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Evidence that Modulation Requires Sequences Downstream of the Promoters of Two *vir*-Repressed Genes of *Bordetella pertussis*

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Gene expression in *Bordetella pertussis* is altered by environmental signals in a process called antigenic modulation. In the presence of modulating signals, expression of several known virulence factors and outer membrane proteins is coordinately reduced. From a bank of Tn*phoA* fusions, we have identified five genes whose expression profiles are reciprocal of those of the major virulence determinants; that is, alkaline phosphatase activity is maximal during growth in the presence of the modulators nicotinic acid and $MgSO_4$ (S. Knapp and J. J. Mekalanos, J. Bacteriol. 170:5059–5066, 1988). We have called these loci *vir*-repressed genes (*vrg*). Two of these gene fusions (*vrg-6* and *vrg-18*) have been cloned in *Escherichia coli*, returned on low-copy-number plasmids to several strains of *B. pertussis*, and found to be regulated similarly to the fusions harbored on the chromosome. Deletions of the two *vrg* promoters were constructed and returned to *B. pertussis*. Regulation was maintained even when all but 24 nucleotides upstream of the *vrg-18* initiation codon and 60 nucleotides upstream of the *vrg-6* initiation codon were deleted, suggesting that *cis*-acting regulatory elements of these genes lie very near or within the coding region. We observed a 21-base palindromic sequence overlapping an 8-base direct repeat within the signal sequence coding region of *vrg-6*; insertion of a 6-bp linker in this region abolished regulation. These repetitive sequences are also at the site of greatest primary sequence identity between *vrg-6* and *vrg-18* and correspond to the signal sequence coding region. We propose models that involve recognition of this region by a *vir*-regulated gene product.

Bordetella pertussis is the causative agent of the human respiratory disease whooping cough. This gram-negative bacterium colonizes the ciliated epithelium of the upper respiratory tract and produces a wide array of virulence factors, which include pertussis toxin, filamentous hemagglutinin, and adenylate cylcase-hemolysin toxin. The production of these and other factors is coordinately regulated in response to environmental signals in a process known as antigenic modulation (13). Growth of *B. pertussis* in the presence of nicotinic acid (5 mM) or $MgSO_4$ (20 mM) or at low temperature (22 to 27°C) leads to negligible expression of the major virulence determinants (11, 13, 23), and the organism is said to be in C mode. In contrast, organisms grown in the absence of these modulators are said to be in X mode.

It is possible to isolate avirulent (phase IV) variants of *B. pertussis* that are unable to express virulence factors regardless of growth conditions (15, 22). A locus called *vir* is altered in these phase variant mutants (30, 31). This locus contains at least three genes, *bvgABC* (for *Bordetella* virulence genes) required for expression of the major virulence factors (1, 18). The *bvgA* and *bvgC* genes encode proteins homologous to the activator and sensory components of bacterial signal transduction systems, respectively (1). In *B. pertussis*, a single base insertion in *bvgC* results in phase variation (27), whereas *B. bronchiseptica* phase variants can arise by small deletions in the *vir* locus (20). Knapp and Mekalanos isolated virulent (phase I) variants constitutive for the expression of virulence factors and called these *mod* (for modulation constitutive) mutants (12). These mutations also mapped to the *vir* region, although the precise locations of the lesions are yet to be determined. Falkow and colleagues have demonstrated that antigenic modulation is also controlled by the *vir* locus. They have shown that *fhaB* expression is regulated by modulators in *Escherichia coli* when the cloned *bvg* genes are present in *trans* (19, 24, 27).

We were interested in identifying other genes under the control of the *vir* locus and antigenic modulation. Transposon Tn*phoA* (17) was used to generate fusions to genes encoding secreted proteins in *B. pertussis*; these were then analyzed for control by modulating signals (12). In this way, several genes were found to be regulated in the same manner as the major virulence factors, that is, maximally expressed in the absence of modulators. These have been designated *vir*-activated genes (*vag*) and some have been identified as the genes for previously described virulence factors (T. M. Finn, S. Knapp, R. Shahin, and J. J. Mekalanos, unpublished data). In addition, five genes expressed maximally in the presence of modulators were identified and have been called *vir*-repressed genes (*vrg*). In this paper we describe the cloning of two *vrg* genes and deletion analysis of their promoters. We have found that essentially all of the *B. pertussis* DNA upstream of the translational start site can be deleted and regulation is maintained. Insertion of a 6-bp linker in the signal sequence coding region abolishes regulation by modulators. These data suggest that *cis*-acting regulatory elements lie within the coding regions of *vir*-repressed genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* and *B. pertussis* strains and plasmids used in this study are listed in Table 1. *B. pertussis* strains were grown on Bordet-Gengou (BG) agar (Difco Laboratories, Detroit, Mich.) supplemented with 10 ml of glycerol and 250 ml of defibrinated sheep blood per liter. Modulators and antibiotics were added by spreading stock solutions onto BG agar plates and drying

