Role of (p)ppGpp in biofilm formation and expression of filamentous structures in *Bordetella pertussis*

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*Bordetella pertussis*, the causative agent of whooping cough, is highly adapted to cause human infection. The production of virulence factors, such as adhesins and toxins, is just part of an array of mechanisms by which *B. pertussis* causes infection. The stringent response is a global bacterial response to nutritional limitation that is mediated by the accumulation of cellular ppGpp and pppGpp [termed together as (p)ppGpp]. Here, we demonstrate that production of (p)ppGpp was controlled by RelA and SpoT proteins in *B. pertussis*, and that mutation-induced loss of both proteins together caused deficiencies in (p)ppGpp production. The (p)ppGpp-deficient mutants also exhibited defects in growth regulation, decreases in viability under nutritionally limited conditions, increases in susceptibility to oxidative stress and defects in biofilm formation. Analysis of the secreted proteins and the respective transcripts showed that lack of (p)ppGpp led to decreased expression of *fim3* and *bsp22*, which encode a fimbrial subunit and the self-polymerizing type III secretion system tip protein, respectively. Moreover, electron microscopic analysis also indicated that (p)ppGpp regulated the formation of filamentous structures. Most virulence genes – including *fim3* and *bsp22* – were downregulated in a (p)ppGpp-deficient mutant, normal expression of *fhaB*, *cyaA* and *ptxA* persisted. Lack of coherence between virulence gene expression and (p)ppGpp production indicated that (p)ppGpp did not modulate the Bvg phase. Taken together, our data indicate that (p)ppGpp may govern an as-yet-unrecognized system that influences *B. pertussis* pathogenicity.

INTRODUCTION

*Bordetella pertussis* is the causative agent of whooping cough (pertussis), a highly infectious and transmissible disease of the human respiratory tract. Pertussis is traditionally described as an acute and life-threatening disease in unvaccinated children (Carbonetti, 2007), and is also common among adolescents and adults, in whom it often manifests as persistent, but less paroxymal, cough with milder and atypical symptoms (Birkebaek *et al*., 1999; Halperin, 2007). However, over the past two decades, pertussis has experienced a worldwide resurgence in individuals of 15 years or older (He & Mertsola, 2008). Many reports implicate adolescents and adults as reservoirs and sources of pertussis transmission (Cherry, 1999; Nelson, 1978).

The signalling molecule (p)ppGpp (pppGpp and pppGpp) is synthesized in bacteria and plants; in bacteria, (p)ppGpp activates the stringent response, which is an adaptive and global physiological response to nutrient deprivation. In beta- and gamma-proteobacteria, intracellular levels of (p)ppGpp are modulated by RelA and SpoT proteins. RelA

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**Abbreviation:** MV, methyl viologen.

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proteins are monofunctional alarmone synthase, and SpoT proteins are bifunctional synthase/hydrolases (Potrykus & Cashel, 2008). Both RelA and SpoT enzymes can synthesize (p)ppGpp, whereas SpoT enzymes can also hydrolyse (p)ppGpp (Chatterji et al., 1998; Mittenhuber, 2001). Upon amino acid starvation, RelA-mediated (p)ppGpp synthesis is activated. SpoT is induced during exponential growth, and can respond to a broad range of stress factors in the environment including deprivation of carbon sources or energy sources (Boes et al., 2008; Magnusson et al., 2005). Intracellular concentrations of (p)ppGpp change in response to growth conditions; the synthesis of (p)ppGpp is activated by RelA, and the balance of (p)ppGpp is regulated by SpoT, which tunes cellular (p)ppGpp levels via hydrolyase activity and weak synthase activity (Xiao et al., 1991).

Expression of virulence factors in Bordetella is regulated by the well-described BvgAS system (Mattoo et al., 2001; Uhl & Miller, 1996). During the Bvg + phase, BvgAS is activated and virulence factors — including filamentous haemagglutinin (FHA), pertussis toxin (PTX) and adenylate cyclase toxin (CyaA) — are expressed; this phase is required to establish an infection in a host organism. In contrast, BvgAS is inactive during the avirulent Bvg − phase, and virulence factors are not synthesized. An intermediate phase (Bvg i) exists between the Bvg + and Bvg − phases; during Bvg i, adhesin genes such as flaB are expressed, and toxin genes such as ptx and cyaA are repressed (Deora et al., 2001). These phases are fully reversible by phenotypic modulation. Other than the Bvg system, little is known about other factors that may regulate virulence in B. pertussis. The host environment is often a hostile and nutritionally deficient environment for bacterial growth; therefore, we investigated the possibility that (p)ppGpp has a role in B. pertussis pathogenesis.

