Published in final edited form as:
Mol Microbiol. 2013 April ; 88(1): 156–172. doi:10.1111/mmi.12177.

In vivo phosphorylation dynamics of the *Bordetella pertussis* virulence-controlling response regulator BvgA

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Summary

We have used protein electrophoresis through polyacrylamide gels derivatized with the proprietary ligand Phos-tag™ to separate the response regulator BvgA from its phosphorylated counterpart BvgA~P. This approach has allowed us to readily ascertain the degree of phosphorylation of BvgA in *in vitro* reactions, or in crude lysates of *Bordetella pertussis* grown under varying laboratory conditions. We have used this technique to examine the kinetics of BvgA phosphorylation after shift of *B. pertussis* cultures from non-permissive to permissive conditions, or of its dephosphorylation following a shift from permissive to non-permissive conditions. Our results provide the first direct evidence that levels of BvgA~P *in vivo* correspond temporally to the expression of early and late BvgA-regulated virulence genes. We have also examined a number of other aspects of BvgA function predicted from previous studies and by analogy with other two component response regulators. These include the site of BvgA phosphorylation, the exclusive role of the cognate BvgS sensor kinase in its phosphorylation in *Bordetella pertussis*, and the effect of the T194M mutation on phosphorylation. We also detected the phosphorylation of a small but consistent fraction of BvgA purified after expression in *Escherichia coli*.

Keywords

Bordetella pertussis; transcriptional activation; RNA polymerase; two-component systems; phosphorylation

Introduction

Throughout their evolution, bacteria faced a number of puzzles that had to be solved to enable their adaptation to multiple, variable environments. One of these was how changes in external conditions could be transduced into relevant adaptive changes taking place in the cytoplasm, particularly changes in gene expression. A highly successful solution to this problem is represented by two-component systems. These systems typically comprise a membrane-spanning sensor kinase protein (SK), the activity of which is responsive to relevant environmental or physiological signals, together with a cognate target response regulator (RR) protein. The degree of phosphorylation of a given RR is generally an indicator of its activity as a transcriptional activator of relevant genes. Two-component systems are found in virtually every species of bacteria and have been found to regulate

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virtually all cellular processes. These processes include essential metabolic and physiologic activities but also secondary characteristics that confer highly adaptive traits for specialized environments, such as virulence factors of bacterial pathogens.

Given their importance, it is not surprising that two-component systems have been the subjects of intensive study. *In vitro* biochemical methods have been crucial in describing basic functional aspects, notably the flow of phosphate (Uhl & Miller, 1994). Genetic approaches, while contributing to these *in vitro* studies, have been particularly useful *in vivo.* For example, while the phosphorylation of response regulators by sensor kinases was first demonstrated *in vitro*, the importance of this *in vivo* can be investigated by altering the conserved aspartate residue that is the site of phosphorylation. Experiments like this demonstrate the importance of phosphorylation in a qualitative sense and indicate the extreme phenotype resulting from a complete lack of phosphorylation (Lukat *et al.*, 1991). But quantifying the level of a phosphorylated protein relative to its unphosphorylated form has been difficult. Response regulators, which are phosphorylated at an aspartate residue, have been particularly challenging, in part because the phospho-Asp acylphosphate bond is easily hydrolysed (Lukat et al., 1991). Although a number of approaches have been successfully used to assess *in vivo* phosphorylation, these have generally been cumbersome or have involved radioactive labeling of response regulators (Head *et al.*, 1998, Idelson & Amster-Choder, 1998, Ladds *et al.*, 2003).

Recently a simpler, less expensive, and more direct procedure has been employed (Barbieri & Stock, 2008). This approach uses electrophoresis of total cellular proteins through a polyacrylamide gel cast from monomers derivatized with a proprietary adduct termed Phos-tag™. Phos-tag™ is a dinuclear metal complex, which together with Zn^{2+} or Mn^{2+}, forms a complex with a phosphomonoester dianion, such as the phosphorylated aspartate of a RR. During SDS-PAGE through this matrix, the phosphorylated protein migrates more slowly than its non-phosphorylated form (Barbieri & Stock, 2008). As a result, a Western blot of total cellular proteins separated on a Phos-tag™ gel can be interrogated using antibody specific to a protein of interest. The relative amounts of the two species can then be estimated.

We report here our application of this method to the BvgAS two-component system involved in regulation of virulence in *Bordetella pertussis*. In this human respiratory pathogen, the BvgAS two-component system is a global regulator that controls the expression of over 100 virulence genes in *Bordetella* species (Cummings *et al.*, 2006, Hot *et al.*, 2003). Different laboratory conditions are known to modulate activity of this system and consequently, the level of virulence gene expression. At least three different modes (Bvg^+, Bvg^i, Bvg^-) correspond to three different expression patterns for virulence genes. In the Bvg^+ mode (non-modulating condition) characterized by growth in the absence of modulating factors, all of the virulence genes are expressed. In the Bvg^- mode, i.e. under “modulating” conditions such as a temperature of 25°C or presence of nicotinic acid or $MgSO_4$, virulence gene expression is off [for review,(Cotter & Jones, 2003, Decker *et al.*, 2012)]. The Bvg^i mode is manifested under growth conditions between these two extremes. Some virulence genes, so called “early genes”, are expressed but others, termed “late genes”, are not. This difference has been attributed to a requirement by late genes for higher levels of BvgA~P. Another feature of the intermediate mode is the maximal expression of another class of genes, most notably *bipA*. This pattern of expression has been shown to be due to the presence of an upstream high-affinity BvgA-binding site that activates expression, even at low BvgA~P levels, together with low-affinity BvgA-binding sites downstream that can repress *bipA* transcription at high levels of BvgA~P (Williams *et al.*, 2005). These features have led to the characterization of the BvgAS system as a “rheostat” rather than an

on/off switch. As such, the ability to determine *in vivo* levels of BvgA phosphorylation is especially relevant.

Previous work has identified mutations in *bvgS* and *bvgA* that affect their behavior in ways that have been interpreted in terms of their hypothetical effects on BvgA phosphorylation. Some *in vitro* work supports these interpretations, but the ability to test their validity by monitoring effects on phosphorylation *in vivo* has heretofore been lacking. Similarly, modulation has been predicted to control the level of BvgA phosphorylation *in vivo*, but this has not been testable until now. In this report we demonstrate the use of the Phos-tag™ gel system to address these and related issues.

Results

BvgA and BvgA~P can be separated by Phos-tag™ SDS-PAGE

To determine if SDS-PAGE with Phos-tag™ could be used to separate BvgA~P from BvgA, we initially followed the protocol reported by Barbieri *et al.* for the analysis of *E. coli* PhoB and PhoB~P (Barbieri & Stock, 2008). PhoB and BvgA have similar molecular weights; 26kDa and 23 kDa, respectively. Initially unsuccessful, we tried recently described modifications including performing electrophoresis at neutral pH and substituting Zn2+ in place of Mn2+. Both have been reported to increase the separation between phosphorylated and non-phosphorylated species (Kinoshita & Kinoshita-Kikuta, 2011). We phosphorylated BvgA *in vitro* with acetylphosphate (Ac~P) or treated with buffer as a control, and performed SDS-PAGE in a gel containing Zn^{2+} and the Phos-tag™ ligand. Different amounts of BvgA (Fig. 1A, lanes 1-3), BvgA~P (lanes 4-6) or an equal mix of both forms (lane 7) were electrophoresed on a Phos-tag™ polyacrylamide gel.

Under these conditions, BvgA and BvgA~P migrated at distinct positions and were easily separated on the gel. The amount of protein quantified in each band was proportional to the amount of protein loaded (data not shown). Treatment of BvgA with Ac~P (Fig. 1A, lanes 4-6) resulted in the majority of the protein being phosphorylated. Mixing BvgA and BvgA~P together prior to electrophoresis (lane 7) gave the expected ratio of nonphosphorylated to phosphorylated protein, indicating no significant loss of BvgA~P during the electrophoresis. Surprisingly, (as discussed further below), the purified, untreated BvgA appeared to contain a trace of phosphorylated protein (lane 3).

SDS-PAGE with Phos-tag™ can be used to determine the levels of BvgA and BvgA~P in vivo

To investigate whether the same approach could be used *in vivo* in *B. pertussis* we lysed cells with formic acid and then neutralized the acid with NaOH as described for *E. coli* (Barbieri & Stock, 2008). After electrophoresis, BvgA was visualized by Western analysis. This analysis demonstrated that BvgA and BvgA~P generated *in vivo* and in the context of a heterogenous sample migrated similarly to their *in vitro* counterparts (Fig. 1B, lanes 1-3 versus lanes 6 and 7). To determine whether the procedure for generating the cell lysate affected the level of BvgA~P observed we tested different conditions for processing the cells. The maximum level of BvgA~P was observed when either no NaOH or 170 mM NaOH was added to the lysate (Fig. 1B, lanes 1 and 2, respectively). BvgA~P was not detected when 500 mM or a higher concentration of NaOH was used (Fig. 1B, lanes 4 and 5). This is unlike the case of PhoB, for which a high level of NaOH could be used (Barbieri & Stock, 2008).

Because of the inherent instability of the phospho-aspartate bond, it was especially important to determine whether the treatments used for cell lysis and subsequent gel electrophoresis affected the level of BvgA~P we detected. To investigate this possibility, we

added a constant amount of BvgA~P, formed by phosphorylation *in vitro* with Ac~P, to cell pellets, lysed the cells as above, and incubated the lysed samples on ice for different times. As shown in Fig. 1C, lanes 4-12, no significant difference in the amount of BvgA~P was observed, indicating that the extent of dephosphorylation of BvgA~P during the process was negligible.

These results demonstrate that the degree of BvgA phosphorylation can be quantitated easily and directly, both *in vivo* and *in vitro,* using Phos-tag™ technology. This experimental approach therefore allowed us to investigate the levels of BvgA~P in *B. pertussis* under a range of relevant growth conditions and genetic contexts.

Modulation of virulence gene expression in B. pertussis by $MgSO_4$ occurs through a reduction in the level of BvgA~P

It is known that growth in the presence of 50 mM $MgSO_4$ results in a Bvg^- state, in which expression of the BvgA-activated genes is turned off. It has been assumed that the effect of $MgSO_4$ and other modulating conditions is to reduce the level of BvgA~P *in vivo*. However, this assumption has not been tested directly. The Phos-tag™ system allowed us to directly observe the levels of BvgA and BvgA~P and to follow the kinetics of phosphorylation after cells were modulated or relieved from modulation.

