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Constitutive Sensory Transduction Mutations in the *Bordetella pertussis bvgS* Gene

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The products of the *bvgAS* locus coordinately regulate expression of the *Bordetella pertussis* virulence regulon in response to environmental signals. Transcription of *bvgAS*-activated genes is nearly eliminated by several modulating conditions, including the presence of sulfate anion or nicotinic acid and growth at low temperature. We have isolated spontaneous mutations that result in the constitutive synthesis of multiple *bvg*-regulated loci. Several of these mutations have been analyzed and were found to result from single-nucleotide substitutions within *bvgS*, in a region encoding a 161-amino-acid segment which links the transmembrane sequence with cytoplasmic domains that appear to be involved in signaling events. The effect of signal transduction mutations in *Escherichia coli* was determined by measuring the expression of an *fhaB-lacZYA* transcriptional fusion, and that in *B. pertussis* was determined by measuring expression of both *fhaB-cat* and *ptxA3201-cat* fusions. The constitutive mutations have little effect on *fhaB-cat* or *fhaB-lacZYA* expression in the absence of modulating signals but result in a nearly complete insensitivity to $MgSO_4$, nicotinic acid, or growth at low temperature. Furthermore, insertion and deletion mutations in *bvgS* sequences encoding the periplasmic domain eliminate activity of the wild-type product, whereas constitutive mutants remain active. In *B. pertussis* cultures grown in Stainer-Scholte broth, expression of *ptxA3201-cat* differed from that of *fhaB-cat* in several respects. In combination with a wild-type *bvgS* allele, *ptxA3201-cat* expression required the addition of heptakis-(2,6-*O*-dimethyl)-β-cyclodextrin, and this requirement was eliminated by the presence of the constitutive mutations.

A majority of the known virulence factors expressed by *Bordetella pertussis*, the etiologic agent of whooping cough, are coordinately regulated by a sensory transduction system encoded by the *bvgAS* (*vir*) locus (3, 36, 40). Transcription of the pertussis toxin operon (*ptxA-E*), the adenylate cyclase toxin-hemolysin gene (*cyaA*), the locus encoding filamentous hemagglutinin (*fhaB*), fimbrial subunit genes (*fim*), and the *bvg* operon itself requires the *bvgAS* gene products in *trans* (6, 10, 18, 22, 28, 30, 42). In addition, expression of dermonecrotic toxin, a 69-kDa outer membrane protein, cytochrome d_{629}, and several other loci is positively controlled by *bvg* by mechanisms which remain to be determined (20, 40). In *Escherichia coli*, the wild-type *bvgAS* locus is sufficient for activation of the *fhaB* and *bvgA* promoters but does not *trans*-activate expression of *ptxA-E* or *cyaA* (9, 22). It has therefore been suggested that there are additional requirements for expression of these two loci (9, 11, 23). Several genes that are negatively controlled by *bvg* have also been identified (4, 15). These genes, which have been called *vrg* loci, are of special interest since they are expressed under conditions that eliminate production of the virulence factors noted above. Although functional roles have not been assigned to the *B. pertussis vrg* genes, recent studies have identified *cis*-acting sequences that are required for repression of two of these loci (4). As described in the accompanying report, the *bvgAS* locus is also involved in negative control of motility and flagellum production by *B. bronchiseptica* (1). It therefore appears that the *Bordetella* virulence regulon is complex, containing both *bvg*-activated and *bvg*-repressed genes.

Expression of the *Bordetella* virulence regulon is affected by a variety of diverse environmental signals, and it is often suggested, but not yet proven, that this sensory response is important in the host-microorganism interaction (20, 21). The presence of sulfate anion or nicotinic acid or growth at low temperature (25 to 28°C) results in decreased expression of *bvg*-activated genes and a concomitant induction of *vrg* loci (15, 17, 22, 41). Although the signals noted above are commonly used in laboratory studies, factors that may influence *bvg*-regulated gene expression in vivo are currently unknown. Sensory transduction and the presence of activated as well as repressed loci are common features of many bacterial virulence control systems (21).

Several lines of evidence indicate that the *bvg* products are involved in both transcriptional activation and sensory transduction. *bvg* regulation of *fhaB* and *bvgAS* expression responds to the same environmental signals in *E. coli* as in *B. pertussis* (22, 28, 35). *bvgA* and *bvgS* encode 23- and 135-kDa proteins with a distinctive pattern of sequence similarity found in members of a family of bacterial proteins that respond to environmental signals (3, 36). These systems consist of at least two regulatory proteins, one of which (the sensor) contains a C-terminal "transmitter" domain, and the other of which (the regulator) carries an N-terminal "receiver" domain (16). Biochemical studies indicate that transmitter proteins are autophosphorylating kinases that respond to environmental stimuli and modulate the activity of receiver proteins through phosphorylation (37). The N terminus of BvgA contains a receiver domain. The predicted amino acid sequence of BvgS is somewhat unusual in that it contains

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both a transmitter and a receiver. The BvgS signaling domains follow a hydrophobic transmembrane sequence and an N-terminal region that is localized in the periplasm, as demonstrated by the analysis of alkaline phosphatase fusion proteins (36). Current models propose that BvgS is a transmembrane protein with an N-terminal periplasmic domain involved in signal recognition and cytoplasmic domains that transduce recognized signals into transcriptional control events via a phosphorylation cascade (3, 36). Recent results suggest that BvgA is a transcriptional control factor which, upon modification by BvgS, binds to specific promoter sequences to activate or repress transcription (26, 27). An interaction between BvgA and repeated sequences found upstream of the *fhaB* and *bvgA* promoters has been reported (26).

In this study we describe the isolation and characterization of a class of mutations that affect sensory transduction by the BvgAS system. Single-amino-acid substitutions in BvgS which result in an insensitivity to environmental signals are identified, providing direct evidence for a role of this protein in signal transduction. Mutations that eliminate the modulating effect of environmental signals also relieve the requirement for an intact periplasmic domain. The phenotypes resulting from constitutive mutations in both *E. coli* and *B. pertussis* were examined to determine whether diverse growth conditions that modulate virulence factor expression act through a common mechanism involving BvgS.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are described in Table 1 and in the figures. *B. pertussis* strains were grown on Bordet-Gengou (BG; Difco Laboratories, Detroit, Mich.) agar plates containing 1% proteose peptone (Difco) and 15% sheep blood. BG agar plates were supplemented as indicated with 20 or 40 mM $MgSO_4$, 5 mM nicotinic acid, 20 μg of rifampin per ml, 20 μg of ampicillin per ml, 10 μg of gentamicin per ml, and 400 μg of streptomycin per ml. *E. coli* strains were grown in L broth or on L agar plates (29). Supplements for *E. coli* strains were as above except for 10 mM nicotinic acid, 100 μg of ampicillin per ml, 30 μg of kanamycin per ml, and 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml.

