JOURNAL OF BACTERIOLOGY, July 1995, p. 3801–3807 Vol. 177, No. 13
0021-9193/95/$04.00+0

Identification of a *Bordetella pertussis* Regulatory Factor Required for Transcription of the Pertussis Toxin Operon in *Escherichia coli*

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Received 13 December 1994/Accepted 25 April 1995

Transcription of the pertussis toxin operon (*ptx*) is positively regulated in *Bordetella pertussis* by the *bvgAS* locus. However, a *ptx-lacZ* transcriptional fusion in *Escherichia coli* cannot be activated by *bvgAS* in *trans*. This suggests that an additional factor(s) is required for transcription of *ptx*. A gene encoding a Bvg accessory factor (Baf) was identified by its ability to activate an *E. coli ptx-lacZ* fusion in the presence of *bvgAS*. The expression of *ptx-lacZ* was decreased by the addition of 40 mM $MgSO_4$, a compound that also modulates *ptx* expression in *B. pertussis*. Baf alone did not activate expression of an *E. coli fhaB-lacZ* fusion, nor did it increase expression of *fhaB-lacZ* in *trans* with *bvgAS*. The gene encoding Baf was localized, sequenced, and found to produce a novel 28-kDa protein. Sequences homologous to *B. pertussis baf* were identified in *Bordetella bronchiseptica* and *Bordetella parapertussis* but not in *Bordetella avium*. When an additional copy of *baf* was integrated into the chromosome of BC75, a *B. pertussis* mutant that produces a low level of pertussis toxin, pertussis toxin production was partially complemented in the cointegrate strain.

Bordetella pertussis, a gram-negative coccobacillus, causes the upper respiratory tract disease pertussis (whooping cough) in humans. *B. pertussis* synthesizes multiple attachment factors and toxins that act in concert to produce disease (8, 39, 48). Filamentous hemagglutinin, pertactin, pertussis toxin (PT), and fimbriae are important in mediating attachment to a variety of cell types. The organism elaborates several factors that are toxic to host cells, including tracheal cytotoxin, adenylate cyclase toxin, and PT.

All of the virulence factors listed above, with the exception of tracheal cytotoxin, are coordinately regulated by the products of the *bvgAS* locus, BvgA and BvgS (6, 22, 34, 37, 51). These molecules are members of a family of transcriptional regulators that utilize two components, a sensor and a regulator, to activate or repress the transcription of genes in response to a variety of environmental stimuli (15, 30). BvgS is a 135-kDa transmembrane sensor protein that regulates the transcription of virulence genes in response to environmental stimuli such as $MgSO_4$, nicotinic acid, or growth at low temperatures (25°C) (26, 27, 29, 46). In the presence of such stimuli, there is no transcription of *B. pertussis* virulence-activated genes (*vag*), a phenomenon termed "phenotypic modulation" (21, 26). In the absence of these signals (e.g., at 37°C), BvgS activates BvgA, a 23-kDa cytoplasmic protein (46). BvgA binds to regulatory sequences upstream of some virulence genes and positively influences their transcription (36). Uhl and Miller (47) have recently shown that the cytoplasmic domain of BvgS phosphorylates BvgA in vitro. The phosphorylation of BvgA correlates with the activation of an *fhaB-lacZ* transcriptional fusion in *Escherichia coli* (47), suggesting that phosphorylated BvgA mediates the transcriptional activation of virulence-associated genes. Similar results were reported by Boucher et al. (2). Gel shift assays revealed that BvgA phosphorylated in vitro exhibited an enhanced ability to bind to target DNA sequences, while a dephosphorylated form had limited binding ability (2).

The BvgAS system is necessary and sufficient for the transcriptional activation of both *fhaB* and *bvgAS* in *E. coli* (31, 32, 37, 38, 45). In contrast, *ptx* and *cya*, the genes encoding PT and adenylate cyclase toxin, respectively, are not normally activated by BvgAS in *E. coli* (14, 31, 32, 37). Scarlato et al. demonstrated that *bvgAS* could activate the PT promoter (P_{tox}) in *E. coli* when P_{tox} was cloned in the plasmid pKK232 but not in pGem-3. (40). However, environmental regulation of P_{tox} was abolished under these conditions (40). This suggests that transactivation of *ptx* and *cya* by BvgAS is either indirect or requires an accessory factor(s).

In this study, we present the cloning and characterization of a Bvg accessory factor, termed Baf. The results indicate that Baf and BvgAS cooperate to transactivate a *ptx-lacZ* fusion in *E. coli.*

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* cultures were grown for 18 to 24 h at 37°C in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 85 mM NaCl [pH 7.5]) or on LB plates. *B. pertussis* cultures were grown for 3 to 4 days at 37°C on Bordet-Gengou agar containing 10% sheep blood or on charcoal agar containing 15% horse blood. A 20-mg/ml stock solution of the chromogenic indicator 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was prepared in *N,N*-dimethylformamide, and 60 μl was spread onto the surface of plate media when necessary. Antibiotics were used at the following concentrations: 100 μg of ampicillin per ml, 25 μg of chloramphenicol per ml, 50 μg of nalidixic acid per ml, and 10 μg of gentamicin sulfate per ml. For modulating conditions, $MgSO_4$ was added to media at a final concentration of 40 mM.

