Melanin Produced by *Bordetella parapertussis* Confers a Survival Advantage to the Bacterium during Host Infection

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ABSTRACT *Bordetella parapertussis* causes respiratory infection in humans, with a mild pertussis (whooping cough)-like disease. The organism produces a brown pigment, the nature and biological significance of which have not been elucidated. Here, by screening a transposon library, we demonstrate that the gene encoding 4-hydroxyphenylpyruvate dioxygenase (HppD) is responsible for production of this pigment. Our results also indicate that the brown pigment produced by the bacterium is melanin, because HppD is involved in the biosynthesis of a type of melanin called pyomelanin, and homogentisic acid, the monomeric precursor of pyomelanin, was detected by high-performance liquid chromatography-mass spectrometry analyses. In an infection assay using macrophages, the *hppD*-deficient mutant was internalized by THP-1 macrophage-like cells, similar to the wild-type strain, but was less able to survive within the cells, indicating that melanin protects *B. parapertussis* from intracellular killing in macrophages. Mouse infection experiments also showed that the *hppD*-deficient mutant was eliminated from the respiratory tract more rapidly than the wild-type strain, although the initial colonization levels were comparable between the two strains. In addition, melanin production by *B. parapertussis* was not regulated by the BvgAS two-component system, which is the master regulator for the expression of genes contributing to the bacterial infection. Taken together, our findings indicate that melanin produced by *B. parapertussis* in a BvgAS-independent manner confers a survival advantage to the bacterium during host infection.

IMPORTANCE In addition to the Gram-negative bacterium *Bordetella pertussis*, the epidemiological agent of pertussis, *Bordetella parapertussis* also causes respiratory infection in humans, with a mild pertussis-like disease. These bacteria are genetically closely related and share many virulence factors, including adhesins and toxins. However, *B. parapertussis* is clearly distinguished from *B. pertussis* by its brown pigment production, the bacteriological significance of which remains unclear. Here, we demonstrate that this pigment is melanin, which is known to be produced by a wide range of organisms from prokaryotes to humans and helps the organisms to survive under various environmental stress conditions. Our results show that melanin confers a survival advantage to *B. parapertussis* within human macrophages through its protective effect against reactive oxygen species and eventually contributes to respiratory infection of the bacterium in mice. This study proposes melanin as a virulence factor involved in the increased survival of *B. parapertussis* during host infection.

KEYWORDS *Bordetella parapertussis*, melanin, brown pigment
counted as pertussis; *B. parapertussis* has been detected at significant rates during pertussis outbreaks (2–4), indicating that it spreads concurrently with *B. pertussis* epidemics. *B. pertussis* and *B. parapertussis* are genetically closely related and share many virulence factors, including adhesins and toxins, except pertussis toxin, which is specific to *B. pertussis* (5, 6). In contrast, *B. parapertussis* can be clearly distinguished from *B. pertussis* by its brown pigment production. In 1952, Ensminger reported that *B. parapertussis*, but not *B. pertussis*, grown on Bordet-Gengou (BG) agar plates produces a melanin-like brown pigment (7), and several subsequent studies also showed that most *B. parapertussis* isolates from humans and sheep produce the brown pigment (8–10). Thus, the pigment production is a characteristic feature of *B. parapertussis* in comparison to *B. pertussis*; however, the nature and biological significance of the pigment remain to be elucidated.

In the present study, by using a transposon mutagenesis technique, we identified a gene of *B. parapertussis*, hppD, responsible for the pigment production. The hppD gene encodes 4-hydroxyphenylpyruvate dioxygenase (HppD), which has been reported to be required for melanin production in other pathogenic bacteria (11–13). An hppD-deficient mutant (ΔhppD) from *B. parapertussis* exhibited no production of the brown pigment and melanin, indicating the pigment was melanin. Our results also showed that the ΔhppD mutant is less able to survive within macrophages and is eliminated more rapidly from mouse respiratory organs. Therefore, we concluded that melanin production by *B. parapertussis* contributes to bacterial survival during host infection.

