Signal transduction-dependent small regulatory RNA is involved in glutamate metabolism of the human pathogen *Bordetella pertussis*

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ABSTRACT

***Bordetella pertussis* is the causative agent of human whooping cough, a highly contagious respiratory disease which despite vaccination programs remains the major cause of infant morbidity and mortality. The requirement of the RNA chaperone Hfq for virulence of *B. pertussis* suggested that Hfq-dependent small regulatory RNAs are involved in the modulation of gene expression. High-throughput RNA sequencing revealed hundreds of putative noncoding RNAs including the RgtA sRNA. Abundance of RgtA is strongly decreased in the absence of the Hfq protein and its expression is modulated by the activities of the two-component regulatory system BvgAS and another response regulator RisA. Whereas RgtA levels were elevated under modulatory conditions or in the absence of *bvg* genes, deletion of the *risA* gene completely abolished RgtA expression. Profiling of the Δ*rgtA* mutant in the Δ*bvgA* genetic background identified the *BP3831* gene encoding a periplasmic amino acid-binding protein of an ABC transporter as a possible target gene. The results of site-directed mutagenesis and in silico analysis indicate that RgtA base-pairs with the region upstream of the start codon of the *BP3831* mRNA and thereby weakens the BP3831 protein production. Furthermore, our data suggest that the function of the BP3831 protein is related to transport of glutamate, an important metabolite in the *B. pertussis* physiology. We propose that the BvgAS/RisA interplay regulates the expression of RgtA which upon infection, when glutamate might be scarce, attenuates translation of the glutamate transporter and thereby assists in adaptation of the pathogen to other sources of energy.**

Keywords: sRNA; signal transduction; *Bordetella*; translational repression; riboregulation

INTRODUCTION

In the last two decades, small noncoding regulatory RNAs (sRNAs) have become recognized as very important modulators of gene expression in bacteria (Gottesman et al. 2006; Wagner and Romby 2015). Importantly, sRNAs are predominantly expressed under various stress conditions and help bacteria to adapt to changing environmental conditions (Hoe et al. 2013; Holmqvist and Wagner 2017). The most studied and best-characterized class of sRNAs acts at the post-transcriptional level by base-pairing with the target mRNAs and thereby modulating their stability and translational efficiency (Storz et al. 2004; Desnoyers et al. 2013). These activities may have either a positive or negative effect on target mRNA translation. Based on their chromosomal location, sRNAs are divided into two classes: *cis*- and *trans*-encoded. The *cis*-encoded sRNAs are transcribed antisense to their targets and therefore share perfect complementarity with the regulated mRNAs. On the other side, *trans*-encoded sRNAs are encoded at different genomic loci as their target genes and share only limited complementarity with target transcripts.

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Of note, the majority of RNA transactions promoted by *trans*-encoded sRNAs result in repression of the protein synthesis as the base-pairing between sRNA and target mRNA leads to inhibition of translation and subsequent RNA degradation (Wagner 2009). In Gram-negative bacteria, the regulation based on imperfect sRNA/mRNA duplexes often requires activity of the RNA chaperones such as Hfq or the recently identified ProQ protein (Smirnov et al. 2017). The Hfq protein forms a ring-shaped hexamer (Moller et al. 2002), which possesses several RNA-binding sites allowing simultaneous interaction with both sRNA and mRNA and stabilization of their interaction (Vogel and Luisi 2011; Updegrove et al. 2016). Besides its role in the stabilization of the RNA duplexes, Hfq can also actively remodel the structure of RNAs and increase or decrease the stability of interacting RNAs (Brennan and Link 2007; Vogel and Luisi 2011). Importantly, Hfq-associated sRNAs were proven to be involved in a wide variety of cellular processes, e.g., iron homeostasis, membrane remodeling, energy metabolism, and quorum sensing (Guillier et al. 2006; Papenfort and Vogel 2010; Bobrovskyy et al. 2015; Wagner and Romby 2015; Carrier et al. 2017). Given that a pathogenic lifestyle often requires a quick response to sudden changes in environmental conditions, it is not surprising that a large number of sRNAs produced by bacterial pathogens are involved in stress adaption and virulence (Wassarman 2002; Papenfort and Vogel 2010). Accordingly, Hfq seems to be important for the physiological fitness and virulence in several bacterial pathogens, including *Bordetella pertussis* (Sonnleitner et al. 2003; Ding et al. 2004; Sittka et al. 2007; Geng et al. 2009; Bibova et al. 2013).

Bordetella pertussis is a Gram-negative strictly human pathogen of the respiratory tract and the etiological agent of whooping cough (pertussis) (Mattoo and Cherry 2005). Despite vaccination programs, pertussis remains one of the 10 most common causes of vaccine preventable deaths (WHO 2006). Furthermore, pertussis incidence is currently on the rise in industrialized countries with highly vaccinated populations (Raguckas et al. 2007; Cherry 2010). While there are several reasons for this phenomenon, two major factors are contributing to the recent increase in pertussis cases: short-lived immunity induced by current acellular vaccines and pathogen adaptation leading to escape from immunity by antigenic variation (Mooi et al. 2014; Burdin et al. 2017). The reemergence of pertussis strongly suggests that a better understanding of the molecular mechanisms underlying the pathogenesis of *B. pertussis* is necessary to tackle the disease.

In order to colonize and damage the epithelial cells of the respiratory tract, *B. pertussis* expresses a complex set of virulence factors, including adhesins and toxins (Locht 1999). The expression of most of the virulence factors is controlled by the BvgAS two-component system, consisting of sensor kinase BvgS and response regulator BvgA, which, upon phosphorylation by BvgS, facilitates expression of the virulence-activated genes, *vags* (Cotter and Jones 2003). In principle, based on the BvgA phosphorylation status, three different phenotypes can be distinguished: Bvg^+ (virulent), Bvg^- (avirulent), and Bvg^i (intermediate). While the environmental signals sensed by BvgS kinase are not known, the BvgAS system can be rendered inactive by a process called phenotypic or antigenic modulation when *B. pertussis* cells are grown in the presence of magnesium sulfate or nicotinic acid (Lacey 1960; Melton and Weiss 1989). In contrast to the closely related *B. bronchiseptica*, the BvgAS system remains active at temperatures below 25°C in *B. pertussis* (Seydlova et al. 2017). The majority of factors expressed in the avirulent Bvg^- state (virulence repressed genes, *vrgs*) is regulated by two factors, BvgR and another response regulator called RisA. While the Bvg-activated *bvgR* gene, lying downstream from the *bvgS* gene, is required for repression of *vrg* genes in the Bvg^+ phase (Merkel and Stibitz 1995; Merkel et al. 1998), the RisA regulator is essential for expression of the *vrg* genes in the Bvg^- mode (Jungnitz et al. 1998; Croinin et al. 2005; Stenson et al. 2005; Coutte et al. 2016). The exact mechanism of the BvgR–RisA interplay was not completely understood; however, recently it was suggested that BvgR is not a typical repressor interacting with promoters of repressed genes but represents a di-guanylate phosphodiesterase degrading the second messenger c-di-GMP to GMP (Chen et al. 2017). It is presumed that in the Bvg^- mode, when BvgR is not produced, the c-di-GMP accumulates and binds to response regulator RisA phosphorylated by its cognate kinase RisK. Phosphorylated RisA in complex with c-di-GMP binds to *vrg* promoters and increases their activity (Coutte et al. 2016; Chen et al. 2017).