We determined levels of BvgA~P and BvgA in *B. pertussis* before, and at various times after, the addition of $MgSO_4$. We also performed the complementary experiment in which cells grown in the presence of $MgSO_4$ were shifted to a permissive medium lacking the modulator. As seen in Fig. 2A, in both cases, the cultures were growing exponentially over the time course of this experiment (with the exception of the 24 hour time point).

After many generations of growth in the presence of $MgSO_4$, phosphorylated BvgA was not detected (Fig. 2B, lane 1) and the amount of BvgA protein was low. With the switch to permissive conditions (removal of $MgSO_4$), BvgA~P could be detected within 5 minutes (lane 2), and the level of phosphorylation increased rapidly as incubation continued (lane 2-9) ultimately reaching a maximum level of 83% after two hours. In addition, the overall level of BvgA increased. This finding is reasonable since BvgA~P is known to activate expression of the *bvgAS* locus itself (Roy & Falkow, 1991, Scarlato *et al.*, 1990). The reason for an apparent decrease in BvgA~P relative to BvgA at 24 hours is unknown at this time. Phosphorylation of all of the BvgA was never observed, even after continued incubation in the absence of $MgSO_4$. Given that our control analyses suggested that BvgA~P is stable under our conditions (Fig. 1C), this result indicated a balance between the kinase activity of BvgS and mechanisms of BvgA dephosphorylation, either spontaneous or mediated by a hypothetical phosphatase activity of BvgS.

The kinetics of virulence gene expression following a shift from modulating to non-modulating conditions have been demonstrated previously, most notably by Scarlatto et al. (1991). Experiments such as this led to the concept of “early” and “late” genes, typified by *fha* and *ptx*, respectively. It has been assumed that the differential expression patterns observed between these two classes were orchestrated by rising levels of BvgA~P following relief of modulation. Indeed, Scarlatto et al. showed, as we have here, that overall levels of BvgA protein were increasing over the time course of their experiment. We have been able, through the use of the electrophoresis system described here, to extend this observation and to demonstrate that, as predicted, the level of BvgA~P correlates with gene expression. The early *fha* promoter can be activated at relatively low levels of BvgA~P, while activation of the late *ptx* promoter does not occur until higher levels have been reached. Since our study was performed somewhat differently from that of Scarlatto et al., we verified that differential expression of *fha* and *ptx* was seen in our hands as well. As shown in Fig. 2A,

generation of light resulting from expression of *lux* fusions to these two promoters followed the same general patterns of early and late expression under our conditions of growth in PLB and induction by removal of MgSO4 as had been observed previously after growth in Stainer-Scholte medium and temperature shift (Scarlatto et al., 1991).

Lysates of cells obtained after long-term growth in permissive conditions contained approximately equal levels of BvgA and BvgA~P (Fig. 2C, lane 1). Upon the addition of $MgSO_4$, there was a rapid decrease in the level of BvgA~P (lane 2-9) with no detectable BvgA~P remaining after 15 minutes of incubation (lane 5). However, the total amount of BvgA protein actually appeared to increase with continued incubation, until 15 minutes after addition of MgSO4, and then remained stable for hours. This finding is consistent with the idea that the BvgA protein itself is stable *in vivo*, but may also indicate a hitherto unappreciated regulatory process that stimulates BvgA synthesis immediately upon modulation. After 24 hours, the level of BvgA had decreased somewhat. This may indicate a very slow return to the low level observed at the zero time point in the panel above, which represents long term (2 days) growth on solid media under modulating conditions.

BvgS is the only kinase that phosphorylates BvgA in B. pertussis

Genetic evidence supports the idea that BvgS is the major effector of BvgA phosphorylation. The *bvgS* gene is the locus affected by mutations that result in a variety of phenotypes, including a null phenotype, resistance to modulation, and the intermediate phase phenotype (Cotter & Miller, 1997, Goyard *et al.*, 1994, Manetti *et al.*, 1994, Miller *et al.*, 1992). However it is not known if any other *B. pertussis* factors can phosphorylate BvgA. To address this directly, we determined the level of BvgA phosphorylation in wild-type and mutant *B. pertussis* (Fig. 3). As observed previously, BvgA~P was present when wild-type cells were grown in permissive conditions (Fig. 3B, lane 1), but only BvgA was observed under non-permissive growth conditions (lane 2). Growth under permissive or non-permissive conditions of a strain in which *bvgS* had been deleted (strain QC2935, depicted in Fig. 3A) also resulted in no BvgA phosphorylation, and the overall amount of BvgA protein was low (lanes 3 and 4), indicating that BvgS is the only kinase that phosphorylates BvgA in *B. pertussis* under these, typical, growth conditions. As expected, no BvgA was detected when *bvgA* was deleted (Fig. 3B, lanes 5 and 6; strain BP1526 depicted in Fig. 3A). The lack of any detectable species in lanes 5 and 6 also demonstrated the high specificity of the BvgA antibody used in these experiments.

Mutation of BvgA residue D54 eliminates BvgA phosphorylation in B. pertussis

Key amino acids involved in BvgA function can be described as belonging to three groups (Dyer & Dahlquist, 2006, Ruiz *et al.*, 2008): a phosphoaccepting aspartate, catalytic residues, and conformational switch residues. These three groups of amino acids have been identified for CheY (Dyer & Dahlquist, 2006). Although BvgA contains 11 aspartic acid residues, D54 has been assumed to be the phospho-accepting aspartate based on protein sequence alignments of BvgA with other well-characterized response regulators, such as CheY, PhoB, and OmpR, as well as NarL, on which the structure of BvgA has been modeled (Fig. 4A, residue shaded in cyan). In addition, this mutation confers a Bvg^- colonial phenotype (flat, non-hemolytic) and renders BvgA incapable of activating *fha-lux* or *ptx-lux* transcriptional fusions *in vivo* (data not shown). Consequently, we introduced D54N substitution into the cloned *bvgA* gene driven by an IPTG inducible promoter on the plasmid pSS4983. This expression plasmid, together with those containing wild-type *bvgA*, or no insert, were transferred to *B. pertussis* strain QC3216, in which *bvgA* together with the *bvgAS* promoter were deleted and replaced with the constitutive promoter $P_{trc\text{-}v5}$ (depicted in Fig. 3A) to drive *bvgS* expression in a constant, non-Bvg-dependent fashion.

Cells were grown in permissive conditions either in the absence of IPTG, which yielded only a very low level of the plasmid-encoded protein (Fig. 4B, lanes 3-5) or in the presence of IPTG, which yielded high levels of protein (lanes 6-8). The wild-type BvgA that was thereby produced *in trans* was phosphorylated (lane 6), but as expected, no phosphorylation was observed of the D54N (lane 7) mutant protein. In addition, no BvgA protein was detected when the empty vector-containing strain was induced (lane 8). A parallel experiment was performed *in vitro*, using Ac~P to phosphorylate BvgA or $BvgA_{D54N}$. As shown in Fig. 4C, phosphorylation of wild-type BvgA was very rapid, with 75% of BvgA phosphorylated in less than 2 minutes. However, no phosphorylation of $BvgA_{D54N}$ was detected, even at the longest time point examined (15 minutes). These results demonstrate directly that BvgA phosphorylation requires D54. Given these results and the sequence homologies among response regulators (Fig. 4A), it is highly likely D54 is the target of phosphorylation.

Effects of mutation at BvgA T194 on phosphorylation by Ac~P

Two different substitutions of the threonine residue at position 194 have been examined *in vivo* in at least two different experimental contexts, with different results. Jones et al. (2005) observed an effect of the T194M mutation on late gene (*cya*) expression, while Boucher et al. (2003) found no impact of the T194C mutation (in the context of C93A and C103A) on *fha* or *ptx* expression. As shown in Fig. 5, in the same genetic context used above for $BvgA_{D54N}$, following IPTG-induction, $BvgA_{T194M}$ was phosphorylated less efficiently than wild-type BvgA. This finding was consistent with our observation that, on BG agar containing IPTG, the $BvgA_{T194M}$-containing strain demonstrated markedly less hemolysis (data not shown).

When expressed in E. coli, BvgA is phosphorylated by a BvgS-independent process

A small amount of BvgA~P was detected in our purified BvgA, which was isolated after high level expression from a plasmid in *E. coli* BL21(DE3) pLysE cells (Fig. 1A, lane 3). Furthermore, during *in vitro* transcription assays, a trace of BvgA~P-dependent transcription has been observed even when the purified BvgA has not been phosphorylated by Ac~P, when high concentrations of BvgA have been used (data not shown). These results suggested that BvgA might be phosphorylated by an *E. coli* kinase or by some other BvgS-independent process.

To test this possibility, we compared the levels of BvgA~P present in *E. coli* containing IPTG-inducible pET21a(+) expression plasmids for wild-type BvgA or $BvgA_{D54N}$ (Fig. 6A). When using low concentrations of IPTG, the level of either wild-type or mutant BvgA protein was low, and no BvgA~P was detected in either case (lanes 3-5 and 8-10). However, at higher levels of IPTG (0.5 or 1 mM), the level of BvgA increased sharply (lanes 6, 7, 11 and 12) and a species that co-migrated with BvgA~P was detected when using the wild-type BvgA plasmid (lanes 6 and 7). This species was not present in the D54N extracts (compare lane 6 to 11 and 7 to 12). Neither BvgA nor BvgA~P was seen when *E. coli* containing the empty vector was analyzed (lanes 13 and 14). We conclude that BvgA can be phosphorylated in *E. coli* and that this phosphorylation occurs at the D54 residue. Under these conditions, BvgA~P constitutes about 5% of the total amount of BvgA.

Uhl and Miller (1995) previously reported that BvgAS-mediated activation of the *ptx* promoter in *E. coli* was dependent on growth medium, being higher when grown in M9-glucose minimal medium than when grown in LB. It was possible that, in those experiments, these different growth conditions might have also affected the background, non-BvgS-mediated phosphorylation we have demonstrated here. To determine if this was the case we repeated our analysis with BvgA-expressing *E. coli* strains grown in LB, PLB, or M9-

glucose broth. As shown in Fig. 6B, lanes 1-3, no effect of growth medium was observed. As shown in lane 4, in the absence of the BvgA expression plasmid, no BvgA or BvgA~P was detected.

