Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 1163–1167, February 1994
Biochemistry

Autophosphorylation and phosphotransfer in the *Bordetella pertussis* BvgAS signal transduction cascade

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Communicated by Stanley Falkow, November 1, 1993

ABSTRACT **Expression of adhesins, toxins, and other virulence factors of *Bordetella pertussis* is under control of the BvgA and BvgS proteins, members of a bacterial two-component signal transduction family. BvgA bears sequence similarity to regulator components, whereas BvgS shows similarity to both sensor and regulator components. BvgA and the cytoplasmic portion of BvgS ('BvgS) were overexpressed and purified. 'BvgS autophosphorylated with the γ-phosphate from [γ-^{32}P]ATP and phosphorylated BvgA. Kinetic analysis indicated that BvgA receives its phosphate from 'BvgS. Mutations in the transmitter, receiver, and C-terminal domains of BvgS were tested for activation of a BvgAS-dependent *fhaB*::*lacZ* reporter fusion *in vivo* and for autophosphorylation and phosphotransfer to BvgA *in vitro*. All mutations abolished activation of the *fhaB*::*lacZ* fusion. A point mutation in the transmitter (H729Q) prevented autophosphorylation of 'BvgS. In contrast to other characterized sensor proteins, autophosphorylation also required sequences in the 'BvgS receiver and C-terminal domains. A 'BvgS receiver point mutation (D1023N) had the novel phenotype of being able to autophosphorylate but unable to transfer the phosphate to BvgA. Autophosphorylation activity of the D1023N mutant protein was kinetically and chemically indistinguishable from wild-type 'BvgS despite an uncoupling of phosphotransfer from autophosphorylation. 'BvgS was shown to contain primarily amidyl phosphate and BvgA an acyl phosphate linkage. We present a model for a phosphorelay controlling virulence gene expression in *B. pertussis*.**

Phosphorylation cascades mediated by bacterial two-component systems provide a conserved mechanism for coordinate regulation in response to signal inputs. In bacteria, diverse processes such as chemotaxis, cell division, metabolic pathways, nitrogen fixation, stress responses, and virulence are regulated by two-component systems. Two distinct modules are typically involved; a sensor recognizes environmental cues and transmits a signal to a response regulator. Signal transduction between the sensor and response regulator is a phosphorelay (1, 2), with the sensor protein autophosphorylating at a conserved histidine residue in the transmitter domain (≈220 aa) and transferring the phosphate group to a conserved aspartic acid in the receiver domain (≈130 aa) of the response regulator.

In *Bordetella pertussis*, the etiologic agent of whooping cough, the BvgAS proteins control a variety of virulence determinants (3–5). In addition to positively regulating adhesins and toxins, BvgAS negatively regulates motility in *Bordetella bronchiseptica* and a collection of genes in *B. pertussis* (6–8). Sequence analysis predicts that BvgA is a 23-kDa protein with an N-terminal receiver domain and a C-terminal helix–turn–helix motif (5). BvgA is cytoplasmically located and binds to specific regions in the *fhaB* and *bvgAS* promoters (9–11). BvgS is a 134-kDa protein localized to the cytoplasmic membrane by means of two transmembrane domains (12). Transcriptional activation mediated by BvgS is decreased by temperature, sulfate anion, and nicotinic acid, a phenomenon termed modulation (13, 14). Both the periplasmic region and the linker, which lies adjacent to the second transmembrane sequence, are involved in sensing and transmitting environmental signals (14). BvgS contains a transmitter domain found in sensor proteins but is distinguished from the majority of sensors by containing a receiver domain typically found in response regulators and a C-terminal region with no significant sequence similarity to other known proteins (5). BvgS therefore belongs to a group of unorthodox sensor proteins with linked transmitters and receivers. The transmitter, receiver, and C-terminal domains are separated by alanine/proline-rich sequences of ≈15 aa (5). In other proteins, similar sequences form flexible hinges to allow interactions between distinct domains (15, 16).