DNA manipulation and sequence analysis. Restriction enzymes, T4 DNA ligase, calf intestinal alkaline phosphatase, and Klenow fragment were purchased from Boehringer Mannheim. Enzymes were used according to the instructions provided by the supplier. DNA sequencing was performed as described previously (10). The frameshift mutation in pDD100BC was constructed by digestion of pDD100 with *Bgl*II, followed by filling in the recessed termini with Klenow reagent and deoxynucleoside triphosphates (24). The vector was then religated, resulting in the addition of 4 bp.

Construction of a *B. pertussis* genomic library. A *B. pertussis* genomic library constructed in pUC18, which had been used in previous studies, was used in these investigations (1, 20). In brief, genomic DNA was isolated from the virulent clinical isolate BP504 by standard procedures (24). The genomic DNA was

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli		
DH5α	α-Complementation; high-efficiency transformation	Bethesda Research Laboratories
SM10	RP4-2 Tc::Mu; conjugation strain	41
JFMC3	λ lysogen containing *fhaB*::*lacZYA* transcriptional fusion; Km^R	31
JFME3	λ lysogen containing *ptxA*::*lacZYA* transcriptional fusion; Km^R	31
B. pertussis		
BP504	Virulent clinical isolate	Food and Drug Administration
BP338	Derivative of Tohama I; Nal^R	49
BP347	Avirulent derivative of BP338; *bvgS*::Tn*5*	49
BC75	Derivative of Tohama I; *zzz*::Tn5tac1 Nal^R	7
BC75C	BC75::pCC201 Gm^R	This study
B. bronchiseptica B205	Wild type	A. Bertschinger[a]
B. parapertussis 231	Wild type	P. Askeloff[b]
B. avium GOBL124	Wild type	C. R. Gentry-Weeks[c]
Plasmids		
pUC18	Ap^R cloning vector; ColE1 origin	52
pDD100	pUC18 with a 2.8-kb *Sau*3A BP504 DNA insert containing *baf*	This study
pDD101	pDD100 derivative lacking the 1.2-kb *Sma*I fragment	This study
pDD102	pDD100 derivative lacking the 1.7-kb *Pst*I fragment	This study
pDD103	pDD100 derivative lacking the 2.1-kb *Bgl*II-*Hin*dIII fragment	This study
pDD104	pDD100 derivative lacking the 0.7-kb *Bgl*II-*Eco*RI fragment	This study
pDD100BC	pDD100 derivative containing a 4-bp insertion at the *Bgl*II site	This study
pDD100R	pUC18 containing the pDD100 insert DNA cloned in the opposite orientation	This study
pHC79	Ap^R Tc^R cosmid cloning vector	17
pDD200	pHC79 with a 40-kb *Sau*3A BP504 DNA insert containing *baf*	This study
pDD201	pUC18 with a 6.5-kb *Sal*I DNA insert from pDD200 containing *baf*	This study
pDD201R	pUC18 containing the pDD201 insert in the opposite orientation	This study
pSS1129	*B. pertussis* suicide vector; Gm^R Ap^R	43, 46
pCC201	pSS1129 containing the 6.5-kb insert from pDD201	This study
pACYC184	Tc^R Cm^R cloning vector; p15A origin	5
pUW1004	pACYC184 with a 14.7-kb *Bam*HI insert containing *bvgAS*; Cm^R	45

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partially digested with *Sau*3A, and DNA fragments between 3 and 10 kb long were recovered from a 10 to 40% sucrose gradient (24). Chromosomal fragments were ligated into the *Bam*HI site in pUC18, and the ligation was used to transform DH5α. This *B. pertussis* pUC18 genomic library was isolated and transformed into JFME3(pUW1004) for these investigations.

β-Gal assays. Overnight *E. coli* cultures were diluted 1:100 in LB broth with appropriate antibiotics, grown at 37°C to an optical density at 600 nm of 1.0 to 2.0, and assayed for β-galactosidase (β-Gal) activity as described previously (28). Where indicated, 40 mM $MgSO_4$ or 5 mM nicotinic acid was added to the culture medium.

In vitro coupled transcription/translation assays. The *E. coli* S30 coupled transcription/translation system was purchased from Promega and was used according to the protocol provided.

Southern blot analysis. The extraction of *B. pertussis*, *Bordetella bronchiseptica*, *Bordetella parapertussis*, and *Bordetella avium* chromosomal DNA and Southern blot analysis were described previously (1).

Western blot (immunoblot) analysis. Bacterial cell lysates were electrophoresed on a 5 to 20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transblotted to nitrocellulose paper. Blots were blocked with 5% skim milk and screened with an anti-PT monoclonal antibody, IB12, that detects the S1 subunit of the toxin (13), followed by addition of horseradish peroxidase-labeled goat–anti-mouse antibody and a reaction buffer containing 4-chloro-1-naphthol and H_2O_2 (13). Band intensity was measured by densitometry scanning with the QGEL electrophoresis gel imaging and analysis system (Kendrick Laboratories, Inc., Madison, Wis.).

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been deposited in the GenBank database under the accession number U12020.

