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Genetic Analysis of a Region of the *Bordetella pertussis* Chromosome Encoding Filamentous Hemagglutinin and the Pleiotropic Regulatory Locus *vir*

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The *vir* locus of *Bordetella pertussis* apparently encodes a *trans*-acting positive regulator that is required for the coordinate expression of genes associated with virulence: pertussis toxin, filamentous hemagglutinin (FHA), hemolysin, and adenylate cyclase toxin. DNA clones of *vir* and of genes required for the synthesis of some of the factors under *vir* control were obtained with DNA probes from the chromosomal DNA surrounding sites of Tn*5* insertion mutations that inactivated those genes. Two *vir* clones were found which also contained genes required for the proper expression of FHA in *B. pertussis*. The plasmids which contained both the *fha* and *vir* genes expressed immunologically reactive FHA in *Escherichia coli*, as detected by colony blots, whereas plasmids which contained only *fha* or *vir* were negative in this assay. The regulation of FHA production in *E. coli*, as in *B. pertussis*, was temperature dependent and inhibited by high concentrations of either magnesium ions or nicotinic acid, indicating that the sequences cloned in *E. coli* contained the information required to preserve the physiological responses seen in *B. pertussis*. Further characterization of the *vir-fha* clones by Tn*5* mutagenesis in *E. coli* and by the return of cloned sequences to *B. pertussis* in *trans* and to the *B. pertussis* chromosome led to the localization of the *vir* locus, the structural gene for FHA, and genes that are possibly required for the synthesis and export of FHA.

Bordetella pertussis, the causative agent of whooping cough, coordinately regulates the production of toxins and other factors that are associated with its ability to cause disease (36). These virulent-phase genes include at least four toxins (pertussis toxin, adenylate cyclase toxin, dermonecrotic toxin, and hemolysin) and an attachment factor, the filamentous hemagglutinin (FHA).

Two forms of regulation control the expression of these genes. The first is a coordinate, reversible repression influenced by the conditions of bacterial growth. *B. pertussis* does not express the virulent-phase genes when grown at temperatures lower than 37°C or in the presence of high concentrations of magnesium ions or nicotinic acid (15, 18). In addition, *B. pertussis* strains undergo a metastable genetic event (phase variation) such that, at a frequency as high as 10^{-3} for some strains, variants arise in the population that no longer express any of the virulent-phase genes (20). These are called avirulent-phase derivatives. This switch is reversible in that these strains can revert to the virulent phase (36). We have previously shown that a single genetic locus, *vir*, can control the expression of the virulent-phase genes by showing that insertion mutations at this locus abolish the expression of multiple virulence-associated traits. We have hypothesized (36) that *vir* encodes a *trans*-acting gene product that acts as an activator for other genes. Virulent-phase bacteria synthesize the proteins under the control of *vir*; avirulent-phase bacteria do not.

Other investigators (4, 21, 27, 31) have reported an inability to detect expression in *Escherichia coli* of the *B. pertussis* factors under *vir* control. We also have been unsuccessful in identifying pertussis toxin clones using assays which required the expression of this gene in *E. coli*. This led us to investigate whether the *trans*-acting inducer encoded by *vir* promotes gene expression in *E. coli* as well as in *B. pertussis*.

As a first step in this investigation, we undertook the cloning of the *vir* locus and other genes that are regulated by the *vir* locus. These cloning experiments led to the isolation of a region of the *B. pertussis* chromosome encompassing the *vir* and *fha* loci. In *E. coli* this region directs the synthesis of a product that is immunoreactive with anti-FHA. This synthesis is modulated by environmental conditions in the same way as it is in *B. pertussis*. Characterization of this region by Tn*5* mutagenesis identified sequences that are essential for the synthesis of FHA in *E. coli*. The return of these cloned sequences to *B. pertussis* allowed the assignment of specific regions to the *vir* locus, to the structural gene for FHA, and to genes for other functions that are possibly required for the synthesis and export of FHA.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* and *B. pertussis* strains used in this study are described in Table 1. BP371-1 was derived from BP370-1 in a manner identical to that described for BP369-3 (36). UW901 was constructed from AB1133 (1) by Ron Gill and involved two P1 transduction steps. One was by selection for thr^+ to introduce *hsdR4*, and the other was by selection for tetracycline resistance to introduce *srl*::Tn*10* and *recA*. The plasmids used in this study are presented in Table 2.

Bacterial conjugations. Techniques for the transfer of plasmids from *E. coli* to *B. pertussis* strains have been described previously (33).

Growth media. *B. pertussis* was grown on Bordet-Gengou agar (26), and *E. coli* was grown on L agar or minimal A agar

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TABLE 1. Bacterial strains used in this study

Strain	Genotype or phenotype	Reference
B. pertussis		
BP338	Parental strain, virulent phase	37
BP347	*vir-1*::Tn*5*, avirulent mutant	37
BP348	*hly-1*::Tn*5*, hemolysin and adenylate cyclase deficient	37
BP353	*fha-1*::Tn*5*, FHA deficient	37
BP354	*fha-2*::Tn*5*, FHA deficient	37
BP357	*ptx-2*::Tn*5*, reduced in pertussis toxin production	37
BP359	*vir-2*::Tn*5*, avirulent mutant	36
BP368-3	Virulent-phase derivative of BP326	36
BP369-3	Avirulent-phase derivative of BP368-3	36
BP370-1	Virulent-phase derivative of BP369-3	33
BP371-1	Avirulent-phase derivative of BP370-1	This study
Tohama III	Avirulent phase	36
E. coli		
HB101	F$^-$ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*	23
UW901	*leu-6 argE3 proA2 his-4 thi-1 galK2 ara-14 xyl-5 mtl-1 supE44 lacY1 str-31 hsdR4 recA srl*::Tn*10*	This study
K802	*hsdR hsdM gal met supE*	23

(24). *E. coli* clones were selected with 100 μg of ampicillin per ml, 50 μg of kanamycin per ml, or 30 μg of chloramphenicol per ml. Ampicillin-resistant *E. coli* was grown on medium containing 1 mg of ampicillin per ml. For studies on the regulation of FHA expression, *E. coli* clones were grown on L agar containing 5.0 mg of $MgCl_2$ per ml and 5.0 mg of NaCl per ml or on L agar supplemented with 0.625 mg of nicotinic acid per ml.

