BvgAS Is Sufficient for Activation of the *Bordetella pertussis* *ptx* Locus in *Escherichia coli*

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BvgA and BvgS, which regulate virulence gene expression in *Bordetella pertussis*, are members of the two-component signal transduction family. The effects of growth conditions on the ability of BvgAS to activate transcription of *phaB* (encoding filamentous hemagglutinin) and *ptxA* (encoding the S1 subunit of pertussis toxin) were assessed in *Escherichia coli* by using chromosomal *phaB-lacZ* and *ptxA-lacZ* fusions. Although it had previously been reported that a *ptxA-lacZ* transcriptional fusion was not activated by *bvgAS* in *E. coli* (J. F. Miller, C. R. Roy, and S. Falkow, J. Bacteriol. 171:6345–6348, 1989), we now present evidence that *ptxA* is activated by *bvgAS* in *E. coli* in a manner that is highly dependent on the growth conditions. Higher levels of β -galactosidase were produced by *ptxA-lacZ* in the presence of *bvgAS* during growth in Stainer-Scholte medium or M9 minimal salts medium with glucose than in Luria-Bertani medium. In contrast, the level of *phaB-lacZ* expression was high during growth in all media. Addition of modulating stimuli which inhibit BvgAS function eliminated expression of *ptxA-lacZ*. Levels of β -galactosidase expressed from the *ptxA-lacZ* fusion correlated with growth rate and with the final optical density at 600 nm, suggesting that the lower growth rate in M9-glucose and Stainer-Scholte media was responsible for greater accumulation of β -galactosidase than was seen in Luria-Bertani medium. Overproduction of BvgA was not sufficient for activation of *ptxA* expression but was sufficient for *phaB* expression. However, overproduction of a constitutive BvgA allele (*bvgA-C1*) or overproduction of BvgA in the presence of BvgS was able to activate *ptxA*. Our results demonstrate Bvg-dependent activation of a *ptxA-lacZ* fusion in *E. coli* and indicate that *bvg* is the only *Bordetella* locus required for *ptxA* activation in this heterologous system.

Bordetella pertussis, the causative agent of whooping cough, synthesizes toxins and adhesins which appear to aid the bacterium in colonization and multiplication (15, 23, 28, 38, 56, 59). Expression of toxins (pertussis toxin, adenylate cyclase-hemolysin, and dermonecrotic toxin) and adhesins (filamentous hemagglutinin, fimbriae, and pertactin) is positively regulated by the products of the *bvg* operon (11, 57, 59). In contrast to the multiple loci activated by *bvg*, *vrg* genes of *B. pertussis* (5, 6, 25), siderophore production in some *Bordetella bronchiseptica* strains (14), and motility genes of *B. bronchiseptica* (1–3) are repressed by *bvg*.

Sequence analysis and in vitro studies have indicated that BvgA and BvgS are members of the two-component family of signal transduction proteins. BvgS is a 135-kDa sensor protein localized to the cytoplasmic membrane, and BvgA is a 23-kDa cytoplasmic response regulator (4, 7, 37, 49, 50–53). The signals sensed by BvgAS in vivo are not known, but BvgAS can be inactivated in vitro by growth at a low temperature or by addition of sulfate anion or nicotinic acid to the culture medium. This reversible inactivation of BvgAS is known as phenotypic modulation (26). The BvgS periplasmic region has been postulated to sense signals and transmit information to the cytoplasmic domains via the BvgS linker, which lies adjacent to the second transmembrane domain (32). The cytoplasmic portion of BvgS effects the enzymatic steps of signal transduction and can autophosphorylate in vitro with the γ -phosphate from ATP and transfer a phosphoryl group to BvgA (53, 54). BvgA contains an N-terminal receiver domain and a

C-terminal helix-turn-helix DNA binding motif (4), and phosphorylation of BvgA has been shown to increase its affinity for specific target sequences (7). The Bvg⁺ phase is required for infection in animal models of bordetellosis, and the in vitro activities of BvgS and BvgA are consistent with a phosphorylation cascade activating *Bordetella* virulence genes in vivo (1, 12, 21, 54, 58).

bvg-activated genes can be placed into distinct classes on the basis of several criteria. These include (i) sensitivity to modulation, (ii) requirements for activation of transcriptional fusions in *Escherichia coli*, and (iii) the time necessary to detect RNA transcripts following a switch from modulating to nonmodulating conditions in *B. pertussis* (7, 18, 33, 39, 41, 44, 46a). Several lines of evidence suggest that *phaB* and *bvg* are directly activated by BvgA. BvgA binds to these promoters in vitro, and *bvg* is the only *Bordetella* locus required for their activation in *E. coli* (33, 39, 50).

