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RESEARCH ARTICLE

The Transcriptional Regulator BpsR Controls the Growth of *Bordetella bronchiseptica* by Repressing Genes Involved in Nicotinic Acid Degradation

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ABSTRACT Many of the pathogenic species of the genus *Bordetella* have an absolute requirement for nicotinic acid (NA) for laboratory growth. These Gram-negative bacteria also harbor a gene cluster homologous to the *nic* cluster of *Pseudomonas putida* which is involved in the aerobic degradation of NA and its transcriptional control. We report here that BpsR, a negative regulator of biofilm formation and Bps polysaccharide production, controls the growth of *Bordetella bronchiseptica* by repressing the expression of *nic* genes. The severe growth defect of the Δ*bpsR* strain in Stainer-Scholte medium was restored by supplementation with NA, which also functioned as an inducer of *nic* genes at low micromolar concentrations that are usually present in animals and humans. Purified BpsR protein bound to the *nic* promoter region, and its DNA binding activity was inhibited by 6-hydroxynicotinic acid (6-HNA), the first metabolite of the NA degradative pathway. Reporter assays with the isogenic mutant derivative of the wild-type (WT) strain harboring deletion in *nicA*, which encodes a putative nicotinic acid hydroxylase responsible for conversion of NA to 6-HNA, showed that 6-HNA is the actual inducer of the *nic* genes in the bacterial cell. Gene expression profiling further showed that BpsR dually activated and repressed the expression of genes associated with pathogenesis, transcriptional regulation, metabolism, and other cellular processes. We discuss the implications of these findings with respect to the selection of pyridines such as NA and quinolinic acid for optimum bacterial growth depending on the ecological niche.

IMPORTANCE BpsR, the previously described regulator of biofilm formation and Bps polysaccharide production, controls *Bordetella bronchiseptica* growth by regulating the expression of genes involved in the degradation of nicotinic acid (NA). 6-Hydroxynicotinic acid (6-HNA), the first metabolite of the NA degradation pathway prevented BpsR from binding to DNA and was the actual *in vivo* inducer. We hypothesize that BpsR enables *Bordetella* bacteria to efficiently and selectively utilize NA for their survival depending on the environment in which they reside. The results reported herein lay the foundation for future investigations of how BpsR and the alteration of its activity by NA orchestrate the control of *Bordetella* growth, metabolism, biofilm formation, and pathogenesis.

KEYWORDS *Bordetella*, metabolism, transcriptional regulation

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Members of the genus *Bordetella* are Gram-negative bacteria that include human, animal, and avian pathogens and many strains isolated from soil. Of the nine species known to date, the three known as the "classical species" are closely related genetically. *Bordetella bronchiseptica* infects humans, animals, and marine mammals. It causes a dramatically diverse range of respiratory diseases and chronic asymptomatic colonization. *Bordetella pertussis*, the agent of whooping cough, is strictly a human pathogen, whereas *Bordetella parapertussis* can infect both humans and sheep (1–3).

The classical species of *Bordetella* have an absolute requirement for either nicotinic acid (NA) or nicotinamide for laboratory growth and are routinely grown in medium containing micromolar concentrations of either of these compounds (4, 5). NA, along with nicotinamide is a precursor for the coenzymes NAD and NADP *in vivo*, which participate in many critical cellular pathways (6). While many bacteria produce NAD *de novo* from aspartic acid, *Bordetella* and other bacteria that lack this pathway utilize the salvage or the recycling pathway. This pathway utilizes NAD precursors (NA or nicotinamide) or breakdown products. NA is converted in three steps to NAD by the conserved "Preiss-Handler pathway" by the conversion to nicotinic acid mononucleotide and nicotinic acid adenine dinucleotide. For the production of NAD from nicotinamide, nicotinamidases convert it to NA for the Preiss-Handler pathway (6, 7). The presence of NA in millimolar concentrations during *Bordetella* growth results in a phenomenon known as phenotypic modulation. This requires the BvgAS signal transduction system and is characterized by large-scale alterations in the expression of genes, including pathogenesis-associated or virulence genes (8–11).

In addition to serving as a source of NAD, NA is also a carbon, nitrogen, and energy source for many bacteria which catabolize it by both anaerobic and aerobic routes (12). In *Pseudomonas putida* strain KT2440, aerobic degradation of NA via the maleamate pathway involves the genes of the *nic* cluster (Fig. 1A and B). The expression of the *nic* cluster is tightly controlled in *P. putida* and involves the activity of two transcriptional repressors, NicR and NicS, and induction by nicotinic acid (Fig. 1). The orthologues of *nic* genes are also present in many soil bacteria that colonize the plant rhizosphere (13, 14).

The conservation of *nic* genes in bacteria associated with the plant rhizosphere is consistent with plants being the major source of NA in the environment. The degradation of NA by the activity of *nic* genes will enable these bacteria to survive in the soil environment. Surprisingly, a search for homologs of the *nic* genes of *P. putida* KT2440 revealed the conservation of the *nic* genes, including *bpsR*, the homolog of *nicR*, in *B. bronchiseptica* (Fig. 1) (13). In *B. bronchiseptica*, BpsR functions as a transcriptional repressor of Bps polysaccharide production and biofilm development (15, 16). The activity of the NA degradation pathway will lead to a depletion of the NA pool in *B. bronchiseptica* and may thus lead to a defect in NAD and NADP synthesis. In addition to *B. bronchiseptica*, the majority of *nic* genes and *bpsR* are intact in *B. parapertussis* and *B. pertussis* (Fig. 1). The speciation of *B. pertussis* and *B. parapertussis* from *B. bronchiseptica* has been driven mainly by large gene losses and inactivation events that have considerably reduced the genomic content, altered regulatory network, and modified the metabolic pathways (17, 18). Thus, evolutionarily, the conservation of the *nic* cluster in *B. pertussis* and *B. parapertussis* suggests a physiological function for these genes. It is important to understand the contribution of the *nic* cluster to the growth and life cycle of *Bordetella*.

In this report, we demonstrate that *B. bronchiseptica* exerts tight control over the expression of *nic* genes by the activity of BpsR. We show that the transcriptional control of *Bordetella* gene expression by NA in the bacterial cell occurs at concentrations that are below those present in animal and humans. The implications of this regulation for bacterial survival in an environmental niche and for the selection of a pyridine source for NAD biosynthesis are discussed. Finally, this work lays the framework for investigating the regulatory mechanisms linking the control of

