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Effect of Mutations Causing Overexpression of RNA Polymerase α Subunit on Regulation of Virulence Factors in *Bordetella pertussis*

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In *Bordetella pertussis*, expression of virulence factors is controlled by the Bvg proteins, which comprise a sensor-regulator two-component signal transduction system. Previously, we described a mutant strain of *B. pertussis* that had reduced transcription of pertussis toxin and adenylate cyclase toxin genes, while other virulence factors were relatively unaffected. We obtained a *B. pertussis* clone that repaired the defect in both this strain and an independent mutant strain with a similar phenotype when introduced onto the chromosome by allelic exchange. Further analysis revealed that the mutations were just upstream of the translational start site of the *rpoA* gene encoding the α subunit of RNA polymerase. We confirmed that these mutations were responsible for the mutant phenotype by site-directed mutagenesis. Our hypothesis that these mutations cause an overexpression of *rpoA* was confirmed by Western immunoblotting and translational fusion analysis. Corroboration of this effect was obtained by overexpressing *rpoA* on a plasmid in wild-type *B. pertussis*, which caused the same phenotype as the mutants showed. Conclusions in regard to the identity of the transcription activator of the toxin genes are discussed.

Regulation of expression of virulence factors in response to environmental stimuli is under the control of two-component signal transduction systems in a number of bacterial pathogens (15). In *Bordetella pertussis*, the agent of the disease whooping cough, the Bvg proteins carry this responsibility. BvgS is a transmembrane sensor protein with environmentally dependent histidine autokinase activity (40, 41), while BvgA is a cytoplasmic response regulator that binds specific DNA sequences and activates transcription of virulence factor genes (29, 30). Phosphorelay from BvgS to BvgA is crucial for transcription activation within this regulon (41). The virulence factors whose expression is regulated by the Bvg system include adhesins such as filamentous hemagglutinin, pertactin (PRN), and fimbriae (FIM) and toxins such as pertussis toxin (PTX) and adenylate cyclase/hemolysin toxin (42). However, while BvgA has been shown to bind upstream from and to activate the promoters of the *bvg* and *fha* genes, the same is not true for the *ptx* and *cya* genes (14, 16, 29, 30). Furthermore, a time course induction assay revealed that *fha* and *bvg* transcripts appear soon (10 min) after an inducing signal, whereas *ptx* and *cya* transcripts are detected much later (after 4 h) (32). Thus, there seem to be at least two subclasses of promoters within the *bvg* regulon. Two alternative hypotheses to explain these differences are the following. (i) BvgA is the universal activator, but different affinities of BvgA for the binding sequences upstream from promoters determine the different subclasses; and (ii) an additional factor(s) is involved in activation of the toxin gene promoters, possibly an intermediate activator forming part of a cascade of regulatory molecules. At present there is little circumstantial evidence and no direct evidence in support of either one of these possibilities.

Previously, we described a mutant strain of *B. pertussis*, BC75, in which transcription of the *ptx* and *cya* genes is reduced, while expression of other *bvg*-regulated genes is normal (8). The phenotype of this strain strongly suggested that it was missing or defective in a factor that binds specific sequences upstream of the toxin gene promoters and activates transcription from these promoters. In this study, we further analyzed BC75 and another mutant strain, RPV3, a spontaneous phase variant of *B. pertussis* with a phenotype very similar to that of BC75. Our results led to the discovery that the mutations in these strains map to the *rpoA* gene encoding the α subunit of RNA polymerase and that the mutations cause an overexpression of α that results in down-regulation of the toxin gene promoters.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *B. pertussis* strains were grown on Bordet-Gengou agar plates supplemented with 20% sheep blood (5) or in Stainer-Scholte liquid medium (37). When necessary, antibiotics were added to the following final concentrations: streptomycin, 400 μg/ml; nalidixic acid, 20 μg/ml; gentamicin, 10 μg/ml; tetracycline, 12.5 μg/ml; chloramphenicol, 20 μg/ml; and kanamycin, 50 μg/ml. Other supplements were added as described in Results. Bacterial conjugations were performed as previously described (18), by using *Escherichia coli* S17.1 or SM10 as the donor (35).

