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A *vir*-Repressed Gene of *Bordetella pertussis* Is Required for Virulence

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Coordinate regulation of gene expression in *Bordetella pertussis* is controlled by the products of the *vir* locus, BvgA and BvgS. In the presence of modulating signals such as $MgSO_4$ and nicotinic acid, expression of *vir*-activated genes (*vag*) is reduced, while expression of *vir*-repressed genes (*vrg*) is maximal. We have cloned one of these *vir*-repressed genes, *vrg-6*, in *Escherichia coli*. DNA sequencing has shown that *vrg-6* is contained on a single *Eco*RI restriction endonuclease fragment and is predicted to code for a protein of 105 amino acids with a molecular weight of 11,441. The predicted protein product appears to have two domains, one consisting of seven hydrophobic proline-rich pentameric repeats and the other consisting of five alkaline trimeric repeats. Southern blot analysis has revealed *vrg-6*-homologous sequences in the chromosomes of *Bordetella bronchiseptica* and *Bordetella parapertussis*, but, unlike *Bordetella pertussis*, these species do not express *vrg-6*-homologous RNA when grown under modulating conditions. In order to assess the role of *vrg* gene products in *B. pertussis* pathogenesis, two 18323 derivatives which harbor Tn*phoA* insertions in *vrg* genes were analyzed in a mouse model of respiratory infection. Strain SK6, which carries a *vrg-6*::Tn*phoA* mutation, failed to induce lymphocytosis and was significantly less able to colonize lungs and trachea than its parent strain 18323 or than SK18, which harbors a Tn*phoA* fusion in the *vrg-18* locus. This is the first evidence that a *vir*-repressed gene may play an important role in the virulence of *B. pertussis* and the pathogenesis of whooping cough.

Coordinate regulation of gene expression in response to environmental signals has been observed in many pathogenic bacteria, including *Bordetella pertussis*, the causative agent of whooping cough. The ability of this organism to successfully colonize the upper respiratory tract depends on the production of a wide array of virulence factors such as pertussis toxin, filamentous hemagglutinin, adenylate cyclase-hemolysin toxin, fimbriae, and other outer membrane proteins (9, 22, 33, 35). In order to express these factors in vitro, *B. pertussis* requires specific growth conditions; the presence of high levels of certain modulating factors (e.g., $MgSO_4$ or nicotinic acid) in the growth medium drastically reduces expression of virulence determinants, a process termed antigenic modulation (12, 26).

We have identified a class of *B. pertussis* genes whose profiles of expression are reciprocal to those of the major virulence factors (11). In the presence of antigenic modulators, these *vir*-repressed genes (*vrg*) are expressed, while levels of the *vir*-activated gene (*vag*) products such as pertussis toxin and filamentous hemagglutinin are negligible. This coordinate loss of virulence factors is also observed in phase variants, which are strains with mutations in the *vir* locus (15, 24, 30, 34). In these strains, the products of the *bvgA* and *bvgS* genes are unable to activate the promoters of *vag* genes or repress the expression of *vrg* genes. Although phase variants are unable to initiate infection (22, 32), they can be isolated from patients recovering from bordetellosis (12). This suggests that *vir*-repressed gene products probably are expressed during the infectious cycle of *B. pertussis* when phase variants are present. However, even in the absence of phase variants, host environmental signals may lead to high-level *vrg* expression during some stage of the infection cycle.

Previously, we have cloned and sequenced the DNA upstream of two *vrg*::Tn*phoA* gene fusions identified in strain 18323 (2). The limited amount of coding sequence obtained predicted that both *vrg-6* and *vrg-18* would code for secreted or surface proteins with typical N-terminal signal sequences. Thus, these two genes were likely to encode surface proteins expressed in strains that had undergone antigenic modulation or in phase variants. In this paper, we report the cloning and sequencing of the *vrg-6* gene and the sequence of its deduced product, a protein composed primarily of repetitive sequence elements. A strain harboring a transposon insertion in the *vrg-6* gene was found to be dramatically impaired in its ability to grow in the lungs and tracheae of mice. This suggests that at least one *vir*-repressed gene plays an important and previously unrecognized role in the pathogenesis of *B. pertussis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *Escherichia coli*, *B. pertussis*, *Bordetella avium*, *Bordetella bronchiseptica*, and *Bordetella parapertussis* strains and plasmids used in this study are listed in Table 1. All *Bordetella* strains were grown on Bordet-Gengou (BG) agar (Difco Laboratories, Detroit, Mich.) supplemented with 10 ml of glycerol and 150 ml of defibrinated sheep blood per liter or in modified Stainer-Scholte broth. Modulators and antibiotics were added by spreading stock solutions onto BG agar plates and drying them 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, 12.5 μg of tetracycline, and 30 μg of cephalexin per ml.

Recombinant DNA methods. Standard methods were used

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TABLE 1. Strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Relevant feature or source	Reference or source
B. pertussis		
18323	Wild type	ATCC 9797
SK6	18323 *vrg-6*::Tn*phoA*	11
SK18	18323 *vrg-18*::Tn*phoA*	11
338	Nal^r Tohama I derivative	34
Other *Bordetella* species		
B. avium 197		C. Gentry-Weeks
B. bronchiseptica IT2		C. Lee
B. parapertussis 17903		M. Peppler
Plasmids		
pUC19		36
pUCP1	pUC19 *vrg-6*::Tn*phoA* insert	2
pDB101	pUC19 *vrg-6*	This study
pLAFR2	IncP1 *cos* cloning vector	10
pL9A2	pLAFR2 *vrg-6*	This study
Phages		
M13mp19		36
M13DB101	M13mp19 (*vrg-6*)	This study

for plasmid and chromosomal DNA purification, 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.