DNA methods. Restriction enzymes, Klenow fragment, calf intestinal alkaline phosphatase, T4 DNA ligase, T4 DNA polymerase, oligonucleotide linkers, and *Bal*31 exonuclease were purchased from Bethesda Research Laboratories, New England BioLabs, or Promega Corp. and were used as specified by the manufacturers. Linkers used for mutagenesis were the *Bam*HI 8-mer (GCCTAGGC), the *Bam*HI 10-mer (GCCCTAGGGC), and the *Bam*HI 12-mer (CGCG GATCCGCG). Plasmid DNA was transformed into *E. coli* by high-voltage electroporation (7) with a Gene Pulser apparatus (Bio-Rad Laboratories). DNA sequence analysis was performed on double-stranded plasmid DNA templates by using a Sequenase II kit as specified by the manufacturer (U.S. Biochemical Corp.).

TOX35, VIR101, VIR102, and isolation of mutant *bvg* loci from *B. pertussis*. pTOX28 carries a 2.8-kb *Eco*RI-*Bgl*II fragment containing the pertussis toxin operon promoter and structural genes extending from *ptxA* to a point within *ptxE*, cloned into the *Eco*RI and *Bam*HI sites of the allelic exchange vector pRTP1 (34). This fragment also carries the *ptxA3201* allele, consisting of a linker insertion in *ptxA* which generates a *Bam*HI site (34). A chloramphenicol acetyltransferase (CAT) gene cartridge (*cat*; Pharmacia, Inc.) lacking transcriptional control signals was inserted into pTOX28 at the *Bam*HI site in *ptxA3201* to construct pTOX35 (*ptxA3201-cat*). The *ptxA3201-cat* transcriptional fusion was introduced into strain BP370 by allelic exchange as described previously (34) to construct TOX35. Triparental matings used HB101 (pRKTV5) to donate transfer functions.

VIR101 (Fig. 1) was constructed by allelic exchange between pVIR101 and TOX35. pVIR101 is a pVIR2 (Fig. 1) derivative carrying the *bvg*Δ*101* deletion. pVIR2 carries the 14-kb *Bam*HI fragment encompassing the *bvgAS* locus and adjacent sequences, cloned into the *Bam*HI site of pRTP1. The *bvg*Δ*101* allele is a 7.6-kb deletion extending from the *bvgA*-proximal *Sal*I site in *fhaB* to the *Xho*I site following *bvgS*. *bvg*Δ*101* removes the entire *bvgAS* operon and results in a Bvg^- phenotype. pVIR2 was introduced into VIR101 by conjugative transfer, and a single homologous recombination event resulted in the cointegrate strain VIR102 (Fig. 1).

Following isolation of Bvg^c mutations in strain VIR102 (see Results), pVIR2 derivatives which had undergone a second homologous recombination event resulting in excision from the VIR102 genome were isolated by mobilization into *E. coli* HB101Nal by triparental mating with HB101 (pRKTV5). Plasmids that were recovered in HB101Nal were returned to VIR101 to determine whether the constitutive mutations were linked to the pVIR2 derivatives.

Construction of Bvg^c *B. pertussis* strains. pJM500 was created by inserting the 2.3-kb *Sfi*I fragment from pDM20, containing an internal portion of *bvgS* which codes for the wild-type linker region, into the *Bam*HI site of pSS1129. The *Sfi*I fragment was modified by treatment with Klenow fragment and addition of *Bam*HI linkers. pJM501 and pJM503 were constructed in the same manner, except that the 2.3-kb *Sfi*I fragments were from pJM214 and pJM215 (Fig. 2) and carry the *bvgS-C1* and *bvgS-C3* sensory transduction mutations, respectively. pJM504 consists of the 3.6-kb *Sac*II fragment from pJM216 (Fig. 2), which carries the *bvgS-C4* allele, inserted into the *Bam*HI site of pSS1129. The *Sac*II fragment was treated with T4 DNA polymerase to remove 3′ overhangs; this was followed by addition of *Bam*HI linkers. The restriction fragments that were inserted into pSS1129 were chosen to place the mutant alleles approximately in the center. *E. coli* SM10 was used as a donor strain to transfer pJM500 (*'bvgS'*), pJM501 (*'bvgS-C1'*), pJM503 (*'bvgS-C3'*), or pJM504 (*'bvgS-C4*) into *B. pertussis* BP370. Allelic exchange (34) resulted in only Bvg^+ colonies (subject to modulation by $MgSO_4$) with pJM500 and a mixture of Bvg^+ and Bvg^c colonies (resistant to modulation by $MgSO_4$) with pJM501, pJM503, and pJM504. SJ301 (*bvgS-C1*), SJ303 (*bvgS-C3*), and SJ304 (*bvgS-C4*) were chosen for further analysis.

fhaB-cat transcriptional fusions were introduced into wild-type and mutant strains by conjugative transfer, and homologous recombination mediated integration of the suicide plasmid p*fhaB-cat* (27). p*fhaB-cat* is a pSS1129 derivative containing the *fhaB* promoter region joined to a *cat* gene cartridge. The *fhaB-cat* derivatives of BP370, SJ301, SJ303, and SJ304 are designated with the suffix FC. *ptxA3201-cat* derivatives were constructed by integration of pTOX35, and these strains are given the suffix TC.

β-Galactosidase assays. *E. coli* JFMC3 (*fhaB-lacZYA* [22]) containing plasmids to be assayed for BvgAS activity were grown overnight in L broth under the conditions indicated in Table 2. Overnight cultures were diluted 1/100 in the same medium and grown to mid-log phase (optical density at 600