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Reference
B. pertussis		
18323 derivatives		
SK6	*vrg-6*::Tn*phoA* Km^r Sm^r	12
SK18	*vrg-18*::Tn*phoA* Km^r Sm^r	12
Tohama I derivatives		
347	*vir-1*::Tn*5* Km^r Sm^r	30
348	*hly-1*::Tn*5* Km^r Sm^r	30
E. coli derivatives		
CC118	Δ(*ara leu*)*7697 lacX74 araD139 phoA20 galE galK thi rpsE rpoB argE recA1*	16
MM294	*endA hsdR pro supF*	24
Plasmids		
pRK2013	IncP1 *tra oriE1* Km^r	5
pLAFR2	IncP1 *cos* Tc^r	7
pBR322	Ap^r Tc^r	3
pUC19	Ap^r	32
pUCP1	pUC19 *vrg-6*::Tn*phoA* Ap^r	This study
pUCP1Δ1-18	Deletion series of pUCP1	This study
pSK18P	pUC19 *vrg-18*::Tn*phoA* Ap^r	This study
pSK18PΔ1-7	Deletion series of pSK18P	This study
pLM6	pLAFR2 *vrg-6*::Tn*phoA* Km^r Tc^r	12
pLSK18	pLAFR2 *vrg-18*::Tn*phoA* Km^r Sm^r	This study
pLUCP1Δ1-18	Replicon fusions of pUCP1 deletions Ap^r Tc^r	This study
pLSK18PΔ1-7	Replicon fusions of pSK18P deletions Ap^r Tc^r	This study
pUCP1Δ12S	*Sma*I linker in *vrg-6*::Tn*phoA* Ap^r	This study
pUCP1Δ18S	*Sma*I linker in *vrg-6*::Tn*phoA* Ap^r	This study
pLUCP1Δ18S	Replicon fusion of pUCP1Δ18S Ap^r Tc^r	This study

to achieve the following final concentrations: 20 mM $MgSO_4$, 5 mM nicotinic acid, and 50 μg of ampicillin, 30 μg of kanamycin, 100 μg of streptomycin, and 12.5 μg of tetracycline per ml.

Cloning of *vrg*::Tn*phoA* fusions. *Bam*HI-digested SK18 chromosomal DNA and pUC19 plasmid DNA were ligated overnight at 15°C and transformed into $CaCl_2$-competent *E. coli*. The transformations were then plated on LB agar containing ampicillin and kanamycin to select for the *bla* gene of pUC19 and the *neo* gene of Tn*phoA*. The resulting plasmid, pSK18, had an insert of approximately 6.5 kbp, including approximately 1.5 kbp of *B. pertussis* DNA. This plasmid was then digested with *Pst*I and ligated, thus eliminating 1.862 kbp of Tn*phoA* DNA, giving rise to plasmid pSK18P for use in generating promoter deletions (see below). The cloning of the *vrg-6*::Tn*phoA* fusion was reported by Knapp and Mekalanos (12). The *Bam*HI fragment containing this fusion was cloned first into plasmid pBR322, and then a 3,077-bp *Pst*I fragment containing the fusion was subcloned into plasmid pUC19. The resulting plasmid, pUCP1, had an insert of 3,077 bp, including 477 bp of *B. pertussis* DNA.

Deletions of *vrg* promoters. Nested deletions of the *vrg* promoters were made with the Erase-a-Base kit (Promega) after the method of Henikoff (10) based on exonuclease III, which will processively digest DNA from blunt or 5′-overhanging ends but not from 3′-overhanging ends. Briefly, plasmid pSK18P was digested with *Sma*I (digestable) and *Sac*I (protected), and plasmid pUCP1 was digested with *Bam*HI (digestable) and *Kpn*I (protected). Restriction endonuclease digests identified clones with deletions of DNA upstream of the *vrg*::Tn*phoA* fusion junction. Only those clones which maintained the *Eco*RI site of the pUC19 polylinker adjacent to the *Sac*I and *Kpn*I sites were chosen for further study, as these would have lost no vector sequences.