Cloning of *vrg-6* gene. The *vrg-6* gene was cloned from a genomic DNA library described by Knapp and Mekalanos (11). This library consists of 20- to 30-kb fragments of *B. pertussis* 18323 genomic DNA cloned into the *Bam*HI site of plasmid pLAFR2 (10). A total of 1,440 transformed colonies were picked to 96-well microtiter plates containing Luria broth supplemented with 20% glycerol and 12.5 μg of tetracycline per ml and grown overnight at 37°C. The plates were then frozen at −70°C. A multiple inoculator was used to transfer culture material from the microtiter plates to nitrocellulose filters (Schleicher & Schuell, Keene, N.H.) placed on the surface of Luria-Bertani agar plates. The filters were incubated overnight at 37°C, and the bacteria were lysed in 0.5 N NaOH–1.5 M NaCl (16).

The filters were probed with a 156-bp *Nae*I fragment of plasmid pUCP1 (2), which contains 24 bp of the *vrg-6* coding region and 142 bp of upstream DNA sequence. The fragment was radioactively labeled with [^{32}P]dCTP (DuPont NEN) by nick translation (Amersham). The probe was allowed to hybridize with the nitrocellulose filters overnight in hybridization solution (16). The filters were then washed twice in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at 65°C for 30 min per wash and autoradiographed. Hybridizing clones were picked from the corresponding wells in the microtiter plates and grown for plasmid preparation. All hybridizing clones were confirmed by using Southern blot analysis (see below).

An 829-bp *Eco*RI fragment common to all hybridizing clones was subcloned into pUC19 and phage M13 (36). This construct was used for the generation of a restriction map and for sequence and Northern (RNA) blot analyses.

Sequencing of the *vrg-6* gene. The parental plasmid pL9A2 and the subclones pDB101 and M13DB101 were sequenced with Sequenase (USB)-modified DNA polymerase. The standard Sequenase protocol for incorporation of ^{35}S-dATP was used with a modification of double-stranded template-primer hybridization as described elsewhere (2). Single-stranded templates were denatured and hybridized by using the Sequenase protocol.

Southern blot analysis. Capillary blotting of agarose gels to nitrocellulose was performed as described previously (16). Hybridization conditions were identical to those used to screen the library. For rescreening of clones recovered from the library, the same 156-bp *Nae*I fragment was used as a probe of minipreparation plasmid DNA digested with a variety of restriction endonucleases. This blot was washed three times in 0.2× SSC–0.1% SDS at 65°C for 30 min per wash and autoradiographed. For analysis of *Bordetella* species, the 198-bp *Nae*I-*Acc*I fragment internal to *vrg-6* was used as a probe of chromosomal DNA digested with *Eco*RI. This blot was treated in the same manner.

Preparation and analysis of RNA. RNA was prepared from cultures of *Bordetella* species grown in the presence of $MgSO_4$ and nicotinic acid by a modification of the hot-phenol method (31). RNA (5 to 10 μg) was subjected to electrophoresis in a denaturing agarose-formaldehyde gel and transferred to nitrocellulose as described elsewhere (16). The 829-bp *Eco*RI fragment containing *vrg-6* was used as a probe. Hybridization was performed in 50% formaldehyde–2× Denhardt's solution–0.1% SDS–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7])–10% dextran sulfate–100 μg of salmon sperm DNA per ml. The blot was washed three times in 2× SSPE–0.1% SDS at 25°C for 10 min each time and twice in 0.5× SSPE–0.1% SDS at 25°C for 10 min each time and autoradiographed.

Aerosol challenge and analysis of respiratory infection. Specific-pathogen-free BALB/cAnNcR mice were obtained on day 17 postpartum from the Animal Production Program, Division of Cancer Treatment, National Cancer Institute, Frederick, Md. Animals were housed in filtered microisolators with free access to food and water. A 21-h culture of bacteria was harvested from BG agar slants and suspended in sterile saline to approximately 10^9 CFU/ml. The challenge inoculum was administered to the mice as an aerosol for 30 min as previously described (9). Upon removal from the aerosol chamber, a group of mice was sacrificed, their lungs and tracheae were removed and homogenized in sterile phosphate-buffered saline, and the homogenate was plated to determine the number of viable *B. pertussis* organisms. All mice tested had approximately 10^4 CFU in the lungs 1 h after aerosol infection. To determine bacterial persistence, mice were sacrificed after 14 days, and CFU counts in the lungs were determined as described above. Mice were bled from the orbital sinus periodically after infection, and leukocyte counts were determined in a model ZM Coulter Counter (Coulter Electronics, Hialeah, Fla.).

CHO cell clustering assay. The CHO cell clustering assay (9) was used to screen for pertussis toxin. Supernatants of 2-day Stainer-Scholte cultures were filtered through 0.2-μm-pore-size polysulfone filters (Acrodisc; Fisher Scientific, Medford, Mass.). After centrifugation, the cell pellets were suspended in 1/10 volume of 50 mM Tris HCl–50 mM EDTA (pH 7.5) containing 2 mg of lysozyme per ml. After incubation for 1 h at 27°C, the pellets were filtered through 0.2-μm-pore-size polysulfone filters. Dilutions of superna-

tants and cell pellet lysates were made in Ham's nutrient mixture F12 (Hazleton Research Products, Inc., Lenexa, Kans.) supplemented with 2% newborn calf serum, 50 U of penicillin, and 50 μg of streptomycin per ml. Confluent 24- to 48-h cultures of CHO cells in plates (60 by 15 mm) were treated with trypsin, harvested, and suspended to 2.5×10^4 cells per ml in F12 plus 2% newborn calf serum. Finally, 200 ml of the CHO cell suspensions was added to serial dilutions of *B. pertussis* cell supernatant preparations in 96-well microtiter plates. Purified pertussis toxin (List Laboratories) was used as a control. Plates were incubated at 37°C in 7% CO_2 for 48 h before being read.