DNA isolation and manipulation. Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs, Inc. (Beverly, Mass.) and Bethesda Research Laboratories (Gaithersburg, Md.) and were used according to the instructions of the manufacturers. Cosmid clone banks were constructed in pHC79 from high-molecular-weight chromosomal DNA which was partially digested with *Sau*3A, as described previously (14).

Complementation of amino acid auxotrophy. *E. coli* UW901 was transduced with the cosmid bank from *B. pertussis* and plated onto L agar containing ampicillin. After overnight growth at 37°C, all colonies were picked onto the same medium, allowed to grow, and then replica plated onto minimal A agar supplemented with three of the four necessary amino acids (arginine, leucine, proline, and histidine) at 10 μg/ml and scored for growth after 48 h at 37°C.

Generation of DNA probes from Tn*5* insertion mutants. In a previous study (37), Tn*5* insertion mutants in the *B. pertussis* chromosome were isolated, and mutants deficient in the ability to produce specific virulence factors were identified. The phenotypes of these mutants are described in Table 1. To obtain clones containing genes of interest without the requirement that those genes be expressed in *E. coli*, we took advantage of the fact that Tn*5* insertions tag a gene with resistance to kanamycin. Chromosomal DNA from several different Tn*5* insertion mutants was isolated and purified on $CsCl_2$ density gradients (14). Random fragments of DNA were generated by partial digestion with *Sau*3A (14), and these fragments were separated by size on an agarose gel. Since Tn*5* is 5.8 kilobases (kb) in length, the cloning of larger fragments ensures that the surrounding *B. pertussis* DNA is included in clones that are selected for kanamycin resistance. Fragments in the size range of 9 to 20 kb were cut from the gel, separated from the agarose by electroelution, and ligated to *Bam*HI-digested pHC79. These ligations were transformed into HB101, and kanamycin-resistant colonies were selected. In this way, plasmids pUW974 and pUW975b were isolated (Table 2).

Cloning of the intact *vir* and *fha* loci. Purified restriction fragments from plasmids pUW974 and pUW975b were ^{32}P

TABLE 2. Plasmids used in this study

Plasmid	Description	Reference
pACYC184	Cloning vector compatible with pHC79	6
pHC79	Cosmid cloning vector based on pBR322	13
pRK290	Broad-host-range cloning vector derived from RK2	8
pRK2013	Tra functions of RK2 cloned in ColE1	10
pRTP1	Vector for return of cloned sequences to the *B. pertussis* chromosome	33
pSS528	pRK290 *Bam*HI subclone of pUW21-26 containing *vir* and *fhaB'*	This study
pSS584	pRK290 *Bam*HI subclone of pUW21-26 containing *vir* and part of Tn*5*	This study
pUW11-25	BP338 clone homologous to pUW974	This study
pUW13-15	BP338 clone homologous to pUW975b	This study
pUW15-26	BP338 clone homologous to pUW975b	This study
pUW21-2	BP338 clone homologous to pUW974 and pUW975b	This study
pUW21-26	BP338 clone homologous to pUW974 and pUW975b	This study
pUW974	Kanamycin-resistant clone from BP347	This study
pUW975b	Kanamycin-resistant clone from BP353	This study
pUW1004	pACYC184 *Bam*HI subclone of pUW21-26 containing *vir* and *fhaB'*	This study
pUW1006	*Bam*HI subclone of pUW21-26 containing *fhaC* and pHC79	This study

labeled by nick translation and used as probes in a colony hybridization assay to detect *E. coli* clones containing gene inserts derived from wild-type strain BP338 (23). Several plasmids homologous to sequences surrounding the site of Tn*5* insertion were identified with each probe. In this way, cosmids pUW11-25, pUW13-15, pUW15-26, pUW21-2, and pUW21-26 were isolated (Table 2).

Colony immunoblot and Western blot analysis. For colony immunoblots, *E. coli* was streaked onto sterile nitrocellulose filters on the surface of L agar containing appropriate antibiotics, other supplements, or both and was allowed to grow. To remove the bacteria, the filters were washed in 0.5% bovine serum albumin in 50 mM Tris hydrochloride (pH 7.5)–0.15 M NaCl–0.25% gelatin–0.15% sodium azide–0.1% Nonidet P-40 (7). For Western blot analysis, an approximately equal mass of *B. pertussis* cells was removed from Bordet-Gengou agar, suspended in sample buffer, boiled, and electrophoresed on polyacrylamide gels as described by Laemmli (19). Transfer of the proteins to nitrocellulose membranes was done as described by Burnette (5). In both cases nitrocellulose membranes were washed, exposed to antiserum, and processed with ^{125}I-labeled staphylococcal protein A to reveal antibody binding, as described previously (7). Polyclonal rabbit antiserum to purified FHA (a gift from J. L. Cowell) and polyclonal rabbit antiserum to pertussis toxin (a gift from E. L. Hewlett) were preadsorbed with avirulent *B. pertussis* BP326 (36) and *E. coli* HB101 containing the plasmid pHC79. The FHA antiserum that was used has previously been shown (16, 37) to inhibit hemagglutination of sheep erythrocytes by FHA and to detect only three major bands in Western blot analysis in a pattern similar to that obtained with monoclonal antibodies to FHA. Monoclonal antibodies F1, F2, and F5 to FHA were provided as mouse ascites fluid and were a gift from L. I. Irons (16).

Southern blotting analysis. Chromosomal DNA samples were digested with restriction enzymes and electrophoresed on agarose gels by standard methods (23). Subsequent transfer to nitrocellulose and hybridization to labeled DNA probes were by the method of Smith and Summers (32).

Mapping of the plasmid pUW21-26 by Tn*5* mutagenesis. HB101 containing the cosmid pUW21-26 was used as the recipient in a transduction assay with a phage lambda::Tn*5* suicide vector, as described by Ruvkun and Ausubel (30), to obtain Tn*5* insertions that were located randomly in this strain. Several separate transductions were performed to ensure the isolation of genetically independent mutations. Kanamycin-resistant transductants from each transduction were pooled, and plasmid DNA was isolated by the method of Birnboim and Doly (2). Tn*5* insertions within the cosmid were isolated by transformation into HB101 and selection for kanamycin resistance. These insertions were assayed by colony blots for their ability to direct the synthesis of immunoreactive FHA. The site of insertion of Tn*5* was determined by restriction analysis with the restriction enzymes *Bam*HI, *Cla*I, *Eco*RI, and *Hin*dIII.