Sequencing of deletions. The parental and promoter deletion plasmids were sequenced by using the Sequenase (USB) modified DNA polymerase and the universal primer (USB) or Tn*phoA* primer (5′-AATATCGCCCTGAGCA-3′, corresponding to bases 71 to 86). The standard Sequenase protocol for incorporation of [^{35}S]dATP was used with a modification of the template-primer hybridization. Double-stranded templates were denatured by adding 40 μl of 0.2 M NaOH–0.2 mM EDTA to 5 μl of miniprep plasmid DNA, followed by incubation at 25°C for 5 min. To this was added 1 μl of primer, 4 μl of 2 M ammonium acetate (pH 4.5), and 80 μl of absolute ethanol; this mixture was vortexed, plunged into a dry ice-ethanol bath for 20 min, and spun for 10 min at 4°C, and the pellet was washed in 70% ethanol, dried, and resuspended in 10 μl of Sequenase buffer. Sequencing reactions were electrophoresed in 7% acrylamide–8 M urea gels at 60 W; gels were dried for 1 h at 75°C and autoradiographed for 12 to 48 h at 25°C.

Insertion of linker in *vrg-6*::Tn*phoA*. Deletion plasmid pUCP1Δ12 was digested with *Nae*I for 48 h at 37°C followed by phenol-chloroform extraction and ethanol precipitation. The pellet containing approximately 5 μg of DNA was resuspended in 10 μl of H_2O–19 μl of *Sma*I linker (150 pmol of phosphorylated hexamer; Pharmacia)–3.5 μl of 10× ligase buffer–2.5 μl of ligase; the ligation mixture was placed at 15°C overnight. The ligation mixture was digested with *Sma*I for 2 h to eliminate multiple linkers, followed by phenol-chloroform extraction and ethanol precipitation. The pellet was resuspended in 8 μl of H_2O–1 μl of 10× ligase buffer–1 μl of ligase, and the ligation mixture was placed at 15°C for 5 h. The ligase was inactivated by incubation at 65°C for 10 min, and the DNA was digested with *Nae*I overnight to eliminate any plasmids that had no linkers inserted at the previous step. This was used to transform *E. coli* CC118, and the transformation was plated on LB agar containing ampicillin and 5-bromo-4-chloro-3-indoyl-phosphate (Bachem); blue colonies were picked for plasmid isolation and analysis of restriction endonuclease digests. A clone containing a plasmid not digested by *Nae*I but with a unique *Sma*I site located within the linker was chosen for further study. This plasmid was called pUCP1Δ12S.

Plasmid pUCP1Δ12S was digested overnight with *Hin*cII, which cuts at two sites flanking the point of linker insertion. This approximately 450-bp fragment was purified from an agarose gel. Likewise, plasmid pUCP1Δ18 (which contains an additional 236 bp of DNA upstream of pUCP1Δ12) was also digested overnight with *Hin*cII, and the approximately 5.0-kbp fragment containing vector, Tn*phoA*, and *B. pertussis* sequences was purified. The two fragments were ligated overnight at 15°C, and this ligation mixture was used to transform *E. coli* CC118, plating on LB agar with ampicillin and 5-bromo-4-chloro-3-indoyl-phosphate. Blue colonies were picked for plasmid isolation and analysis of restriction digests. A clone containing a plasmid with the digest pattern of pUCP1Δ18 but with a new *Sma*I site provided by the linker was chosen for further study; this plasmid was called pUCP1Δ18S.

Construction of replicon fusions. Replicon fusions of deletion plasmids and pLAFR2 were made by digestion of each with *Hin*dIII for 4 h at 37°C, followed by phenol-chloroform extraction and ethanol precipitation. The pellets were each resuspended in 3.5 μl of H_2O and mixed; to each mix was added 2 μl of 5× ligase buffer and 1 μl of T4 DNA ligase, and the mixtures were incubated overnight at 15°C. The ligation mix was used to transform *E. coli* CC118, and the transformation mix was plated on LB agar containing ampicillin and tetracycline. Resulting colonies were picked for plasmid preparation and analysis of restriction endonuclease digests to identify those with equimolar amounts of pLAFR2 and deletion plasmid-derived DNA, indicating replicon fusions rather than cotransformed plasmids.

Plasmid transfer to *B. pertussis*. Derivatives of the broad-host-range plasmid pLAFR2 were transferred into strains of *B. pertussis* by triparental matings. *E. coli* strains containing the plasmid to be transferred and MM294 containing pRK2013 to provide transfer functions were grown to mid-log phase, and approximately 5×10^7 cells of each strain were added directly to a plate of *B. pertussis*. The strains were mated for 4 to 6 h at 37°C and then harvested and washed in 0.15 M NaCl. Cells were then plated on BG agar plates supplemented with the appropriate antibiotics and incubated at 35°C.

Recombinant DNA methods. Standard methods were used for purification of plasmid and chromosomal DNA, restriction enzyme digestion, agarose gel electrophoresis, band purification, and ligation (16). Restriction endonucleases and T4 DNA ligase were purchased from either New England BioLabs or Bethesda Research Laboratories.

