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Integration of multiple domains in a two-component sensor protein: the *Bordetella pertussis* BvgAS phosphorelay

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BvgS and BvgA, a two-component system, regulate virulence gene expression in *Bordetella pertussis*. BvgS is a transmembrane sensor protein that can autophosphorylate and phosphorylate BvgA. Phosphorylated BvgA activates transcription of virulence genes. The cytoplasmic region of BvgS contains three domains separated by alanine/proline-rich sequences—the transmitter, receiver and C-terminus. We report that the C-terminal domain, like the transmitter and receiver, is an essential part of the phosphorelay from BvgS to BvgA. The BvgS C-terminal domain is phosphorylated in *trans* via a phosphotransfer mechanism by the cytoplasmic portion of BvgS, and *trans*-phosphorylation of the C-terminal domain requires both the transmitter and receiver. We also demonstrate that phosphorylated, purified C-terminal domain alone is sufficient for phosphotransfer to BvgA. A point mutation in the C-terminal domain (His1172→Gln) abolishes BvgS activity *in vivo* and eliminates detectable phosphorylation of BvgA *in vitro*. Activity of BvgS His1172→Gln could be restored by providing the wild-type C-terminal domain in *trans*. Our results indicate an obligatory role for an alternate phosphodonor module in the BvgAS phosphorelay.

Keywords: *Bordetella*/BvgAS/phosphorylation/signal transduction/two-component systems

Introduction

In many bacteria, adaptation to extracellular signals is coordinated by a family of sensory transduction molecules known as two-component regulatory systems. Two-component systems are widely distributed among prokaryotes and have been identified in eukaryotes as well (Parkinson and Kofoid, 1992; Chang *et al.*, 1993; Ota and Varshavsky, 1993). These systems are so named because they typically consist of a sensor protein and a response regulator protein. According to the basic paradigm, input signals induce a phosphorylation cascade that leads to one or more output signals. Sensor proteins autophosphorylate in response to specific inputs; the site of phosphorylation in most cases is a conserved histidine residue located in a domain known as the transmitter. The sensor protein transduces signals by transferring a phosphoryl group to a second component, the response regulator. A conserved aspartic acid residue in the receiver domain is the site of phosphorylation in response regulators (Stock *et al.*, 1995). Phosphorylation of the response regulator leads to an output signal such as transcriptional activation or protein–protein interaction (Stock *et al.*, 1989).

Transmitters and receivers have been defined as modules that integrate signaling circuits (Parkinson and Kofoid, 1992). In hybrid sensor proteins, these modules are incorporated within a single molecule. Three families of hybrid sensors have been described thus far; these are represented by AsgA of *Myxococcus xanthus*, VirA of *Agrobacterium tumefaciens* and BvgS of *Bordetella pertussis*. AsgA is the sole member of the first category of hybrid sensors, which is distinguished by a receiver N-terminal to the transmitter. Members of the VirA family are characterized by a receiver C-terminal to the transmitter. The third class of sensor proteins, the BvgS family, contains not only a transmitter and receiver but an additional C-terminal domain. Biochemical characterization of hybrid sensor proteins and their cognate response regulators has demonstrated that they also communicate by a phosphorylation cascade (Jin *et al.*, 1990a,b; McCleary and Zusman, 1990; Iuchi and Lin, 1992; Uhl and Miller, 1994). It is not entirely clear how these additional signal transduction domains regulate and influence the cascade, but the receiver in VirA has been suggested to be an autoinhibitory domain (Pazour *et al.*, 1991; Chang and Winans, 1992).

BvgS and its cognate response regulator, BvgA, are required for production of proteins necessary for respiratory tract colonization by *Bordetella* species (Weiss *et al.*, 1983; Aricò *et al.*, 1989; Roy *et al.*, 1989; Weiss and Goodwin, 1989; Guierard and Guiso, 1993; Cotter and Miller, 1994; Akerley *et al.*, 1995). BvgA is similar to other response regulators, with an N-terminal receiver domain and C-terminal helix–turn–helix motif (Aricò *et al.*, 1989; Figure 1). The receiver of BvgA contains the conserved aspartic acids (Asp9, Asp10 and Asp54) and the conserved lysine (Lys109) characteristic of these modules. Based on other systems, Asp54 is presumed to be the site of phosphorylation. BvgS is a 135 kDa sensor protein localized to the cytoplasmic membrane (Aricò *et al.*, 1989; Stibitz *et al.*, 1989; Stibitz and Yang, 1991). BvgS has a periplasmic region and several distinct cytoplasmic domains: the linker, transmitter, receiver and C-terminus (Figure 1). The BvgS linker is the site of mutations that render BvgS insensitive to environmental signals (Miller *et al.*, 1992; Goyard *et al.*, 1994; Manetti *et al.*, 1994). The transmitter of BvgS contains a conserved histidine (His729) which is the proposed site of autophosphorylation. We have shown previously that mutation of this histidine eliminates detectable autophosphorylation (Uhl and Miller, 1994). Similar to the BvgA receiver, the BvgS receiver contains conserved aspartic acid and lysine residues, with Asp1023 corresponding to the predicted site of phosphorylation. Mutation of Asp1023 does not

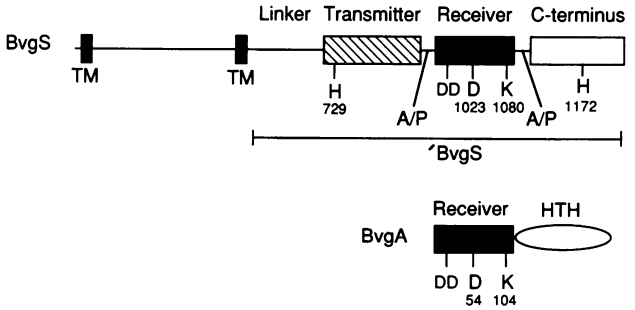


Fig. 1. Domain organization of BvgA and BvgS (Aricò *et al.*, 1989). The transmembrane sensor protein BvgS contains several distinct domains delineated by hydrophobic transmembrane sequences (TM) and alanine/proline-rich regions (A/P). The cytoplasmic portion of BvgS ('BvgS) utilized for *in vitro* experiments is noted. Amino acids conserved among the transmitter and receiver domains are shown for BvgS and BvgA. The C-terminal domain of BvgS contains a histidine residue conserved among BvgS family members, while a helix–turn–helix motif (HTH) is present in the C-terminal region of BvgA. Further details are given in the text.

affect autophosphorylation of BvgS, but completely abolishes BvgA phosphorylation (Uhl and Miller, 1994). These results suggest that the BvgS transmitter is the initial site of autophosphorylation and that phosphorylation of the BvgS receiver is required for phosphotransfer to BvgA.