The presence of DNA but not RNAP stimulates in vitro *phosphorylation of BvgA by Ac~P*

Non-phosphorylated BvgA has been shown to bind the promoter regions of some *B. pertussis* virulence genes, including *fhaB* (Boucher et al., 1997, Roy & Falkow, 1991), and *fim3* (data not shown). Previous work has also demonstrated that DNA binding stimulates phosphorylation of the RR OmpR (Ames *et al.*, 1999, Head et al., 1998). We wondered if DNA binding could either accelerate or impair phosphorylation of BvgA or whether the presence of BvgA's transcriptional partner, RNA polymerase (RNAP), might affect phosphorylation. We used *E. coli* RNAP for this study since previous work has documented that it behaves similarly to *B. pertussis* RNAP in terms of promoter binding and transcriptional activation by BvgA~P (Baxter *et al.*, 2006, Boucher et al., 1997, Decker *et al.*, 2011, Steffen & Ullmann, 1998), We found that the kinetics of BvgA phosphorylation by Ac~P in the presence of RNAP were the same as those observed with BvgA alone. However, as shown in Fig. 7, in the presence of plasmid DNA consisting of either the BvgA-regulated promoter $P_{fim3\text{-}15C}$ or P_{fhaB} cloned into pTE103 (Chen *et al.*, 2010, Decker et al., 2011) BvgA phosphorylation was stimulated as previously observed for OmpR (Ames *et al.*, 1999). Approximately 50% of the BvgA was phosphorylated 20 seconds after addition of Ac~P in the presence of $P_{fim3\text{-}15C}$ or P_{fhaB} while only 16% was phosphorylated in absence of DNA (Fig. 7). Interestingly, stimulation of BvgA phosphorylation was observed to the same degree even when empty pTE103 vector was added, indicating that the stimulation did not depend on the presence of sequences known to interact with BvgA.

Discussion

It has been nearly three decades since the recognition of two-component regulatory systems as a distinct paradigm for environmentally responsive gene regulation (Ninfa & Magasanik, 1986). Since that time a great deal of research has given us a significantly more detailed and comprehensive understanding of the structure/function relationships involved in their action. Much of the more precise physicochemical description of these mechanisms has come from structural determination and from biochemical studies performed *in vitro*. But there has also been a synergy between these and genetic and functional studies performed *in vivo*. The latter have helped to put mechanistic knowledge into a relevant context and have provided a "reality check" to test the implications and conclusions of *in vitro* studies. In this regard, it has been frustrating that arguably the most salient feature of two-component systems, i.e. the activation of the response regulator component by transfer of phosphate from the sensor histidine component, has been difficult to monitor in *in vivo* systems. This is perhaps particularly true with two-component systems such as BvgAS, where phosphorylation is thought to be not simply an on/off switch, but rather to produce different effects at different promoters at different levels of BvgA~P. In this regard, at least three different classes of *B. pertussis* Bvg-activated genes (early, late, and intermediate) have been described.

Studies of protein kinases that have tyrosine, or serine as targets, i.e. mostly eukaryotic systems, have benefited greatly from the availability of specific antibody reagents to detect and measure these modifications *in vivo*. However analogous tools have not been developed for two-component systems. A number of other approaches to this issue have been employed. Head et al (1998) separated purified OmpR from OmpR~P using HPLC and were able to quantify the extent of phosphorylation *in vitro*. Ladds et al (2003) demonstrated that Spo0A of *B. subtilis* was phosphorylated *in vivo* when produced in *E. coli*. In this case, Spo0A and Spo0A~P were separable by gel filtration chromatography or native gel electrophoresis of trypsin-digested purified protein. SacY of *B. subtilis* was shown to be

reversibly phosphorylated *in vivo* by ^{35}S-labeling following specific induction of a cloned *sacY* gene and subsequent separation of SacY and SacY~P by 2-D gel electrophoresis (Idelson & Amster-Choder, 1998). In all of these examples, demonstration of RR phosphorylation did not readily lend itself to routine *in vivo* examination of multiple conditions or time points.

In contrast, using the recently available Phos-tag™ ligand in gel electrophoresis, we were able to readily ascertain the level of BvgA~P *in vivo* and *in vitro*, under multiple conditions and at varying time points. As we began these studies, an initial overriding concern, given the intrinsic lability of the aspartyl-phosphate bond, was the stability of BvgA~P. Failure to maintain the modification during the time and under the conditions required for sample processing could confound a reliable assessment of *in vivo* BvgA~P levels. Gratifyingly, simple control experiments have indicated that, under the conditions we employed, the amount BvgA~P in our samples did not significantly decline over the time course of a typical experiment. In addition, in some samples (see Fig. 2B, lane 6) a majority of the BvgA was found to be phosphorylated, again indicating that significant degradation did not occur.

As a dynamic application of this technique, we monitored the kinetics of BvgA phosphorylation following a shift of *B. pertussis* from permissive (i.e. non-modulating) to non-permissive (i.e. modulating) growth conditions, or the reverse. It has previously been observed that after a shift from non-permissive to permissive conditions, so-called “early genes” such as *fha* were expressed within minutes, while other, “late genes”, such as *ptx* and *cya*, were expressed only after several hours (Scarlato *et al.*, 1991). A presumably related observation is the separation of early and late gene expression at different concentrations of modulating agents (Stibitz, 1998). Scarlato et al. interpreted the lag in late gene expression in their time course experiment to be the time required for BvgA~P levels to reach a higher threshold required for *ptx* and *cya* activation, compared to that required for *fha* activation. Indeed these researchers observed an increase in total BvgA concentration with time following induction, consistent with the known autoregulation of the *bvgAS* locus. In this study we have been able to recreate this scenario, but with the added ability to monitor BvgA phosphorylation. Although none was observed at the zero time point, at 5 minutes after a shift to permissive conditions, a significant amount of BvgA~P was present. This corresponds to a minimal level sufficient to activate the “early” *fha* promoter. Both the total amount of BvgA, as well as the level of BvgA~P increased dramatically over the following minutes and hours, reaching a maximum at 2-6 hours. These results are in marked agreement with prediction, and offer the first direct demonstration that the timing and degree of BvgA phosphorylation coincides with early and late promoter activity.

In the reverse experiment, involving a shift to non-permissive conditions, a number of unexpected observations were made. One was that the initial steady state level of total BvgA, after long-term growth under permissive conditions, was lower than expected (Fig. 2C, lane 1) and was in fact comparable to the initial amount after long term growth under non-permissive conditions. As expected, BvgA~P declined rapidly and was absent by 15 minutes. Unexpectedly, the total amount of BvgA appeared to increase to a maximum at the same time. This higher level appeared quite stable, remaining elevated at 2 hours, and declining somewhat by 24 hours. Both the mechanism leading to, and the utility of, this increase are unknown at this time. However, we can speculate that it could represent an adaptation involved in inter-host transmission. Transmission of *B. pertussis* is likely to involve a brief sojourn in aerosolized particles; assuming non-permissive conditions in these droplets, the total level of BvgA could increase during this time. Upon subsequent restoration of permissive conditions after redeposition in a human respiratory tract, rapid

phosphorylation would lead to an initially high level of BvgA~P, thus favoring early synthesis of crucial virulence factors.

We have been able to directly test several other prevailing assumptions regarding BvgAS function predicted by analogy with other two-component systems. For example, we have shown that BvgS is necessary and sufficient for phosphorylation of BvgA *in vivo*. Crosstalk between two-component systems, i.e. the ability of a sensor kinase to phosphorylate a non-cognate response regulator, although found infrequently, is an important issue to address. Our finding that deletion of the *bvgS* gene abolished BvgA phosphorylation indicates that, under standard laboratory growth conditions, BvgA does not receive inputs from other sensor kinases. This experiment also provides evidence against *in vivo* phosphorylation of BvgA by small molecule phospho-donors such as Ac~P, as was reported for the *E. coli* RR CpxR (Lima *et al.*, 2012). Based on protein sequence alignment of BvgA with more fully characterized response regulators, it is predicted that aspartic acid residue number 54 is the site of phosphorylation. In support of this prediction, we have shown that substitution with asparagine abolished phosphorylation of BvgA *in vivo* and *in vitro*. Another property of interest is one that has been reported for OmpR of *E. coli*, but is not necessarily predicted for BvgA. This is the stimulation of phosphorylation by interaction with a cognate DNA binding site. In cases where this has been shown, it has been posited that binding to a high affinity site might shift the equilibrium of the phosphorylation reaction by shifting a conformational equilibrium from a non-activated toward an activated structure. We used the Phos-tag™ gel system to monitor phosphorylation of BvgA *in vitro* in the absence and presence of plasmid DNA containing either the *fim3* or the *fhaB* promoter, each of which contains a high affinity BvgA binding site. Phosphorylation was significantly stimulated by the presence of the DNA. Even at the zero time point, corresponding to simply addition of Ac~P, mixing and freezing, phosphorylation was approximately 20% complete. Interestingly, the empty vector DNA had the same effect, suggesting that this stimulation was not the result of BvgA interacting with specific binding sites, but rather to interactions with the DNA backbone, i.e. in a non-sequence-specific way. This is not altogether surprising when one recalls that the crystal structure of the C-terminal domain of the homologous NarL RR bound to DNA indicated the presence of extensive backbone contacts (Maris *et al.*, 2002).

Similarly, we looked for stimulation by the presence of RNAP in the phosphorylation reaction. In this case no stimulation of phosphorylation with Ac~P was observed. However, the possibility remains that phosphorylation by BvgS might respond to such conditions.

Mutations at the threonine residue at position 194 have been examined in at least two different experimental contexts. Jones et al. (2005) isolated the BvgA T194M mutation after an *in vivo* screen for mutants that failed to express Bvg^+ mode genes but did express Bvg^- and Bvg^i genes. When kinetics of expression were examined, it was found that the early gene *fha* and the intermediate gene *bipA* were somewhat delayed but eventually reached wild type levels. These observations were suggestive of a defect in phosphorylation. *In vitro* phosphorylation studies using either Ac~P or truncated BvgS supported this. In contrast, the T194C mutation, in the context of the C93A and C103A mutations did not adversely affect BvgAS-mediated activation of *fha* or *ptx in vivo*, and when conjugated with Fe-BABE, this protein successfully formed productive transcription complexes with RNAP at BvgA-regulated promoters *in vitro* (Boucher et al., 2003, Chen et al., 2010). In this study, we found that, while $BvgA_{T194M}$ phosphorylation *in vitro* using Ac~P (probably at higher concentrations than used by Jones et al.) was not impaired (data not shown), the kinetics of $BvgA_{T194M}$ *in vivo* were indeed somewhat impaired. Taken together, these results support a role for the T194 residue in efficient phosphorylation at D54, perhaps by a conformational effect.

