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Mutations in the *Bordetella pertussis bvgS* Gene That Confer Altered Expression of the *fhaB* Gene in *Escherichia coli*

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The *bvg* locus of *Bordetella pertussis*, required for coordinate regulation of virulence genes in response to environmental signals, encodes two proteins, BvgS and BvgA, that belong to the bacterial two-component signal transduction systems. We have isolated spontaneous mutations of the *bvg* locus in *Escherichia coli* and analyzed their effects on the expression of *fhaB*::*lacZY* transcriptional fusions. The mutations, localized in the linker and transmitter domain of BvgS, result in increased activation of *fhaB* and/or in insensitivity to a modulating agent, nicotinic acid.

Bordetella pertussis, the etiologic agent of whooping cough, produces a number of virulence factors, including pertussis toxin, filamentous hemagglutinin, and adenylate cyclase toxin, whose expression is coordinately regulated at the transcription level by the *bvg* locus (5, 8, 14). This locus encodes two proteins, BvgA and BvgS, of 23 and 135 kDa, respectively, which show a high degree of similarity with the constituent proteins of several bacterial two-component signal transduction systems (1). These systems generally consist of a pair of a sensory transmembrane protein and a cytoplasmic DNA-binding protein which communicate with each other by a conserved mechanism involving protein phosphorylation (12, 20). On the basis of similarities between *bvg* and other two-component systems, substantiated recently by in vitro phosphorelay studies (21), a model in which the periplasmic N-terminal domain of the cytoplasmic membrane-anchored BvgS protein senses external signals has been proposed. Under permissive conditions, BvgS would phosphorylate the transcriptional activator BvgA, which in its phosphorylated form would regulate transcription of various loci. Under nonpermissive conditions, called modulation, the phosphorylation cascade would be abolished (7, 9, 17).

Under ex vivo conditions, signals responsible for modulation, such as sulfate ions, nicotinic acid, and low temperature of growth, have been identified. However, in vivo signals which would coordinate the expression of virulence factors during the disease are not yet known.

The identification of *bvg* mutants altered in their response to known modulating agents could provide important information about the molecular mechanisms involved. Miller et al. (9) have previously shown that single-amino-acid substitutions in the BvgS protein render the system insensitive to environmental signals. Those mutations are confined to a region designated linker, located between the transmembrane domain and the cytoplasmic kinase—formerly designated transmitter—domain of the protein. In this study, we describe the isolation and characterization of *bvgS* mutants with altered regulation in *Escherichia coli* and more particularly mutants that show high-level expression of a target gene and/or are affected in their response to a modulating agent, nicotinic acid.

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Isolation of *bvg* mutants with altered response in *Escherichia coli*. It has been well documented that *bvg* regulation of the genes encoding filamentous hemagglutinin (*fhaB*) and *bvgAS* expression responds to the same environmental signals in *E. coli* as in *B. pertussis* (10, 14, 15). Therefore, to obtain a large collection of mutants, the *bvgAS* locus, carried on a multicopy plasmid (pDM20 [Ap^r]) (10), was introduced into a powerful mutator *E. coli* strain (carrying *mutD*) (2) and grown for 20 generations. The mutagenized pDM20 pool was introduced into an *E. coli* Δ*lac* strain (NM554) (13), harboring an *fhaB*::*lacZY* transcriptional fusion constructed on a low-copy-number plasmid (pRKZ [Tc^r]) (4). The screening of functionally altered *bvg* mutants was carried out on Luria-Bertani (LB)–X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates on which the strain harboring the *fhaB*::*lacZY* fusion and wild-type pDM20 gave a light blue phenotype. Clones that showed a dark blue phenotype corresponded, most likely, to *bvg* mutants that confer high levels of *fhaB* expression. Screening for *bvg* mutants altered in sensory transduction was carried out on LB–X-Gal plates supplemented with 4 mM nicotinic acid or 30 mM $MgSO_4$. Light or dark blue clones growing on these plates were likely candidates for mutants insensitive to these modulating agents. Plasmids carrying the mutated *bvg* locus were purified and introduced into the tester strain harboring the *fhaB*::*lacZY* fusion. Transformants were grown on LB–X-Gal plates and tested for their ability to respond to modulating factors: nicotinic acid (4 mM) and $MgSO_4$ (30 mM). All the transformants exhibited the same phenotype as the original mutant clones, indicating that the observed phenotype was due to the plasmid carrying the *bvg* locus. On the basis of phenotypic criteria, three classes of mutants could be obtained: (i) high-level *fhaB* expression, sensitive to modulating agents, (ii) wild-type or high-level *fhaB* expression, resistant to nicotinic acid and sensitive to $MgSO_4$, and (iii) wild-type or high-level *fhaB* expression, insensitive to both modulating factors. This latter class of mutants probably corresponded to the constitutive mutants described by Miller et al. (9) and was not further analyzed. It has to be noted that we were unable to isolate *bvg* alleles specifically insensitive to $MgSO_4$ while sensitive to nicotinic acid; more than 150 clones isolated on 30 mM $MgSO_4$ turned out to be fully constitutive.