The relationship between the complex structure of BvgS and its function in controlling *Bordetella* virulence in response to environmental signals is unknown. Although the biochemical activities of transmitter and receiver domains have been characterized, the effects of multiple domains present in the same sensor protein have yet to be determined. To address the individual and combinatorial roles of the transmitter, receiver, and C terminus in signal transduction, we examined BvgS *in vitro* and *in vivo*. BvgS domains were altered by site-directed mutagenesis of conserved amino acids and by introducing small in-frame insertions and deletions. The mutated proteins were analyzed for their ability to activate an *fhaB*::*lacZ* fusion in *Escherichia coli*. For *in vitro* studies, BvgA, BvgS, and mutant derivatives were purified and studied in phosphorylation reactions. Roles for BvgS domains in signal transduction are proposed based on *in vitro* and *in vivo* observations.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. For BvgS overexpression, a 2.5-kb *Eco*RI–*Pst* I fragment from pDM20 (17) encoding the linker, transmitter, receiver, and C terminus (aa 532–1238, designated 'BvgS) was cloned into the *Eco*RI/*Pst* I site of pTrc99A (Pharmacia). For BvgA overexpression, we introduced an *Afl* III site after the initial methionine codon in *bvgA*. The *Afl* III-cut fragment was cloned into *Nco* I-digested pTrc99A. The resulting plasmid was then digested with *Bst*BI and *Sma* I, and a *Bst*BI–*Sph* I fragment from pDM20 comprising the rest of BvgA was inserted.

The oligonucleotide-directed mutagenesis method (Amersham) was used for site-directed mutagenesis. Oligonucleotides were hybridized to single-stranded DNA created from portions of pDM20 cloned into M13mp19 (18). The mutations were introduced into pDM20 and sequenced.

Plasmid pMU304, which contains bp 3431–5210 of pDM20 in pBR322 (18), was used to construct BvgS receiver mutations. pMU304 was digested either with *Bsm* I or with *Bsm* I

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and *Pfl*MI. After treatment of the ends with T4 DNA polymerase, a *Bam*HI octamer (Promega) was inserted. The portion containing the mutation was cloned back into pDM20. The BvgS C-terminal Δ15C mutation was created by digesting pDM20 with *Mun* I and ligating the two large fragments. This deleted bp 4233–4278 of pDM20. The BvgS C-terminal linker insertion, pSJ60, has been described (14).

Purification of BvgA and 'BvgS. *E. coli* strain DH5α cultures containing the 'BvgS- or BvgA-overproducing plasmids were grown to midlogarithmic phase, induced with 2 mM isopropyl β-D-thiogalactopyranoside, and grown 2 hr at 37°C. Proteins were purified by the methods of McCleary and Zusman (15) and Jin *et al.* (19).

Phosphorylation Reactions. Reaction conditions were 30 mM Hepes, pH 7.2/50 mM KCl/10 mM $MgCl_2$/30 μM [γ-^{32}P]ATP (specific activity, 0.15–0.30 Ci/mmol; 1 Ci = 37 GBq). 'BvgS wild-type or mutant derivatives were present at 0.3 μM and BvgA at 0.3 μM. Reactions proceeded for 5 min at room temperature and were terminated by adding 0.33 volume of 4× sample buffer (320 mM Tris, pH 6.8/40% glycerol/100 mM EDTA/8% SDS) and dithiothreitol to 0.1 M. After heating to 55°C for 5 min, proteins were separated by SDS/PAGE and transferred to Immobilon filter (Millipore). The filter was dried and exposed to film at −70°C. Identical specific activities of [α-^{32}P]ATP or [γ-^{32}P]GTP were added when substituted for [γ-^{32}P]ATP.

For pulse–chase experiments, 'BvgS was incubated in a standard phosphorylation reaction mixture for 5 min. BvgA and 6 mM unlabeled ATP were then added. As a control, 'BvgS was incubated with 6 mM ATP and 30 μM [γ-^{32}P]ATP for 5 min before BvgA addition. The experiment was repeated with 'BvgS and 'BvgS D1023N; after the phosphorylation mixtures were incubated for 5 min, 6 mM ATP was added. Samples were taken prior to and at various time points after addition of unlabeled ATP. A mock chase in which the proteins were incubated with both 30 μM labeled ATP and 6 mM unlabeled ATP was performed as a control. ^{32}P incorporation was estimated by autoradiography with preflashed film followed by laser densitometry of a range of exposures.

***In Vivo* Analysis.** All *in vivo* assays were performed in the *E. coli* strain JFMC3, which contains an *fhaB*::*lacZ* fusion carried by a λ prophage present in the chromosome (17). BvgS mutations were introduced into pDM20 (14). β-Galactosidase levels were assayed in triplicate according to Miller (20).