RESULTS

Identification of a plasmid conferring a β-Gal$^+$ phenotype on a *ptx-lacZ* fusion in *E. coli* containing BvgAS. Previous studies have demonstrated that the BvgAS regulatory system is both necessary and sufficient for transactivation of *fhaB* and *bvgAS* in *E. coli* (31, 32, 36–38, 45). In contrast, the BvgAS system is not sufficient for transactivation of *ptx* or *cya* in *E. coli* (14, 31, 32, 37), suggesting that an additional regulatory factor(s) is needed for their expression. In an attempt to identify this putative factor, we utilized *E. coli* JFME3 (31), a strain containing a chromosomal *ptx-lacZ* transcriptional fusion. JFME3 was transformed with pUW1004 (45), a pACYC184 derivative containing the *bvgAS* locus. JFME3(pUW1004) colonies were white on LB plates containing X-Gal, confirming that transcription of *ptx-lacZ* is not activated by *bvgAS* in *trans*. As a control, pUW1004 and pACYC184 were transformed into JFMC3 (31), a strain of *E. coli* containing a *fhaB-lacZ* transcriptional fusion. JFMC3(pUW1004) colonies were blue on LB plates containing X-Gal, while JFMC3(pACYC184) colonies were white, demonstrating that *bvgAS* is expressed from pUW1004 in *E. coli* (31, 45) (Table 2).

A *B. pertussis* genomic library constructed in pUC18 was

TABLE 2. Effect of plasmid constructs in *trans* on β-Gal production by *E. coli* strains containing *fhaB-lacZ* (JFMC3) or *ptx-lacZ* (JFME3) chromosomal transcriptional fusions

Strain (plasmid)	β-Gal units[a]	
	No $MgSO_4$	40 mM $MgSO_4$
JFMC3(pACYC184)	2.3 ± 1	1.7 ± 0.2
JFMC3(pUW1004)	79 ± 10	2 ± 0.3
JFMC3(pACYC184, pDD100)	0.2 ± 0.3	1.0 ± 0.9
JFMC3(pUW1004, pUC18)	76 ± 9.1	2.4 ± 1.5
JFMC3(pUW1004, pDD100)	64 ± 19	2.1 ± 0.4
JFME3(pUW1004, pUC18)	0.2 ± 0.2	0.7 ± 0.7
JFME3(pACYC184, pDD100)	0.2 ± 0.3	1 ± 0.9
JFME3(pUW1004, pDD100)	72 ± 12	1 ± 1.5

[a] Values are presented as Miller units and were obtained from strains grown in LB broth with the appropriate antibiotics at 37°C to an optical density at 600 nm of 1.0 to 2.0. The means ± standard deviations of three separate experiments are shown.

transformed into JFME3(pUW1004), and transformants were selected on LB plates containing chloramphenicol, ampicillin, and X-Gal. After 24 h of incubation at 37°C, six blue colonies were detected among a total of 1,050 transformants. When these colonies were reisolated on plates, only one consistently yielded blue colonies. This clone contained a recombinant plasmid, which was designated pDD100 (Fig. 1A). This plasmid was isolated and used to retransform JFME3(pUW1004), which resulted in blue colonies.

β-Gal assays were performed with JFME3 and JFMC3 containing various plasmid constructs grown in LB broth to confirm the phenotypes observed on plates. The results are presented in Table 2.

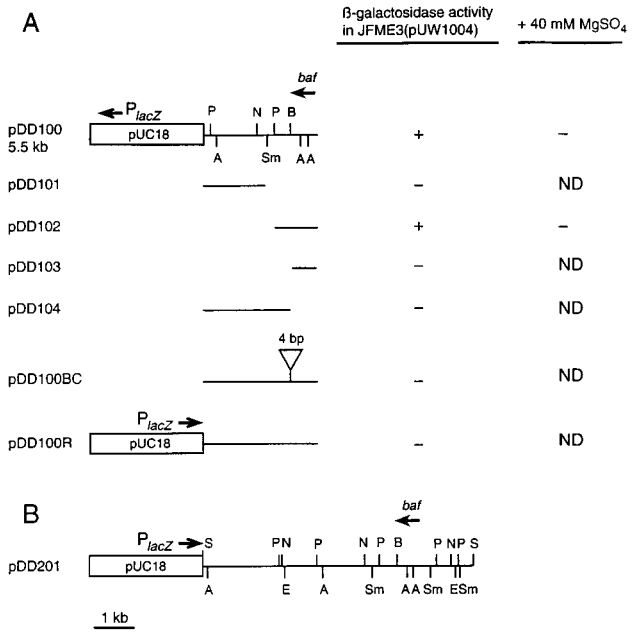

FIG. 1. Restriction maps of *baf*-containing plasmids. (A) Restriction map of pDD100 and its derivatives. (B) Restriction map of pDD201. The open box represents the vector pUC18, and the thin horizontal lines represent *B. pertussis* DNA. The arrow above pUC18 shows the direction of transcription of the *lacZ* promoter (P_{lacZ}). The location and direction of transcription of *baf* are represented by an arrow above the *B. pertussis* DNA. The insertion of 4 bp at the *Bgl*II site of pDD100, creating pDD100BC, is shown schematically with an inverted triangle above the point of insertion. ND, not determined; A, *Apa*I; B, *Bgl*II; E, *Eco*RV, N, *Not*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I.

JFME3(pUW1004, pDD100) produced a 360-fold increase in β-Gal activity compared with the controls JFME3 (pUW1004, pUC18) and JFME3(pACYC184, pDD100) (Table 2). JFMC3(pUW1004) produced a 34-fold increase in β-Gal activity compared with the control JFMC3(pACYC184). JFMC3(pDD100) produced background levels of β-Gal activity, while JFMC3(pUW1004, pDD100) produced levels of β-Gal activity comparable to those of JFMC3(pUW1004). These results demonstrate that both pUW1004 and pDD100 are required for the transcription of *ptx-lacZ* in JFME3, while only pUW1004 is required for transcription of *fhaB-lacZ* in JFMC3.