Localization and sequence analysis of the *bvg* mutations. Mutations were first localized by restriction fragment exchange between the mutants and the wild-type plasmid. The reconstituted plasmids were checked for the initial mutant phenotype,

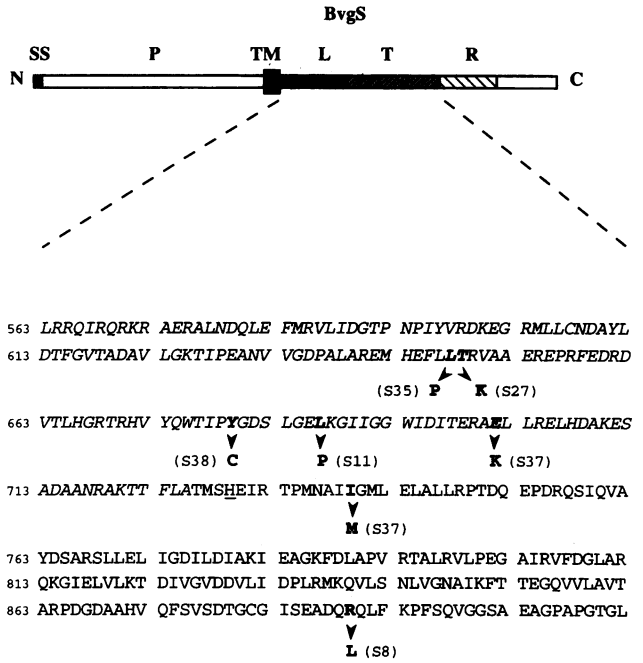

FIG. 1. Location of amino acid substitutions in the BvgS protein. The structure of the BvgS protein is indicated. The amino acid sequence of the linker region is shown in italics. His-729, the initial site of autophosphorylation, is underlined. Abbreviations: N, N terminus; SS, signal sequences; P, periplasmic domain; TM, transmembrane region; L, linker; T, transmitter; R, receiver; C, C terminus. Arrowheads and boldface type indicate amino acid substitutions; the corresponding BvgS alleles are shown in parentheses.

and the corresponding fragments were subsequently sequenced. Surprisingly, most of the mutations causing insensitivity of *fhaB* expression to nicotinic acid were localized in the cytoplasmic domain of BvgS in a region extending from amino acid residues 648 to 739, overlapping the linker (amino acid residues 569 to 724), defined as a region lying between the transmembrane sequence and the transmitter domain (9). Interestingly, the constitutive *bvgS* mutations isolated by Miller et al. (9) were localized in the same region. In one of the *bvgS* mutants (*bvgS8*, Fig. 1) that conferred high-level *fhaB* expression while retaining sensitivity to modulating agents, the mutation was localized at position 889, within the histidine kinase domain (1, 21). A sequence analysis of the mutants is shown in Fig. 1. All the mutants were single-amino-acid substitutions, except one (*bvgS37*) which was a double-amino-acid substitution.

