INFECTION AND IMMUNITY, Dec. 1989, p. 3757–3764
0019-9567/89/123757-08$02.00/0

Vol. 57, No. 12

Lethal Infection by *Bordetella pertussis* Mutants in the Infant Mouse Model

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Received 5 June 1989/Accepted 5 September 1989

Different aspects of lethal infection of infant mice with *Bordetella pertussis* were examined. Mutants deficient in *vir*-regulated genes were tested for the ability to cause a lethal infection in the infant mouse model. Adenylate cyclase toxin-hemolysin and pertussis toxin were required to cause a lethal infection at low doses. Mixed infection caused by challenging the mice with an equal number of pertussis toxin and adenylate cyclase toxin-hemolysin mutants at a dose at which neither alone was lethal was also unable to cause a lethal infection. Production of the filamentous hemagglutinin and the dermonecrotic toxin was not required to cause a lethal infection. Nine other mutants in *vir*-regulated genes whose phenotypes have yet to be determined were also tested. Only two of these mutants were impaired in the ability to cause a lethal infection. Expression of fimbriae does not appear to affect the dose required to cause a lethal infection; however, fimbrial expression was correlated with the later stages of a nonlethal, persistent infection. Growth of the bacteria in $MgSO_4$, a condition which reversibly suppresses expression of the genes required for virulence, did not alter the ability of the bacteria to cause a lethal infection. Auxotrophic mutants deficient in leucine biosynthesis were as virulent as the parental strain; however, mutants deficient in methionine biosynthesis were less virulent. A *B. parapertussis* strain was much less effective in promoting a lethal infection than any of the wild-type *B. pertussis* strains examined. A persistent infection in the lungs was observed for weeks after challenge for mice given a sublethal dose of *B. pertussis*, and transmission from infected infants to the mother was never observed.

Despite decades of research, the events in the natural course of infection by *Bordetella pertussis* remain obscure. There are very few animal models, and much of what we know of the disease is based on vaccine efficacy studies. The most widely used animal model for vaccine studies is intracerebral challenge. Mice are injected with a lethal dose of live bacteria in the brain. The current whole-cell vaccine protects mice from death in this assay. However, the assay does not serve as a model for disease in human infants. Infants are infected in the respiratory tract, not the brain. Interestingly, only one strain of *B. pertussis*, 18-323, kills by the intracerebral route (34); however, this is not a typical strain and may be more closely related to *B. parapertussis* or *B. bronchiseptica* than to clinical isolates of *B. pertussis* (20). While the intracerebral challenge has served an important need as a way to measure vaccine potency, we can learn little about the actual disease process with this model.

Respiratory infection by *B. pertussis* has been studied in mice, rats, rabbits, and great apes (34). Great apes hold the best promise for a realistic model of disease but are prohibitively expensive for all but the most focused of studies. Rabbits become infected but do not become ill. New studies using rats appear to be promising (40), but much investigation remains to be done toward characterization of this disease model.

The most widely used and well-characterized model of the disease is intranasal challenge of mice. Infant mice can succumb to a lethal challenge. Older mice become infected and display symptoms such as lymphocytosis but do not die (21). Experimentally, it is easier to monitor survival of mice than parameters such as lymphocytosis or numbers of bacteria in the lungs.

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We have begun to exploit intranasal challenge of infant mice as a first approximation of human disease by studying the abilities of various bacterial mutants to cause a lethal infection in this model. *B. pertussis* is an excellent model system by which to study pathogenesis because all of the genes required for virulence are coordinately expressed and regulated by growth conditions. One gene (called *vir* for virulence regulating) acts as a positive transcriptional inducer for expression of virulence genes (19, 27, 33). Previously (36), we have shown that bacteria with a mutation in *vir* were unable to express any of the *vir*-regulated genes and were also unable either to persistently infect or cause a lethal infection at a challenge dose of 10^7 CFU, whereas the wild-type strains killed mice with a dose of about 10^3 CFU. Bacterial mutants deficient in a single virulence factor were also tested for the ability to cause disease in this model. Mutants deficient in pertussis toxin or adenylate cyclase toxin-hemolysin production were severely diminished in the ability to cause a lethal infection, suggesting that these factors are required to cause disease (36). However, mutants deficient in production of filamentous hemagglutinin (FHA) were not severely affected in the ability to cause a lethal infection in this model.