We have shown that the Hfq protein significantly affects the expression of more than 10% of the *B. pertussis* genes (Bibova et al. 2015) and that the Hfq protein is required for *B. pertussis* virulence (Bibova et al. 2013). These observations suggested that sRNAs could play an important role in the physiological fitness of this pathogen. So far, several sRNAs have been identified in *B. pertussis* (Hot et al. 2011); however, their function and possible targets remained unknown. The extensive impact of the *hfq* gene deletion on transcriptomic profiles in *B. pertussis* motivated us to search for regulatory RNAs on a genome-wide scale. A recently determined primary transcriptome of *B. pertussis* (Amman et al. 2018) revealed a large variety of noncoding RNAs, including sRNAs. In this study, we characterize one of the identified sRNAs, which, based on its possible function, was designated RgtA (repressor of glutamate transport). We determined its chromosomal location, transcriptional start site, and size. We show that RgtA synthesis is substantially induced under modulating conditions and that its expression requires the activity of RisA transcriptional regulator. Furthermore, we present a possible target

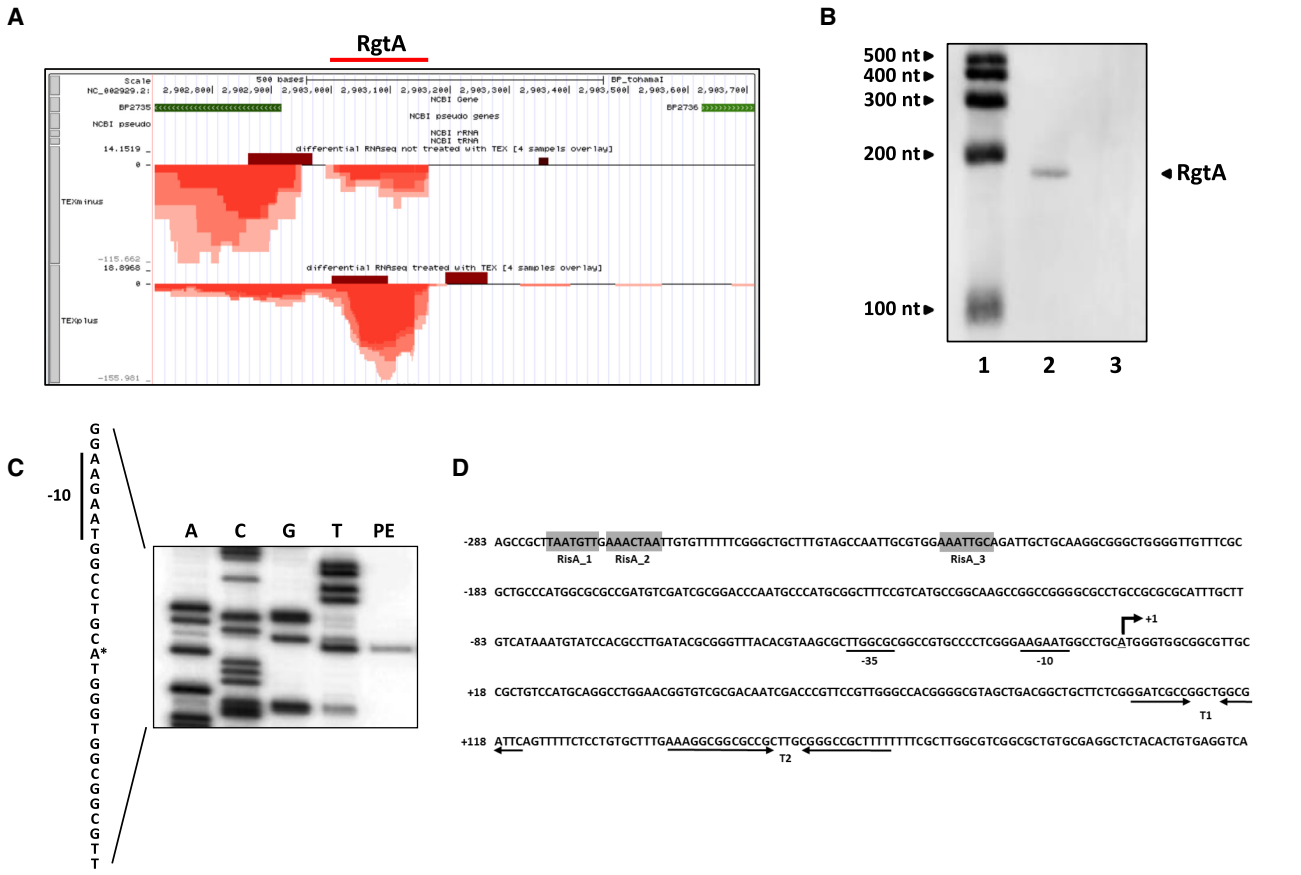

FIGURE 1. Identification and characterization of RgtA sRNA in *B. pertussis*. (*A*) Visualization of the dRNA-seq normalized data set (±treatment with terminal exonuclease) showing the region between the *BP2735* and *BP2736* genes. Graphs display sequencing depth of the positive (dark red) and negative (light red) strands. (The different color intensities depict the different library replicates.) Gene annotations are depicted as green arrows. Red bar *above* the picture denotes the genomic position of RgtA RNA. (*B*) Detection of RgtA by northern blot analysis. Total RNA isolated from *B. pertussis* Tohama I (lane *2*) and Δ*rgtA* (lane *3*) strains was separated on 8% PAA-8M urea gel, transferred to membrane, and probed with biotinylated oligo specific to RgtA. Biotinylated Century-Plus RNA ladder was loaded as a molecular size marker (lane *1*). (*C*) Determination of the transcriptional start site of RgtA by primer extension analysis. The product of the primer extension reaction (PE, lane *5*) was separated together with sequencing reactions performed with the primer used for primer extension (lanes *1*–*4*) on a 6% polyacrylamide–8 M urea gel. Only the relevant part of the autoradiograph is presented. The sequence of the coding strand is shown on the *left* with the transcriptional start site (asterisk) and the plausible −10 sequence (*vertical* line). (*D*) Nucleotide sequence map of the *rgtA* locus. The transcriptional start site identified by primer extension and dRNA-seq analysis (rectangular arrow, +1), plausible −35 and −10 promoter sequences (underlined), putative RisA binding sites (gray boxes), and two possible terminators (convergent arrows) are depicted.

of RgtA RNA, the mRNA encoding the periplasmic amino acid-binding protein BP3831.