HeLa cell adhesion assay. HeLa cells were cultured in RPMI 1640 medium (Irvine Biochemical) supplemented with 5 mM glutamine (GIBCO Laboratories, Grand Island, N.Y.) and 5% fetal calf serum (GIBCO). Monolayers grown on glass cover slips in glass vials were washed twice with phosphate-buffered saline, and fresh medium was replaced. Approximately 10^7 bacteria were grown in Stainer-Scholte medium (17), washed once, suspended in phosphate-buffered saline, and added to the HeLa cells in 0.05-ml portions. Vials were centrifuged gently (1,500 rpm [J-6; Beckman Instruments, Inc., Fullerton, Calif.] for 10 min); and HeLa cell monolayers were then washed 5 times with phosphate-buffered saline, fixed in methanol for 2 min, and stained with Giemsa stain.

Introduction of Tn*5* insertions in *fhaB* into the *B. pertussis* chromosome. Insertion of Tn*5* into the approximately 10-kb *Eco*RI fragment of pUW21-26 containing the *fhaB* locus created a fragment of approximately 16 kb. This fragment from a number of derivatives of pUW21-26 containing Tn*5* inserted at different positions was cloned into the cloning vector pRTP1. The recombinant plasmids were introduced into BP338 by conjugation. Kanamycin-resistant exconjugants should have had the recombinant plasmid integrated into the *B. pertussis* chromosome via recombination, such that the pRTP1 vector was flanked by direct repeats of the cloned segment. These strains were maintained on Bordet-Gengou agar containing kanamycin, and individual colonies were screened for the loss of the ampicillin resistance determinant of pRTP1 by recombination between these repeats. Such ampicillin-sensitive derivatives should have had the chromosomal *fhaB* locus replaced with that containing the specified Tn*5* insertion.

Deletion analysis of pUW21-26. *B. pertussis* DNA contains very few *Hin*dIII sites. The cosmid pUW21-26 contained no *Hin*dIII sites in the inserted DNA, and thus, it had only the single *Hin*dIII site present in the vector sequences. We took advantage of this and the fact that the Tn*5* insertion introduces two *Hin*dIII sites, to create deletions of pUW21-26. Different Tn*5* insertion derivatives of pUW21-26 were digested with *Hin*dIII, religated, and transformed into HB101. Ampicillin-resistant transformants that contained the recircularized fragment containing vector sequences were isolated. These made up a set of deletion derivatives that contained various amounts of pUW21-26. These derivatives were assayed for their ability to allow the detection of immunoreactive FHA, both in the presence and absence of the plasmid pUW1004, containing the *vir* locus and a truncated *fhaB* locus.

RESULTS

Identification of *B. pertussis* cosmid clones which complement amino acid deficiencies in *E. coli*. In preliminary experiments, we attempted to clone the pertussis toxin gene of *B. pertussis* into *E. coli*. A cosmid gene bank prepared from *B. pertussis* BP338 chromosomal DNA and the cosmid cloning vector pHC79 was screened for the production of pertussis toxin by an immunological screening method (9). Among thousands of clones that were screened, none producing pertussis toxin was detected. This result led us to examine whether other genes, specifically, those involved in amino acid biosynthesis, could be expressed in *E. coli*.

The cosmid bank screened previously for pertussis toxin expression was transduced into *E. coli* UW901, which is auxotrophic for four amino acids. Arginine-complementing clones were detected at a frequency of 1/70, and leucine-complementing clones were detected at a frequency of 1/382. No clones complemented defects in histidine or proline pathways, even though *B. pertussis* does not require either amino acid for growth. We have subsequently demonstrated that clones of *B. pertussis* DNA can complement a mutation in the *E. coli recA* gene (C. Lory, S. Stibitz, B. Black, and S. Falkow, unpublished data). These results indicate that at least some genes from *B. pertussis* can be expressed directly in *E. coli*.

Since a genetic locus, *vir*, is required for the expression of *B. pertussis* virulence factors (36), we investigated whether

the *vir* gene product is also required in *E. coli* for the expression of virulence-associated traits.

Cloning and expression of *B. pertussis* virulence-associated loci. Approximately 1,000 colonies from a gene bank of *B. pertussis* BP338 were screened for homology with the four *B. pertussis* probes representing *vir*, *fha*, *ptx* (pertussis toxin), and *adc* (adenylate cyclase). Several independent, DNA probe-positive cosmids were obtained in each case. Most of the positive clones were homologous to only one probe. However, two of the four *fha* cosmids and two of the three *vir* cosmids were homologous with both the *vir* and *fha* probes. This indicates that *vir* and *fha* map close enough on the *B. pertussis* chromosomal DNA to be included on a single cosmid of about 45 kb.

The clones were tested for production of their associated gene products. The *ptx* cosmids were negative for the production of pertussis toxin when assayed by an immunological assay (colony blots with pertussis toxin antisera) and by a biological assay for functional toxin (using Chinese hamster ovary cells) (11). This was consistent with results of our previous screening of random clones. The *adc* cosmids were negative for the production of adenylate cyclase toxin when screened by an enzyme assay in vitro (12) and by assays for toxic activity in mouse S49 lymphoma cells in culture (38). Different results were obtained when the *fha* cosmids were screened.

The clones which hybridized with the *fha* probe were assayed for the production of FHA by colony immunoblots with antiserum to FHA. The two cosmids pUW21-2 and pUW21-26 which were DNA probe positive for both *vir* and *fha* directed the production of a substance that was immunologically cross-reactive to FHA. Strains containing those cosmids which hybridized with only the *vir* probe (pUW11-25) or the *fha* probe (pUW13-15 and pUW15-26) produced no material that reacted with FHA antiserum. The control strain containing only the vector pHC79 was also negative.

The FHA product synthesized in *E. coli* had no detectable hemagglutinating activity, and our attempts to detect it by Western blot analysis of polyacrylamide gels were not successful. It also did not react with monoclonal antibodies to FHA (data not shown). The exact nature of this product is therefore unknown. However, in spite of these limitations, we were able to use this assay to begin to define the genetic and molecular elements of the *vir* regulatory system in *E. coli*.