While it is clear that *bvg* is required for expression of *ptxA* and *cya*, it has not been resolved whether BvgA is directly or indirectly involved in their activation. *ptxA* and *cya* transcripts are not detected until hours after a switch from modulating to nonmodulating conditions, in contrast to *phaB* and *bvg* transcripts, which are observed within a few minutes (44). This observation is consistent with results indicating that *ptxA* and *cya* are more sensitive to modulation than *phaB* and *bvg* (46a, 48). Promoter elements for *ptxA* and *cya* have been defined, but early attempts to demonstrate BvgA binding to these promoters were not successful (18–20, 27, 35, 39). Furthermore, initial reports indicated that *bvgAS* was not sufficient for activation of *ptxA* and *cya* transcriptional fusions in *E. coli* (17, 33). These results suggested that an intermediate factor was required for expression of pertussis toxin and adenylate cyclase; however, such a factor has not yet been described (9, 31, 48).

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TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
MC4100	F ⁻ <i>araD139</i> Δ (<i>argF-lac</i>) <i>U169 rpsL 150 relA1</i>	33
JFMC3	MC4100 <i>recA1</i> λ <i>fhaB-lacZYA</i> lysogen	33
JFME3	MC4100 <i>recA1</i> λ <i>ptxA-lacZYA</i> lysogen	33
Plasmids		
pTrc99A	<i>trc</i> promoter-based expression vector; Amp ^r	Pharmacia
pBR322	Cloning vector; Amp ^r Tet ^r	42
pACYC184	Cloning vector; Cam ^r Tet ^r	42
pDM20	<i>bvgA</i> ⁺ S ⁺ ; pBR322 derived	32
pJM660	BvgA under control of IPTG-inducible <i>trc</i> promoter in pTrc99A	53
pJMB21	<i>bvgA</i> ⁺ <i>bvgS-C3</i> (insensitive to modulators); pBR322 derived	32
pMU281	<i>SspI-NotI</i> fragment containing <i>bvgAS</i> from pDM20 inserted into <i>EcoRV-EagI</i> -digested pACYC184	This study
pMU260	<i>SspI-NotI</i> fragment containing <i>bvgA</i> and <i>bvgS-C3</i> from pJMB21 inserted into <i>EcoRV-EagI</i> -digested pACYC184	This study
pMU112	pJM660 with the <i>bvgA-C1</i> allele (Val-13→Ile)	This study
pMU352	In-frame deletion in BvgA generated by <i>XcmI</i> digestion of pMU281	This study

In this report, we demonstrate *bvg*-dependent activation of a pertussis toxin transcriptional fusion (*ptxA-lacZYA*) in *E. coli*. Activation of *ptxA-lacZYA* was sensitive to the presence of modulating signals which eliminate *ptxA* expression in *B. pertussis*. Growth in M9 minimal salts or Stainer-Scholte (SS) medium (47) was required for optimal *ptxA* expression. The lower growth rate of *E. coli* in M9 minimal salts or SS medium than in Luria-Bertani (LB) medium correlated with increased levels of β -galactosidase. Additionally, expression of the *ptxA* and *fha* promoters had different requirements for BvgA. While overproduction of BvgA was sufficient for *fha* expression, *ptxA* required activation of BvgA either by a mutation conferring constitutive activity or by the presence of BvgS. These results indicate that *bvg* is sufficient for activation of a *ptxA-lacZYA* fusion in *E. coli* and that growth conditions have a major effect on the ability to detect *ptxA* expression in this heterologous host.

