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Tn5-Induced Mutations Affecting Virulence Factors of *Bordetella pertussis*

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Transposon Tn5 was used to isolate mutants of *Bordetella pertussis*. Strains with Tn5 insertions were screened for loss of virulence-associated factors, including filamentous hemagglutinin, hemolysin, and pertussis toxin. Several mutants deficient for hemolysin production were obtained. All produced dermonecrotic toxin, pertussis toxin, and filamentous hemagglutinin, but were found to vary with respect to adenylate cyclase production. One hemolysin mutant had no detectable adenylate cyclase activity; others had 0.6% or 16% wild-type activity, whereas a fourth seemed to be unaffected in terms of adenylate cyclase activity. Mutants deficient in the ability to hemagglutinate sheep erythrocytes were also isolated. These mutants either failed to synthesize or produced reduced amounts of three protein species of 200,000, 130,000, and 100,000 daltons, all of which reacted with antiserum to filamentous hemagglutinin. Pertussis toxin mutants were identified by screening culture supernatants for failure to induce a clustered growth pattern in Chinese hamster ovary cells, and identification was confirmed by the standard histamine-sensitizing assay in mice. These mutants will be useful to determine the relative contribution of each virulence factor to pathogenicity as well as to determine the identity of the antigens important in protective immunity.

The bacterial vaccine for whooping cough (pertussis) is known to cause local reactions in the majority of recipients and, less frequently, serious systemic toxicity reactions including brain damage and death (7, 28). The vaccine consists of whole killed *Bordetella pertussis* cells in which some of the toxic factors of the microorganism are still active (14, 15). These toxic factors include pertussis toxin (23, 38) (also called islet-activating protein, lymphocytosis-promoting factor, or pertussigen) and extracytoplasmic adenylate cyclase (9, 17, 18, 42). Although the current vaccine affords significant protection, the identity of the antigens necessary and sufficient to confer immunity has not been established (34). Recent efforts to develop a safe and effective vaccine have been frustrated by lack of information on the pathogenesis of the natural infection (32, 33). We have attempted to study *B. pertussis* pathogenesis by generating a series of mutants, each deficient in a single factor believed to be important for virulence. Studies with mutations in a single gene would be useful for determining the contribution of a particular microbial determinant in the disease process.

B. pertussis has the ability to undergo a form of phase variation in which the cells no longer produce several determinants associated with the disease (12, 21, 22, 30, 31). These factors include pertussis toxin, extracytoplasmic adenylate cyclase, filamentous hemagglutinin (FHA), pili, cytochrome *d*-629, and several outer membrane proteins (12). We have found that the high frequent of occurrence of this phase shift makes it difficult to characterize mutants genetically by recombinational methods due to the high background of spontaneous negative mutants (41). The advantage of transposon mutagenesis is that usually only a single gene is mutated and the site of the insertion is marked both physically by the transposon DNA and genetically with an antibiotic resistance. In a previous study we described a plasmid vector that could be used to introduce transposons into the *B. pertussis* chromosome (41). In the present study, we used a similar transposon delivery system and transposon Tn5 as a mutagen, and herein we describe several mutants with single-site mutations that are deficient in virulence-associated factors of *B. pertussis*.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. The *B. pertussis* strains used in this study are listed in Table 1. The bacteria were grown in Stainer-Scholte (SS) broth

TABLE 1. Bacterial strains

Strain	Genotype or phenotype[a] (reference)
E. coli plasmid pUW964	pRK2013 *kan*::Tn*7* *xyz*::Tn*5* (this study)
B. pertussis	
Tohama I	Virulent phase (34)
Tohama III	Avirulent phase (34)
BP338	*nal-1*, derivative of Tohama I
BP326	*str-2 rif-2*, derivative of Tohama III
BP321	*str-3 rif-3*, derivative of Tohama I

[a] Allelic designations and resistance genes of pRK2013 are as follows: *nal*, nalidixic acid resistance; *str*, streptomycin resistance; *rif*, rifampin resistance; *kan*, kanamycin resistance.

or on Bordet-Gengou (BG) plates as described previously (40, 41). During the course of this study it became apparent that not all commercially available lots of BG agar permitted the identification of hemolysis. In this study we used Difco BG agar base (Difco Laboratories, Detroit, Mich.) lot 689592 supplemented with 15% sheep erythrocytes to detect hemolysis. Genetic matings were performed as previously described (41). When selecting for resistant bacteria, BG plates were supplemented with antibiotics at the following concentrations: kanamycin, 25 μg/ml; and nalidixic acid, 50 μg/ml.