Alkaline phosphatase activity assays. Alkaline phosphatase activity expressed by strains of *B. pertussis* was determined after 2 days of growth on BG plates at 35°C. Cells were harvested in 0.15 M NaCl, washed, and suspended in 1 M Tris hydrochloride, pH 8.0. Activity expressed by strains of *E. coli* was determined after overnight growth in LB broth at 37°C. Cells were centrifuged, resuspended, and washed in 1 M Tris hydrochloride, pH 8.0. The activity was determined as described before (17) and is expressed as units per optical density at 600 nm.

Outer membrane protein enrichment and protein electrophoresis. Whole-cell protein extracts were enriched for outer membrane proteins by the method of Hankte (9). Briefly, cells were harvested from BG plates in 0.15 M NaCl, centrifuged in a JA-20 rotor for 10 min at 10,000 rpm, washed in 0.2 M Tris hydrochloride (pH 8), transferred to Microfuge tubes, and spun in a Microfuge for 2 min at 4°C. Pellets were resuspended in 50 μl of 0.2 M Tris hydrochloride and placed on ice, and the following were added: 100 μl of 0.2 M Tris hydrochloride–1 M sucrose, 10 μl of 10 mM EDTA (pH 8.0), 10 μl of 2 mg of lysozyme (Sigma) per ml, and 320 μl of H_2O. After 10 min at 25°C, 10 μl of 1 mg of DNase I (Bethesda Research Laboratories) per ml and 500 μl of 2% Triton–10 mM $MgCl_2$–50 mM Tris hydrochloride were added and the solution was cleared by two cycles of freezing-thawing (−70°C, 10 min/37°C, 10 min). The cleared solution was spun in a Microfuge for 30 min at 4°C, and the resulting pellet was washed three times in H_2O. The washed pellet was resuspended in 100 μl of 2× loading buffer, boiled for 5 min, and spun for 5 min. The supernatant was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 15% slab gels as described previously (14). Aliquots of 25 μl of supernatant were loaded in each gel lane. Gels were stained with Coomassie brilliant blue.

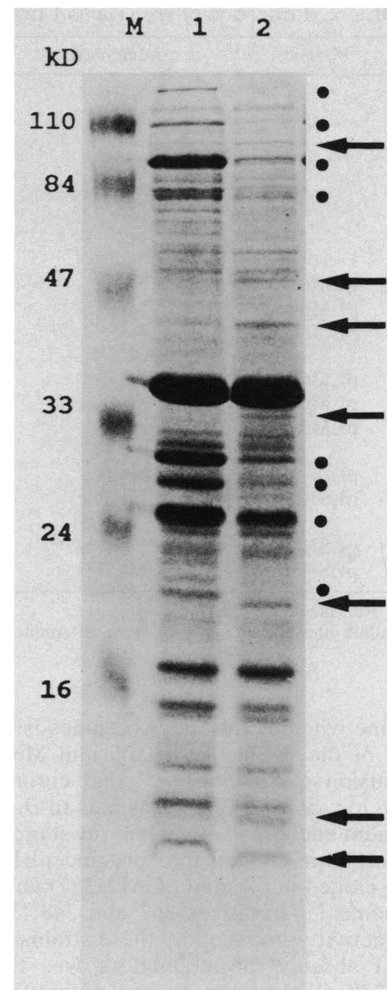

FIG. 1. Modulation of *B. pertussis* proteins. Strain 18323 was grown on BG agar plates in the absence and presence of modulators, and whole-cell protein extracts were enriched for other membrane proteins, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and stained with Coomassie brilliant blue. Lane 1, 18323. Lane 2, 18323 grown in the presence of $MgSO_4$ (20 mM) and nicotinic acid (5 mM). Arrows indicate proteins whose expression is increased under modulating conditions; circles indicate those decreased under modulating conditions.

RESULTS

Identification of *vir*-repressed genes. Starting with a set of 99 Tn*phoA* fusions in *B. pertussis* 18323 expressing alkaline phosphatase activity, Knapp and Mekalanos (12) identified five fusions expressing increased activity when grown on BG agar plates supplemented with either $MgSO_4$ or nicotinic acid. Under these conditions, colonies are nonhemolytic and produce negligible levels of the known virulence factors and X-mode antigens (11, 13, 23). These fusions, therefore, identified genes regulated by the same environmental signals but in a reciprocal fashion. Figure 1 shows a Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel of outer membrane protein-enriched extracts of *B. pertussis* 18323 grown under nonmodulating and modulating conditions. Several bands appear to increase when this strain is grown in the presence of $MgSO_4$ and nicotinic acid (arrows), demonstrating the same pattern of regulation as the genes identified by the Tn*phoA* fusions.