Nucleotide sequence accession number. The GenBank accession number for the *vrg-6* DNA sequence is M77374.

RESULTS

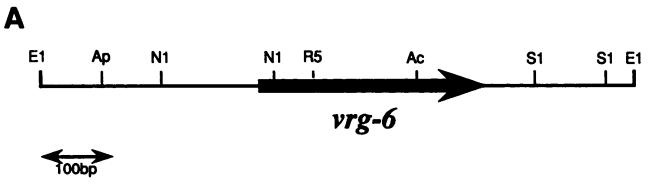
Cloning of the *vrg-6* gene. We have previously reported the cloning of two *vir*-repressed Tn*phoA* gene fusions from *B. pertussis*. We were interested in the nature of these gene products, so we sought to clone the wild-type *vrg-6* gene. A library of *B. pertussis* chromosomal DNA fragments cloned in the vector pLAFR2 was screened by hybridization with a 156-bp *Nae*I fragment from the promoter region of the *vrg-6* gene (2). Autoradiography for 18 h revealed 5 strongly hybridizing clones along with 8 more weakly hybridizing ones of the 1,440 that were screened. The five positive clones were recovered from the frozen library plates, and plasmid DNA was isolated (see Materials and Methods). Southern blot analysis using the *vrg-6* probe showed that all five clones had the same hybridizing fragments, including an approximately 800-bp *Eco*RI fragment (data not shown).

The clone designated 9A2 was chosen for further study; the plasmid carried in this clone was designated pL9A2. Plasmid pL9A2 was digested with *Eco*RI, and an 829-bp fragment that hybridized to the *vrg-6* probe was subcloned into phage M13mp19 to give M13DB101. This same fragment was further subcloned from M13DB101 into pUC19 to give pDB101.

Sequence of the *vrg-6* gene. Figure 1A shows the restriction map of the *vrg-6* locus as determined by endonuclease digests and dideoxy sequencing of the entire *Eco*RI fragments from M13DB101 and pDB101. The *vrg-6* nucleotide sequence and the deduced sequence of its 105-amino-acid product are shown in Fig. 1B. The coding sequence (established by gene fusions; 2) begins at bp 205 of the *Eco*RI restriction fragment and terminates at bp 622. We have previously reported (2) that the first 21 residues of Vrg-6, which includes a long hydrophobic region, is sufficient to direct the export of alkaline phosphatase. It is likely, therefore, that the Vrg-6 protein itself is secreted past the bacterial cytoplasmic membrane.

The most striking features of the predicted amino acid product of the *vrg-6* gene are two regions of repeated sequences, which are underlined in Fig. 1B. The first is a proline-rich pentamer repeated seven times that has the consensus sequence Pro-Ala-Pro-Val-Trp. A comparison of these repeats reveals that the second and third positions are the most variable, whereas the first, fourth, and fifth positions are nearly invariant. The other repeated sequence is a highly basic trimer repeated five times with the consensus Arg-Gly-His. A comparison of these repeats shows that there is only one rather conservative change, an arginine to a lysine.

The two sets of repeated sequences appear in distinctly different domains of the Vrg-6 protein. A plot of hydrophi-



B

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GAATTCCGTCTGCTGAACCAGAACGGGCATTGATCCTCGTGGCGGACGGGGCACG      55
CAAGGTGTGGAAATGTAACCCCACCTGGGGCCCGCCTGGCTCCCCCCGCGTTTCA     110
ACCCATTTCAAAGCCGCGCCCGCCCCAGCGGGACAAATTTGCCGCAACGCAGCAA     165
ACGCCGGCGTCCCGGACTGCGGGCGGTTGCGGATTGTCGCCATTGCGGGGGCTGT     220
TTTCACAAATTTACGCGCAAAACAGCCGAGAATAGGCCCGTCGATTAGTCAACTT     275