Here, we constructed a (p)ppGpp-deficient mutant to assess the roles of (p)ppGpp in B. pertussis. Our findings demonstrated that (p)ppGpp was synthesized by RelA and SpoT and that it mediated the bacterial response to oxidative stress and the adaptation to nutrient limitation. Moreover, it is likely that the (p)ppGpp regulated biofilm development on an abiotic surface and autoaggregation of B. pertussis because it induced the expression of extracellular filamentous structures that are related to pathogenesis.

This is the first report, to our knowledge, investigating the role of (p)ppGpp in B. pertussis. Based on our findings, we suggest that pathogenesis of B. pertussis is mediated by synergistic mechanisms, specifically that (p)ppGpp works alongside the BvgAS two-component system to regulate virulence.

METHODS

Bacterial strains and growth conditions. B. pertussis strains (Table 1) were grown at 35 °C on Bordet–Gengou (BG) agar supplemented with 15 % defibrinated sheep’s blood (Bordet & Gengou, 1906) or in modified Stainer–Scholte defined medium (SS) (Stainer & Scholte, 1910) with 0.5 % Casamino acids. When required, 10 μg gentamicin ml −1 and 12.5 μg tetracycline ml −1 were added to the medium. Escherichia coli DH5α strain was used for routine plasmid propagation. Bacterial growth was monitored by measurement of OD600 at the time points indicated in the figures.

Construction of (p)ppGpp synthase deletion mutants and complementation strains. Each fragment, which contained the ΔrelA or ΔspoT allele, was constructed by PCR overlap extension and was used to disrupt the respective target gene by homologous recombination (Ho et al., 1989). Briefly, fragments carrying the 5’ and 3’ terminal and flanking regions of relA were amplified by PCR with the primer pairs relA-F1 / relA-R1 and relA-F2 / relA-R2, respectively (Table S1; available in Microbiology Online); these PCR products were used as templates to generate a single 848 bp fragment that encoded a ΔrelA allele with relA-F1 and relA-R2 primers. The resulting 848 bp fragment was digested with BamHI and EcoRI, and cloned into the pSS1129 vector to yield pSSK23. Similarly, fragments carrying upstream and downstream regions of spoT were each amplified using the primer pairs spoT-F1/spoT-R1 and spoT-F2/spoT-R2, respectively; a single 926 bp fragment encoding the ΔspoT allele was synthesized using the spoT-F1 and spoT-R2 primers, and was then cloned into pSS1129 to yield pSSK24.

A single strain carrying two in-frame non-polar deletions – a relA deletion and a spoT deletion – was constructed by double homologous recombination as described previously (Stibitz, 1994). Briefly, the relA deletion in the strain designated PMK19 was verified by PCR using the primer pair relA-F-Bam/spoT-R-Eco, and the ΔrelA ΔspoT double mutant was constructed from this PMK19 strain by integration and excision of pSSK24. The ΔspoT mutation in the resultant double mutant strain, designated PMK21, was verified by PCR using the primer pair spoT-F-Bam/spoT-R-Eco.

For complementation study, a wild-type relA was synthesized by PCR with the primer pair relA-F-Bam/spoT-R-Eco and was cloned into pRK415. The resultant plasmid was designated pRKT01 and was used for routine plasmid propagation. A single strain carrying two in-frame non-polar deletions – a relA deletion and a spoT deletion – was constructed by double homologous recombination as described previously (Stibitz, 1994). Briefly, the relA deletion in the strain designated PMK19 was verified by PCR using the primer pair relA-F-Bam/spoT-R-Eco, and the ΔrelA ΔspoT double mutant was constructed from this PMK19 strain by integration and excision of pSSK24. The ΔspoT mutation in the resultant double mutant strain, designated PMK21, was verified by PCR using the primer pair spoT-F-Bam/spoT-R-Eco.

Detection of intracellular (p)ppGpp. Assays of (p)ppGpp were performed as described previously (Cashel, 1994), but with modifications for B. pertussis. Briefly, each overnight culture of bacterial cells was washed twice with low phosphate SS medium (final concentration 0.4 mM); each cell pellet was resuspended to OD600 0.15 in the same medium. To elicit the stringent response, serine hydroxamate, which binds to and interferes with seryl-tRNA synthetases, was added to a final concentration of 1 mM 4 h before addition of [32P] monophosphonate phosphate (MP Biomedicals). When the OD600 of each culture was between 0.2 and 0.25 (early exponential phase), [32P] phosphate was added at a final concentration of 0.1 μCi (3.7 MBq) ml −1. After being labelled for 4 h, bacterial cells in a 100 μl sample of the culture were centrifuged at 11 000 g for 1 min, and resuspended in 20 μl of 13 M formic acid. The bacterial cells were lysed by three freeze-thawing cycles to extract (p)ppGpp. Cell debris was removed by centrifugation at 11 000 g for 2 min, and 20 μl of supernatant was spotted onto a polyethylenimine cellulose TLC plate (PEI cellulose-F; Merck). The samples were developed in 1.5 M KH2PO4 (pH 3.4) at room temperature, and (p)ppGpp was visualized on an autoradiograph by exposure to Hyperfilm ECL (GE Healthcare). Identification of ppGpp and pppGpp was achieved by comparison of the rate of flow values with (p)ppGpp produced by E. coli K-12 strain as a marker (Cashel & Gallant, 1969; Cashel, 1994).
Table 1. Bacterial strains and plasmids used in this study