MATERIALS AND METHODS

Media and culture conditions. The strains and plasmids used are listed in Table 1. All strains were grown in either LB medium, SS medium, or M9 minimal salts medium with 0.2% (wt/wt) glucose unless noted otherwise (34, 47). When necessary, the medium was supplemented with antibiotics at final concentrations of 100 μ g/ml for ampicillin, 40 μ g/ml for chloramphenicol, 10 μ g/ml for novobiocin, and 10 μ g/ml for rifampin. NaCl was added to the media at a final concentration of 250 mM for LB medium and 100 mM for SS or M9 medium. M9 medium was supplemented with 0.25 mM MgSO₄, 0.3 mM MnSO₄, 0.45 μ M FeSO₄, and 4.5 μ M CaCl₂. M9-acetate was prepared by addition of sodium acetate to 0.2% (wt/wt). Other additions are noted in the text and figure legends. For SS medium without NaCl, NaCl was omitted and the pH was adjusted with *ortho*-phosphoric acid instead of HCl. This resulted in a final Cl⁻ concentration of approximately 8 mM.

E. coli strains to be assayed for β -galactosidase activity were grown in LB broth and then subcultured in various media and grown overnight. For determination of exponential-phase activity, overnight cultures were diluted to an optical density at 600 nm (OD₆₀₀) of 0.04 and grown to mid-exponential phase (OD₆₀₀ of approximately 0.8). Growth curves were determined by subculturing LB or SS medium with an aliquot of an overnight culture so that the starting OD₆₀₀ was 0.02 (LB medium) or 0.01 (SS and M9-glucose media).

To test the effect of overproduction of BvgA on *ptxA-lacZYA* and *fhaB-lacZYA*

expression, overnight cultures grown at 22°C were used to inoculate LB medium at a starting OD₆₀₀ of 0.07; the cultures were then incubated at 37°C. After 1.5 h, BvgA expression was induced by addition of IPTG (isopropyl- β -D-thiogalactopyranoside) at a final concentration of 2 mM. The cultures were incubated for an additional 4 h and then assayed for β -galactosidase activity.

β -Galactosidase assays and DNA methods. β -Galactosidase activities were determined with cells permeabilized with sodium dodecyl sulfate (SDS)-CHCl₃ as described by Miller (34). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Pharmacia, or Promega Corp. and were used according to the manufacturer's instructions.

SDS-PAGE and Western blot (immunoblot) analysis. Cultures of JFME3/pDM20 and JFME3/pBR322 were grown to stationary phase in LB medium, M9 minimal salts medium with 0.2% glucose, or SS medium. Aliquots (OD₆₀₀, 1.5) from these cultures were pelleted and resuspended in sample buffer (60 mM Tris, 2% SDS, 10% glycerol, 0.005% bromophenol blue, 0.1 M dithiothreitol). Prior to being loaded, the samples were boiled for 10 min and spun at 17,000 $\times$ g for 5 min. The samples contained 0.2 OD₆₀₀ units of whole-cell lysates and were loaded on two separate 10% acrylamide SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels along with prestained molecular weight markers (Sigma). One gel was transferred to Immobilon for Western blot analysis, and the other gel was stained with Coomassie brilliant blue and destained with 35% methanol–10% acetic acid. Western blot analysis was performed by the enhanced chemiluminescence technique (Amersham) according to the manufacturer's instructions. The membrane was probed with monoclonal antibodies directed against either BvgS or BvgA, both at a 1:5,000 dilution. Horseradish peroxidase-conjugated sheep anti-mouse antibody was used as the secondary antibody at a 1:5,000 dilution.

RESULTS

Expression of a *ptxA-lacZYA* transcriptional fusion in *E. coli*.

In a previous study utilizing mid-exponential-phase cultures of *E. coli* JFME3 (λ *ptxA-lacZYA*) grown in LB medium, activation of a *ptxA-lacZYA* transcriptional fusion by *bvgAS* was not detected (33). Since BvgA and BvgS are the only regulatory factors that have been determined to be necessary for pertussis toxin production in *B. pertussis*, we reexamined strain JFME3 for conditions permissive for *ptxA* expression. SS medium (47) allowed high levels of β -galactosidase expression in a Bvg-dependent manner. In stationary-phase cultures, expression was induced nearly 100-fold in the presence of BvgAS encoded by pDM20 (Fig. 1). Addition of modulating agents (40 mM MgSO₄ and 10 mM nicotinic acid), which eliminate pertussis toxin expression in *B. pertussis* through inhibition of BvgS (26, 29, 30, 32), reduced β -galactosidase levels to background. Removal of chloride from the medium was also sufficient to modulate expression of BvgS in JFME3/pDM20 or JFMC3/pDM20 (*fhaB-lacZYA*; data not shown). It has not been determined if removal of chloride anion sensitizes BvgS to modulators or if chloride is required for activity (26, 30). Figure 1 demonstrates that the pertussis toxin transcriptional fusion contained in JFME3 can be expressed in a *bvg*-dependent manner.