TABLE 2. Regulation of *vrg*::Tn*phoA* fusions

Strain	Plasmid	Addition(s)[a]	Activity[b]
SK6		None	17
SK6		Mg	284
SK6		Nic	505
SK18		None	23
SK18		Mg	178
SK18		Nic	199
348	pLM6	None	400
348	pLM6	Mg	2,000
348	pLM6	Nic	2,100
347	pLM6	None	2,300
347	pLM6	Mg	2,650
347	pLM6	Nic	2,300
348	pLSK18	None	45
348	pLSK18	Mg/Nic	415
347	pLSK18	None	248
347	pLSK18	Mg/Nic	314

[a] Mg, $MgSO_4$; Nic, nicotinic acid.
[b] Units of alkaline phosphatase activity were determined as previously described (17).

To determine whether this class of genes is regulated by the products of the *vir* locus, Knapp and Mekalanos (12) cloned the fusion carried on the SK6 chromosome and returned it on low-copy-number plasmids to *B. pertussis*. A *Bam*HI fragment encoding kanamycin resistance (due to the *neo* of Tn*phoA*) was cloned into plasmid pBR322 (3) and subsequently cloned in plasmid pLAFR2 (7) and conjugated into the Tohama I derivatives 347 and 348 (30). Alkaline phosphatase activity produced by these strains grown in the presence and absence of modulators was then assayed (Table 2). In 348, alkaline phosphatase activity was found to be regulated by modulators like the chromosomal fusion. In 347, which has a Tn*5* insertion in *bvgC* (1), activity was expressed at a high level and was unregulated. Based on these data, the genes represented by the five fusions were named *vir*-repressed genes (*vrg* [12]).

We wished to extend this analysis to at least one other member of this class of genes, so the same approach was used to characterize the *vrg-18* fusion carried in SK18. A *Bam*HI fragment encoding kanamycin resistance was first cloned in pUC19 (33) and subsequently cloned into pLAFR2. Again we observed regulated expression in 348 and high unregulated expression in 347 (Table 2). This suggests that both *vrg-6* and *vrg-18* are repressed directly or indirectly by the products of the *vir* locus.

The BvgG protein could interact with either BvgA or a homologous protein to achieve repression of this class of genes. To analyze the role of BvgA in the expression of *vrg* genes, the cloned *vrg-6* fusion was conjugated into the Tohama I derivative 3707 which contains an in-frame deletion in *bvgA* (24). We observed high unregulated levels of alkaline phosphatase activity comparable to the results seen with the *bvgC* mutant, 347 (data not shown). This suggests that both *bvgA* and *bvgC* are required for regulation of *vir*-repressed genes.

Deletion analysis of *vrg* promoters. We were interested in localizing *cis*-acting regulatory elements in the cloned *vrg* fusions. Thus, we constructed nested deletions (10) starting in the polylinker of the cloning vector and proceeding towards the fusion junction in both *vrg-6*::Tn*phoA* and *vrg-18*::Tn*phoA*. The resulting plasmids had the same 5'-distal end and differed only in the amount of *vrg* DNA remaining upstream of the junction with Tn*phoA*. Using the *phoA* and universal primers, we obtained approximately 600 nucleotides of sequence upstream of Tn*phoA* of each *vrg* fusion and determined their respective initiation codons (see below). Replicon fusions with pLAFR2 were constructed for conjugation into *B. pertussis* 348. The exconjugants were grown on BG agar plates in the presence and absence of modulators and assayed for alkaline phosphatase activity. These data are presented in Fig. 2. We observed a steady drop in enzyme activity as the deletions approached the initiation codon, suggesting the loss of promoter elements. However, modulation of enzyme activity was maintained in all deletion clones except those that terminated within the translated region. Indeed, regulation was observed even in pLSK18PΔ7.13, which regained only 24 nucleotides upstream of the *vrg-18* initiation codon. These data suggest that the regulatory region of *vrg* genes lie very close to or within the translated sequence itself.

Sequences of *vrg-6*::Tn*phoA* and *vrg-18*::Tn*phoA*. The nucleic acid sequences of the *vrg-6* and *vrg-18* fusions and the deduced amino acid sequences of the leader peptides are presented in Fig. 2 and 3. Based on the sequence data and Western blots (immunoblots) using anti-alkaline phosphatase serum (data not shown), we have determined that the Tn*phoA* fusion junction of each of these inserts is at the 22nd codon, producing fusion proteins that are 2.1 kDa larger than the alkaline phosphatase moity. The deduced amino acid sequences suggest strongly hydrophobic leader peptides followed by charged residues before the fusion junctions. This is consistent with the phosphatase activity of these fusions and suggests that the products of *vrg-6* and *vrg-18* are membrane, periplasmic, or secreted proteins. Other than the initiator methionine, the two leader peptides have only two amino acids in common, a lysine at position 2 and an alanine in the hydrophobic domain at position 12, yielding an identity of only 14% including the methionine.