In this study, we examined the role of other *vir*-regulated genes in lethal infection. We generated a panel of mutants deficient in *vir*-regulated genes by using a promoter fusion transposon, Tn*5 lac* (14), by screening for mutants that express β-galactosidase when *vir* genes are expressed but not when they are suppressed (38). In this study, we examined the abilities of these mutants to cause lethal infections in infant mice. These studies will surely give us information on the pathogenesis of this infection and, we hope, reveal insights into the human disease.

TABLE 1. Fimbrial serotypes and approximate 50% lethal doses for *B. pertussis* and *B. parapertussis* strains

Strain (source)	Relevant characteristic(s) (source)	Fimbriae 2[a]	Fimbriae 3/6[a]	LD_{50}[b]
Wild-type *B. pertussis* isolates				
Tohama	Clinical isolate (20)	−	−	$<3 \times 10^4$
BP325	Tohama; increased hemolysis (32)	+	−	$<2 \times 10^4$
BP338	Nal^r Tohama (35)	+	−	2×10^3–20×10^3
BP338-5	Spontaneous, afimbrial BP338 (38)	−	−	$<5 \times 10^3$
BP370	Vir^+ derivative of Vir^- BP326 (26, 33)	+	−	$<2 \times 10^4$
18-323	Used for intracerebral challenge (20)	+	−	$<2 \times 10^4$
B. parapertussis 77	*B. parapertussis* (20)	ND[c]	ND	$>3 \times 10^6$
Tn*5* insertion mutants of BP338				
BP347	Vir^- (35)	−	−	$>1 \times 10^7$
BP356	Pertussis toxin mutant (35)	+	−	5×10^4–20×10^4
BP357	Pertussis toxin mutant (35)	−	−	4×10^5–10×10^5
BP348	Adenylate cyclase-hemolysin mutant (35)	−	−	$\approx 10^6$
BPG50	Leucine auxotroph (this study)	ND	ND	$\approx 2 \times 10^4$
BPF43	Methionine auxotroph (this study)	ND	ND	$>2 \times 10^5$
Tn*5* *lac* insertion mutants of BP338 (38)				
BPM433	Adenylate cyclase-hemolysin mutant	−	−	$>2 \times 10^7$
BPM3183	Adenylate cyclase-hemolysin mutant	+	+	8×10^4–800×10^4
BPM1809	Dermonecrotic toxin mutant	−	−	$<2 \times 10^3$
BPM1821	FhaA mutant	−	−	$<2 \times 10^4$
BPM409	FhaB mutant	−	−	$<4 \times 10^3$
BPM3171	Unknown; pertussis toxin linked	−	−	2×10^4–30×10^4
BPM2041	*vir* regulated; unknown function	−	−	2×10^4–20×10^4
BPM177	*vir* regulated; unknown function	−	−	$<1 \times 10^4$
BPM245	*vir* regulated; unknown function	−	−	$<2 \times 10^4$
BPM1579	*vir* regulated; unknown function	−	−	$<1 \times 10^4$
BPM2055	*vir* regulated; unknown function	−	−	$<1 \times 10^4$
BPM2119	*vir* regulated; unknown function	−	−	$<3 \times 10^4$
BPM2123	*vir* regulated; unknown function	+	−	$<3 \times 10^4$
BPM2859	*vir* regulated; unknown function	−	−	$<4 \times 10^4$
BPM2375	Pleiotropic	+	−	$>1 \times 10^6$
BPM3447	Pleiotropic	−	+	$>6 \times 10^4$

[a] Determined by agglutination.
[b] When the LD_{50} was bracketed, values are expressed as the range between the lowest lethal dose and the highest sublethal dose tested. Other values are the lowest lethal dose or the largest sublethal dose tested, depending on whether the mice died or lived, respectively.
[c] ND, Not done.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are described in Table 1. *B. pertussis* strains were grown on Bordet-Gengou agar (BBL Microbiology Systems, Cockeysville, Md.) as previously described (38). Auxotrophic mutants were identified as mutants that grow on Bordet-Gengou agar but not Stainer-Scholte (24) minimal agar. The nutritional defect was determined by trial and error.