DNA manipulations. All DNA manipulations were carried out by standard methods (31). Routine cloning and subcloning was done in *E. coli* DH5α with pBluescript vectors. Constructs were introduced into *B. pertussis* either by using the broad-host-range vector pLAFR2 (11) or pMMB206 (27) or by allelic

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant feature(s)	Source or reference(s)
Strains		
E. coli K-12		
DH5α	High-efficiency transformation	GIBCO BRL
SM10	Tra functions of IncP plasmid integrated into chromosome, kanamycin resistant	35
S17.1	Tra functions of IncP plasmid integrated into chromosome, kanamycin sensitive, r_K^- m_K^+	35
B. pertussis		
Tohama I	Wild type	24
BC75	Mutant derivative of Tohama I, reduced expression of *ptx* and *cya* genes	8, 10
RPV3[a]	Spontaneous mutant derivative of Tohama I, reduced expression of *ptx* and *cya* genes	Rino Rappuoli
BP347	Derivative of Tohama I, *bvgS*::Tn*5*	42
BP359	Derivative of Tohama I, *bvgA*::Tn*5*	42
B. bronchiseptica		
BB7865	Wild type	2
Plasmids		
pBluescript	Cloning vector	Stratagene
pSS1129	Mobilizable suicide vector	40
pLAFR2	Broad-host-range, mobilizable cosmid cloning vector	11
pMMB206	Broad-host-range, mobilizable cloning vector	27
pNM482	Translational *lac* fusion vector	26
pMAL-c2	Contains adjacent *lacI*^{q} gene and *tac* promoter	New England Biolabs
pNMD99	pBluescript containing 1.7-kb insert from Tohama I α operon	This study
pNMD100	pBluescript containing 7-kb *Bam*HI fragment with Tohama I α operon	This study
pNMD101	pSS1129 containing 7-kb *Bam*HI insert from pNMD100	This study
pNMD120	pLAFR2 containing the *rpoA* gene under the control of a *tac* promoter and a *lacI*^{q} gene	This study
pT*lac*	pMMB206 containing *rpoA*::*lacZ* translational fusion with wild-type *rpoA*	This study
pR*lac*	pMMB206 containing *rpoA*::*lacZ* translational fusion with RPV3 *rpoA*	This study
pB*lac*	pMMB206 containing *rpoA*::*lacZ* translational fusion with BC75 *rpoA*	This study

[a] Previously called TIII (16).

exchange, using the mobilizable suicide vector pSS1129 (40). Site-directed mutagenesis was performed by a modified version of the PCR megaprimer method as described previously (1). Preparation of *B. pertussis* DNA in agarose plugs and pulsed-field gel electrophoresis were performed as previously described (39). DNA sequencing was performed with the Sequenase kit (United States Biochemical, Cleveland, Ohio) or by cycle sequencing on a model 373A automated sequencer (Applied Biosystems Inc., Foster City, Calif.). Sequence analysis was performed with the University of Wisconsin Genetics Computer Group programs.

Plasmid constructions. For construction of pNMD120, we used PCR to amplify a fragment from pMAL-c2 (New England Biolabs, Beverly, Mass.) containing the *lacI*^{q} gene and the *tac* promoter (bp 1 to 1479), with *Cla*I and *Xba*I sites incorporated into the primers, and also a fragment from pNMD100 containing the *rpoA* gene (bp 206 to 1242; see Fig. 2), with *Xba*I and *Bam*HI sites incorporated into the primers. These fragments were then suitably digested and ligated with *Cla*I-*Bam*HI-digested pLAFR2 to produce pNMD120.

For construction of translational fusions between wild-type and mutant alleles of *rpoA* and *lacZ*, we used PCR to amplify a fragment containing part of the *rpoA* coding region from the start to the *Sal*I site (see Fig. 2) and 350 bp of upstream sequence, with an *Eco*RI site incorporated into the upstream primer. This fragment was digested with *Eco*RI and *Sal*I and ligated with appropriately digested pNM482 (26), generating in-frame fusions of the *rpoA* and *lacZ* genes. From these constructs, an *Eco*RI-*Nco*I fragment which contained the complete *rpoA-lacZ* fusion and the downstream *lacY* gene was isolated. After generation of blunt ends by a filling-in reaction with Klenow enzyme, the fragments were cloned into the *Sma*I site of the broad-host-range vector pMMB206 so that the *rpoA-lacZ* fusions were under the control of the *lac* promoter present on pMMB206. These constructs were transformed into SM10 and conjugated into Tohama I with selection for chloramphenicol and streptomycin resistance. After induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), the β-galactosidase activities of the various strains were assayed as described previously (31).