The nucleic acid sequences, however, show substantial identity in this region. The 64 bases have an overall identity of 58%, but much of this is clustered into a 24-base subsequence having 74% identity (Fig. 3). There is no comparable level of similarity between the upstream DNA that was shown to be unnecessary for regulation in the two deletion series. In addition, we have identified repetitive elements within this region of *vrg-6*, a 21-base palindrome which overlaps with an 8-base direct repeat, both of which are indicated by arrows in Fig. 3. A 4-base repeated element observed in this region of *vrg-6* is found within and surrounding the corresponding region of *vrg-18*, as represented by the shaded sequences in Fig. 3. These data suggest that the sequences that provide regulation in *cis* may be those with the greatest primary sequence identity.

Insertional mutagenesis of *vrg-6*::Tn*phoA*. To analyze the regulatory properties of the translated region of *vrg-6*, we inserted a 6-bp linker into the repetitive elements mentioned above. A *Sma*I hexamer (CCCGGG) encoding proline-glycine was cloned into the unique *Nae*I site at base pair 24 of the *vrg-6* coding sequence in plasmid pUCP1Δ12, resulting in plasmid pUCP1Δ12S. As pUCP1Δ12 had low levels of alkaline phosphatase activity, even in the presence of modulators, a *Hinc*II fragment containing this insertion mutation was used to replace the corresponding fragment in pUCP1Δ18, giving rise to plasmid pUCP1Δ18S.

Replicon fusions of these plasmids (pLP1Δ18 and

vrg-6

```
                                       16]
CTGCAGGCCATCAACGAGGCCGACGCGGCGGCCGAAGTTCGCGCCGCATCCCGGCTGGAT
                   1]                                    15]
GAATTCCTGCCGGAAACCCCGCCCCAGGCCGCGTTTGCGCTGGCCTGGTGCGAACGCTAC
                                                18]     10]
GGCGCGCGCGCCTGGGCGGCCAACCCCTGGGTATGGGTGCTGGAATTCCGTCTGCTGAAC
                   13]                                 6]
CAGAACGGGCATTGATCCTCGTGGCGGACGGGCCACGCAAGGTGTGGAAATGTAACCCCA

CCTGGGGCCCGCCTTGGCTCCCCCCGCGTTTCAACCCATTTCAAAGCCGCGCCCGCCCCA

GCGGGACAAATTTGCCGCAACGCAGCAAACGCCGGCGTCCCGGACTGCGGGCGGTTGCGG
   8]                                       12]
ATTGTCGCCATTGCGGGGGCTGTTTTCACAAATTTACGCGCAAAACAGCCGAGAATAGGC

CCGTCGATTAGTCAACTTGGCTGCACGCGCAGCAAGGCAGGTGAAAAATGAAAAAGTGGT
  14]                                          11]
TTCGTTGCTGCCGGCATCGGCGCTGCCGGACTCATGCTCTCCAGCGCCGCCCCphoA
```

Plasmid	Alkaline Phosphatase Activity		
	No Additions	$MgSO_4$	Ratio
pLP1	502	2612	5.2
pLP1Δ16	489	1774	3.8
pLP1Δ1	463	1145	2.5
pLP1Δ15	652	1842	2.8
pLP1Δ18	249	1401	5.6
pLP1Δ10	200	971	4.9
pLP1Δ13	161	696	4.3
pLP1Δ6	51	135	2.6
pLP1Δ8	6	21	3.5
pLP1Δ12	5	23	4.6
pLP1Δ14	2	2	1.0
pLP1Δ11	3	3	1.0

vrg-18

```
GTGCGGCAAAACCCCTTCGAACTGATCTGCGGCGACGTGTTCGCGCTGGAAGACGCCACG

CTGGCCGCCTGCGCCGGCGTGTACGACCGCGCCGCGCTGGTGCGCGTGCCCGAGCCCATG

CGCAAGCGCTACGCTTGCGAGGTCTATGGCCGCCTGGGCCGGGGCTGCCGGGGCATCCTC
                7.5]                            6.12]
ATCACGCTGGACTATCCGCAGACCAGATGGAAGGTCCGCCCTTTTCCGTGGACGACGCCG
                                      6.10]
AAGTGCAGGCGCTGTATGCCGGCCATACCGAAGCCCGCCTGATCGACCGGCGCGACATCC
               7.12]
TCGACAAGGAGCCCAAGTTCAACCAGCGCGGCGTGGCCGGCTGGACACGCTGGTATACCG
6.6]
GCTCGAACGCCTGGGCTGAGCGCGGCGATGCTGCCGCTTGTTACCGACATCCATGCCTGG
                            7.13]
TAGCGATGGGCGAGCCTACACTTGCGCCGTCTGGAACAACAGGAATATGGGCATGAAATC
                                                        8.1]
GATACTCGGGCTGTTGCTTGCCGCGGCGCTGCTGGGCGGCTGTGCCGTCTATACCCphoA
```