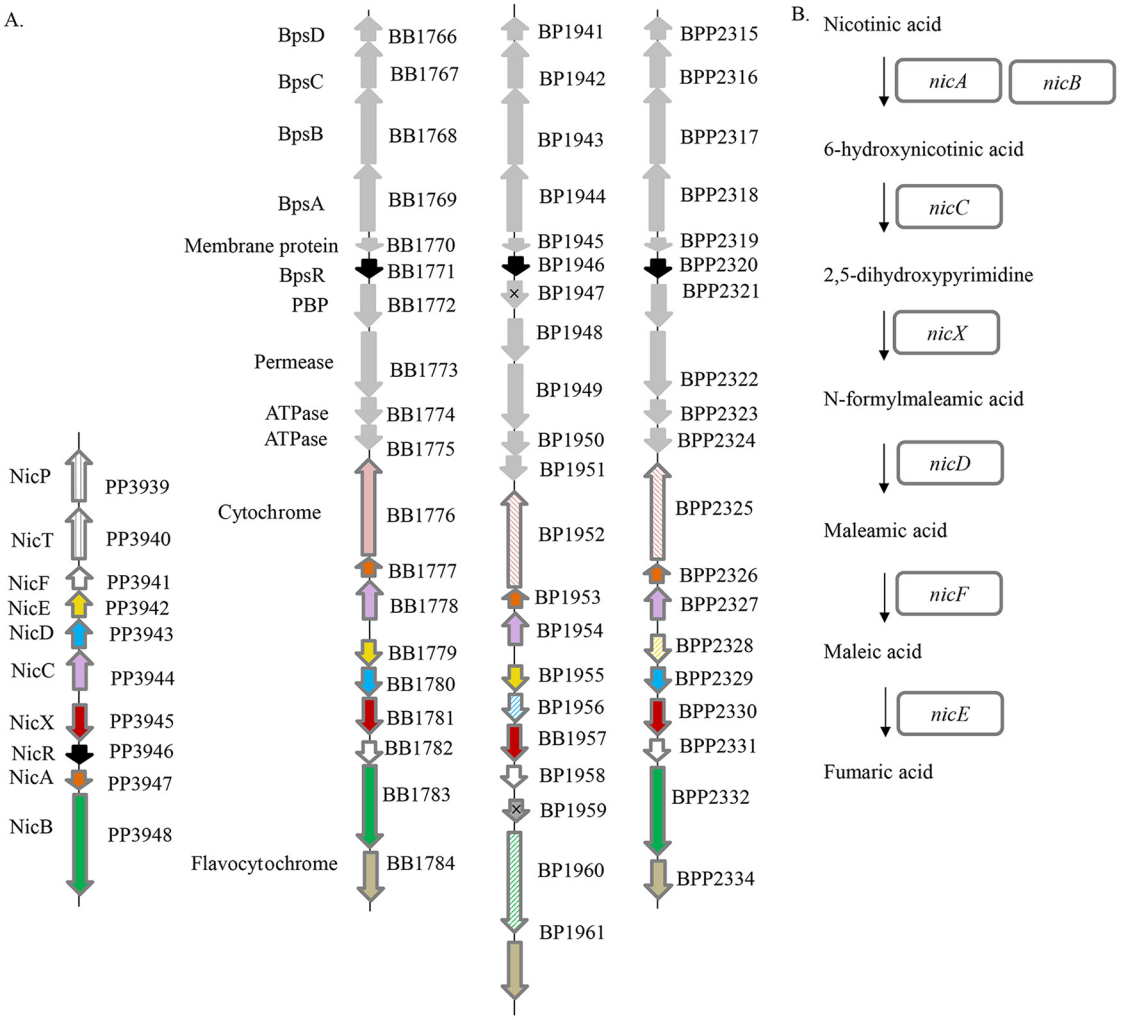

FIG 1 (A) Comparison of the *nic* cluster of *P. putida* strain KT2440 with homologous clusters from the classical *Bordetella* species. Each arrow indicates an ORF. For the *Bordetella* species, the gene organization is extended to include the adjacent *bpsA-D* locus that is regulated by *bpsR* (15, 39, 40). Homologous genes are indicated by the same color, and the protein designation of the *Bordetella nic* genes is based on their amino acid homology with the corresponding Nic proteins of *P. putida* KT2440 (13). For the other *Bordetella* genes, the functions are assigned on the basis of the annotation in the genomic databases. Diagonal stripes indicate annotations as pseudogenes. Cross marks represent insertions of a transposase and thus inactive genes. (B) Predicted aerobic NA degradation pathway in *Bordetella bronchiseptica* based on its homology to *nic* cluster in *P. putida* KT2440 (13, 14).

growth, metabolism, and biofilm formation in *B. bronchiseptica* and expands the role of BpsR in this process.

RESULTS

Severe growth defect of the Δ*bpsR* strain can be recovered by transfer to fresh growth medium. In a previous report, we made an in-frame deletion of *bpsR* in the *B. bronchiseptica* wild-type (WT) strain RB50. In that paper and further described here, we found that culturing RB50 and its derivative, the Δ*bpsR* mutant (15), for 24 h in Stainer-Scholte (SS) medium, a simple chemically defined medium used for the routine growth of *B. bronchiseptica*, led to a decreased growth of the mutant strain (Fig. 2A). This phenotype persisted out to 40 h in SS medium, with the *bpsR* mutant failing to reach WT growth levels (data not shown). However, when these strains were grown in LB medium, there were no significant differences in growth (Fig. 2B).

To understand the biochemical mechanism of the growth defect, we first tested the hypothesis that the mutant strain was growing poorly due to the production of an inhibitory factor(s) during growth. Experiments were performed with the spent medium

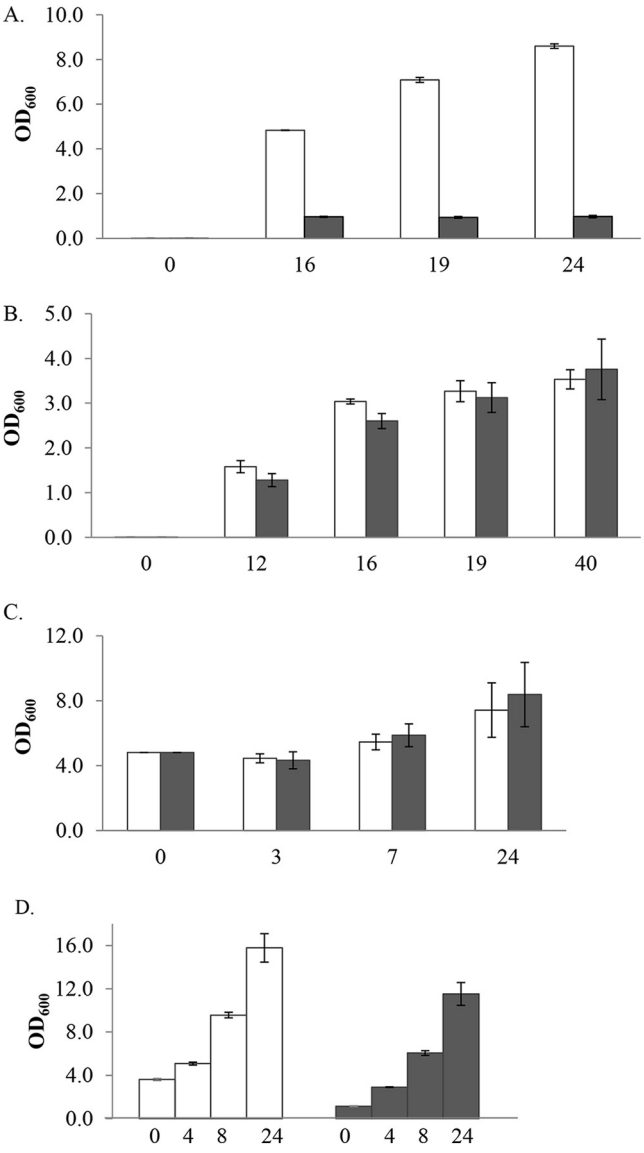

FIG 2 Growth yields of RB50 (open bars) and Δ*bpsR* (closed bars) strains grown in various media. *x* axes, time in hours. (A) Strains grown in SS medium. (B) Strains grown in LB broth. (C) RB50 grown first in SS medium for 12 h and then washed, resuspended in adjusted spent medium (described in Materials and Methods) harvested from either strain RB50 or the Δ*bpsR* strain, and grown additionally for the indicated times. (D) Strains grown in regular SS medium for 12 h and then resuspended in fresh SS medium and grown additionally for the indicated times. Each data point represents the average from triplicates from one of two or more independent experiments.

collected at 12 h of growth, a time point when the growth of the Δ*bpsR* strain has stopped. RB50 cells actively growing for 12 h in regular SS medium were resuspended in reconstituted spent medium (as described in Materials and Methods) from either the RB50 or Δ*bpsR* strain and grown for an additional 24 h. As shown in Fig. 2C, the WT strain grew to a similar optical density at 600 nm (OD_{600}) in spent medium collected from either of the strains. This suggests that the growth defect of the Δ*bpsR* strain in SS medium is not due to the production of a growth inhibitory factor(s).