A unique feature of the BvgS family is the presence of a C-terminal domain in addition to transmitter and receiver modules. Seven BvgS family members have been identified thus far, including ArcB, BarA and EvgS of *Escherichia coli* (Iuchi *et al.*, 1990; Nagasawa *et al.*, 1992; Parkinson and Kofoid, 1992; Utsumi *et al.*, 1994). The C-terminal domain contains limited sequence similarity among BvgS family members (Ishige *et al.*, 1994). The most notable region of similarity includes an invariant histidine residue (His1172, Figure 1) with conservation of flanking sequences. While phosphorylation of this domain has been reported for ArcB and BarA, the functional significance of phosphorylation is not known (Ishige *et al.*, 1994).

We report that the BvgS C-terminus has an integral role as a phosphotransfer domain in the BvgAS signal transduction cascade. The BvgS C-terminal domain can be phosphorylated in *trans* by BvgS. Phosphorylation of the C-terminal domain by BvgS occurs by a phosphotransfer reaction and requires the proposed sites of phosphorylation in the transmitter and receiver. Purified, phosphorylated C-terminal domain is sufficient for phosphotransfer to BvgA. Mutation of the conserved histidine in the C-terminal domain inactivates BvgS *in vivo* and results in an inability to phosphorylate BvgA *in vitro*. While transmitter and receiver domains have long been known to govern signal transduction responses, we now show that the BvgS C-terminal domain represents a new type of communication module incorporated in two-component systems.

Results

Phosphorylation of the C-terminal domain of BvgS

Previous results suggested that the BvgS transmitter was necessary for autophosphorylation, while the BvgS receiver mediated phosphotransfer to BvgA (Uhl and Miller, 1994). To examine further the role of BvgS receiver in signal transduction, we purified the receiver and C-terminal domain of BvgS as a fusion to glutathione-S-transferase (GST–RC). As shown in Figure 2, purified GST–RC could be phosphorylated in *trans* by the cytoplasmic portion of BvgS ('BvgS, as in Figure 1), but only low levels of phosphorylation were detected (Figure 2, lane 2).

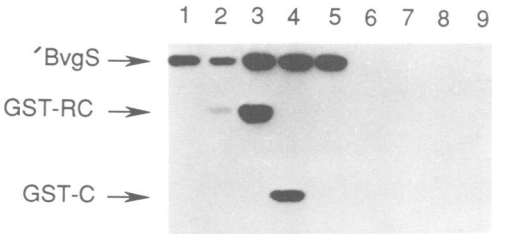


Fig. 2. Phosphorylation of the BvgS C-terminus. 'BvgS (~75 kDa) was incubated in a phosphorylation reaction with [γ-^{32}P]ATP alone (lane 1) or with addition of GST fused to the BvgS receiver and C-terminal domain (~53 kDa; GST–RC, lane 2), GST–RC with the Asp1023→Asn mutation (lane 3), GST fused to the BvgS C-terminal domain (~38 kDa; GST–C, lane 4), or GST–C with the His1172→Gln mutation (lane 5). Lanes 6–9 are identical to lanes 2–5 except that 'BvgS was not present.

Asp1023 of BvgS corresponds to the conserved site of receiver phosphorylation. To determine if this could be the site of phosphorylation in the receiver/C-terminal domain, we purified a GST–RC derivative containing an Asp1023→Asn mutation (GST–RC D1023N) and tested it for phosphorylation by 'BvgS. The Asp1023→Asn mutation did not eliminate phosphorylation by 'BvgS (Figure 2, lane 3); in contrast, levels of phosphorylation were substantially greater compared with GST–RC. This indicated that Asp1023 was not the sole site of phosphorylation in the receiver/C-terminal domain of BvgS and suggested that one or more additional sites of phosphorylation exist. It is worth noting that not only were levels of phosphorylated GST–RC decreased relative to GST–RC D1023N (Figure 2, lanes 2 and 3), but levels of phosphorylated 'BvgS were reduced when GST–RC was added (lane 2) as compared with either no addition or addition of GST–RC D1023N (lanes 1 and 3 respectively). This will be discussed in more detail below.

We next purified the BvgS C-terminal domain alone as a GST fusion (GST–C) and examined it for BvgS-dependent phosphorylation. The BvgS C-terminal domain was phosphorylated efficiently in *trans* by 'BvgS (Figure 2, lane 4). The C-terminal domains of BvgS family members all contain an absolutely conserved histidine corresponding to His1172 in BvgS (Ishige *et al.*, 1994; Uhl and Miller, 1995a). This histidine is a likely site of phosphorylation, based on conservation among family members and the similarity of the flanking region to the site of phosphorylation in CheA of *E.coli* (Hess *et al.*, 1988; Ishige *et al.*, 1994). Phosphorylation of the BvgS C-terminal domain requires His1172, as no phosphorylation of GST–C with a His1172→Gln (H1172Q) mutation was observed (Figure 2, lane 5). Addition of 'BvgS was obligatory for phosphorylation of GST–RC, GST–RC D1023N and GST–C since no phosphorylation of these molecules in the absence of 'BvgS was detected (Figure

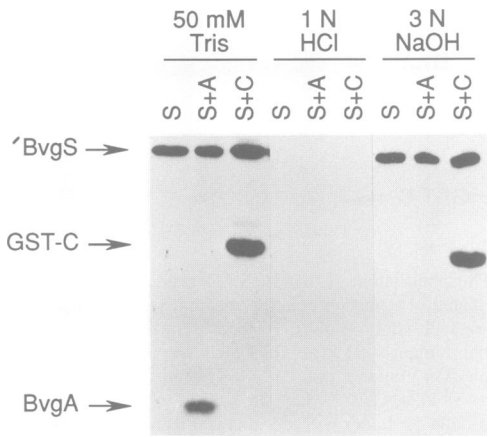


Fig. 3. Chemical stability of phosphorylated BvgS C-terminal domain. Phosphorylation reactions containing 'BvgS (S), 'BvgS with BvgA (S+A) or 'BvgS with GST–C (S+C) were transferred to Immobilon membranes (Millipore). Membranes were incubated with 50 mM Tris (pH 7.6), 1 M HCl or 3 M NaOH. 'BvgS and BvgA (23 kDa) are included as controls for base-stable and base-labile phosphoryl group linkages (Uhl and Miller, 1994).