Plasmid	Alkaline Phosphatase Activity		
	No Additions	$MgSO_4$/Nic	Ratio
pLSK18P	44	415	9.4
pLSK18PΔ7.5	20	224	11.2
pLSK18PΔ6.12	16	248	15.5
pLSK18PΔ6.10	13	185	14.2
pLSK18PΔ7.12	13	176	13.5
pLSK18PΔ6.6	14	122	8.7
pLSK18PΔ7.13	6	43	7.2
pLSK18PΔ8.1	6	6	1.0

FIG. 2. Sequence and regulation of *vrg-6*::Tn*phoA* (top) and *vrg-18*::Tn*phoA* (bottom) gene fusions and nested deletions. Nested deletions of *vrg* promoters were constructed and used for DNA sequencing and analysis of regulation fusion protein enzyme activity in *B. pertussis* 348. Activity was determined after growth on BG agar plates in the absence and presence of $MgSO_4$ (20 mM) and nicotinic acid (5 mM), and regulation is represented as the ratio of activity expressed from modulated and unmodulated cultures. Bars above sequence indicate the deletion endpoint in the respective deletion plasmid. Underlined sequences are translated in the fusion proteins. The endpoint of pLSK18PΔ8.1 is at base pair 148 of Tn*phoA*.

vrg-6

```
-51                                                         +1
AGGCCCGTCGATTAGTCAACTTGGCTGCACGCGCAGCAAGGCAGGTGAAAAATGAAAAAG
                                     SD          MetLysLys

+10
TGGTTCGTTGCTGCCGGCATCGGCGCTGCCGGACTCATGCTCTCCAGCGCCGCCCphoA
TrpPheValAlaAlaGlyIleGlyAlaAlaGlyLeuMetLeuSerSerAlaAlaProAsp
          Linker
```