Regulation of FHA expression in *E. coli*. Since the expression of virulence-associated traits is regulated by environmental conditions in *B. pertussis*, we undertook a study to determine whether this form of regulation is also seen in the expression of FHA in *E. coli*. Strain HB101 containing both *vir* and *fha*-specific sequences was assayed by the colony blot procedure for the expression of FHA under the conditions of reduced temperature or increased magnesium ion or nicotinic acid concentrations. When it was grown at 23°C no FHA expression was detected. The synthesis of FHA was also found to be inhibited by high concentrations of magnesium ions or nicotinic acid. Thus, conditions that promote the phenotypic repression of FHA in *B. pertussis* have the same effect in *E. coli*.

Mapping of the essential sequences for FHA expression. A restriction map of one of the FHA-positive cosmids, pUW21-26, was obtained. The sites of the original Tn*5* insertions in strains BP347 and BP359 (Vir^-) and in strains BP353 and BP354 (FHA deficient) were determined by Southern blotting analysis. After digestion with the restriction enzymes *Bam*HI, *Cla*I, and *Eco*RI, chromosomal DNA from these mutant strains was transferred to nitrocellulose and probed with purified restriction fragments of pUW21-26, which was labeled with ^{32}P by nick translation. The sites of these four insertions are shown in Fig. 1 with reference to the physical map of pUW21-26.

The sequences necessary for FHA expression in *E. coli* were further defined by Tn*5* mutagenesis of pUW21-26. Tn*5* insertions which affected the FHA phenotype fell into the four separate regions shown in Fig. 1. We named these regions, from left to right, *vir*, *fhaB*, *fhaA*, and *fhaC*. *vir* and *fhaB* were separated by a single Tn*5* insertion which did not cause a loss of the FHA phenotype. Since Tn*5* insertions are polar, this implies that *vir* and *fhaB* represent two separate transcriptional units. *fhaA* is defined by a group of insertions spanning approximately 4 kb, which caused an apparent increase in FHA production, as assayed by colony immunoblots. A small cluster of insertions just to the right of *fhaA* gave a negative phenotype and identified *fhaC*. These mapping data suggest that both *fha*- and *vir*-associated sequences are required for FHA expression in *E. coli* and that at least three transcriptional units are involved. Each of the loci identified is discussed below.

The *vir* locus. The region on the map of cosmid pUW21-26, which we termed the *vir* locus, encodes a factor which acts in *trans*, in a positive fashion, to regulate the expression of multiple virulence-associated traits. Three lines of evidence support this claim. First, as described above, this region encompassed the sites of a cluster of Tn*5* insertion mutations which abolished the FHA phenotype in *E. coli* and which were not within the structural gene for FHA. Second, this region encompassed the sites of two Tn*5* insertion mutations in the *B. pertussis* chromosome which gave a Vir^- phenotype. Third, we were able to return this locus in *trans* to strains of *B. pertussis* that had a Vir^- phenotype and to show that their ability to synthesize multiple virulence-associated determinants was restored. This last set of experiments is discussed below.

To perform these experiments, we cloned the 14.7-kb *Bam*HI fragment of pUW21-26, which contains the *vir* locus, into the *Bgl*II site of pRK290 to create pSS528. The transfer of pSS528 to avirulent *B. pertussis* BP369-3 was achieved after conjugation with *E. coli* K802 containing pSS528 and pRK2013 and selection for the acquisition of the tetracycline resistance determinant of pRK290. The *B. pertussis* exconjugants arising from this mating had a Vir^+ phenotype, as determined by colonial morphology, in that colonies were domed in appearance and were hemolytic on Bordet-Gengou agar. If this merodiploid strain was streaked onto medium that lacked tetracycline, Vir^- derivatives arose at a high rate (approximately 50% in the population). These derivatives were shown to be tetracycline sensitive and had apparently lost the pSS528 plasmid. Thus, the restoration of the Vir^+ phenotype is apparently not due to recombination with the chromosome but to an effect that occurs in *trans*. These results demonstrate that at least one virulence-associated factor (hemolysin) is activated by the cloned *vir* locus. We therefore sought to determine whether other virulence factors were also affected.

To test for the expression of pertussis toxin, whole-cell extracts of *B. pertussis* strains were examined by Western blot analysis. The BP369-3 exconjugants containing pSS528 synthesize pertussis toxin that is indistinguishable either by gel migration or level of expression from that of the Vir^+ derivatives BP368-3 and BP370-1 (Fig. 2).

The ability of *B. pertussis* strains to adhere to cultured human cells is another characteristic that is specific to the