SS medium contains sources for multiple amino acids and vitamins, many of which are essential for robust growth (5). Next, we hypothesized that as the Δ*bpsR* cells grow, the essential nutrients are consumed and eventually exhausted, leading to a slowing and ultimate cessation of growth. To test this hypothesis, we repeated the growth experiment using fresh medium. Bacterial cells were grown in SS medium for 12 h and

then washed and resuspended in fresh SS medium and grown for an additional 24 h. As shown in Fig. 2D, both the WT and Δ*bpsR* cells continued to grow and reached OD_{600}s of ~16 and 12, respectively, by 24 h. The lower OD_{600} of the Δ*bpsR* strain compared to the WT strain is probably due to a slight depletion of the nutrients. These results suggest that the growth defect of the Δ*bpsR* strain in SS medium can be recovered by transferring to fresh medium.

BpsR controls the expression of genes involved in diverse cellular processes in *B. bronchiseptica*. Previously, we reported that BpsR is homologous to the MarR family of transcriptional regulators. BpsR functions as a repressor of transcription of the *bpsA-D* operon, a gene locus that encodes the enzymatic machinery for the synthesis of the Bps polysaccharide (15). This family of regulators controls diverse cellular processes, including those for the survival of bacterial cells under various environmental stresses (15, 19, 20). To understand the mechanism of the growth defect of the Δ*bpsR* strain, whole-genome transcriptome analysis was conducted to determine which genes are differentially expressed between the WT and Δ*bpsR* strains grown in LB broth, a medium chosen to avoid any effects on gene expression due to growth differences. The expression levels of 31 genes were elevated and 33 were reduced in the Δ*bpsR* mutant relative to that in the RB50 strain (see Table S1 in the supplemental material). The genes highly expressed in the mutant strain included *fim2* (BB3674), which encodes one of the several fimbrial subunits expressed by *B. bronchiseptica* (fimbriae have been implicated in cellular adhesion in *Bordetella*) (21, 22), a transcriptional regulator of the LysR family (BB0088) (a family of protein regulators involved in oxidative stress responses, cell wall shape, quorum sensing, efflux pumps, secretion, motility, nitrogen fixation, and virulence) (23), 13 cellular metabolism associated genes, 3 genes encoding periplasmic/exported lipoproteins, and 2 genes encoding proteins with folding and chaperone activities. The genes identified to have reduced expression in the Δ*bpsR* mutant included 8 genes categorized as having roles in metabolism and 10 in substrate transport. Additionally, 3 genes encoding membrane proteins, one gene encoding a transcriptional regulator of the LysR family (BB3731), and 5 genes encoding periplasmic/exported lipoproteins were downregulated in the Δ*bpsR* mutant (Table S1).

Expression of genes homologous to those involved in aerobic catabolism of NA and biosynthesis of NAD is elevated in the Δ*bpsR* strain. Because of the differential regulation of multiple genes involved in metabolism, we focused on this group for further analyses. *nadC*, a gene encoding a quinolinate phsophoribosyl transferase, was expressed at higher levels in the mutant strain. In *B. bronchiseptica*, *nadC* is involved in the conversion of quinolinic acid to nicotinic acid mononucleotide, which is a precursor for NAD synthesis (24). The expression of a cluster of genes (BB1772 to BB1783) located adjacent to *bpsR* (BB1771) (Fig. 1) was elevated (2.14- to 38.22-fold) in the Δ*bpsR* mutant. Previously, this cluster of genes was designated as homologous to the *P. putida nic* cluster (13). In *P. putida* strain KT 24440, the *nic* cluster is involved in the degradation of NA via the maleamate pathway (13). The first step of NA degradation is its conversion to 6-hydroxynicotinic acid (6-HNA) by a nicotinic acid hydroxylase encoded by the *nicA* and *nicB* genes. 6-HNA is then converted to 2,5-dihydroxypyrimidine by a 6-HNA monooxygenase encoded by the *nicC* gene which is further degraded in a stepwise manner via the maleamate pathway by the involvement of the *nicX*, *nicD*, *nicF*, and *nicE* gene products (Fig. 1) (13, 25, 26).

The enhanced expression of two genes of the *nic* cluster (BB1781 and BB1772) and the *nadC* (BB4286) gene in the Δ*bpsR* strain was independently confirmed by real-time reverse transcriptase PCR (RT-PCR). The expressions of BB1772, BB1781, and *nadC* were elevated 20.89-, 14.73-, and 38.13-fold, respectively (Fig. 3). As a positive control, the expression of the *bpsA* gene (Fig. 1) was tested. Consistent with the previous report (15), the expression of *bpsA* was 2.41-fold higher in the mutant strain.

Addition of NA and nicotinamide restores the growth of the Δ*bpsR* strain in SS medium. We hypothesized that due to the increased expression of the *nic* genes in the Δ*bpsR* strain, NA in the growth medium is depleted earlier than in the growth medium of the WT strain, resulting in the observed slowing/cessation of growth (Fig. 2). If this

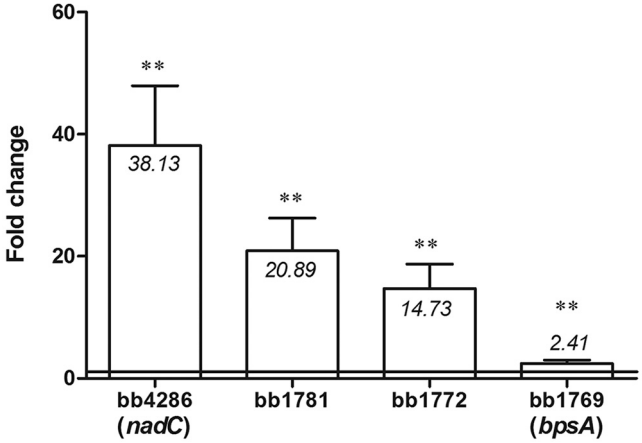

FIG 3 Relative mRNA expression of BpsR-regulated genes. Relative expression of *nadC*, BB1781, BB1772, and *bpsA* was assessed by real-time RT-PCR in RB50 and Δ*bpsR* strains. The strains were harvested at mid-log phase of growth. Expression in strain RB50 is set to 1. *rpoD* was used as the standardization control. Fold changes were calculated using the Pfaffl method. Statistical difference was analyzed by a pair-wise fixed reallocation randomization test, run by Rest2009 software (Qiagen). Average values are presented from two biological duplicates with reciprocal technical triplicates and their respective standard deviations. **, $P < 0.01$.

hypothesis is valid, then the supplementation of SS medium with NA should improve the growth. To test this, RB50 and Δ*bpsR* strains were grown in either SS medium (base concentration of NA, 0.03 mM) or in media supplemented to contain 0.16, 0.32, and 0.81 mM NA, and the OD_{600} was monitored at various intervals. In support of our hypothesis, the supplementation of SS medium with NA at 0.16 and 0.32 mM resulted in better growth of the Δ*bpsR* strain (Fig. 4B and C), whereas 0.81 mM nicotinic acid restored the growth of the mutant strain to that of the RB50 strain (Fig. 4D). As a control, the supplementation of SS media with any of these concentrations of NA did not affect the growth of the WT strain (compare Fig. 4A to D).

In addition to NA, nicotinamide also supports the robust growth of *B. bronchiseptica*. Thus, we tested if supplementation with nicotinamide would also restore the growth of the *bpsR* mutant. The addition of nicotinamide also resulted in the restoration of growth of the mutant strain to that of the WT strain. The growth of the *bpsR* mutant in SS media containing 0.16 and 0.32 mM nicotinamide was better that that in media containing the same concentrations of NA (Fig. 4B and C). This is likely due to the degradation of NA but not nicotinamide by the gene products of the *nic* cluster.

Deletion of *nicA* in the *bpsR* mutant results in the restoration of growth. If the hypothesis that the growth defect of the *bpsR* mutant is due to an excessive degradation of NA by the activity of genes of the *nic* cluster is correct, then the prevention of NA conversion in the *bpsR* mutant should ameliorate the growth defect. To test this, we deleted the *nicA* gene from the *bpsR* mutant strain, resulting in the Δ*bpsR* Δ*nicA* strain, and compared its growth with that of the Δ*bpsR* and WT strains. As shown in Fig. 4E, the growth of the Δ*bpsR* Δ*nicA* strain was similar to that of the WT strain, further supporting our hypothesis.