In vitro experimentation with BvgA has invariably utilized recombinant protein produced in *E. coli*. A working assumption has been that this protein, produced in the absence of BvgS, is not phosphorylated. However, we have shown in this study that a low, but constant, fraction of BvgA purified from *E. coli* is in fact phosphorylated by a BvgS-independent process. It is quite possible that this represents two-component crosstalk, i.e. phosphorylation of BvgA by a non-cognate sensor kinase protein. The observation that this phosphorylation requires the aspartate 54 residue is consistent with this, but not dispositive. We are currently working to identify the *E. coli* factor responsible. However, whatever the source, the presence of this low level of BvgA~P requires us to examine more closely activities ascribed to BvgA preparations heretofore assumed to be devoid of phosphorylation. An example of this is the ability of BvgA to bind to the high-affinity binding site in the *fha* promoter, first shown by Roy and co-workers (Roy & Falkow, 1991). Similarly, in *in vitro* transcription assays, we have consistently observed low basal levels of activation of the *fhaB* and *fim3* promoters, even in the absence of acetyl phosphate (Chen et al., 2010). We consider it quite possible that these activities are due to the low level of BvgA~P in such preparations. Once the source of this *E. coli* kinase activity has been identified and inactivated, we will be able to produce truly unphosphorylated BvgA to test this hypothesis.

Experimental Procedures

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in LB broth, on LB agar, or in M9 medium supplemented with 2% glucose (Miller, 1992). *B. pertussis* strains were grown on Bordet Gengou (BG) agar described in Chen, et al., (2010). For the BvgAS modulation and induction kinetics assays, *B. pertussis* strains were grown in liquid Pertussis LB (PLB) (Vanderpool & Armstrong, 2001), in which the LB broth was supplied with 0.12% Dimethyl β cyclodextrin (Cyclodextrin Technologies Development Inc.) and 0.2% bovine serum albumin (BSA, Sigma). Unless indicated elsewhere, the antibiotics in the culture media were used in following concentrations in LB for *E. coli* strains: ampicillin, 100 μg/mL; gentamicin, 5 μg/mL; kanamycin, 50 μg/mL. Antibiotic concentrations used in BG agar or PLB for *Bordetella* strains were: streptomycin, 50μg/mL; gentamicin, 5 μg/mL; kanamycin, 20 μg/mL.

Plasmid and strain constructions

The allelic exchange plasmid pSS4661 was used for the introduction of defined deletions into the *B. pertussis* BP536 chromosome. This vector can be transferred to *B. pertussis* strains by conjugation but is incapable of replication. Thus, integration by recombination can be forced after transfer by selection for kanamycin resistance. A second recombination event leading to allelic exchange can then be forced by initiating cleavage of an I-*Sce*I site within the vector, in a manner similar to that described by Posfai et al. (1999). The I-*Sce*I restriction enzyme catalyzing this cleavage is encoded within the vector but is not expressed until the *ptx* promoter driving its expression is activated by removal of $MgSO_4$ from the growth medium. Details of the construction of pSS4661 and related plasmids will be published separately. Plasmids constructed to introduce specific deletions in the *B. pertussis* chromosome consisted of sequences flanking and defining the desired deletion endpoints, cloned between the *Not*I and *Bam*HI sites of pSS4661. To create the *bvgS* deletion strain QC2935, the plasmid pQC1805 was used. The deletion in this construct removed all but the first five and last five codons of the *bvgS* orf and was flanked on both sides by segments of approximately 800 bp (Fig. 3A). In the construct pSS4871, similarly sized segments flanked a similarly designed inframe deletion of the *bvgA* gene. In addition, a segment that extended from 61 bp upstream of the *bvgA* start codon to the nearby *Eco*RI site and which included

the native *bvgAS* promoter was replaced with the sequence GAATTCagctgTTGACAattaatcatccggctcgTATAAGgtgtggaattgtgaGTCGAC, which contains a variant of the *trc* promoter ($P_{trc\text{-}v5}$). This plasmid was used to perform allelic exchange on BP536 resulting in strain QC3216 (Fig. 3A).

The *Bordetella* expression plasmid pQC1883 was derived by the addition of a PCR fragment containing the *lac* promoter and the *lacI*^{q} gene from the *relA* expression plasmid pALS10 (Svitil *et al.*, 1993) to pTM203, itself derived from the broad host-range vector pBBR1 (Antoine & Locht, 1992) by the addition of a kanamycin resistance gene and transcription terminators flanking a multiple cloning site. Plasmid pSS4983 consists of pQC1883 plus a PCR-generated fragment comprising the wild-type *bvgA* orf together with an AAGGAG ribosome binding site, downstream of, and driven by, the IPTG-inducible *lac* promoter of pQC1883. The D54N and T194M substitutions were introduced into pSS4983 to create pSS5027 and $pBvgA_{T194M}$, respectively, by a variation on the method of Stemmer and Morris (1992) or through the use of the QuickChange kit (Agilent). These pQC1833-based expression plasmids were introduced into *B. pertussis* strain QC2316 by conjugation using *E. coli* strain SM10 as a host donor strain. For the expression of wild-type BvgA and mutant derivatives in *E. coli,* preparatory to purification, derivatives based on the pET21a+ vector (Novagen) were constructed.

Plasmid pQC1552 contains a PCR-generated *ptx* promoter fragment cloned between the upstream *Eco*RI and *Sal*I sites upstream of *luxCDABE* in the promoter assay vector pSS3967 (Chen et al., 2010). Similarly, plasmid pQC1557 contains a PCR-generated *fhaB* promoter fragment. The resulting pSS3967-based plasmids were integrated as single copies at a specific ectopic chromosomal location in *B. pertussis* BP536 following conjugation using *E. coli* SM10 strain as a host donor strain as described previously (Chen et al., 2010).

Proteins

E. coli RNAP core was purchased from Epicentre Technologies. Purification of σ^{70} was done using a modification of the method of Gribskov and Burgess (Gerber & Hinton, 1996, Hernandez *et al.*, 1996) using *E. coli* BL21(DE3)/pLysS (Studier *et al.*, 1990) containing the plasmid pLHN12 (Hernandez et al., 1996, Nguyen, 1996), which expresses *rpoD*.

Wild type, D54N, and T194M, BvgA were purified as previously described (Chen et al., 2010) except that the white precipitate formed after dialysis of the Hi-Prep 16/10 Q Sepharose flow-through fraction and centrifugation was resuspended in the inclusion body storage buffer [50 mM HEPES-OH (pH 7.5), 0.1 M NaCl, 1 mM EDTA, 1 mM DTT, 20% glycerol] and diluted to a final concentration of 0.15 mg/mL in 50 mM Na_2HPO_4 (pH 6.8), 6 M guanidinium-HCl, 2 mM $MgCl_2$, 1 mM DTT. The protein was then dialyzed as previously described (Chen et al., 2010) and stored at −80°C. BvgA storage buffer contained 20 mM HEPES pH 7.4, 10 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, and 50 % glycerol.

Phosphorylation of BvgA

BvgA was phosphorylated by incubation in the presence of 20 mM acetyl phosphate, (lithium potassium acetyl phosphate from Sigma-Aldrich dissolved in 20 mM Tris-Cl, pH 8) for the indicated times at room temperature. Non-phosphorylated BvgA was incubated for the same period of time in the presence of 20 mM Tris-Cl (pH 8) only. Samples were collected on dry ice.

To investigate the effect of RNAP, P_{fim3} or P_{fhaB} DNA on BvgA phosphorylation, 25 pmol BvgA was mixed either with reconstituted RNAP [previously reconstituted using 3.8 pmol RNAP core and 9.4 pmol purified σ^{70} at 37°C for 10 min], RNAP buffer (33 mM Tris-Cl (pH 8), 0.1 mM EDTA, 355 mM NaCl, 0.6 mM DTT and 50% glycerol), and 1 pmol

pP*fim3*-15C supercoiled DNA, 1 pmol pP*fhaB* supercoiled DNA, 1 pmol pTE103 (50 nM DNA) or the same volume of H_2O. After 10 minutes incubation at 37°C, 20 mM Ac~P was added to start the reaction and samples were put back at 37°C for the indicated amount of time. To determine kinetics of phosphorylation of wild-type BvgA mutants, protein (25 pmol) was first incubated for 10 min at 37°C. Ac~P (20 mM) was then added and the solution incubated for the indicated amount of time at 37°C.

Sample preparation for in vivo *detection of BvgA phosphorylation*

B. pertussis strain BP536 and its derivatives were grown at 37° C for 2 days on BG agar supplemented with streptomycin (50 μg/mL) with or without $MgSO_4$ (50 mM). To prepare cell lysates for the Phos-tag™ gel assay, cells were swabbed from the plate with a polyester-tipped applicator (Puritan Medical Products Company LLC.) and resuspended in 1.5 mL of Phosphate Buffered Saline (PBS, GIBCO) to an OD_{600} of 0.15. A 1 mL aliquot was centrifuged for 1 min at RT, the supernatant was removed, and the pellet was frozen in dry ice. Unless otherwise indicated, the frozen cell pellet was then lysed on ice by the addition of 33 μL of ice-cold 1M formic acid (0.55 M final concentration), immediately followed by the addition of 2 μL of 5 N NaOH (0.17 N final concentration) to neutralize the solution, 10 μL of H_2O and 15 μL of 5X Loading Solution (1% SDS, 65 mM Tris-Cl (pH 8), 25% glycerol, 5% Bromophenol Blue). Resulting cell lysates (4 μl) were immediately loaded onto a Phos-tag™ gel for electrophoresis as described below. *E. coli* strains were grown overnight in LB with appropriate antibiotics before being diluted in fresh medium +/− IPTG, at an OD_{600} of 0.1. Cells were grown at 37°C for indicated amount of time, pelleted by centrifugation at 16,000 × g for 1 min, frozen on dry ice, and lysed as described above.

Phos-tag™ gel electrophoresis

BvgA and BvgA~P were separated on polyacrylamide gels containing acrylamide-Phos-tag™ ligand (Wako Pure Chemical) as previously described (Barbieri & Stock, 2008, Kinoshita & Kinoshita-Kikuta, 2011). Gels were composed of a 10% resolving solution [10% (w/v) 29:1 acrylamide:N,N'-methylene-bis-acrylamide (deionized 3 min with Ag 501-X8 Resin (Biorad) before filtration); 350 mM Tris-Cl (pH 6.8); 0.1 % SDS; 75 μM Phos-tag™ acrylamide; and 150 μM $Zn(NO_3)_2$] and a 4 % stacking solution [4% (w/v) 29:1 acrylamide:N, N'-methylene-bis-acrylamide (deionized 3 min with Ag 501-X8 Resin before filtration); 350 mM Tris-Cl (pH 6.8 @ 4°C); and 0.1 % SDS]. Protein samples were mixed with 5X loading solution (1% SDS, 65 mM Tris-Cl (pH 8), 25% glycerol, 5% Bromophenol Blue) to a final concentration of 1 × before loading on the gel. Electrophoresis was performed at 150 V at 4°C for 1 h 20 min in MOPS running buffer (pH 8) (0.1 M Tris-Cl, 0.1 M MOPS, 0.1% SDS and 5 mM sodium bisulfite). After being washed in water, gels were stained with Coomassie blue or used for Western blot analyses as described below. Stained gels were scanned using a Powerlook 2100XL densitometer and analyzed using Quantity One software from Bio-Rad, Inc.