Construction of a plasmid for generating Tn*5* insertion mutants. We have previously shown that ColE1 plasmids were not maintained in *B. pertussis*, and chimeric plasmids containing a ColE1 replication origin and P incompatibility plasmid conjugation genes could be used to introduce transposons Tn*7* and Tn*501* into *B. pertussis* (41). We constructed a similar chimeric plasmid, pUW964, for introducing Tn*5* into *B. pertussis* (Fig. 1). Initially, we used plasmid pRKTV5 (from N. Panapoulos, University of California, Berkeley), a derivative of plasmid pRK2013 (13) in which a different, poorly transposable kanamycin resistance determinant had been inactivated by a Tn*7* insertion. Tn*7* encodes resistance to the antibiotics trimethoprim, spectinomycin, and streptomycin. Tn*5* was transposed onto plasmid pRKTV5 by using a defective lambda derivative by the method of Ruvkun and Ausubel (36).

Hemagglutination assays. *B. pertussis* cells were grown in SS broth for 2 days. A 100-μl sample of cells was mixed with 100 μl of sheep erythrocytes, washed, and suspended to 0.5% in phosphate-buffered saline (pH 7.4) in microtiter plates. The plates were incubated overnight at 4°C and then scored for hemagglutination.

Adenylate cyclase assays. Enzymatic adenylate cyclase assays, measuring [α-^{32}P]ATP conversion to ^{32}P-labeled cyclic AMP, were performed as described by Hewlett et al. (17) with and without 1 μM calmodulin.

SDS-PAGE and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% acrylamide slab gels was performed as described by Laemmli (27). Bacteria from 3-day-old SS cultures were concentrated by centrifugation and suspended in final sample buffer to approximately 4.8 optical density units at 580 nm. The samples were boiled, and 25-μl samples were loaded on each lane. Western blotting (4) with electrophoretic transfer to nitrocellulose, incubation with antisera, and incubation with ^{125}I-labeled staphylococcal protein A (New England Nuclear Corp., Boston, Mass.) was performed as described by Cohen and Falkow (8). Rabbit immunoglobulin G specific for FHA was a gift from J. Cowell, Division of Bacterial Products, National Center for Drugs and Biologics. The antiserum was further purified by adsorbing with the avirulent strain BP326.

Dermonecrotic toxin assay. The dermonecrotic toxin was assayed in infant mice by nuchal injection of whole organisms as described previously (10).

Biological assays for pertussis toxin. The Chinese hamster ovary (CHO) cell assay (16) was used to test for the presence of pertussis toxin. Ten microliters of supernatant SS medium from 48-h cultures of each mutant was added to CHO cells in microtiter wells containing 200 μl of F-12 medium (GIBCO Laboratories, Grand Island, N.Y.) with 1% serum. Nontoxigenic mutants were identified by their failure to elicit the characteristic cell cluster morphology. All mutants altered in toxin activity were further characterized by injecting supernatant culture medium (0.4 ml) intravenously into the tail vein of female CFW mice and testing for histamine sensitization 48 h later by intraperitoneal injection of 1 mg of histamine base (29).

Restriction digests and filter hybridization. Chromosomal DNA was prepared as previously described (19). Restriction endonucleases were used under conditions recommended by the supplier (Bethesda Research Laboratories, Gaithersburg, Md.). Electrophoresis of cleaved DNA in 0.7% agarose with Tris-acetate buffer, transfer to nitrocellulose, and hybridization with a ^{32}P-labeled DNA probe were performed as previously described (41). The Tn*5*-specific DNA probe was prepared from the internal *Hin*dIII fragment of Tn*5*, which contains all of the