vrg-18

```
-51                                                         +1
AGCGATGGGCGAGCCTACACTTGCGCCGTCTGGAACAACAGGAATATGGGCATGAAATCG
                                     SD          MetLysSer

          GCTGCCGGCATCGGCGCTGCCGG
+10       * ***  **  ********* **
ATACTCGGGCTGTTGCTTGCCGCGGCGCTGCTGGGCGGCTGTGCCGTCTATACCCphoA
IleLeuGlyLeuLeuLeuAlaAlaAlaLeuLeuGlyGlyCysAlaValTyrThrProAsp
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FIG. 3. DNA and deduced amino acid sequence of *vrg-6*::Tn*phoA* and *vrg-18*::Tn*phoA*. Putative Shine-Dalgarno sequences are underlined, and the site of insertion of the *Sma*I linker in *vrg-6* is indicated by the vertical arrow. The 21-base palindrome and 8-base direct repeat in *vrg-6* are indicated by horizontal arrows. The 4-base repeat found in both sequences is shaded. The region of greatest primary identity is aligned over the *vrg-18* sequence; identical bases are starred.

pLP1Δ18S) were conjugated into *B. pertussis*, and the alkaline phosphatase activity was assayed (Fig. 4). While the enzyme activity expressed from pLP1Δ18 is about fivefold greater when grown in the presence of modulators, no regulation is observed with the mutant pLP1Δ18S. This construct is apparently resistant to repression by the *vir* system.

ALKALINE PHOSPHATASE ACTIVITY (□ BG ▨ BG+MODS.)
1400 1300 1200 1100 1000 900 800 700 600 500 400 300 200 100 0
pLP1Δ18 pLP1Δ18S

FIG. 4. Regulation of *vrg-6*::Tn*phoA* and insertion mutant. Strain 348 containing pLP1Δ18 or pLP1Δ18S (*Sma*I linker insertion mutant) was grown on BG agar plates in the absence (open bars) or presence (hatched bars) of $MgSO_4$ (20 mM) and nicotinic acid (5 mM), and alkaline phosphatase activity was determined.

One might expect that a mutation rendering a *vir*-repressed gene resistant to repression would lead to an unregulated and high level of expression, rather than the low level seen with pLP1Δ18S. However, the addition of the *Sma*I linker inserted proline-glycine to the signal sequence, which could affect secretion of the fusion protein. Indeed, we observed that this mutant expressed less alkaline phosphatase activity in *E. coli* (2 units versus 19 units for the wild-type clone), suggesting that there is a secretion defect. Taking into account this 10-fold difference in secretion in *E. coli*, the data suggest that this mutant is constitutively derepressed in *B. pertussis*.

DISCUSSION

Like many pathogenic bacteria, *B. pertussis* possesses a complex system for sensing and responding to environmental signals. This response results in a drastic modulation of secreted and surface-exposed antigens, giving rise to a very different antigenic state of the organism (10, 13, 23). Transposon mutagenesis by Tn*phoA* and Tn*5lac* (32) has enabled the identification of previously unknown regulated genes and the analysis of *vir* regulation. While much is known about the virulence factors that disappear when *B. pertussis* undergoes phase change or antigenic modulation, very little is known about the new proteins expressed in phase IV or C mode. We have identified at least five genes of the *vir* regulon that are maximally expressed in phase variant and modulated strains. Tn*phoA* fusions to these genes are positive for alkaline phosphatase activity, indicating that their gene products are periplasmic, membrane associated, or extracellular; thus, the *vir*-repressed genes are the most likely candidates identified so far to be the antigens preferentially expressed in phase IV and C-mode strains. Our sequence

analysis of the *vrg-6* and *vrg-18* Tn*phoA* fusions supports the conclusion that these two genes encode products with hydrophobic N-terminal signal sequences, again demonstrating the utility of Tn*phoA* in identifying secreted proteins in a wide variety of bacterial systems.

The *vir*-repressed genes clearly require an intact *vir* locus for repression; what other gene products are needed is uncertain. Unlike the *fhaB* gene, other *vir*-activated genes and these *vrg* genes are unresponsive to *bvgABC* when present in *trans* in *E. coli* (19; T. M. Finn, D. T. Beattie, and J. J. Mekalanos, unpublished observations). This indicates that other loci are essential to effect regulation; in the case of *vrg* genes, this might be an unlinked *vir*-regulated repressor. In this study, we have localized potential *cis*-acting regulatory sites in two *vrg* genes by deletion analysis and insertional mutagenesis. Our data suggest that the site of action of this hypothetical *vir*-regulated repressor is very near or within the coding sequence of the two *vrg* genes and may involve conserved repetitive elements.

There are a number of mechanisms by which a repressor could effect regulation within the translated region of a gene. Binding may occur to DNA and effect premature transcriptional termination; this has been demonstrated with the *lac* repressor and artificial placement of the operator site downstream from the *lac* promoter (3). Alternatively, binding could occur to the mRNA, preventing initiation or elongation of the translation product, comparable to the binding of T4 RegA protein to the Shine-Dalgarno sequence of gene 44 RNA (29). These two models assume constant levels of transcripts. Regulation could also be achieved by varying mRNA levels through increased degradation under repressing conditions or stabilization during modulation. The products of both the *ompA* (21) and *ermC* (2, 26) genes are regulated at the level of mRNA stability. Finally, the protein products could be selectively degraded to effect regulation (8). To test these models, we are analyzing transcript levels by Northern (RNA) blot analysis, attempting to identify the transcriptional start sites, and searching for repressor-resistant point mutations in *vrg-6* and *vrg-18*.

Lacey noted that proteins expressed during the C mode of growth were far less immunogenic than those expressed in X mode (13). Indeed, we have observed only one protein upregulated in modulating conditions on Western blots with serum raised against the Tn*5*-bearing phase IV mutant 347 (D. T. Beattie and J. J. Mekalanos, unpublished observations). Likewise, the deduced amino acid sequence of the *vrg-6* gene product (data not shown) predicts a small hydrophobic protein with a poor antigenic index. It is possible that the C mode of growth is involved in persistence of *B. pertussis* in the host and that the lack of immunogenicity of the *vrg* products is advantageous to the bacteria. Lacey observed that most people convalescent from whooping cough produced sera that would agglutinate an X-mode suspension of *B. pertussis*, but that a small percentage produced sera capable of agglutinating a C-mode suspension. These sera were from adults with mild coughs and positive nasopharyngeal swabs for up to 5 months, suggesting that the production of C-mode antigens is associated with growth in an environment not encountered in the acute phase of infection (13). With the recent in vitro observation of invasion and survival within epithelial cells by *B. pertussis* (6, 14a), we speculate that this may be a modulating environment that the bacterium encounters in vivo during maintenance of a carrier state. Efforts are under way to detail the significance of the C mode of growth and the contribution of *vir*-repressed genes in persistence of *B. pertussis* after recovery from whooping cough.

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