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or description</th>
<th>Source or reference</th>
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<tr>
<td>Strain</td>
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<td>B. pertussis</td>
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<tr>
<td>UT25Sm1</td>
<td>Spontaneous streptomycin-resistant derivative of wild-type</td>
<td>Brickman &amp; Armstrong (1996); Field &amp; Parker (1978)</td>
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<td>PMK19</td>
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<td>Keen et al. (1988)</td>
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<td>Expression vector for His-tagged peptide</td>
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<td>Allelic exchange vector, Gen'&lt;sup&gt;a&lt;/sup&gt;, Amp'&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>pQEElhaB</td>
<td>1.9 kb truncated fhaB cloned into pQE31</td>
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Viability in PBS and sensitivity to oxidative stress. To assess susceptibility to nutrient-deficient conditions, B. pertussis cells were grown overnight, harvested, washed/pelleted and resuspended in PBS. Viability under nutrient starvation conditions was verified by monitoring c.f.u. ml<sup>-1</sup> during incubation at 35 °C after adjustment to OD<sub>600</sub> 1.0 (1.6 ± 0.4 × 10<sup>9</sup> c.f.u. ml<sup>-1</sup>). Sensitivity to oxidative stress was assessed with a disc-diffusion assay. A bacterial suspension prepared at OD<sub>600</sub> 0.1 (1.7 ± 0.2 × 10<sup>9</sup> c.f.u. ml<sup>-1</sup>) from an overnight culture was spread onto BG agar with rayon swabs (COPAN Italia), and paper discs (8 mm diameter) containing 10 μl of 500 μl aliquot of bacterial suspension was carefully collected from the surface of the liquid in the first tube, and the OD<sub>600</sub> of each aliquot was measured. To measure OD<sub>600</sub> of bulk suspension, the entire 2 ml suspension in the second tube was mixed with a vortex to disrupt cell aggregates, and the OD<sub>600</sub> value was measured. Autoaggregation rate was defined as: [1 − (OD<sub>600</sub> supernatant/OD<sub>600</sub> total bacterial suspension)] × 100.

SDS-PAGE and immunoblot analyses of proteins in culture supernatants. Bacterial cells in exponentially growing cultures were removed from suspension by centrifugation at 9000 g for 10 min at 4 °C; each supernatant was passed through a filter (pore size, 0.45 μm) to remove all remaining bacterial cells. Proteins in filtered supernatants were precipitated with 10% TCA–acetone. Proteins were resuspended in loading buffer and then separated by SDS-PAGE (12.5% polyacrylamide). Anti-CyaA (sc-13582) and anti-PTX (sc-57639) monoclonal antibodies were purchased from Santa Cruz Biotechnology. Polyclonal anti-FHA antibody was prepared from the serum of a rabbit immunized with a His-tagged and truncated fragment of FHA. 1.5 kb of fhaB (nucleotides 1656–3252) fragment encoding FhaB (amino acids 553–1084) was synthesized by PCR with fhaBUpBam and fhaBLowHin primers; the fragment was cloned into pQE31 (Qiagen) to create pQEElhaB (Table 1). The tagged FHA fragment was expressed in and purified from E. coli JM109 treated with pQEElhaB, according to Qiagen protocols. Reactivity to the mature FHA was verified using standard FHA (List Biological Laboratories) and immunoblots. For each immunoblot, proteins were transferred onto PVDF membranes, and incubated with 250- or 400-fold dilutions of monoclonal or polyclonal antibodies. After...
incubation with and removal of primary and then secondary antibodies, the blots were developed with ECL Prime Western blotting Detection Reagents (GE Healthcare), and images were analysed with ImageQuant LAS-4000 (GE Healthcare).

**Mass spectrometry.** Protein bands of interest were excised from a Coomassie blue stained SDS-PAGE gel and treated with trypsin. Digested proteins were run through a mass spectrometer system (LTQ-Orbitrap Velos mass spectrometer) that was coupled with a direct nanoLC system (DINa, KYA Technologies). The Mascot search engine was used to access the NCBI bacteria database and analyse the data.