RESULTS

Identification and characterization of the RgtA sRNA

Recently, we applied high-throughput sequencing approaches (RNA-seq and differential RNA-seq) to decipher the transcriptional landscape of *B. pertussis* (Amman et al. 2018). One of the sRNAs discovered by dRNA-seq analysis was identified as an unknown transcript located within the intergenic region between BP2736 and BP2735 genes and originating from the negative strand (Fig. 1A). To validate the RNA-seq data, we assayed the synthesis of RgtA in the wt and Δ*rgtA* strains by northern blot analysis using a probe specific to the *rgtA* locus. The result of the northern blot analysis was in agreement with our transcriptomic data since we detected an approximately 180 nt long transcript that seemed to be specific as the RNA isolated from Δ*rgtA* mutant did not hybridize with the probe (Fig. 1B). Next, we determined the transcriptional start site of the RgtA RNA by primer extension analysis as an A residue at position 2,903,162 within the *B. pertussis* genome (Parkhill et al. 2003), again fully in agreement with the results of the dRNA-seq analysis (Fig. 1C). Upstream of the transcriptional start site we identified plausible –35 (TTGGCG) and –10 (AAGAAT) promoter sequences optimally spaced by 17 bp (Fig. 1D). Two possible terminator structures were predicted at the 3′ end of *rgtA*, and using

northern blot analysis we determined that both structures are present in the RgtA transcript (data not shown). This observation allowed us to conclude that the size of RgtA is 174 nt. Nucleotide sequence alignment revealed that the *rgtA* locus is well-conserved (98% identity) in closely related species *B. bronchiseptica* and *B. parapertussis* (Supplemental Fig. S1A). In support, northern blot analysis proved that both related species produce RgtA homologs (Supplemental Fig. S1B).

Expression of RgtA is repressed by the BvgAS two-component system

Most of the regulatory small RNAs are induced in response to stress stimuli and often require Hfq for their stability and function. Interestingly, northern blot analysis revealed that when compared to wt strain, the abundance of RgtA is strongly reduced in the Δ*hfq* strain, indicating that Hfq protein may contribute to stability of this sRNA (Fig. 2A). Next, we used several stress conditions, including cold and heat shock, oxidative, and membrane stresses, but we did not observe any significant changes in RgtA expression under tested conditions (data not shown). Interestingly, cells grown under modulatory conditions known to induce avirulent Bvg$^-$ phenotype (50 mM magnesium sulfate or 20 mM nicotinic acid) produced increased amounts of RgtA sRNA (Fig. 2B). These results suggested that *rgtA* belongs to virulence repressed genes and its expression is up-regulated in the absence of a functional *bvg* system. Of note, growth at low temperature did not affect RgtA production (Fig. 2B), which is in line with our recent observation that in contrast to *B. bronchiseptica*, the *bvg* system of *B. pertussis* is active at temperatures below 25°C (Seydlova et al. 2017). To corroborate our observations, we decided to genetically inactivate the *bvg* system by construction of Δ*bvgA*, Δ*bvgS*, and Δ*bvgR* strains and to determine the RgtA synthesis in these mutants by northern blot analysis. As shown in Figure 2C, the expression of RgtA was increased in all the *bvg* deletion mutants. Collectively, these results clearly showed that under chemically or genetically induced Bvg$^-$ conditions, the abundance of the RgtA is substantially increased.

RisA regulator activates the expression of RgtA

It has been demonstrated that under Bvg$^-$ conditions the response regulator RisA is required for efficient expression of *vrg* genes. Thus, repression of RgtA synthesis by the *bvg* system suggested that expression of the *rgtA* gene could be RisA-dependent. To test this assumption, the RgtA

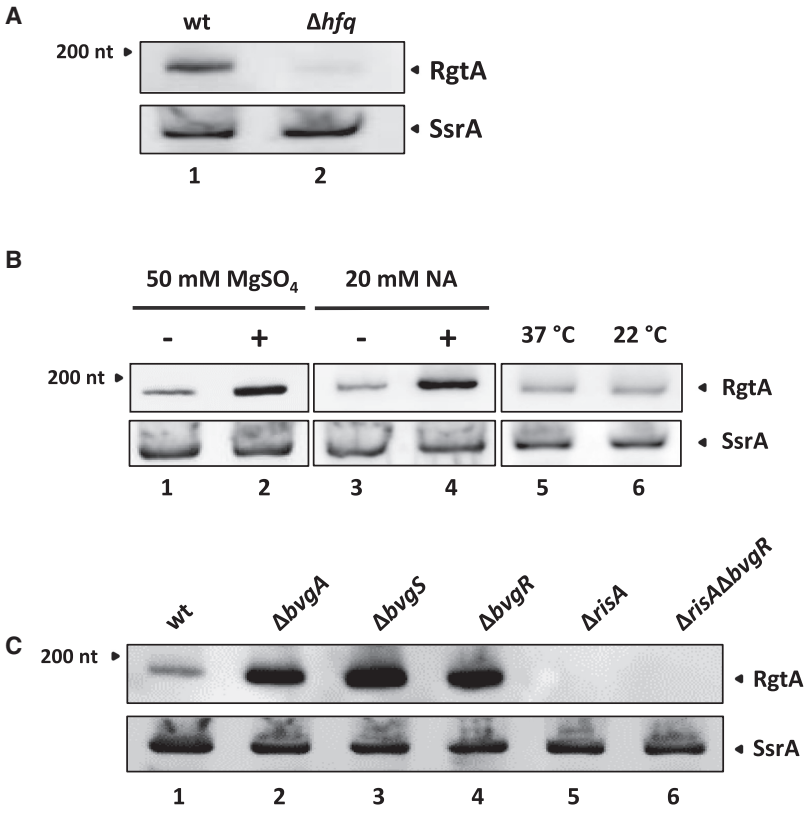

FIGURE 2. Abundance of RgtA is affected by the Hfq protein and phenotypic modulation. (*A*) Northern blot analysis was performed using total RNA isolated from Tohama I strain (lane *1*) and its isogenic Δ*hfq* mutant (lane *2*) probed with biotinylated probes specific to RgtA (*upper* panel) and to SsrA RNAs (*lower* panel, loading control). Only relevant parts of the membranes are shown. The result is a representative of three independent experiments. (*B*) *B. pertussis* Tohama I strain was grown in SS medium in the absence or presence of magnesium sulfate (lanes *1*,*2*) and nicotinic acid (lanes *3*,*4*) for 2 h. In another experiment, Tohama I cells were grown in parallel at 37°C and 22°C for 48 h in SS medium (lanes *5*,*6*). At these time points cells were harvested and isolated RNA was probed with biotinylated RgtA-specific probe and subjected to northern blot analysis (*upper* panels). Signals obtained upon rehybridization of the membrane with SsrA-specific probe (*lower* panels) served as loading control. Only relevant parts of the membranes are shown. The result is a representative of four experiments. (*C*) *B. pertussis* Tohama I strain (lane *1*) and its isogenic mutants carrying deletions of *bvgA* (lane *2*), *bvgS* (lane *3*), *bvgR* (lane *4*), Δ*risA* (lane *5*), and Δ*risA*Δ*bvgR* (lane *6*) genes were grown in SS medium to exponential phase. RNA isolated from harvested cells was probed with biotinylated RgtA-specific probe and subjected to northern blot analysis. Signals obtained upon rehybridization of the membrane with SsrA-specific probe (*lower* panels) served as loading control. Only relevant parts of the membranes are shown. The result is a representative of three experiments.