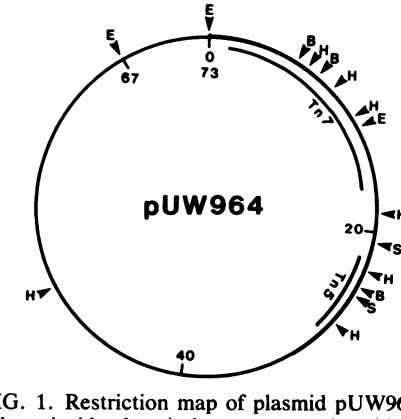

FIG. 1. Restriction map of plasmid pUW964. The numbers inside the circle represent plasmid coordinates in kilobases starting from the *Eco*RI site used to join ColE1 with the pRK212 sequences in the construction of pRK2013 (13). The inner arcs represent Tn*7* and Tn*5* insertion sequences. Restriction sites: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; S, *Sal*I.

neomycin phosphotransferase gene and some inverted repeat sequences (35). To prepare this fragment, ColE1::Tn*5* plasmid DNA was restricted with *Hin*dIII, and the two fragments were separated by electrophoresis. The fragment containing the Tn*5* sequence was cut from the gel, electroeluted, and nick translated as previously described (41).

Nomenclature. Genotypes and phenotypes of Tn*5* insertion mutants were named according to the guidelines of Campbell et al. (5). Insertions in sites with undefined phenotypes were named according to the convention of Chumley et al. (6). We have attempted to use three-letter mnemonics for genetic alleles according to the standard nomenclature for *Escherichia coli* (1). When analogous genes exist in *E. coli*, we have attempted to use the same mnemonic. Genes unique to *B. pertussis* have been given distinctive mnemonics. Phenotypic mnemonics were used for antibiotic resistances (such as *nal* instead of *gyrA* for nalidixic acid resistance) to avoid implying a mechanism of resistance since no biochemical data are available for *B. pertussis*.

We have chosen to use the terms virulent and avirulent phase to designate the different phases of *B. pertussis*. We believe this is a concise description of the single-step genetic change that occurs naturally at a high frequency (22, 31). Previous designations such as phase I versus phase III (21, 22), fresh versus degraded isolate (30), or domed hemolytic versus flat nonhemolytic (31) do not seem to be appropriate because they either depend on serological identification (even though the reference antiserum is not generally available [21]), refer to how the strains were handled, or describe limited physical properties of the cells. Our use of the term virulent phase does not, however, reflect the behavior of a particular strain in an experimental infection. Rather we use the term to refer to the potential of a strain to synthesize a constellation of factors associated with virulence (including pertussis toxin, extracellular adenylate cyclase, FHA, hemolysin, dermonecrotic toxin, pili, cytochrome *d*-629, two outer membrane proteins, and perhaps other factors [12]). When a particular strain had simultaneously lost three or more of these virulent phase-associated traits we referred to this as a phase change mutant. The allele has been denoted with the mnemonic *vir*, for virulent phase.

RESULTS

Isolation and characterization of Tn*5* insertion mutants. The frequency of spontaneous phase change in *B. pertussis* can interfere with attempts to select mutants deficient in a single characteristic. To reduce this difficulty we used transposon-mediated mutagenesis with transposon Tn*5*, which encodes resistance to kanamycin. We constructed a Tn*5*-containing chimeric plasmid which has a ColE1 origin of replication and the broad-host-range conjugation genes from RK2. The restriction map of plasmid pUW964 is shown in Fig. 1. This plasmid, like a similar plasmid used to deliver transposon Tn*501* (41), was able to replicate in *E. coli*, but not *B. pertussis*, because of the limited host range for ColE1 incompatibility group plasmids.

The Tn*5*-containing "suicide plasmid" pUW964 was introduced into *B. pertussis* strain BP338 by plate matings with the *E. coli* donor for 4 to 6 h on BG plates (41). We found that the efficiency of mating was increased when the BG plates were supplemented with 10 mM $MgSO_4$. Tn*5* insertions into the *B. pertussis* chromosome were selected on BG plates containing nalidixic acid and kanamycin. Presumptive Tn*5* insertion mutants were purified by streaking for single colony isolates on BG. To confirm that the plasmid was not maintained in the recipient cells, the loss of trimethoprim and spectinomycin resistance encoded by the other plasmid marker, Tn*7*, was scored. To insure that each insertion was independent, only one kanamycin-resistant mutant from a single mating was retained. The properties of the Tn*5* mutants that we chose to characterize are summarized in Table 2.