**RNA isolation and quantitative RT-PCR (qRT-PCR).** Total RNA was isolated from mid-exponential-phase bacterial cultures with a commercial NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer’s protocols. Copy DNA was synthesized with a PrimeScript reagent kit (Takara Bio); total RNA (1 µg) and 5 µM of random 6-mers were used in a 20 µl reaction mixture according to the manufacturer’s protocol. PCR was performed with SYBR Premix Ex Taq GC (Takara Bio). Reactions were run in triplicate on a 7500 Real-Time PCR system (Applied Biosystems) using the universal thermal cycling parameters (30 s at 95 °C, 40 cycles of 5 s at 95 °C and 35 s at 60 °C; dissociation curve: 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C, and a final dissociation at 60 °C for 1 min). Data were captured with the sequence detection software Primer Express (Applied Biosystems) and analysed. Primers used for qRT-PCR are listed in Table S1. Relative transcript levels were calculated with the comparative threshold cycle ($C_t$) method (Livak & Schmittgen, 2001). Fold changes were calculated with the $\Delta C_t$ method. Briefly, $C_t$ values for each target gene were normalized by subtracting the $C_t$ value for the recA gene, which is Bvg independent (Sukumar et al., 2007), as the reference ($\Delta C_t$). Relative expression values ($R$) were calculated using the equation: $R=2^{-\Delta C_{\text{relA}}-\Delta C_{\text{relA wild-type}}}$. All real-time PCRs were performed in triplicate in three independent experiments.

**Statistical analysis.** Student’s $t$-test was used to analyse the data; $P$-values less than 0.05 were considered statistically significant. Results are expressed as the mean $\pm$ SD.

**RESULTS**

**Production of pppGpp and ppGpp by B. pertussis**

A BLAST search of the Tohama I genome sequence (http://www.genedb.org) recovered no other sequences exhibiting high homology with any (p)ppGpp synthase other than relA or spoT homologues. Therefore, deletion mutations of relA and spoT in B. pertussis were generated to investigate the production of (p)ppGpp. Unlike the wild-type strain (UT25Sm1), which produced (p)ppGpp under amino-acid starvation conditions, the double ΔrelAΔspoT mutant (PMK21) did not produce any (p)ppGpp (Fig. 1a). Production of (p)ppGpp was restored to PMK21 by complementation with a wild-type relA gene (Fig. 1b). Remnant (p)ppGpp production in PMK19 may have been due to the synthase activity of SpoT. Unidentified spots between pppGpp and ppGpp were detected in each sample, though the levels of these spots were not affected by (p)ppGpp production. Taken together, these results indicated that (p)ppGpp was produced in the presence of relA and/or spoT, and that RelA was primarily responsible for (p)ppGpp synthesis in vitro. Therefore, we used the PMK21 strain to investigate the physiological effect(s) of (p)ppGpp.

The interaction between RNA polymerase and stringent promoters is easily modified by RNA polymerase missense mutations; consequently, suppressor mutations often arise in ppGpp$^0$ strains, and most of these strains are resistant to rifampicin (Murphy & Cashel, 2003; Zhou & Jin 1998). So, we used the primers BPprpB-F and BPprpB-R to sequence the region containing the sites in the rpoB gene in which suppressor mutations are detected. For each mutant strain, the rpoB sequence was same as those for the wild-type strain (data not shown). In addition, the rifampicin resistance of B. pertussis strains was assessed. We found that each strain formed colonies on BG plates supplemented with 0.25 or 0.5 µg rifampicin ml$^{-1}$ within the same time frame, but none of the strains formed colonies on plates containing more than 1 µg rifampicin ml$^{-1}$ until 48 h after plating. These findings indicated that none of the strains
had any suppressor mutations that would confer rifampicin resistance (data not shown).

Notably, production of ppGpp in the absence of SpoT is lethal to E. coli cells because these cells cannot degrade ppGpp (Xiao et al., 1991); nevertheless, we recovered ΔrelAΔspoT double mutant cells with a wild-type relA gene and ΔspoT single mutant cells. Thermus thermophilus is a Gram-negative, aerobic thermophile that has an orthologue of Rsh (RelA/SpoT); Rsh can both synthesize and degrade (p)ppGpp in response to nutritional starvation. In addition to Rsh, a ppGpp degradation pathway mediated by Ndx8 is present in T. thermophilus (Ooga et al., 2009). Additionally, Listeria monocytogenes strains that harbour a transposon insertion in gppH, which encodes a metal-dependent phosphohydrolase, are cold sensitive and contain increased levels of ppGpp (Liu et al., 2006). Neither Ndx8 nor GppH exhibits any similarity to RelA or SpoT in amino-acid sequence. Thus, it may be that B. pertussis exploits a currently unknown system that can degrade ppGpp. Further investigation of this possibility is necessary.