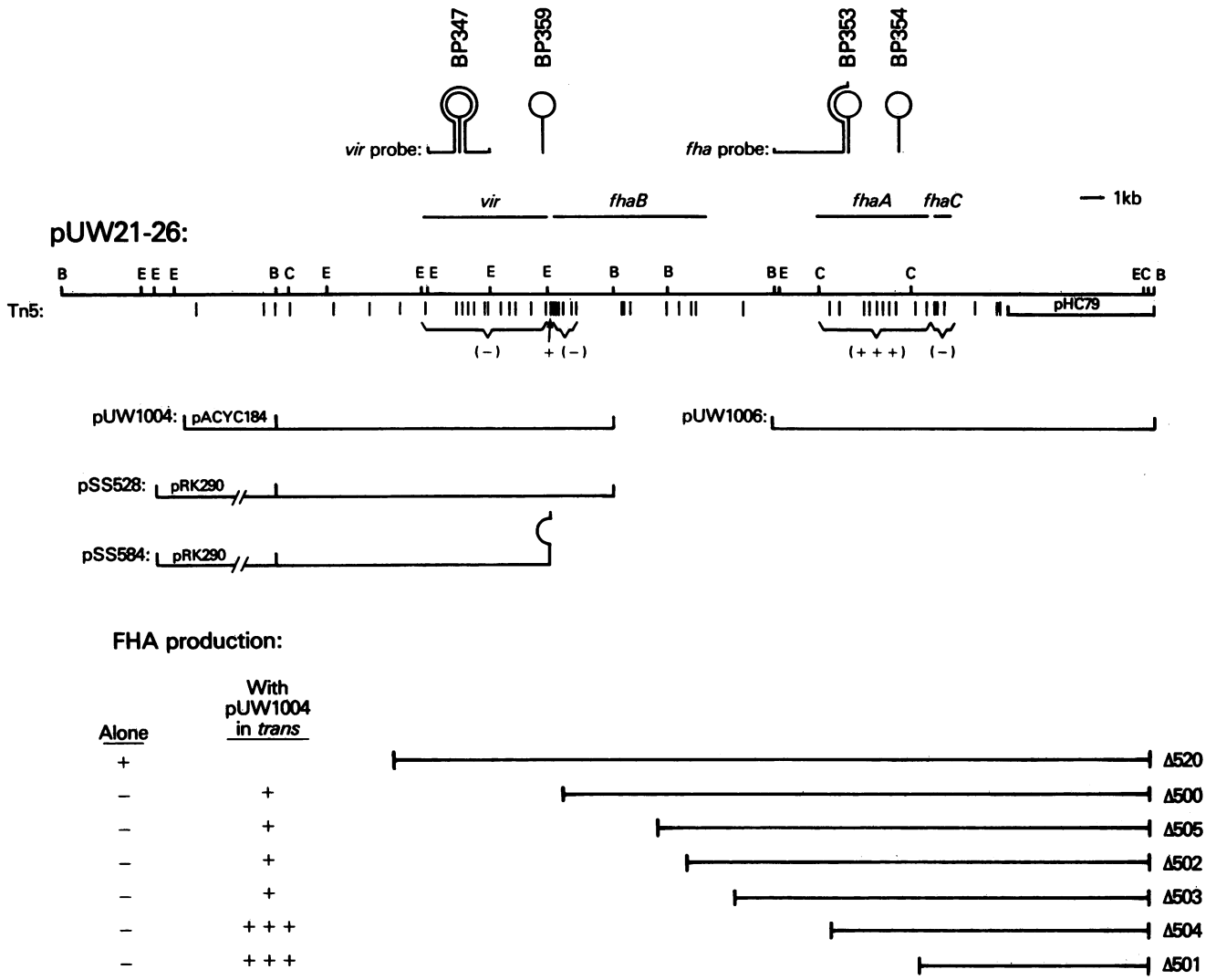

FIG. 1. Composite map of the *fha-vir* region. A restriction map of pUW21-26 is shown. Abbreviations for the restriction enzymes are as follows: B, *Bam*HI; C, *Cla*I; and E, *Eco*RI. The site of insertion of Tn*5* in the *B. pertussis* chromosome is shown for the mutant strains BP347 and BP359 (Vir$^-$) and for BP353 and BP354 (FHA deficient). The *Eco*RI fragment from pUW974 and the *Bam*HI fragment from pUW975b that were used as probes to detect pUW21-26 are shown in relation to these Tn*5* insertions. The sites of Tn*5* insertions isolated in pUW21-26 are depicted by short vertical lines below the map of pUW21-26. These insertions have a normal (+) phenotype, unless indicated otherwise in parentheses (−). Other plasmids derived from pUW21-26 are shown in register with the sequences that they contain. The vertical line and half circle on pSS584 represent the part of Tn*5* that was included in this plasmid. Shown in the lower part of the figure are deletions of pUW21-26. Solid lines indicate the extent of DNA sequences contained in the various derivatives. These plasmids in HB101 were assayed by colony immunoblotting for the production of FHA, in the presence and absence of the *vir*- and *fhaB'*-containing plasmid pUW1004, to give the results shown.

virulent phase. BP369-3 containing pSS528 apparently adhered as well as the Vir$^+$ strain BP368-3 did to cultured HeLa cells, while BP369-3 adhered poorly (Fig. 3).

Taken together, these results demonstrate that the presence in *trans* of the cloned 14.7-kb *Bam*HI fragment encompassing the *vir* locus is sufficient to render Vir$^-$ *B. pertussis* strains Vir$^+$. To delineate further the extent of the sequences required for Vir activity, we repeated the experiments described above with a derivative of pSS528 in which the region which we called *fhaB* was deleted. The effects of plasmid pSS584 (Fig. 1) on the expression of hemolysin and pertussis toxin were indistinguishable from those seen with pSS528 (data not shown). Thus, *fhaB* is not required for Vir activity.

The *fhaB* locus. The region on the map of pUW21-26 that we called *fhaB* contains the structural gene for FHA. Early evidence for this was seen when we examined the *vir* merodiploid strain BP369-3(pSS528) for expression of FHA. A Western blot of whole *B. pertussis* cells that was probed with rabbit antisera to purified FHA is shown in Fig. 4. Figure 4, lane a, shows the pattern that we usually obtained with BP338, the parental Vir$^+$ strain of *B. pertussis*, with major bands detectable at positions corresponding to polypeptides of approximately 200, 130, and 100 kilodaltons (kDa). When BP369-3(pSS528) was examined, only a single major band was observed, and this was at a position corresponding to a polypeptide of approximately 80 kDa. One explanation for this result is that the structural gene for FHA starts at the left end of *fhaB* and extends to the right. Thus, in pSS528 the gene is interrupted by cloning at the *Bam*HI