transcript levels in the Δ*risA* strain were analyzed by northern blot method. When compared to wt strain and especially to all *bvg* mutants, the RgtA synthesis was completely abolished in the Δ*risA* background (Fig. 2C). The absolute requirement of RisA factor for RgtA production became even more evident when we determined the RgtA levels in the Δ*bvgR* genetic background. As shown in Figure 2C, when compared to Δ*bvgR* mutant (increased abundance of RgtA due to lack of BvgR), the RgtA levels in the Δ*risA*Δ*bvgR* double mutant were also completely ablated. Apparently, the absence of a functional *bvg* system is not sufficient for RgtA expression when the RisA activator is lacking. To further characterize the RisA requirement for RgtA expression we searched for possible RisA-binding motifs in the promoter region of the *rgtA* gene. The consensus sequence of the RisA-binding site has been determined as the 7-bp motif 5′-AAAT(G/T)TA-3′ (Croinin et al. 2005). We identified several nearly perfect matches to this RisA-binding motif in the promoter region of the *rgtA* gene. In fact, the 60-bp region spanning nt −277 to −217 relative to the transcriptional start site contains three RisA-binding motifs in close proximity (Figs. 1D, 3A). Thus, we created a deletion mutant termed *rgtA*Δ277/217 lacking this 60-bp region and analyzed its ability to produce RgtA sRNA. Indeed, the synthesis of the RgtA in this mutant was totally abolished as already observed in the Δ*risA* strain, thereby indicating that this 60-bp region is required for RisA-dependent activation of *rgtA* expression (Fig. 3B).

BP3831 is a possible target of RgtA-mediated regulation

Our results proved that expression of RgtA is controlled by two different signal transduction systems and thereby indicated that this sRNA may play a significant role in the physiology of *B. pertussis*. Therefore, we decided to characterize its possible function and identify its target genes. First, we constructed the Δ*rgtA* mutant of *B. pertussis* in the Δ*bvgA* background with the rationale that expression of the *rgtA* gene is increased in the Bvg$^-$ mode and under these conditions we may better identify biologically relevant targets. Therefore, we compared transcript profiles of the double Δ*bvgA*Δ*rgtA* mutant (no RgtA synthesis) with that of Δ*bvgA* strain (increased synthesis of RgtA) by DNA microarray technique. Among the very short list of genes that showed significant deregulation (Supplemental Table S1), the most prominent change in expression was found for the *BP3831* gene (+1.2 $\log_2$ fold change). BP3831 is annotated as a periplasmic amino acid-binding protein of a putative ABC transporter within the *B. pertussis* genome (Parkhill et al. 2003). Microarray data suggested that presence of RgtA would display a negative effect on *BP3831* transcript levels and consequently, on BP3831 protein levels. To verify this hypothesis, the levels of BP3831 protein were examined by immunoblotting. Western blot analysis with protein samples isolated from cells of the wild-type, Δ*rgtA*, Δ*bvgA*, and Δ*bvgA*Δ*rgtA* strains revealed that when compared to Δ*bvgA* strain (increased expression of RgtA RNA), the amounts of BP3831 protein in Δ*bvgA*ΔRgtA (lack of RgtA expression) were significantly increased (Fig. 4A). There was no significant difference between the wild-type and Δ*rgtA* strain most probably due to overall lower production of RgtA in the wt strain (see Fig. 2C). This result was in agreement with our microarray data and confirmed the negative effect of the RgtA RNA on the BP3831 protein production. In many cases, the *trans*-encoded sRNAs exhibit their negative effects

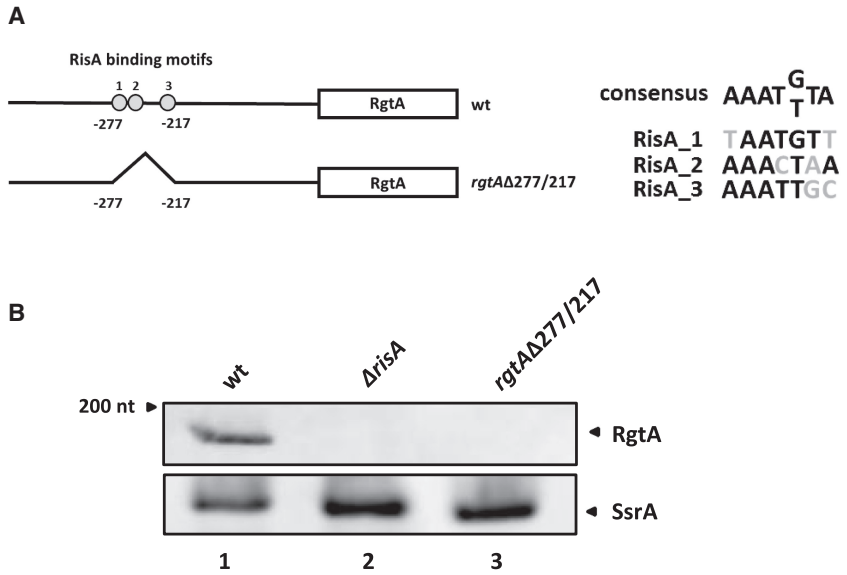

FIGURE 3. The requirement of the response regulator RisA for *rgtA* expression. (*A*) Schematic representation of RisA binding motifs in *rgtA* promoter region. (*Left*) Three plausible RisA binding sites (gray circles *1*–*3*) are present in a 60-bp region (−277 to −217) upstream of the *rgtA* transcriptional start site. This 60-bp region has been deleted, resulting in the *rgtA*Δ277/217 mutant. (*Right*) Sequences of canonical RisA-binding motif as well of three RisA-binding motifs found upstream of the *rgtA* promoter are depicted; nucleotides different from consensus are in gray. (*B*) *B. pertussis* Tohama I strain (lane *1*) and its isogenic Δ*risA* (lane *2*) and *rgtA*Δ277/217 (lane *3*) mutants were grown in SS medium to exponential phase. RNA isolated from harvested cells was probed with biotinylated RgtA-specific probe and subjected to northern blot analysis (*upper* panel). Signals obtained upon rehybridization of the membrane with SsrA-specific probe (*lower* panel) served as loading control. Only relevant parts of the membranes are shown. The result is a representative of two experiments.

