the *B. pertussis* 18323 genome sequence (NC_018518.1).
[b]Length of the IGR containing sRNA coding genes.
[c]The number of sequenced reads corresponding to each Bpr obtained by *in vivo* RNA-seq using three murine tracheas colonizing *B. pertussis*.
[d]Bpr1 and Bpr2 carry complementary sequences to each other.

including BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and sRNAMap (http://srnamap.mbc.nctu.edu.tw).

In vitro and *in vivo* expression of Bpr1–9 were compared by qRT-PCR analyses. Total RNA was extracted and purified from the tracheas of mice independently infected with *B. pertussis* Tohama, a vaccine strain, and two clinical strains (BP139 and BP143 gifted from K. Kamachi, National Institute for Infectious Diseases)[20] in the same manner as *B. pertussis* 18323. Total RNA was also prepared from the four strains of *B. pertussis* and Bvg^+- and Bvg^--locked mutants derived from *B. pertussis* 18323 grown *in vitro* using the PureLink RNA Mini Kit and RNase-Free DNase according to the manufacturer's instructions. The Bvg^+- and Bvg^--locked mutants, which constitutively express the Bvg^+ and Bvg^- phenotypes, respectively, were constructed by the site-directed mutagenesis of BvgS to replace Arg with His at position 570 and to delete the region of amino acid positions from 542 to 1020, respectively,[21] using double-crossover homologous recombination as described previously.[22] In brief, the plasmids *bvgS*-C3-pABB-CRS2-Gm and Δ*bvgS*-pABB-CRS2-Gm[22] were introduced into *Escherichia coli* DH5α λ*pir*, and then transconjugated into *B. pertussis* 18323 by triparental conjugation with the helper strain *E. coli* HB101 harboring pRK2013,[23] which was provided by K. Minamisawa (Tohoku University). Total RNA samples (1 μg) from bacteria recovered from murine tracheas and *in vitro*-cultured bacteria were reverse transcribed into cDNA using the PrimeScript RT Reagent Kit (Takara Bio) with random hexamers in a total volume of 20 μL. The transcription levels of target RNAs were estimated from the amounts of the resultant cDNA measured with the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster, CA) using the Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) and the primers listed in Table 2 under the following conditions: initial denaturation at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. qRT-PCR analyses revealed that the expression levels of Bpr4, 5, 8, and 9 in *B. pertussis* 18323 colonizing murine tracheas were significantly higher (118-, 64-, 9-, and 6-fold, respectively) than those in *in vitro*-cultured bacteria (Figure 1a). By contrast, no significant differences in the expression of Bpr1–3, 6, or 7 were observed between *in vitro* and *in vivo* conditions. Similar results were obtained with *B. pertussis* Tohama and two clinical strains (Figure 1b). The *in vitro* expression levels of Bpr1–7 and 9 in *B. pertussis* 18323 were largely unaffected by the absence or presence of 40 mM $MgSO_4$. In addition, the Bvg^+- and Bvg^--locked mutants equally expressed these sRNAs (Figure 1c). By contrast, the expression of Bpr8 was negligible in *B. pertussis* 18323 wild-type grown in the presence of 40 mM $MgSO_4$ (i.e. Bvg^- phase condition) and the Bvg^--locked mutant. These results indicate that the expression of Bpr1–7 and 9 is independent of the BvgAS regulatory system, whereas that of Bpr8 is BvgAS dependent.