Western Blot analyses

For Western Blot analyses, 4 μL of cell lysate (prepared as described above) and 1 pmol of purified BvgA (unphosphorylated or phosphorylated *in vitro*) were loaded onto the gel. After electrophoresis, the Phos-tag™ gel was washed 10 min at RT with Transfer Buffer (25 mM Tris base, 0.192 M glycine, 20% methanol) supplied with 1 mM EDTA to remove Zn^+ from the gel, followed by a 20 min wash with the Transfer Buffer to remove the chelated metal. Transfer of the gel to the PVDF filter (Invitrogen) was carried out using MINI-PROTEIN II (Bio-Rad) at constant voltage 100V for 1 h at RT, using a cooling system at 4° C. The PVDF filter was blocked with 1% BSA in PBS, washed with PBS and then incubated with BvgA monoclonal antibody diluted 1:5000 in PBS containing 1% BSA at RT for 1 h, followed by 3 washes (15 min. each) with PBS + 0.05% Tween. The filter was then

incubated with goat anti-mouse IgG-HRP conjugated (1:20,000, Santa Cruz) in PBS containing 1% milk at RT for 1 h. After 3 washes (15 min. each) with PBS + 0.05% Tween, the filter was developed using the Amersham ECL Plus Western Blotting Detection System (GE Healthcare), and the signal was detected using a FUJI LAS-3000 imaging system (Fuji).

Induction and modulation kinetics of BvgA~P in vivo

Strain QC1416, a derivative of BP536 (Chen et al., 2010) was used in these studies. Although not relevant to this work, it also harbors a chromosomal insertion of the vector pSS3967 containing an ectopic transcription fusion of $P_{fim3\text{-}15C}$.

For repression kinetics, strain QC1416 was grown on BG plates supplemented with streptomycin and gentamicin at 37°C in the absence of $MgSO_4$ for 3 days to produce Bvg^+ mode cells. After restreaking on the same medium and growth at 37°C overnight, cells were swabbed from plates and resuspended in PLB plus streptomycin and gentamicin. Cultures were incubated at 37°C with shaking until the OD_{600} had doubled (~3 h). An aliquot of cells, corresponding to time t=0 was removed, harvested and frozen on dry ice as described above. $MgSO_4$ was added to the culture to a final concentration of 50 mM and growth was continued at 37°C.

For induction kinetics, strain QC1416 was grown on BG plates supplemented with streptomycin, gentamicin, and $MgSO_4$ (50mM) at 37°C for 3 days, resulting in Bvg^- mode cells. After restreaking on the same medium and growth at 37°C overnight, cells were swabbed from plates and resuspended in PLB plus streptomycin, gentamicin and 50 mM $MgSO_4$. Cultures were incubated at 37°C with shaking until the OD_{600} had doubled (~3 h). An aliquot of cells, corresponding to time t=0 was removed, harvested and frozen on dry ice as described above. The remainder of the culture was harvested by centrifugation at 1200 × g for 10 min at RT, and resuspended in the same volume of pre-warmed PLB supplemented with streptomycin and gentamicin but no $MgSO_4$. Cell growth was continued at 37°C.

In both cases aliquots were then taken at indicated time points, cell pellets were obtained by centrifugation, and held frozen on dry ice. Samples were normalized based on OD_{600} readings and processed together for electrophoresis as described above.

Induction kinetics of fhaB *and* ptx in vivo

B. pertussis BP536 harboring plasmids pQC1552, pQC1557, or pSS3967 vector integrated in the chromosome were cultured and induced as described above. A 200μl aliquot was removed at the zero time point before induction and at indicated times after. Aliquots were transferred to individual wells of a 96-well white microtiter plate with clear bottom. The OD_{600} reading and measurement of luminescence activity of each aliquot were carried out using Gen5 software on a Synergy 2 plate reader (BioTek Instruments, Inc.).

Acknowledgments

The authors thank A. Battesti, L. Knipling, T. James, S. Jha, L. Abell, and J. Kassis for helpful discussions, Ilana Cohen for technical assistance, and J. P. Castaing for the construction of pET21a+ D54N. This research was supported in part by the Intramural Research Program of the NIH, NIDDK.

Abbreviations

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
RR	response regulator

HK	histidine kinase
HPLC	high pressure liquid chromatography
BSA	bovine serum albumin
BG	Bordet Gengou agar
LB	Luria Broth
orf	open reading frame
Ac~P	acetyl phosphate

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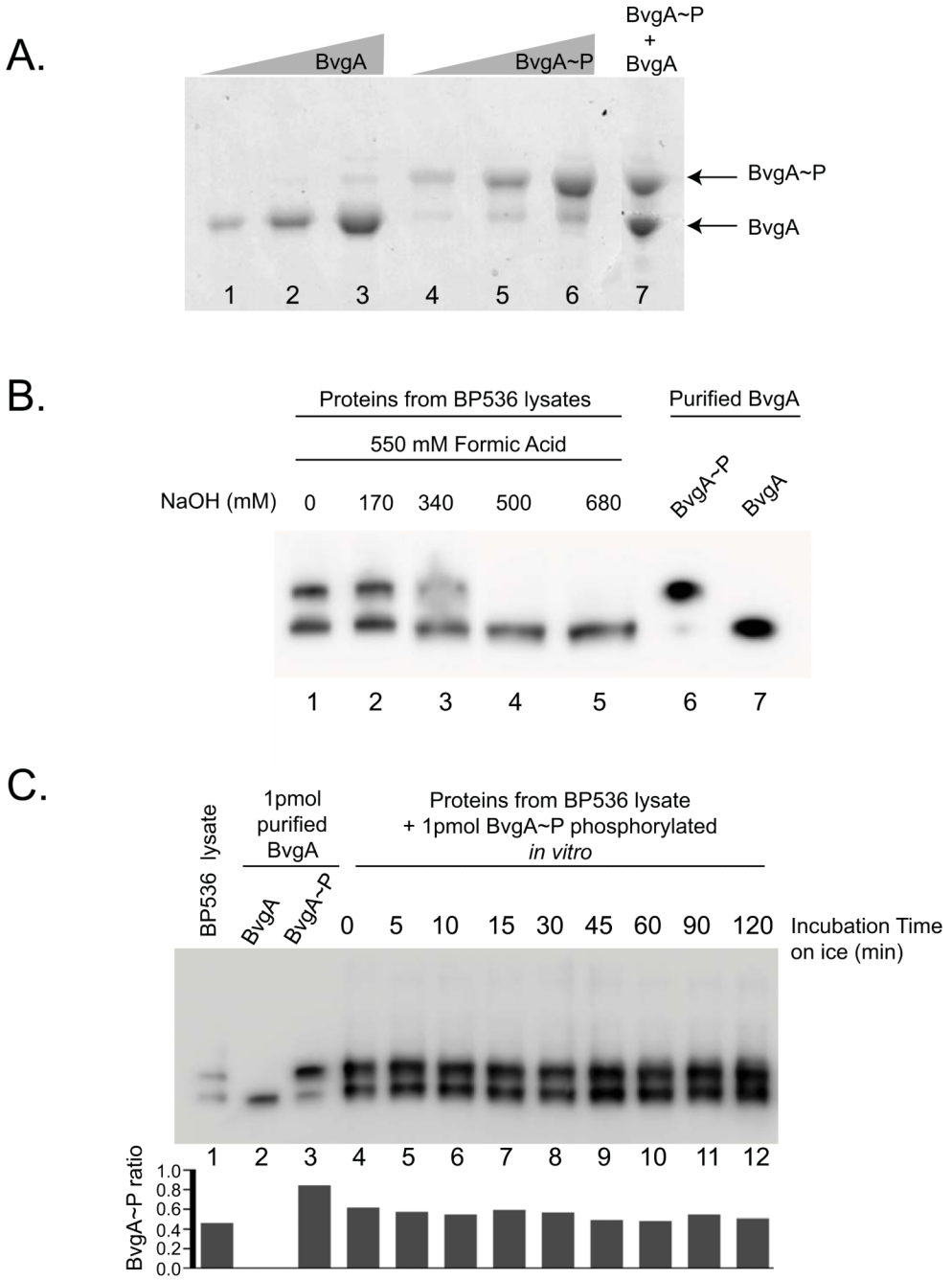

Figure 1. BvgA and BvgA~P can be separated by Phos-tag™ polyacrylamide gel electrophoresis and detected *in vivo* by Western analysis

A. Phosphorylation of BvgA *in vitro*. A Coomassie-stained gel shows different amounts **[**10 pmol (lane 1 and 4), 25 pmol (lane 2 and 5), 50 pmol (lane 3 and 6)] of BvgA after treatment with buffer alone (lanes 1-3) or buffer plus 20 mM Ac~P (lanes 4-6), followed by SDS-PAGE with Phos-tag™. Lane 7 contains a mix of 40 pmol of BvgA and 40 pmol of BvgA~P.

B. BvgA phosphorylation in *B. pertussis*. Cells were lysed and processed as described in Experimental Procedures using different amounts of NaOH (lanes 1-5) before

electrophoresis on a Phos-tag™ gel and Western analysis. As a control, 1 pmol of purified BvgA~P (lane 6) and BvgA (lane 7) was loaded on the gel.

C. Stability of BvgA~P during cell lysate processing. One frozen aliquot of *B. pertussis* BP356 cells (harvested from 1 mL culture at OD_{600} 0.15) was mixed with 15 pmol of *in vitro* phosphorylated BvgA. This combination of purified BvgA~P and *B. pertussis* cells was treated with 550 mM formic acid and 170 mM NaOH in a total volume of 60 μl for lysis (as described in Experimental Procedures) and incubated on ice for the indicated amount of time. From this mixture, 4 μl (containing 1 pmol of purified BvgA~P plus the *in vivo B. pertussis* BvgA~P) were electrophoresed on the Phos-tag™ gel. BvgA was detected by Western analysis. Lane 1 shows the pellet lysate with no purified BvgA~P added, to indicate the starting ratio of BvgA~P to BvgA. Lanes 2 and 3 correspond to 1 pmol of purified BvgA or BvgA~P, respectively. The graph represents the ratio of BvgA~P on the total amount of BvgA protein for each lane.