Infant mouse model. In past studies, BALB.K mice were used (36). This inbred line is not commercially available, and in this study we used BALB/cByJ mice (Jackson Laboratory, Bar Harbor, Maine). Intranasal challenge of 6-day-old infant mice was performed essentially as described previously (36), except that 20 μl of bacteria was used instead of 25 μl to challenge the mice. Twenty microliters of bacteria at an optical density of 0.2 (the highest challenge dose used) contains approximately 10^7 bacteria (CFU). Survival for 25 days after infection was reported as a sublethal infection. Minced lung sections were plated on Bordet-Gengou agar to recover bacteria.

Serotyping of strains. Recently, the identities of many of the serotype antigens have been determined. By using Eldering typing sera, serotype 1 has been shown to be composed of lipooligosaccharide (16). Fimbrial production has been associated with serotypes 2 and 6. Serotype 2 is correlated with the presence of fimbriae with a subunit molecular mass of 22 kilodaltons (kDa), and serotype 6 is correlated with the presence of fimbriae with a subunit molecular mass of 21.5 kDa (15). A 69-kDa outer membrane protein is associated with serotype 3 (4). Monoclonal antibodies kindly provided by Michael Brennan, Bureau of Biologics, Food and Drug Administration, Bethesda, Md., were used to type the strains. Fimbrial agglutinogen 2 (22-kDa subunit size) was detected with monoclonal antibody BPF2 (15), and fimbrial agglutinogen 3/6 (21.5-kDa subunit size) was detected with monoclonal antibody BPC10 (15). Agglutination tests were performed as previously described (38), by mixing the bacteria and the sera, and scored as positive if the characteristic clumping appeared.

RESULTS

To determine whether the BALB/cByJ mouse strain gives results similar to those obtained with the BALB.K strain, we repeated several of our previous experiments. In the current study, as previously found, BP347, an avirulent mutant, was unable to cause a lethal infection at a dose of 10^7 CFU (Table 1), while the virulent-phase parental strain, BP338, was able to cause a lethal infection at a dose of 2×10^4 CFU (Table 1; Fig. 1) but not at 2×10^3 CFU, which was the lethal dose