Characterization of wild-type and *bvgS* mutants in *E. coli*. The two categories of *bvg* mutants, phenotypically classified as showing high-level *fhaB*::*lacZY* expression or insensitivity of *fhaB* expression to nicotinic acid, were further characterized by measuring levels of β-galactosidase in strains grown in LB medium in the absence or in the presence of 4 mM nicotinic acid or 30 mM $MgSO_4$. Results presented in Table 1 show that *fhaB*::*lacZY* expression was still modulated by $MgSO_4$ in all *bvg* mutants tested, although in some to a lesser extent than in the wild type. In contrast, *fhaB*::*lacZY* expression in all mutants, except *bvgS8*, exhibited partial loss of modulation by nicotinic acid. To quantify the effects of the modulating agents on these *bvgS* mutants, we performed dose-response curves in the presence of 0.1 to 100 mM nicotinic acid or 0.5 to 50 mM $MgSO_4$ and calculated the concentrations that reduced the *fhaB*::*lacZY* expression to 50%. The results presented in Table 2 show that the mutants required 8- to 40-fold-higher nicotinic acid concentrations to yield 50% inhibition than did the wild-type *bvg*$^+$ strain. One of the mutants showed no inhibition of *fhaB*::*lacZY* expression even at 100 mM nicotinic acid. $MgSO_4$ concentrations required for 50% inhibition of *fhaB*::*lacZY* expression in the wild-type and mutant *bvgS* strain did not differ by more than a factor of 5.

TABLE 1. Expression of *fha*::*lacZY* fusion in strains carrying plasmids with different *bvg* alleles

bvgS allele	Amino acid substitution	β-Galactosidase activity (U/mg [dry weight] of bacteria)[a] in indicated medium		
		LB	NA[b]	$MgSO_4$[c]
Wild type	None	200	1	1
bvgS8	P-889→L	1,900	40	37
bvgS11	L-686→P	3,000	2,000	371
bvgS27	T-648→K	2,500	900	77
bvgS35	L-647→P	295	105	1
bvgS37	E-701→K, I-739→M	1,500	800	120
bvgS38	Y-679→C	315	540	1

[a] β-Galactosidase activities were determined in toluenized bacterial suspensions of overnight cultures at 37°C in LB and represent the average values obtained from at least three independent assays that differed by less than 10%. One unit of enzyme corresponds to 1 nm of substrate formed per min at 28°C (11).
[b] LB plus 4 mM nicotinic acid.
[c] LB plus 30 mM $MgSO_4$.

Unexpectedly, one of the *bvg* mutants (*bvgS38*) in which *fhaB* expression was apparently not inhibited by nicotinic acid showed activation of *fhaB* expression when grown in the presence of high nicotinic acid concentrations (Fig. 2). In addition, nicotinic acid could overcome the inhibitory effect of $MgSO_4$, suggesting either that there was a common interaction site for the two modulating agents or that nicotinic acid could induce a distinct, active, conformational state of the BvgS38 protein and binding of $MgSO_4$ at an independent site would shift the protein in an inactive state.

To assess the pleiotropic effects of the different *bvg* mutants, we measured their effects on *bvgA*::*lacZY* fusions. In line with previously reported results, the effects of *bvgS* mutations on *bvgA* expression were comparable with those observed on *fhaB* (data not shown). In contrast, none of the *bvg* mutants, even those which increased *fhaB* expression more than 10-fold, activated the *cyaA* promoter in *E. coli*.

TABLE 2. Concentrations of modulating agents that cause 50% decrease in *fhaB*::*lacZY* expression

bvgS allele	50% inhibitory concn (mM)[a]	
	NA	$MgSO_4$
Wild type	0.4	1.2
bvgS8	0.6	1.4
bvgS11	15	7
bvgS27	3	2.5
bvgS35	3	2.5
bvgS37	9	5
bvgS38	No inhibition at 100 mM	1.5

[a] Determined from dose-response curves after overnight growth of cultures at 37°C in LB plus increasing concentrations (0.1 to 100 mM) of nicotinic acid (NA) or 0.5 to 50 mM $MgSO_4$. The values are the averages obtained from at least three independent assays that differed by less than 10%.