GGCTGCACGCGCAGCAAGGCAGGTGAAAA  ATG AAA AAG TGG TTC GTT      322
                               M   K   K   W   F   V
GCT GCC GGC ATC GGC GCT GCC GGA CTC ATG CTC TCC AGC GCC      364
 A   A   G   I   G   A   A   G   L   M   L   S   S   A
GCC CTG GCC CGT GTG GAT ATC GGG GTG TCG ATC GGC ATT CCG      406
 A   L   A   R   V   D   I   G   V   S   I   G   I   P
GGC GTG GTG TAC CCG GCG CCC GTG TAT GTG GCG CCC GCC CCG      448
 G   V   V   Y   P   A   A   V   Y   V   A   P   A   P
GTG TAT GCC CCG CCG CCC GTG GTG CAC TAC CCG GCG CCG GTC      490
 V   Y   A   P   P   P   V   V   H   Y   P   A   P   V
TAT GTG CGC CCG CAG GTG GTG TAT CCG GCG CCC GTC TAC TAT      532
 Y   V   R   P   Q   V   V   Y   P   A   P   V   Y   Y
GGC GGC CCC CGC TAC TAC AAG GGC CAT CGG CAC TAT GAC CGC      574
 G   G   P   R   Y   Y   K   G   H   R   H   Y   D   R
GGC CAT CGC GGC CAC CGC GGC CAT GGC CGC GGC CAC TGG CGC      616
 G   H   R   G   H   R   G   H   G   R   G   H   W   R
GAT TGA    CCGGCGATCGCCGCAAATCAATGAAAAAGCCGCCCGGATCGGCG      666
 D   *
TTTTTTTGCGGCACGGCTGGGTTCAGGCCTGTTCGGCCGGGACCTTGGCGGCTGG     721
CGCGGCCGTTTGCCCCTCGGCGGGCTTGTCGCTGGCATGGCGTCAAGCCAGCGCG     776
GCGGCGCGCTGGAGGCCTTCCACCAGCCGTTATGCGGACGCGTGTGCGAATTC       829
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FIG. 1. Restriction map and nucleotide sequence of the *vrg-6* locus. (A) Direction of translation and protein-coding sequence are indicated by the arrow. The following abbreviations represent restriction enzyme sites: Ac, *Acc*I; Ap, *Apa*I; E1, *Eco*RI; N1, *Nae*I; R5, *Eco*RVI; S1, *Stu*I. (B) The deduced amino acid sequence of the *vrg-6* gene is shown in bold type below the nucleotide sequence of the 829-bp *Eco*RI fragment cloned out of *B. pertussis* 18323. Proline-rich repeats are underlined, and base-rich repeats are double underlined.

licity (6) shows that the proline repeats occur in a highly hydrophobic region while the basic repeats occur in a highly charged and hydrophilic region (Fig. 2). The proline repeat region is predicted to have a very low surface probability and a relatively poor antigenic index. The alkaline repeats, on the other hand, appear to be much more antigenic.

Expression of *vrg-6* in other *Bordetella* species. We were interested in the possibility that *vir*-repressed genes may be common to other *Bordetella* species. Chromosomal DNA was prepared from *B. pertussis* SK6 and SK18 (isogenic derivatives of 18323 harboring Tn*phoA* insertions in *vrg-6* and *vrg-18*, respectively), *B. avium* 197, *B. bronchoseptica* IT2, and *B. parapertussis* 17907. A Southern blot was performed on these DNAs digested with *Eco*RI, and it was probed with an *Nae*I-*Acc*I fragment internal to *vrg-6* (Fig. 3). Lane 2 of Fig. 3 contains DNA from SK18, which has a wild-type *vrg-6* locus; the 829-bp fragment which carries the *vrg-6* gene can be seen. Lane 1 contains DNA from SK6 and

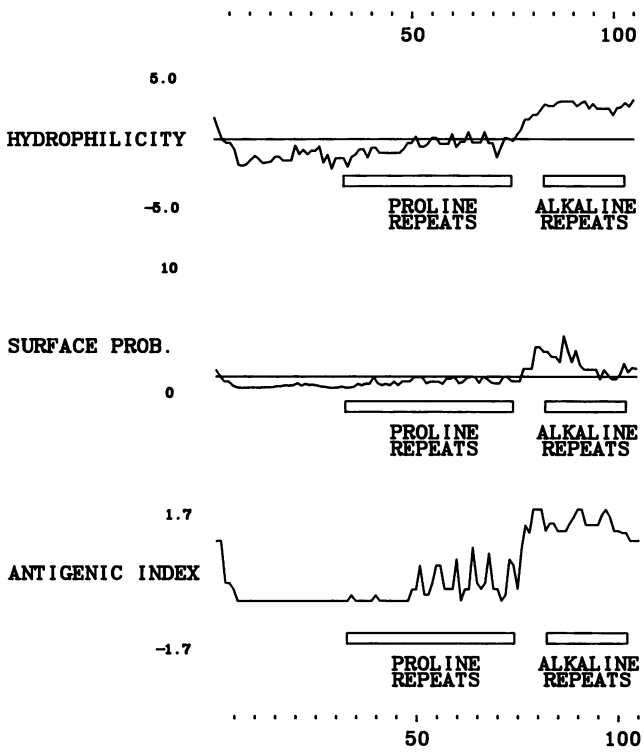

FIG. 2. Sequence analysis of Vrg-6, with hydrophilicity, surface probability, and antigenic index of the deduced amino acid product of *vrg-6*. Repeated sequences are indicated by the boxed regions.

shows the effects of the transposon insertion into the *vrg-6* gene. The 829-bp fragment is gone and a larger 7,099-bp fragment appears because of the *Eco*RI sites in Tn*phoA*.

Lanes 3 to 5 in Fig. 3 contain *Eco*RI-digested DNA from other *Bordetella* species. Lane 3 shows that the *B. avium* chromosome has no hybridizing bands at this stringency. Lane 4 demonstrates that *B. bronchiseptica* has a hybridizing band nearly identical in size to that of *B. pertussis*.

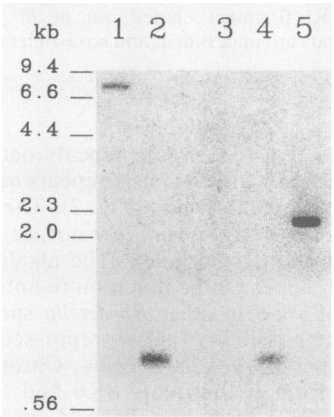

FIG. 3. Southern blot of *Bordetella* species. A Southern blot of *Eco*RI-digested chromosomal DNAs from various *Bordetella* species was probed with the 198-bp *Nae*I-*Acc*I fragment internal to the *vrg-6* gene. Lane 1, *B. pertussis* SK6; lane 2, *B. pertussis* SK18; lane 3, *B. avium* 197; lane 4, *B. bronchiseptica* IT2; lane 5, *B. parapertussis* 17903. The reduced intensity of the signal in lane 4 reflects the fact that less of the *B. bronchiseptica* DNA was loaded on the gel, as determined by ethidium bromide staining.