Micromolar concentrations of NA induce the expression of *nic* genes. The induction of catabolic genes involved in a specific pathway in the presence of the metabolite is a common theme in bacteria (27). To test the induction of the *nic* genes by NA, the levels of β-galactosidase activity produced by RB50 strains carrying single-copy chromosomally integrated *nicC-lacZ* or *nicE-lacZ* transcriptional fusions were determined. These reporter strains were grown in SS media supplemented with a range of concentrations of NA. Compared to those in regular SS medium, the expressions of both *nicC-lacZ* and *nicE-lacZ* fusions increased by ~3-fold in SS media containing 0.16 and 0.32 mM nicotinic acid. The addition of higher concentrations of nicotinic acid

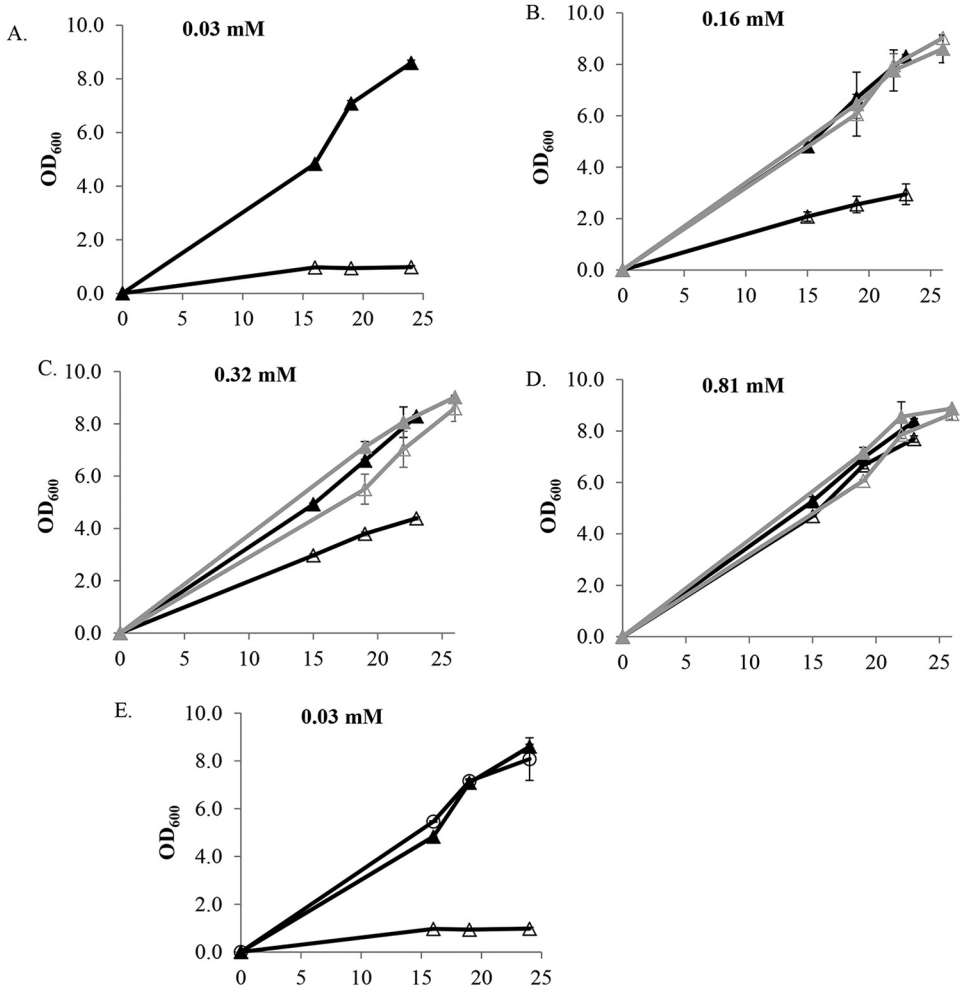

FIG 4 Growth yields of RB50 (▲), Δ*bpsR* (△), and Δ*bpsR* Δ*nicA* (○) strains in SS medium supplemented with NA (black) or nicotinamide (gray). (A and E) SS media containing regular concentrations (0.03 mM) of NA. (B to D). SS media supplemented with indicated (top) concentrations of NA or nicotinamide. *x* axes, time in h. Each data point represents the average from triplicates from one of two or more independent experiments.

(0.81 and 1 mM) resulted in further increases (~4-fold and ~5-fold, respectively) in β-galactosidase activity (Fig. 5A and B).

The concentration of NA in the SS medium is 0.03 mM. Thus, it was not possible to measure if NA in concentrations lower than 0.03 mM could serve as an inducer. Given that nicotinamide can replace NA for growth (Fig. 4), we determined the lowest concentration of NA that is sufficient for the induction of *nic* expression. RB50 derivatives harboring the reporter fusions were grown in SS medium either containing 0.03 mM nicotinamide alone or supplemented with different concentrations of NA. There was a slight but statistically significant induction of both the transcriptional fusions at 1 μM NA and higher levels of induction at 5 and 10 μM (Fig. 5). These results suggest that low micromolar concentrations of NA are sufficient to induce *nic* gene expression.

The induction of the *nic* genes by NA was also measured in the Δ*bpsR* strain. The expressions of both *nicC* and *nicE* were higher in the *bpsR* mutant (Fig. 6A). This result is in agreement with results from microarray and real-time RT-PCR assays (Table S1 and Fig. 3). While the presence of NA in the growth medium of the *bpsR* mutant resulted in a slight increase (1.7-fold) in the expression of *nicC*, there was no significant difference in the expression of *nicE* (Fig. 6B). This result suggests that the induction of *nicC* and *nicE* gene expression by NA is primarily dependent on the presence of an intact *bpsR* gene.

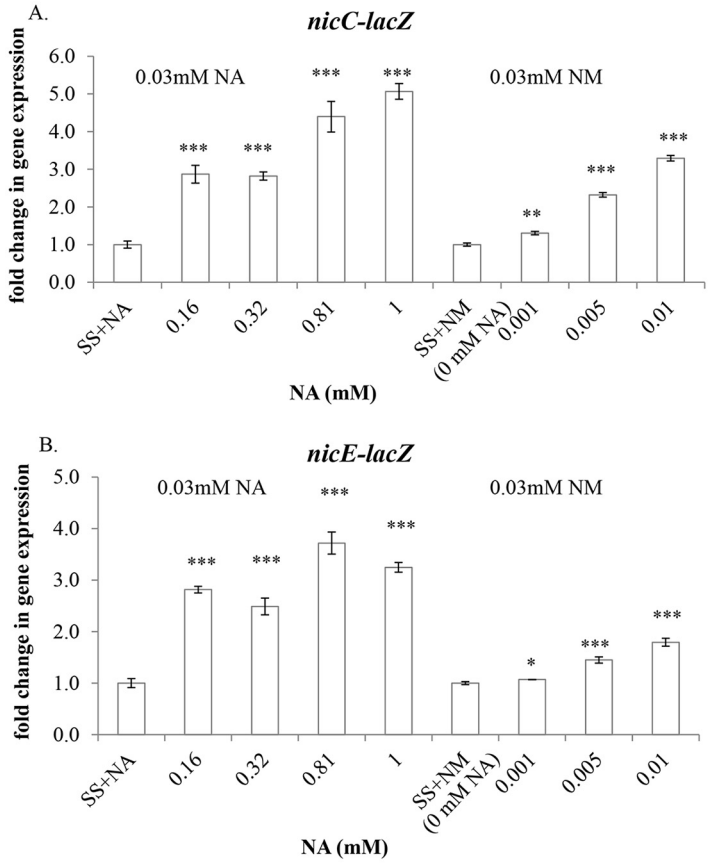

FIG 5 Expression of the *nicE* and *nicC lacZ* fusions integrated in RB50 strain. Fold changes in the β-galactosidase activity were calculated by dividing β-galactosidase units after growth in SS media supplemented with indicated concentrations of nicotinic acid by β-galactosidase activity units after growth in SS medium supplemented with 0.03 mM NA (SS+NA) or nicotinamide (SS+NM). Each data point represents the average of triplicates from one of two or more independent experiments. The error bars indicate the standard deviations. Statistical significance was calculated from unpaired Student's *t* tests. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