Effects of growth media on *fhaB-lacZYA* and *ptxA-lacZYA* expression in *E. coli*. Strains JFME3 and JFMC3 (*fhaB-lacZYA*) carrying plasmid pDM20 were grown to stationary phase in LB, M9-glucose, or SS medium to determine the effects of different culture media on *ptxA* and *fha* expression. LB medium is a complex medium with multiple nutrient sources which allows rapid growth of *E. coli*. M9-glucose and SS media are poorer substrates for growth than LB medium and result in lower growth rates. M9 minimal salts medium is a defined minimal medium with glucose added as the sole carbon source (34). SS medium is typically employed for liquid culture of *B. pertussis* and is a complex defined medium which contains amino acids as the carbon source (47).

The *fhaB-lacZYA* fusion was expressed well in all media, differing less than twofold between LB, M9-glucose, and SS media (Fig. 2B). The levels of β -galactosidase expressed from *ptxA-lacZYA* were approximately sixfold higher in M9-glucose and 20-fold higher in SS medium than in LB medium (Fig. 2A). Under all conditions, the level of *ptxA-lacZYA* expression was

FIG. 1. Expression and modulation of a *ptxA-lacZYA* fusion in *E. coli* JFME3/pDM20 (*BvgA*⁺*S*⁺). Values are means $\pm$ standard deviations (SD) (error bars) for three experiments performed with stationary-phase cultures grown in SS medium (47). JFME3/pDM20 was grown in SS medium with no additions, with the addition of 40 mM MgSO₄ (SO₄) or 10 mM nicotinic acid (Nic), or without chloride (-NaCl) (see Materials and Methods). Plasmid pBR322 was used as the vector control.

lower than that of *fhaB-lacZYA*. Both *ptxA-lacZYA* and *fhaB-lacZYA* had an absolute requirement for *bvg* in all media (Fig. 2, pDM20 versus pBR322). Differences in expression were not due to changes in pH, as growth of JFME3 in SS, LB, and M9-glucose media did not alter the pH by more than 0.4 pH

units (data not shown). While *fhaB-lacZYA* expression was not greatly affected by growth in the different media, *ptxA-lacZYA* expression varied significantly depending on the growth medium utilized.

Modulation does not account for the variation of *ptxA-lacZYA* expression in different media. Potential explanations for the differences in *ptxA-lacZYA* expression are that LB medium contains components that inhibit BvgS activity and that the *ptxA* promoter may be more sensitive to modulation than the *fhaB* promoter. We assayed JFME3/pMU260 (*bvgA*⁺ *bvgS*-C3), JFME3/pMU281 (*bvgA*⁺ *S*⁺), and JFME3/pACYC184 (vector control) for *ptxA* expression in LB, SS, or M9-glucose medium. The *bvgS*-C3 allele contained on pMU260 encodes a BvgS protein that is insensitive to modulating signals (12, 32). When contained on a pBR322-based plasmid, the *bvgS*-C3 allele led to poor growth in SS or M9-glucose medium (data not shown); consequently, pACYC184 derivatives containing wild-type *bvgS* or *bvgS*-C3 were utilized. JFME3/pMU260 and JFME3/pMU281 had nearly identical patterns of β -galactosidase expression in the different media (Fig. 3). β -Galactosidase levels were approximately 8-fold higher in M9-glucose and 20-fold higher in SS medium than in LB medium for both JFME3/pMU260 and JFME3/pMU281, similar to what was detected with JFME3/pDM20 (Fig. 2A). JFME3/pACYC184 did not activate *ptxA-lacZYA* under any culture conditions. It is worth noting that pMU281 (pACYC184 derived) promoted overall lower levels of expression of *ptxA-lacZYA* than pDM20 (pBR322 derived). This is consistent with the reported lower copy number of pACYC184 derivatives (ca. 10 to 12 copies per cell) in comparison with that of pBR322 derivatives (ca. 15 to 20 copies per cell) (42), although the effect was not strictly proportional to the estimated gene dosage of *bvg*. Since no significant difference was observed between constitutive and wild-type *bvgS* alleles, we conclude that variation of *ptxA-lacZYA* expression in SS, M9-glucose, and LB media is not due to modulation of BvgS. This suggests that the varying levels of *ptxA* expression are due to different growth characteristics as opposed to medium components modulating BvgS.

