NOTE

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

Yukihiro Hiramatsu¹ | Koichiro Suzuki¹,² | Daisuke Motooka³ | Shota Nakamura³ | Yasuhiko Horiguchi¹

¹Department of Molecular Bacteriology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan
²The Research Foundation for Microbial Diseases of Osaka University (BIKEN), Suita, Osaka, Japan
³Department of Infection Metagenomics, Genome Information Research Center, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan

Correspondence
Yukihiro Hiramatsu, Department of Molecular Bacteriology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan. Email: yhiramatsu@biken.osaka-u.ac.jp

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 MgSO₄ (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.

Yukihiro Hiramatsu and Koichiro Suzuki contributed equally to this work.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2020 The Authors. *Microbiology and Immunology* published by The Societies and John Wiley & Sons Australia, Ltd
found that several Bvg-activated genes were repressed in *B. pertussis* colonizing the respiratory tracts of mice. van Beek et al. also reported that approximately 30% of all genes were differentially expressed between *in vitro* and *in vivo* conditions. 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. 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 non-coding RNA molecules that range between 50 and 500 nucleotides in length. 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). 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. A wide variety of physiological processes, including metabolism, stress responses, and the expression of virulence genes, are regulated by sRNAs. 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*. 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-PETON (Nihon Pharmaceutical, Tokyo, Japan), 1% glycerol, 15% defibrinated horse blood, and 10 μg/mL cefitubten (BG plate). The bacteria recovered from the colonies on BG plates were suspended in Stainer–Scholte (SS) broth 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 × 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. Homologous sRNAs to Bpr1–9 were not found in the public databases.
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) 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, using double-crossover homologous recombination as described previously. In brief, the plasmids bvgS-C3-pABB-CRS2-Gm and ΔbvgS-pABB-CRS2-Gm were introduced into Escherichia coli DH5α λpir, and then transconjugated into B. pertussis 18323 by triparental conjugation with the helper strain E. coli HB101 harboring pRK2013, 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 MgSO₄. 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 MgSO₄ (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 1: Genetic localization of Bpr in Bordetella pertussis 18323

| sRNA     | Coordinate | Predicted sRNA size (base) | IGR length | Adjacent genes       | Number of reads |
|----------|------------|-----------------------------|------------|----------------------|-----------------|
|          | Start      | Stop                        |            |                      | 1   | 2   | 3   | Average |
| Bpr1⁴    | 615294     | 615326                      | 33         | 253                  | 536 | 369 | 485 | 463     |
| Bpr2⁴    | 615326     | 615294                      | 33         | 253                  | 93  | 57  | 125 | 92      |
| Bpr3     | 3280265    | 3280530                     | 266        | 893                  | 157 | 30  | 62  | 83      |
| Bpr4     | 971049     | 971071                      | 23         | 231                  | 69  | 26  | 92  | 62      |
| Bpr5     | 3280631    | 3280424                     | 208        | 893                  | 113 | 22  | 9   | 48      |
| Bpr6     | 1174521    | 1174937                     | 417        | 727                  | 87  | 19  | 33  | 46      |
| Bpr7     | 1175217    | 1174967                     | 251        | 727                  | 76  | 21  | 8   | 35      |
| Bpr8     | 410106     | 409944                      | 163        | 595                  | 77  | 19  | 9   | 35      |
| Bpr9     | 1432753    | 1432869                     | 117        | 308                  | 43  | 18  | 8   | 23      |
| recA     | 2148903    | 2147842                     | 1062       | recX/ompR            | 30  | 46  | 45  | 40      |

⁴Coordinate in the B. pertussis 18323 genome sequence (NC_018518.1).

Length of the IGR containing sRNA coding genes.