BpsR binds to *nicC-nicE* intergenic region and 6-HNA inhibits its DNA binding activity. We have previously shown that BpsR is a DNA binding protein (15). We hypothesized that BpsR represses the transcription of the *nic* genes by directly binding to DNA. Additionally, on the basis of the induction of the activity of the transcriptional fusions by NA (Fig. 5), we hypothesized that its presence will interfere with the DNA binding activity of BpsR. To examine these hypotheses, we performed electrophoretic mobility shift assays (EMSAs) with purified BpsR and a DNA fragment spanning the intergenic region between the divergently transcribed *nicC* and *nicE* genes. As shown in Fig. 7A and B, purified BpsR bound to this fragment. When an EMSA was conducted with NA present in the reaction buffer, there was no observable effect on the DNA binding activity of BpsR (Fig. 7A). Since in the bacterial cell, the catabolism of NA starts with its hydroxylation to 6-HNA (13), we reasoned that 6-HNA is the actual inducer. The inclusion of 0.5 mM 6-HNA in the EMSA reaction buffer resulted in an observable inhibition of the DNA binding activity of BpsR, whereas 1 mM 6-HNA completely prevented the DNA binding (Fig. 7B).

6-HNA is the *in vivo* inducer of *nic* genes. On the basis of the inhibition of the DNA binding activity of BpsR by 6-HNA, we hypothesized that it is the *in vivo* inducer of *nic* gene expression. To test this, the *nicC-lacZ* transcriptional fusion was introduced into the RB50 Δ*nicA* strain (Fig. 8). In this strain, since the *nicA* gene is missing, NA cannot be converted to 6-HNA. Therefore, only 6-HNA should function as the inducer of *nic* genes. In the WT strain, *nicC-lacZ* expression was strongly

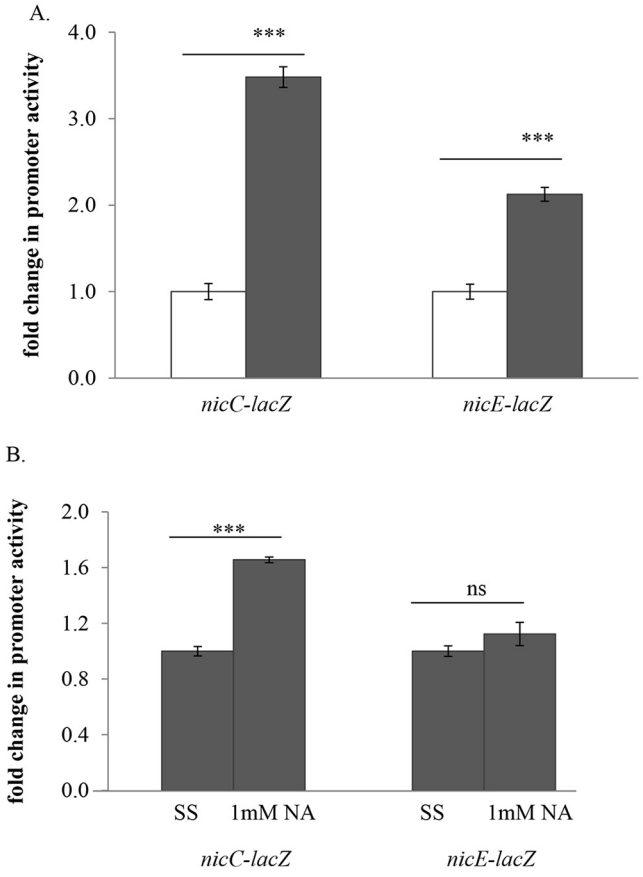

FIG 6 Expression of the *nicE* and *nicC lacZ* fusions integrated in RB50 (open bars) or Δ*bpsR* (closed bars) strains. Fold change was calculated by dividing β-galactosidase units of the Δ*bpsR* strain with that of RB50 after growth in SS media (A) or β-galactosidase units of the Δ*bpsR* strain after growth in SS medium supplemented with 1 mM NA by β-galactosidase activity units after growth in SS medium (B). Each data point represents the average from triplicates from one of two or more independent experiments. The error bars indicate the standard deviations. Statistical significance was calculated from unpaired Student's *t* tests. ***, $P < 0.0005$; ns, not significant.

induced by both NA and 6-HNA. In comparison, in the RB50 Δ*nicA* strain, the expression of *nicC-lacZ* was induced only by 6-HNA. These results suggest that 6-HNA, the product of the first pathway of NA degradation, serves as the *in vivo* inducer of *nic* gene expression.

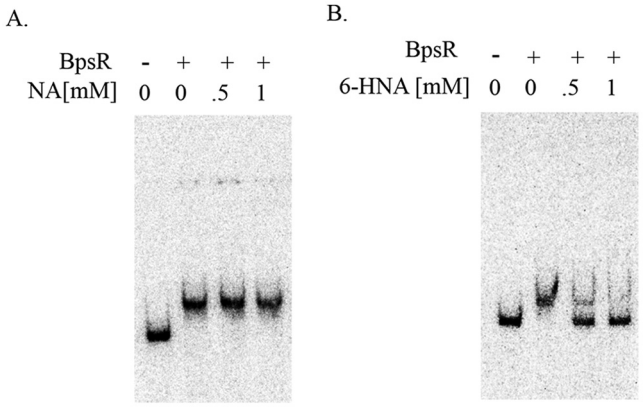

FIG 7 Electrophoretic mobility shift assays. ^{32}P-labeled DNA fragment spanning the region overlapping the *nicC* and *nicE* genes was incubated with 0.4 μg of BpsR (+) and the indicated concentrations of either NA (A) or 6-HNA (B).

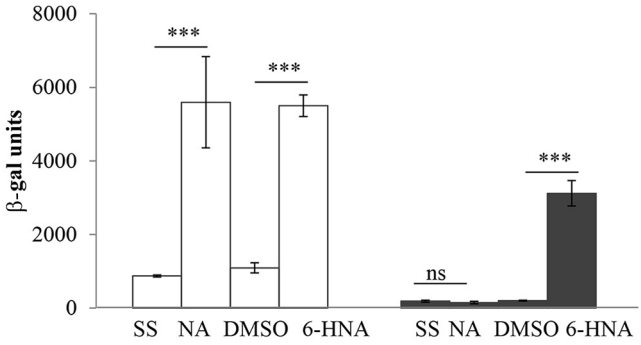

FIG 8 Expression of the *nicC-lacZ* fusions integrated in RB50 (open bars) or Δ*nicA* (closed bars) strains. Strains were grown in SS medium alone (SS) or supplemented with 1 mM NA, 6-HNA, or 0.3% DMSO as a solvent control for 6-HNA. Each data point represents the average from triplicates from one of two or more independent experiments. The error bars indicate the standard deviations. Statistical significance was calculated from unpaired Student's *t* tests. ***, $P < 0.0005$; ns, not significant.