Isolation of an avirulent-phase mutant. One Tn*5* insertion mutant, BP347, did not produce hemolysin and when further characterized was also negative for hemagglutination, pertussis toxin production, dermonecrotic toxin production, and adenylate cyclase production (Tables 2 and 3) and seemed to be identical to an avirulent-phase mutant. We used this mutant and BP326, a derivative of Tohama III, as negative controls when we assayed for biological activities associated with virulence.

Identification of hemolysin mutants. Several

TABLE 2. Tn*5* insertion derivatives of BP338

Strain	Genotype[a]	Phenotypic characteristics[b]			
		Hly	Fha	Ptx	Dnt
BP347	*vir-1*::Tn*5*	−	−	−	−
BP348	*hly-1*::Tn*5*	−	+	+	+
BP349	*hly-2*::Tn*5*	−	+	+	+
BP350	*hly-3*::Tn*5*	−	+	+	+
BP351	*hly-4*::Tn*5*	−	+	+	+
BP352	*hly-5*::Tn*5*	+/−	+	+	+
BP353	*fha-1*::Tn*5*	+	−	+	+
BP354	*fha-2*::Tn*5*	+	−	+	+
BP356	*ptx-1*::Tn*5*	+	+	−	+
BP357	*ptx-2*::Tn*5*	+	+	−	+
BP358	*zzz-1*::Tn*5*	+	+	+	+

[a] Alleles defined by Tn*5* are as follows: *vir*, virulent phase; *hly*, hemolysin; *fha*, FHA; *ptx*, pertussis toxin.

[b] The phenotypes were characterized as follows: Hly, hemolysis on BG plates; Fha, FHA (assayed by hemagglutination of sheep erythrocytes); Ptx, pertussis toxin (assayed by CHO cells or histamine-sensitizing assay); Dnt, dermonecrotic toxin (assayed in infant mice). Strains designated + had demonstrable activity, those designated − were negative, and +/− means that the strain had an intermediate phenotype.

TABLE 3. Adenylate cyclase activity in *B. pertussis* mutants[a]

Strain	nmol of cyclic AMP/min per mg of protein			
	Whole cells		Supernatant	
	+ Cal	− Cal	+ Cal	− Cal
BP338 (Vir$^+$)	44.1	0.03	22.5	0.12
BP348 (Hly)	<0.01	<0.01	<0.01	<0.01
BP349 (Hly)	7.1	<0.01	5.8	<0.01
BP350 (Hly)	0.26	0.02		
BP351 (Hly)	0.65	<0.01		
BP352 (Hly)	144.8	1.76		
BP353 (Fha)	21.4	0.18	266	1.9
BP354 (Fha)	25.2	0.072	249	1.1
BP357 (Ptx)	116.6	5.3		
BP358 (Kan^r)	45.4	0.24	22.0	0.24
BP347 (Vir$^-$)	<0.01	<0.01	<0.01	<0.01
BP326 (Vir$^-$)	<0.01	<0.01	<0.01	<0.01

[a] Two-day-old, SS-grown cells or supernatants were used. Assays were performed in the presence (+ Cal) or absence (− Cal) of 1 μM calmodulin.

other nonhemolytic mutants were obtained (Table 2). One mutant, BP352, seemed to have reduced hemolytic activity, but was not totally negative. Unlike BP347, all of these hemolysin mutants were positive when assayed for production of pertussis toxin, dermonecrotic toxin, and FHA. However, when these nonhemolytic mutants were assayed for adenylate cyclase production, variable results were obtained (Table 3). The adenylate cyclase activity of *B. pertussis* is characterized by the unique properties of being localized to an extracytoplasmic compartment (17) and of being stimulated by the eucaryotic protein calmodulin (42). Whole cell and supernatant fractions were assayed for adenylate cyclase in the presence and absence of calmodulin. The parental strain, BP338, contained high levels of enzyme activity in both fractions. Two nonhemolytic mutants, strains BP349, and BP352, had significant levels of enzyme activity. Mutants BP350 and BP351 had only trace levels of activity, and strain BP348 was totally negative for adenylate cyclase activity. The avirulent-phase mutants BP326 and BP347 were also devoid of adenylate cyclase activity. A Tn*5* insertion mutant with no obvious change in phenotype, BP358, had nearly wild-type levels of activity, both cell associated and in the supernatant fraction, demonstrating that the change in adenylate cyclase levels was not due to the presence of Tn*5* alone.