The number of sequenced reads corresponding to each Bpr obtained by in vivo RNA-seq using three murine tracheas colonizing B. pertussis.
TABLE 2  Primers used in the present study

| Primers  | Sequence (5′–3′)          | Application                      |
|----------|---------------------------|-----------------------------------|
| Bpr1, 2-Fw  | ACCCTGCATTAAACCACCTGCA    | qRT-PCR for Bpr1 and Bpr2        |
| Bpr1, 2-Rv  | GCCAACCCTGGAAATGCTC       |                                   |
| Bpr3-Fw  | AACCTGAATAACGCCCTTC       | qRT-PCR for Bpr3                  |
| Bpr3-Rv  | ATTTCAACCCAGGCTGTTC       |                                   |
| Bpr4-Fw  | ATCGGCCGCCAAATCCCTTG      | qRT-PCR for Bpr4                  |
| Bpr4-Rv  | CCGGCGCGCTATTTATCAC       |                                   |
| Bpr5-Fw  | CCGTTTTGGTTGGACCTTTTC     | qRT-PCR for Bpr5                  |
| Bpr5-Rv  | CCGCAAGGCTCATTCCAC        |                                   |
| Bpr6-Fw  | AGGCAGGCAATGCAAAAG       | qRT-PCR for Bpr6                  |
| Bpr6-Rv  | CCAAAATCGCCACAAAAACCC     |                                   |
| Bpr7-Fw  | CAGCAT7TCACGCATGAG        | qRT-PCR for Bpr7                  |
| Bpr7-Rv  | TTTATCCGATGGGTTGAG        |                                   |
| Bpr8-Fw  | TCGTCCAGGATGATTTG         | qRT-PCR for Bpr8                  |
| Bpr8-Rv  | GTCCGAAGAGGCGTTGAG        |                                   |
| Bpr9-Fw  | TTGGATATGGAATGCGTTTC      | qRT-PCR for Bpr9                  |
| Bpr9-Rv  | AACCAGGCAACGGCTATTGG      |                                   |
| recA-Fw  | CCAATGCGTCGCAAAAGTCC      | qRT-PCR for recA                   |
| recA-Rv  | ATGCCCTATTTCTGTGCTC       |                                   |
| Bpr4_RACE-S  | GATTACGCGCAAGCTTATCGCCCTCCTGCAGCTT | RACE for Bpr4         |
| Bpr4_RACE-AS | GATTACGCCAAGCTTCCCGCCGCTATTTCAC |                                      |
| Bpr5_RACE-S  | GATTACGCCAAGCTTCCCGCCGCTATTTCAC | RACE for Bpr5                  |
| Bpr5_RACE-AS | GATTACGCCAAGCTTCCCGCCGCTATTTCAC |                                      |
| Bpr8_RACE-S  | GATTACGCCAAGCTTATCGCCCTCCTGCAGCTT | RACE for Bpr8                  |
| Bpr8_RACE-AS | GATTACGCCAAGCTTCCCGCCGCTATTTCAC |                                      |
| Bpr9_RACE-S  | GATTACGCCAAGCTTATCGCCCTCCTGCAGCTT | RACE for Bpr9                  |
| Bpr9_RACE-AS | GATTACGCCAAGCTTATCGCCCTCCTGCAGCTT |                                      |
| Bpr4_EcoRI-S | AGGGAGACCGGAATTCCCGCCGCTATTTCATT | RNA probe for Bpr4             |
| Bpr4_BamHI-AS | CGACTCTAGAGGCTATCCGGCCGCAATCCGC |                                      |
| Bpr5_EcoRI-S | AGGGAGACCGGAATTCCGGCCGCTATTTCATT | RNA probe for Bpr5             |
| Bpr5_BamHI-AS | CGACTCTAGAGGCTATCCGGCCGCAATCCGC |                                      |
| Bpr8_EcoRI-S | AGGGAGACCGGAATTCCGGCCGCTATTTCATT | RNA probe for Bpr8             |
| Bpr8_BamHI-AS | CGACTCTAGAGGCTATCCGGCCGCAATCCGC |                                      |
| Bpr9_EcoRI-S | AGGGAGACCGGAATTCCGGCCGCTATTTCATT | RNA probe for Bpr9             |
| Bpr9_BamHI-AS | CGACTCTAGAGGCTATCCGGCCGCAATCCGC |                                      |
| recA_EcoRI-S | AGGGAGACCGGAATTCCGGCCGCTATTTCATT | RNA probe for recA             |
| recA_BamHI-AS | CGACTCTAGAGGCTATCCGGCCGCAATCCGC |                                      |