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli		
HB101	F^- *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*	29
HB101Nal	Nal^r derivative of HB101	This study
SM10	RP4-2-Tc::Mu, Km^r	32
MC4101	F^- *araD139* Δ*(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR recA1*	22
JFMC3	λJMC3 lysogen of MC4101 (λJMC3 carries the *fhaB-lacZYA* reporter fusion)	22
B. pertussis		
BP370	Bvg^+ Rif^r Sm^r	34
BP347	*vir1*::Tn*5* (Bvg^-) Km^r Sm^r	39
TOX35	BP370 *ptxA3201-cat*	This study
VIR101	TOX35 *bvg*Δ*101* (*bvg*Δ*101* is a 7.6-kb deletion from the *Sal*I site in *fhaB* to the *Xho*I site following *bvgS*)	This study
VIR102	VIR101::pVIR2	This study
SJ301	BP370 *bvgS-C1*	This study
SJ303	BP370 *bvgS-C3*	This study
SJ304	BP370 *bvgS-C4*	This study
BP370FC	BP370 *fhaB-cat*	This study
SJ301FC	SJ301 *fhaB-cat*	This study
SJ303FC	SJ303 *fhaB-cat*	This study
SJ304FC	SJ304 *fhaB-cat*	This study
BP370TC	BP370 *ptxA3201-cat*	This study
SJ301TC	SJ301 *ptxA3201-cat*	This study
SJ303TC	SJ303 *ptxA3201-cat*	This study
SJ304TC	SJ304 *ptxA3201-cat*	This study
Plasmids		
pBR322	Ap^r Tc^r cloning vector	5
pUC19	Ap^r high-copy cloning vector	29
pRTP1	Allelic exchange vector, Ap^r *rpsL* $oriT_{IncP}$	34
pSS1129	Allelic exchange vector, Gm^r Ap^r *rpsL* $oriT_{IncP}$	36
pLAFR2	IncP1 Tra^+ *cos* Tc^r	8
pRKTV5	Tra^+ (IncP1) Sm^r Sp^r Tm^r	34
pTOX28	pRTP1 with 2.8-kb *Eco*RI-*Bgl*II insert carrying *ptxA-E* promoter and *ptxA3201-ptxE'* loci	This study
pTOX35	pTOX28 derivative with *ptxA3201-cat* transcriptional fusion	This study
p*fhaB-cat*	pSS1129 with *fhaB-cat* transcriptional fusion	27
pVIR2	pRTP1 with 14-kb *Bam*HI insert carrying *bvgAS* and adjacent sequences	This study
pVIR201	pVIR2 *bvgS-C1*	This study
pVIR203	pVIR2 *bvgS-C3*	This study
pVIR204	pVIR2 *bvgS-C4*	This study
pVIR101	pVIR2 *bvg*Δ*101*	This study
pDM20	pBR322 with 5.995-kb *Eco*R1-*Xho*I insert carrying *bvgAS*	22
pJM201-219	Fig. 2; Fragment exchange derivatives; pDM20 × pVIR201, pVIR203, pVIR204	This study
pSJ30-34	Fig. 4; pDM20, pJM214, pJM215, pJM216 derivatives with *Bam*HI linker insertion at *Dra*III site in *bvgS*	This study
pSJ40-44	Fig. 4; pDM20, pJM214, pJM215, pJM216 derivatives with *Bam*HI linker insertion at *Sac*I site in *bvgS*	This study
pSJ50-54	Fig. 4; pDM20, pJM214, pJM215, pJM216 derivatives with 1.1-kb deletion and *Bam*HI linker insertion in *bvgS*	This study
pSJ60-64	Fig. 4; pDM20, pJM214, pJM215, pJM216 derivatives with *Bam*HI linker insertion at *Sna*BI site in *bvgS*	This study
pJM500	pSS1129 with 2.3-kb *Sfi*I fragment carrying *'bvgS'*	This study
pJM501	pJM500 *bvgS-C1*	This study
pJM503	pJM500 *bvgS-C3*	This study
pJM504	pSS1129 with 3.6-kb *Sac*II fragment carrying 'bvgS-C4	This study
pVI62	pBR322 with 14-kb *Bam*HI insert carrying *bvgAS*	15
pLAF-C1	pLAFR2 cosmid clone with *bvgS(mod-1)* allele	15
pVI-C1	pUC19 with 14-kb *Bam*HI insert carrying $bvgA^+$ *bvgS(mod-1)*	This study
pVI-R1/2	Reassortants of pVI62 and pVI-C1 at *Bgl*II site in *bvgS*	This study
pVI-C1455	pUC19 with 455-bp *Pst*I-*Eco*RI fragment of pVI-C1	This study

nm [OD_{600}] of approximately 0.6). β-Galactosidase activities were determined with cells permeabilized with sodium dodecyl sulfate (SDS)-$CHCl_3$ as described by Miller (24).

CAT assays. *B. pertussis* strains were grown in liquid Stainer-Scholte medium (33) with 1% Casamino Acids and 0.04% ascorbic acid. Additions included $MgSO_4$ (20 mM), nicotinic acid (5 mM), and heptakis-(2,6-*O*-dimethyl)-β-cyclodextrin (MeβCD; 1 mg/ml) as indicated. Cultures were harvested in mid-logarithimic growth phase, and 1-ml aliquots were permeabilized by addition of 30 μl of $CHCl_3$ and

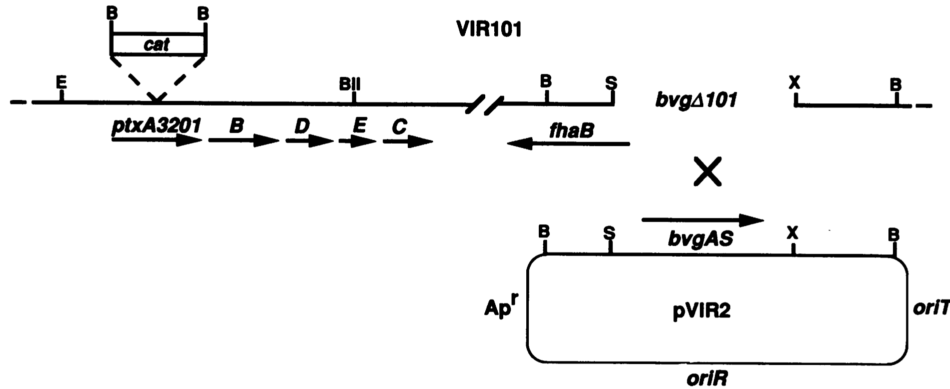

FIG. 1. *B. pertussis* VIR101 and VIR102. The top line represents the genomic structure of the Bvg⁻ strain VIR101, which contains the *ptxA3201-cat* fusion and the *bvgΔ101* deletion. VIR102 (Bvg⁺) resulted from a single crossover between pVIR2 and homologous sequences in VIR101. Further details are given in Materials and Methods and in Table 1.

15 μl of 0.1% SDS. CAT assays were performed at 22°C by the colorimetric method of Shaw (31) with 5,5′-dithiobis-2-nitrobenzoic acid (Sigma) as the chromogenic reagent. Cultures were normalized by OD_{600}, and CAT activities are expressed as $[\Delta OD_{412}/(OD_{600})(min)(ml)] \times 500$.

Characterization of the *mod-1* allele. The 14-kb *Bam*HI fragment containing the *bvg* locus from strain SK100 (15) was subcloned from pLAF-C1 into pUC19 to give pVI-C1. The 5.5-kb *Bgl*II-*Hin*dIII fragments (coding for BvgA and the N terminus of BvgS) of pVI-C1 and pVI62 were exchanged in a reciprocal manner, giving rise to plasmids pVI-R1, coding for BvgS with a wild-type C terminus, and pVI-R2, coding for BvgS with a C terminus from the *mod-1* strain. Replicon fusions (4) with pLAFR2 were constructed