Immunoblotting procedures. Whole-cell lysates were prepared by resuspending cells from Bordet-Gengou agar plates in Stainer-Scholte medium, measuring the A_{600}, making appropriate dilutions and then adding sample buffer. Trichloroacetic acid precipitates of culture supernatants were prepared by addition of sodium deoxycholate and trichloroacetic acid to final concentrations of 0.04 and 6%, respectively, followed by centrifugation and resuspension of the pelleted proteins in 1 M Tris-HCl, pH 6.8, containing 0.5 M NaOH. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer of proteins to nitrocellulose (Micron Separations Inc., Westborough, Mass.) were performed by standard methods with standard equipment as described by the manufacturer (Bio-Rad Laboratories, Melville, N.Y.). Detection was performed with the enhanced chemiluminescence system (Amersham Corp., Arlington Heights, Ill.), and band intensity was measured by laser scanning of autoradiographs. Antibodies used were monoclonal antibody (MAb) 6G7 against the PTX S1 subunit (4), MAb BPH2 against FIM (Fim2 subunit; kindly provided by Mike Brennan), polyclonal antiserum against PRN (kindly provided by Rino Rappuoli), MAb 54G8 against GroEL of *B. pertussis* (kindly provided by Drusilla Burns), MAb 4RA2 against the *E. coli* α subunit (cross-reacts with the *B. pertussis* α subunit; kindly provided by Richard Burgess), and polyclonal antisera

against the α and β subunits of *E. coli* RNA polymerase (kindly provided by H. Heumann).

Nucleotide sequence accession number. The nucleotide sequence presented in this paper has been submitted to GenBank and EMBL under accession number Z26647.

RESULTS

Identification of a clone that repaired the mutations in BC75 and RPV3. In a previous study, we showed that the nonhemolytic mutant strain BC75 had reduced transcription of the pertussis toxin (*ptx*) and adenylate cyclase toxin/hemolysin (*cya*) genes, while expression of other virulence factors was relatively unaffected (8). This led us to the hypothesis that it contains a mutation in a novel transcription factor involved in the activation of the toxin gene promoters of *B. pertussis*. Another spontaneous mutant, RPV3, which had a very similar phenotype, was previously identified, though the reduction of toxin expression was less pronounced (with a faint hemolytic halo occasionally seen on Bordet-Gengou agar plates). The first approach to clone the factor mutated in these strains was an attempt at transcomplementation to a wild-type phenotype, by introduction of a pLAFR2 cosmid library of Tohama I DNA and screening for hemolytic colonies. However, we were repeatedly unsuccessful using this approach, and so we mapped the locations of the mutations in order to facilitate the cloning. We were able to locate the approximate positions of the mutations on the chromosomes of BC75 and RPV3 by using the Hfr mapping system developed by Stibitz, as described in the accompanying paper (38). The linkage data suggested that the mutations lay within the *Xba*H fragment, close to the *Xba*I site between fragments *Xba*H and *Xba*L4 (38, 39). Therefore, we performed pulsed-field gel electrophoresis on Tohama I DNA digested with *Xba*I and recovered the *Xba*H-*Xba*I fragments (which migrate too closely to be separated). The DNA was then further digested with *Cla*I, and fragments were cloned into pBluescript. One such clone (pNMD99; Fig. 1) originating from *Xba*H proved to be from the desired region, and it was used as a hybridization probe to clone a 7-kb *Bam*HI fragment from Tohama I DNA into pBluescript to produce plasmid pNMD100 and into pSS1129 to produce plasmid pNMD101 (Fig. 1). pNMD101 was introduced into BC75 and RPV3 by conjugation, and after allelic replacement by recombination, approximately 20% of the colonies were hemolytic. These hemolytic strains were found to secrete wild-type levels of PTX into the medium (data not shown). Thus, the insert on pNMD101 could repair the mutants and restore a wild-type phenotype, but only when allelic replacement occurred and not by transcomplementation. Consistent with this observation, a plasmid consisting of pLAFR2 with the *Bam*HI insert of pNMD101 did not complement the mutants when introduced by conjugation from S17.1. We concluded that either there was insufficient DNA on this *Bam*HI fragment (a partial operon or gene) for transcomplementation or the mutations in BC75 and RPV3 were dominant. This may have explained why our previous efforts to identify a clone on the basis of transcomplementation of the mutants had been repeatedly unsuccessful.