*Bpr1 and Bpr2 are amplified with Bpr1, 2-Fw and Bpr1, 2-Rv, respectively.*
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 SalI, and DIG-labeled RNA probes were

**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 MgSO₄ (c). The relative amount of each Bpr was assessed by qRT-PCR with the ΔΔCₜ 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 MgSO₄ (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
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. In B. pertussis, 14 types of sRNAs designated as BprA–N were identified by an in silico analysis and Northern blotting; 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. 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. Li et al. also reported that Ysr170, a strongly expressed sRNA in Yersinia pestis invading host cells, contributed to the bacterial intracellular survival. 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, recent studies demonstrated that the expression profiles of Bvg-regulated genes were largely different between in vitro and in vivo conditions, suggesting a complex mechanism that regulates in vivo gene expression. Several groups reported the PhrSR 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. 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.

ORCID
Yukihiro Hiramatsu http://orcid.org/0000-0002-6340-864X
Yasuhiko Horiguchi http://orcid.org/0000-0002-1592-5861

REFERENCES
1. Mills KH, Ross PJ, Allen AC, Wilk MM. Do we need a new vaccine to control the re-emergence of pertussis? Trends Microbiol. 2014;22:49-52.
2. Tan T, Dalby T, Forsyth K, et al. Pertussis across the globe: recent epidemiologic trends from 2000 to 2013. Pediatr Infect Dis J. 2015;34:e222-32.
3. Melvin JA, Scheller EV, Miller JF, Cotter PA. Bordetella pertussis pathogenesis: current and future challenge. Nat Rev Microbiol. 2014;12:274-88.
4. van Beek LF, de Gouw D, Eleveld MJ, et al. Adaptation of Bordetella pertussis to the respiratory tract. J Infect Dis. 2018;217:1987-96.
5. Wong TY, Hall JM, Nowak ES, et al. Analysis of the transcriptome of Bordetella pertussis during infection of mice. mSphere. 2019;4:e00154-19.
6. Hiramatsu Y, Yoshino S, Yamamura Y, et al. The proline residue at position 319 of BvgS is essential for BvgAS activation in Bordetella pertussis. Pathog Dis. 2017;75:ftx011.
7. Gottesman S. Micros for microbes: non-coding regulatory RNA is involved in glutamate metabolism of the human pathogen Bordetella bronchiseptica. RNA. 2018;24:1530-41.
8. Papenfort K, Vanderpool CK. Target activation by regulatory RNAs in bacteria. FEMS Microbiol Rev. 2015;39:362-78.
9. Keidel K, Amman F, Bibova I, et al. Signal transduction-dependent small regulatory RNA is involved in glutamate metabolism of the human pathogen Bordetella pertussis. RNA. 2018;24:1530-41.
10. Waters LS, Storz G. Regulatory RNAs in bacteria. Cell. 2009;136:615-28.
11. Papenfort K, Vogel. Regulatory RNA in bacterial pathogens. Cell Host Microbe. 2010;8:116-27.
12. Westermann AJ, Förstner KU, Amman F, et al. Dual RNA-seq unveils noncoding RNA functions in host-pathogen interactions. Nature. 2016;529:496-501.
13. Gómez WA, Desnoyers G, Bouchard MP, Masse E. New insights into small regulatory RNA in bacteria. Trends Genet. 2005;21:399-404.