We tested all of the nonhemolytic mutants for phenotypic complementation. If a particular process, such as hemolysis, requires the interaction of two proteins, two mutants each deficient in a different factor will regain the activity when mixed together if each supplies the factor the other lacks. We cross-streaked the nonhemolytic mutants as well as the avirulent-phase mutant, BP347, in all possible combinations against each other on BG plates and scored for hemolysis production. No hemolysis was observed with any combination of nonhemolytic strains, indicating that complementation did not occur under these experimental conditions.

Isolation and characterization of non-hemagglutinating mutants. *B. pertussis* possesses two protein factors which mediate hemagglutination (20, 37), FHA and pertussis toxin. The presence of two factors with the same activity could obscure an assay for single mutants. We avoided this difficulty by assaying for hemagglutination with sheep erythrocytes, which efficiently detect hemagglutination mediated by FHA, but not that mediated by pertussis toxin (20). Kanamycin-resistant strains were grown in SS medium and then assayed for hemagglutination. Two mutants, BP353 and BP354, were negative for hemagglutination and were further characterized.

Both mutants were positive for pertussis toxin production by the CHO cell assay and also produced hemolysin and adenylate cyclase (Tables 2 and 3). However, when we compared the levels of adenylate cyclase activity with that of the parental strain, BP338, both mutants had reduced cell-associated activity, but higher levels of enzyme activity, in the supernatant fraction. Unlike the nonhemolytic mutants, the adenylate cyclase activity in these mutants appears to be quite high, but the distribution of the activity between the different cellular compartments has been altered.

The protein profiles of whole cell preparations of the mutants and wild-type strains were characterized by SDS-PAGE (Fig. 2). The virulent-phase parental strain produced a large polypeptide at molecular mass of about 200,000 daltons detected by Coomassie straining. The protein was present in reduced amounts in mutant BP354 and was absent in mutant BP353 and the avirulent phase mutants BP326 and BP347. Based on previous data (11) we presumed that this protein was the FHA. To confirm this, we used the Western blotting technique (Fig. 3). The rabbit antiserum to FHA was able to block hemagglutination when the parental strain, BP338, was incubated with sheep erythrocytes. When whole cell protein preparations of BP338 were separated by electrophoresis, two bands of 130,000 and 100,000 daltons in addition to the 200,000-dalton protein, were detected which reacted specifically with the antiserum to FHA (Fig. 3, lane b). These bands were missing or reduced in the mutants BP353 and BP354 as well as in the avirulent-phase strain BP326 (Fig. 3, lanes c, d, and a, respectively). These data suggest that mutants BP353 and BP354 are de-