Lack of (p)ppGpp resulted in abnormal growth under nutrient limitation and decreased viability under starvation conditions in B. pertussis

During nutrient limitation, bacteria activate the stringent response to adopt a slow mode of growth. Therefore, we predicted that (p)ppGpp production-deficient B. pertussis mutants would not have an altered growth rate during nutrient limitation. B. pertussis does not have the full set of enzymes for the TCA cycle, and sodium glutamate is used extensively as the main carbon source in catabolic states. To test whether (p)ppGpp mediated the growth arrest caused by depletion of sodium glutamate in B. pertussis, growth of wild-type and PMK21 cells was assayed. Cells were cultured in SS media prepared with 0 %, 20 %, or 100 % of the standard sodium glutamate supply. SS medium contained 10.72 mg sodium glutamate ml\(^{-1}\) and 5 mg Casamino acids ml\(^{-1}\), and therefore contained approximately 1 mg sodium glutamate ml\(^{-1}\) derived from Casamino acids even when sodium glutamate was not added to the media (Justo et al., 2004). Under nutrient-rich conditions with 100 % sodium glutamate, PMK21 cultures grew as well as wild-type cultures (Fig. 2a). Growth of wild-type cultures depended on the amount of added sodium glutamate; specifically, cultures grew more slowly with less added sodium glutamate (Fig. 2b). In contrast, growth trajectories of PMK21 cultures supplemented with 20 % sodium glutamate did not differ from those supplemented with 100 %; moreover, culture growth was only slightly diminished when sodium glutamate was not provided (Fig. 2b). These findings indicated that depletion of (p)ppGpp caused by the relA-spoT mutations in the double mutant cells resulted in loss of B. pertussis growth control under starvation conditions.

This loss of growth arrest under nutrient limitation in PMK21 cultures indicated low adaptation ability and decreased viability associated with the inhibition of the stringent response. Survival of cells subjected to nutrient starvation was monitored by counting the number of viable cells during incubation in PBS. In wild-type cultures, there were 1.2 \(\times\) 10\(^6\) \(\pm\) 0.2 \(\times\) 10\(^6\) c.f.u. ml\(^{-1}\) and 5.1 \(\times\) 10\(^6\) \(\pm\) 0.3 \(\times\) 10\(^6\) c.f.u. ml\(^{-1}\) at 24 h and 48 h, respectively, and some cells...
(1.1 \times 10^4 \pm 0.2 \times 10^4 \text{ c.f.u. ml}^{-1}) \text{ at } 24 \text{ h and none at } 48 \text{ h. These results indicated that (p)ppGpp contributed to prolonged survival under conditions of nutrient limitation.}

**Deletion of (p)ppGpp synthases increased sensitivity to oxidative stress in *B. pertussis***

Reportedly, the stringent response is correlated with resistance to stress in vitro. The disc-diffusion assay was used to assess bacterial sensitivity to MV, which induces oxidative stress. The diameter of the inhibition zone was directly proportional to MV concentration in each strain assayed; however, PMK21 cells showed significantly higher sensitivity to MV than did wild-type cells (Fig. 2c). Plasmid-derived relA was used to assess the effects of overproduction of relA on sensitivity to oxidative stress in wild-type cells. The multicopy relA plasmid raised the bacterial tolerance to MV (Fig. 2d). Therefore, in *B. pertussis*, (p)ppGpp production is probably important for resistance to oxidative stress.

**Fig. 3. (a) Semi-quantification of biofilm formation (OD_{550}) and growth (OD_{600}) of wild-type or PMK21 (ΔrelAΔspoT) *B. pertussis* cells on abiotic surfaces at 24, 48, or 72 h. Open circles indicate growth (OD_{600}). Biofilm mass was measured 24, 48 or 72 h after inoculation (black bars). The asterisks indicate a significant difference compared to the data at 24 h (*, \text{P}<0.05; **, \text{P}<0.01; NS, not significant). (b) Complementation of biofilm formation defects of PMK21. Biofilm mass formed by wild-type cells (WT), PMK21, or PMK21 carrying pRKT01 (PMK21+relA) were measured 24 or 96 h after inoculation. The asterisks indicate significant differences between a mutant and the respective complementation strain (*, \text{P}<0.05; **, \text{P}<0.01). The data are representative of three independent experiments. (c) Autoaggregation of *B. pertussis* cells. Autoaggregation of *B. pertussis* wild-type (●) or PMK21 (ΔrelAΔspoT, ▲) strains was measured by the decrease in OD_{600} of the upper layer of the bacterial suspensions over time. The OD_{600} value of the remaining suspension was defined as 100%. Asterisks indicate statistically significant differences from wild-type (*, \text{P}<0.05; **, \text{P}<0.01). Error bars in biofilm measurement (a and b) and in aggregation ratio (c) represent ±SD from three and three independent experiments, respectively.