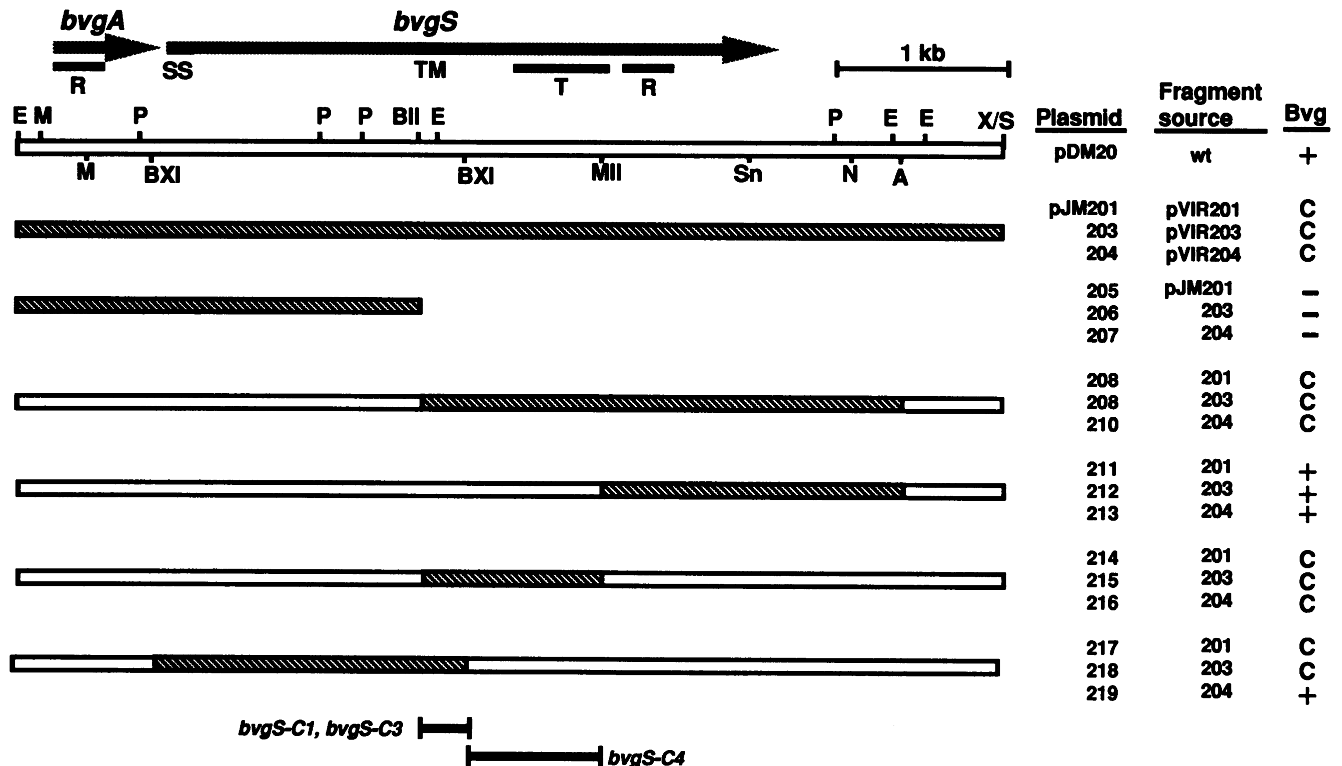

FIG. 2. Localization of Bvg^c mutations. The top line represents the structure of the 5,995-bp *Eco*RI-*Xho*I fragment from *B. pertussis* containing the *bvgAS* locus. The restriction map is derived from the nucleotide sequence (3). Sequences encoding the BvgS signal sequence (SS), transmembrane domain (TM), and transmitter (T) are indicated, along with the receiver domains (R) in BvgA and BvgS. pDM20 (open bar) contains a wild-type *bvgAS* locus, and pJM201, pJM203, and pJM204 (hatched bar) contain mutant *bvg* loci with constitutive mutations. For pJM208 to pJM219, restriction fragments were exchanged as indicated. In each case the fragment source pertains to the hatched restriction fragment. The phenotypes of the resulting plasmids in JFMC3 (*fhaB-lacZYA*) were determined, and the locations of the *bvgS-C1*, *bvgS-C3*, and *bvgS-C4* mutations are shown. In the Bvg column, +, C, and – designate Bvg⁺, Bvg^c, and Bvg⁻ phenotypes, respectively. Restriction sites: E, *Eco*RI; M, *Mlu*I; P, *Pst*I; BXI, *Bst*XI; BII, *Bgl*II; MII, *Mst*II; Sn, *Sna*BI; N, *Nde*I; A, *Aoc*I; X/S, *Xho*I joined to the *Sal*I site in pBR322.

TABLE 2. β-Galactosidase activities of JFMC3 (*fhaB-lacZYA*) derivatives carrying plasmids with wild-type and constitutive *bvg* alleles

Plasmid	Description	β-Galactosidase activity[a]			
		LB	SO_4^{-}[b]	NA[c]	28°C[d]
pVIR2	*bvgAS*$^+$	2,045	19		
pVIR201	*bvgS-C1*	1,972	1,890		
pVIR203	*bvgS-C3*	2,204	2,105		
pVIR204	*bvgS-C4*	3,436	2,580		
pRTP1	Vector	22	24		
pDM20	*bvgAS*$^+$	6,661	59	58	137
pJM214	*bvgS-C1*	5,044	5,600	4,934	12,737
pJM215	*bvgS-C3*	5,891	5,451	5,640	12,505
pJM216	*bvgS-C4*	10,029	8,041	8,706	11,779
pBR322	Vector	15	21	18	20

[a] β-Galactosidase activities are expressed in Miller units (24) and represent average values obtained from at least three independent assays that differed by less than 15%. Measurements were done with *E. coli* JFMC3 grown in 37°C in L broth unless otherwise indicated (see below).
[b] L broth plus 40 mM $MgSO_4$.
[c] L broth plus 10 mM nicotinic acid.
[d] L broth and growth at 28°C.

and mobilized into *B. pertussis* 347. Both plasmids yielded hemolytic transconjugants, confirming the transfer of *bvgA* and *bvgS*. Only pVI-R2 gave rise to hemolytic colonies when the transconjugants were grown in the presence of $MgSO_4$ and nicotinic acid.

A 455-bp *Pst*I-*Eco*RI fragment from pVI-C1 which spans the *Bgl*II site was subcloned into pUC19 to give plasmid pVI-C1455. This was used for double-stranded sequencing as described above.

RESULTS

Isolation of Bvg^c mutations in *B. pertussis*. A selective scheme was designed to facilitate the isolation and recovery of mutations at the *bvg* locus that specifically affect sensory transduction. The desired class of mutations was that which confers constitutive transcription of *bvg*-regulated virulence determinants in the presence of environmental signals that normally modulate expression. To accomplish this, a *cat* structural gene cartridge was introduced into the *ptxA* locus to construct TOX35, a *B. pertussis* BP370 derivative carrying the *ptxA3201-cat* transcriptional fusion (see Materials and Methods). The *cat* cartridge present in TOX35 is located 450 bp downstream from the site of *ptx* transcription initiation. To demonstrate that the *ptxA3201-cat* fusion was under *bvg* control, the *bvg*Δ*101* deletion, which eliminates the entire *bvgAS* operon, was introduced into TOX35 to construct strain VIR101 (Fig. 1). The parental strain BP370 (*ptx*$^+$ *bvg*$^+$), TOX35 (*ptxA3201-cat bvg*$^+$), and VIR101 (*ptxA3201-cat bvg*Δ*101*) were assayed for CAT activity following growth on BG agar. TOX35 produced more than 18 times the activity of the other two strains, which gave activity essentially at background levels (data not shown). We also examined the MIC of chloramphenicol for TOX35 and found it to be reduced from 6.0 μg/ml on BG agar to less than 0.6 μg/ml on BG agar containing $MgSO_4$ or nicotinic acid as modulating agents. The hemolytic (Hly) phenotypes of TOX35 and VIR101 coincided with expression of *ptxA3201-cat*. These results demonstrated that the *ptxA3201-cat* fusion is subject to *bvg* regulation and phenotypic modulation.

A VIR101 derivative was constructed to allow conjugative transfer of *bvg* sequences following selection for the constitutive phenotype. Plasmid pVIR2 (Fig. 1), which contains the entire *bvgAS* locus, was introduced into VIR101 by conjugative transfer. Homologous recombination between sequences that flank *bvg* resulted in the formation of strain VIR102 (Fig. 1). VIR102 is phenotypically Bvg$^+$ and contains a wild-type *bvgAS* allele linked to plasmid sequences present in tandem with the *bvg*Δ*101* deletion.