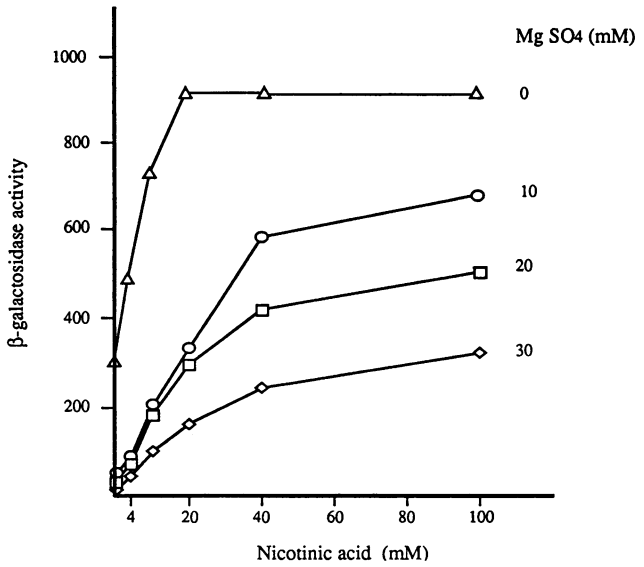

FIG. 2. Combined effects of nicotinic acid and $MgSO_4$ on *fhaB*::*lacZY* expression in the *bvgS38* mutant. The strain was grown overnight at 37°C in LB in the absence or in the presence of different concentrations of the two modulating agents. β-Galactosidase activities, expressed in units per mg (dry weight) of bacteria, were determined in toluenized bacterial suspensions (11) and represented the average values obtained from at least three independent assays that differed by less than 10%.

The *bvgAS* system bears extensive sequence and functional similarities to other bacterial two-component regulatory systems which play a central role in signal transduction coupled to gene control. It now seems established that the mechanism of communication in these systems involves phosphotransfer reactions. The sensor proteins act as protein kinases: after autophosphorylation at a histidine residue, the phosphate group is transferred to the regulatory protein. Moreover, it has been shown that several members of the histidine protein kinase family also act as phosphatases, catalyzing dephosphorylation of the associated response regulator (3, 6). Regulation may therefore take place by modulating either the kinase or the phosphatase activity of the protein. The transcription of the target gene will reflect the level of phosphorylation of the regulatory protein (16). According to this paradigm, external signals interact with the periplasmic domain of the sensor, are then communicated to the cytoplasmic domain by propagation of conformational changes through the transmembrane and linker regions, and finally act to control the kinase or phosphatase activities.

The molecular basis for the BvgAS phosphorelay has recently been established (21). The modular structure of BvgS inferred from amino acid sequence analysis (1) and localization studies (19) has been substantiated by the demonstration that the transmitter domain is involved in autophosphorylation and both the receiver and the C-terminal domains are required for phosphotransfer to BvgA (21).

Several *bvgS* mutants, reported here, exhibit a previously unobserved phenotype: compared with wild-type *bvg*, they confer 10-fold-higher expression of the target gene, *fhaB*. It is likely that the mutations lead to an alteration in the phosphorylation state of BvgA, due to an increased kinase or decreased phosphatase activity. Indeed, in one of these mutants (*bvgS8*), which is still sensitive to modulating agents, sequence analysis identified an amino acid substitution in position 889, located in the C-terminal part of the transmitter domain, in between the residues involved in autophosphorylation (His-729) and phosphate transfer to the receiver (Asp-1023) (21). All mutations exhibiting varying degrees of insensitivity to nicotinic acid and conferring high-level expression on the target gene are located within the 160-amino-acid long linker, and none of the mutations map in the region corresponding to the periplasmic domain of BvgS. Mutations in the linker region could stabilize the protein in a conformational state that would affect the ability of the periplasmic domain to communicate the detected signal. However, the possibility that the binding site for the modulating agents is located in the linker region itself and that the mutations decrease the affinity for nicotinic acid and, to a lesser extent, for $MgSO_4$ cannot be excluded. This latter hypothesis would account for the results showing that (i) high concentrations of modulating agents can restore the inhibitory response and (ii) nicotinic acid can overcome the inhibitory effect of $MgSO_4$ in one of the mutants (*bvgS38*).

It is noteworthy that constitutive *bvgS* mutants, which have been characterized by Miller et al. (9) and which exhibit phenotypes different from those described here, are also located in the linker region. Those authors proposed that the mutations would stabilize BvgS in an oligomeric conformation required for activity (18, 19), regardless of the presence of modulating agents. We would rather suggest that the linker region, besides its structural role in promoting association of the BvgS monomers, has a regulatory function as the target for binding the modulating agents. Once bound, they would then affect the phosphorylation cascade by modulating kinase or phosphatase activities. It may be relevant to this point that the His-729 residue, involved in autophosphorylation, is in the immediate vicinity of the linker region. It has to be emphasized, however, that in vivo signals that would modulate the expression of virulence factors in *B. pertussis* have not yet been identified. It is conceivable, therefore, that these signals might act through a different mechanism.

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