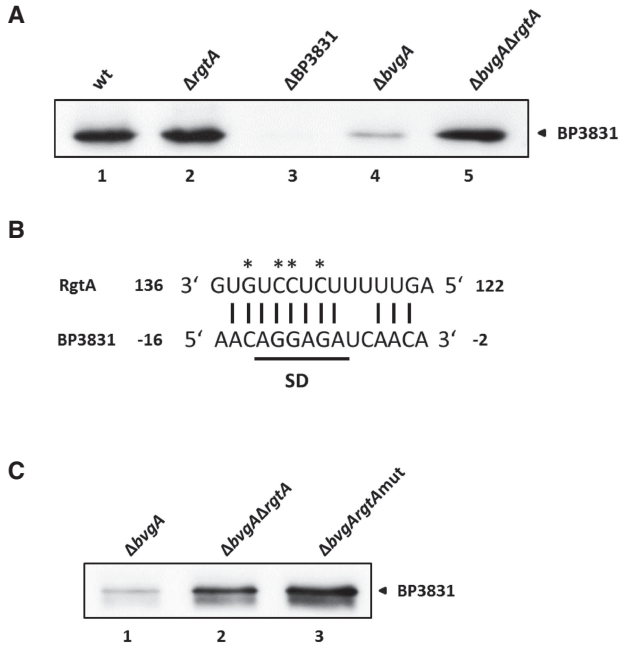

FIGURE 4. Inhibitory effect of the RgtA sRNA on BP3831 protein production. (*A*) *B. pertussis* Tohama I (lane *1*) and its isogenic Δ*rgtA* (lane *2*) Δ*BP3831* (lane *3*), Δ*bvgA* (lane *4*), and Δ*bvgA*Δ*rgtA* (lane *5*) mutants were grown in SS medium to exponential phase. Samples of cell lysates equivalent to 0.1 OD_{600} unit were separated on 12.5% SDS-PAGE gels and analyzed by immunoblotting using anti-BP3831 antibodies. Only relevant parts of the membranes are shown. The result is a representative of three experiments. (*B*) Schematic representation of the putative base-pairing between the RgtA RNA and *BP3831* mRNA. Nucleotides 122 to 136 of RgtA are complementary to −2 to −16 region of *BP3831* mRNA containing the Shine and Dalgarno sequence (SD). Nucleotides marked with an asterisk were mutated to disrupt the possible base-pairing between the RgtA and *BP3831* transcripts in the Δ*bvgArgtA*mut strain. (*C*) The effect of the mutations introduced into RgtA RNA on BP3831 protein levels. *B. pertussis* Tohama I Δ*bvgA* (lane *1*), Δ*bvgA*Δ*rgtA* (lane *2*), and Δ*bvgArgtA*mut (lane *3*) mutants were grown in SS medium to exponential phase. Samples of cell lysates equivalent to 0.1 OD_{600} unit were separated on 12.5% SDS-PAGE gels and analyzed by immunoblotting using anti-BP3831 antibodies. Only relevant parts of the membranes are shown. The result is representative of two experiments.

on translation by blocking the ribosome loading. We searched in silico for possible interactions between the RgtA sRNA and the *BP3831* mRNA. Using the program CopraRNA (http://rna.informatik.uni-freiburg.de/CopraRNA/Input.jsp) (Wright et al. 2014), we identified a 13-nt region within the RgtA sequence that shared partial complementarity to the region immediately upstream of the BP3831 start codon and that also included the Shine and Dalgarno sequence (Fig. 4B). As depicted in Supplemental Figure S2, this 13-nt region within the RgtA is predicted by mfold RNA folding server (Zuker 2003) to be single-stranded. To prove this predicted RNA–RNA interaction, we introduced four chromosomally located point mutations within this 13-bp region into the Δ*bvgA* strain yielding the Δ*bvgArgtA*mut strain. As shown in Figure 4C, when compared to the Δ*bvgA* strain, the Δ*bvgArgtA*mut strain produced increased amounts of BP3831 protein that were similar to those found in the Δ*bvgA*Δ*rgtA* (lacking the *rgtA* expression completely). Collectively, these results indicated that RgtA may interfere with the BP3831 production most likely by blocking the ribosome binding.

BP3831 is presumably involved in glutamate uptake

BP3831 is tentatively annotated as a putative periplasmic amino acid-binding protein of an ABC transporter in the *B. pertussis* genome. BLAST search unveiled that the BP3831 protein exhibits high homology with the entry 4Z9N in the PDB protein database (10.2210/pdb4Z9N/pdb). This entry contains X-ray diffraction data of an ABC transporter periplasmic binding protein from *Brucella ovis* that had been cocrystallized with glutathione. Interestingly, the binding pocket of the *B. ovis* protein shares nine of 11 amino acids with the BP3831 transporter (data not shown). Glutathione (GSH) is a tripeptide consisting of three amino acids, glutamate, cysteine, and glycine, and in the SS medium serves as an important antioxidant due to its reducing capacity. Nevertheless, glutathione was recently demonstrated as a suitable source of sulfur for *B. pertussis* and as such can replace cysteine (Branco Dos Santos et al. 2017). Therefore, to decipher if BP3831 is responsible for GSH binding, we cultivated both wt and Δ*BP3831* cells in the medium with or without cysteine with the rationale that if the GSH would be the specific substrate for BP3831, the mutant strain should not grow in medium lacking cysteine (the source of sulfur would be missing). Interestingly, when compared to wt strain, the mutant strain manifested observable growth deficit; however, it grew equally well in the media with or without the cysteine thereby ruling out the GSH as a substrate (Fig. 5A). Therefore, we focused on the other two amino acids that are included in the SS medium, glutamate and proline. First, the observed growth defect of the Δ*BP3831* strain was reproducible regardless if proline was present or absent in the medium and therefore, we excluded also proline as the substrate of BP3831 transporter (Fig. 5B). However, when we compared growth of the wild-type and Δ*BP3831* strains in the medium lacking glutamate (proline was the sole source of the carbon and nitrogen), we did not observe any significant difference (Fig. 5C), suggesting that in the absence of glutamate the BP3831 transporter does not confer any selective advantage. Hence, we concluded that the BP3831 transporter most likely exhibits the affinity for glutamate. Based on the observed negative effect of RgtA sRNA on BP3831 production (Fig. 4), we inspected the possible role of RgtA in the regulation of the glutamate uptake. Therefore, we analyzed the growth properties of the Δ*bvgA* (high levels of RgtA) and Δ*bvgA*Δ*rgtA* (no RgtA expression) strains in