VIR102 was plated on BG agar containing nicotinic acid and $MgSO_4$ as modulating agents and chloramphenicol (6 μg/ml) at a concentration sufficient to inhibit the growth of modulated cells. At a frequency of approximately 10^{-6}/CFU, mutants that were Cm^r Hly$^+$ and displayed a Bvg$^+$ colony morphology were recovered. These mutants were able to express several phenotypes characteristic of the Bvg$^+$ state under modulating conditions, and their phenotype is designated Bvg^c (Bvg constitutive). Cm^r Hly$^-$ colonies with a Bvg$^-$ morphology were also isolated at a similar frequency. These were presumed to carry mutations that allow *bvg*-independent expression of the *ptxA3201-cat* fusion and were not examined further.

Recovery of *bvgAS* loci from Bvg^c *B. pertussis* strains. VIR201, VIR203, and VIR204 are spontaneous, independent Bvg^c mutant derivatives of VIR102 that were chosen for further study. The *bvg* locus from each strain was conjugatively recovered following homologous recombination-mediated excision of the plasmid from *B. pertussis* and mobilization into *E. coli* HB101Nal (Fig. 1; see Materials and Methods). Plasmids pVIR201, pVIR203, and pVIR204 contain the *bvgAS* loci from VIR201, VIR203, and VIR204, respectively. Upon reintroduction into strain VIR102 by conjugative transfer and homologous recombination, each of these plasmids transferred the Bvg^c phenotype. These results demonstrated that the mutational alterations associated with the Bvg^c phenotype are present on the transferred plasmids and are therefore linked to the *bvg* locus. By restriction mapping, no gross alterations have been found in the plasmids encoding the Bvg^c phenotype.

We previously demonstrated that the intact *bvgAS* locus is sufficient for transcriptional activation of a chromosomal *fhaB-lacZYA* operon fusion carried by an integrated bacteriophage lambda derivative (λJMC3) present in *E. coli* JFMC3 (22). Furthermore, activation measured with the wild-type *bvgAS* locus in *trans* is nearly eliminated by growth in the presence of $MgSO_4$ or nicotinic acid or at low temperature (22). The *bvgAS* system is therefore modulated in *E. coli* by the same signals that affect expression of *bvg*-regulated genes in *B. pertussis*. The phenotypes of pVIR2 and derivatives containing the Bvg^c mutations were therefore tested in the *E. coli* system.

Plasmids pVIR2, pVIR201, pVIR203, and pVIR204 activated *fhaB-lacZYA* transcription in JFMC3 to approximately equal levels, which represent a nearly 100-fold increase in β-galactosidase activity over the basal level of expression observed with the vector plasmid pRTP1 (Table 2). The level of expression measured with pVIR2 was decreased to background by the addition of $MgSO_4$, yet the Bvg^c plasmids were completely unaffected by this modulating signal. On agar plates containing the chromogenic indicator X-Gal, JFMC3 derivatives containing Bvg^c plasmids formed dark-blue colonies in both the presence and absence of $MgSO_4$, whereas wild-type pVIR2 resulted in dark-blue colonies in the absence of $MgSO_4$ and white to pale-blue colonies in its presence. These data demonstrate that the Bvg^c phenotype is reproduced in our *E. coli* system.

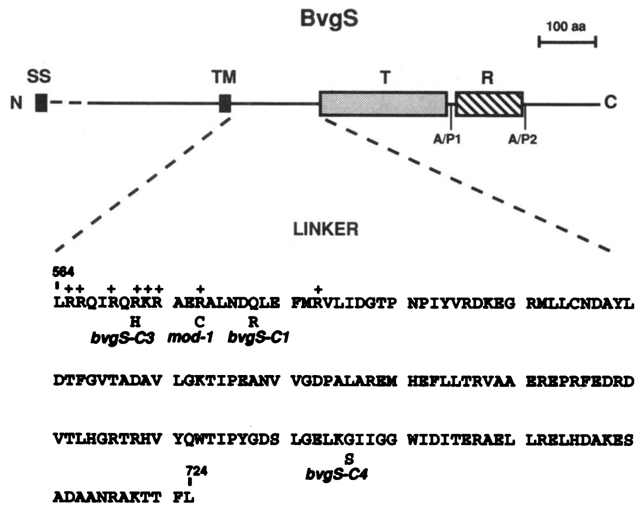

FIG. 3. Sensory transduction mutations in the BvgS linker region. The structure of the BvgS protein is indicated. The amino acid sequence of the linker region is shown along with substitutions resulting from the constitutive mutations in *bvgS*. Abbreviations: N, N terminus; SS, signal sequence; TM, transmembrane region; T, transmitter; A/P1, alanine-proline-rich sequence AAPPAAATAAT PSHP; R, receiver; A/P2, AALPTPPSPQAAAPA; C, C terminus; aa, amino acids.

Localization and sequence analysis of Bvg^c mutations. To map the Bvg^c mutations, we took advantage of the ability to detect their phenotype in *E. coli*. The 5.99-kb *Eco*RI-*Xho*I fragment containing the *bvgAS* operon from pVIR201, pVI203, and pVI204 was subcloned into pBR322 to construct pJM201, pJM203, and pJM204, respectively. As shown in Fig. 2, these plasmids retained the Bvg^c phenotype in JFMC3 as indicated by the appearance of blue colonies on X-Gal agar containing $MgSO_4$. pDM20, which contains the wild-type locus, was subject to modulation (Bvg$^+$).

Deletion of *bvgS* sequences from the Bvg^c plasmids gave a Bvg$^-$ phenotype (pJM205 to pJM207 [Fig. 2]), showing that none of the mutations resulted in an altered *bvgA* product that no longer requires BvgS for activity. Restriction fragments were then exchanged between mutant and wild-type plasmids (Fig. 2). All three of the Bvg^c mutations were found to be located in *bvgS*, within the region encoding the transmembrane and histidine kinase domains. Further localization placed the Bvg^c alleles originally isolated from VIR201 and VIR203, designated *bvgS-C1* and *bvgS-C3*, within a 275-bp *Bgl*II-*Bst*XI fragment. The *bvgS-C4* mutation derived from VIR204 was located on an adjacent 935-bp *Bst*XI-*Mlu*II segment.

Figure 3 shows the sequence alterations associated with the Bvg^c phenotypes, as well as several functional domains that appear to be present within BvgS (see Discussion). The *bvgS-C1*, *bvgS-C3*, and *bvgS-C4* mutations are due to single-nucleotide substitutions at positions 2557 (A to G), 2527 (G to A), and 2880 (G to A), respectively, resulting in amino acid replacements of arginine for glutamine at position 580, histidine for arginine at position 570, and serine for glycine at position 688. All of these alterations lie between the BvgS transmembrane domain and cytoplasmic domains that are likely to be involved in phosphorylation events, in a region that we now designate as the linker. Comparison of the BvgS linker with similar regions in other sensor proteins did not reveal obvious sequence similarities except for the presence of several positively charged residues immediately following the transmembrane sequence. The *bvgS-C3* alteration lies within this positively charged region, and *bvgS-C1* results in the addition of an arginine at a nearby position. The *bvgS-C4* mutation is located in a distal portion of the linker.