Analysis of pNMD100. In order to locate more precisely the region on the pNMD100 insert that could repair the mutations in BC75 and RPV3 (and thereby to determine the positions of the mutations in these strains), we mapped a number of restriction sites on the plasmid and then constructed a set of subclones in pSS1129 as shown in Fig. 1. These were each introduced into BC75 and RPV3 by conjugation from S17.1, and their ability to repair the mutations by allelic exchange was assayed. The results localized the mutations to a 1.3-kb segment of DNA between the leftmost *Sca*I site and the rightmost *Bgl*II site (Fig. 1). This region was subjected to DNA sequence analysis, and searches of sequence databases revealed that the region around the *Bgl*II site had strong homology with the *rpoA* genes of *E. coli* and *Salmonella typhimurium*, which encode the α subunit of RNA polymerase. In addition, the sequence around the *Sca*I site had homology with the *rpsD* gene of *E. coli*, which encodes the ribosomal protein S4 and is also immediately upstream of *rpoA* in *E. coli*. Therefore, this region could not contain a gene encoding a novel transcription factor specific for toxin gene activation in *B. pertussis* (despite our previous conclusions). However, a mutation in the *rpoA* gene seemed feasible because the α subunit of RNA polymerase is known to interact with a number of transcription factors and is therefore intimately involved in transcription activation at specific loci (22). On the basis of our subcloning data, the mutations mapped to a region near the start of the *rpoA* gene. We therefore amplified a segment of DNA from BC75 and RPV3 that included the start of the *rpoA* open reading frame and the upstream region, and this was cloned into pBluescript and sequenced. Single nucleotide changes were found in each of the mutants in the noncoding region upstream from the *rpoA* open reading frame as shown in Fig. 2. In BC75, the change was an A-to-G transition 9 bp upstream from the ATG codon of *rpoA*, whereas in RPV3 the change was a C-to-T transition 48 bp upstream from the ATG, within the relatively long intergenic region (212 bp versus 25 bp in *E. coli*). Cloning and sequencing of the entire *rpoA* genes and upstream regions from BC75 and RPV3 confirmed that these were the only changes in the mutants. In order to confirm that these nucleotide changes were the mutations responsible for the phenotype of BC75 and RPV3, we created the same changes by

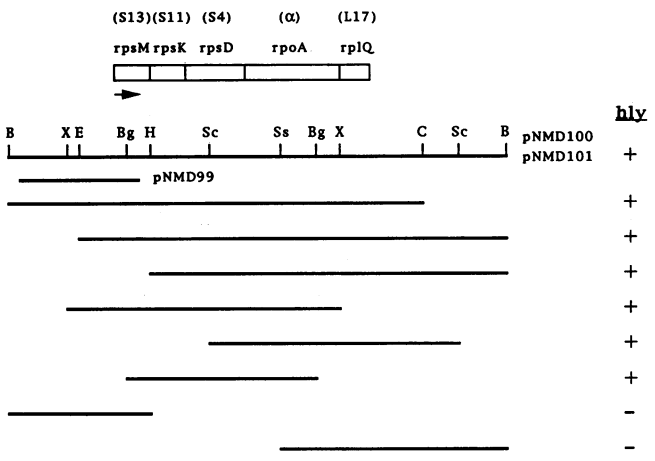

FIG. 1. Restriction map of the 7-kb *Bam*HI insert fragment of pNMD100 and pNMD101. Shown below the restriction map are the insert fragments in pNMD99 and a series of subclones used to locate the mutations in BC75 and RPV3. At the right under the heading hly is indicated the ability (+) or inability (−) of the subclone to restore a hemolytic phenotype when introduced into the mutants by allelic exchange (pNMD99 was not tested). Above the restriction map is shown the genetic organization of the central region of the pNMD100 insert fragment, with the open bars representing the genes with their respective names shown above. Above the gene names, in parentheses, are the predicted protein products of the genes, based on homologies to the equivalent genes in *E. coli*. The arrow below the *rpsM* gene indicates the direction of transcription of the operon. Restriction sites are as follows: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Sc, *Sca*I; Ss, *Ssp*I; and X, *Xho*I.