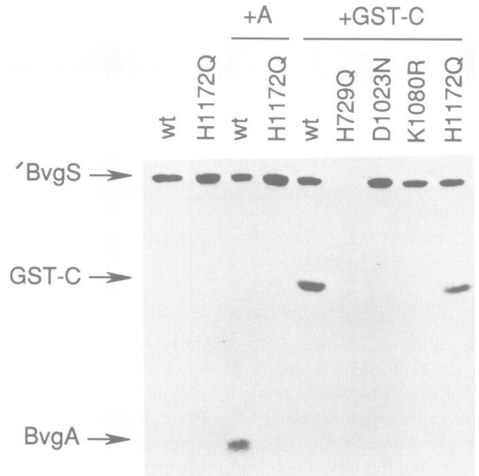


Fig. 4. Phenotypes of BvgS mutations *in vitro*. 'BvgS wild-type or mutants as indicated were incubated with [γ-^{32}P]ATP, separated by SDS–PAGE and transferred to an Immobilon membrane. 'BvgS and mutant derivatives were assessed for their ability to autophosphorylate (first two lanes), phosphorylate BvgA (+ A) or phosphorylate GST–C (+ GST–C).

2, lanes 6–8). These results demonstrate 'BvgS-dependent phosphorylation of the BvgS C-terminal domain.

The chemical stability profile of the phosphorylated C-terminal domain should be characteristic of amidyl phosphate if His1172 is a site of phosphorylation. Phosphoamidyl linkages (phosphohistidine or phospholysine) are base stable and acid labile, while phosphoacyl linkages (phosphoaspartate or phosphoglutamate) are labile to both acid and base (Koshland, 1951; Hultquist, 1968; Fujitaki and Smith, 1984). As shown in Figure 3, the C-terminal domain of BvgS is stabile to base and labile to acid. This is consistent with phosphorylation via a phosphoamidyl linkage.

The BvgAS phosphorelay can be separated into distinct steps by mutations in the BvgS transmitter, receiver or C-terminal domains

The H1172Q mutation was introduced into full-length BvgS to determine the effect of this mutation *in vivo*. BvgS activity can be measured *in vivo* by activation of a chromosomal *fhaB–lacZYA* fusion in *E.coli* (Miller *et al.*, 1989). Wild-type *bvgAS* in *trans* produced 12 400 ± 700 units of β-galactosidase, while only 7 ± 5 units of β-galactosidase were produced with pBR322 (vector control) in *trans*. *bvgA*$^+$*bvgSH1172Q* in *trans* resulted in 16 ± 10 units of β-galactosidase. Therefore, His1172 is required for both phosphorylation of the C-terminal domain *in vitro* and for BvgS activity *in vivo*.

We hypothesized that the inability of BvgS H117Q to activate *fhaB–lacZYA* was due to a defect in the phosphorylation cascade. 'BvgS H1172Q was compared with wild-type 'BvgS *in vitro* for autophosphorylation and phosphorylation of BvgA. 'BvgS H1172Q was able to autophosphorylate to wild-type levels but was deficient for BvgA phosphorylation (Figure 4), consistent with its Bvg$^-$ phenotype *in vivo*. 'BvgS H1172Q and other BvgS mutations were also examined for *trans*-phosphorylation of the C-terminal domain. 'BvgS H729Q, containing a His729→Gln mutation, was unable to autophosphorylate or phosphorylate the C-terminal domain (Figure 4). 'BvgS containing mutations in conserved receiver amino acids, Asp1023→Asn and Lys1080→Arg (D1023N and K1080R), was deficient for *trans*-phosphorylation of the C-terminal domain despite wild-type levels of autophosphorylation. These mutant 'BvgS derivatives are also unable to phosphorylate BvgA (Uhl and Miller, 1994, 1995a). Although 'BvgS H1172Q lost the capacity to phosphorylate BvgA, it retained the ability to *trans*-phosphorylate the C-terminal domain (Figure 4).

The phenotypes of mutations in the transmitter, receiver and C-terminal domain *in vitro* suggest that the BvgAS phosphorelay consists of discrete ordered steps. His729 of the BvgS transmitter is required for autophosphorylation of BvgS, while Asp1023 and Lys1080 are necessary for *trans*-phosphorylation of the BvgS C-terminal domain. Finally, His1172 in the BvgS C-terminal domain is essential for BvgA phosphorylation.

Phosphorylation of the C-terminal domain by a phosphotransfer reaction

Figure 5A depicts two potential mechanisms for phosphorylation of the C-terminal domain. One possibility is that the kinase activity of the BvgS transmitter may have a dual specificity, phosphorylating both the transmitter histidine and the C-terminal domain histidine. Alternatively, the C-terminal domain may be phosphorylated by either the transmitter or receiver via a phosphotransfer mechanism. These possibilities were distinguished by testing whether phosphorylation of GST–C by GST–'BvgS was dependent on free ATP (Figure 5B). GST–'BvgS was incubated in a standard phosphorylation reaction and purified away from ATP. GST–'BvgS is indistinguishable from 'BvgS with regards to autophosphorylation and phosphorylation of BvgA or the BvgS C-terminal domain (unpublished data). GST–'BvgS was incubated with [γ-^{32}P]ATP (Figure 5B, lane P), bound to glutathione–agarose beads, washed extensively and eluted with glutathione (Figure 5B, lane E).