The *mod-1* allele is a *bvgS* linker mutation. In a previous study (15), a search for constitutive mutations similar to those described here resulted in the isolation of the *mod-1* allele in strain SK100, a *B. pertussis* 18323 derivative. Phenotypic characterization of SK100 indicated that the *mod-1* mutation confers a Bvg^c phenotype, as demonstrated by the production of hemolytic activity, filamentous hemagglutinin, and pertussis toxin in the presence of $MgSO_4$ or nicotinic acid. Furthermore, *mod-1* was shown to be *trans*-dominant to the wild-type allele.

To establish the relationship between the *mod-1* allele and the Bvg^c mutations described here, the sequence encoding the *mod-1* mutation was identified first by restriction fragment reassortment experiments and then by DNA sequencing (see Materials and Methods). Our analysis indicates that the *mod-1* mutation resides within the *bvgS* gene and is a substitution at nucleotide position 2541 (C to T). This predicts a cysteine-for-arginine replacement at amino acid position 575 within the BvgS linker region. As shown in Fig. 3, *mod-1* maps between the *bvgS-C1* and *bvgS-C3* mutations within a stretch of basic amino acids that immediately follow the BvgS transmembrane segment.

Characterization of wild-type and mutant *bvgS* alleles in *E. coli*. Plasmids pDM20 (*bvg*$^+$), pJM214 (*bvgS-C1*), pJM215 (*bvgS-C3*), and pJM216 (*bvgS-C4*) activated *fhaB-lacZYA* to similar levels during exponential growth in L broth (Table 2). As expected, $MgSO_4$ had little effect on the activity of the Bvg^c plasmids, while decreasing activation by pDM20 to near background levels. The resistance to modulation extends to include nicotinic acid and growth at low temperature. Although growth at low temperature resulted in a nearly 50-fold decrease in expression with pDM20, there was an increase in activity resulting from the presence of the Bvg^c mutations. Whether this is due to the combination of constitutive activity and an increased plasmid copy number during growth at low temperature or to a specific effect of temperature on the function of the mutant BvgS molecules in *E. coli* has not been determined. The results in Table 2 show that single-amino-acid substitutions in the BvgS linker result in an altered response in *E. coli* to three very different environmental stimuli, indicating that BvgS is directly involved in the response to these signals.

Mutagenesis of sequences encoding the BvgS periplasmic and C-terminal domains. Current models for the function of BvgS suggest that the periplasmic domain is involved in signal recognition (3, 36). To examine the requirement for this region, we introduced in-frame linker insertion and deletion mutations into wild-type and mutant *bvgS* loci at positions between the signal sequences and transmembrane domains. Linker insertions at the *Dra*III or *Sac*I sites eliminated the activity of the wild-type *bvgS* gene product as judged by the inability to activate *fhaB-lacZYA* expression (Fig. 4). In combination with the constitutive alleles, however, these linker insertions had virtually no effect on BvgAS activity. As a further test, nearly all of the sequences encoding the periplasmic domain were deleted. This resulted in a Bvg$^-$ phenotype in combination with the wild-type allele (pSJ50), as expected. Remarkably, the presence of the constitutive mutations compensated for the effect of this deletion. pSJ51, pSJ53, and pSJ54, which carry the periplasmic deletion and the *bvgS-C1*, *bvgS-C3*, and *bvgS-C4* alleles,

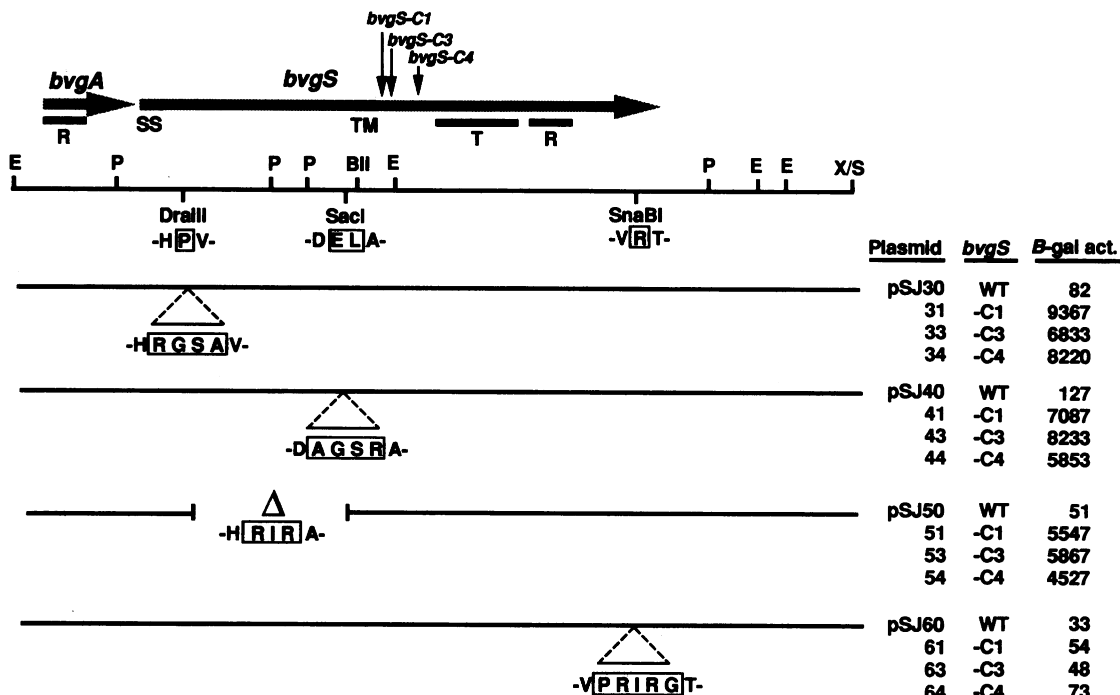

FIG. 4. Mutational analysis of wild-type and constitutive *bvgS* loci. Amino acid sequence alterations resulting from in-frame linker insertions at the *Dra*III (*Bam*HI 12-mer), *Sac*I (*Bam*HI 10-mer), and *Sna*BI (*Bam*HI 12-mer) sites in *bvgS* are shown. For pSJ50 to pSJ54, sequences between the *Dra*III and *Sac*I sites were deleted and replaced with a *Bam*HI (8-mer) linker. T4 DNA polymerase was used to remove 3′ overhangs generated by *Dra*III and *Sac*I. The locations of the *bvgS-C1*, *bvgS-C3*, and *bvgS-C4* mutations are also shown. Each plasmid carries the indicated insertion or deletion mutation in the wild-type *bvgS* locus (WT) or in the *bvgS-C1*, *bvgS-C3*, or *bvgS-C4* mutant loci (for example, pSJ31 carries the *bvgS-C1* mutation and an insertion mutation at the *Dra*III site). The β-galactosidase activity expressed with the plasmid present in JFMC3 (*fhaB-lacZYA*) is indicated. β-Galactosidase activities are as described in Materials and Methods and Table 2, footnote *a*. Abbreviations and restriction sites are described in the legend to Fig. 2.

respectively, were capable of high-level activation of the reporter fusion (Fig. 4). The presence of the constitutive mutations therefore allows BvgS to function in the absence of an intact periplasmic domain.