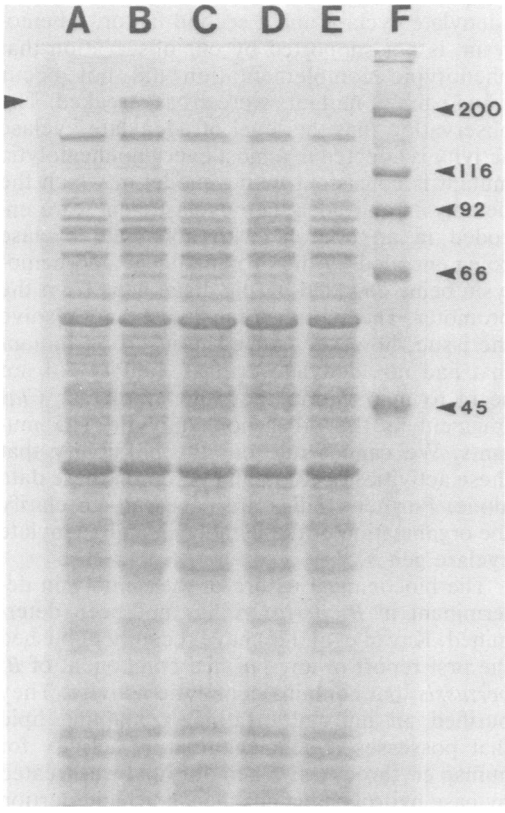

FIG. 2. Identification of FHA in *B. pertussis* by SDS-PAGE with Coomassie staining of total cellular proteins. Lanes: A, BP326; B, BP338; C, BP353; D, BP354; E, BP347. Lane F contains the following protein molecular mass standards (from top to bottom): 200,000 daltons (myosin); 116,250 daltons (β-galactosidase); 92,500 daltons (phosphorylase B); 66,200 daltons (bovine serum albumin); 45,000 daltons (ovalbumin).

fective in the structural gene for FHA or a gene necessary for its expression.

Pertussis toxin mutants. Kanamycin-resistant Tn*5* insertion strains were screened for pertussis toxin production by the CHO cell assay. Two mutants, BP356 and BP357, had no detectable activity in this biological assay. These mutants also failed to produce histamine sensitization in CFW mice (Table 4). Further characterization of these mutants, however, detected cell-associated pertussis toxin. Since the amount of material employed was 5 to 100 times that required to elicit responses with the wild-type, parental strain, it appears that these mutants are partially deficient in toxin production or are unable to process and transport it normally.

Physical characterization of the Tn*5* insertion mutants. The location of Tn*5* insertion mutations can be determined by using a ^{32}P-labeled DNA probe specific for Tn*5* to hybridize to chromosomal DNA restriction fragments separated by electrophoresis. The hybridizations were performed on various combinations of mutants to determine whether the mutant phenotypes were due to single insertions and whether the insertions occurred within the same restriction fragment for mutants that had the same phenotype.

Large fragments of chromosomal DNA were prepared and restricted with endonuclease *Eco*RI or *Cla*I. These two enzymes were used because Tn*5* does not encode the restriction sequence for either of these enzymes. Tn*5* insertions occurring within the same restriction fragment in the *B. pertussis* chromosome should comigrate.

All of the mutants analyzed contained a single *Eco*RI restriction fragment that was homologous to Tn*5* (Fig. 4), including BP347 (Fig. 4f), the avirulent-phase mutant that had lost several of the determinants associated with pathogenicity. The FHA-deficient mutants BP353 and BP354 had restriction fragments containing Tn*5* that appeared to comigrate (Fig. 4a and b), as did the pertussis toxin mutants BP356 and BP357 (Fig. 4g and h).

Different results were obtained when three nonhemolytic mutants, BP348, BP349, and BP350, were compared. The Tn*5*-containing fragment of BP348 (Fig. 4c) did not appear to comigrate with that of BP349 and BP350 (Fig. 4d and e), which appeared to comigrate with each other. The phenotype of mutants BP349 and BP350 was different from that of BP348. BP349

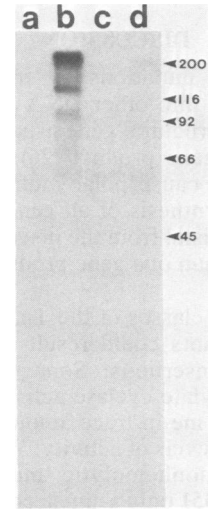

FIG. 3. Western blot of FHA mutants. SDS-PAGE samples were transferred to nitrocellulose and blotted with FHA antisera. Lanes a through d are the same as lanes A through D, respectively, in Fig. 2. The arrows indicate molecular mass markers in kilodaltons.

TABLE 4. Histamine-sensitizing assay for pertussis toxin[a]

Sensitizing material	No. of deaths/total
Phosphate-buffered saline	0/4
BP338 (Vir$^+$)	4/4
BP348 (Hly)	4/4
BP356 (Ptx)	1/4
BP357 (Ptx)	1/4
BP326 (Vir$^-$)	1/4

[a] CFW female mice were injected intravenously in the tail with culture medium or phosphate-buffered saline alone. Mice were challenged 48 h later by the intraperitoneal injection of 1 mg of histamine.

and BP350 had adenylate cyclase activity, even though the levels of activity were quite different from each other and from the parental strain, BP338. BP348, however, was the nonhemolytic mutant that totally lacked adenylate cyclase activity. From these data we conclude that the site of the Tn*5* insertion in BP348 is separated by at least one *Eco*RI restriction site from the site of insertion in BP349 and BP350, and these two regions may not be genetically linked at all.