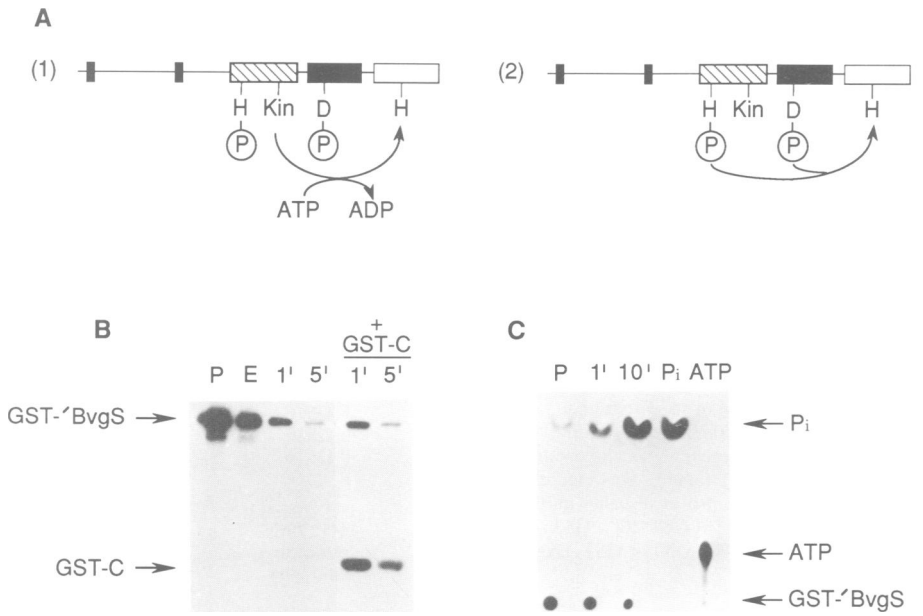


Fig. 5. Mechanism of phosphorylation of the C-terminal domain. (**A**) In model (1), the C-terminal domain is phosphorylated by the kinase activity of the BvgS transmitter. Phosphorylation of the BvgS receiver is necessary for this process. Model (2) depicts phosphorylation of the C-terminal domain by a phosphotransfer mechanism, with either the conserved histidine in the transmitter or conserved aspartic acid in the receiver serving as a phosphodonor. (**B**) GST–'BvgS (98 kDa) was incubated in a phosphorylation reaction (lane P) and purified by affinity chromatography from ATP (lane E). Purified phospho-GST–'BvgS was incubated in buffer alone for 1 min or 5 min or in the presence of GST–C (+ GST–C) for 1 min or 5 min. (**C**) Thin-layer chromatography of purified phospho-GST–'BvgS. 1×10^3 c.p.m. of phospho-GST–'BvgS, $^{32}PO_4$ (P_i) or [γ-^{32}P]ATP was denatured in SDS and spotted on a PEI-cellulose TLC plate. Samples of phospho-GST–'BvgS were also incubated in a buffer containing Mg^{2+} (see Materials and methods) for 1 min or 10 min before denaturation. The TLC plate was resolved with LiCl/acetic acid, dried and exposed to film.

Phospho-GST–'BvgS was able to phosphorylate the C-terminal domain (Figure 5B, + GST-C).

To assess the amount of bound ATP remaining after purification, a sample of phosphorylated GST–'BvgS was denatured in SDS and separated on a polyethyleneimine (PEI) cellulose thin layer chromatography plate as shown in Figure 5C. Inorganic phosphate (P_i) and ATP were run as standards and migrated with R_f values of 0.68 and 0.2 respectively. No significant amount of ATP bound to GST–'BvgS was detected (Figure 5C, lane P). Purified phosphorylated GST–'BvgS contained two species; one co-migrated with inorganic phosphate and the other remained at the origin and is presumably phospho-'BvgS. When phospho-'BvgS was incubated with a buffer containing Mg^{2+} before denaturation, the amount of phospho-'BvgS decreased with an accompanying increase in the amount of inorganic phosphate (Figure 5C, 1′ and 10′). This is due to hydrolysis of the phosphoryl group on BvgS to form inorganic phosphate. Figure 5B and C demonstrates that *trans*-phosphorylation of the C-terminal domain by phospho-'BvgS does not require ATP and occurs by a phosphotransfer mechanism.

The C-terminal domain of BvgS is a phosphodonor for BvgA

In vitro, the BvgS H1172Q mutant was able to autophosphorylate but was unable to phosphorylate BvgA. This suggested that phosphorylation of the C-terminal domain may be necessary for BvgA phosphorylation. In Figure 6, the BvgS C-terminal domain fused to GST was phosphorylated by 'BvgS (lane P) and purified from 'BvgS and free ATP by affinity chromatography (lane E). Incubation of phosphorylated BvgS C-terminal domain with BvgA for 45 s resulted in loss of label from the C-terminal domain with appearance of label on BvgA (lane +A). The BvgS C-terminal domain is therefore able to serve as a phosphodonor to BvgA.

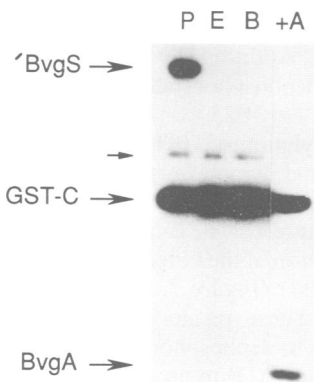


Fig. 6. Phosphorylation of BvgA by the BvgS C-terminal domain. GST–C was phosphorylated by 'BvgS in a standard phosphorylation reaction (lane P). GST–C was then purified from 'BvgS (lane E) and incubated in buffer alone for 45 s (lane B) or with BvgA for 45 s (lane +A). The smaller arrow denotes a minor species that had identical properties to GST–C. This species bound to glutathione–agarose and was also dephosphorylated by BvgA and the BvgS receiver.

Dephosphorylation of the C-terminal domain by the BvgS receiver

We had noted that levels of phosphorylation of GST–RC were decreased relative to GST–RC D1023N (Figure 2, lanes 2 and 3). Additionally, levels of phosphorylated 'BvgS were decreased upon addition of GST–RC. This

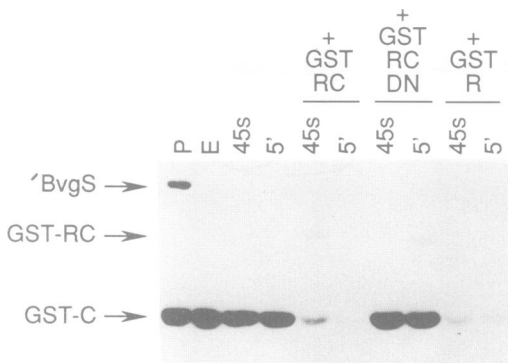
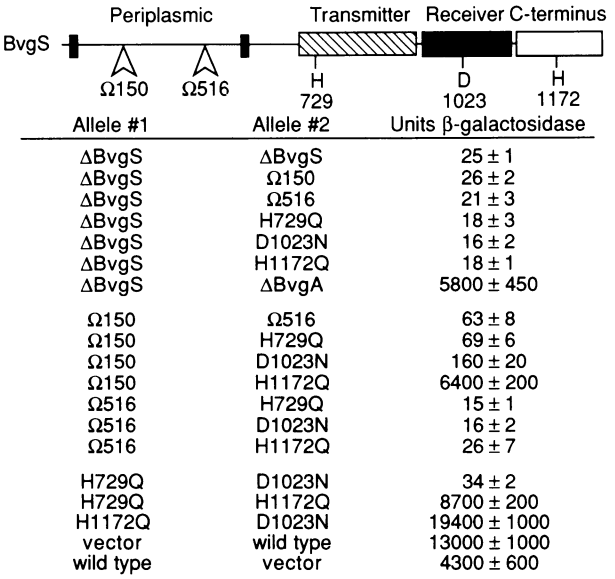


Fig. 7. Receiver-mediated dephosphorylation of the C-terminal domain. GST–C was purified by affinity chromatography with glutathione–agarose from phosphorylation reactions which contained 'BvgS and GST–C (lane P). Purified GST–C (lane E) was incubated in buffer alone for 45 s or 5 min. GST–C was also incubated with the GST–BvgS receiver and C-terminal domain (+ GST–RC), GST–RC with the Asp1023→Asn mutation (+ GST–RC DN) or GST–BvgS receiver (+ GST–R).