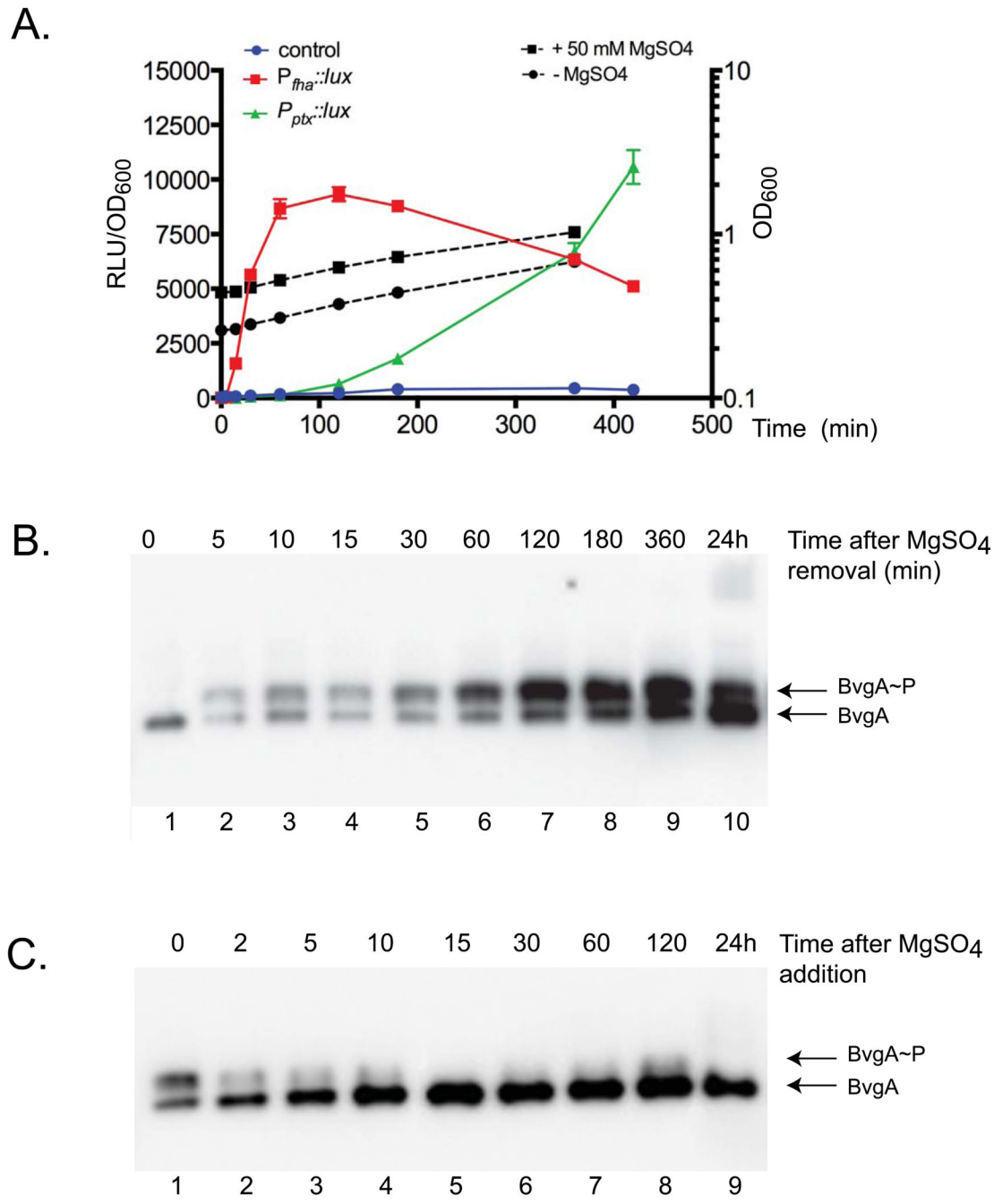

Figure 2. Kinetics of BvgA phosphorylation and gene expression in *B. pertussis* following modulation, or relief of modulation, of BvgAS kinase

A. Level of gene expression and cell growth at different time points after removal of 50 mM $MgSO_4$.

A vector carrying a transcriptional fusion of P_{fhaB}, P_{ptx}, or no promoter (control) with the *luxCDABE* operon was integrated in single copy in the BP536 chromosome. At the zero time point cells in liquid PLB medium were shifted from a non permissive (+$MgSO_4$) to a permissive medium (−$MgSO_4$) and OD_{600} as well as luminescence were monitored. The results shown here represent the assay repeated 4 times with triplicate sample reading at each time point. The left Y-axis corresponds to the relative luminescence in arbitrary units

(RLU) normalized by OD_{600}. Dashed black lines represent growth curves of both cultures monitored by OD_{600}, with reference to the right Y-axis.

B., C. Levels of BvgA and BvgA~P at different time points after removal (B) or addition (C) of 50 mM $MgSO_4$. At t=0, cells were shifted from a permissive ($-MgSO_4$) to a non-permissive medium ($+MgSO_4$) or vice versa. At the indicated times, cells were harvested. Cell lysates were subjected to Phos-tag™ gel electrophoresis and Western blot.

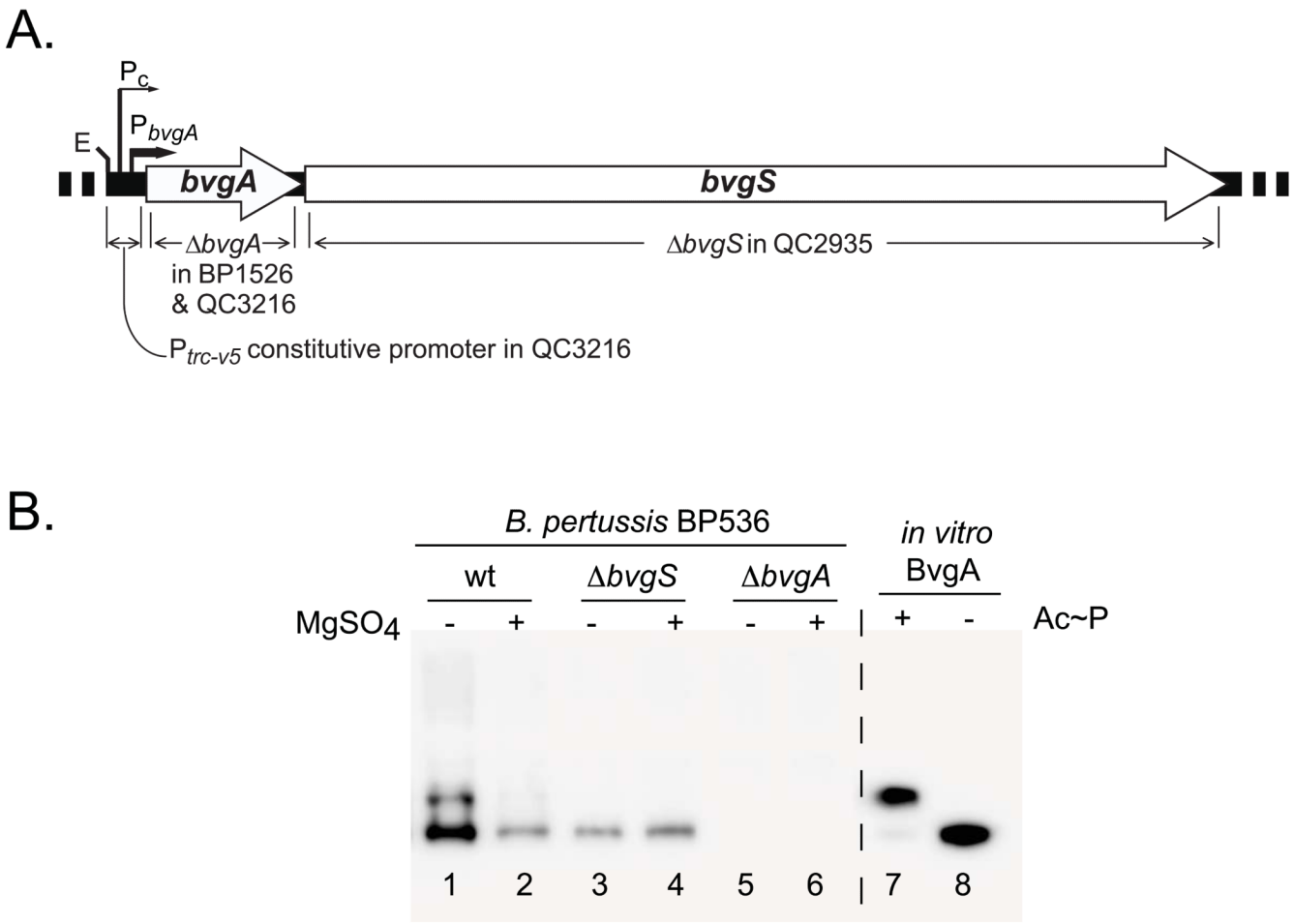

Figure 3. BvgS is the only kinase that phosphorylates BvgA in *B. pertussis* under typical growth conditions

A. Schematic illustration of the *B. pertussis bvgAS* locus showing specific deletions of *bvgA* and *bvgS* used in this study. Also shown are the locations of the native BvgA-inducible and constitutive promoters and the $P_{trc\text{-}v5}$ constitutive promoter added to the *bvgA* deletion strain QC3216 to drive *bvgS* expression.

B. Effect of specific chromosomal deletions on BvgA phosphorylation. *B. pertussis* strains BP536 (wild-type, lanes 1 and 2), QC2935 (*ΔbvgS*, lanes 3 and 4) and BP1526 (*ΔbvgA* lanes 5 and 6) grown in the absence (lanes 1, 3, and 5) and presence (lanes 2, 4, and 6) of MgSO4 were analyzed by Phos-tag™ gel-electrophoresis and Western blot. Control lanes contained purified wild-type BvgA incubated with (lane 7), or without (lane 8) Ac~P.