The presence of Bpr4, 5, 8, and 9 in *B. pertussis* 18323 was confirmed by rapid amplification of cDNA end (RACE) and Northern blotting. For the determination of the transcription start and termination sites of the Bpr, 5′- and 3′-RACE were performed using a SMARTer RACE 5′/3′ Kit (Takara Bio) according to the manufacturer's instructions. In brief, total RNA was extracted from *in vitro*-cultured *B. pertussis* 18323 and polyadenylated by poly(A) polymerase (New England Biolabs). After reverse transcription by

TABLE 2 Primers used in the present study

Primers	Sequence (5′–3′)	Application
Bpr1, 2-Fw[a]	ACCCTGCATTAAACCACTGC	qRT-PCR for Bpr1 and Bpr2
Bpr1, 2-Rv[a]	GCAAACCCCTGGAAATGCTC	
Bpr3-Fw	AACTGAAAACACGCCCTTCC	qRT-PCR for Bpr3
Bpr3-Rv	ATTTCAACCCAGGGTCGTTC	
Bpr4-Fw	ATCGGCCGCGCAATCCGCTTG	qRT-PCR for Bpr4
Bpr4-Rv	CCGGGCCCGCTATTTATTCAC	
Bpr5-Fw	CCGGTTTTGGTTGGACTTTC	qRT-PCR for Bpr5
Bpr5-Rv	CCCGAAGGCTCATTCCAC	
Bpr6-Fw	AGGCAGGCAAGTAGCAAAGC	qRT-PCR for Bpr6
Bpr6-Rv	CACAAATCGCCCAAAACACC	
Bpr7-Fw	CAGCATTTCACGCATGAGG	qRT-PCR for Bpr7
Bpr7-Rv	TTTATCCGATGCGGGTGTAG	
Bpr8-Fw	TGCGTCCCAGGATGATTTG	qRT-PCR for Bpr8
Bpr8-Rv	GTGCGAGGAGTGCGTTGAG	
Bpr9-Fw	TTGATGTAGAATGCTGGGTTTGC	qRT-PCR for Bpr9
Bpr9-Rv	AACCAGGCAACGGCTATTGG	
recA-Fw	CCAATGTGGTCGACAAGTCC	qRT-PCR for *recA*
recA-Rv	ATGGCCATTTCCTTGTGCTC	
Bpr4_RACE-S	GATTACGCCAAGCTTATCGGCCGCGCAATCCGCTTG	RACE for Bpr4
Bpr4_RACE-AS	GATTACGCCAAGCTTCCGGGCCCGCTATTTATTCAC	
Bpr5_RACE-S	GATTACGCCAAGCTTCCGGTTTTGGTTGGACTTTC	RACE for Bpr5
Bpr5_RACE-AS	GATTACGCCAAGCTTCCCGAAGGCTCATTCCAC	
Bpr8_RACE-S	GATTACGCCAAGCTTTGCGTCCCAGGATGATTTG	RACE for Bpr8
Bpr8_RACE-AS	GATTACGCCAAGCTTGTGCGAGGAGTGCGTTGAG	
Bpr9_RACE-S	GATTACGCCAAGCTTTTGATGTAGAATGCTGGGTTTGC	RACE for Bpr9
Bpr9_RACE-AS	GATTACGCCAAGCTTAACCAGGCAACGGCTATTGG	
Bpr4-*Eco*RI-S	AGGGAGACCGGAATTCCCGGGCCCGCTATTTATT	RNA probe for Bpr4
Bpr4-*Bam*HI-AS	CGACTCTAGAGGATCCATCGGCCGCGCAATCCGC	
Bpr5-*Eco*RI-S	AGGGAGACCGGAATTCCCCGAAGGCTCATTCCAC	RNA probe for Bpr5
Bpr5-*Bam*HI-AS	CGACTCTAGAGGATCCCCGGTTTTGGTTGGACTTT	
Bpr8-*Eco*RI-S	AGGGAGACCGGAATTCGTGCGAGGAGTGCGTTGA	RNA probe for Bpr8
Bpr8-*Bam*HI-AS	CGACTCTAGAGGATCCTGCGTCCCAGGATGATTTG	
Bpr9-*Eco*RI-S	AGGGAGACCGGAATTCAACCAGGCAACGGCTATTG	RNA probe for Bpr9
Bpr9-*Bam*HI-AS	CGACTCTAGAGGATCCTTGATGTAGAATGCTGGGTT	
recA-*Eco*RI-S	AGGGAGACCGGAATTCATGGCCATTTCCTTGTGCT	RNA probe for *recA*
recA-*Bam*HI-AS	CGACTCTAGAGGATCCCCAATGTGGTCGACAAGTC	

[a]Bpr1 and Bpr2 are amplified with Bpr1, 2-Fw and Bpr1, 2-Rv, respectively.