Allele #1	Allele #2	Units β-galactosidase
ΔBvgS	ΔBvgS	25 ± 1
ΔBvgS	Ω150	26 ± 2
ΔBvgS	Ω516	21 ± 3
ΔBvgS	H729Q	18 ± 3
ΔBvgS	D1023N	16 ± 2
ΔBvgS	H1172Q	18 ± 1
ΔBvgS	ΔBvgA	5800 ± 450
Ω150	Ω516	63 ± 8
Ω150	H729Q	69 ± 6
Ω150	D1023N	160 ± 20
Ω150	H1172Q	6400 ± 200
Ω516	H729Q	15 ± 1
Ω516	D1023N	16 ± 2
Ω516	H1172Q	26 ± 7
H729Q	D1023N	34 ± 2
H729Q	H1172Q	8700 ± 200
H1172Q	D1023N	19400 ± 1000
vector	wild type	13000 ± 1000
wild type	vector	4300 ± 600

Fig. 8. *In vivo* complementation of the BvgS H1172Q mutation. *E.coli* strain JFMC3 (*fhaB-lacZYA*; Miller *et al.*, 1989) was used to assess mutant *bvgS* alleles for intermolecular complementation. *bvgA* is wild-type unless otherwise noted. The first column designates alleles present on a pBR322-derived plasmid, and the second column designates alleles present on a pACYC184-derived plasmid (Sambrook *et al.*, 1989). Ω150 and Ω516 alleles are small in-frame insertions of approximately four amino acids (Miller *et al.*, 1992). Units of β-galactosidase are expressed as means of triplicate assays ± standard deviation.

suggested that the presence of the BvgS receiver may decrease the levels of phosphorylation of BvgS in a manner that requires Asp1023. To investigate further, we phosphorylated GST–C with 'BvgS and purified phosphorylated GST–C from 'BvgS and ATP. We assayed GST–RC, GST–RC D1023N and GST–R (the BvgS receiver fused to GST) for their ability to dephosphorylate GST–C. As shown in Figure 7, addition of GST–RC to the phosphorylated BvgS C-terminal domain resulted in a rapid loss of label as compared with incubation in buffer. Addition of GST–RC D1023N did not significantly dephosphorylate the C-terminal domain, indicating that Asp1023 was required for dephosphorylation. Phosphorylation of GST–RC and GST–RC D1023N by the C-terminal domain was detected, indicating that the C-terminal domain could phosphorylate these molecules at a low level (Figure 7). The BvgS receiver was sufficient for dephosphorylation of the C-terminal domain, as addition of the BvgS receiver (Figure 7, + GST–R) resulted in rapid loss of label. These results demonstrate that the BvgS receiver serves to dephosphorylate the C-terminal domain in the absence of ATP in an Asp1023-dependent manner.

Intermolecular complementation of the BvgS H1172Q mutation in vitro and in vivo

Intermolecular complementation of mutations has been demonstrated for some sensor proteins, including BvgS (Yang and Inouye, 1991; Swanson *et al.*, 1993; Beier *et al.*, 1995). In Figure 8, BvgS alleles containing mutations in the periplasmic domain, transmitter, receiver or C-terminal domain were assessed for intermolecular complementation. These alleles individually inactivate BvgS (Miller *et al.*, 1992; Uhl and Miller, 1994) and were not complemented by a *bvgA*$^+$Δ*bvgS* allele (Figure 8). Mutations in the periplasmic domain of BvgS, the Ω150 and Ω516 alleles, were not complemented by mutations in the transmitter (H729Q) or receiver (D1023N). We also observed no intermolecular complementation between the *bvgS* H729Q mutation and the *bvgS* D1023N mutation. This would suggest either that transfer from His729 to Asp1023 is intramolecular or that the H729Q or D1023N mutations lock the molecule into an inactive conformation.

bvgS H1172Q was complemented by Ω150, H729Q or D1023N. These results show that some *bvgS* alleles with an intact C-terminal domain are able to complement the *bvgS* H1172Q mutation and are consistent with a model in which *trans*-phosphorylation of the BvgS C-terminal domain occurs within a multimeric complex. The inability of the Ω516 allele to complement *bvgS*H1172Q indicates that this allele may have a dominant signal-negative phenotype, although this has not yet been explored further. Our results with intermolecular complementation agree with findings of Beier *et al.* (1995) who have demonstrated that a mutation in the C-terminal domain of BvgS can be complemented by overexpression of a wild-type C-terminal domain.

Since the C-terminal domain was able to phosphorylate BvgA, we reasoned that addition of the C-terminal domain to 'BvgS H1172Q may restore BvgA phosphorylation *in vitro*. As shown in Figure 9, addition of the BvgS C-terminal domain (GST–C) to 'BvgS H1172Q resulted in phosphorylation of BvgA (Figure 9, lane 4). His1172 was required for complementation, as a mutant C-terminal domain, GST–C H1172Q, did not restore phosphorylation of BvgA by 'BvgS H1172Q (Figure 9, lane 6). In all cases examined, mutations that result in a Bvg$^-$ phenotype *in vivo* have a defect in the phosphorylation cascade *in vitro*. Rescue of these mutations results in restoration of the phosphorylation cascade from BvgS to BvgA.

Discussion

It is clear from our analysis that BvgAS signal transduction involves multiple phosphorylation steps. The BvgS transmitter, receiver and C-terminal domain have distinct

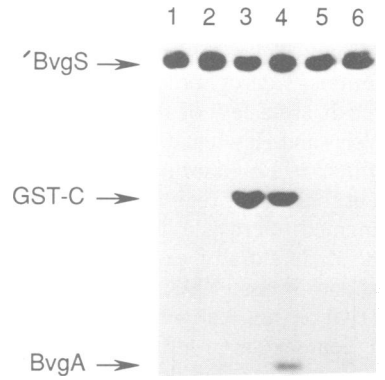


Fig. 9. *In vitro* complementation of 'BvgS H1172Q. 'BvgS H1172Q was incubated in a phosphorylation reaction (lane 1) with addition of BvgA (lane 2) or GST–C (lane 3) or BvgA and GST–C (lane 4). Lanes 5 and 6 are identical to lanes 3 and 4 except that GST–C contains the H1172Q mutation.