**Biofilm formation is an important strategy for bacterial survival in hostile environments.** We predicted that *B. pertussis* modulates biofilm formation via (p)ppGpp. To examine this prediction, mutant and wild-type cells were compared with regard to biofilm formation. There was no significant difference in growth rate between the wild-type and PMK21 cultures (Fig. 3a). The mass of biofilm in wild-type cultures increased over time until 48 h post-inoculation (Fig. 3a) and was sustained from 48 h to 72 h; biomasses of wild-type biofilms at 96 h and at 48 h did not differ significantly from one another (data not shown). However, significantly fewer PMK21 cells remained on the coverslips at 24 h when compared to wild-type cells (\text{P}<0.01), and PMK21 biofilm mass did not increase significantly over 48 h (Fig. 3a). Even after 72 h incubation, PMK21 biofilm mass was much lower than wild-type biofilm mass. Exogenous wild-type relA was introduced into PMK21 cells, and the transformed mutants could form biofilms (Fig. 3b).

Several factors relating to aggregation are important to biofilm formation. We found that (p)ppGpp regulated biofilm formation by *B. pertussis*; therefore, (p)ppGpp may also affect autoaggregation of *B. pertussis*. Aggregation rates increased over time in wild-type cultures, and reached a steady state after 12 h (Fig. 3c). Autoaggregation was reduced in PMK21 cultures; at 24 h, the mean aggregation rate of PMK21 cultures (40.9\pm7.3\%) was significantly lower than that of wild-type cultures (68.7\pm2.5\%).

### Morphology of biofilms formed on abiotic surfaces by wild-type or (p)ppGpp-deficient mutant *B. pertussis*

To further understand the role of (p)ppGpp in biofilm formation, the microstructures of biofilms formed within
24 h of culture were analysed via scanning electron microscopy (Fig. 4). After inoculation, wild-type cells attached to the surface of coverslips, and subsequently added bacterial cells seemed to attach preferentially to previously attached cells. In addition, several kinds of filamentous structures had formed on the surface of wild-type bacterial cells. Filaments were observed on the surface of wild-type biofilms; 187 such filaments were randomly chosen and measured, and the filament lengths were analysed statistically (Fig. S1). Filaments were classified into three groups according to their length (Table 2). The filaments longer than 150 nm ($495.3 \pm 7.7$ nm) varied greatly in size. The length of the intermediate sized filaments ranged between 80 and 150 nm ($101.7 \pm 13.2$ nm), and short filaments ranged between 30 and 80 nm ($49.8 \pm 8.0$ nm). For the PMK21 biofilms that had attached to the surface of coverslips, 130 filaments were randomly chosen and analysed. In mutant biofilms, the short filaments (30–80 nm) were most abundant and were very straight (Fig. 4). In contrast, intermediate-sized filaments were rare (4 of 130 filaments), and no long filaments were evident in any PMK21 biofilm (Fig. S1). Among the filaments on the surface of wild-type biofilms, the short and straight filaments had relatively uniform lengths ($49.8 \pm 8.0$ nm) that were very similar to the length of FHA filaments (Makhov et al., 1994). FHA is encoded by $fhaB$, and is a major mediator of cell–cell adhesion in $B. pertussis$ (Menozzi et al., 1994; Serra et al., 2011). Therefore, $fhaB$ transcript levels and FHA levels in wild-type cultures were compared with those in PMK21 cultures; $fhaB$ transcript levels and FHA levels did not differ significantly between wild-type and mutant cultures (Table 3 and Fig. 5b).

### Analysis of secreted proteins whose expression is affected by (p)ppGpp

To find (p)ppGpp-dependent factor(s) involved in biofilm formation, extracellular proteins in the culture fluids were isolated, separated by SDS-PAGE and analysed (Fig. 5a). For several distinct bands, the amount of protein in the band differed between PMK21 and wild-type samples. Notably, p20 levels and p24 levels were significantly lower in PMK21 samples than in wild-type samples (Fig. 5a); moreover, wild-type $relA$ restored each of these proteins back to wild-type levels in PMK21 + $relA$ cells. The p20 and p24 proteins were analysed via mass spectrometry and identified as Fam3 (coverage rate, 86.83 %; number of unique peptides, 15; score, 18662.5) and Bsp22 (coverage rate, 53.85 %; number of unique peptides, 7; score, 1372.1), respectively. Fam3 is a subunit of fimbria and Bsp22 is the tip of the type III secretion system (TTSS) apparatus. Alterations in gene expression during the stringent response occur because transcription is reorganized. To investigate whether the reduced expression of Fam3 and of Bsp22 in PMK21 was due to decreased transcription of these genes, each mRNA was quantified by qRT-PCR (Table 3). In PMK21 cultures, transcription of $fim3$ and of $bsp22$ was reduced from wild-type levels to $0.03 \pm 0.003$ (32.04-fold reduction) and $0.55 \pm 0.05$ (1.82-fold reduction), respectively. These data indicated that $fim3$ was strongly repressed and $bsp22$ was moderately repressed in (p)ppGpp-deficient mutants, and

### Table 2. Lengths of filamentous structures observed in $B. pertussis$ biofilms

<table>
<thead>
<tr>
<th>Type</th>
<th>Short (nm)</th>
<th>Intermediate (nm)</th>
<th>Long (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>$49.8 \pm 8.0$</td>
<td>$101.7 \pm 13.2$</td>
<td>$495.3 \pm 388.1$</td>
</tr>
<tr>
<td>PMK21</td>
<td>$46.6 \pm 7.7$</td>
<td>$97.1 \pm 5.0$</td>
<td>ND*</td>
</tr>
</tbody>
</table>

*Not detected.