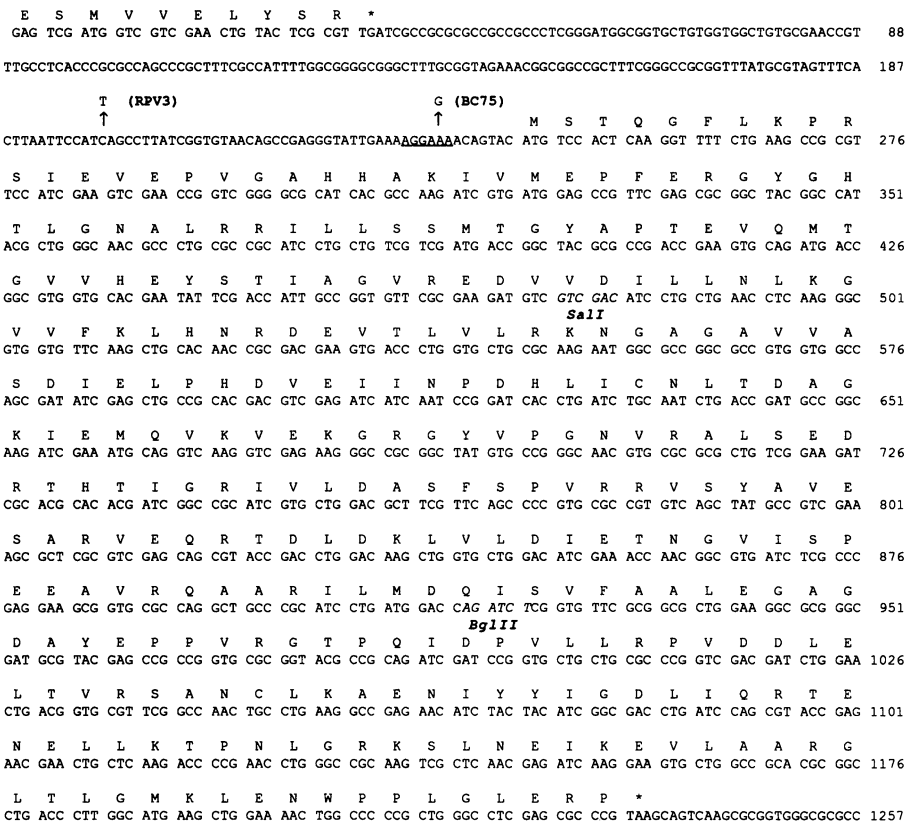

FIG. 2. Nucleotide and amino acid sequences of the *rpoA* gene of Tohama I. Numbers of nucleotides are indicated at the right. The sequence starts with the last few codons of the upstream *rpsD* gene. The arrows in the intergenic sequence before the *rpoA* gene indicate the mutations in BC75 and RPV3. The putative SD region of *rpoA* is underlined. Relevant restriction sites are shown (in italics). Stop codons are indicated by asterisks.

site-specific mutagenesis and introduced them into Tohama I by allelic exchange. Approximately 50% of the exconjugants were nonhemolytic, and when assayed by Western blotting (immunoblotting), these strains were found to secrete very low levels of PTX but to produce normal levels of PRN and FIM (data not shown), which was similar to the case with BC75 and RPV3. The region around the start of the *rpoA* gene was amplified from two hemolytic colonies and two nonhemolytic colonies from each conjugation, and sequencing of these amplified products revealed that the hemolytic colonies had the wild-type sequence in this region, whereas the nonhemolytic colonies had the appropriate mutation, i.e., that found in BC75 and RPV3. This confirmed that these mutations were responsible for the phenotype of the mutants.

The complete sequence of the *rpoA* gene and upstream region of Tohama I is shown in Fig. 2. The DNA is typically (for *B. pertussis*) GC rich (63%). The *rpoA* gene sequence has 63% identity with those of the *rpoA* genes of *E. coli* and *S. typhimurium*, and the predicted proteins have 60% identity and 91% similarity (the *B. pertussis* protein [328 amino acids] is 1 residue shorter than that of *E. coli*). Further sequence analysis of pNMD100 revealed that the genetic organization in this region in *B. pertussis* (Fig. 1) appears to be very similar to that in *E. coli* (3).

Overexpression of α in BC75 and RPV3. The position of the mutation in BC75 appeared to be in the putative Shine-Dalgarno (SD) sequence of *rpoA*, and a change of A to G altered the sequence towards the *E. coli* consensus SD sequence (AGGAGG) (13). Therefore, we predicted that the effect of this mutation was an increase in ribosome binding and a corresponding increase in translation initiation efficiency, leading to overproduction of the *rpoA* gene product. On first examination of the sequence, the mutation in RPV3 had no obvious predictable effect, but analysis of the predicted stability of RNA folding patterns in the intergenic *rpsD-rpoA* region revealed a potential effect of the mutation on a predicted hairpin structure, causing a "bubble" in the region of the SD sequence (Fig. 3). Therefore, the SD sequence may be more accessible for binding by the ribosome, and this would have the same overall effect of increasing translation initiation efficiency for *rpoA*. We tested our hypothesis that overexpression of α was the result by performing Western blotting of whole-cell lysates of wild-type and mutant strains of *B. pertussis*, using an MAb (4RA2) raised against the α subunit of *E. coli* RNA polymerase that cross-reacts with a *B. pertussis* protein of the size (36 kDa) predicted for α. Results of a typical blot are shown in Fig. 4. A slight increase in the level of α was apparent in BC75 (threefold; determined by laser densitometry) and RPV3 (twofold) compared with Tohama I or BP359 (*bvg* mutant). We attempted to use as an internal control an antibody that recognizes the β subunit of RNA polymerase (antiserum raised against the *E. coli* protein that cross-reacts with the *B. pertussis* protein), but this produced inconsistent results. We also used an MAb (54G8) specific for the GroEL-like protein of *B. pertussis* (7), and although the level of this protein appeared relatively constant between strains (Fig. 4), the relatively high level of expression did not allow for accurate comparisons. For further confirmation and quantitative assess-