Restriction fragments of chromosomal DNA were also prepared by using *Cla*I and hybridized with the Tn*5* probe. Both *Eco*RI and *Cla*I gave essentially identical results, that is, each lane contained only a single band homologous to Tn*5*, and bands that comigrated when restricted with *Eco*RI also comigrated with *Cla*I. However, the sizes of the fragments were different for the different digests (data not shown).

DISCUSSION

Tn*5* induces mutations by insertion of its DNA sequence into other DNA sequences. Insertion into a structural gene usually results in a nonfunctional gene product (26). Tn*5* has also been shown to cause polar mutations, that is, Tn*5* disrupts synthesis of all gene products encoded at sites distal from the insertion site when there is more than one gene product encoded in an operon (3).

The different classes of the Tn*5*-induced nonhemolytic mutants could result from polar effects of Tn*5* insertions. Some mutants were devoid of adenylate cyclase activity, some produced this enzyme in trace amounts, and some had significant levels of activity. We have shown that for the nonhemolytic mutants BP349, BP350, and BP351 only a single copy of Tn*5* was present in the chromosome. Therefore, the simultaneous loss of two activities, hemolysin and adenylate cyclase, for mutant BP348 cannot be due to double insertion events. The hypothesis that hemolytic activity requires two proteins, adenylate cyclase and a second factor, a hemolysin, is not supported by our observation that phenotypic complementation did not occur when pairs of mutants were cross-streaked. The observation that the level of adenylate cyclase activity is affected in almost every nonhemolytic mutant is consistent with a model in which the hemolysin and adenylate cyclase genes are encoded in an operon, with adenylate cyclase being encoded by the proximal gene and hemolysin being encoded by the distal gene from the promoter. The hybridization data did not resolve the issue, however, since the insertion mutant that had no adenylate cyclase activity did not seem to map within the same *Eco*RI or *Cla*I fragment as the other nonhemolytic Tn*5* mutants. We cannot rule out the possibility that these activities occur in an operon on these data alone. Further studies are necessary to clarify the organization of the hemolysin and adenylate cyclase genes.

The biochemical nature of the hemolysin determinant in *B. pertussis* has not been determined. Kawai et al. (25) have recently published the first report of any purified component of *B. pertussis* that contains hemolytic activity. They purified an unusual ornithine-containing lipid that possesses a hemagglutinating activity for human erythrocytes. When this lipid was treated by base hydrolysis, a hexadecanoic acid portion

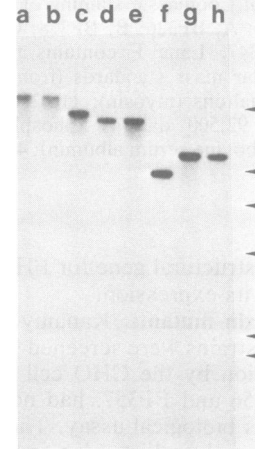

FIG. 4. Detection of Tn*5*-specific sequences by Southern hybridization. *Eco*RI digests of chromosomal DNA were separated by electrophoresis in 0.7% agarose buffered with Tris-acetate and hybridized with a ^{32}P-labeled probe specific to Tn*5*. Lanes a, BP353 (Fha$^-$); b, BP354 (Fha$^-$); c, BP348 (Hly$^-$); d, BP349 (Hly$^-$); e, BP350 (Hly$^-$); f, BP347 (Vir$^-$); g, BP356 (Ptx$^-$); and h, BP357 (Ptx$^-$). The arrows indicate the mobility of the following lambda *Hin*dIII size standards determined by ethidium bromide staining of the same gel (from top to bottom): 23.7, 9.46, 6.67, 4.26, 2.25 and 1.96 kilobases.

was removed from the lipid. The remaining aminolipid could no longer mediate hemagglutination, but had acquired hemolytic activity. Whether this activity is related to the hemolysis observed on BG plates is unknown; however, both virulent- and avirulent-phase bacteria synthesize this lipid (24), but only virulent-phase bacteria are hemolytic. The availability of the nonhemolytic mutants isolated in this study should help clarify the biochemical nature of the hemolysin determinant.