### Table 3. Quantitative RT-PCR analysis of transcripts encoding virulence factors in $B. pertussis$

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative transcripts levels*</th>
</tr>
</thead>
<tbody>
<tr>
<td>$fhaB$</td>
<td>$0.80 \pm 0.02$</td>
</tr>
<tr>
<td>$fim3$</td>
<td>$0.03 \pm 0.003$</td>
</tr>
<tr>
<td>$bsp22$</td>
<td>$0.55 \pm 0.05$</td>
</tr>
<tr>
<td>$cyaA$</td>
<td>$1.85 \pm 0.14$</td>
</tr>
<tr>
<td>$ptxA$</td>
<td>$1.15 \pm 0.11$</td>
</tr>
</tbody>
</table>

*Relative transcript levels were calculated via the $\Delta \Delta CT$ method described in Methods. The ratio of the gene expression level in the PMK21 strain compared to that of the wild-type strain was determined. Each value is shown as a mean ± SD of three independent assays.
were each detected as described in Methods. The SDS-PAGE gel to PVDF membrane, FHA, CyaA, and PTX analysis of FHA, CyaA and PTX. After transferring the proteins in Proteins (p20, p24) that were significantly reduced in the cultures of wild-type, PMK21 (ΔrelAΔspoT), or PMK21 carrying pRKT01 (PMK21ΔrelA) cells as described in Methods. Measurements for each sample were normalized relative to the number of bacteria in the culture as estimated by OD$_{600}$ measurement of original cultures (2 OD$_{600}$ units per lane). Proteins (p20, p24) that were significantly reduced in the (p)ppGpp mutant are indicated with arrowheads. (b) Immunoblot analysis of FHA, CyaA and PTX. After transferring the proteins in the SDS-PAGE gel to PVDF membrane, FHA, CyaA, and PTX were each detected as described in Methods.

therefore, that these two genes were differentially regulated during the stringent response.

Expression of toxins in PMK21 cells

The expression of Fim3 and of Bsp22 was lower in PMK21 than in the wild-type strain, as was that of FHA, which itself was not changed (Fig. 5). CyaA and PTX are expressed during the Bvg$^+$ phase; however, these toxins are not synthesized in the Bvg$^-$ or the Bvg$^g$ phase. To investigate the effect of (p)ppGpp on the expression of Bvg-dependent genes, expression of each toxin was analysed. For cyaA and ptxA, the amount of each transcript was similar between mutant and wild-type cells (Table 3), and production of each protein (CyaA and PTX) was also similar between the strains (Fig. 5b).

DISCUSSION

Biofilm formation is a multi-step process that includes initial attachment, microcolony formation, architectural development via cell–cell attachments and production of extracellular polysaccharides for protection (Stanley & Lazazzera, 2004). Recent studies show that biofilm formation in the host respiratory tract is important for adaptation and survival of B. pertussis during an infection (Serra et al., 2007, 2008). Here, we demonstrated that in B. pertussis, (p)ppGpp was synthesized by RelA and SpoT, and that these (p)ppGpp had an important role in B. pertussis biofilm formation. The (p)ppGpp-deficient mutant strain, PMK21, lost viability in nutrient-depleted conditions and showed increased susceptibility to oxidative stress. Therefore, PMK21 may have a lower general fitness than wild-type bacteria in the biofilm-inducing environments. On the other hand, physiological change caused by the accumulation of (p)ppGpp in bacterial cells would invoke the expression of substances that promote biofilm formation. In Agrobacterium, biosynthesis of curdlan, a homo-exopolysaccharide involved in biofilm formation, is positively regulated during the stringent response (Ruffing & Chen, 2012). Moreover, (p)ppGpp synthases are necessary for transcription of vpsR and vpsT, which encode transcriptional regulators of exopolysaccharide synthesis in Vibrio cholerae (He et al., 2012). Additionally, (p)ppGpp-mediated regulation of biofilm formation is associated with expression of type 1 fimbriae in E. coli (Aberg et al., 2006). Taken together, these observations indicate that the stringent response promotes biofilm formation in many bacteria.