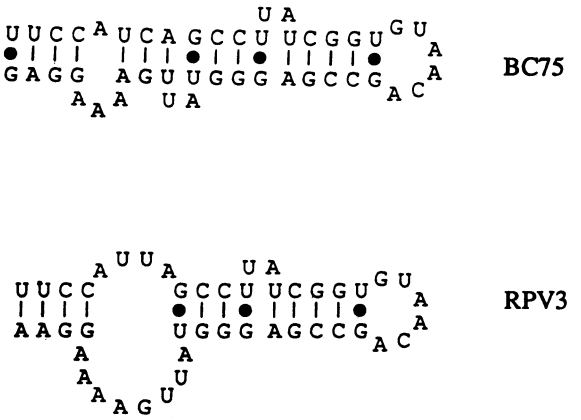

FIG. 3. Predicted folding patterns of a stem-loop structure in the mRNA in the region just upstream from the *rpoA* gene. The last nucleotide shown (lower left) is 9 nucleotides upstream from the start codon of *rpoA* (nucleotide 238 in Fig. 2), within the putative SD sequence. The structure of the equivalent region of the wild-type sequence (not shown) is identical to that of the BC75 sequence. Note the larger bubble in the stem of the RPV3 structure as a result of the mutation in that strain. Folding was predicted with the Mulfold program (23). The calculated free energies for the complete folded intergenic region that includes the structures shown here are as follows: −83.9 kcal/mol (1 cal = 4.184 J) for the wild-type sequence; −83.4 kcal/mol for the BC75 sequence; and −82.5 kcal/mol for the RPV3 sequence.

ment of the overexpression of *rpoA* in the mutants, we constructed translational fusions of the *rpoA* open reading frames of wild-type and mutant *B. pertussis* strains with the *lacZ* gene under the control of the *lac* promoter on the vector pMMB206 (Fig. 5) and measured the β-galactosidase activities of *E. coli* and *Bordetella* strains carrying these constructs. However, the fusion constructs were highly unstable in *B. pertussis* and so were transferred to the *Bordetella bronchiseptica* BB7865 strain, in which they were relatively stable. In both *E. coli* and *B. bronchiseptica*, we observed similar significant increases in β-galactosidase activity from the mutant constructs compared with that from the wild-type construct ($P < 0.005$)

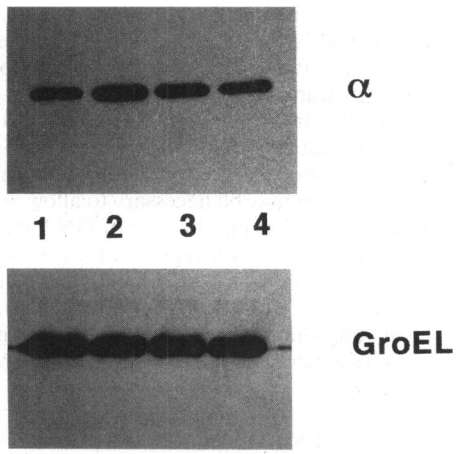

FIG. 4. Western immunoblot showing the overexpression of α in BC75 and RPV3. The top panel shows the 36-kDa α protein, and the bottom panel shows the 63-kDa GroEL-like protein of *B. pertussis*. Protein samples were whole-cell lysates of Tohama I (lane 1), BC75 (lane 2), RPV3 (lane 3), and BP359 (lane 4).