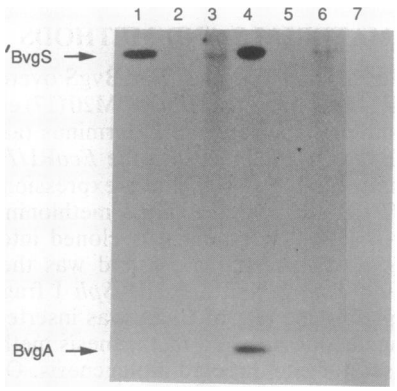

FIG. 1. Characterization of substrates for 'BvgS and BvgA phosphorylation *in vitro*. 'BvgS (lanes 1–3), 'BvgS and BvgA (lanes 4–6), or BvgA alone (lane 7) was incubated with [γ-^{32}P]ATP (lanes 1, 4, and 7), [α-^{32}P]ATP (lanes 2 and 5), or [γ-^{32}P]GTP (lanes 3 and 6) in the standard phosphorylation mixture. Reaction products were separated by SDS/PAGE and transferred to Immobilon prior to autoradiography. The location of the 75-kDa cytoplasmic portion of BvgS ('BvgS) and the 23-kDa BvgA protein are denoted with arrows.

Chemical Stability of Phosphorylated Proteins. Phosphorylated proteins on Immobilon (Millipore) filters were either untreated or treated for 2 hr at 22°C with 50 mM Tris (pH 7.4), 3 M NaOH, or 1 M HCl. Strips were rinsed with double-distilled water, dried, and exposed to preflashed film at −70°C. Filter strips were stained with amido black to ensure that the removal of label was not due to loss of proteins from the membranes. Densitometry was used for quantitative analysis.

RESULTS

Phosphorylation of 'BvgS and BvgA. To assay the biochemical activities of BvgA and BvgS, we overexpressed and purified the cytoplasmic portion of BvgS (aa 532–1238, 'BvgS) and full-length BvgA. The cytoplasmic portion of BvgS was chosen for overexpression to avoid hydrophobic stretches of amino acids. The 75-kDa 'BvgS protein autophosphorylated with [γ-^{32}P]ATP but not with [α-^{32}P]ATP and only slightly with [γ-^{32}P]GTP (Fig. 1, lanes 1–3). The smearing noted with [γ-^{32}P]GTP (lane 3) was repeatedly observed. When BvgA was added to the 'BvgS phosphorylation reaction it was phosphorylated (lane 4); however, BvgA in the absence of 'BvgS did not detectably autophosphorylate (lane 7). BvgA phosphorylation paralleled that of 'BvgS, as BvgA was labeled with [γ-^{32}P]ATP but not with [α-^{32}P]ATP (lanes 4 and 5). [γ-^{32}P]GTP did not give rise to a labeled BvgA product (lane 6). This demonstrates that the cytoplasmic portion of BvgS is sufficient for autophosphorylation ability and for phosphorylation of BvgA *in vitro*. 'BvgS uses [γ-^{32}P]ATP preferentially as a substrate for autophosphorylation, and labeling of the protein is due to removal of the γ-phosphate from ATP as opposed to ATP binding.

Kinetics of BvgA Phosphorylation. A pulse–chase experiment was performed to determine whether BvgA was phosphorylated from the free nucleotide pool or from phosphorylated 'BvgS. 'BvgS was incubated in a phosphorylation mixture with 30 μM [γ-^{32}P]ATP for 5 min, and then an excess