**RESULTS**

*B. parapertussis* produces melanin. *B. parapertussis* strain 12822, but not *B. pertussis* strain Tohama, grown on BG agar plates or in Stainer-Scholte (SS) broth (14) produced the brown pigment (Fig. 1A and B), as reported previously (7–10). The brown pigment was found in the culture supernatant (Fig. 1C), indicating that it is released from the bacterial cells. To quantify the produced brown pigment, we analyzed the absorption spectra of the bacterial culture supernatants at visible wavelengths ranging

![Image](https://msphere.asm.org/)

**FIG 1** Brown pigment and melanin production by *B. parapertussis*, but not *B. pertussis*. (A to C) Brown pigment production by *B. pertussis* strain Tohama and *B. parapertussis* strain 12822 grown on BG agar plates (A) or in SS broth (B). The bacterial culture supernatants were collected by centrifugation (C). (D) Absorption spectra of the bacterial culture supernatants at visible wavelengths ranging from 340 to 800 nm. (E and F) The A₃₄₀ values (E) and melanin concentrations (F) in the culture supernatants of the bacteria incubated for the indicated times. Values are means and standard errors of the means (SEM) (n = 3).
from 340 to 800 nm, and the maximum absorbance was obtained at 340 nm ($A_{340}$) in the culture supernatant of *B. parapertussis* (Fig. 1D). Therefore, we estimated the amount of the brown pigment by determining the $A_{340}$ of the bacterial culture supernatants and confirmed the time-dependent increase in level of the brown pigment in cultures of *B. parapertussis*, but not *B. pertussis* (Fig. 1E). As a previous study postulated that the brown pigment is melanin (7), we measured melanin concentrations in the bacterial culture supernatants by enzyme-linked immunosorbent assay (ELISA). Similar to the brown pigment, melanin accumulated in the cultures of *B. parapertussis*, but not *B. pertussis*, with incubation time (Fig. 1F).

**hppD gene is essential for melanin production.** To identify the bacterial gene(s) required for brown pigment production by *B. parapertussis*, we generated transposon-based random mutants from *B. parapertussis* strain 12822 and screened for production of the brown pigment. One of the 1,000 transposon-integrated mutants, designated 8-26, grown on BG agar plates did not produce the brown pigment. DNA sequence analysis indicated that the mutant 8-26 carries the transposon at nucleotide position 853 in the *hppD* gene (locus tag BPP_RS19020) (Fig. 2A). We generated a Δ*hppD* mutant derived from *B. parapertussis* 12822 by homologous recombination and examined it for brown pigment production. Although it proliferated similar to the wild-type (WT) strain during in vitro culture (Fig. 2B), the Δ*hppD* mutant exhibited no production of the brown pigment (Fig. 2C to E). In contrast, the Δ*hppD* mutant complemented with an *hppD*-carrying plasmid, p*hppD*, but not the empty vector, produced the brown pigment to a similar extent to the WT strain (Fig. 2F). Similarly, the Δ*hppD* mutant did not produce melanin, which was restored by *hppD* complementation (Fig. 2G). In addition, we generated Δ*hppD* mutants from other *B. parapertussis* isolates from humans (23054 and CN8234) and a sheep (CZ77) and examined them for brown pigment and melanin production. All parental WT strains for each mutant produced the brown pigment and melanin, while the corresponding Δ*hppD* mutants did not (Fig. 2H and I). In high-performance liquid chromatography (HPLC) analyses, a peak at 290 nm corresponding to a reference standard of homogentisic acid (HGA), the monomeric precursor of a type of melanin called pyomelanin (15, 16), was detected from the culture of *B. parapertussis* 12822 WT strain, but not the Δ*hppD* mutant (Fig. 2J). Mass spectrometry (MS) analyses of the elution fraction containing the HGA peak from the WT strain detected a fragment at m/z 167 and 123 corresponding to the molecular ion [HGA-H]$^-$ and its fragment (17) (Fig. 2K). These results indicate that *hppD* is essential for brown pigment and melanin production by *B. parapertussis*. Here, we refer to the brown pigment as melanin for the reasons mentioned in the Discussion.