FIG. 2. Effect of culture conditions on expression of *ptxA-lacZYA* (A) and *fhaB-lacZYA* (B). Stationary-phase cultures were grown in triplicate in the indicated media and assayed for β -galactosidase activity. (A) JFME3/pDM20 (solid bars) and JFME3/pBR322 (hatched bars). (B) JFME3/pDM20 (solid bars) and JFME3/pBR322 (hatched bars).

FIG. 3. Effect of the *bvgS-C3* constitutive allele on *ptxA-lacZYA* expression in LB, M9, or SS medium. Triplicate cultures of JFME3 with pMU281 (solid bars; *bvgS* wild type), pMU260 (striped bars; *bvgS-C3*, insensitive to modulating signals), or pACYC184 (open bars; vector used for construction of pMU281 and pMU260) were grown to stationary phase and assayed for β -galactosidase activity.

Activation of *ptxA-lacZYA* correlates with growth rate. We routinely noted that β -galactosidase levels were increased in stationary-phase cultures of JFME3/pDM20 in comparison with those in exponential-phase cultures. As cultures reached late exponential phase or early stationary phase, the β -galactosidase levels increased fivefold over those of early exponential phase and threefold over those of late exponential phase (Fig. 4A). This was not limited to cultures grown in LB medium, as a similar increase (ca. fivefold) was seen in exponential-phase versus stationary-phase cultures of JFME3/pDM20 grown in SS or M9-glucose medium. By comparison, levels of β -galactosidase expressed from the *fhaB-lacZYA* fusion in the presence of *bvg* remained relatively constant in early exponential, late exponential, and stationary phase (Fig. 4C). Neither *fhaB-lacZYA* (Fig. 4D) nor *ptxA-lacZYA* (Fig. 4B) was activated in the absence of *bvg*, as expected.

Expression of *ptxA* in relation to growth phase differed depending on the growth medium. When JFME3/pDM20 was grown in M9-glucose medium, β -galactosidase activity increased at a linear rate throughout most of the growth curve (Fig. 5A). A similar pattern was seen in cultures grown in SS medium (Fig. 5B). This suggested that the *ptxA* promoter was not being specifically activated in late exponential or stationary phase but that β -galactosidase levels increased when growth was slowed.

Relation of *ptxA* expression to growth rate and final optical density. We compared β -galactosidase levels at mid-exponential phase and stationary phase with the growth rate and final optical density of JFME3/pDM20 grown in LB, SS, and M9-glucose media. The rates of growth of JFME3/pDM20 in SS and M9-glucose media were equivalent, and the β -galactosidase levels at mid-exponential phase ($OD_{600} = 0.6$) were similar (Table 2). The higher levels of *ptxA-lacZYA* observed in stationary-phase cultures of JFME3/pDM20 grown in SS compared with M9-glucose medium cannot be explained by growth rate differences between SS and M9-glucose media but correlate instead with the higher final optical density reached in SS medium. While stationary-phase cultures of JFME3/pDM20

grown in SS medium have eightfold-higher levels of β -galactosidase than cultures grown in LB medium (Table 2), the cultures reach similar final optical densities but have different growth rates. This suggests that both the final optical density and the growth rate influence the levels of β -galactosidase expressed by JFME3/pDM20, although growth rate has the greater effect.

We tested the relationship between final optical density and β -galactosidase levels by changing the total amount of glucose in M9-glucose medium. Decreasing the amount of glucose from 0.2 to 0.04% did not change the growth rate during early exponential growth phase (Table 3) but did reduce the final OD_{600} and the β -galactosidase levels at stationary phase. Increasing the glucose concentration from 0.2 to 1% did not affect the growth rate or β -galactosidase levels at late exponential phase (Table 3 and data not shown), but cultures grown in 1% glucose did reach a slightly higher final OD_{600} and demonstrated a small increase in the levels of β -galactosidase in stationary phase. When we slowed the growth of JFME3/pDM20 by substitution of acetate for glucose as a carbon source, M9-acetate-grown cultures had increased levels of β -galactosidase compared with glucose-grown cultures at a similar OD_{600} (Table 3, M9 plus 0.04% glucose and M9 plus 0.2% acetate). These results suggest that levels of β -galactosidase increase as the growth rate of the cell decreases and as the cell density reached in stationary phase increases.