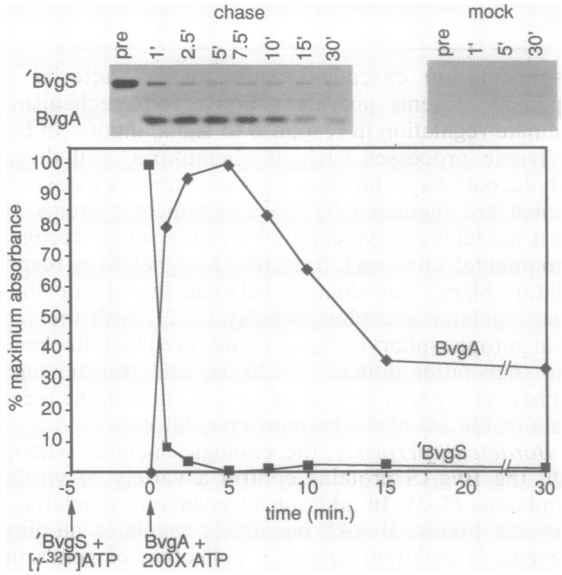

FIG. 2. Pulse–chase analysis of BvgA phosphorylation. 'BvgS was preincubated with [γ-^{32}P]ATP for 5 min before addition of BvgA and a 200-fold excess of unlabeled ATP. Samples were taken at various times, subjected to SDS/PAGE, transferred to an Immobilon membrane, and exposed to preflashed film. Percent maximum absorbance of bands corresponding to 'BvgS (■) and BvgA (◆) was determined by densitometry of the autoradiogram. The data shown are representative of results obtained from several independent experiments.

of unlabeled ATP (6 mM) and BvgA were simultaneously added. Labeling of BvgA coincided with dephosphorylation of 'BvgS (Fig. 2). Incorporation of ^{32}P into BvgA was not initially inhibited by the addition of excess unlabeled ATP. When 'BvgS was incubated for 5 min with both 30 μM [γ-^{32}P]ATP and 6 mM ATP, followed by addition of BvgA in a mock chase reaction, efficient labeling of 'BvgS and BvgA was not detected (Fig. 2, mock). This demonstrates that BvgA phosphorylation is not due to stimulation of the reaction by high concentrations of ATP and that incorporation of label can be inhibited by an excess of unlabeled ATP added at the beginning of the reaction. If BvgA is phosphorylated from the free nucleotide pool, then an excess of unlabeled ATP should compete effectively with labeled ATP for BvgA phosphorylation in a pulse–chase reaction. Conversely, if BvgA is phosphorylated by 'BvgS, then the label should be chased from 'BvgS to BvgA. BvgA phosphorylation is initially resistant to competition from excess ATP, and it therefore appears that BvgA phosphate is directly obtained from 'BvgS in a phosphotransfer reaction. 'BvgS autophosphorylation ability and phospho group transfer to BvgA parallel observations made with canonical sensor proteins containing transmitter but not receiver domains (1, 2).

***In Vivo* Characteristics of BvgS Mutants.** The *bvg* operon has previously been shown to function in *E. coli*, directly activating the promoter for the *fhaB* adhesin gene and responding to the same modulation signals as in *B. pertussis* (17). Mutations were introduced into the BvgS transmitter, receiver, and C-terminal regions, and activity of the mutant proteins was measured *in vivo* by assessing activation of a chromosomal *fhaB*::*lacZ* fusion in the presence of a wild-type *bvgA* allele (Fig. 3). His^{729} of BvgS corresponds to the conserved histidine found in all transmitters and is analogous to His^{48} of CheA, which has been demonstrated to be the site of autophosphorylation (21). His^{729} was changed to Gln (BvgS H729Q), a conservative amino acid substitution that

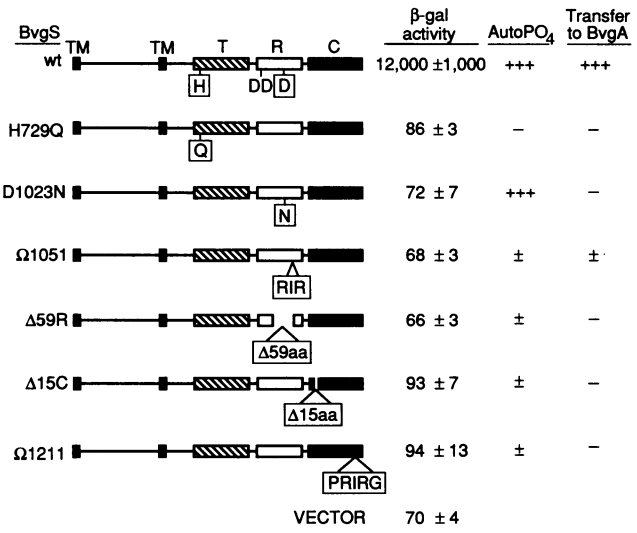

FIG. 3. *In vivo* activity of mutant BvgS derivatives. The BvgS transmembrane regions (TM), transmitter (T), receiver (R), and C-terminal domain (C) are noted. Conserved histidine (H) and aspartic (D) residues are also labeled, with potential sites of phosphorylation (H729 and D1023) boxed. Allele designations are listed beside the representation of the mutation. Mutations were present on plasmid pDM20 derivatives (14) in cis to a wild-type *bvgA* allele. β-Galactosidase (β-gal) activities resulting from transcriptional activation of a chromosomal *fhaB*::*lacZ* fusion are given in Miller units (20) with standard deviation indicated. Autophosphorylation ($AutoPO_4$) and phosphotransfer capabilities of mutant proteins are summarized as follows: +++, full activity; ±, slight activity (<2% of wild type); –, no detectable activity.