Modulation of *ptx-lacZ* expression in *E. coli* by $MgSO_4$. There is a decrease in the production of most virulence-associated traits when *B. pertussis* is grown at 25°C or at 37°C in the presence of high concentrations of sulfate ions, perchlorate ions, nicotinic acid, or benzoic acid (26, 27). This phenomenon has been termed phenotypic modulation and is mediated by the *bvgAS* locus (29, 31). The BvgAS system is not functional in the presence of these stimuli and does not activate transcription of the *B. pertussis* virulence regulon (8, 39). Table 2 shows the effect of 40 mM $MgSO_4$ on β-Gal production by JFMC3 (pUW1004) and JFME3(pUW1004, pDD100). The expression of *fhaB-lacZ* by JFMC3(pUW1004) was modulated by $MgSO_4$, which confirmed the results obtained by Miller et al. (31). The expression of *ptx-lacZ* by JFME3(pUW1004, pDD100) was also modulated by $MgSO_4$ (Table 2). Nicotinic acid, at a concentration of 5 mM, also was found to modulate expression of *ptx-lacZ* by JFME3(pUW1004, pDD100) (data not shown). This indicates that transcription of *ptx-lacZ* in *E. coli* is regulated by the same environmental stimuli that regulate virulence gene expression in *B. pertussis.*

Restriction mapping and deletion analysis of pDD100. A restriction map of the 2.8-kb DNA insert of pDD100 is shown in Fig. 1A. Deletion derivatives of pDD100 were constructed and transformed into JFME3(pUW1004). Transformants were screened for their β-Gal phenotype on LB plates with X-Gal. The gene necessary for transactivation of *ptx-lacZ* was localized to a 1.2-kb region on the right end of the pDD100 insert, as depicted in Fig. 1A (pDD102). Deletion of DNA on either side of the unique *Bgl*II site resulted in plasmids unable to transactivate *ptx-lacZ* (Fig. 1A, pDD103 and pDD104). In addition, the insertion of 4 bp at the *Bgl*II site resulted in a plasmid (pDD100BC) that was unable to activate *ptx-lacZ* expression. These results indicated that the *Bgl*II site was located either within the gene encoding the *trans*-acting factor or in an adjacent regulatory region.

Interestingly, when the orientation of the pDD100 insert DNA was reversed (pDD100R), there was no activation of *ptx-lacZ* (Fig. 1A). This suggests that the gene on pDD100 is transcribed from a vector promoter, probably the *lacZ* promoter (P_{lacZ}). Thus, it is unlikely that BvgAS is involved in the expression of the gene on pDD100. This implies that the *trans*-acting factor and BvgAS cooperate in some manner to activate *ptx-lacZ* transcription in *E. coli.*

Analysis of pDD100 and derivatives with an in vitro coupled transcription/translation system. The *E. coli* S30 coupled transcription/translation system (Promega) was utilized to analyze the gene products of pDD100 and its derivatives. The *E. coli* S30 extract lacks template DNA and methionine but contains all other components necessary for transcription and translation of cloned genes. Plasmid DNA and [^{35}S]methionine were added to the S30 extract, and the products were analyzed by SDS-PAGE (Fig. 2). As expected, the β-lactamase gene prod-

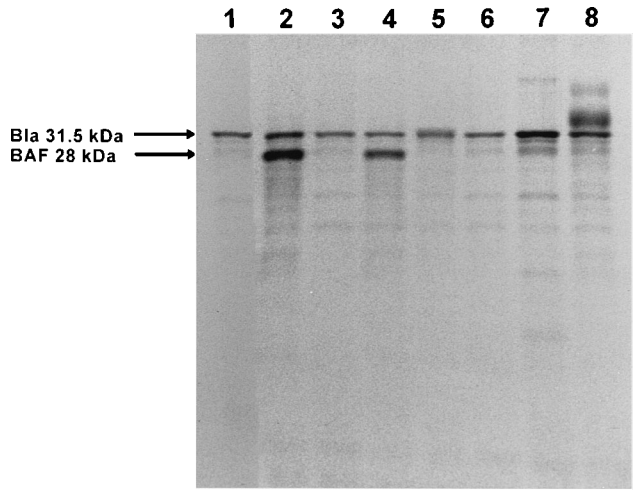

FIG. 2. Autoradiograph of the ^{35}S-labeled gene products synthesized in vitro from pDD100 and its derivatives. Lanes: 1, pUC18; 2, pDD100; 3, pDD101; 4, pDD102; 5, pDD103; 6, pDD104; 7, pDD100R; 8, pDD100BC.

uct (Bla) was present in all of the assays performed (Fig. 2, lanes 1 to 8). A unique 28-kDa protein was produced when pDD100 (Fig. 2, lane 2) or pDD102 (Fig. 2, lane 4) was added to the *E. coli* S30 extract but was not produced by the other pDD100 derivatives. Thus, the production of a 28-kDa protein correlates with the transactivation of *ptx-lacZ* in JFME3 (pUW1004) (Fig. 1A and 2). When pDD100BC was added to the extract, a protein with an apparent molecular mass of 34 kDa was produced (Fig. 2, lane 8). The protein band immediately below Bla in Fig. 2, lane 7, was present as background in all samples but was accentuated in this sample because of overloading. In addition, this protein migrates more slowly on SDS-PAGE gels than the 28-kDa protein present in Fig. 2, lanes 2 and 4.