**Melanin confers a survival advantage to *B. parapertussis* during host infection.** Previous studies reported that melanin protects some pathogenic bacteria from reactive oxygen species (ROS)-mediated bacterial killing within macrophages, and increases the bacterial persistence in mouse models of infection (12, 18–20). Therefore, we investigated the involvement of melanin in survival of *B. parapertussis* within macrophages by comparing the intracellular survival of *B. parapertussis* 12822 WT strain and the Δ*hppD* mutant using the human monocyte cell line THP-1 that had been differentiated into macrophage-like cells by treatment with phorbol 12-myristate 13-acetate (PMA). The numbers of bacteria that survived in the cultures of differentiated THP-1 cells did not differ significantly after the first 1 and 6 h of incubation; however, the Δ*hppD* mutant showed less recovery compared to the WT strain during 24 to 72 h of incubation (Fig. 3A). We also confirmed that the WT strain harboring an empty vector (WT/vector) and the *hppD*-complemented mutant (Δ*hppD*/p*hppD*), but not the Δ*hppD* mutant harboring empty vector (Δ*hppD*/vector), showed similar rates of survival within the differentiated THP-1 cells after 48 and 72 h of incubation (Fig. 3B). Furthermore, the decreased intracellular survival rate of Δ*hppD* mutant was reversed by treatment of the cells with apocynin, an NADPH oxidase inhibitor, or N-acetyl-L-cysteine, a free radical scavenger, which have been reported to reduce production and accumulation of ROS followed by intracellular killing of bacteria by macrophages (21, 22) (Fig. 3C). In addition, the Δ*hppD* mutant was found to be more sensitive to the H$_2$O$_2$-mediated bactericidal
Identification of the \textit{hppD} gene required for brown pigment and melanin production by \textit{B. parapertussis}. (A) The transposon insertion site in the transposon-integrated mutant 8-26, derived from \textit{B. parapertussis} strain 12822. The transposon (Continued on next page)
effect than the WT strain, while hppD complementation restored resistance to H_2O_2 (Fig. 3D). These results indicate that melanin protects *B. parapertussis* from ROS-mediated bacterial killing inside macrophages without affecting the intracellular uptake of the bacteria.

In mouse infection experiments, the WT strain and ΔhppD mutant, which were inoculated intranasally, showed equivalent colonization of the nasal septum, trachea, and lungs on day 3, while the ΔhppD mutant was eliminated more rapidly from the respiratory organs than the WT strain on days 9 and 15 (Fig. 4A). In contrast, ΔhppD/ppppD, but not ΔhppD/vector, showed equivalent colonization of the mouse respiratory organs compared to WT/vector 9 days after bacterial inoculation (Fig. 4B). These results indicate that melanin is not involved in the initial colonization of *B. parapertussis*, but confers a survival advantage to the bacterium during infection in the mouse respiratory tract.

**Melanin production is independent of the BvgAS system.** *Bordetella* species, including *B. parapertussis*, exhibit two distinct phenotypic phases, which are regulated by the BvgAS two-component system in response to environmental alterations (23). At 37°C in standard *Bordetella* media, the BvgAS system promotes the transcription of various virulence genes. Conversely, this system is inactivated in the presence of MgSO_4 or nicotinic acid, and transcription of the virulence genes is repressed. The former bacterial state is called the Bvg + phase, which is generally considered to represent the virulent phenotype, and the latter is the Bvg − phase. We examined the involvement of the BvgAS system in melanin production by *B. parapertussis*. The Bvg + - and Bvg −-locked mutants of *B. parapertussis* 12822, which constitutively exhibit the Bvg + and Bvg − phenotypes, respectively, were found to produce melanin, similar to the WT strain, as determined by the color of the colonies on BG agar plates and ΔA_{450} values and melanin levels in the bacterial culture supernatants (Fig. 5A and B; see Fig. S1 in the supplemental material). The ability of the WT strain to produce melanin was largely unaffected in the presence of MgSO_4 (Fig. 5B).

In addition, no significant differences in the expression levels of hppD transcript were observed between *B. parapertussis* strains exhibiting the Bvg + phenotype and those exhibiting the Bvg − phenotypes (Fig. 5C). These results indicate that melanin production by *B. parapertussis* is independent of the BvgAS system.

**B. pertussis HppD is functional.** The genome of *B. pertussis* strain Tohaman harbors a gene encoding HppD protein that is nearly identical to HppD of *B. parapertussis* strain 12822 (98.4% identity), and hppD transcript was detected at similar levels in both bacteria (Fig. 6A). In addition, ectopic expression of hppD from both *B. pertussis* (hppD<sub>pp</sub>) and *B. parapertussis* (hppD<sub>ppp</sub>) restored the ability to produce melanin by the 12822 ΔhppD mutant, but not strain Tohaman (Fig. 6B), indicating that *B. pertussis* produces functional HppD.