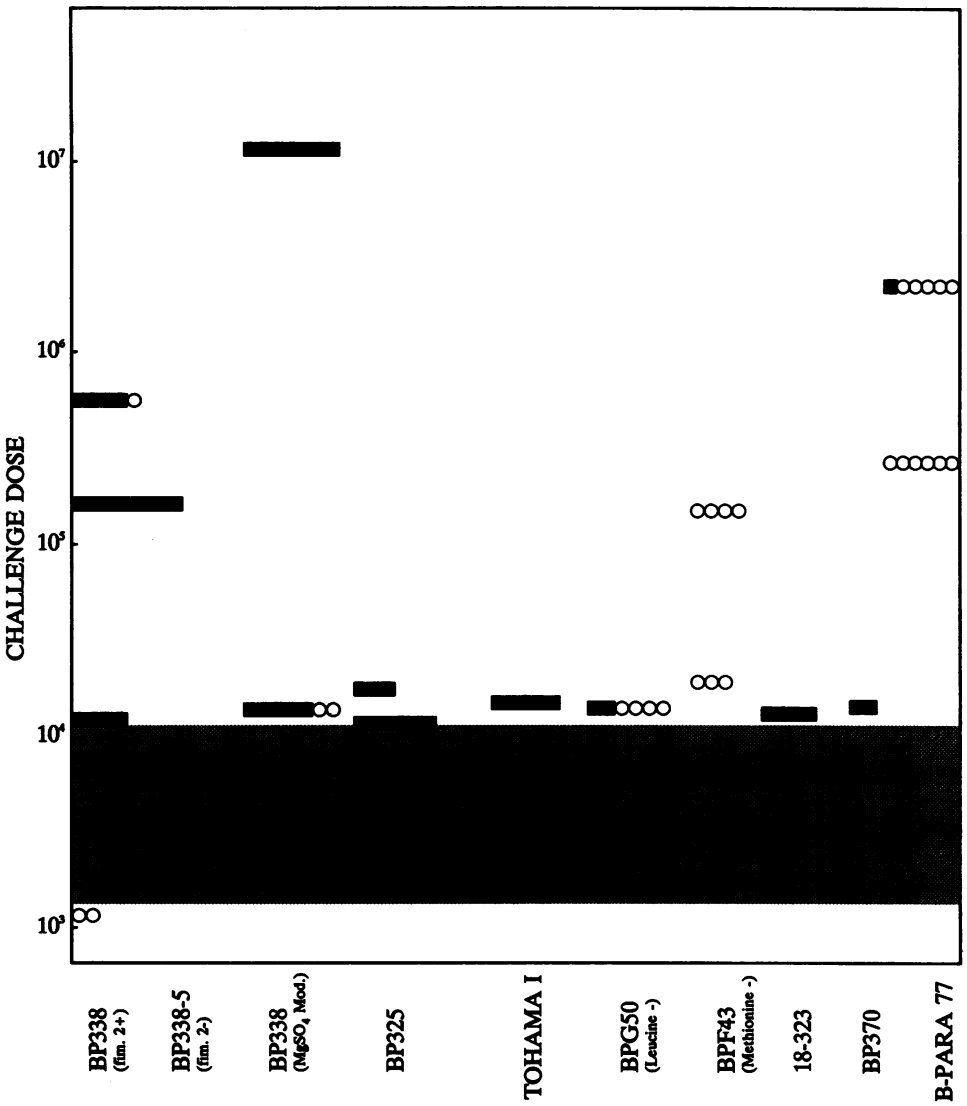

FIG. 1. Challenge of infant mice (BALB/cByJ) with various *Bordetella* strains. The number of symbols indicates the number of mice challenged at that dose. Closed symbols indicate that the mice died, and open symbols indicate that the mice survived the challenge. The hatched areas indicate the LD_{50} zone for the wild-type strain. Abbreviations: fim. 2^+, expresses fimbriae 2; fim. 2^-, does not express fimbriae 2; $MgSO_4$ mod., expression of the *vir*-regulated genes was modulated by pregrowth in 10 mM $MgSO_4$; B-PARA 77, *B. parapertussis* 77.

with the BALB.K strain. This lethal challenge dose is slightly higher than that previously observed (36). Other wild-type strains, including Tohama I, 18-323, BP325, and BP370, were also tested at a dose of about 10^4 CFU, and were virulent; however, *B. parapertussis* 77 did not cause a lethal infection at 3×10^6 CFU (Table 1; Fig. 1).

To test whether growth under conditions which reversibly inhibit expression of *vir*-regulated genes would prevent lethal infection, we grew BP338 in 20 mM $MgSO_4$ before the challenge, which completely but reversibly suppressed expression of *vir*-regulated genes in vitro (19). This did not result in a remarkable difference in the lethal dose, since a dose of 2×10^4 CFU was lethal to most but not all of the mice, while a challenge of 10^7 CFU was uniformly fatal (Fig. 1). These data suggest that *vir*-regulated genes can be synthesized before host defenses can eradicate the infection.

Additionally, two auxotrophic mutants obtained by Tn*5* mutagenesis of strain BP338 were tested for the ability to cause a lethal infection. Mutant BPG50, which requires leucine for growth, was able to cause a lethal infection at a dose of 2×10^4 CFU in two of six mice challenged. Mutant BPF43, which requires methionine for growth, seemed to be more impaired, since all mice survived a challenge with 2×10^5 CFU (Fig. 1). This suggests that certain auxotrophic markers can have some impact on the ability to cause a lethal infection in this model, as in studies with salmonellae, in which some but not all auxotrophic mutations affected growth in vivo (6).