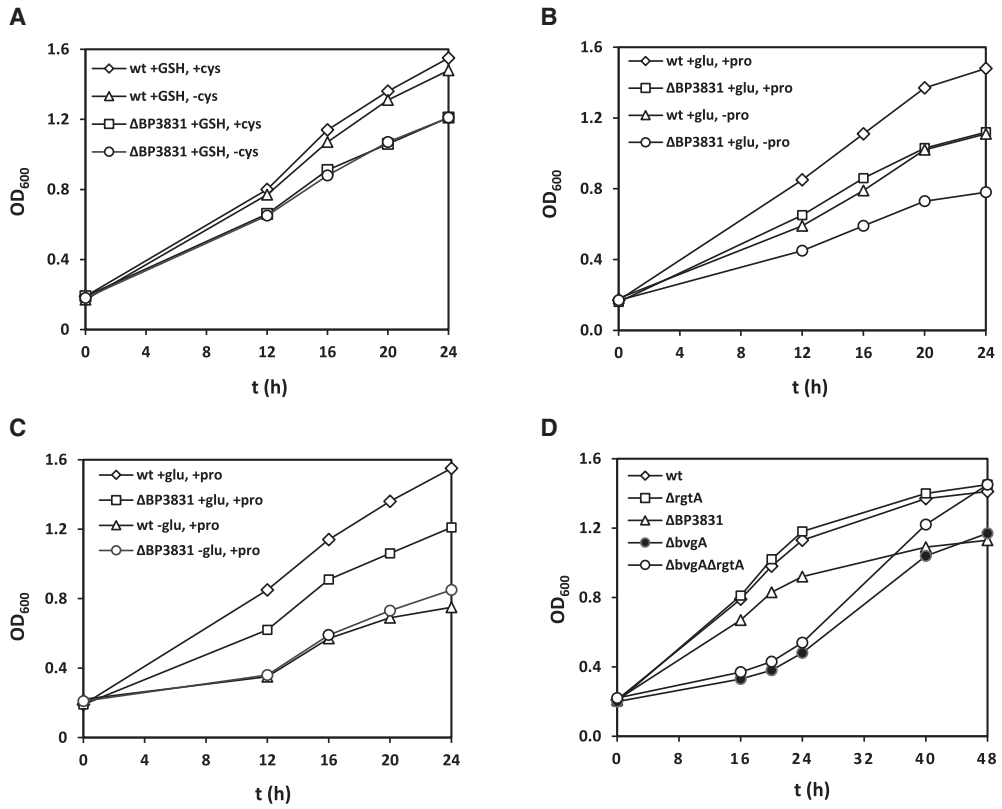

FIGURE 5. Requirement of the BP3831 transporter for growth of *B. pertussis* in chemically defined media. (*A*) *B. pertussis* Tohama I strain and its isogenic Δ*BP3831* mutant were grown in chemically defined SS medium in the presence (diamonds and squares, respectively) or absence (triangles and circles, respectively) of cysteine. (*B*) *B. pertussis* Tohama I strain and its isogenic Δ*BP3831* mutant were grown in chemically defined SS medium in the presence (diamonds and squares, respectively) or absence (triangles and circles, respectively) of proline. (*C*) *B. pertussis* Tohama I strain and its isogenic Δ*BP3831* mutant were grown in chemically defined SS medium in the presence (diamonds and squares, respectively) or absence (triangles and circles, respectively) of glutamate. (*D*) *B. pertussis* Tohama I strain (diamonds) and its isogenic Δ*rgtA* (squares), Δ*BP3831* (triangles), Δ*bvgA* mutant (filled circles), and Δ*bvgA*Δ*rgtA* (empty circles) mutants were grown in chemically defined SS medium with the glutamate as the sole source of carbon and nitrogen (no proline). The results in all panels are representative of three experiments.

the medium with glutamate as the sole source of energy (no proline). As shown in Figure 5D, wild-type (producing low amounts of RgtA) and Δ*rgtA* strains grew very similarly whereas as expected, the Δ*BP3831* strain showed impaired growth (see Fig. 5A). In agreement with our assumptions, the double Δ*bvgA*Δ*rgtA* mutant lacking RgtA RNA displayed better growth properties and reached higher OD values (equal to wild-type strain) when compared to Δ*bvgA* strain.

DISCUSSION

In this work we present the first fully characterized regulatory sRNA RgtA in the human pathogen *B. pertussis*. Furthermore, we identified its putative target gene as well as the possible RgtA-dependent regulatory mechanism and we propose that this sRNA is involved in control of transport of glutamate, an important source of carbon and nitrogen.

Importantly, RgtA displays several characteristics typical for a *trans*-encoded sRNA as it is (i) encoded within the intergenic region between genes *BP2735* and *BP2736*, (ii) its size is 174 nt, and (iii) its abundance is Hfq-dependent. Primer extension and northern blot analyses revealed that RgtA is transcribed from the negative strand and that the 5′ end of RgtA starts at nt 2,903,162 in the *B. pertussis* Tohama I genome (accession number: NC 002929.2). Importantly, the sequence of the *rgtA* gene including promoter region and putative RisA-binding sites is highly conserved in the closely related species *B. bronchiseptica* RB50 and *B. parapertussis* 12822 (Supplemental Fig. S1A). Likewise, the region upstream of the initiation codon including the Shine and Dalgarno sequence is well preserved in the *BP3831* gene and its homologs (data not shown). These observations indicate that the suggested sRNA–mRNA interaction might be functional also in related *Bordetella* species. Northern blot analysis confirmed that both related species produce RgtA RNA, although the *B. parapertussis* RgtA homolog seems to be smaller in size.

While we could not observe any significant changes in RgtA expression under several stress conditions, we

discovered that under modulatory conditions, inducing avirulent Bvg$^-$ phase, the abundance of this sRNA is substantially increased. This finding was corroborated using mutants created in the *bvgASR* locus and identified RgtA as a virulence repressed gene. The expression of most of the *vrgs* was shown to be dependent on another response regulator, RisA, which in its phosphorylated form binds c-di-GMP as a cofactor and activates expression of *vrg* genes (Coutte et al. 2016; Chen et al. 2017). Therefore, our data on *rgtA* expression nicely fit in the proposed model integrating the roles of BvgAS, BvgR, and RisA regulatory systems. RisA requirement was indirectly confirmed by deletion of three putative RisA-binding sites within a 60-bp region between positions −277 and −217 relative to the start site of the *rgtA* transcript, which completely abolished transcription. Interestingly, deletions between positions −271 and −159 relative to the transcription start site completely disrupted RisA-dependent regulation of the *vrg6* gene expression (Croinin et al. 2005). These observations suggest that regions located ~270 bp upstream of the start of transcription may be essential for RisA-dependent activation of the *vrg* genes. Nevertheless, additional experiments would be necessary to define the specific sequences contributing to binding of RisA and to demonstrate direct RisA interaction with the *rgtA* promoter.