was predicted to abolish autophosphorylation. Asp^{1023} of BvgS is conserved in all receivers and corresponds to the sites of phosphorylation in the CheY (Asp^{57}) and VirG (Asp^{52}) response regulators (22, 23). This residue was converted to Asn in the BvgS D1023N mutant. To assess the role of the C terminus of BvgS, a 12-bp insertion between codons for aa 1211 and 1212 (BvgS Ω1211, ref. 14) and a 15-aa deletion from aa 1140 to aa 1154 (BvgS Δ15C) were constructed. As expected, the H729Q mutation rendered BvgS inactive *in vivo* (Fig. 3). BvgS D1023N, BvgS Ω1211, and BvgS Δ15C were also unable to activate the *fhaB*::*lacZ* transcriptional fusion, suggesting that the BvgS receiver and C terminus participate in the activation of BvgA.

It has been hypothesized that receivers on sensor proteins can act as autoinhibitory domains (24–26), with phosphorylation relieving inhibition of phosphotransfer. If BvgS D1023N is locked into an inhibitory state, further disruption of the receiver may eliminate inhibition. The BvgS Δ59R mutant contains an in-frame deletion removing approximately half of the receiver as well as the Asp at position 1023. This mutant and an additional mutant containing a small insertion at aa 1051 (BvgS Ω1051) are inactive *in vivo*, arguing against the BvgS receiver acting exclusively as an autoinhibitory component. These results indicate that the predicted sites of phosphorylation of BvgS, His^{729} of the transmitter and Asp^{1023} of the receiver, are required for activation of BvgA. Both the BvgS receiver and C terminus are required for *in vivo* activity.

BvgS Autophosphorylation and Phosphotransfer to BvgA Are Genetically Separable. Although all *bvgS* mutations tested had identical phenotypes *in vivo*, we reasoned that defects could be due either to an inability of BvgS to autophosphorylate or to a loss of phosphotransfer to BvgA. The cytoplasmic portion of BvgS for each of the mutants was purified, and phosphorylation reactions were carried out as described for the wild-type 'BvgS protein. Mutants were assayed in the presence or absence of BvgA to distinguish between autophosphorylation and phosphotransfer.

'BvgS with the His → Gln substitution in the transmitter at the proposed site of autophosphorylation ('BvgS H729Q) did not label with [γ-^{32}P]ATP (Fig. 4, lane 2). Autophosphorylation of the 'BvgS Ω1051, Δ59R, and Δ15C (lanes 4–6) and Ω1211 (data not shown) mutant proteins was substantially reduced. Longer exposures and densitometry revealed residual activities of <2% that of the wild type. In contrast to the complete lack of activity *in vivo*, the 'BvgS D1023N receiver mutant retained full autophosphorylation ability *in vitro* (lane 3).

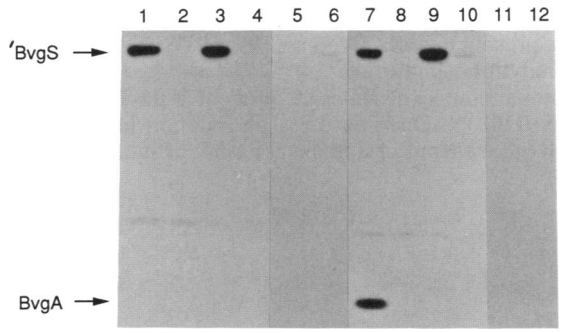

FIG. 4. *In vitro* activity of 'BvgS and mutant derivatives. Wild-type 'BvgS and mutant derivatives were phosphorylated in the absence (lanes 1–6) or presence (lanes 7–12) of BvgA. Lanes 1 and 7, wild type 'BvgS; lanes 2 and 8, 'BvgS H729Q; lanes 3 and 9, 'BvgS D1023N; lanes 4 and 10, 'BvgS Δ15C; lanes 5 and 11, 'BvgS Δ59R; lanes 6 and 12, 'BvgS Ω1051. Locations of 'BvgS and BvgA are marked with arrows.