Nucleotide sequence analysis of *baf*. The nucleotide sequence of the pDD102 insert revealed an open reading frame (ORF) situated 17 bp away from the pUC18 polylinker, immediately downstream of P_{lacZ} (Fig. 1A and 3). This 804-bp ORF is capable of encoding a 267-amino-acid (28-kDa) protein. The unique *Bgl*II site of pDD100 is present within this ORF at nucleotide position 654 (Fig. 3). The addition of 4 bp at this *Bgl*II site (pDD100BC) causes a frameshift mutation in this ORF. Sequence analysis suggests that this addition would result in the production of a protein with a size of at least 40 kDa, yet, SDS-PAGE analysis of the *E. coli* S30 extract of pDD100BC detected a protein product with an apparent molecular mass of 34 kDa (Fig. 2, lane 8). The reason for the differences in the observed and predicted protein size is presently not known.

Taken together, the data presented strongly suggest that the 804-bp ORF encodes a 28-kDa accessory factor necessary for BvgAS-dependent transcription of *ptx-lacZ* in *E. coli*. We propose to call the gene encoding this regulatory factor "*baf*," for Bvg accessory factor.

The G + C content of *baf* is 74%, which is higher than those of all other previously characterized *B. pertussis* genes. The majority of *B. pertussis* genes in the GenBank database (release 82.0) have G + C content of 62 to 68%. Two notable exceptions are *prn* (6) and *aroA* (25), which have 71% G + C content.

There were no identifiable ribosome binding sites upstream of *baf*, but the sequence 5′TCCGG3′ was found 9 bp upstream

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BamHI
GGATCCCGCCCCAAGCATGATTATCCTCATCGACTCCGGCAACAGCCGCC 50
              M  I  I  L  I  D  S  G  N  S  R

TCAAAGTCGGCTGGTTTGACCCGGACGCGCCGCAGGCGGCGCGCGAGCCC 100
L  K  V  G  W  F  D  P  D  A  P  Q  A  A  R  E  P

GCCCCCGTCGCCTTCGACAATCTCGACCTGGACGCGCTGGGCCGCTGGCT 150
 A  P  V  A  F  D  N  L  D  L  D  A  L  G  R  W  L

GGCCACCCTGCCCAGGCGCCCGCAACGGGCGCTGGGCGTGAACGTCGCCG 200
  A  T  L  P  R  R  P  Q  R  A  L  G  V  N  V  A

GGCTTGCCCGCGGCGAAGCCATTGCCGCCACGCTGCGCGCGGGCGGTTGC 250
G  L  A  R  G  E  A  I  A  A  T  L  R  A  G  G  C
          ApaI
GACATCCGGTGGCTGCGGGCCCAGCCCCTGGCCATGGGGCTGCGCAACGG 300
 D  I  R  W  L  R  A  Q  P  L  A  M  G  L  R  N  G

CTATCGCAATCCCGACCAACTGGGCGCCGACCGCTGGGCGTGCATGGTGG 350
  Y  R  N  P  D  Q  L  G  A  D  R  W  A  C  M  V

GCGTGCTGGCGCGCCAGCCGTCCGTGCACCCGCCGCTGCTGGTGGCCAGT 400
G  V  L  A  R  Q  P  S  V  H  P  P  L  L  V  A  S
                         ApaI
TTCGGCACGGCCACCACGCTGGACACCATCGGGCCCGACAATGTCTTTCC 450
 F  G  T  A  T  T  L  D  T  I  G  P  D  N  V  F  P

CGGCGGGCTGATCCTGCCCGGCCCCGCCATGATGCGCGGCGCGCTGGCCT 500
  G  G  L  I  L  P  G  P  A  M  M  R  G  A  L  A

ACGGCACCGCCCACCTGCCCCTGGCCGACGGCCTGGTGGCCGACTACCCC 550
Y  G  T  A  H  L  P  L  A  D  G  L  V  A  D  Y  P

ATCGACACCCATCAGGCCATCGCCAGCGGCATCGCCGCCGCCCAGGCCGG 600
 I  D  T  H  Q  A  I  A  S  G  I  A  A  A  Q  A  G

CGCGATCGTGCGGCAATGGCTGGCCGGCCGCCAACGCTACGGCCAGGCGC 650
  A  I  V  R  Q  W  L  A  G  R  Q  R  Y  G  Q  A
     BglII
CGGAGATCTATGTCGCCGGCGGCGGGTGGCCCGAAGTGCGGCAGGAAGCC 700
P  E  I  Y  V  A  G  G  G  W  P  E  V  R  Q  E  A