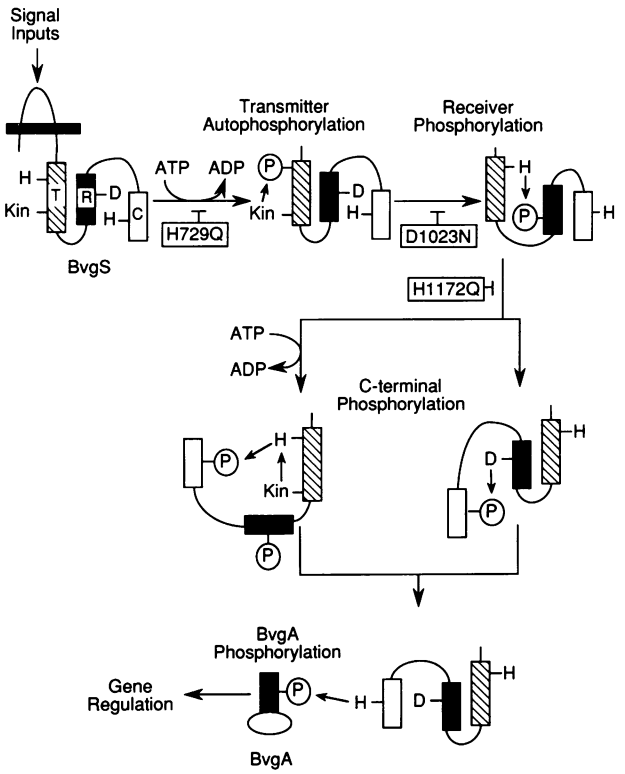


Fig. 10. Model of BvgAS signal transduction. Proposed steps in the phosphorylation cascade involved in *Bordetella* virulence gene activation are depicted above, with details described in the text. Only the cytoplasmic steps of signal transduction are depicted for BvgS, which is localized to the cytoplasmic membrane (Stibitz and Yang, 1991). BvgS domains are abbreviated as follows: T, transmitter; R, receiver; C, C-terminus. The phosphoryl group is denoted by an encircled P, and the points at which loss of function mutations (H729Q, D1023N, H1172Q) interrupt the phosphorylation cascade are shown.

roles in signal transduction that can be separated by loss of function mutations. Figure 10 depicts a model of the BvgAS phosphorylation cascade and our proposed roles for BvgS cytoplasmic domains in this process. Although BvgS is depicted as a monomer, it is likely that multimerization plays an integral role in its function. Multimerization of BvgS is supported by intermolecular complementation of BvgS mutations as well as genetic evidence for dimerization (Beier *et al.*, 1995).

Following proper signal inputs, BvgS autophosphorylates at His729 by means of the transmitter kinase activity (Figure 10). Since the BvgS H729Q mutation prevents autophosphorylation and other signaling events, our model predicts that His729 of the transmitter is the initial site of phosphorylation. After autophosphorylation, the phosphoryl group is transferred from the transmitter to the BvgS receiver at Asp1023. The D1023N and K1080R mutations prevent *trans*-phosphorylation of the C-terminal domain and BvgA phosphorylation and are predicted to interfere with receiver phosphorylation and activation. For this reason, we suggest that receiver phosphorylation is necessary for all subsequent steps in the cascade. Determination of whether the BvgS receiver can serve directly as a phosphodonor for the C-terminal domain has been hampered by the instability of the phosphorylated BvgS receiver as well as its ability to dephosphorylate the C-terminal domain rapidly. Consequently, we present two potential roles for the BvgS receiver in signal transduction. The receiver may serve as a phosphodonor for the C-terminal histidine (H1172). Alternatively, receiver phosphorylation may relieve an autoinhibitory effect, allowing the transmitter to re-autophosphorylate and transfer a phosphoryl group to the C-terminal domain. Phosphorylation and dephosphorylation of the C-terminal domain by the BvgS receiver are both dependent on Asp1023.

The BvgS C-terminal domain is required for phosphorylation of BvgA. The C-terminal domain alone is able to serve as a phosphodonor for BvgA (Figure 7), and the BvgS H1172Q mutation prevents BvgA phosphorylation but not autophosphorylation or phosphotransfer to GST–C. Phosphorylation of BvgA increases its affinity for specific BvgA binding sites upstream of the *fhaB* promoter, and phosphorylation of BvgA presumably leads to activation of *Bordetella* virulence genes (Boucher *et al.*, 1994).

If the path of the phosphoryl group is from the BvgS transmitter to the receiver to the C-terminal domain and then finally to BvgA, then BvgAS signal transduction may be analogous to the phosphorylation cascade involved in initiating *Bacillus subtilis* sporulation (Burbulys *et al.*, 1991; Hoch, 1995). KinA or KinB, sensor proteins with transmitter domains, autophosphorylate and transfer a phosphoryl group to the receiver of Spo0F. Spo0F is a phosphodonor for Spo0B, which has no strong sequence similarity to other known proteins. Chemical stability analysis suggests the site of phosphorylation of Spo0B is a histidine or lysine (Burbulys *et al.*, 1991). Spo0B phosphorylates Spo0A, a response regulator with a C-terminal helix–turn–helix motif. So, KinA and KinB may be thought of as functional equivalents of the BvgS transmitter, while Spo0F is analogous to the BvgS receiver. Spo0B and the BvgS C-terminal domain both serve to receive a phosphoryl group and transfer it to Spo0A or BvgA, respectively.

An alternative mechanism for phosphorylation of the C-terminal domain is re-autophosphorylation of the BvgS transmitter followed by transfer of the phosphoryl group from the transmitter to the C-terminal domain (Figure 10). Phosphotransfer between the domains requires phosphorylation of the BvgS receiver. Histidine to histidine phos-

photransfer has not been described in two-component systems, but has been well characterized in phosphotransferase system (PTS) enzymes involved in sugar transport (Saier, 1989). The two proposed pathways for the flow of phosphate through BvgS differ not only by the domain which transfers the phosphoryl group to the C-terminus, but also by the stoichiometric requirement for ATP hydrolysis (Figure 10). Hydrolysis of two ATP molecules would be required prior to phosphorylation of the C-terminal domain by the transmitter. The first pathway, in which the receiver phosphorylates the C-terminal domain, requires hydrolysis of only one ATP molecule. Recent results support the first pathway and suggest that the receiver has a direct role in C-terminal domain phosphorylation. *Trans*-phosphorylation of the C-terminal domain is eliminated *in vitro* by precise deletion of the BvgS receiver but not by deletion of the BvgS C-terminal domain. Additionally, genetic evidence supports the notion that phosphorylation and not merely activation of the receiver is necessary for virulence gene expression. Several independent mutations which suppress the *bvgS*D1023N phenotype *in vivo* have been isolated, and all of these mutations result in reversion of Asn1023 to Asp (Uhl and Miller, unpublished data).