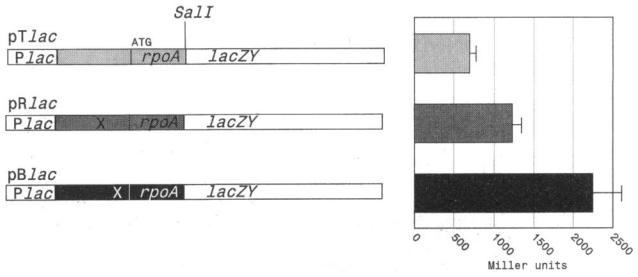

FIG. 5. Translational fusion constructs used for the determination of the translational efficiency of the *rpoA* upstream regions of BC75 and RPV3. pT*lac*, pR*lac*, and pB*lac* contain the wild-type, RPV3, and BC75 sequences, respectively. The relative positions of the mutations in the upstream untranslated regions are indicated by X in pR*lac* and pB*lac*, the *lac* promoter of pMMB206 is designated P*lac*, and the fusion site between *rpoA* and *lacZ* is marked by the *Sal*I site. The β-galactosidase activities of *Bordetella* strains carrying the various constructs are shown at the right; data are means of results from six independent experiments. Bars indicate the standard deviations ($P < 0.005$).

(Fig. 5). The construct pR*lac*, derived from RPV3, showed a 1.5-fold increase, and the BC75 construct, pB*lac*, showed a 2.8-fold increase in activity compared with the Tohama I construct pT*lac*.

Overexpression of α in Tohama I. To test the effect of excess α on *B. pertussis* directly, we reproduced the overexpression of *rpoA* in Tohama I by introducing a plasmid construct, pNMD120, on which expression of the *rpoA* gene is controlled by a *tac* promoter and the *lacI*^{q} gene product (the repressor of the *tac* promoter). Tohama I containing this plasmid was nonhemolytic and produced undetectable levels of PTX but normal levels of PRN and FIM (Fig. 6), and therefore it has the same phenotype as BC75 (8). This was true even in the absence of IPTG induction, suggesting some leakiness of repression of the promoter that allowed low-level expression of the *rpoA* gene. When repression of the *tac* promoter was relieved by growth of the strain on medium containing 1 μg (4.2 μM) or 10 μg (42 μM) of IPTG per ml, overexpression of α was easily visible by Western blotting, but expression of PRN and FIM was also substantially reduced at 10 μg of IPTG per ml (Fig. 6). These results demonstrate that a mild overexpression (2- to 3-fold) of α is responsible for reduced expression of PTX and adenylate cyclase/hemolysin toxin, but greater overexpression of α (5- to 10-fold) has an effect on other *bvg*-regulated virulence factors as well.

DISCUSSION

In this study we have shown that a modest (less-than-threefold) overexpression of the α subunit of RNA polymerase in *B. pertussis* causes significant loss of expression of PTX and adenylate cyclase toxin, while other *bvg*-regulated virulence factors are expressed normally. The overexpression of α in the mutant strains BC75 and RPV3 was (i) predicted from the nucleotide sequence changes, (ii) detected by Western immunoblotting, (iii) shown to be due to increased translation of *rpoA* by translational fusion analysis, and (iv) shown to cause the mutant phenotype by reproducing the overexpression from a plasmid construct in wild-type *B. pertussis*. The dominant nature of the mutations in BC75 and RPV3 probably explains why we were consistently unable to complement these strains by introducing libraries of wild-type DNA. However, that the defect was in a novel transcription factor specific for the toxin gene promoters, as we previously predicted (8), turned out not

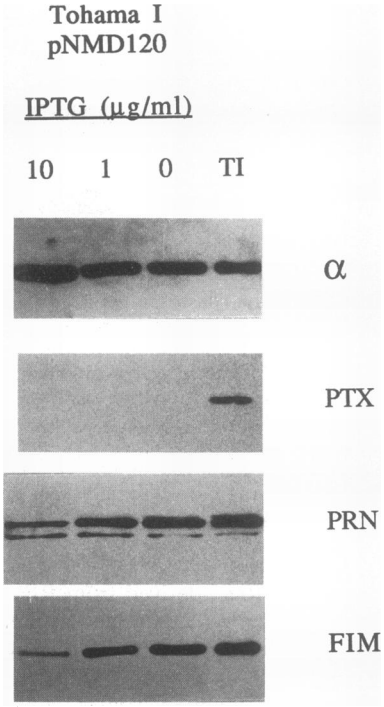

FIG. 6. Western immunoblots of whole-cell lysates (α, PRN, and FIM) or trichloroacetic acid-precipitated supernatant proteins (PTX) of Tohama I (TI) and Tohama I carrying pNMD120. Tohama I pNMD120 was grown on medium containing the indicated concentrations of IPTG (Tohama I was grown without IPTG). The proteins detected in each panel are indicated at the right. Sizes were as follows: α, 36 kDa; PTX (S1 subunit), 26 kDa; PRN, 69 kDa; and FIM (Fim2 subunit), 18 kDa.

to be the case, and the identity of the activator of these promoters remains in doubt.