DISCUSSION

Nicotinic acid is essential for the growth of *B. bronchiseptica*, since it serves as a source for NAD synthesis by the salvage pathway. NAD and its reduced and phosphorylated derivatives (NADH, NADP, and NADPH) have important roles in electron transfer and multiple metabolic processes by functioning as hydride acceptors and donors. NA is also a carbon, nitrogen, and energy source for many bacteria, which can degrade this molecule by both anaerobic and aerobic routes (13, 28). *B. bronchiseptica* harbors the genes orthologous to the *nic* cluster of *P. putida*, which is involved in aerobic degradation of NA. The presence of both the salvage and degradation pathways for nicotinic acid suggests that this bacterium has evolved to metabolize NA by one of these two pathways and has the ability to regulate its utilization route. In this report, we show that the transcriptional regulator BpsR represses the expression of the *nicC* and *nicE* genes. This discovery was prompted by the finding that the *bpsR* mutant had a severe growth defect in SS medium. Multiple transcriptional assays showed that the expression of the *nic* genes was elevated in the *bpsR* mutant. The supplementation of the SS medium with NA resulted in the recovery of the growth of the *bpsR* mutant, and the deletion of the *nicA* gene from this mutant did not result in a growth defect. Thus, the poor growth of the *bpsR* mutant in regular SS medium containing low levels of NA is likely due to the enhanced degradation of NA as a result of the higher expression and activity of the *nic* degradation pathway. The supplementation of the growth medium with NA also induced the expression of *nic* genes in the WT strain. Thus, NA serves both as a substrate and as an inducer of the *nic* pathway.

The induction of the *nic* operon by NA required BpsR, since the induction of the *nicE-lacZ* fusion by NA was lost and only a slight induction of the *nicC-lacZ* fusion occurred in the Δ*bpsR* strain. Purified BpsR bound to the intervening region of DNA between *nicC* and *nicE* genes, suggesting that it represses the transcription of the *nic* cluster by binding to the promoter regions. The DNA binding activity of BpsR was prevented by 6-HNA and not by NA. Consistent with this result, in the WT strain harboring a deletion of the *nicA* gene, only 6-HNA but not NA induced the expression of the *nicC-lacZ* fusion. In the bacterial cell, the catabolism of NA begins with its hydroxylation to 6-HNA by a nicotinic acid hydroxylase encoded by the *nicA* and *nicB* genes (13). Thus, we suggest that 6-HNA is the actual *in vivo* inducer of the *nic* operon. We propose a regulatory model of the *Bordetella nic* cluster (Fig. 9). In this model, in the absence of NA, BpsR functions as a repressor of the *Bordetella nic* genes by binding to the *nicE* and *nicC* intergenic region. 6-HNA, after conversion inside the bacterial cell from NA, interacts with BpsR. This interaction then prevents the binding of BpsR to the DNA and alleviates the repression, resulting in the induction of expression of the *nic* cluster.

Results very similar to ours were recently reported by Brickman and Armstrong (29).

nicC

CTGGGTTTGCCTTGCACGGTGTTTCTCCTGGAGTGAGGCGTGGAGCGGACCTGCGGTGCTCATGAAAATA
GACCCAAACGGAAC**GTG**CCACAAAGAGGACCTCACTCCGCACCTCGCCTGGACGCCACGAGTACTTTTA

AAGTGTACACACTCAAAAATGGCAAGTAAACAGGGAATCGCCAGGCGGTTCGTTCGAGGCTGTCCGGTAT
TTTCACATGTGTGAGTTTTTACCGTTCATTTGTCCCTTAGCGGTCCGCCAAGCAAGCTCCGACAGGCCATA
-10 -35

GGCGATGGTTTGCAGCAAGCTGTTGTCTATATGGAACTTTTATGCGGGTTTTCCCTTAGACGGATGATCC
CCGCTACCAAACGTCGTTCGACAACAGATATACCTTGAAAATACGCCCAAAAGGGAATCTGCCTACTAGG

-35 -10
GGAAAGGCGTGATCCGATCTATTGCCATGCCGTGGCATAGCTGTGAAAATAAAGCGTACACATCTTATTA
CCTTTCCGCACTAGGCTAGATAACGGTACGGCACCGTATCGACACTTTTATTTCGCATGTGTAGAATAAT

AGGGCGTCTTTCGGCGTCCGGTTACCGGGAGCAGGGC**GTG**CAGAAGACCTACC
TCCCGCAGAAAGCCGCAGGCCAATGGCCCTCGTCCCGCACGTCTTCTGGATGG

nicE

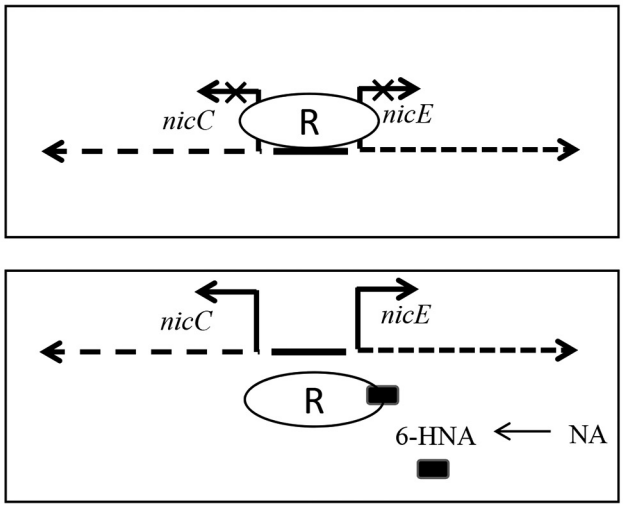

FIG 9 Model for repression of the *Bordetella nic* cluster by BpsR. The DNA sequence of the intergenic region between *nicC* and *nicE* genes along with the putative −10 and −35 promoter elements and translational codons (underlined and bolded) are shown. (Top rectangle) When NA is absent or its levels are low, BpsR (designated by oval) binds to the intergenic region of *nicC* and *nicE* and prevents transcription. (Bottom rectangle) When NA is present at sufficient levels, it enters the cell and is converted to 6-HNA, which then interacts with BpsR. BpsR cannot bind DNA in the presence of 6-HNA, and transcription of the genes of the *nic* cluster occurs.

However, these authors did not report a restoration of the growth of the *bpsR* mutant due to the deletion of the *nicA* gene, the binding of BpsR to the *nic* promoter, and the effect of NA and 6-HNA on this binding.

It was previously shown that the presence of NA in the medium markedly increases *nadC* gene (a quinolinate phosphoribosyl transferase) expression and that this induction is under the control of the NadQ regulator (24), but the regulation of *nadC* by BpsR has not been reported. In addition to the genes of the *nic* cluster, we found that the expression of *nadC* also increased in the Δ*bpsR* strain, suggesting that BpsR represses *nadC* expression. It needs to be determined if the NA-mediated induction of *nadC* is also dependent on *bpsR*. Additionally, the results of our study and those reported previously by Brickman et al. (24) raise the possibility of an overlap and cross-regulation of *Bordetella* genes by NadQ and BpsR and require further study.

In light of the absolute requirement of NA for growth, it is reasonable to ask why *B. bronchiseptica* would maintain the expression of genes involved in its degradation and evolve a regulatory mechanism to control such a process. *B. bronchiseptica* can survive for extended periods in the environment. RB50, the strain of *B. bronchiseptica* used here, can grow efficiently on soil extracts (30). We propose that because of the high levels of NA available in the soil, the BpsR-mediated repression of the *nic* cluster is relieved and the bacterium can then utilize NA as a nutrient source. This then leads to its transient survival in soil of animal farms until it is able to infect its relevant hosts.

Since *B. bronchiseptica* can infect and cause disease in both animals and humans, what is the physiological relevance of the observed regulation of *nic* genes in the mammalian hosts? It is reasonable to postulate a tight control over the catabolism of NA because of its utilization for NAD synthesis. We propose that by the BpsR-mediated negative regulation of the *nic* cluster, *B. bronchiseptica* prevents unwarranted depletion of NA, thereby ensuring the synthesis of NAD and optimal growth. While the concentration of NA in the mammalian respiratory tract is unknown, its concentration in the whole blood of various animal species (dog, pig, sheep, and horse) and humans is 40 to 70 μM (31, 32). *B. bronchiseptica* can utilize NA from the plasma transduced into the respiratory epithelium. We show that the induction of *nic* gene expression by NA occurs at concentrations lower than those present in animals and humans. Thus, it is likely that *nic* genes are expressed in host organs. Consequently, the degradation of NA by the activity of the *nic* cluster may pose a survival problem, since sufficient NA may not be available for NAD synthesis. The repression of the *nadC* gene by BpsR and its subsequent induction by NA may provide *Bordetella* with an alternative mechanism to produce NAD, if and when the *nic* cluster is induced in the presence of NA. While the concentration of quinolinic acid in human blood is relatively low, stimulation of the immune system is associated with increased concentrations of quinolinic acid in various organs, including the lungs (33). Additionally, activated macrophages increase the production of quinolinic acid (34). We propose that by exerting control over both biosynthetic and degradative processes involved in NAD synthesis, *B. bronchiseptica* controls the utilization of pyridines for ensuring optimal growth. In comparison to that in *B. bronchiseptica* strain RB50, many of the *nic* orthologs in *B. pertussis* strain Tohama I and *B. parapertussis* strain 12822 share a very high sequence identity ($\leq$98%), but some of the *nic* genes are pseudogenes (Fig. 1). Determining if the *nic* pathway is functional in *B. pertussis* and *B. parapertussis* will reveal if it is required for their growth.