Mutant proteins with abolished or decreased autophosphorylation ability, 'BvgS H729Q, Δ59R, Δ15C, and Ω1051 (lanes 8, 10, 11, and 12), as well as Ω1211 (data not shown), were defective in phosphotransfer to BvgA, in contrast to the high level of BvgA phosphorylation by the wild-type 'BvgS (lane 7). Longer exposures showed that the Ω1051 protein retains slight phosphotransfer activity, phosphorylating BvgA at <1% of the wild-type level. Efficient 'BvgS autophosphorylation is therefore prerequisite for subsequent phosphorylation of BvgA.

Although autophosphorylation of the wild-type and D1023N 'BvgS proteins occurred at similar levels, their phosphotransfer capabilities were quite different. In contrast to wild-type 'BvgS, phosphorylation of BvgA by the D1023N 'BvgS protein was not detected even after extended periods of autoradiography. This demonstrates that Asp^{1023} in the BvgS receiver is specifically required for phosphotransfer to BvgA but is not necessary for autophosphorylation. The BvgS D1023N mutation genetically separates autophosphorylation from phosphotransfer in the signal transduction cascade.

Chemical Stability of Phosphorylated Proteins. Amidyl phosphates, such as histidine or lysine phosphate, are stable to base but labile to acid. Acyl phosphates (aspartate and glutamate) are acid labile and base labile (1). To compare wild-type 'BvgS, 'BvgS D1023N, and BvgA chemical stability, phosphorylation reactions were performed for each, and the products were subjected to SDS/PAGE and transferred to Immobilon. Immobilon strips were either untreated or treated with acid, base, or a neutral buffer. BvgA was labile to both acid and base (Fig. 5, lanes 5 and 8), characteristic of an acyl phosphate. 'BvgS and 'BvgS D1023N were stable to base and labile to acid (lanes 4–6 and 7–9), indicating that these two proteins are primarily amidyl phosphates. This is consistent with BvgA being phosphorylated at an aspartic residue and 'BvgS wild-type and D1023N at a histidine residue.

Despite the lack of apparent qualitative difference between the wild-type and D1023N 'BvgS proteins in Fig. 5, we reasoned that at least a small portion of the wild-type protein may be phosphorylated at the receiver, since the receiver was genetically indispensable. The two proteins should be chemically distinct upon treatment with base if the 'BvgS D1023N receiver mutation is phosphorylated at only His^{729} and the wild-type 'BvgS is phosphorylated to significant levels at both His^{729} and Asp^{1023}. We therefore repeated the chemical stability test with base treatment for quantitative analysis. Compared with the untreated control, the wild-type 'BvgS retained 81 ± 6% of its label after base treatment, whereas the D1023N 'BvgS mutant retained 91 ± 5% of its label. Baselability results therefore suggest that the majority of phosphate present in both 'BvgS and 'BvgS D1023N is in the form of amidyl phosphate.

Comparison of the Rates of Decay of Wild-Type 'BvgS and 'BvgS D1023N. Once autophosphorylation has occurred in the 'BvgS transmitter domain a variety of subsequent events

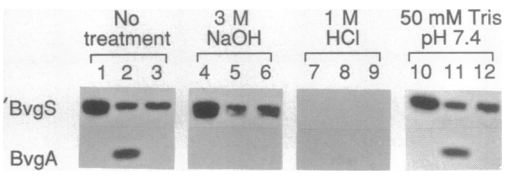

FIG. 5. Chemical stability of phosphorylated proteins. Immobilon strips containing phosphorylated proteins were incubated with 3 M NaOH, 1 M HCl, or 50 mM Tris (pH 7.4) or were left untreated for 2 hr at 22°C. Wild-type 'BvgS (lanes 1, 4, 7, and 10), 'BvgS D1023N (lanes 3, 6, 9, and 12), and wild-type 'BvgS with BvgA (lanes 2, 5, 8, and 11) were treated and exposed to film.