In the majority of two-component systems, a transmitter domain is sufficient for both autophosphorylation and phosphotransfer to a receiver domain. However, three domains are necessary for BvgS phosphotransfer to BvgA. It is not yet understood how the requirement for the receiver and C-terminal domain in BvgAS signal transduction relates to the biology of *Bordetella* infection of mammalian hosts. These domains may serve to adjust the response of BvgS by serving as control points that gauge the intensity or duration of environmental signals. On the other hand, the BvgS C-terminal domain could receive alternative signal inputs. The *Bacillus subtilis* Spo0BG protein, which binds GTP, has been proposed to sense GTP levels and relay this information to Spo0B and the sporulation initiation cascade (Kok *et al.*, 1994). Alternative signal inputs have also been proposed for ArcB, a BvgS family member, since the C-terminal domain of ArcB does not appear to be required for ArcB-mediated repression of lactate dehydrogenase (Iuchi *et al.*, 1990; Ishige *et al.*, 1994). While the requirement of various BvgS domains for *bvg*-mediated repression of genes has not yet been examined, the BvgS C-terminal domain is essential for BvgS-mediated activation of virulence genes. It is becoming increasingly evident that two-component systems can utilize domains other than transmitters and receivers in phosphorylation cascades. In addition to initiation of sporulation in *B.subtilis* and virulence gene activation in *Bordetella*, establishment of mucoidy in *Pseudomonas aeruginosa* is another example of a two-component system that includes a non-traditional domain (Roychoudhury *et al.*, 1992). Additionally, genetic evidence in *Saccharomyces cerevisiae* and *Arabidopsis thaliana* indicates that two-component systems in these organisms interact with characterized eukaryotic signal transduction pathways which include Raf and MAP kinase (Chang *et al.*, 1993; Maeda *et al.*, 1994). The means of communication between two-component systems and these pathways are not known.

BvgS is clearly a principal processor of information for *Bordetella* species. Colony morphology, cell shape, production of toxins and adhesins, survival under nutrient-limiting conditions, siderophore production and motility/flagellar synthesis are a few of the phenotypes controlled by BvgAS (Weiss and Hewlett, 1986; Akerley *et al.*, 1992; Cotter and Miller, 1994; Giardina *et al.*, 1995). Although the requirement for *bvg* for regulation of these phenotypes is well documented, there is evidence that *bvg*-regulated genes may be differentially expressed (Scarlato *et al.*, 1991; Melton and Weiss, 1993; Carbonetti *et al.*, 1994; Stibitz, 1994). It is not known if particular classes of *bvg*-activated genes require different levels of BvgAS activation or accessory factors. Incorporation of additional types of phosphorelay domains may represent a mechanism that BvgAS and other two-component systems utilize to couple distinct signals to diverse outputs.

Materials and methods

Bacterial strains, β-galactosidase assays, plasmids and growth conditions

Escherichia coli strain DH5α (Miller, 1992) was utilized for overexpression of proteins for purification. For quantitation of *bvg* activity *in vivo*, *E.coli* strain JFMC3 (MC4100 *recA* λ*fhaB-lacZYA*) was employed (Miller *et al.*, 1989). Cultures were grown in Luria–Bertani media (Miller, 1992); when necessary media were supplemented with antibiotics at concentrations of 100 μg/ml for ampicillin and 40 μg/ml for chloramphenicol. β-Galactosidase activities were determined with cells permeabilized with SDS/$CHCl_3$ as described (Miller, 1992).

Plasmid pDM20 which carries the entire *bvgAS* locus on a pBR322-derived plasmid has been described previously, as have plasmids pSJ30 (pDM20 with the Ω150 mutation) and pSJ40 (pDM20 with the Ω516 mutation) (Miller *et al.*, 1992). Plasmids pMU212 (pDM20 with the *bvgS* D1023N mutation) and pMU222 (pDM20 with the *bvgS* H729Q mutation) have also been described (Uhl and Miller, 1994). pMU230, $bvgA^{+}\Delta bvgS$, was constructed by digesting pDM20 with *Stu*I, resulting in deletion of the BvgS cytoplasmic domains as well as the majority of the periplasmic domains (Uhl and Miller, 1995b).

For intermolecular complementation experiments, the *bvg* locus (wild-type or mutant derivatives) was cloned as an *Ssp*I–*Not*I fragment into pACYC184 (Sambrook *et al.*, 1989) which had been digested with *Eag*I and *Eco*RV. pMU352 ($\Delta bvgAbvgS^{+}$) was created by digesting pMU281 (wild-type *bvgAS* on pACYC184-derived plasmid; Uhl and Miller, 1995b) with *Xcm*I and religating the plasmid. This created an in-frame deletion in the C-terminal portion of BvgA.

pMU155 (Uhl and Miller, 1994) contains the BvgS linker, transmitter, receiver and C-terminal domain cloned as an *Eco*RI–*Pst*I fragment into pTrc99A (Pharmacia). pMU155 was utilized for overexpression of 'BvgS. Plasmids used for overexpression of 'BvgS containing point mutations were constructed in a similar fashion to pMU155. Construction of pJM660, the BvgA overexpression vector, has been described previously (Uhl and Miller, 1994). pJM660 contains the entire *bvgA* gene in pTrc99A.

Construction of GST–BvgS fusion proteins and the bvgS H1172Q mutation

pMU100 contains 'BvgS as a C-terminal fusion to GST (GST–'BvgS). pMU100 was constructed by digesting pDM20 with *Eco*RI, isolating the 2.7 kb fragment, ligating *Bam*HI 8mers (Promega), digesting with *Bam*HI and cloning the resulting fragment into *Bam*HI-digested pGEX2T (Pharmacia). pMU502 is the BvgS receiver and C-terminal domain fused to GST (GST–RC). pMU502 was constructed by digesting pMU304 (Uhl and Miller, 1994) with *Nco*I and blunting the ends by treatment with the Klenow fragment of DNA polymerase. The 1.1 kb fragment was cloned into pGEX2T which had been digested with *Eco*RI–*Bam*HI and treated with the Klenow fragment of DNA polymerase. pMU506 was the vector used for overexpression of GST–RC D1023N, and is pMU502 containing the BvgS D1023N mutation (GST–RC D1023N). pMU516, a fusion between GST and the BvgS C-terminal domain (GST–C) was constructed by introducing a *Bam*HI–*Eco*RI fragment from pMU647 into *Bam*HI–*Eco*RI-digested pGEX3X. pMU647 is pBluescript KS+ (Stratagene) with an *Xma*I–*Pvu*II fragment from pMU209 inserted

into the *Xma*I–*Eco*RV sites in the polylinker. The *Xma*I–*Pvu*II fragment contains the alanine/proline linker between the BvgS receiver and C-terminal domains as well as the entire C-terminal domain. pMU209 is identical to pDM20 (Uhl and Miller, 1994), except that it contains a single nucleotide change (A4106→C) that creates a *Xma*I site at the junction of the receiver and C-terminal domain. This nucleotide change is a silent mutation and does not affect BvgS activity *in vivo* (unpublished data). The GST–C H1172Q overexpression plasmid, pMU527, was constructed in a similar fashion to pMU516. pMU525, the plasmid used for overexpression of the BvgS receiver (GST–R), was constructed by replacing an *Sph*I–*Sna*BI fragment from pMU502 with an identical fragment from pHB50delC. pHB50delC was created by digesting pMU209 with *Xma*I and inserting the self-annealing oligonucleotide 5′-CCGGGGGTAAAAGCTTTTACCC-3′ which creates an in-frame stop codon at the end of the BvgS receiver.