**DISCUSSION**

In this study, we concluded that the brown pigment produced by *B. parapertussis* is melanin for the following reasons: (i) the brown pigment and melanin increased in parallel in the culture supernatants of *B. parapertussis*, (ii) neither phenotype was observed in *B. pertussis*, (iii) the hppD gene encoding an enzyme that is involved in melanin synthesis is necessary to produce the brown pigment, (iv) HGA was detected in the culture of the *B. parapertussis* WT strain, but not the ΔhppD mutant, and (v) production of the
brown pigment and melanin showed no regulation by the BvgAS system. Moreover, we found that melanin confers a survival advantage to *B. parapertussis* during host infection. To our knowledge, this is the first report indicating a function of melanin in *B. parapertussis* infection.

Melanin is produced by diverse organisms ranging from bacteria to humans and is classified into four categories based on the intermediates in the formation process: allo-melanin, eumelanin, pheomelanin, and pyomelanin. Among them, pyomelanin is formed from tyrosine through deamination by aromatic amino acid aminotransferases and conversion into 4-hydroxyphenylpyruvate, from which HppD generates HGA. Subsequently, HGA is released from the cells, and pyomelanin is formed extracellularly through several steps, including auto-oxidation and self-polymerization of HGA (15, 16). Our results show that the deletion of *hppD* results in no production of melanin in *B. parapertussis*, indicating that the bacterial melanin is pyomelanin. Consistent with our observations, *hppD* was reported to be required for pyomelanin production by some pathogenic bacteria, such as *Burkholderia cenocepacia*, *Pseudomonas aeruginosa*, and *Legionella pneumophila* (11–13). On the other hand, *B. pertussis* did not produce melanin despite the production of functional HppD. Previous studies showed that biosynthesis of pyomelanin in bacteria is

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**FIG 3** Increased survival of *B. parapertussis* within macrophages through antioxidative effect of melanin. (A to C) Intracellular survival of *B. parapertussis* 12822 and its mutant derivatives in THP-1 cells differentiated into macrophage-like cells. THP-1 cells were infected with the bacteria, treated with polymyxin B, and then further incubated in the absence (A and B) or presence of apocynin or N-acetyl-L-cysteine (C) for the indicated periods (A and B) or 72 h (C). The bacterial numbers within the THP-1 cells are expressed as CFU per well. Values are means and SEM (n = 3). The significance of differences was analyzed by two-way ANOVA with Sidak’s multiple-comparison test. *, P < 0.05; **, P < 0.01; ns, no significant differences. (D) Sensitivity of *B. parapertussis* 12822 and its mutant derivatives to H$_2$O$_2$-mediated bactericidal effect.
mediated by various enzymes, including HppD, to form HGA and its transporter (11, 15, 16, 24), suggesting that B. pertussis does not produce melanin due to any deficiency in the gene(s) required for melanin production other than hppD.

B. parapertussis is not considered an intracellular pathogen, but has been reported to survive for several days within human phagocytes, including macrophages and neutrophils. Two molecules, O antigen of lipopolysaccharide (LPS) and adenylate cyclase toxin (ACT), have been reported to be involved in survival of B. parapertussis within phagocytes by inhibiting production of ROS, which exhibit bactericidal activity (25, 26).

The present study indicates that melanin, which is known to scavenge ROS (12, 15), also protects B. parapertussis from intracellular killing by phagocytes and eventually contributes to bacterial infection. We also confirmed that the ΔhppD mutant from B. parapertussis produces LPS and ACT comparable to the WT strain (see Fig. S2 in the supplemental material), suggesting that the protective effects of melanin have no relation to LPS and ACT. In addition, we found that melanin production is not regulated by the BvgAS system, which is consistent with the previous report of BvgAS-independent production of the brown pigment by most B. parapertussis isolates from humans and sheep (8). Considering that B. parapertussis produces melanin regardless of the Bvg phase, melanin may contribute to bacterial survival not only during host infection but also in the extrahost environments, where the bacteria are in the Bvg− phase. This hypothesis was supported by previous observations that melanin helps bacteria to

**FIG 4** Contribution of melanin to B. parapertussis colonization in mouse respiratory organs. B. parapertussis 12822 and its mutant derivatives were intranasally inoculated into mice, and the bacteria recovered from the nasal septum, trachea, and lungs were counted on days 3, 9, and 15 in panel A or 9 alone in panel B. Each horizontal bar represents the geometric mean. The significance of differences was analyzed by the Mann-Whitney test (A) or Kruskal-Wallis test with Dunn’s multiple-comparison test (B). *, P < 0.05; **, P < 0.01.
survive under various environmental stress conditions through multiple functions, such as electron transfer, heavy metal binding, iron reduction/acquisition, and resistance to oxidative and hyperosmotic stresses (15, 16).