Levels of BvgA and BvgS in JFME3/pDM20 grown in different media. The lower growth rates of M9-glucose and SS medium-grown cultures may allow a greater accumulation of activated BvgA per cell, leading to increased transcription of *ptxA-lacZYA*. JFME3/pDM20 and JFME3/pBR322 were grown to stationary phase in LB, M9-glucose, or SS medium, and the levels of BvgA and BvgS were assessed. Protein profiles of JFME3/pDM20 normalized by OD_{600} were relatively similar under the different growth conditions (Fig. 6A), with the exception of a somewhat smaller amount of total protein in JFME3/pDM20 grown in M9-glucose medium.

Western blots using monoclonal antibodies directed against either BvgA or BvgS demonstrated that cultures of JFME3/pDM20 grown in SS medium had slightly elevated levels of BvgS and BvgA compared with cultures grown in M9-glucose or LB medium (Fig. 6B). As measured by densitometry, the levels of BvgA in SS cultures were twofold higher than those in LB or M9-glucose cultures. BvgS levels were 2.4-fold (SS) or 1.4-fold (M9) higher than those in LB cultures. Neither BvgA nor BvgS was detected in cultures of JFME3/pBR322 grown under any conditions (Fig. 6B). The approximately twofold difference in levels of BvgA and BvgS contrasts with the eightfold difference in β -galactosidase levels observed between cultures of JFME3/pDM20 grown in SS versus LB medium (Table 2). Although the difference in BvgA levels may be one of the factors that contributes to higher levels of *ptxA-lacZYA* expression during growth of JFME3/pDM20 in SS medium, it does not appear to be the sole reason for increased amounts of β -galactosidase.

Effects of overproduction and activation of BvgA on *ptxA-lacZYA* expression levels. Overproduction of BvgA in *E. coli* has previously been shown to be sufficient for expression of *fhaB-lacZYA* but not *ptxA-lacZYA* (40). However, as shown in Fig. 7, overproduction of BvgA with activating mutations or overproduction of BvgA in the presence of BvgS activates *ptxA-lacZYA* expression in stationary-phase *E. coli* cultures grown in LB medium. Expression of BvgA from the IPTG-inducible *trc* promoter did not detectably activate *ptxA* (Fig. 7A, pJM660 with and without IPTG) but was able to activate *fha* (Fig. 7B, pJM660 with and without IPTG). In fact, the

FIG. 4. Expression of *ptxA-lacZYA* and *fhaB-lacZYA* as a function of growth phase. Triplicate cultures were grown in LB medium, and samples were periodically assayed for β -galactosidase activity. (A) JFME3/pDM20; (B) JFME3/pBR322; (C) JFMC3/pDM20; (D) JFMC3/pBR322.

leaky expression from the *trc* promoter under noninduced conditions was sufficient for high levels of *fha* activation by BvgA alone (Fig. 7B, pJM660 without IPTG). The BvgA constitutive mutation, *bvgA-C1*, contains a Val-13-to-Ile mutation. *bvgA-C1* in a pBR322 vector is able to activate an *fhaB-lacZYA* fusion approximately 50-fold over pBR322 background levels in the absence of BvgS and 300-fold in the presence of BvgS (55). Overexpression of BvgA-C1 and overexpression of BvgA in the presence of BvgS were able to induce expression of both *ptxA-lacZYA* and *fhaB-lacZYA* (Fig. 7, pMU112 and pJM660 + pMU352). Our data are consistent with activation and overproduction of BvgA from pJM660 being required for *ptxA-lacZYA* expression but with only overproduction of BvgA from pJM660 being required for *fhaB-lacZYA* expression.

DISCUSSION

It has been well established that *bvg* is required for both *ptxA* and *fhaB* expression in *B. pertussis* (40, 57). However, *fhaB* and

ptxA have different temporal patterns of expression following a shift from modulating to nonmodulating conditions (44) and distinct requirements for expression in *E. coli* (33). This has led to speculation that the *bvg* virulence control system may have a downstream activator that participates in *ptxA* expression, much like the ToxRS-ToxT regulatory system of *Vibrio cholerae* (13). Several mutations conferring an *Fha*⁺ *Cya*⁻ *Ptx*⁻ phenotype have been isolated and mapped (9, 48). These mutations are not located in a putative downstream transcriptional activator but are instead found upstream of the translational start site of *rpoA* or in the C terminus of BvgA, invoking a model in which BvgA interacts with RpoA at the *ptxA* promoter. This model is supported by recent findings of Boucher and Stibitz, who have clearly demonstrated binding of RpoA and BvgA to repeat sequences upstream of the *ptxA* promoter (7a). Phosphorylation of BvgA appears to enhance binding to the upstream repeat sequences, which contain recognizable BvgA consensus binding sites. The involvement of additional