GAGCGCCTGCTGGCGGTCACCGGCGCCGCCTTCGGCGCCACGCCGCAGCC 750
 E  R  L  L  A  V  T  G  A  A  F  G  A  T  P  Q  P

CACTTACCTCGACAGCCCCGTGCTCGACGGCCTGGCGGCGCTCGCCGCGC 800
  T  Y  L  D  S  P  V  L  D  G  L  A  A  L  A  A

AAGGCGCGCCAACGGCCTGACCCCCCACACCCGGCGGATTTCCCATGCGC 850
Q  G  A  P  T  A  *                        M  R

ATCCTGTTCCTCCTGATCGTCGCGGCCAACCTGTGGGTCTATGCGCTGGG 900
 I  L  F  L  L  I  V  A  A  N  L  W  V  Y  A  L  G

CCAGGGCTGGCTGGGCCTGCGTCCCGCCGACGAAGGACGCGACGCCGCTC 950
  Q  G  W  L  G  L  R  P  A  D  E  G  R  D  A  A

GCCTGAACCAGGAAATGAAGGCCGACCAGGTCAAGGTGCTGCGGCCCTGA 1000
R  L  N  Q  E  M  K  A  D  Q  V  K  V  L  R  P  *

CGAACCGCACGGCGGGCTATCCCAGCCAATCCTCCAGCGCGTCCAGCGTG 1050

CGCCGCGTGGCGCCGGCATGCGCGGCGGTCCAGGCGCGCGCGGCCTCGCT 1100
                              PstI
CATGGCCTGCCGGCGTGCCGGCTCGGCCAGCAGCTGCAG
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FIG. 3. Annotated nucleotide sequence of *baf*. The deduced amino acid sequence is shown beneath the nucleotide sequence in the one-letter code. Relevant restriction endonuclease recognition sites are indicated above the sequence. The putative translational regulatory sequence 5′TCCGG3′ is shaded. The *Bam*HI site at position 1 was generated by ligation of the 2.8-kb *Sau*3A *B. pertussis* insert DNA into *Bam*HI-digested pUC18 during genomic library construction. A 156-bp ORF starting at the ATG at position 845 is also shown. *, Opal stop codon.

of the ATG start codon (Fig. 3). This sequence is found 8 to 12 nucleotides upstream of the genes encoding the PT subunits S2 to S5 (23, 33). Only the first gene of the PT operon, encoding subunit S1, contains a Shine-Dalgarno sequence (24, 33, 34). Nicosia et al. (33) proposed that the sequence 5′TCC(T)GG3′ might be involved in the regulation of translation of the down-

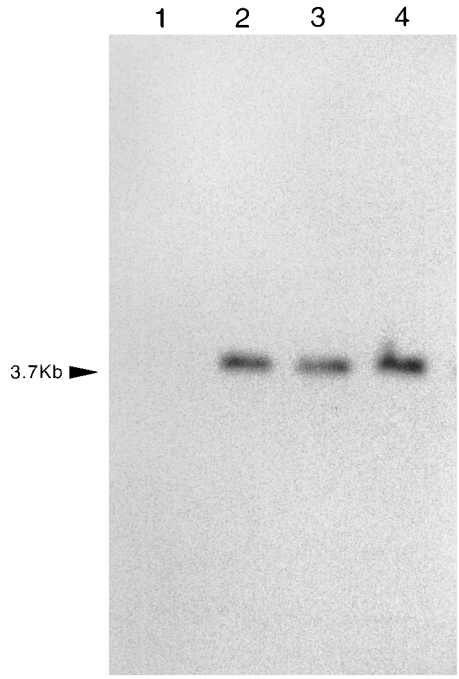

FIG. 4. Southern blot analysis of *Bordetella* chromosomal DNA. The 650-bp *Bam*HI-*Bgl*II fragment from pDD100 was hybridized to *Bgl*II-digested chromosomal DNA. Lanes: 1, *B. avium* GOBL124; 2, *B. bronchiseptica* B205; 3, *B. parapertussis* 231; 4, *B. pertussis* BP338.

stream genes in the PT operon. This sequence may also be involved in the translation of *baf*. There were no potential rho-independent transcriptional terminators in the nucleotide sequence downstream of *baf*, but there was a small (156-bp) ORF beginning 25 nucleotides downstream of *baf* (Fig. 3). The significance of this ORF is presently unknown.

The DNA sequence and deduced amino acid sequence of *baf* were compared with the sequences held in the GenBank database (release 82.0), and no significant homology to any known bacterial transcriptional regulator was found. However, homology (27.6% identity, 49.3% similarity) was found to a hypothetical 26.2-kDa protein (YacB), of unknown function, encoded by an ORF in the *ftsh-cysk* intergenic region of *Bacillus subtilis* (35). In addition, the Baf amino acid sequence was analyzed with the GCG sequence analysis software package (Genetics Computer Group, Madison, Wis.) available through the University of Arizona Biotechnology computer facilities. No protein motifs were identified with the programs MOTIFS and PROFILESCAN. Thus, Baf may represent a new class of bacterial transcriptional regulator.

Southern blot analysis of *Bordetella* spp. The 650-bp *Bam*HI-*Bgl*II fragment from pDD100, which contains most of *baf* (Fig. 3), was used as a probe in Southern blot analysis to determine if sequences homologous to *baf* are present in *B. avium*, *B. bronchiseptica*, and *B. parapertussis*. The results presented in Fig. 4 demonstrate that *B. bronchiseptica* and *B. parapertussis* contain sequences homologous to *B. pertussis baf* (Fig. 4, lanes 2 to 4). The *baf* probe hybridized to a 3.7-kb *Bgl*II fragment in all three species. On the other hand, a *baf* homolog was not detected in *B. avium* (Fig. 4, lane 1).