The requirement for the C-terminal portion of BvgS which follows the receiver domain was examined next. Although a specific function has not been assigned to this region, we previously showed that deletion of sequences encoding the C-terminal 27 amino acids of BvgS results in a Bvg$^-$ phenotype in *E. coli* (22). Linker insertions at this site eliminated activity with both wild-type and constitutive alleles (Fig. 4), suggesting that this portion of BvgS is essential for activity.

Construction and characterization of isogenic *B. pertussis* sensory transduction mutants. To determine whether the single-amino-acid changes identified in *E. coli* were indeed responsible for the Bvg^c phenotype in *B. pertussis*, we constructed isogenic *B. pertussis* strains differing only by single-nucleotide substitutions within *bvgS*. Strains SJ301 (*bvgS-C1*), SJ303 (*bvgS-C3*), and SJ304 (*bvgS-C4*) were derived from BP370 (*bvg*$^+$) by allelic exchange (see Materials and Methods). These mutant strains formed hemolytic colonies with a Bvg$^+$ morphology on BG agar in the presence of $MgSO_4$, indicating a Bvg^c phenotype.

To quantitate the effect of the constitutive mutations when present in single copy at the *B. pertussis bvg* locus, we constructed *fhaB-cat* and *ptxA3201-cat* fusion derivatives of BP370 and the mutant strains. Expression at both the *fhaB* and *ptxA* loci was measured since our previous data suggested that *fhaB* is directly activated by the *bvgAS* gene products, whereas the wild-type *bvgAS* locus is not sufficient for transcriptional activation of the *ptxA-E* promoter (22, 23).

Liquid growth in Stainer-Scholte medium at 37°C in the absence of modulating signals resulted in similar levels of *fhaB-cat* expression in strains carrying either wild-type or constitutive *bvgS* alleles (Fig. 5A). The addition of $MgSO_4$ or nicotinic acid or growth at low temperature (26°C) nearly eliminated expression by the *bvg*$^+$ strains, whereas the constitutive mutants continued to express high levels of CAT activity. These results correspond well to measurements that were previously made with *E. coli* (Table 2).

In contrast, the pattern of expression observed with the *ptxA3201-cat* fusion strains during liquid growth in Stainer-Scholte medium differed dramatically from that with the *fhaB-cat* fusions (Fig. 5B). At 37°C, growth conditions that resulted in high CAT activity from BP370FC (*bvgS*$^+$ *fhaB-cat*) gave only background levels with BP370TC (*bvgS*$^+$ *ptxA3201-cat*). The lack of expression with the wild-type strain was completely reversed by the presence of the constitutive mutations and partially reversed by the addition of MeβCD. Although the mechanism is not yet understood, this compound has been shown to increase pertussis toxin production in Stainer-Scholte broth with little effect on cell growth (12). In the presence of MeβCD, *ptx3201-cat* expression by the wild-type strain was increased severalfold and approached the level expressed by the constitutive mutants. Furthermore, BP370TC was modulated by $MgSO_4$, by nicotinic acid, and by growth at low temperature in Stainer-Scholte plus MeβCD, whereas the constitutive mutants remained unaffected by these modulating signals. In contrast

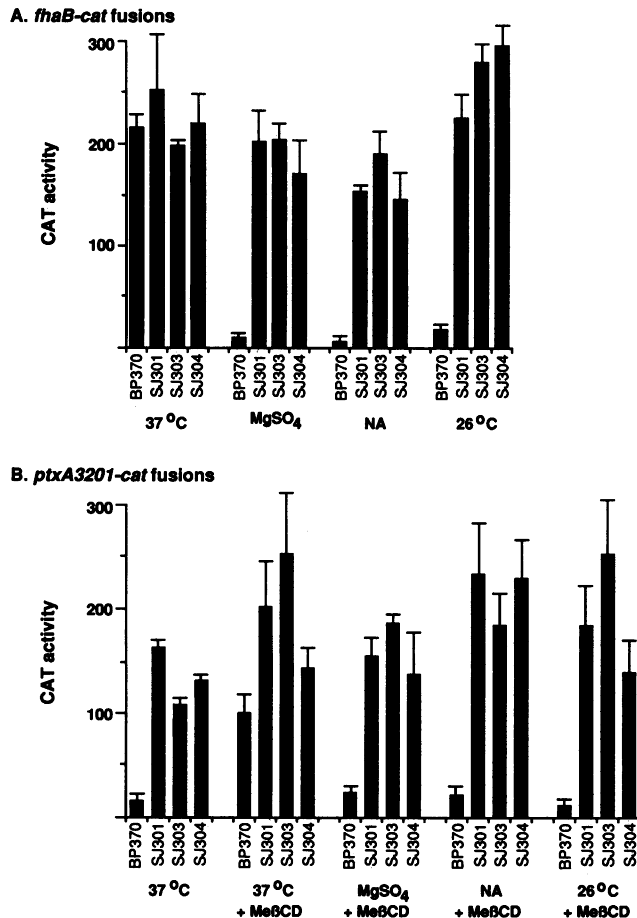

FIG. 5. CAT activities expressed by mutant and wild-type *B. pertussis* strains. Strains containing wild-type (BP370) or constitutive (SJ301 *bvgS-C1*, SJ303 *bvgS-C3*, SJ304 *bvgS-C4*) *bvgS* alleles and *fhaB-cat* (FC) or *ptxA3201-cat* (TC) fusions were assayed as described in Materials and Methods. Strains were grown in Stainer-Scholte broth at 37°C unless otherwise noted. $MgSO_4$ (20 mM), nicotinic acid (NA; 5 mM), and MeβCD (1 mg/ml) were present as indicated. Average values from three independent determinations are shown, with the standard error.

to liquid growth, both the *ptxA3201-cat* and *fhaB-cat* fusions were expressed at similar levels by wild-type and constitutive strains during growth on BG agar in the absence of modulation (data not shown).

DISCUSSION

The response of the *bvgAS* system to environmental signals is likely to be an important aspect of *Bordetella* virulence control. To study the genetic requirements for this response, we have used a selective scheme to isolate mutations in *B. pertussis* that specifically affect sensory transduction. In the course of this analysis, we have also clarified the nature of the *mod-1* mutation. Although an initial report suggested that *mod* and *vir* were distinct loci (15), we now show that *mod-1* is a mutant allele of *bvgS*. Mapping and sequence analysis identified single-nucleotide substitutions within *bvgS* that lead to amino acid replacements, resulting in constitutive expression of multiple *bvg*-regulated loci. The *bvgS-C1*, *bvgS-C3*, *bvgS-C4*, and *mod-1* mutations therefore provide direct evidence that BvgS is involved in sensory transduction.