The BvgS H1172Q mutation was constructed with mismatch oligonucleotides and PCR using *Pfu* DNA polymerase (Stratagene). PCRs were with the primers H1172Q 5′ (5′-GACATGGCGCAGAGGCTGGCCGGCGGC-3′) and p4592 (5′-CCAATTATCCGTAAGGTCC-3′) or H1172Q 3′ (5′-CGGCCAGCCTCTGCGCCATGTCCGAGAC-3′) and p4192 (5′-GACGGCGTTCTCGGCCGAGTC-3′). The resulting PCR products were mixed and PCR was repeated with the primers p4592 and p4192. The PCR product was digested with *Eag*I and introduced into pMU304. Digestion of pMU304 with *Nco*I and introduction of the 1.1 kb fragment into pDM20 gave rise to pMU709, which is pDM20 with the BvgS H1172Q mutation. The portion of pMU709 derived from the PCR fragment was sequenced to ensure that no other mutations had arisen.

Protein purification

GST–′BvgS, ′BvgS and BvgA were purified by a previously described method (Jin *et al.*, 1990a; McCleary and Zusman, 1990; Uhl and Miller, 1994). Briefly, cultures of *E.coli* containing plasmids overexpressing BvgA or BvgS were grown to mid-exponential phase (OD_{600} of 0.6–0.8) and expression induced by addition of 1 mM IPTG. Cultures were grown an additional 2 h, then pelleted and washed with Wash Buffer #1 [20 mM HEPES (pH 7.4), 50 mM KCl, 10% glycerol]. Cultures were resuspended in 0.02 vol of Wash Buffer #1 and sonicated with 30 s bursts followed by 2 min rests. The sonicate was centrifuged for 10 min at 4°C, 18 000 *g*. The insoluble pellet fraction was washed with Wash Buffer #2 [10 mM HEPES (pH 7.2), 50 mM KCl, 0.1% Triton X-100], solubilized in denaturation buffer [6 M guanidine–HCl, 50 mM Na_2HPO_4 (pH 7.2)] and dialyzed against 200× volume of renaturation buffer [20 mM HEPES (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 50% glycerol] for 18 h without stirring. Renaturation buffer was changed and dialysis continued for another 18 h with stirring. Proteins were stored at –70°C.

For purification of GST–RC, GST–C and their mutant derivatives, cultures were grown, induced and sonicated as above. The majority of the GST fusion proteins remained soluble following sonication. Following separation of the sonicate into soluble and insoluble fractions, the supernatant was loaded on a glutathione–Sepharose column (Pharmacia). The column was prepared according to the manufacturer's instructions. The column was washed with phosphate-buffered saline (PBS) (Sambrook *et al.*, 1989) and GST fusion proteins were eluted with elution buffer [50 mM Tris–HCl (pH 8.0), 10 mM glutathione]. Positive fractions were diluted 1:1 in a buffer containing 10 mM DTT and 90% glycerol and stored at –70°C.

Phosphorylation reactions and chemical stability of phosphorylated proteins

Reactions were in a buffer containing 30 mM HEPES (pH 7.2), 50 mM KCl, 10 mM $MgCl_2$, 30 μM ATP (sp. act. 0.15–0.30 Ci/mmol). BvgA and ′BvgS wild-type or mutant derivatives were present at 0.3 μM. GST–RC, GST–R and GST–C were present at 1 μM. Reactions proceeded for 5 min at room temperature and were terminated by addition of 4× sample buffer [0.32 M Tris (pH 6.8), 40% glycerol, 0.1 M EDTA (pH 8.0), 8% SDS, 0.4 M DTT] and heated to 55°C for 5 min. Samples were separated by SDS–PAGE and transferred to an Immobilon membrane (Millipore). The membrane was dried and exposed to film at –70°C. Chemical stability of phosphorylated proteins was determined by treating membranes with either 50 mM Tris (pH 7.6), 3 M NaOH or 1 M HCl for 2 h at room temperature. Treated membranes were rinsed with double-distilled water, dried and exposed to film.

For phosphotransfer experiments, phosphorylation reactions were performed as above except GST–′BvgS or ′BvgS was present at 0.6 μM and ATP was at 60 μM. Following a 5 min incubation, 150 μl of glutathione–agarose was added. Binding of GST fusion proteins proceeded for 10 min on ice. Agarose beads were pelleted with a brief spin in a microfuge and washed 4–6 times with PBS. Proteins were eluted with elution buffer for 10 min on ice. The eluant was then incubated at room temperature for various lengths of time in a buffer that contained 50 mM Tris–HCl (pH 7.5), 50 mM KCl and 10 mM $MgCl_2$.

Thin-layer chromatography

The method of Hess *et al.* (1987) was used for thin layer chromatography. 1×10^3 c.p.m. of labeled GST–′BvgS denatured in sample buffer, ATP or inorganic phosphate were spotted on a polyethyleneimine (PEI) cellulose plate (Brinkmann) and resolved with 0.8 M LiCl and 0.8 M acetic acid. A sample of phosphorylated GST–′BvgS was incubated in 100 mM Tris–HCl (pH 7.5), 100 mM KCl and 20 mM $MgCl_2$ for either 1 min or 10 min before denaturation in sample buffer. The PEI cellulose plate was dried and wrapped in polyvinyl chloride before autoradiography at –70°C.

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Note added in proof

While this article was in press, Tsuzuki *et al.* described phosphorylation of the ArcB C-terminal domain by a phosphotransfer mechanism, although the conserved aspartate in the ArcB receiver that corresponds to Asp1023 of BvgS was not required for this process (Tsuzuki *et al.*, *Mol. Microbiol.*, **18**, 953–962). Similarly to BvgS, the C-terminal domain of ArcB is able to serve as a phosphodonor to the cognate response regulator, ArcA.