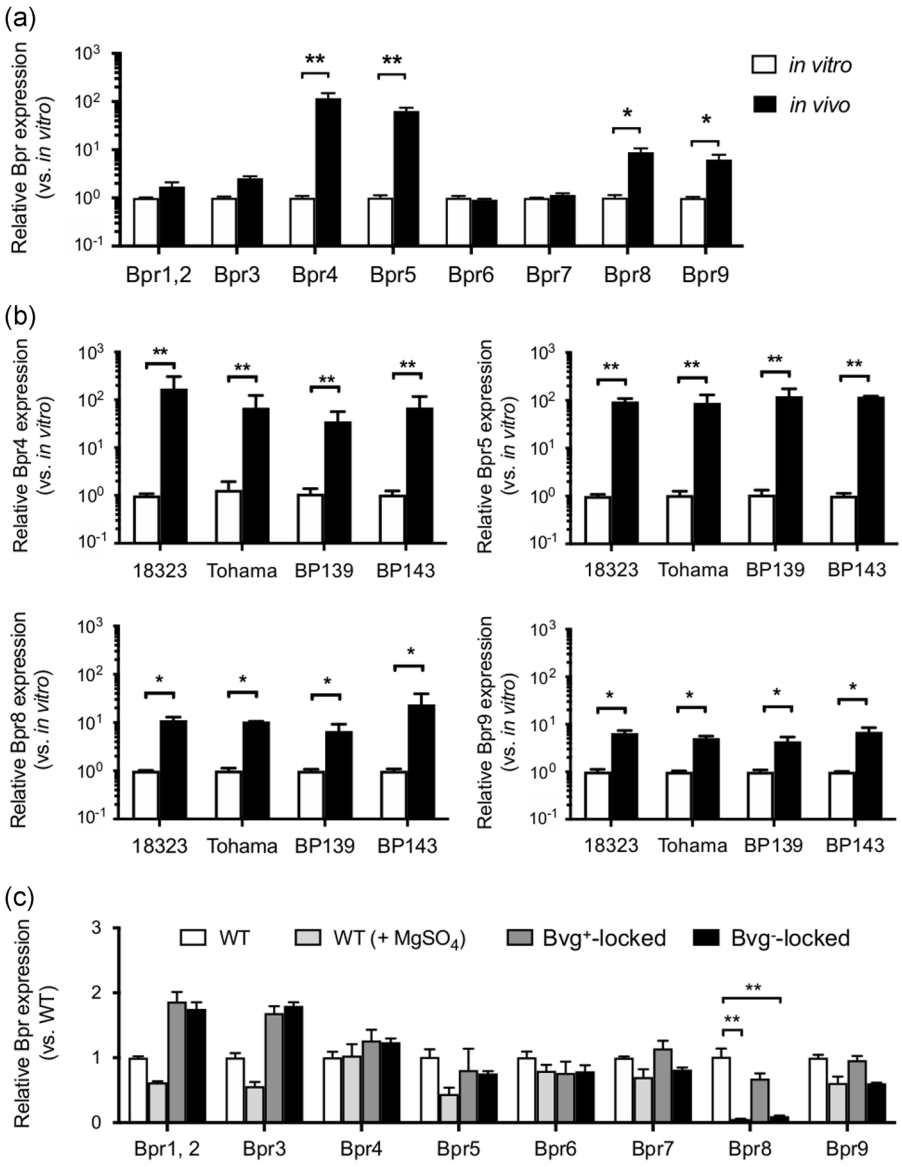

FIGURE 1 *In vivo* and *in vitro* expression of *Bordetella pertussis* sRNAs. (a-b) Mice were intranasally inoculated with *B. pertussis* 18323 (a and b), Tohama, BP139, or BP143 (b). Total RNA was extracted from the bacteria recovered from murine tracheas (*in vivo*) and *in vitro*-cultured bacteria (*in vitro*) (a and b) or *B. pertussis* 18323 WT and Bvg^+- and Bvg^--locked mutants cultured in SS broth with or without 40 mM $MgSO_4$ (c). The relative amount of each Bpr was assessed by qRT-PCR with the $\Delta\Delta C_t$ method normalized to that of *recA* mRNA as an internal control for each sample. Data are represented as fold changes in expression from that observed in *in vitro*-cultured bacteria (a and b) or WT grown in the absence of $MgSO_4$ (c). Values are means and SEM ($n = 3$). Statistical analyses were performed by a two-way analysis of variance and Tukey's multicomparison test using Prism 8 (GraphPad Software). $^*P < 0.05$, $^{**}P < 0.01$. mRNA, messenger RNA

SMARTScribe Reverse Transcriptase, the resultant cDNA was used as a template for PCR with Universal primer and each *bpr*-specific primers (Table 2). The PCR products were then cloned into linearized pRACE and five individual clones were sequenced. The precise transcription start and termination sites of Bpr4, 8, and 9 were poisoned at 971000–971155 (156 base), 410138–409796 (343 base), and 1432692–1433084 (393 base), respectively, in the *B. pertussis* 18323 genome sequence. By contrast, two start sites (3280825 and 3280639) and one termination site (3280298) were detected in the transcripts