14. Wassarman KM, Repolia F, Rosenow C, Storz G, Gottesman S. Identification of novel small RNAs using comparative genomics and microarrays. Genes Dev. 2001;15:1637-51.
15. Hot D, Slupek S, Wulbrecht B, et al. Detection of small RNAs in Bordetella pertussis and identification of a novel repeated genetic element. BMC Genomics. 2011;12:207.
16. Gómez-Lozano M, Marvig RL, Molin S, Long KS. Identification of bacterial small RNAs by RNA sequencing. Methods Mol Biol. 2014;1149:433-56.
17. Amman F, D’Halluin A, Antoine R, et al. Primary transcriptome analysis reveals importance of IS elements for the shaping of the transcriptional landscape of Bordetella pertussis. RNA Biol. 2018;15:967-75.
18. Desnoyers G, Bouchard MP, Masse E. New insights into small RNA-dependent translational regulation in prokaryotes. Trends Genet. 2013;29:92-8.
19. Papenfort K, Vanderpool CK. Target activation by regulatory RNAs in bacteria. FEMS Microbiol Rev. 2015;39:362-78.
20. Keidel K, Amman F, Bibova I, et al. Signal transduction-dependent small regulatory RNA is involved in glutamate metabolism of the human pathogen Bordetella pertussis. RNA. 2018;24:1530-41.
21. Waters LS, Storz G. Regulatory RNAs in bacteria. Cell. 2009;136:615-28.
22. Papenfort K, Vogel. Regulatory RNA in bacterial pathogens. Cell Host Microbe. 2010;8:116-27.
23. Westermann AJ, Förstner KU, Amman F, et al. Dual RNA-seq unveils noncoding RNA functions in host-pathogen interactions. Nature. 2016;529:496-501.
24. Li N, Hennelly SP, Stubben CJ, et al. Functional and structural analysis of a highly-expressed Yersinia pestis small RNA following infection of cultured macrophages. PLoS One. 2016;11:e0168915.
25. Stainer DW, Scholte MJ. A simple chemically defined medium for the production of phase I Bordetella pertussis. J Gen Microbiol. 1970;63:211-20.
26. Hiramatsu Y, Osada-Oka M, Horiguchi Y. Bordet-Gengou agar medium supplemented with albumin-containing biologics for cultivation of bordetellae. Microbiol Immunol. 2019;63:513-6.
27. Martinez de Tejada G, Cotter PA, Heininger U, et al. Neither the Bvg phase nor the vrg6 locus of Bordetella pertussis is required for respiratory infection in mice. Infect Immun. 1998;66:2762-8.
28. Nishikawa S, Shinzawa N, Nakamura K, Ishigaki K, Abe H, Horiguchi Y. The bvg-repressed gene btrA, encoding biofilm-associated surface adhesin, is expressed during host infection by Bordetella bronchiseptica. Microbiol Immunol. 2016;60:93-105.
29. Figurski DH, Helinski DR. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci USA. 1979;76:1648-52.
30. Bone MA, Wilk AJ, Perault AI, et al. Bordetella PlrSR regulatory system control BvgAS activity and virulence in the lower respiratory tract. Proc Natl Acad Sci USA. 2017;114: E1519-27.
31. Kurushima J, Kuwae A, Abe A. The type III secreted protein BspR regulates the virulence genes in Bordetella bronchiseptica. PLoS One. 2012;7:e38925.
32. Ahuja U, Shokeen B, Cheng N, et al. Differential regulation of type III secretion and virulence genes in Bordetella pertussis and Bordetella bronchiseptica by a secreted anti-σ factor. Proc Natl Acad Sci USA. 2016;113:2341-8.
33. Nakamura K, Shinoda N, Hiramatsu Y, et al. BspR/BtrA, an anti-σ factor, regulates the ability of Bordetella bronchiseptica to cause cough in rats. mSphere. 2019;4:e00093-19.