*B. pertussis*, *B. parapertussis*, and *Bordetella bronchiseptica*, which are genetically related and share many virulence factors, are often collectively called the “classical *Bordetella.*” Among them, only *B. parapertussis* produces the brown pigment (i.e., melanin) (8, 9). We also confirmed that *B. bronchiseptica* exhibits no production of melanin, similar to *B. pertussis* (data not shown). In other *Bordetella* species that are phylogenetically distinct from the classical *Bordetella*, *Bordetella holmesii* is also known to produce melanin-like brown pigment, while the closely related species *Bordetella avium*, does not (9, 27). Thus, the ability to produce melanin (or melanin-like pigments) is not phylogenetically conserved, but sporadically acquired in *Bordetella* species, although identification and phylogenetic analyses of multiple genes involved in melanin production in the genus are needed. Given that *B. holmesii* also causes respiratory infection in humans with a mild pertussis-like disease, similar to *B. parapertussis* (28), melanin (or melanin-like pigments) may play a specific role in the pathogenicity of *B. parapertussis* and *B. holmesii*. Further analyses of the role of melanin in *Bordetella* infections are required to clarify this hypothesis.

**MATERIALS AND METHODS**

**Bacterial strains.** *B. pertussis* strain Tohama was maintained in our laboratory. *B. parapertussis* strains 12822 (ATCC BAA-587), 23054, CN8234, and CZ77 were provided by A. Abe (Kitasato University).
Bordetella strains were grown at 37°C on BG agar (Becton Dickinson) containing 1% hypolypepton (Nihon Pharmaceutical), 1% glycerol, 15% defibrinated horse blood, and 10 μg/ml ceftibuten. The bacteria recovered from colonies on BG agar plates were suspended in SS broth to an optical density at 650 nm (OD650) of 0.1 and incubated at 37°C with shaking. The number of CFUs of B. parapertussis was estimated from the OD650 values of fresh cultures according to the following equation: 1 OD650 unit = 6.8 × 10^8 CFU/ml. Escherichia coli was grown on Luria-Bertani agar or broth. E. coli strains DH5α, Apir and HB101 harboring pRK2013 (29) were provided by K. Minamisawa (Tohoku University). The growth media were supplemented with antibiotics as necessary at the following concentrations: ampicillin, 50 μg/ml; gentamicin (Gm), 10 μg/ml; kanamycin (Km), 25 μg/ml.

**Construction of mutant strains.** Mutants derived from B. parapertussis strains 12822, 23054, CN8234, and CZ77 were constructed by double-crossover homologous recombination as described previously (30, 31). The primers used in this study are listed in Table S1 in the supplemental material. For the generation of ΔhppD mutants derived from the four strains of B. parapertussis, DNA fragments of ~1 kbp of the upstream and downstream regions of the hppD gene were amplified by PCR using genomic DNA (gDNA) from B. parapertussis 12822 as the template with primers hppD-U-S and hppD-U-AS, and hppD-D-S and hppD-D-AS, respectively. The PCR products were ligated and inserted into the ScaI site of pABB-CRS2-Gm (32), which was provided by A. Abe (Kitasato University), using an In-Fusion HD Cloning kit (TaKaRa Bio). The resultant plasmids designated pABB-CRS2-Gm, was introduced into E. coli DH5α, Apir and transconjugated into B. parapertussis strains 12822, 23054, CN8234, and CZ77 by triparental conjugation with a helper strain, E. coli HB101 harboring pRK2013. The resultant ΔhppD mutants were isolated after confirming the gene deletion by appropriate PCR followed by agarose gel electrophoresis.