of Bpr5 (528 and 342 bases), indicating the presence of two overlapping transcripts of different length. Northern blotting was performed using a DIG Northern Starter Kit (Sigma-Aldrich, St Louis, MO). For production of digoxigenin (DIG)-labeled RNA probes, partial antisense strands of *bpr* and *recA* genes were amplified from *B. pertussis* 18323 using appropriate primers (Table 2), and cloned into the downstream of T7 promoter on pSPT18 (Sigma-Aldrich). The resulting plasmids were linearized with *Sal*I, and DIG-labeled RNA probes were

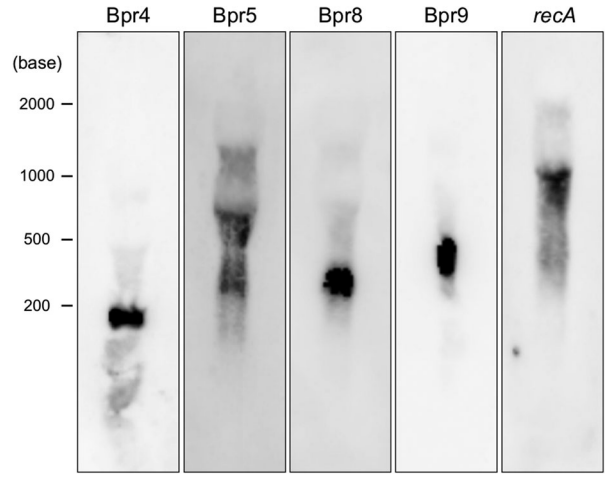

FIGURE 2 Experimental validation of the sRNAs expression in *Bordetella pertussis*. Total RNA extracted from *in vitro*-cultured *B. pertussis* 18323 was subjected to Northern blotting using RNA probes for the detection of Bpr4, 5, 8, and 9, and *recA*

generated by *in vitro* transcription with T7 polymerase. Next, total RNA (20 μg) extracted from *in vitro*-cultured *B. pertussis* 18323 was subjected to electrophoresis in a 1.5% denaturing formaldehyde agarose gel, transferred to a positively charged membrane (Hybond-N+; GE Healthcare, Piscataway, NJ), and UV cross-linked to the membrane. The membrane was then independently incubated with DIG-labeled RNA probes for each Bpr and *recA*, respectively, followed by alkaline phosphate-conjugated sheep anti-DIG immunoglobulin G, and visualized with CDP-*Star*. Northern blotting using the RNA probes for Bpr4, 8, and 9 detected a single band, whereas Bpr5 migrated as two bands (Figure 2). The mobility of each Bpr corresponded to that estimated from its length determined by RACE.