Currently available next-generation sequencing technologies as well as improved bioinformatics tools led to the discovery of myriads of sRNAs in numerous bacteria. Nevertheless, characterization of their regulatory roles and identification of their interaction partners is strenuous and time consuming. Importantly, in our study we identified a possible target of the RgtA-mediated regulation, the periplasmic amino acid-binding protein of an ABC transporter BP3831. Transcriptomic profiling of the wt and Δ*rgtA* strains did not reveal any significantly modulated genes (data not shown) most probably due to lower production of RgtA under Bvg$^+$ conditions. Nevertheless, when the Bvg$^-$ conditions were introduced by deletion of the *bvgA* gene, comparative analysis of the Δ*bvgA*Δ*rgtA* and Δ*bvgA* strains unveiled *BP3831* as the most deregulated gene. Down-regulation of the BP3831 production in the presence of RgtA in vivo, as well as in silico prediction of the possible RgtA sRNA–*BP3831* mRNA interaction, suggested a negative effect of RgtA on BP3831 protein levels. We verified this assumption by site-directed mutagenesis of several bases in RgtA involved in the predicted base-pairing with the *BP3831* mRNA. Notably, the levels of the BP3831 protein in this mutant were similar to those observed in the Δ*rgtA* mutant, suggesting that in line with the in silico modeling, the RgtA RNA may overlap with the Shine and Dalgarno sequence of the *BP3831* transcript and impair the ribosome loading.

Finding the target of the RgtA-dependent regulation encouraged us to characterize the function of the BP3831 transporter in the cellular metabolism and, consequently, to decipher the possible biological role of RgtA. The BP3831 protein has been only tentatively annotated as a periplasmic amino acid-binding protein. Interestingly, it has been shown that multiple periplasmic binding proteins of the ABC transporters family are part of the Hfq-regulon in several organisms and a direct interaction between various sRNAs and ABC transporter mRNAs has been proven as well (Antal et al. 2005; Sharma et al. 2007; Wilms et al. 2012). The growth analysis of strains lacking *BP3831* or *rgtA* genes suggests that BP3831 is most likely involved in the uptake of glutamate, although we are aware of rather subtle effects caused by these deletions. Only partially impaired growth of the Δ*BP3831* strain in the medium with glutamate as the sole source of energy indicates that BP3831 may have yet another substrate specificity or that there are still other glutamate transporters produced by *B. pertussis* cells. Indeed, several genes within the Tohama I genome are predicted to encode periplasmic binding proteins of unknown specificity, e.g., BP0558 and BP1529 proteins share 61% sequence identity and 81% sequence positivity with BP3831 transporter. Therefore, it is very well possible that some of these proteins can deliver the glutamate also in the absence of BP3831 and mask the effects conferred by RgtA-mediated repression or *BP3831* deletion. Recently, increased abundance of the BP3831 protein in the secretome of the Australian epidemic strain was documented and suggested to be linked with increased fitness of this strain (Luu et al. 2018). Furthermore, BP3831 protein was identified within the surface proteome of *B. pertussis* and was also found to be immunogenic (Tefon et al. 2011; Williamson et al. 2015). *B. pertussis* as a strictly human pathogen faces upon infection several stresses resulting from nutrient scarcity as well as from the immune response of the host. In this respect, the RgtA-dependent reduction of BP3831 levels may help the pathogen to adapt to new sources of nutrients (other than glutamate) and/or decrease the antigenicity by reduction of the amounts of immunogenic protein. In support, the expression of *BP3831* was significantly reduced in mouse-passaged *B. pertussis* cells recovered 12 d post infection (Bibova et al. 2015). Furthermore, glutamate starvation was shown to result in reduced production of virulence factors (Nakamura et al. 2006) and may be then considered as a signal to reprogram the metabolism in order to persist within the host. Indeed, multiple metabolic pathways are up-regulated in the Bvg$^-$ phase and potentially help to overcome the environmental and nutritional stress (Moon et al. 2017). Interestingly, *bvg*$^-$ mutants have been shown to accumulate among persistent *B. pertussis* cells within the upper respiratory tract of experimentally infected rhesus monkeys (Karataev et al. 2016). In this respect, it is possible that RisAK of *B. pertussis* may play similar roles as the RisAS system of *B. bronchiseptica*, which controls

expression of *vrgs* required for intracellular survival and persistence in the mouse model of infection (Jungnitz et al. 1998; Zimna et al. 2001). In conclusion, the RisA-dependent small regulatory RNA RgtA represents a regulatory factor that is functionally related to the Bvg^- mode of the *B. pertussis* life cycle. We speculate that RgtA-mediated riboregulatory activity helps the pathogen to adapt to nutritional stress and reduce the host immune responses and, consequently, to establish persistent infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The *Bordetella pertussis* Tohama I strain (Kasuga et al. 1954) and its isogenic deletion mutants as well as *Bordetella bronchiseptica* RB50 (Cotter and Miller 1994) and *Bordetella parapertussis* 12822 (Heininger et al. 2002) strains were grown on Bordet–Gengou agar (BGA) plates supplemented with 15% sheep blood for 3 to 4 d at 37°C. For liquid cultures, bacteria were grown in Stainer–Scholte (SS) medium (Stainer and Scholte 1970) supplemented with 0.1% cyclodextrin and 0.5% casamino acids (Difco) at 37°C. In experiments where the growth was monitored in chemically defined media, the casamino acids were omitted. For phenotypic modulation experiments with magnesium sulfate or nicotinic acid an overnight culture of *B. pertussis* was diluted to OD of 0.4. After reaching OD 0.5, the culture was divided into two flasks. To modulated cultures, either magnesium sulfate or nicotinic acid was added to 50 mM and 20 mM final concentrations, respectively. Cultures in second flasks without modulators served as controls. Samples for RNA isolation were taken 2 h after addition of modulators. For growth at 22°C, the *B. pertussis* cultures were cultivated at 37°C for 14 h and then further passaged either at 37°C (control) or at 22°C to achieve at least three cell divisions (>48 h). *Escherichia coli* strains were cultured on Luria–Bertani (LB) agar or in LB broth.

Construction of deletion mutants

The deletions or point mutations were introduced into *B. pertussis* Tohama I strain chromosome as already described (Bibova et al. 2013). In general, two DNA fragments of ~750 bp corresponding either to the upstream or the downstream flanking regions of the corresponding gene were created using PCR. The upstream fragment (containing the start codon of the gene at the 3′-end) and the downstream fragment (containing the stop codon of the gene at the 5′-end) were cleaved at the 3′-end and the 5′-end, respectively, with the same restriction enzyme and then ligated. The ligation mixture was then used as a template to create ~1.5 kb PCR product. This amplified PCR product was then ligated into the allelic exchange plasmid pSS4245. The resulting plasmid was transformed into the *E. coli* SM10 strain (donor strain) and transferred to *B. pertussis* Tohama I (recipient strain) by conjugation, as described elsewhere (Inatsuka et al. 2010). After two recombination events, the strain carrying the desired markerless in-frame deletion (where only the start and stop codons of the deleted gene separated by the restriction site were preserved) was obtained. Successful chromosomal deletions, point mutations, and the sequences of the flanking regions were confirmed by DNA sequencing. *Bordetella* sp. strains used in this study are listed in Supplemental Table 2. Primers used for the construction of the mutants are listed in Supplemental Table 3.