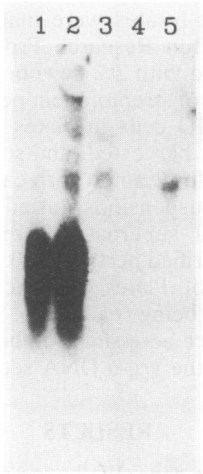

FIG. 4. Northern blot of RNAs prepared from various *Bordetella* species grown in the presence of $MgSO_4$ and nicotinic acid probed with the 829-bp *Eco*RI fragment containing the *vrg-6* gene. Lane 1, *B. pertussis* 338; lane 2, *B. pertussis* 18323; lane 3, *B. avium* 197; lane 4, *B. bronchiseptica* IT2; lane 5, *B. parapertussis* 17903.

Finally, *B. parapertussis* in lane 5 shows a hybridizing band with a restriction fragment length different from those of *B. pertussis* and *B. bronchiseptica*. These data suggest that sequences with similarity to *vrg-6* are present on the chromosomes of other *Bordetella* species and that in the case of *B. bronchiseptica*, the locus has a chromosomal structure similar to that seen in *B. pertussis*.

The presence of hybridizing sequences does not mean that this gene is expressed in these other species. To address this, we performed Northern blot analysis of RNAs prepared from several *Bordetella* species grown under modulating conditions (Fig. 4). RNAs prepared from a Tohama 1 derivative (lane 1) and an 18323 (lane 2) derivative hybridize with the *vrg-6* probe, whereas RNAs from *B. avium*, *B. bronchiseptica*, and *B. parapertussis* do not (lanes 3 to 5). We conclude that the chromosomal loci which hybridize to *vrg-6* in these species do not produce hybridizing mRNA detectable by Northern blotting.

Effect of a *vrg-6* mutation on the virulence of *B. pertussis*. Except for lipopolysaccharide and the tracheal cytotoxin, a fragment of peptidoglycan, all previously identified virulence factors of *B. pertussis* are in the category of *vir*-activated genes (33). Mutants lacking these two factors do not exist, so their respective contributions to pathogenicity have not been established. Thus, only strains with mutations in *vir*-activated genes have been tested in animal models. The availability of a transposon insertion in several *vir*-repressed genes afforded us the opportunity to test *vrg* mutants in a mouse model of respiratory infection (9).

We analyzed the abilities of strains 18323, SK6 (*vrg-6*::Tn*phoA*), and SK18 (*vrg-18*::Tn*phoA*) to persist in mouse lungs and tracheae 14 days after aerosol inoculation and to induce lymphocytosis during the course of infection (see Materials and Methods). Figure 5 shows that all strains had been successfully delivered to the mice in comparable doses; after 30 min, between 10^4 and 10^5 organisms were present in the lungs and approximately 10^3 organisms were present in the tracheae.

After 14 days, however, the strains showed very different abilities for persisting. In the lungs, 18323 and SK18 both increased by a factor of about 10^4, i.e., to 8.7×10^7 and 1.3

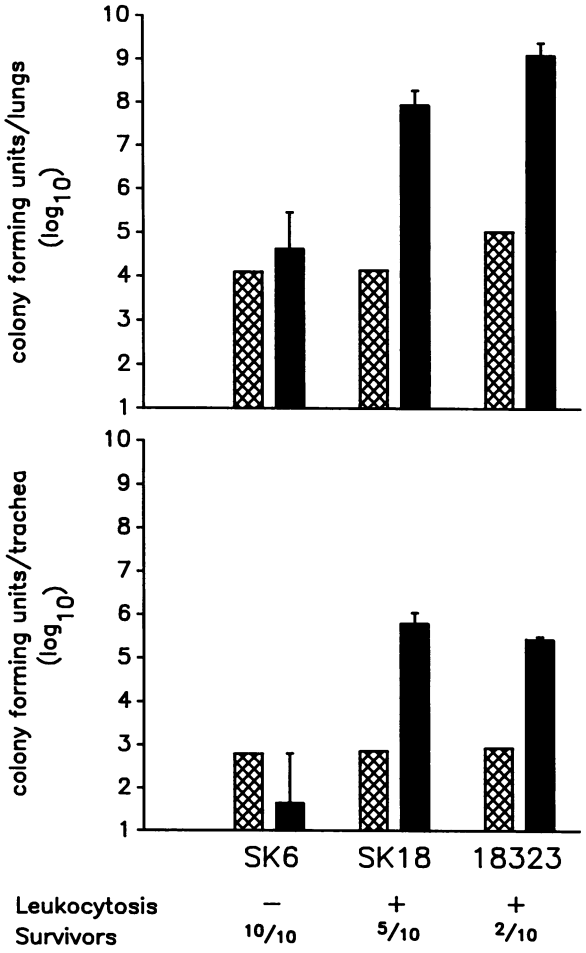

FIG. 5. Persistence of *B. pertussis* in mice following aerosol challenge. Log_{10} CFU was measured in the lungs (top) and tracheae (bottom) of mice subjected to aerosol challenge with *B. pertussis* SK6, SK18, and 18323. Hatched bars indicate mean CFU 1 h after challenge, and solid bars indicate mean CFU and standard deviation after 14 days. Positive leukocytosis (beneath lower panel) indicates $>75 \times 10^3$ leukocytes per μl of blood at 14 days after infection; negative leukocytosis indicates $<25 \times 10^3$ leukocytes per μl of blood. Survival was determined at day 21.