The presence of millimolar concentrations of NA in *Bordetella* growth medium results in phenotypic modulation, which is characterized by the loss of many of the virulence traits and requires the BvgAS signal transduction system (8–11). When modulated, *Bordetella* is in the Bvg$^-$ phenotypic phase, and in this phase, the expression of the majority of the virulence factors is inhibited, resulting in an attenuation of bacterial virulence. It has been shown that *Bordetella* can exist in the Bvg$^-$ phase *in vivo*, and a Bvg$^-$ phase gene, *brtA*, is expressed in the rat trachea (35–37). Although as stated above, plasma concentrations of NA in animals and humans are only in the micromolar range, given that NA concentrations in the respiratory tract are not known, one cannot exclude the possibility that the local concentration of NA at this site is in the millimolar range and results in the NA-mediated modulation of gene expression and the switching of *Bordetella* to the Bvg$^-$ phase.

In addition to the genes involved in NAD synthesis and NA degradation, the gene expression analyses reported herein demonstrate that BpsR can activate and repress a number of other genes. These *bpsR*-regulated genes are predicted to encode proteins serving a diversity of cellular functions. Some of these proteins are periplasmic/membrane/exported proteins and others have function in pathogenesis, transcriptional regulation, cellular metabolism, the binding and transport of substrates, and electron transport. These results suggest that in addition to the previously identified role in regulating polysaccharide production and biofilm formation (15), BpsR controls the expression of genes critical for the growth and pathogenesis of *B. bronchiseptica*.

TABLE 1 Bacterial strains and plasmids used in the study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Plasmids		
pEGZ	Suicide plasmid carrying the promoterless *lacZ* gene; Ap^r Gm^r	10
pMF2	−344 to +49 region of the *nicE* promoter cloned into the EcoRI and BamHI sites of pEGZ	This study
pMF3	−328 to +44 region of the *nicC* promoter cloned into the EcoRI and BamHI sites of pEGZ	This study
pRK2013	Helper plasmid containing RK2 transfer gene linked to ColE1 replicon, Km^r	53
Strains		
DH5α	*E. coli* strain, high-efficiency transformation	Laboratory strain
RB50	Wild-type strain of *B. bronchiseptica*	10
RB50 Δ*bpsR*	RB50 derivative containing an in-frame deletion in the *bpsR* gene	15
RB50 Δ*nicA*	RB50 derivative containing an in-frame deletion in the *nicA* gene	This study
RB50 Δ*bpsR* Δ*nicA*	RB50 derivative containing an in-frame deletion in the *bpsR* and *nicA* genes	This study
RB50$^{nicC\text{-}lacZ}$	RB50 derivative containing pMF3	This study
Δ*bpsR*$^{nicC\text{-}lacZ}$ strain	RB50 Δ*bpsR* derivative containing pMF3	This study
RB50$^{nicE\text{-}lacZ}$	RB50 derivative containing pMF2	This study
Δ*bpsR*$^{nicE\text{-}lacZ}$ strain	RB50 Δ*bpsR* derivative containing pMF2	This study
RB50 Δ*nicA*$^{nicC\text{-}lacZ}$	RB50 Δ*nicA* derivative containing pMF3	This study
RB50 Δ*bpsR* Δ*nicA*$^{nicC\text{-}lacZ}$	RB50 Δ*bpsR* Δ*nicA* derivative containing pMF3	This study

[a]Km^r, kanamycin resistance; Ap^r, ampicillin resistance; Gm^r, gentamicin resistance.

Continued studies will expand our understanding of the molecular mechanisms by which BpsR mediates the control of diverse cellular processes in *Bordetella*.

On the basis of the induction of the *nic* genes by NA, it can be hypothesized that it will also regulate the expression of other *bpsR*-regulated genes and phenotypes. We previously showed that BpsR represses the expression of the Bps polysaccharide and biofilm formation in *B. bronchiseptica* (15). Bps is critical for biofilm formation and respiratory tract colonization by both *B. bronchiseptica* and *B. pertussis* (38–40). Cummings et al. previously reported that high concentrations of NA led to only a moderate induction in the expression of *bpsA*, the first gene of the *bpsA-D* locus (9). Millimolar concentrations of NA can also result in increased biofilm formation in *B. bronchiseptica* (41, 42). Experiments are under way to determine if NA can regulate Bps polysaccharide levels and influence BpsR-dependent regulation of biofilm formation and the impact of the degradation of NA by the *nic* cluster in these processes.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. For growth on solid media, Bordet-Gengou ([BG] Becton Dickinson Microbiology Systems) agar supplemented with 7.5% defibrinated sheep blood and LB agar were used for *Bordetella* and *Escherichia coli*, respectively. For growth in liquid media, *Bordetella* and *E. coli* strains were grown in Stainer-Scholte (SS) (5) and LB media, respectively. Media were supplemented with the following antibiotics when necessary: ampicillin (100 μg/ml), kanamycin (25 μg/ml), gentamicin sulfate (25 μg/ml), and streptomycin (100 μg/ml). Nicotinic acid and 6-hydroxynicotinic acid solutions were prepared in water and dimethyl sulfoxide (DMSO), respectively. The strains and plasmids used in this study are listed in Table 1. A list of the primers used in this study is included in Table 2. NA and nicotinamide at the indicated concentrations were added to the medium when necessary.

Bacterial growth. *Bordetella* strains were grown for 12 to 14 h in either LB or SS medium at 37°C at 90 rpm in a roller drum. Overnight cultures were diluted in fresh medium to yield an OD_{600} of 0.005. The OD_{600} was measured at the indicated time points. For medium changes and growth in spent medium, cells of RB50 and Δ*bpsR* strains were collected by centrifugation (8,000 rpm for 3 min) at 4°C. For growth in spent medium, the supernatant was collected after centrifugation, pH adjusted to 7.5, and filtered through a 0.22-μm syringe filter. For the replenishment of supplements containing essential components, three volumes of adjusted spent medium was mixed with one volume of 4× fresh medium containing the supplement. This medium was designated the reconstituted spent medium. For medium changes, the cells were resuspended in the original volume of fresh SS medium and grown for the indicated times.