FIG. 5. Growth curves of JFME3/pDM20 grown in triplicate in M9 (A) and SS (B) media and assayed for β -galactosidase activity.

mechanisms in *ptxA* regulation, such as histone-like proteins, DNA supercoiling, or alternate sigma factors, cannot be excluded at this time (10, 22, 24, 36, 45), although a protein homologous to the H1 histone protein has been identified in *B. pertussis* and this protein does not appear to influence *ptxA* expression (43). Recently, a locus that allows Bvg-dependent expression of *ptxA-lacZYA* in *E. coli* has been identified. This locus, *baf*, increases expression of *ptxA-lacZYA* approximately 360-fold when present on a high-copy-number plasmid and expressed from a vector promoter (12a). *baf* may represent a transcription factor that is specific for *ptxA*, or it could be a nonspecific component of the transcriptional apparatus. Alternatively, high-level expression of *baf* may result in lower growth rates, mimicking growth in M9 or SS medium. Further experiments should reconcile the role of *baf* in *ptxA* transcription with our data, which indicate that the only *B. pertussis* locus required to activate *ptxA* expression in *E. coli* is *bvgAS*. Any additional factors involved in regulation of *ptxA* expression are likely conserved between *B. pertussis* and *E. coli*.

The effect of *bvg* on activation of *ptxA* and *cya* transcriptional fusions in *E. coli* has previously been examined (16, 17, 33, 45). Earlier reports proposed that BvgAS was not sufficient for activation of *ptxA* or *cya* in *E. coli*. In those experiments,

ptxA-lacZYA and *cya-lacZY* expression in cultures grown in LB medium was assayed (16, 17, 33). We have shown that exponential-phase cultures grown in LB medium are suboptimal for *ptxA* expression in *E. coli*, and this may be true for *cya-lacZY* expression as well. Scarlato et al. reported Bvg-dependent activation of *ptxA* in *E. coli*, employing a plasmid-based *ptxA-CAT* fusion (45). Although not quantitated, activation of *ptxA* in the presence of BvgAS was demonstrated, but the response to modulating signals was variable. Scarlato et al. suggested that DNA supercoiling was an important factor in *ptxA* activation on the basis of studies using a DNA gyrase inhibitor, novobiocin. Cultures of JFME3/pDM20 grown in LB medium with novobiocin demonstrated a slight increase (1.4-fold) in β -galactosidase levels compared with growth in LB medium without novobiocin (data not shown). We were unable to determine if the slight increase was due to inhibition of DNA gyrase or the decreased growth rate, since novobiocin inhibited cell growth. β -Galactosidase levels of JFME3/pDM20 cultured in LB medium with novobiocin were nevertheless substantially lower than those of JFME3/pDM20 grown in M9-glucose or SS medium.

TABLE 2. Growth rates, final OD₆₀₀, and β -galactosidase activity for JFME3/pDM20 grown in various media

Medium	Growth rate (<i>k</i>) ^a	U of β -galactosidase activity ^b		Final OD ₆₀₀
		Mid-exponential phase	Stationary phase	
LB	1.1	130	390 ± 10	3.5
M9-glucose	0.24	550	1,400 ± 100	1.4
SS	0.23	750	3,100 ± 240	3.2

^a Growth rates (*k*, generations per hour) are derived from growth curves done in triplicate.

^b Mid-exponential-phase (OD₆₀₀ = 0.6) β -galactosidase activity is extrapolated from β -galactosidase levels as a function of OD (Fig. 4 and 5). Stationary-phase values are means ± SD from experiments performed in triplicate.

TABLE 3. Growth rates, final OD₆₀₀, and β -galactosidase activity for JFME3/pDM20 grown in M9 medium with different concentrations of glucose or acetate

M9 medium supplement	Growth rate (<i>k</i>) ^a		U of β -galactosidase activity (stationary phase) ^b	Final OD ₆₀₀
	Early exponential phase	Late exponential phase		
1% glucose	0.48	0.25	1,400 ± 150	2.2
0.2% glucose	0.48	0.23	1,100 ± 150	1.8
0.04% glucose	0.48	ND ^c	160 ± 30	0.53
0.2% acetate	0.15	ND ^c	1,100 ± 100	0.59

^a Growth rates are derived from growth curves done in triplicate.