$\times 10^9$ CFU, respectively. SK6 showed a marked inability to proliferate, only increasing from 1.2×10^4 to 4.3×10^4 CFU. In the tracheae, SK18 and 18323 both increased by factors of about 10^3, to 6.2×10^5 and 2.3×10^5 CFU, respectively. Again, SK6 was unable to grow and actually decreased from 6.1×10^2 to 4.5×10^1 CFU. Furthermore, SK6 did not induce lymphocytosis despite its ability to produce pertussis toxin in vitro (see below).

Because SK6 exhibited a pronounced virulence defect, we wished to establish that other *vir*-activated virulence factors were being produced by strain SK6. Hemolysis was detected on a BG blood agar plate, indicating the production of adenylate cyclase-hemolysin toxin. Filamentous hemagglutinin was detected on SDS-polyacrylamide gels (data not shown). Serotyping of fimbriae with monoclonal antibodies indicated that both type 2 and type 3 fimbriae are produced. The Chinese hamster ovary cell clustering assay for pertussis toxin indicated that supernatants of SK6 cultures contained 6.8 μg/ml per optical density unit at 600 nm compared with supernatants of 18323, which contained 4.8 μg/ml per optical density unit at 600 nm. Therefore, we conclude that strain SK6 is probably wild type with respect to *vir*-activated virulence factors and that its defect in colonization does not reflect the loss of these particular gene products. Instead, these data suggest that the *vrg-6* gene product may play an important role in the virulence of *B. pertussis*.

DISCUSSION

We have previously described a set of five genes from *B. pertussis* that are repressed by the virulence gene regulators BvgA and BvgS (2, 11). These *vir*-repressed genes are expressed when the principal virulence factors are eliminated during antigenic modulation or in phase variants. We report here the cloning and sequencing of one of these genes, *vrg-6*. We found that the product of the *vrg-6* gene is required for full virulence of *B. pertussis* in a mouse model of respiratory infection.

The *vrg-6* gene does not appear to be involved in the pathogenesis of other members of the genus *Bordetella*. While Southern blot analysis shows that the chromosomes of *B. bronchiseptica* and *B. parapertussis* contain sequences homologous to the *vrg-6* gene, these loci are not expressed. This is reminiscent of the pertussis toxin (*ptx*) operon, which is expressed only from the chromosome of *B. pertussis*. Both *B. parapertussis* and *B. bronchiseptica* bear intact *ptx* operons which code for enzymatically functional S1 subunits when expressed in *E. coli*, yet these genes are not expressed in these strains because of a number of mutations in their respective promoters (1). We believe that this also is the reason that the *vrg-6* loci are silent in the other *Bordetella* species. We have found that *vrg-6*::Tn*phoA* and *vrg-18*::Tn*phoA* gene fusions produce modulatable levels of alkaline phosphatase activity when conjugated into *B. bronchiseptica* (data not shown), suggesting that the *trans*-acting regulatory elements required for *vrg* regulation occur in species other than *B. pertussis*. Therefore, mutations in the *vrg-6* loci of these other species that prevent their expression must be present.

Many pathogenic bacteria possess genetic regulatory mechanisms for controlling the expression of toxins, pili, hemolysins, and other coordinately regulated virulence factors. Like the *vir* system in *B. pertussis*, these regulons often include repressed genes. The *toxR* gene of *Vibrio cholerae* codes for the major regulator in a complex cascade of gene expression that leads to production of cholera toxin, TCP pili, and other coregulated factors. ToxR also negatively controls the expression of a number of proteins, including the major outer membrane protein OmpT (21), either directly or through the action of other regulatory proteins such as ToxT (7). Similarly, in *Salmonella typhimurium*, the two-component regulatory system of *phoP* and *phoQ* controls virulence and macrophage survival through the action of PhoP-activated genes (*pag*) (19, 20). The PhoP regulon also includes PhoP-repressed genes (*prg*), and a mutation in one of these, *prgH*, leads to an approximately 10-fold increase in the 50% lethal dose of *S. typhimurium* when mice are injected intraperitoneally (3, 18). The gram-positive organism *Staphylococcus aureus* possesses a regulatory locus called *agr* which is required for post-exponential-phase expression of several secreted proteins involved in *S. aureus* pathogenesis. However, null mutations in *agr* lead to elevated expression of protein A and coagulase, both of which are needed for full virulence (23, 27). It is not clear in any of these systems at what stage in the infection cycle the

repressed gene products are required or what the specific nature of their contribution to virulence and their role in pathogenesis are.

The sequences of the *vrg-6* gene and its predicted protein product may provide insights into the importance of *vir*-repressed genes in the pathogenesis of whooping cough. One notable feature of the Vrg-6 protein is the five alkaline-rich repeated sequences found in the C terminus and predicted to be exposed on the surface of the protein (Fig. 2). It is tempting to speculate that this Arg-Gly-His repeat is functionally related to the Arg-Gly-Asp repeat found in filamentous hemagglutinin (28), pertactin (4, 14), and a number of other prokaryotic and eukaryotic proteins that bind to cell matrix proteins of the integrin superfamily and thus facilitate adherence to host cells (25). It is possible that the Arg-Gly-His repeats of Vrg-6 provide an analogous function and allow this protein to act as an accessory colonization factor for *B. pertussis*.