For complementation experiments, a DNA fragment of ~1.6 kbp of the entire hppD gene, including the putative promoter and termination sites (300-bp upstream and 80-bp downstream regions), was amplified by PCR using the gDNA of B. parapertussis 12822 as the template with primers PthppD-S and PthppD-D. The PCR product was digested and ligated into the BamHI-EcoRI sites of pBBR1-MCS5 (33) using an In-Fusion HD Cloning kit. The resultant plasmid, designated pthppD, was introduced into E. coli DH5α and transconjugated into B. parapertussis 12822 ΔhppD by triparental conjugation. The resultant strain was designated 12822 ΔhppD/pthppD and used as hppD-complemented mutant. For ectopic expression of hppD, a DNA fragment of ~0.2 kbp of the tac promoter (P_tac) was amplified by PCR using pBBR1-Ptac-gfp (31) as the template with primers Ptac-S and Ptac-AS. A DNA fragment of ~1.2 kbp of the hppD gene was also amplified by PCR using the DNA of B. pertussis Tohama or B. parapertussis 12822 as the template with primers hppD-S and hppD-D. The PCR products of Ptac and each hppD gene were ligated and inserted into the BamHI-EcoRI sites of pBBR1-Ptac-gfp using an In-Fusion HD cloning kit. The resultant plasmids designated Ptac-hppD/p and Ptac-hppD/p, respectively, were introduced into E. coli DH5α and transconjugated into B. pertussis Tohama and B. parapertussis 12822 ΔhppD by triparental conjugation, respectively. The empty vector (pBBR1-MCS5) was introduced into E. coli S17-1 Apir and transconjugated into B. pertussis Tohama and B. parapertussis 12822 WT strain and ΔhppD mutant by biparental conjugation.

Bvg− and Bvg+ locked mutants were constructed by site-directed mutagenesis of bvgS to replace Arg with His at position 570 and to delete the region from amino acid positions 542 to 1020, respectively (34). Briefly, the plasmids bvgS-C3-pABB-CS2-Gm and ΔbvgS-pABB-CS2-Gm (30) were introduced into E. coli DH5α Apir and transconjugated into B. parapertussis 12822 by triparental conjugation. The mutation (G→A at nucleotide position 1709) or deletion of the bvgS gene in the resultant Bvg− and Bvg+...
B. parapertussis strains were incubated in SS broth for 24 h, and the number of CFU was determined after cultivation of the inocula on BG agar plates. Bacterial survival was estimated from the bacterial growth on the plates after incubation at 37°C for 3 to 4 days, and the number of CFU was counted after cultivation on BG agar plates. Mice were euthanized with pentobarbital on days 3, 9, or 15 postinoculation, and the nasal septum, trachea, and lungs were aseptically excised, minced, and homogenized in Dulbecco’s modified phosphate-buffered saline (PBS) with a BioMasher (Nippi) and a Polytron PT1200E (Kinematica), respectively. The resultant tissue extracts were digested with Sau3AI. The digested products were circularized with T4 DNA ligase (Promega) and used as a template for PCR with primers Gmr-R and MariS. The PCR products were then ligated using an In-Fusion HD cloning kit. The resultant plasmid designated pMariG, carrying Gmr in place of the Km resistance gene in the pMarIK backbone, was introduced into E. coli S17-1 Apir and transconjugated into B. parapertussis 12822 by biparental conjugation.

**Bacterial colonization in mice.** Seven-week-old male C57BL/6J mice (CLEA, Japan) were anesthetized with a mixture of medetomidine (Kyoritsu Seiyaku), midazolam (Teva Takeda Pharma), and butorphanol (Meiji Seika Pharma) at final doses of 0.3, 2, and 5 mg/kg body weight, respectively. B. parapertussis was incubated in SS broth for 24 h and intranasally inoculated at 1 × 108 CFU/25 μl (SS broth) into anesthetized mice using a micropipette with a needle-like tip. The amounts of bacteria were confirmed by counting colonies after cultivation of the inocula on BG agar plates. Mice were euthanized with pentobarbital on days 3, 9, or 15 postinoculation, and the nasal septum, trachea, and lungs were aseptically excised, minced, and homogenized in Dulbecco’s modified phosphate-buffered saline (PBS) with a BioMasher (Nippi) and a Polytron PT1200E (Kinematica), respectively. The resultant tissue extracts were used for DNA isolation using the Blood and Tissue kit (Qiagen) and digested with Sau3AI. The digested products were circularized with T4 DNA ligase (Promega) and used as a template for PCR with primers Gmr-R and MariS. The PCR products were then ligated using an In-Fusion HD cloning kit. The resultant plasmid designated pMariG, carrying Gmr in place of the Km resistance gene in the pMarIK backbone, was introduced into E. coli S17-1 Apir and transconjugated into B. parapertussis 12822 by biparental conjugation.

**Measurement of melatonin concentrations.** Bordetella strains were incubated in SS broth for the indicated periods. The culture supernatants of the bacteria were collected by centrifugation at 12,000 × g for 5 min and filtered through 0.2-μm-pore membranes (Thermo Fisher Scientific). The concentrations of melanin in the culture supernatants were measured using a human melanin ELISA kit (Cusabio).