The α subunit has been shown to be the portion of RNA polymerase that interacts with transcription factors at a number of positively regulated prokaryotic promoters (22). It is therefore not surprising that a regulatory mutation affecting positively activated promoters in *B. pertussis* should map to the *rpoA* gene. Whereas many other mutations in *rpoA* that cause loss of promoter activation are located in the C-terminal one-third of the gene (22), those in BC75 and RPV3 mapped instead to the upstream region and affect translation efficiency. This nature of *rpoA* mutation was suggested, though not demonstrated, in a previous study of OmpR-activated promoters in *E. coli* (36). Indeed, in a separate study, overexpression of α was shown to reduce OmpR-dependent activation of the *ompF* and *ompC* promoters (6).

Why excess α causes preferential loss of activation of the toxin gene promoters in *B. pertussis* is unclear. One possibility is that α interacts with a transcription factor specific for the toxin gene promoters and that excess α, free of RNA polymerase holoenzyme, effectively titrates out this transcription factor. This seems feasible, since α has been shown to associate, though somewhat weakly, with the *E. coli* transcription factor catabolite gene activator protein in solution free of promoter DNA (19). The existence of such a toxin promoter-specific factor (Act) in *B. pertussis* has been suggested (20) but never formally demonstrated, and repeated attempts in our laboratory and others to identify this factor by mutagenesis in *B. pertussis* or cloning in *E. coli* have failed. An alternative possibility is that the excess α titrates out the transcription activator BvgA so that the concentration of free BvgA falls below a threshold level needed to activate the toxin promoters, while remaining high enough for activation of other *bvg*-dependent promoters. This model of virulence gene activation in *B. pertussis*, in which the different affinities of the various promoter regions for BvgA and the concentration of the activator determine expression, was previously suggested by Scarlato and coworkers (32). Our observation that greater overproduction of α from pNMD120 caused reduction also of PRN and FIM expression is consistent with this model, though it does not provide compelling evidence for it. BvgA has not been shown to interact with and activate the *prn* and *fim* promoters, and it is possible that an alternative activator (that interacts with α) acts at these promoters also. BvgA has been shown to activate the *fha* promoter (30), but we did not include results showing the effect of excess α on filamentous hemagglutinin expression, since Western blots of filamentous hemagglutinin show many degradation products and therefore comparisons between strains are difficult.

A third possibility to explain the effect of excess α is that α may bind specifically to sequences in the *ptx* and *cya* promoter regions and thereby inhibit transcription. Sequence-specific binding of free α subunit has been demonstrated to occur upstream of the *rrnB* P1 promoter in *E. coli* (where, however, it stimulates transcription) (28). This highly AT-rich α binding sequence is not present upstream of the *ptx* and *cya* promoters, though a consensus α binding sequence has not been established. In addition, our previous data concerning transcription interference, according to which BC75 appeared to have lost a factor that binds upstream of the *ptx* promoter (8), are not consistent with the binding of α to this region. Instead, these data suggest that titration of the transcription factor is the most likely mechanism by which excess α interferes with toxin promoter activity. We have made preliminary attempts to counteract the excess α in BC75 and RPV3 by overexpression of BvgA in these strains, but so far this has not restored wild-type toxin promoter activity, as measured by a hemolytic phenotype (21). In addition, attempts at in vivo experiments to demonstrate a direct interaction between BvgA and α by using the Matchmaker system (Clontech, Palo Alto, Calif.) have so far been unsuccessful (12).

Evidence that BvgA, rather than a novel factor, is the transcription activator of the toxin promoters has been difficult to obtain (14, 25, 33). Activation of the toxin gene promoters may be complicated by the fact that the direct repeats that are crucial for *ptx* activation (18) and probably represent the binding site of the activator (17) are located at positions −117 to −157 from the transcription start site of *ptx*. This is considered too far upstream from the promoter to allow a direct contact of the activator bound at the repeats with the RNA polymerase (9), and so some form of topological change in the DNA in this region may be necessary to allow activation. Preliminary evidence concerning the role of DNA topology in *ptx* promoter activation has been reported (33). Looping and bending of DNA have been shown to be important in the activation of a number of prokaryotic promoters (34). We are currently investigating the *ptx* promoter and upstream region in more detail to help elucidate the mechanisms involved in its activation in *B. pertussis*.

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