The other notable feature of the Vrg-6 sequence is the seven proline-rich repeated sequences found in the middle of the protein. These repeated sequences are predicted to be highly hydrophobic and to have a very low surface probability, suggesting that this major structural feature is poorly immunogenic (Fig. 2). In fact, we have been unable to raise antibodies to any *vir*-repressed gene product when immunizing with modulated or phase variant strains or when immunizing with conjugated peptides of Vrg-6, even though high titers of antibodies to *vag* gene products were apparent (data not shown). These results suggest that the function of *vrg-6* and other *vir*-repressed gene products may be to provide relatively nonantigenic surface proteins for the bacterium, which would facilitate evasion of the host immune response.

A goal of considerable importance is identifying the specific stage of the infection cycle at which *vrg-6* and other *vrg* genes contribute to the virulence of *B. pertussis*. The *vrg* genes may be expressed early in the infection cycle, when the organism is at low numbers and when high-level expression of the highly immunoreactive *vir*-activated gene products could lead to premature clearance by the host immune system. At this stage, the *vrg* products might provide an adherence function when other factors such as pertactin and filamentous hemagglutinin are underproduced. Alternatively, the *vir*-repressed gene products may operate at a late stage in infection, when the host immune response is maximal and when there would be selective pressure favoring phase variants that have lost the ability to express *vir*-activated genes. At this time, it would be advantageous for the bacterium to be nonimmunogenic. This is consistent with the isolation of phase variants from convalescent individuals and the apparently poor antigenicity of phase variant bacteria and of the Vrg-6 protein itself. If Vrg-6 acts as an alternative colonization factor, as proposed above, its functioning late in the infection cycle combined with the poor antigenic state of the organism may be an effective strategy in prolonging colonization even in the face of an effective host immune response.

A third possibility is that the *vir*-repressed gene products are produced in specific niches during the course of infection and that in such niches, modulating signals are provided by the host and serve to elevate *vrg* expression. One such site is within the cytoplasm of host cells. *B. pertussis* has been shown to enter into and survive within eukaryotic cells in tissue culture (8, 13, 29) and in mice and rabbits (5, 29), and the intracellular environment appears to be a modulating one (17). The *vrg* gene products may function to facilitate intra?cellular survival and allow the persistence of the organism within the host.

The possibilities described above are based on the assumption that the absence of the *vrg-6* gene product has a direct effect on the ability of *B. pertussis* to colonize and persist in the lungs of mice. Therefore, the low level of bacteria in the lung of an SK6-infected mouse would be insufficient to produce lymphocytosis, despite the ability of this strain to produce pertussis toxin in vitro. However, the product of the *vrg-6* gene may promote colonization in an indirect fashion, perhaps by facilitating the delivery of *vir*-activated gene products to the epithelium of the respiratory tract. In such a case, the ability of SK6 to produce virulence factors in vitro would not reflect its ability to produce and deliver these factors at the site of infection. Both scenarios suggest that an intact *vrg-6* gene is critical to the pathogenesis of *B. pertussis*.

The existence of regulatory systems that respond to environmental signals and control virulence gene expression implies that pathogenic bacteria must regulate the production of their virulence factors in a precise way in order to produce infection. In this paper, we have reported that the production of *vir*-activated virulence factors by *B. pertussis* is not sufficient to promote infection and that *vir*-repressed gene products are important. Independent support for this conclusion comes from preliminary observations that mutant strains of *B. bronchiseptica* unable to undergo antigenic modulation in response to environmental signals (*bvgS* constitutive) are deficient relative to wild-type strains in their abilities to colonize the respiratory tracts of guinea pigs (17a). Together, these data indicate that *B. pertussis* must express both *vag* and *vrg* genes through the process of modulation in order to successfully parasitize the host. Efforts are currently under way in this laboratory to determine the mechanism by which *vrg* gene products facilitate infection by *B. pertussis*.

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REFERENCES

1. **Arico, B., and R. Rappuoli.** 1987. *Bordetella parapertussis* and *Bordetella bronchiseptica* contain transcriptionally silent pertussis toxin genes. J. Bacteriol. **169**:2847–2853.
2. **Beattie, D. T., S. Knapp, and J. J. Mekalanos.** 1990. Evidence that modulation requires sequences downstream of the promoters of two *vir*-repressed genes of *Bordetella pertussis*. J. Bacteriol. **172**:6997–7004.
3. **Behlau, I., and S. I. Miller.** 1991. Negative regulation of *Salmonella* virulence by the PhoP/PhoQ two component regulatory system. Abstr. 91st Gen. Meet. Am. Soc. Microbiol. B-4, p. 26.
4. **Charles, I. G., G. Dougan, D. Pickard, S. Chatfield, M. Smith, P. Novotny, P. Morrissey, and N. F. Fairweather.** 1989. Molecular cloning and characterization of protective outer membrane protein P.69 from *Bordetella pertussis*. Proc. Natl. Acad. Sci. USA **86**:3554–3558.
5. **Cheers, C., and D. F. Gray.** 1969. Macrophage behavior during