Deletion of *nicA* gene. An in-frame deletion of the *nicA* gene (BB1777) was constructed using a previously published allelic exchange method (43). A 503-bp KpnI-HindIII fragment including the 5′ region and the first 7 codons of *nicA* was amplified from the RB50 chromosome using primers MGnicAdnF1Hind and MGnicAdnR1Kpn (Table 2). A 589-bp XbaI-HindIII region corresponding to the 3′ region and the last 27 codons of *nicA* was amplified using MGnicAupF2Xba and MGnicAupR2Hind (Table 2). These fragments were then ligated into the vector pRE112 to yield the *nicA* deletion vector pMG26. The resulting plasmid was mobilized from pRK2013 into the RB50 strain and the Δ*bpsR* strain, and

TABLE 2 List of primers

Primer	Sequence
MGRBNicCprom5′	5′-GCCGAATTCGTATTCGAACTCGGCACGA
MGRBNicCprom3′	5′-CGCGGATCCGAGACCGGCGCCGATGACA
MGRBNicEprom5′	5′-CGCGGATCCGTATTCGAACTCGGCACGA
MGRBNicEprom3′	5′-CCGGAATTCGAGACCGGCGCCGATGACA
MGnicAupF2Xba	TGCTCTAGAGGTCCAGCAAGGCGGCCAGTTCG
MGnicAupR2Hind	CCCAAGCTTACCCATATCGAAATCATG
MGnicAdnF1Hind	CCCAAGCTTCGCGAGTGGCTGCAAGCAT
MGnicAdnR1Kpn	CGGGGTACCAGGCGCTCATCGAGGCCACG
BB1778-79F	5′-CCGGAATTCGAGAGGCGCCGATGACA
BB1778-79R	5′-CGCGGATCCCGTAGAACTCGGCACGA
rpoD-FwRT	ATGGGCATCCGCTTCACG
rpoD-RvRT	CTTCGTCCAACACCCAC
bb4286-FwRT	TCGGCCACATGGTCAAGATC
bb4286-RvRT	GTCCATGTTGTCCAGCAGG
bb1781-FwRT	CTGACGGTGGAAAACGGCTA
bb1781-RvRT	CCTGCGGGTCCTTGAACG
bb1772-FwRT	CTGAAGCTGACCATCCCGTT
bb1772-RvRT	AAATCGACCATCACGCCCTT
bb1769-FwRT	CGCTGCTGACCATGGATTT
bb1769-RvRT	CTGGTGTACAGCATGGTGTTGA

exconjugants were selected on BG agar plates containing streptomycin and chloramphenicol. The loss of the plasmid following a second recombination event was confirmed by sucrose counter selection, and the sensitivity to chloramphenicol was determined as described previously (39, 43, 44). The genotype of the Δ*nicA* deletion strain was confirmed by PCR.

***lacZ* transcriptional fusions and β-galactosidase assays.** To construct *nicC-lacZ* and *nicE-lacZ* transcriptional fusions, DNA fragments encompassing 328 bp upstream and 44 bp downstream of the annotated translational start site of *nicC* and 344 bp upstream and 49 bp downstream of annotated translational start site of *nicE* were amplified from the RB50 strain using primers MGRBNicCprom5′ and MGRBNicCprom3′ (*nicC*) and MGRBNicEprom5′ and MGRBNicEprom3′ (*nicE*), respectively. The PCR fragments were digested with EcoRI and BamHI and cloned into the suicide *lacZ* reporter plasmid pEGZ (44), resulting in pMF2 (*nicE-lacZ*) and pMF3 (*nicC-lacZ*). This scheme results in the cloning of the respective promoter fragments upstream of the promoterless *lacZ* gene. The transcriptional fusion constructs were then integrated into the genomes of RB50 and Δ*bpsR* strains by single crossovers at the *nicC* and *nicE* loci, as described previously (10, 45), resulting in the strains RB50$^{nicC\text{-}lacZ}$, RB50$^{nicE\text{-}lacZ}$, Δ*bpsR*$^{nicC\text{-}lacZ}$, and Δ*bpsR*$^{niE\text{-}lacZ}$. β-Galactosidase assays were performed as previously described (10, 45). Two-tailed Student's *t* tests were used for testing the statistical significance of the data.

RNA extraction. RNA for real-time RT-PCR was prepared from log-phase (A_{600} of ~1.0) cultures using a Qiagen RNeasy kit according to the manufacturer's instructions. RNA for microarray analysis was isolated from log-phase cultures using a TRIzol (Life Technologies) extraction according to the manufacturer's instructions.

Real-time RT-PCR. RNA for real-time RT-PCR was prepared from log-phase (A_{600} of ~1.0) cultures using a Qiagen RNeasy kit following manufacturer's instructions. Purified RNA was treated with RQ1 DNase I (Promega), and cDNA was synthesized with random hexamers and SuperScript III reverse transcriptase (Invitrogen). Real-time PCR was then performed using a SYBR green system (Applied Biosystems). Differential gene expression was analyzed by means of the Pfaffl method (46) using REST2009 software (Qiagen). The *rpoD* gene was included as a housekeeping gene for normalization. Real-time PCR analysis was performed with two biological and three technical replicates.

Preparation of labeled cDNA and microarray analysis. RNA isolated from strains RB50 and the Δ*bpsR* mutant was used in microarray experiments. A 2-color hybridization format was used for microarray analysis. For each biological replicate, RNA extracted from the RB50 strain was used to generate Cy5-labeled cDNA, and RNA extracted from the Δ*bpsR* mutant was used to generate Cy3-labeled cDNA. Additionally, dye-swap experiments were performed analogously, in which the fluorescent labels were exchanged to ensure that an uneven incorporation did not confound our results. Fluorescently labeled cDNA copies of the total RNA pool were prepared by direct incorporation of fluorescent nucleotide analogs during a first-strand reverse transcription reaction, followed by buffer exchange, purification, and concentration as described previously (47, 48). The two differentially labeled reactions to be compared were combined and hybridized to a *B. bronchiseptica* strain RB50-specific long-oligonucleotide microarray (47, 48). The slides were then scanned using a GenePix 4000B microarray scanner and analyzed with GenePix Pro software (Axon Instruments, Union City, CA). Spots were assessed visually to identify those of low quality, and arrays were normalized so that the median of ratios across each array was 1.0. Spots of low quality were identified and filtered out prior to analysis. Ratio data from the biological replicates were compiled and normalized on the basis of the total Cy3% intensity and Cy5% intensity to eliminate slide-to-slide variability. Gene expression data were then normalized to 16S rRNA. The statistical significance of the gene expression changes was assessed using the significant analysis of microarrays (SAM) program (49). A one-class unpaired SAM analysis using a false discovery rate of 0.04% was performed.

BpsR protein expression and purification. The gene fragment encoding the BpsR protein was inserted into a modified pET19 expression vector (Novagen) which encodes an N-terminal polyhistidine tag followed by a rhinovirus 3C protease cleavage site, which permits the removal of the affinity tag (PreScission protease; GE Healthcare). The pET19-*bpsR* vector was transformed into *E. coli* C41(DE3) cells (50) for expression. One liter of LB broth supplemented with 50 μg/ml of ampicillin was inoculated with 10 ml of an overnight culture of the C41 cells containing the pET19-*bpsR* vector. The cells were grown at 37°C to an OD_{600} of 0.5 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 16°C for 20 h. Prior to the induction with IPTG, the cells were rapidly cooled on ice to 20°C to bring the temperature of the culture close to the induction temperature. The induction of the cells at low temperature was necessary for protein solubility during overexpression. Overexpressed BpsR was purified from cells by a combination of nickel affinity and ion-exchange chromatography (51). After applying the protein to the Ni-nitrilotriacetic acid (Ni-NTA) column, BpsR was treated with PreScission protease according to the manufacturer's directions to remove the His tag. The purity of the peak fractions after each step was verified by SDS-PAGE. Purified BpsR was dialyzed against a buffer containing 50 mM Bis-Tris pH 6.5, 150 mM NaCl, and 5% glycerol.

Electrophoretic mobility shift assays. A 390-bp fragment spanning −328 and +43 relative to the translational start site of the *nicC* open reading frame (ORF) was generated by PCR using the primers BB1778-79F and BB1778-79R. End-labeling of the PCR products and EMSAs were conducted as previously described (15, 51, 52). Briefly, a [γ-^{32}P]ATP-labeled fragment was incubated with 0.4 μg of purified BpsR protein alone or in the presence of indicated concentrations of NA or 6-HNA at 37°C for 15 min. The samples were then electrophoresed in 4% polyacrylamide gels and visualized by autoradiography.

Data availability. All microarray data are available in Table S1 in the supplemental material and have been deposited in ArrayExpress under accession number E-MTAB-2881.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00712-17.

SUPPLEMENTAL FILE 1, XLSX file, 0.4 MB.

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