The filamentous structures in wild-type B. pertussis biofilms exhibited diversity in lengths and morphologies, but those in (p)ppGpp-deficient mutants did not (Table 2 and Fig. S1). FHA showing filamentous structure is also an important virulence factor in respiratory tract colonization, induction of biofilm formation and autoaggregation because it mediates inter-bacterial adhesion (Serra et al., 2011). Filaments similar to the short, straight filaments on the surface of wild-type bacteria like FHA also formed on the surface of (p)ppGpp-deficient mutant B. pertussis cells. Additionally, the amount of transcription of fhaB and FHA protein were retained in (p)ppGpp-deficient cells (Table 3 and Fig. 5b).

Here, we demonstrated that Fim3 and Bsp22, which are the subunits of long filamentous structures, were decreased by (p)ppGpp depletion in ΔrelAΔspoT double mutant (Table 3 and Fig. 5a). These filamentous structures may be necessary to stabilize cell–cell attachments and biofilm architecture. Bordetella fimbriae belong to the family of bacterial adhesins and are assembled and secreted by the apparatus encoded by fimBCD. B. pertussis can produce two serologically different fimbriae – Fim2 and Fim3 (Ashworth et al., 1982; Zhang et al., 1985). Transcription of fim2 and of fim3, which each encode different fimbrial subunits, is regulated by phase variation; mutations in the C-stretch, a C-rich region of each promoter (fim2 and fim3), cause on/off switching that leads to phase variation (Willems et al., 1990). The wild-type strain used in this study expresses only Fim3-type fimbriae. Transcription of fim2 in this wild-type strain was not changed by the deletion of the (p)ppGpp synthases (data not shown). In addition to this regulation, binding of BvgAS to the promoter is required for fim2 and fim3 expression (Chen et al., 2010). A fimBCD mutant of Bordetella bronchiseptica that expresses no fimbriae has severely defective biofilm formation in the Bvg$^+$ phase in spite of the fact that FHA
contributes to biofilm development in the Bvg+ phase of B. bronchiseptica (Irie et al., 2004). In the same study, it was found that the fimBCD mutant shows only a slight decrease in biofilm formation in the Bvg+ phase. This observation indicates that the components necessary for biofilm formation differ between the Bvg+ and Bvg− phases, and that fimbrae contribute biofilm formation during Bvg− phase.

Bsp22 is an abundant polypeptide secreted into Bordetella culture fluid and is part of the TTSS apparatus. Bsp22 belongs to a recently identified subfamily of TTSS tip complex proteins; Bsp22 forms self-polymerizing, flexible filaments of variable lengths that are essential for TTSS-mediated killing of eukaryotic cells (Kuwae et al., 2006; Medhekar et al., 2009). Antibodies against Bsp22 result in reduced bacterial colonization in a mouse model of B. pertussis infection; therefore, in vivo expression of Bsp22 is probably important for pathogenicity of B. pertussis (Medhekar et al., 2009). Intriguingly, elevated type III secretion mediates bacterial aggregation, and this phenomenon may have applications for engineered biofilm formation in Salmonella enterica serovar Typhimurium (Jennings et al., 2012). Regulators and effectors of TTSS are also synthesized during the Bvg+ phase in Bordetella, but not during the Bvg− phase (Yuk et al., 1998). The bsp22 promoter is recognized by BtrS, an extracytoplasmic function sigma factor encoded by btr, which is regulated by BvgA (Mattoo et al., 2004). The mechanisms by which (p)ppGpp affected fim3 expression and bsp22 expression in B. pertussis remain unclear; nevertheless, the effects of (p)ppGpp deletion on fim3 differed from those on bsp22, indicating that the regulatory mechanisms also differ (Table 3 and Fig. 5a). (p)ppGpp is a global regulator of transcription, translation, and DNA replication in bacteria and plants (Nomura et al., 2012; Srivatsan & Wang, 2008). TTSS is regulated by BtrS (Mattoo et al., 2004), and sigma factor competition is one of the pleiotropic effects of (p)ppGpp because alternative sigma factor usage is increased as part of the stringent response (Österberg et al., 2011). Reduced amounts of transcription in (p)ppGpp-deficient pertussis mutants could have been due to a decrease in alternative sigma factor usage. Translational regulation may also have occurred.

B. pertussis is a highly adapted human pathogen, and has not been found in the external environment thus far. Therefore, the stringent response may be critical for adaptation to the host environment; this adaptation includes regulation of bacterial pathogenicity and subsequent niche acquisition. Toxins are only produced in the Bvg+ phase. Interestingly, mutation-induced (p)ppGpp depletion did not result in reduced expression of FHA, CyaA or PTX; therefore, (p)ppGpp may be a previously unrecognized factor that affects pathogenesis in parallel with the well-documented Bvg system. Deployment of multiple systems for regulating virulence factors could facilitate bacterial infection. Further investigation will focus on the relationship between the stringent response and Bvg regulation.

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