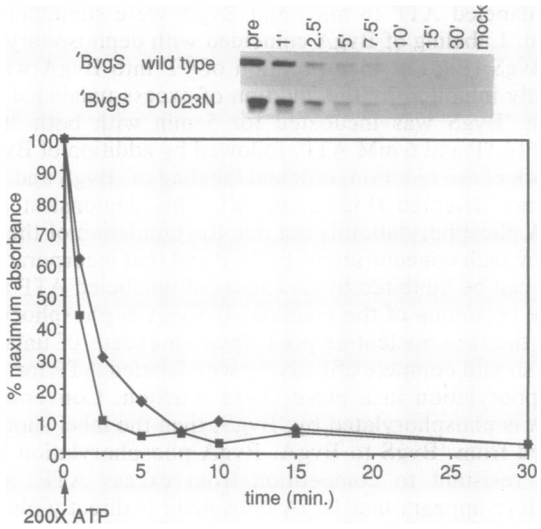

FIG. 6. Kinetic analysis of 'BvgS and 'BvgS D1023N proteins. After 5 min of incubation in a standard phosphorylation mixture, aliquots were removed at the times indicated. After SDS/PAGE, proteins were transferred to Immobilon and exposed to preflashed film. The *y* axis is normalized absorbance of densitometry readings from bands corresponding to 'BvgS (■) and 'BvgS D1023N (◆). The data shown are representative of results obtained from several independent trials.

are conceivable. The phosphate group could be transferred to water (hydrolysis) or to the 'BvgS receiver. The phosphorylated receiver could in turn be stable or subject to further hydrolysis. Since Asp^{1023} is the most likely site for intramolecular phosphotransfer within BvgS, we compared the kinetics of decay of the phosphorylated forms of 'BvgS and the 'BvgS D1023N protein.

A pulse–chase experiment comparing 'BvgS and 'BvgS D1023N is shown in Fig. 6. The wild-type and D1023N proteins were labeled for 5 min before the addition of a 200-fold excess of unlabeled ATP. Aliquots were removed both before and after the chase, and incorporation was measured as a function of time. Label from the wild-type and D1023N 'BvgS proteins was chased rapidly and with similar kinetics in both cases, with a $t_{1/2}$ of ≈2 min. This contrasts to the $t_{1/2}$ of ≈30 sec for BvgS in the presence of BvgA (Fig. 2). From this experiment we conclude that the primary mechanism of phosphate removal from the BvgS transmitter *in vitro*

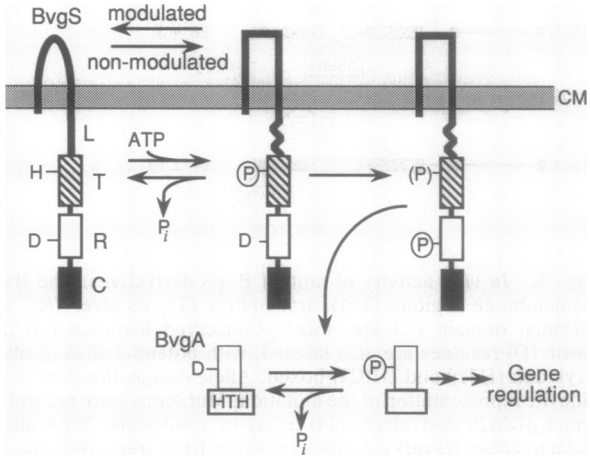

FIG. 7. Model for signal transduction by the BvgS and BvgA proteins. Abbreviations are as in Fig. 3. Also noted is the BvgS linker (L), in which constitutive signal transduction mutations have previously been mapped (14). HTH, helix–turn–helix. See *Discussion* for details.

in the absence of BvgA is not by intramolecular transfer to Asp^{1023} of the receiver but rather by hydrolysis.

DISCUSSION

BvgA and BvgS have long been recognized as central regulators of virulence in *Bordetella* species (3), but their mechanism of action has been inferred from other systems. Although BvgA and BvgS contain easily recognizable signaling domains, it is apparent from our results that the combination of transmitter and receiver modules present in the same protein can give rise to signal transduction pathways with unconventional characteristics.