We have characterized mutants deficient in FHA that do not produce three large-molecular-mass proteins present in the wild-type strain. These proteins migrate at 200,000, 130,000, and 100,000 daltons and cross-react with antisera to FHA. Cowell et al. have purified FHA and have shown that the purified material was separated by SDS-PAGE as three high-molecular-mass polypeptide species at 160,000, 115,000, and 90,000 daltons (11). The differences in the molecular mass determinations may be due to different conditions of electrophoresis. Alternatively, the differences could be due to the fact that we characterized FHA isolated from whole cell preparations as opposed to the FHA purified from the supernatant that was characterized by Cowell et al. (11). The three protein bands may correspond to aggregates of FHA with other proteins, or processed forms of FHA. One of the mutants that failed to hemagglutinate sheep erthrocytes did not produce these proteins, whereas the other synthesized them in reduced amounts. Both mutants seemed to contain a single Tn*5* insertion within the same restriction fragment and may be encoded in an operon. The reduced protein levels in one mutant could be due to an insertion that inactivated a region necessary for the expression of the structural gene, whereas the other mutant could have an insertion within the structural gene itself. Watanabe and Nakase (39) using UV mutagenesis, have also isolated mutants that failed to produce hemagglutination. They did not characterize their mutants biochemically.

All of the mutant phenotypes that we have isolated are associated with secreted products. One might expect that negative phenotypes could be due to either defects in the structural gene or secretory defects, and that the loss of one factor could influence the level of expression of another. The FHA mutants (BP353 and BP354) were able to produce hemolysin, pertussis toxin, and adenylate cyclase; however, they seemed to contain more adenylate cyclase activity in the supernatant fraction and less whole cell-associated activity than did the parental strain, BP338. This difference seems to reflect a difference in compartmentalization of adenylate cyclase activity and not a reduction in total enzyme activity. In contrast, a nonhemolytic mutant (BP349) appeared to have proportionately reduced activity in both the supernatant and cellular compartments. Since both FHA and adenylate cyclase are extracytoplasmic proteins, the loss of one activity could result in more efficient release of the other by either an active or passive process. Bassford and Beckwith (2) have shown that the levels of secretion of extracellular proteins are interrelated. When synthesis of a β-galactosidase fusion protein which contained a leader sequence, but which could not be effectively secreted, was induced, secretion of other proteins was also impaired.

The use of transposons as mutagens in *B. pertussis* has provided us with a series of strains deficient in a single virulence determinant. We believe that this is the first report demonstrating that mutants deficient in a single virulence determinant can be isolated for several of the different *B. pertussis* virulence factors. The different classes of mutants that we have isolated provide preliminary evidence for the genetic organization of the virulence genes of *B. pertussis*. The series of Tn*5* mutants that had lost only a single trait, such as BP353 (the FHA mutant), BP356 (the pertussis toxin mutant), and BP349 (the nonhemolytic mutant), demonstrate that hemolysin, FHA, and pertussis toxin can be mutated independently. These mutants still retained dermonecrotic toxin, indicating that this gene is also independent from the others. The loss of all virulent phase-associated traits due to a single Tn*5* insertion in mutants such as BP347 supports the hypothesis that there is a single genetic region, presumably *trans* acting, that is required for the expression of the other genes. We hope to use the Tn*5*-encoded kanamycin resistance as a genetic marker to clone the restriction fragment containing the gene of interest. The surrounding *B. pertussis* sequences can then be used as a DNA probe to isolate the wild-type genes. The cloned genes should help answer many questions concerning the molecular organization of the *B. pertussis* virulence determinants.

The Tn*5* mutants should also prove useful in experimental infections to determine the precise contribution of each individual virulence factor to the pathogenesis of *B. pertussis* infection. We hope that such studies will help define which determinants of *B. pertussis* are important for generating host immunity to disease and thus would be candidates to be included in an acellular pertussis vaccine.

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