Cosmid subclones of *baf*, containing additional flanking DNA, do not confer a β-Gal$^+$ phenotype on *E. coli* JFME3 (pUW1004). For the *baf* integration studies described below, a clone containing additional flanking DNA was obtained. A

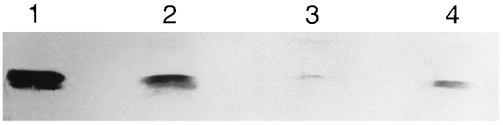

FIG. 5. Western blot of whole-cell bacterial lysates. Lanes 2 to 4 contain lysates from BP338, BC75, and BC75C, respectively. Whole-cell lysates (62 μg of protein) were analyzed by immunoblotting with the anti-PT monoclonal antibody IB12. Lane 1 contains a purified PT preparation.

cosmid clone containing *baf* (pDD200) was isolated from a *B. pertussis* genomic library constructed in pHC79. A 6.5-kb *Sal*I fragment containing *baf* was subcloned into pUC18, yielding pDD201 (Fig. 1B). This plasmid contained 1.1 kb of DNA upstream and 4.5 kb of DNA downstream of *baf*. The 6.5-kb DNA insert of pDD201 (Fig. 1B) was also cloned into pUC18 in the opposite orientation, creating pDD201R. JFME3 (pUW1004) was transformed with pDD201, pDD201R, pUC18, and pDD100, and transformants were screened for their β-Gal phenotype on LB plates with X-Gal. Only JFME3 (pUW1004, pDD100) colonies were blue, suggesting that *baf* is not expressed from pDD201 or pDD201R in *E. coli.*

Integration of *baf* into the chromosome partially complements PT production by *B. pertussis* BC75. Carbonetti et al. (4) characterized a mutant strain of *B. pertussis* (BC75) that produced low levels of PT and adenylate cyclase toxin but exhibited wild-type production of filamentous hemagglutinin, fimbriae, and pertactin. The phenotype of this mutant was due to reduced transcription of *ptx* and *cya*. Allelic exchange experiments demonstrated that the mutation in BC75 was not located in the *ptx*, *cya*, or *bvgAS* loci but was in an unknown locus (4). A BC75 derivative containing an additional copy of *baf* integrated into the chromosome was constructed to determine if this would complement the defect in PT production. The 6.5-kb pDD201 insert DNA (Fig. 1B) was cloned into the vector pSS1129 (46), creating pCC201. *E. coli* SM10 was transformed with pCC201, and conjugation between SM10 (pCC201) and BC75 was carried out as described previously (43). Cointegrates (BC75::pCC201) were selected on Bordet-Gengou plates containing gentamicin sulfate and nalidixic acid. Several colonies grew up, and one, BC75C, was selected for further analysis.

Western blot analysis was performed with the anti-PT monoclonal antibody IB12 (13) to determine the level of PT production by BP338, BC75, and BC75C (Fig. 5). As described previously (4), the mutant strain BC75 produced relatively little PT compared with the wild-type strain, BP338 (Fig. 5, lanes 2 and 3). However, the cointegrate strain, BC75C, exhibited a 2.8-fold increase in the amount of PT that was produced compared with BC75, as determined by densitometry analysis (Fig. 5, lane 4).

These observations were confirmed with the CHO cell cytotoxicity assays, which were performed as described by Hewlett et al. (16). The maximum dilutions of supernatant resulting in CHO cell cytotoxicity were 1:4,096, 1:3, 1:30, and 1:100 for strains BP338, BP347, BC75, and BC75C, respectively. (Results are averages from three experiments performed in duplicate.) Note that the BC75C supernatant exhibited a threefold increase in CHO cell toxicity compared with the BC75 supernatant. These results demonstrate that integration of pCC201, containing *baf*, into the BC75 chromosome partially complements the defect in PT production.

DISCUSSION

The data in this report clearly demonstrate that BvgAS and Baf are required in *trans* to activate the transcription of a chromosomal *ptx-lacZ* fusion in *E. coli*. The expression of *ptx-lacZ* is responsive to the same environmental stimuli that modulate *ptx* expression in *B. pertussis*. The *baf*-containing plasmid pDD100 was identified by its ability to confer a β-Gal$^+$ phenotype on JFME3(pUW1004). However, when the orientation of the pDD100 insert DNA was reversed, the resulting plasmid (pDD100R) did not confer a β-Gal$^+$ phenotype. This result indicated that *baf* was expressed from the *lacZ* promoter of pUC18 and that BvgAS alone was not sufficient for *baf* transcription from pDD100. Nonetheless, pDD100 was unable to activate *ptx-lacZ* in JFME3 without pUW1004 in *trans*. These results imply that BvgAS and Baf must cooperate to activate the transcription of *ptx* in *E. coli*. Yet, the addition of *baf* to JFMC3, which contained pUW1004 in *trans*, did not increase transcription of *fhaB-lacZ*. This demonstrates that Baf is specifically involved in the expression of the *ptx* gene.

Other investigators have tried to isolate genomic clones containing *baf* but have been unsuccessful (4, 32). Miller et al. (32) constructed a *B. pertussis* genomic library in the cosmid pHC79, infected JFME35(pUW1004), and plated the transfectants on selective media containing X-Gal. No blue colonies were detected among greater than 10,000 transfectants. Miller et al. suggested that a single member of the cosmid library was not sufficient for BvgAS-dependent transcription of *ptx* in *E. coli*. These results, along with the data presented in this study, are consistent with the hypothesis that *baf* expression in *E. coli* requires an additional factor(s) that is not closely linked to the *baf* locus. This would explain why Miller et al. (32) were unable to identify a single cosmid clone capable of activating *ptx-lacZ* in JFME35(pUW1004). As mentioned above, pDD100 contained a *lacZ-baf* transcriptional fusion. Thus, the transcription of *baf* occurs from the *lacZ* promoter of pUC18. This is most likely the reason we were able to identify a *baf*-containing clone while others were unsuccessful.