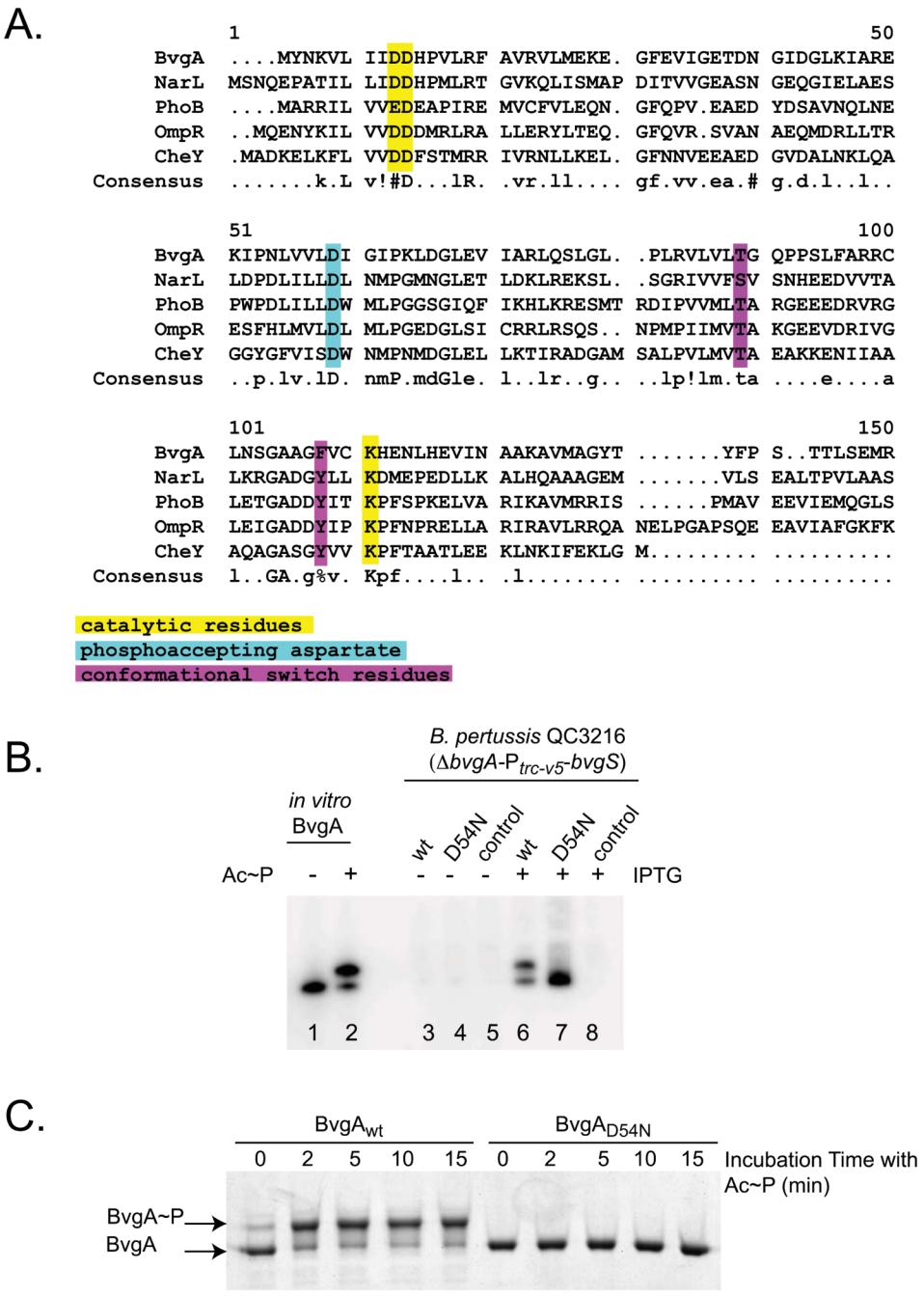

Figure 4. Mutation of the aspartic residue at position 54 eliminates BvgA phosphorylation in *B. pertussis*

A. Alignment of different response regulator protein sequences using the Multalin web program. (http://multalin.toulouse.inra.fr/multalin/). The parameters used correspond to the default parameters of the program. Consensus symbols are:! for L, M; % for F, Y; and # for N, D, Q, E, B, Z. Catalytic residues are highlighted in yellow, the phosphoaccepting aspartate in teal, and conformational switch residues in purple, with reference to Dyer & Dahlquist (2006) and Ruiz *et al.* (2008).

B. $BvgA_{D54N}$ is not phosphorylated in *B. pertussis*. Strains of *B. pertussis* strain QC3216 (*ΔbvgA*-$P_{trc\text{-}v5}$-*bvgS*) harboring plasmids directing the synthesis of wild-type BvgA

(pSS4983, lanes 3 and 6), $BvgA_{D54N}$ (pSS5027, lanes 4 and 7), or the empty pQC1883 vector control (lanes 5 and 8) were analyzed for BvgA synthesis and phosphorylation without (lanes 3-5) and with (lanes 6-8) IPTG-induction. Control lanes contained 1 pmol of purified BvgA (lane 1) or BvgA~P, (lane 2)

C. Kinetics of *in vitro* phosphorylation of wild-type BvgA and $BvgA_{D54N}$. Purified wild-type BvgA and $BvgA_{D54N}$ (25 pmol each) were phosphorylated by treatment with Ac~P for the indicated amount of time prior to sample preparation and Phos-Tag™ gel electrophoresis. Proteins were visualized by Coomassie staining.

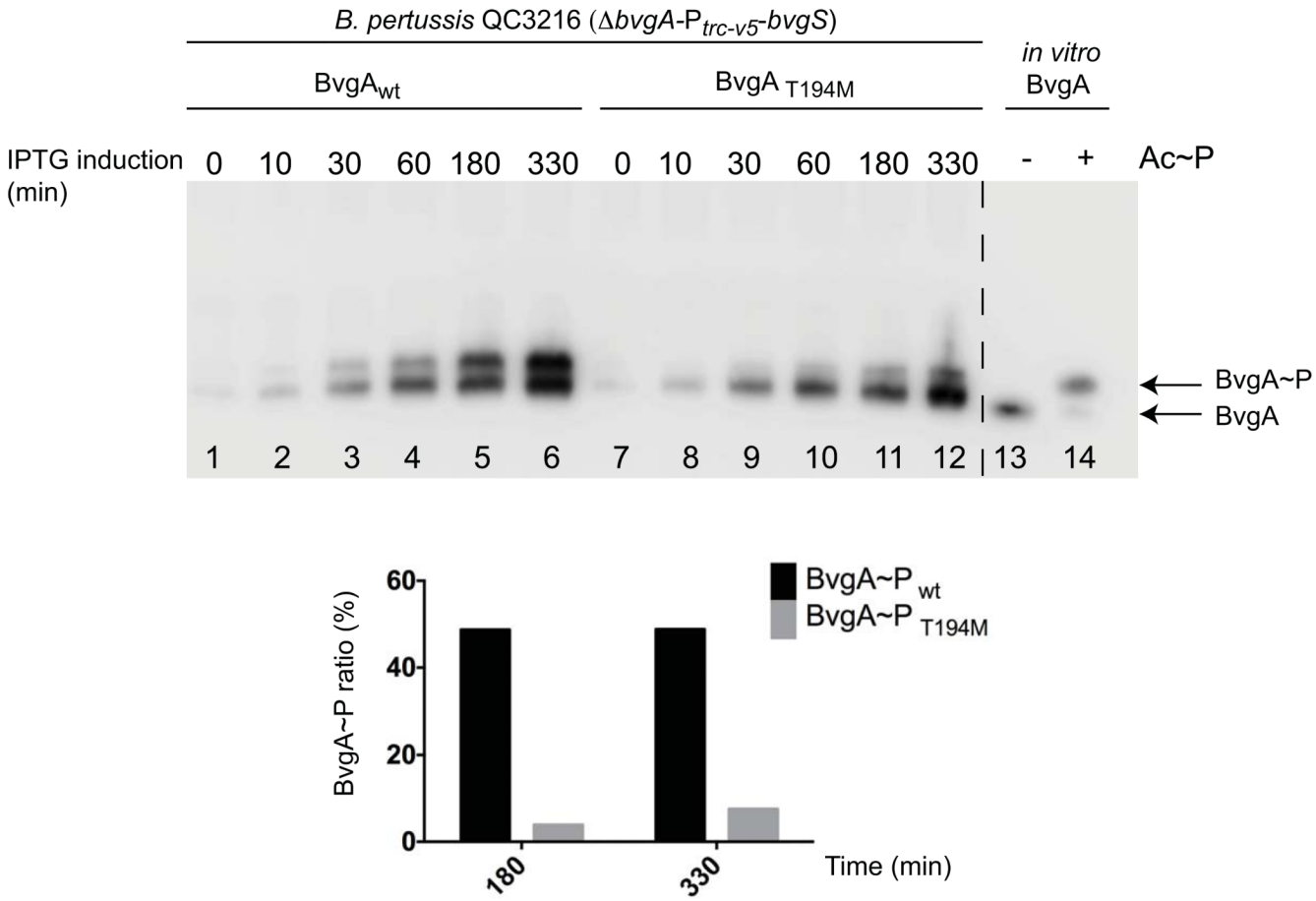

Figure 5. Kinetics of *in vivo* phosphorylation of wild-type and mutant BvgA

Liquid PLB cultures of *B. pertussis* strain QC3216 (*ΔbvgA*-$P_{trc\text{-}v5}$-*bvgS*) harboring plasmids directing the synthesis of wild-type BvgA (pSS4983, lanes 1-6), or $BvgA_{T194M}$ ($pBvgA_{T194M}$, lanes 7-12), were induced with 1 mM IPTG at t=0, and sampled at various times post induction. Cells were harvested by centrifugation, samples were prepared, and Phos-tag™ gel electrophoresis followed by Western blot analysis was performed as described above. Control lanes contained 1 pmol of purified BvgA (lane 13) or BvgA~P, (lane 14). The graph represents the ratio of BvgA~P relative to the total amount of BvgA protein for lanes 5, 6, 11 and 12.