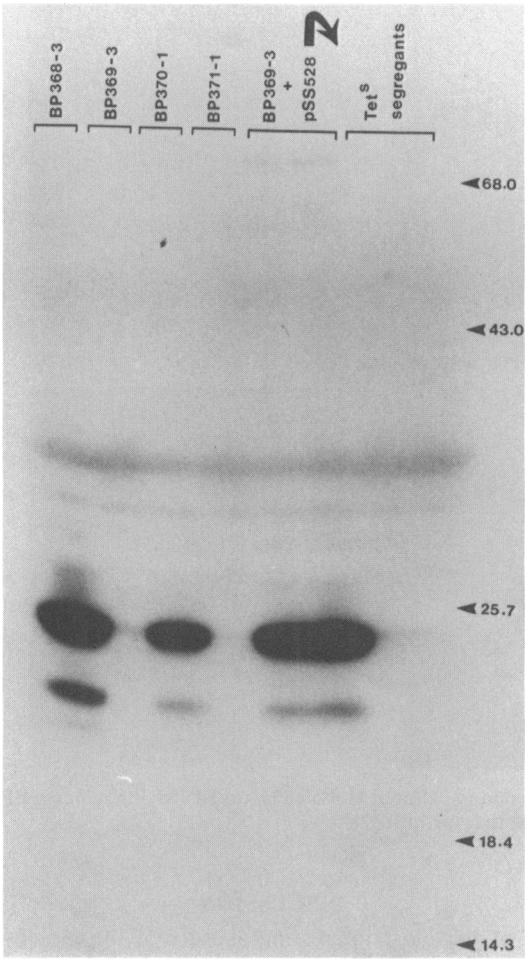

FIG. 2. Western blot analysis of *B. pertussis* strains for the production of pertussis toxin. Samples were as follows, from left to right: BP368-3 (Vir$^+$), BP369-3 (Vir$^-$), BP370-1 (Vir$^+$), BP371-1 (Vir$^-$), two exconjugants of BP369-3 containing pSS528, and two segregants of those two exconjugants which lost the pSS528 plasmid. Samples were treated and subjected to electrophoresis and transfer to a nitrocellulose membrane as described in the text. Pertussis toxin was detected by using rabbit antiserum against purified pertussis toxin. The migration of protein size standards is shown at the right. The molecular masses (in kilodaltons) of the standards are given on the right.

site and might be expected to yield a truncated FHA product. If the start of the FHA structural gene is located near the *Eco*RI site at the left end of *fhaB*, the expected size of a truncated polypeptide would be approximately 85 kDa, which is in good agreement with the observed Western blot pattern.

To test this hypothesis, we undertook to recombine into the *B. pertussis* chromosome Tn*5* insertions that were isolated previously in pUW21-26 and that were within the region expected to contain the FHA structural gene. If the hypothesis were true, for each insertion we would have expected to see a truncated FHA product with a size dependent on the site of the Tn*5* insertion.

The 10-kb *Eco*RI fragment from pUW21-26 contains the entire putative FHA structural gene. Fragments corresponding to this fragment but containing different Tn*5* insertions were cloned from the corresponding pUW21-26 derivatives into the cloning vector pRTP1. The resulting plasmids were then transferred by conjugation into BP338. As described above, recombinants were eventually obtained in which the cloned sequences containing the Tn*5* insertions replaced those that were originally present in the chromosome. The structure of the *fhaB* locus in these *B. pertussis* strains was verified by Southern blotting (data not shown).

Western blot analysis of these recombinant strains with FHA antiserum is shown in Fig. 5. Figure 5, lane a, shows BP338, the parental virulent-phase strain. It can be seen that for BP338 a variety of different-sized polypeptides that cross-reacted with anti-FHA were detected, with the largest being approximately 200 kDa. The other lanes show different derivatives of BP338 in which a Tn*5* insertion in *fhaB* was recombined into the chromosome. In all cases but Tn*5*-16 (Fig. 5, lane d), the largest product that was cross-reactive with anti-FHA was reduced in size relative to that seen for BP338 by an amount which depended on the site of the Tn*5* insertion. Tn*5*-16 was the insertion farthest to the right and was probably outside the 3′ end of the FHA gene. When the size of the largest product seen in the Western blot analysis for each strain was plotted versus the map position of the corresponding Tn*5* insertion, a linear relationship between these two parameters was observed. When one extrapolates to an FHA product of zero size, a map position for the start of the FHA gene is derived, as shown in Fig. 5. This position coincides with the leftmost limit of the cluster of Tn*5* insertions that were originally used to detect the *fhaB* gene. These results suggest that an approximately 6-kb open reading frame is present in the DNA sequence located just to the right of *vir* and that a polypeptide of approximately 200 kDa is encoded therein, from left to right, on the map of pUW21-26. We conclude that this is the structural gene for FHA.

The *fhaA* locus. *fhaA* encompasses approximately 4 kb on the map of pUW21-26 and includes the sites of Tn*5* insertions which resulted in an apparent overproduction of FHA in *E. coli*. It also includes the sites of insertion of Tn*5* in the two *B. pertussis* mutants BP353 and BP354 which had an FHA-deficient phenotype (37). We and others (3, 35) have observed that these mutant strains produce much lower but detectable levels of FHA. This FHA is apparently normal in size and retains biological activity. We therefore conclude, as have others (3), that this locus is involved in regulating the synthesis of FHA, the export of FHA, or both in *B. pertussis*.

The *fhaC* locus. *fhaC* is defined by three Tn5 insertions in pUW21-26 that eliminate the detection of FHA cross-reactive material in *E. coli* when assayed by colony immunoblot analysis. *fhaC* is able to complement *vir* and *fhaB′* (truncated by cleavage at the *Bam*HI site) to allow the detection of FHA in a colony immunoblot. When pUW1004 containing *vir* and part of *fhaB* was coresident with pUW1006 in *E. coli* HB101, FHA was detected in the colony immunoblot assay. Plasmid derivatives of pUW21-26 that were deleted to various extents were also tested for their ability to allow the detection of FHA when they were coresident with pUW1004. Nearly all of the insert of pUW21-26 was deleted without deleting *fhaC* and without affecting the detection of FHA (Fig. 1). However, when the deletion removed the *fhaC* locus (as was the case for pHC79 alone), the ability to complement pUW1004 was also removed and FHA detection was abolished (data not shown). As export would be necessary to detect FHA in the assay that we used, and as this locus was apparently neither *vir* nor the structural gene for FHA, we suggest that it may be required for the proper export of FHA to the outside of the cell.