^b Values are means ± SD from experiments performed in triplicate.

^c Growth rates at late exponential phase were not determined (ND) for M9 plus 0.04% glucose and M9 plus 0.2% acetate because these cultures reached a limited final OD₆₀₀.

FIG. 6. (A) Protein profiles of JFME3/pDM20 and JFME3/pBR322 grown in different media. Results for whole-cell lysates of JFME3/pDM20 grown to stationary phase in SS (lane 1), M9-glucose (lane 3), or LB (lane 5) medium and whole-cell lysates of JFME3/pBR322 grown to stationary phase in SS (lane 2), M9-glucose (lane 4), or LB (lane 6) medium are shown. Sizes of prestained molecular mass markers (Sigma) (lane M) are shown in kilodaltons on the left. (B) The whole-cell lysates described above were subjected to Western blot analysis using monoclonal anti-BvgS and monoclonal anti-BvgA antibodies.

Our results suggest that lower growth rates are required for detection of maximal expression of *ptxA-lacZYA* in JFME3/pDM20. Altering the growth rate of bacteria is known to affect multiple parameters, including the rate of protein synthesis and cell size (8). We have not determined the mechanism by which growth rate affects *ptxA-lacZYA* expression but hypothesize that two parameters are influential. SS medium-grown cultures have slightly increased amounts of BvgS and BvgA. This may lead to higher levels of transcription of *ptxA-lacZYA*. It is also possible that *ptxA-lacZYA* is transcribed at a low level and that we are detecting accumulation of stable β -galactosidase at lower growth rates. This may also account for the higher levels of β -galactosidase in cultures that reach a higher final optical density. However, the total amount of β -galactosidase produced per unit time in M9-glucose or SS medium-grown cultures is greater than that in LB medium-grown cultures, indicating that greater amounts of β -galactosidase are being produced in the M9-glucose and SS medium-grown cultures.

Our data support a model proposed by Scarlato et al. in which the *fhaB* and *ptxA* promoters have different sensitivities to BvgA (44). In this model, low levels of activated BvgA permit *fhaB* but not *ptxA* expression. For maximal *ptxA* expression, larger amounts of activated BvgA are predicted to be necessary. Higher levels of BvgA have been correlated with activation of *ptxA* in *B. pertussis*, and it has been suggested that *ptxA* expression requires a threshold level of BvgA (44). Our results have defined a system which reproduces activation of *ptxA* by *bvg* and will now permit further analysis of conditions

FIG. 7. Ability of BvgA overexpression to activate *ptxA-lacZYA* (A) and *fhaB-lacZYA* (B) transcriptional fusions in LB medium. *E. coli* JFME3 (*ptxA-lacZYA*) and JFMC3 (*fhaB-lacZYA*) were transformed with various plasmids and assayed for β -galactosidase activity in the presence or absence of IPTG as described in Materials and Methods. Plasmids are as follows: pJM660 contains BvgA under control of the IPTG-inducible *trc* promoter, pMU112 is a derivative of pJM660 with the *bvgA-C1* mutation, and pMU352 is the autoregulated *bvg* locus with an in-frame BvgA mutation. pDM20 contains the entire positively autoregulated wild-type *bvg* locus (41, 46), and pTrc99A is the vector used to create pJM660 and pMU112 and contains neither *bvgA* nor *bvgS*. Values are means $\pm$ SD of assays done in triplicate.

which influence and regulate expression of the pertussis toxin operon.

ACKNOWLEDGMENTS

We thank Brian Akerley, Peggy Cotter, and Guillermo Martinez for helpful comments on the manuscript. We are grateful to Scott Stibitz for providing anti-BvgS and anti-BvgA antibodies and to Philip Boucher for communicating results prior to publication. We also thank Nick Carbonetti for advice and consultations.

This work was supported by National Institutes of Health grants AI31548 and AI38417 to J.F.M. M.A.U. is supported by National Institutes of Health Predoctoral Training Grant AI07323. J.F.M. is a Pew Scholar in the Biomedical Sciences.

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