**HPLC-MS analyses.** Melanin production by B. parapertussis was validated by detection of HGA as described previously, with slight modifications (35). Briefly, gDNA was extracted from the transposon-integrated mutant using a DNeasy blood and tissue kit (Qiagen) and digested with Sau3AI. The digested products were circularized with T4 DNA ligase (Promega) and used as a template for PCR with primers Gmr-R and MariS. The PCR products were subjected to direct sequencing using the same primers. The obtained sequences were aligned to the B. parapertussis 12822 genome sequence (NCBI accession no. NC_002928.3), and the transposon insertion sites were identified.

**Assay for sensitivity of bacteria to H2O2.** B. parapertussis cells were incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) at 37°C under 5% CO2 in air. The resultant suspensions were serially diluted with Hanks’-acetyl-L-cysteine (Hanks’-HEPES BSA) at a concentration of 1 × 108 CFU/ml, and the medium of THP-1 cells was replaced with 0.5 ml of the bacterial suspension (multiplicity of infection of 100). After centrifugation of the plates at 500 × g for 5 min, the cells were incubated with the bacteria for 1 h, washed three times with RPMI 1640 medium, and treated with 100 μg/ml polymyxin B for 1 h to eliminate the extracellular bacteria. The cells were further incubated in RPMI 1640 medium supplemented with 10% FBS and 10 μg/ml polymyxin B in the absence or presence of 100 μM apocynin (Abcam) or 10 mM N-acetyl-L-cysteine (Nacalai Tesque) for 1, 6, 24, 48, or 72 h and then lysed with 0.1% saponin in Hanks’-HEPES BSA. The resultant suspensions were serially diluted with Hanks’-HEPES BSA and spread onto BG agar plates. The bacteria on the plates were incubated at 37°C for 3 to 4 days, and the number of CFU was counted.

**Bacterial survival under both conditions (Fig. S1), indicating that the Bvg-phased locked mutants used in this study constitutively exhibit the Bvg- and Bvg- phenotypes, respectively.**
serially diluted with PBS, and CFU were enumerated as described above. All animal experiments were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University, in accordance with the Regulations on Animal Experiments at Osaka University.

Quantitative reverse transcription-PCR. For quantitative reverse transcription-PCR (qRT-PCR), total RNA was extracted from *B. parapertussis* incubated in SS broth with or without 50 mM MgSO₄ for 14 h using a PureLink RNA minikit (Thermo Fisher Scientific) and treated with RNase-Free DNase (TaKaRa Bio) to degrade contaminating DNA. Aliquots of 1 μg of total RNA were then reverse transcribed into cDNA using a PrimeScript RT reagent kit (TaKaRa Bio) with random hexamers in a total volume of 20 μl. The transcription levels of target mRNAs were estimated from the amounts of the resultant cDNA measured with a StepOnePlus real-time PCR system (Applied Biosystems), using Power SYBR green PCR master mix (Thermo Fisher Scientific) and the primer sets listed in Table S1 under the following conditions: initial denaturation at 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min.

RT-PCR. Total RNA and cDNA were prepared from *B. pertussis* and *B. parapertussis* incubated in SS broth for 14 h as described above. PCR was then performed using the cDNA as the template with the primers listed in Table S1 under the following conditions: initial denaturation at 94°C for 2 min and 30 cycles of 98°C for 10 s, 60°C for 30 s, and 68°C for 40 s. PCR products were subjected to 2% agarose gel electrophoresis with ethidium bromide staining.

Statistical analysis. Statistical analyses were performed using Prism 8 (GraphPad Software). Significance is expressed as follows: *, P < 0.05; **, P < 0.01; and ***, P < 0.001. In all analyses, P < 0.05 was taken to indicate statistical significance.

Supplemental material is available online only.

**Supplemental material**

**TEXT S1**, DOCX file, 0.1 MB.

**FIG S1**, TIF file, 0.3 MB.

**FIG S2**, TIF file, 0.3 MB.

**TABLE S1**, DOCX file, 0.1 MB.

**Acknowledgments.**

We thank A. Abe for *B. parapertussis* strains and K. Minamisawa for *E. coli* HB101 harboring pRK2013.

This work was supported by JSPS KAKENHI grant numbers 19K16638, 20H03485, and 21K07003.

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