sRNAs regulate the expression of genes involved in a wide variety of physiological processes in bacteria, including the adaptation to host environments and virulence.[14–18] In *B. pertussis*, 14 types of sRNAs designated as BprA–N were identified by an *in silico* analysis and Northern blotting[9]; however, these sRNAs have not yet been characterized. Recent studies performed an RNA-seq analysis using *B. pertussis* grown *in vitro* and identified an sRNA designated as RgtA (repressor of glutamate transport) that was found to reduce the translation of BP3831, a periplasmic amino acid-binding protein of an ABC transporter, by base pairing with the 5′ untranslated region of *BP3831* mRNA.[11,14] Although this protein is related to the transport of glutamate, it currently remains unclear whether RgtA is involved in the pathogenesis of *B. pertussis*. In the present study, we identified nine types of novel sRNAs that were strongly expressed during the bacterial colonization, and demonstrated that the expression of four types of sRNAs (Bpr4, 5, 8, and 9) was stronger *in vivo* than *in vitro*. To the best of our knowledge, this is the first study to identify the *in vivo* strongly expressed sRNAs of *B. pertussis*. Bpr4, 5, 8, and 9, which were strongly expressed *in vivo*, may be involved in regulating the expression of genes necessary for the bacterial colonization or infection. In *Salmonella enterica* serovar Typhimurium, PinT, a PhoP-induced sRNA, was shown to be upregulated by up to 100-fold during the infection, and regulated the expression of the invasion-associated effectors and virulence genes required for intracellular survival.[17] Li *et al.* also reported that Ysr170, a strongly expressed sRNA in *Yersinia pestis* invading host cells, contributed to the bacterial intracellular survival.[18] These findings support our hypothesis that the sRNAs strongly induced during infection are involved in the adaptation and/or pathogenesis of *B. pertussis*. In addition, we found that the expression of Bpr1–7 and 9 was not regulated by the BvgAS system. Although the BvgAS system was previously considered to be the master virulence regulator in *B. pertussis*,[3] recent studies demonstrated that the expression profiles of Bvg-regulated genes were largely different between *in vitro* and *in vivo* conditions,[4,5] suggesting a complex mechanism that regulates *in vivo* gene expression. Several groups reported the PlrSR two-component system and BspR/BtrA, an anti-σ factor, as accessory regulatory systems downstream of BvgAS, which may play a part in this complex gene regulatory system *in vivo*.[24–27] Besides these regulators, the sRNAs identified in the present study may function as another regulator for gene expression during *B. pertussis* infection. Further research is currently in progress in our laboratory to identify genes whose expression is regulated by the sRNAs and elucidate the mechanisms by which the sRNAs regulate the gene expression.

ACKNOWLEDGMENTS

We thank K. Kamachi for the *B. pertussis* clinical strains and K. Minamisawa for the *E. coli* strain carrying pRK2013. We also thank N. Shinzawa for technical support with RNA-seq. This work was supported by JSPS KAKENHI Grant Numbers 17H04075, 19K16638, and 19K23845.

DISCLOSURE

The authors declare that there are no conflict of interests.

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How to cite this article: Hiramatsu Y, Suzuki K, Motooka D, Nakamura S, Horiguchi Y. Expression of small RNAs of *Bordetella pertussis* colonizing murine tracheas. *Microbiology and Immunology*. 2020;64: 469–475. https://doi.org/10.1111/1348-0421.12791