the complaisant phase of murine pertussis. Immunology 17:875–887.
6. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12:387–395.
7. **DiRita, V. J., C. Parsot, G. Jander, and J. J. Mekalanos.** 1991. Regulatory cascade controls virulence in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA 88:5403–5407.
8. **Ewanowich, C. A., A. R. Melton, A. A. Weiss, R. K. Sherburne, and M. S. Peppler.** 1989. Invasion of HeLa 229 cells by virulent *Bordetella pertussis*. Infect. Immun. 57:2698–2704.
9. **Finn, T. M., R. Shahin, and J. J. Mekalanos.** 1991. Characterization of *vir*-activated Tn*phoA* gene fusions in *Bordetella pertussis*. Infect. Immun. 59:3273–3279.
10. **Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium meliloti*. Gene 18:269–296.
11. **Knapp, S., and J. J. Mekalanos.** 1988. Two *trans*-acting regulatory genes (*vir* and *mod*) control antigenic modulation in *Bordetella pertussis*. J. Bacteriol. 170:5059–5066.
12. **Lacey, B. W.** 1960. Antigenic modulation of *Bordetella pertussis*. J. Hyg. 58:57–93.
13. **Lee, C. K., A. L. Roberts, T. M. Finn, S. Knapp, and J. J. Mekalanos.** 1990. A new assay for invasion of HeLa 229 cells by *Bordetella pertussis*: effects of inhibitors, phenotypic modulation and genetic alterations. Infect. Immun. 58:2516–2522.
14. **Leninger, E., M. Roberts, J. G. Kenimer, I. G. Charles, N. Fairweather, P. Novotny, and M. J. Brennan.** 1991. Pertactin, an Arg-Gly-Asp-containing *Bordetella pertussis* surface protein that promotes adherence of mammalian cells. Proc. Natl. Acad. Sci. USA 88:345–349.
15. **Leslie, P. H., and A. D. Gardner.** 1932. The phases of *Haemophilus pertussis*. J. Hyg. 31:423–434.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Masure, H. R., and J. Barrett.** 1991. The adenylate cyclase toxin undergoes phenotypic modulation when *Bordetella pertussis* enters human macrophages. Abstr. 91st Gen. Meet. Am. Soc. Microbiol. B-284, p. 73.
17a. **Miller, J. F., D. Monack, and S. Falkow.** Personal communication.
18. **Miller, S. I.** 1991. PhoP/PhoQ: macrophage specific modulators of *Salmonella* virulence. Mol. Microbiol. 5:2073–2078.
19. **Miller, S. I., A. M. Kukral, and J. J. Mekalanos.** 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. USA 86: 5054–5058.
20. **Miller, S. I., and J. J. Mekalanos.** 1990. Constitutive expression of the PhoP regulon attenuates *Salmonella* virulence and survival within macrophages. J. Bacteriol. 172:2485–2490.
21. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. 170:2575–2583.
22. **Mooi, F. R.** 1988. Virulence factors of *Bordetella pertussis*. Antonie von Leeuwenhoek 54:465–474.
23. **Patel, A. H., P. Nowlan, E. D. Weavers, and T. Foster.** 1987. Virulence of protein A-deficient and alpha-toxin-deficient mutants of *Staphylococcus aureus* isolated by allele replacement. Infect. Immun. 55:3103–3110.
24. **Peppler, M. S.** 1982. Isolation and characterization of isogenic pairs of domed hemolytic and flat nonhemolytic colony types of *Bordetella pertussis*. Infect. Immun. 35:840–851.
25. **Pierschbacher, M. D., and E. Ruoslahti.** 1984. Variants of the cell recognition site of fibronectin that retain attachment-promoting activity. Proc. Natl. Acad. Sci. USA 81:5985–5988.
26. **Pusztai, Z., and I. Joo.** 1967. Influence of nicotinic acid on the antigenic structure of *Bordetella pertussis*. Ann. Immunol. Hung. 10:63–70.
27. **Recsei, P., B. Kreiswirth, M. O'Reilly, P. Schlievert, A. Gruss, and R. Novick.** 1985. Regulation of exoprotein expression by *agr*. Mol. Gen. Genet. 202:59–61.
28. **Relman, D. A., M. Domenighini, E. Tuomanen, R. Rappuoli, and S. Falkow.** 1989. Filamentous hemagglutinin of *Bordetella pertussis*: nucleotide sequence and crucial role in adherence. Proc. Natl. Acad. Sci. USA 86:2637–2641.
29. **Saukkonen, K., C. Cabellos, M. Burroughs, S. Prasad, and E. Tuomanen.** 1991. Integrin-mediated localization of *Bordetella pertussis* within macrophages: role in pulmonary colonization. J. Exp. Med. 173:1143–1149.
30. **Stibitz, S., W. Aaronson, D. Monack, and S. Falkow.** 1989. Phase variation in *Bordetella pertussis* by frameshift mutation in a gene for a novel two-component system. Nature (London) 338:266–269.
31. **von Gabain, A., J. G. Belasco, J. L. Schottel, A. C. Y. Chang, and S. N. Cohen.** 1983. Decay of mRNA in *Escherichia coli*: investigation of the fate of specific segments of transcripts. Proc. Natl. Acad. Sci. USA 80:653–657.
32. **Weiss, A. A., and M. S. Goodwin.** 1989. Lethal infection by *Bordetella pertussis* in the infant mouse model. Infect. Immun. 57:3757–3764.
33. **Weiss, A. A., and E. L. Hewlett.** 1986. Virulence factors of *Bordetella pertussis*. Annu. Rev. Microbiol. 40:661–686.
34. **Weiss, A. A., E. L. Hewlett, G. A. Myers, and S. Falkow.** 1983. Tn*5*-induced mutations affecting virulence factors of *Bordetella pertussis*. Infect. Immun. 42:33–41.
35. **Weiss, A. A., A. R. Melton, K. E. Walker, C. Andraos-Selim, and J. J. Meidl.** 1989. Use of the promoter fusion transposon Tn*5* *lac* to identify mutations in *Bordetella pertussis vir*-regulated genes. Infect. Immun. 57:2674–2682.
36. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119.