Fig. 7 details a model for the BvgAS phosphorelay. We have demonstrated that the cytoplasmic portion of BvgS autophosphorylates preferentially with the γ-phosphate of ATP *in vitro*. We propose that the full-length BvgS also autophosphorylates *in vivo* at His^{729} of the transmitter. According to the model, BvgS then transfers the phosphate to Asp^{1023} of its own receiver, and this event is prerequisite for subsequent phosphorylation of BvgA. Following phosphotransfer to the BvgS receiver, His^{729} may reautophosphorylate, and one or both of the phosphorylated residues (His^{729} and Asp^{1023}) could donate a phosphate group to BvgA. Phosphorylation of BvgA *in vitro* correlates with activation of gene expression *in vivo*. BvgA and BvgS are depicted as monomers, but it is likely that multimerization is involved in their activity (see below).

The effects of mutations on BvgS function are summarized in Fig. 3. The mutations can be grouped into two broad categories. The first class comprises alterations that markedly decrease or eliminate both autophosphorylation and phosphotransfer. The H729Q mutation has the most pronounced effect. Introduction of a glutamine at the putative primary site of autophosphorylation completely eliminates autophosphorylation and consequently prevents phosphotransfer to BvgA. The BvgS Δ59R, Δ15C, and Ω1211 mutations decrease autophosphorylation to <2% of wild type, and these mutants are apparently unable to phosphorylate BvgA to levels detectable even after extended autoradiography. The Ω1051 mutant also shows a residual level of autophosphorylation activity but is able to phosphorylate BvgA at a low level (≈1%). This low level of phosphotransfer does not result in increased *in vivo* activity (Fig. 3). Precise deletion of the BvgS receiver and C terminus ablates *in vivo* activity, as well as autophosphorylation and phosphate-group transfer to BvgA *in vitro* (data not shown). In addition to the transmitter, the receiver and C terminus of BvgS therefore appear to be required for autophosphorylation, distinguishing BvgS from other sensor proteins. The autophosphorylation defect resulting from receiver and C-terminal mutations could potentially be due to a lack of multimerization, since intermolecular complementation of mutations has been observed for BvgS (24) as well as EnvZ and CheY (25, 26).

The BvgS D1023N substitution comprises the second category of mutations. The 'BvgS D1023N protein can fully autophosphorylate but is unable to detectably phosphorylate BvgA. This mutation is unique in that it uncouples autophosphorylation from phosphotransfer in the signal transduction process and allows us to study the BvgAS phosphorelay stepwise. Although the *in vivo* phenotype of the D1023N mutation is quite different from the wild type, 'BvgS D1023N and wild type are remarkably similar *in vitro* in regard to rate of decay and phosphate bond stability. We propose that activated 'BvgS wild type and 'BvgS D1023N can donate a phosphate group to water with equal efficiency but that only BvgS with a phosphorylated receiver can act as a BvgA substrate. This may explain the similar decay kinetics of 'BvgS and 'BvgS D1023N. Asp^{1023} of the receiver is crucial for BvgA phosphorylation, although stable phosphorylation of the 'BvgS receiver is not readily detectable *in vitro*.

Mutational analysis of BvgS has revealed two functions of the receiver. In combination with the transmitter and C terminus, the BvgS receiver is required for autophosphorylation. Additionally, the receiver of BvgS mediates phosphotransfer to BvgA, either directly or indirectly. The BvgS receiver does not appear to act simply as an autoinhibitory domain, unlike the sensor proteins VirA of *Agrobacterium tumefaciens* and ArcB of *E. coli*, which contain transmitters and receivers but no C-terminal domains (27–29). Although in several cases transmitters are sufficient to act as both kinases and substrates for phosphorylation of response regulators (2), the complex organization of BvgS and the multiple domain requirements for autophosphorylation indicate that BvgS does not follow this paradigm. Further analysis of BvgAS-mediated signal transduction should lead to a better understanding of virulence control by *B. pertussis* and to an appreciation of the diversity of signaling mechanisms catalyzed by two-component bacterial signal transduction systems.

We thank members of the laboratory, Olaf Schneewind, and Virginia Miller for helpful discussions and for reviewing the manuscript. We are grateful to Scott Stibitz for reagents and discussions, Marisa Galvan for plasmid constructions, and Ellen Shang for mutagenesis. This work was supported by National Institutes of Health Grant AI31548 (to J.F.M.). M.A.U. is supported by National Institutes of Health Predoctoral Training Grant GM07185. J.F.M. is a Pew Scholar in the Biomedical Sciences.

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