DNA microarrays

B. pertussis Δ*bvgA* and Δ*bvgA*Δ*rgtA* cells were grown in three independent biological replicates in SS medium and harvested by centrifugation (6000 rpm, 10 min, 4°C) at an exponential phase of growth. Total RNA was extracted from pellets using Tri Reagent (Sigma) as recommended by the manufacturer and further treated with DNase I (Sigma) to remove any contaminating genomic DNA. Purified RNA was checked for concentration and integrity using the Bioanalyzer 2100 device (Agilent Technologies). Labeling with Cyanine-5 and Cyanine-3 was performed as already described (Bibova et al. 2015). For each sample, a Cy5/Cy3 dye-swap technical replicate starting from the same RNA preparation was prepared and hybridized with DNA oligonucleotide probes spotted onto microarray slides for 14 to 16 h at 52°C under agitation. Oligonucleotide probes were designed for 3554 annotated coding sequences of *Bordetella pertussis* Tohama I strain (BX470248.1, GI:33591069) and were spotted in duplicate (non-adjacent) on aldehydsilane coated glass slides (Nexterion Slide AL, Schott) using a QArray II spotter (Genetix) equipped with 12 ArrayHit 946MP3 pins. After hybridization, slides were subsequently washed at room temperature in 2× SSC, 0.2% SDS for 5 min, 0.5× SSC for 10 min, 0.05× SSC for 5 min, and 0.01× SSC for 2 min before being dried and scanned using InnopScan700 (Innopsys). Background correction as well as within- and between-array normalization (Yang et al. 2002) was performed from raw data using LIMMA (Linear Models for Microarray Data) R package (Smyth et al. 2003). After normalization, identification of genes with significantly modulated expression was performed using a moderated Student's *t*-test with empirical Bayes shrinkage of standard errors (Lonnstedt and Britton 2005). Statistics were corrected for multiple testing using a false-discovery rate approach. In order to select genes significantly affected by *rgtA* deletion, absolute log fold change values (log_2FC) of transcript abundance were set to at least 0.5 and threshold for adjusted *P*-value (P) of less than 0.025 was considered as indicative of significance.

Northern blot and primer extension analyses

For northern blot analysis, 8 µg of total RNA was mixed with 2× RNA loading dye, and heated for 5 min at 65°C before electrophoretic separation on an 8% polyacrylamide-8 M urea denaturing gel prepared in 0.5× TBE buffer. Biotinylated RNAs transcribed in vitro using RNA Century-Plus Marker Template (Invitrogen) served as size markers. The RNA was then transferred onto a Zeta-Probe nylon membrane (Bio-Rad) by electroblotting and UV crosslinked. After blocking with salmon sperm DNA, the membrane was hybridized overnight at 55°C with a biotinylated RgtA-specific probe RgtA_NB or after rehybridization with biotinylated SsrA-specific probe SsrA_NB. Signals obtained with SsrA-specific probe detecting tmRNA served as loading controls. Blots were developed using chemiluminescent detection with the

BrightStar BioDetect kit (Ambion). Hybridization signals were visualized using a G:Box Chemi XRQ device (Syngene).

For primer extension analysis, 20 pmol of RgtA_PE primer were labeled at the 5′ end with γ^{32}P-ATP by T4 polynucleotide kinase (Fermentas) as described by the manufacturer and purified with QIAquick Nucleotide Removal Kit (Qiagen). Two picomoles of labeled primer was precipitated with 30 µg of RNA, 2.5 vol 100% ethanol, and 0.1 vol 3 M sodium acetate (pH 4.8) overnight at −20°C. The reaction mixture was centrifuged (30 min, 12,000*g*, 4°C) and washed with 70% ethanol. The pellet was dried, suspended in 12 µL RNase-free water and reverse transcribed with Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's recommendations. The sequencing reactions were performed with the fmol DNA Cycle Sequencing System (Promega) using pSK_IGR2735/36 as template and RgtA_PE primer as sequencing primer according to the manufacturer's recommendations. Primer extension and sequencing reaction were separated on a 6% polyacrylamide-8 M urea gel. The gel was dried and subjected to autoradiography, and primer extension signals were visualized on Molecular Imager FX Pro Plus (Bio-Rad) instrument.

Western blot analysis

B. pertussis cells were cultivated in SS medium at 37°C and cell pellets from 1-mL aliquots were lysed and boiled in sample buffer. Samples equivalent to 0.1 OD_{600} unit were separated on 12.5% SDS-PAGE gels and transferred on nitrocellulose membrane. Gels were stained after the transfer to evaluate the transfer efficiency and served also as loading controls. Membranes were blocked with 5% skim milk and probed with mouse polyclonal antibodies raised against BP3831 followed by incubation with anti-mouse IgG antibodies conjugated with horseradish peroxidase. The antibody–antigen complexes were visualized using SuperSignal West Femto chemiluminescent substrate (Thermo) according to standard protocol on a G:Box Chemi XRQ device (Syngene).

Purification of BP3831 protein and production of anti-BP3831 antibodies

The gene encoding BP3831 transporter was amplified using forward BP3831fw and reverse BP3831rev primers and then cloned into pET42 vector (Novagen) in order to produce N-terminally His-tagged BP3831 protein. The construct was used to transform *E. coli* BL21 (DE3) strain (Promega) which was then cultivated in LB medium at 37°C. The production of recombinant BP3831 protein was induced by 1 mM IPTG at OD ≈ 1.0 and after 3 h the cells were harvested by centrifugation (7000*g*, 20 min at 4°C), washed in TS buffer (50 mM Tris pH 8, 300 mM NaCl) containing 10 mM EDTA, TS buffer without EDTA and finally resuspended in 30 mL of TS buffer and lysed by sonication. Inclusion bodies were pelleted (20,000*g*, 30 min at 4°C); pellet was washed by 50 mM Tris pH 8 containing 4 M urea without resuspension to remove residues of broken cells and then dissolved in TU buffer (50 mM Tris pH 8, 8 M urea). The urea extract was cleared by centrifugation (20000*g*, 30 min at room temperature) and loaded onto an equilibrated Ni-NTA column (Qiagen). After washing the column with TU buffer, the recombinant protein was eluted by increasing concentrations of imidazole (50–300 mM) in TU buffer. The fraction of BP3831 protein eluted by 100 mM imidazole was concentrated and transferred into TU buffer using an Amicon Ultra Centrifugal Filter Unit, 10000 MW cut off size (Merck Millipore). Production of BP3831-specific mouse sera was approved by the Animal Welfare Committee of the Institute of Microbiology of the ASCR, Prague, Czech Republic. A total of 30 µg of BP3831 protein in 180 µL of PBS was mixed with 20 µL of 2% aluminum hydroxide hydrate (Sigma) and left for 30 min on ice prior to injection. In total three female 4-wk-old BALB/c mice (Charles Rivers Genetic Models, Inc.) were injected intraperitoneally twice at a 2-wk interval. Mice were bled 14 d after the last injection for the assessment of anti-BP3831 antibodies.

DATA DEPOSITION

Microarray data described in this work are available in the Gene Expression Omnibus database under accession number GSE113382.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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