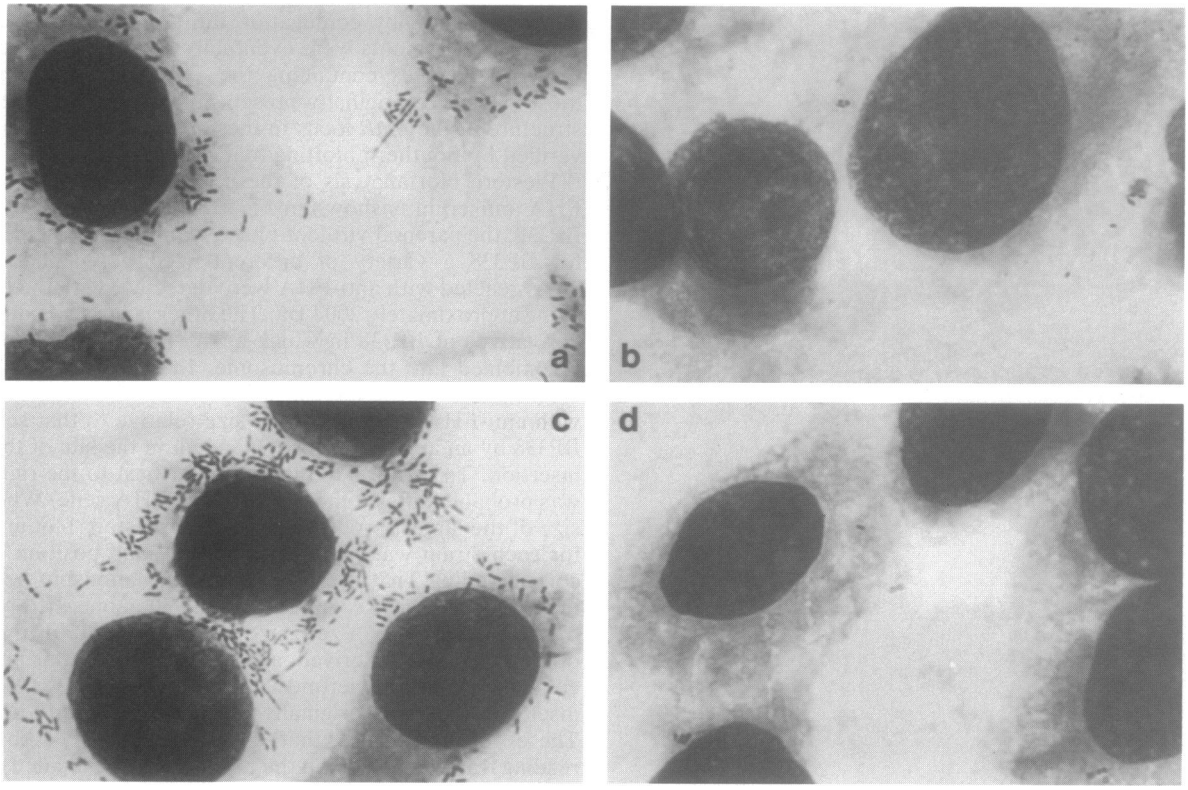

FIG. 3. HeLa cell adhesion assay. *B. pertussis* strains were assayed for adhesion to cultured HeLa cells. (a) BP368-3 (Vir^+); (b) BP369-3 (Vir^-); (c) BP369-3 harboring pSS528 (vir^+); (d) a segregant of BP369-3(pSS528) that lost pSS528.

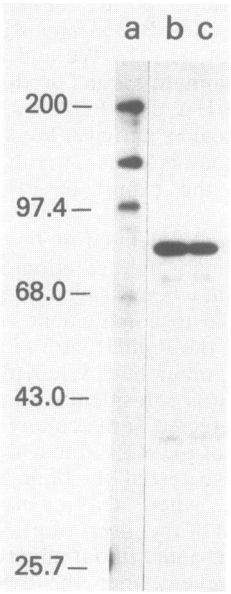

FIG. 4. Western blot analysis of *B. pertussis* strains for the production of FHA. Samples were treated as described in the text. Lane a, BP338; lanes b anc c, two exconjugants of BP369-3 harboring pSS528. The migrations of the protein size standards and their molecular masses (in kilodaltons) are shown on the left.

DISCUSSION

One of the most interesting aspects of the biology and pathogenesis of *B. pertussis* concerns the ability of this highly adapted pathogen to regulate the synthesis of the multiple factors that are involved in its interaction with the human host. In this report we have described our first steps in elucidating these regulatory mechanisms. We cloned from *B. pertussis* DNA sequences encompassing a locus whose role appears central in this system of regulation. Specifically, this *vir* locus is required for the expression of multiple virulence-associated traits, and its introduction in *trans* is sufficient to restore this expression to spontaneously occurring avirulent strains. In *E. coli* this locus is required for the expression of the cloned gene for FHA, a major *B. pertussis* adhesin.

The region which we have identified as the *vir* locus is approximately 5 kb. Although the biochemical nature of the *vir* gene product (Vir) remains to be determined, several possibilities are evident. Vir could be a positive regulatory DNA-binding protein analogous to the catabolite gene activator protein of *E. coli* or the *c*I repressor of phage lambda (28). Another attractive hypothesis is that Vir is an alternate sigma factor which, by associating with the core RNA polymerase, can change its promoter specificity such that virulence-associated genes are expressed. This would be similar to the system of regulation of sporulation seen in *Bacillus subtilis* (22). Given the ability of the *vir*-dependent regulatory system to respond to environmental influences, it also seems reasonable that Vir might be analogous to the *toxR* gene product of *Vibrio cholerae*, a membrane-spanning positive regulatory protein (25). However, the region we identified seems larger than that which would be necessary