All of our constitutive mutations map to a specific region of BvgS located between the transmembrane sequence and putative cytoplasmic signaling domains. We have designated this 161-amino-acid region of BvgS as the linker since it appears to be functionally analogous to similar regions in methyl-accepting chemotaxis receptors such as the *E. coli* Tsr protein (2). Ames and Parkinson characterized mutations in the Tsr linker that result in output signals in the absence of overt stimuli (2). As a result of this analysis, it was proposed that communication between the periplasmic receptor and cytoplasmic signaling domains occurs by direct propagation of conformational changes through the linker. Within members of the two-component family, there is no discernible amino acid sequence homology between linker regions of sensor proteins; however, constitutive mutations in NarX, an *E. coli* sensor involved in regulation of anaerobic respiratory genes, also map to its linker region (14). It therefore appears that linker segments of bacterial sensor proteins play a central and perhaps conserved role in signal transduction. Three of four Bvg[c] linker mutations are clustered within a positively charged segment immediately following the BvgS transmembrane sequence. This portion of the linker might be expected to interact with surface phosphates on the cytoplasmic face of the membrane and may be important in propagating ligand-induced transmembrane conformational changes. Since the BvgS mutations affect the response to three very diverse signals, it seems likely that the linker is required for events that occur subsequent to signal recognition.

Figure 3 depicts several BvgS domains that have been inferred from localization studies (36), examination of the amino acid sequence (3), and our current results. Stibitz and Yang recently demonstrated that sequences preceding the transmembrane region are periplasmic and that sequences that follow are located in the cytoplasm (36). Three cytoplasmic domains in addition to the linker are located between the transmembrane sequence and the C terminus. The transmitter and receiver were described above, and these are probably involved in a phosphorylation cascade. Using a purified glutathione-*S*-transferase–BvgS fusion protein, we have recently found that the cytoplasmic region of BvgS is capable of autophosphorylation (38). The transmitter and receiver domains are followed by a 140-amino-acid region of unknown function. Both deletions and in-frame linker insertion mutations within this segment eliminate the activity of wild-type and constitutive *bvgS* loci (22) (Fig. 4). Located between the transmitter and receiver and between the receiver and the C terminus are alanine- and proline-rich sequences designated A/P1 and A/P2 in Fig. 3. Similar sequences have been identified in other proteins, and several lines of evidence suggest that these motifs form conformationally flexible regions allowing interactions between discrete functional domains (25). Although the presence of both transmitter and receiver modules in a single molecule is unusual, similar arrangements are present in the VirA, ArcB, and FrzE signal transduction proteins (19, 37). We speculate that the BvgS transmitter is capable of intermolecular phosphorylation of BvgA and that phosphorylation of the BvgS receiver may first be required. Evidence for this comes from the initial characterization of the *mod-1* mutation. Knapp and Mekalanos (15) originally assigned *mod-1* to a new gene because inactivation of the *vir* (*bvg*) locus by integration of plasmid pVI7-2 produced a strain that was still Bvg[c] as long as a wild-type copy of the *vir* region was

supplied in *trans*. On the basis of the sequence of the *vir* locus (3), it is now apparent that integration of pVI7-2 should truncate 85 amino acids from the BvgS carboxy terminus and possibly inactivate the nearby receiver domain of BvgS. This truncated BvgS carrying the *mod-1* mutation is apparently partially functional but needs a full-length BvgS molecule to display its Bvg^c phenotype. This suggests that the *mod-1* mutation (and probably all constitutive mutations in BvgS) activate the transmitter domain of BvgS but that BvgS cannot phosphorylate the receiver domain of BvgA unless it first phosphorylates its own receiver. The latter reaction may be specifically affected by truncation of the C terminus of BvgS.

Constitutive mutations in the BvgS linker eliminate the requirement for an intact periplasmic domain. The *bvgS-C1*, *bvgS-C3*, and *bvgS-C4* alleles, in combination with a deletion that removes nearly 75% of the periplasmic region, still produce a functional product. Deletion or linker insertion mutations in the wild-type locus result in a complete loss of activity. On the basis of these observations, we favor a model in which the periplasmic domain is required to maintain the active state of the BvgS transmitter domain via a linker-dependent process, and modulating signals interact with periplasmic sequences to inhibit this function. The constitutive mutations would then lock the protein into an active conformation, regardless of the status of the periplasmic region or the presence of modulating signals. It has previously been suggested that multimerization is required for BvgS activity and that modulatory signals encountered in the periplasm could disrupt this association (36). A role for multimerization in signal transduction has also been proposed for the *Vibrio cholerae* ToxR/ToxS system (5a).

Although the ability of *B. pertussis* to adjust virulence gene expression in response to environmental signals has been known for many years (17), it had not previously been demonstrated that the *bvg*-encoded system actually mediates the response in *Bordetella* species. It has also been difficult to differentiate signals that specifically affect *bvg*-regulated genes from those that might have more pleiotrophic effects. In comparing Bvg$^+$ and Bvg^c strains that differ only by single-nucleotide substitutions within *bvgS*, we have provided direct evidence that $MgSO_4$, nicotinic acid, and low temperature are specifically recognized by the *bvg* sensory system. In contrast, a recent analysis by Melton and Weiss did not show a *bvg*-specific response to growth at low temperature (28°C), as judged by the ratio of *ptx* mRNA to *recA* mRNA (20). We have also found that there is little difference in *fhaB-cat* expression between Bvg$^+$ and Bvg^c *B. pertussis* strains grown at this temperature (data not shown). The critical temperature required for modulation may therefore be lower than 28°C, since we observed clear differences between Bvg$^+$ and Bvg^c strains at 26°C. *bvg*-regulated factors in *B. bronchiseptica* also respond to temperature, as shown by the BvgS-dependent induction of motility and flagellum synthesis during growth at room temperature (1). It should also be noted that growth at 28°C results in a marked modulation of *bvgAS* activity in *E. coli* (Table 2).

Analysis of *fhaB-cat* and *ptxA3201-cat* expression in *B. pertussis* revealed interesting differences between these loci. The Bvg$^+$ and Bvg^c strains expressed nearly identical levels of *fhaB-cat* activity in Stainer-Scholte broth; however, for the *ptxA3201-cat* fusions only the constitutive mutants were active. MeβCD, which has been shown to increase pertussis toxin production in liquid cultures (12), significantly increased *ptxA3201-cat* expression by the *bvgS*$^+$ strain. MeβCD was not needed for expression of *fhaB-cat*. Although there are several possible explanations for these observations, it appears that the constitutive mutations convert BvgS into a form that is able to activate *ptx* expression under conditions in which the wild-type product is inactive. Although we had previously been unable to directly activate *ptx* transcription in *E. coli* by the wild-type *bvgAS* locus, we have recently found that the constitutive alleles are sufficient for low-level *ptx* activation (13). The mechanism responsible for this effect is currently under investigation. It will also be interesting to determine whether the differences between *fhaB* and *ptx* expression observed in BP370 are representative of expression patterns in other *B. pertussis* strains.

A hallmark of our current understanding of the *Bordetella* virulence regulon is the observation that the *bvg* regulatory apparatus is designed to sense and respond to environmental signals. Despite this, we have no indication of the role of this sensory transduction system during infection, disease, or any other stage of the *Bordetella* life cycle. If modulation occurs, when and where it occurs is unknown, and the relationship between signals used in the laboratory and those which may operate in vivo also remain a mystery. Characterized mutations that specifically affect sensory transduction should be useful for future in vitro and in vivo studies of *Bordetella* virulence control.

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