It is also interesting that *baf* was not expressed in *E. coli* from plasmids pDD201 and pDD201R. The plasmid pDD201R contains 1.1 kb of DNA between *baf* and the upstream P_{lacZ}, whereas there are fewer than 100 bp separating these elements on pDD100. There are several potential reasons why *baf* is not expressed from pDD201R. A transcriptional terminator may be present in the 1.1 kb between *baf* and P_{lacZ} on pDD201R, resulting in little or no *baf* expression. In addition, the native *baf* promoter may not be recognized by the *E. coli* transcriptional machinery or may not be present on pDD201R.

BC75, a *B. pertussis* strain that exhibits decreased *ptx* transcription (4), was partially complemented by the integration of a 6.5-kb *baf*-containing fragment from pDD201 into the chromosome. While these studies were in progress, it was reported that BC75 contains a point mutation upstream of *rpoA*, the gene encoding the α subunit of RNA polymerase (3, 44). This mutation results in the overproduction of the α subunit of RNA polymerase in BC75 (3). The defect in *ptx* expression was fully reversed by returning wild-type *rpoA* to the BC75 chromosome via allelic exchange. Thus, the partial complementation of the defect in BC75 appears to be the consequence of an additional copy of the wild-type *baf* gene. Since the *E. coli* RNA polymerase α subunit interacts with a variety of transcriptional activators (18), we hypothesize that the overexpression of *rpoA* in BC75 leads to the titering out of Baf, leaving an insufficient quantity to transactivate *ptx*. The fact that the presence of two copies of *baf* on the chromosome partially restores *ptx* regulation in BC75C is consistent with this hypothesis.

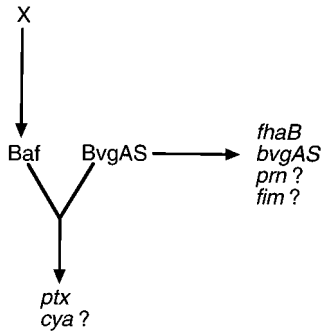

FIG. 6. Schematic model for virulence gene regulation in *B. pertussis*. Arrows indicate a positive influence on gene expression. BvgA specifically binds to the promoter regions of *fhaB* and *bvgAS* and activates transcription from these loci. Both Baf and BvgAS are needed in *trans* for transcription of *ptx* (and possibly *cya*). The BvgAS system is necessary for activation of *prn* and *fim* in *B. pertussis*, but it is not presently known if *fim* and *prn* require an accessory factor for expression or if activation by Bvg is direct. The requirement of an additional factor (X) in the production of Baf is implied from the results presented here and in other studies.

Thus, the optimal transcription of *ptx* in *B. pertussis* probably requires a precise ratio of protein factors (BvgAS, Baf, and RNA polymerase).

In preliminary studies, Western blot analysis and adenylate cyclase enzyme assay results suggest that BC75C has increased levels of adenylate cyclase toxin. However, colonies of BC75C were nonhemolytic on Bordet-Gengou plates (12). Thus, further investigations are currently under way to clarify whether Baf positively regulates *cya*. If it does, one would predict that a mutation in *baf* would result in a phenotype similar to that of BC75 (i.e., decreased expression of *ptx* and *cya* but normal expression of factors such as *bvgAS* and *fhaB*). We have used gene replacement techniques in this laboratory to construct *sodB* (9), *katA* (11), and *btr* (1) mutants of *B. pertussis*. In initial experiments, we have been unable to construct a *baf* mutant by gene replacement. It is interesting that no such mutants have been identified in studies utilizing transposon mutagenesis of the *B. pertussis* chromosome (7, 19, 49, 50). Stibitz recently used chemical mutagenesis in an effort to identify a putative Bvg accessory factor (42). While no accessory factor mutants were identified, two mutants were found that exhibited wild-type *fhaB* expression but decreased *ptx* and *cya* expression. Both mutations mapped to the C terminus of BvgA. Stibitz suggested that *ptx* and *cya* expression may require the interaction of an accessory factor with the C terminus of BvgA (42). This proposed factor may be Baf.

Taken together, the information presented above suggests that a *baf* mutation may be lethal. Thus, in addition to *ptx*, Baf may regulate the expression of some essential *B. pertussis* gene(s) or may itself be an essential protein. Another possibility is that our attempts to construct a *baf* mutant have caused polar effects leading to inactivation of multiple downstream genes and therefore loss of cell viability. Further studies are in progress to address this issue.

A schematic model of *B. pertussis* virulence gene regulation is presented in Fig. 6. The regulation of *fhaB* and *bvgAS* by the BvgAS regulatory system is direct (32, 36, 38). BvgA activated by BvgS binds to specific regulatory sequences upstream of these genes and positively influences transcription by RNA polymerase. It is currently unknown if *fim* and *prn* are directly activated by the BvgAS system or if their activation requires an accessory factor such as Baf. The transcription of *ptx* (and possibly *cya*) requires interaction between both Baf and BvgAS.

Baf may interact with BvgA only, BvgS only, or both BvgA and BvgS. The data presented here suggest that Baf also interacts with the α subunit of RNA polymerase. The exact nature of these interactions is presently unknown. The production of Baf may require an additional factor(s), represented by X in Fig. 6.

ACKNOWLEDGMENTS

We thank Jeff F. Miller for helpful discussions and for supplying plasmids and strains used in this study. We also thank William E. Goldman for *B. pertussis* BC75.

This work was partially supported by a grant from the University of Arizona Small Grants Program to R.L.F.

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