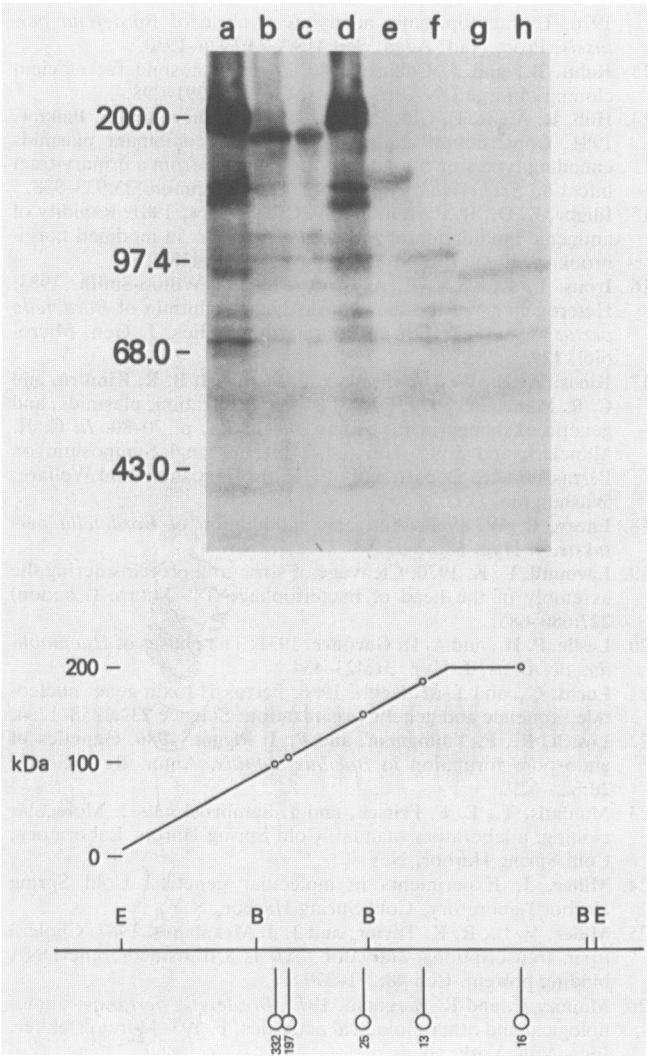

FIG. 5. Western blot analysis of *B. pertussis* strains for the production of FHA. Samples were treated as described in the text. Lane a, BP338; lanes b and c, BP338 Tn*5*-13; lane d, BP338 Tn*5*-16; lane 3, BP338 Tn*5*-25; lane f, BP338 Tn*5*-197; lanes g and h, BP338 Tn*5*-332. The migration of protein size standards is shown at the left. The molecular masses (in kilodaltons) of the size standards are given on the left. Shown below is a plot of the size of truncated FHA product versus the point of Tn*5* insertion. Restriction enzyme abbreviations: E, *Eco*RI; B, *Bam*HI.

to code for any one of these possibilities, and in fact, a combination of these or other activities may well be encoded. We are currently determining the DNA sequence of this region in the hope that we can learn more about the biochemical nature of Vir and the sequences which, we suppose, control its expression.

The synthesis of FHA in *E. coli*, which is under the control of *vir*, responds to the environmental signals of increased magnesium ion concentration, increased nicotinic acid concentration, and reduced temperature in a manner similar to that seen in *B. pertussis*. It is thus apparent that in the cloning of the FHA genes and the *vir* locus, we included the elements necessary for this control. There are many ways to envisage the role of *vir* in this control. In one the level of *vir* transcription and translation would respond to environmental signals, and Vir would thus be seen as an intracellular messenger of these signals. In this case *vir*-regulated genes would be sensitive only to the level of Vir. Alternatively, Vir might act together with these signals to influence gene expression. In this case *vir*-regulated genes would respond to Vir only under certain environmental conditions. Vir would thus be acting as a signal transducer. A third possibility is that Vir is only one factor that is necessary for gene expression at regulated loci and that environmental signals impinge in a relatively independent fashion through other mediators. It is hoped that further characterization will lead to a more complete understanding of how Vir acts and will allow us to distinguish between these possibilities.

Genes coding for and required for the synthesis and export of FHA are very close to and possibly contiguous with those for Vir. We localized the structural gene for FHA to a position just to one side of the *vir* locus. The term structural gene is really one of convenience here since FHA, as a biochemical entity, has resisted characterization. Other researchers have reported seeing a variety of sizes of polypeptides in purified preparations of FHA and in Western blot analyses with sera against purified FHA and with monoclonal antibodies to FHA (16). It is therefore difficult to say which proteins are actually required for FHA activity and which therefore constitute true structural components. It is apparent from the results of this study, however, that a large polypeptide, approximately 200 kDa, is encoded within a gene of approximately 6 kb and that this polypeptide is immunoreactive with anti-FHA.

Recently, Brown and Parker (3) have reported the cloning and expression of the structural gene for FHA. Our results are largely in agreement with theirs, and by comparing the two restriction maps of this region for *Eco*RI and *Cla*I, it is clear that we cloned overlapping regions. Our results differ from theirs, however, in terms of our assignment of the positions of the beginning and end of the FHA gene. Our assignment for both is shifted by approximately 3 kb in the left direction on both maps. It is worth noting that one of our insertions, Tn*5*-16, is within the FHA gene as assigned by Brown and Parker (3); however, it had no effect on the size of the FHA product seen in our Western blot analysis. At this time we are unable to explain the discrepancy between our results and those of Brown and Parker (3). We feel that these differences can be resolved by examining the expression of the *fha* locus directly in *B. pertussis*, in the single copy of the chromosome, and from native promoters.

At least two other loci, *fhaA* and *fhaC*, appear to be involved in the proper expression of FHA in *B. pertussis* and *E. coli*. On first examination, *fhaA* presents us with something of a paradox. Tn*5* insertions within this region in *B. pertussis*, as shown for BP353 and BP354, resulted in a much reduced expression of FHA. The FHA which is detected appears normal in size (3, 35) and is functional (35). However, insertions within the same region in plasmid pUW21-26 in *E. coli* caused an apparent overproduction of FHA, and in fact, the entire *fhaA* locus could be deleted without abolishing FHA expression. In fact, these results can be reconciled if one considers that BP353 and BP354 were characterized as FHA deficient because they had lower levels of FHA associated with the bacterial cell. The Tn*5* insertions in *fhaA* in *E. coli*, on the other hand, were assayed by a method which detects extracellular FHA. Both of these results are consistent with a proposed role for the product of *fhaA* locus being involved in the association of FHA with the bacterial cell surface.

fhaC was necessary for the detection of FHA in *E. coli* in our assay, but we have not yet analyzed the effect of

mutations in this locus in *B. pertussis*. It thus remains to be seen whether this locus affects the production of FHA or, possibly, other virulence-associated determinants in its native genetic environment.

The *vir*-dependent system of regulation is an impressive example of what is emerging as a major theme in the biology of pathogenic bacteria, that is, the coordinate regulation of multiple factors involved in pathogenesis by a positive regulatory element. Other examples include the *toxR* system in *V. cholerae* (34) and *agr* in *Staphylococcus aureus* (29). The full implications of these positive regulatory systems that are found to control pathogenic determinants is still unknown. However, it is clear that what we are learning about the regulation of pathogenesis underscores the notion that bacterial parasites are highly adapted for a life within the changing environments necessarily associated with a pathogenic life-style.

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