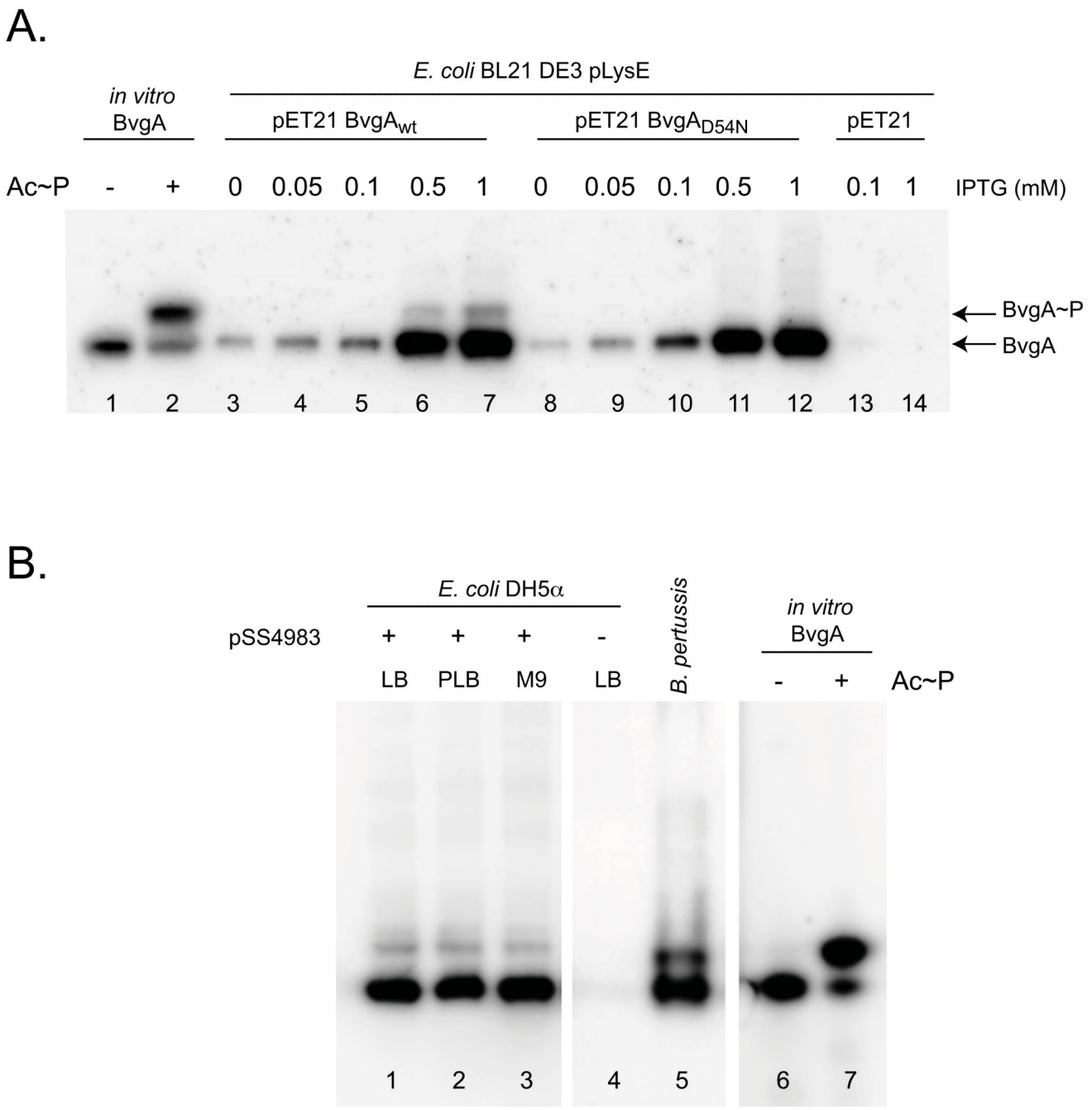

Figure 6. BvgA is phosphorylated in *E. coli* in the absence of BvgS

A. Phosphorylation of BvgA in *E. coli* is dependent on D54. Cultures of *E. coli* BL21(DE3)/ pLysE cells containing pET21a(+) alone (lanes 13-14), or pET21a(+) expressing wild-type *bvgA* (lanes 3-7) or $bvgA_{D54N}$ (lanes 8-12) were incubated for one hour with increasing concentrations of IPTG and analyzed by Phos-tag™ gel electrophoresis and Western blot as described above. Control lanes contained 1 pmol of purified BvgA (lane 1) or BvgA~P (lane 2).

B. Effect of growth medium on BvgS-independent phosphorylation of BvgA in *E. coli.* Cell lysates of *E. coli* DH5α with (lanes 1-3) or without (lane 4) pSS4983 (expressing wild-type

BvgA under IPTG induction) were assessed for phosphorylation of BvgA *in vivo* after growth for three hours in LB (lanes 1 and 4), PLB (lane 2), or M9-glucose (lane 3) media, each supplemented with 2 mM IPTG. A *B. pertussis* culture (lane 5), purified BvgA (lane 6), and *in vitro* phosphorylated BvgA (lane 7) are shown for comparison.

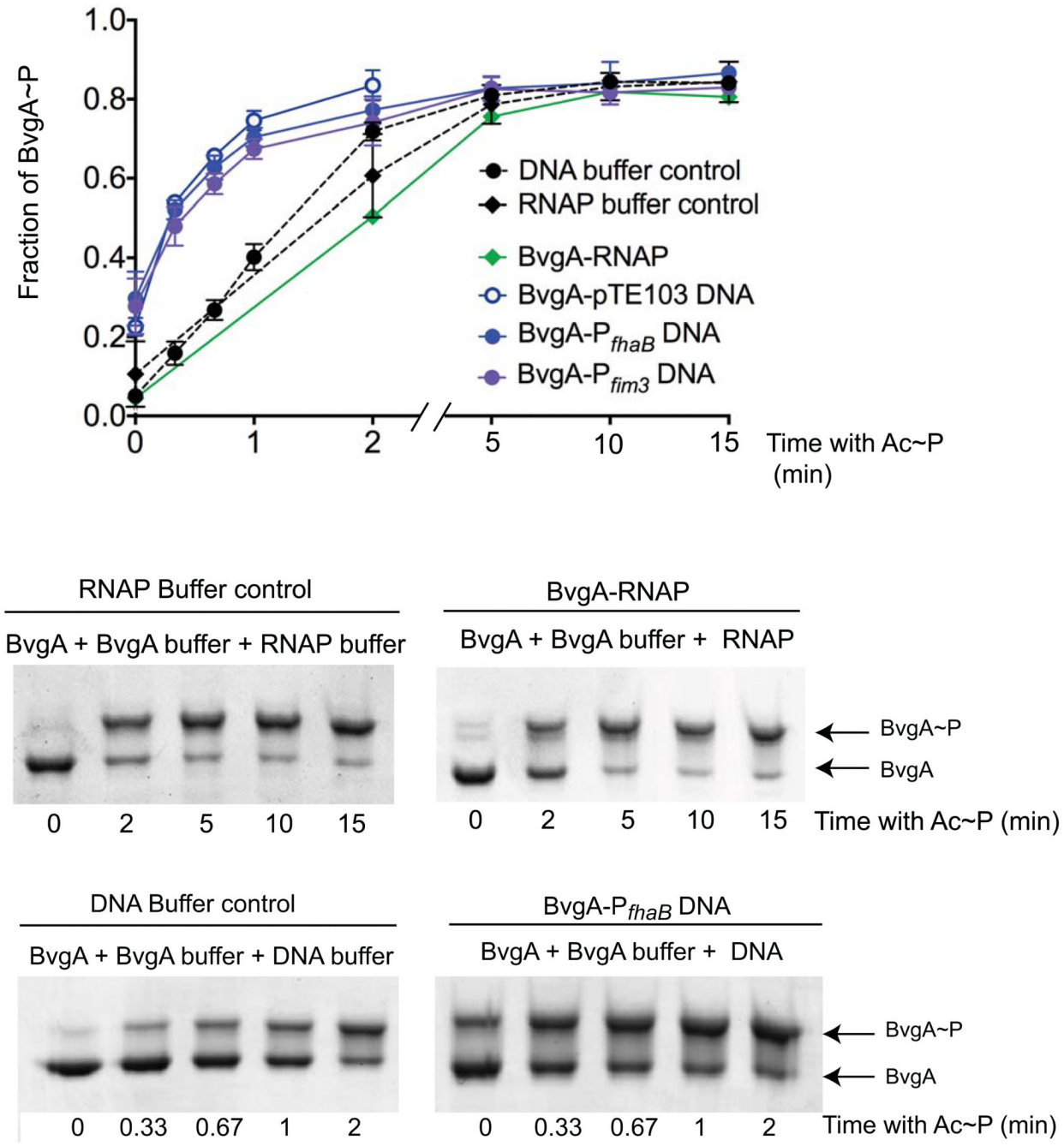

Figure 7. The presence of DNA but not RNAP stimulates *in vitro* phosphorylation of BvgA by Ac~P

BvgA phosphorylation *in vitro* was followed by Phos-tag™ gel electrophoresis and Coomassie staining. Shown below are gels from typical experiments in which BvgA was supplemented with either buffer alone, as control, or buffer plus RNAP or pP*fhaB* plasmid DNA. Since the two buffers differed, a separate buffer control for each is presented. The graph represents the combined data (mean and standard error) from three independent experiments for each set of conditions and also includes the results of using a P*fim3* clone and the pTE103 vector alone.

Table 1
Strains and plasmids used in this work

Strain or plasmid	Relevant features	Source or reference
E. coli		
DH5α	High-efficiency transformation	Bethesda Research Laboratories
BΛ21(ΔE3)[πΛψσE]	Expression strain for pET vectors, CamR	Studier *et al.*, 1990
BΛ21(ΔE3)[πΛψσS]	Expression strain for pET vectors, CamR	Studier *et al.*, 1990
SM10	Mobilization of RK2 *oriT* plasmids	Simon *et al.*, 1983
B. pertussis		
Tohama I	Patient isolate	Kasuga, *et al.* (1954)
BP536	Tohama I, Str^R, Nal^R	Stibitz and Yang (1991)
QC2935	BP536, Δ*bvgS*	This study
BP1526	BP536, Δ*bvgA*	Chen *et al.* (2010)
QC1416	BP536, pSS3967::$P_{fim3\text{-}i5c}$	Chen *et al.* (2010)
QC3216	BP536, Δ*bvgA*-$P_{trc\text{-}v5}$-*bvgS*	This study
Plasmids		
pSS1827	Helper plasmid,for triparental mating, RP4 *tra* genes, Amp^R	Stibitz and Carbonetti (1994)
pQC1883	*B. pertussis* expression vector, Kan^R	This study
pSS4983	pQC1883::*bvgA*-wild-type, Kan^R	This study
$pBvgA_{D54N}$	pQC1883::$bvgA_{D54N}$, Kan^R	This study
$pBvgA_{T194M}$	pQC1883::$bvgA_{T194M}$, Kan^R	This study
pSS4661	Vector for allelic exchange in *Bordetella*, Kan^R	This study
pQC1805	pSS4661::Δ*bvgS*, kan^R	This study
pSS4871	pSS4661::Δ*bvgA*-P^c-*bvgS*, Kan^R	This study
pET21a(+)	Expression vector for BvgA in *E. coli*	EMD Millipore/Novagen
pET21a(+)-$BvgA_{wt}$	pET21a(+)::*bvgA*	This study
pET21a(+)-$BvgA_{D54N}$	pET21a(+)::$bvgA_{D54N}$	This study
pTE103	*In vitro* transcription vector	Elliot and Geiduscheck (1984)
p*Pfim3*-15C	pTE103::P*fim3*-15C	Chen *et al.* (2010)
p*PfhaB*	pTE103::P*fhaB*	Boucher *et al.* (1997)
pSS3967	*luxCDABE* promoter assay vector	Chen *et al.* (2010)
pQC1552	pSS3967::P*ptx*	This study
pQC1557	pSS3967::P*fhaB*	This study