Testing mutants in other *vir*-regulated genes. We generated a series of mutants deficient in different *vir*-regulated genes by using Tn*5* *lac* (38). Like Tn*5* mutants (36), Tn*5* *lac* mutants deficient in adenylate cyclase toxin-hemolysin, BPM433 (Fig. 2) and BPM3183 (Table 1), were less virulent than the wild-type strain. Recent evidence suggests that adenylate cyclase and hemolysin are encoded by a single gene (10). Mutants deficient in FHA (BPM409 and BPM1821) were not impaired in the ability to cause a lethal infection.

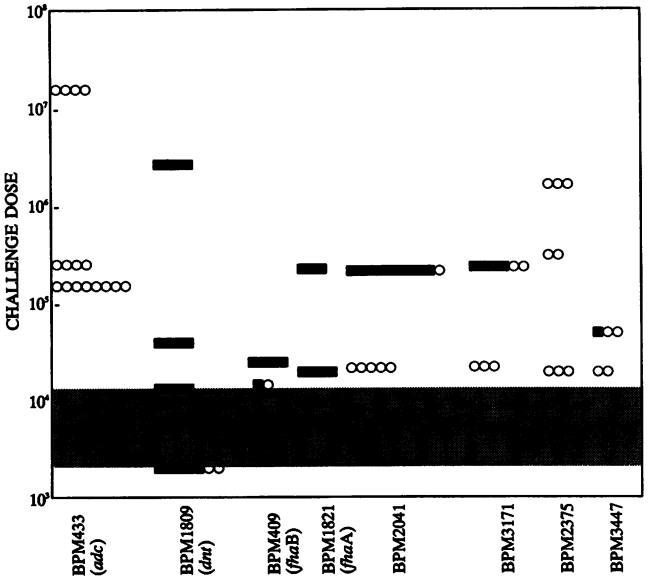

FIG. 2. Determination of the lethal challenge dose for various mutants with *vir*-regulated gene mutations. The properties of the various mutants are described in Table 1. The symbols are as in Fig. 1. Genetic symbols: *ptx*, pertussis toxin; *adc*, adenylate cyclase toxin; *fha*, FHA; *dnt*, dermonecrotic toxin.

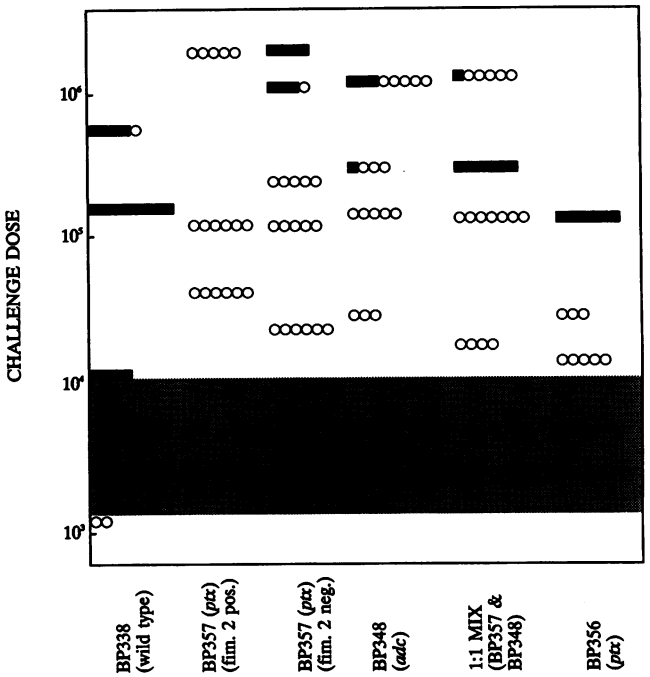

FIG. 3. Phenotypic complementation with adenylate cyclase toxin-hemolysin and pertussis toxin mutants. The challenge was performed as indicated in the legend to Fig. 1, and the symbols are defined in the same way.

A mutant deficient in a previously untested phenotype, dermonecrotic toxin production, was tested for the ability to cause a lethal infection. An unexpected result was that BPM1809 (dermonecrotic toxin mutant) was unimpaired in the ability to cause a lethal infection (Fig. 2).

The phenotypes of most of the mutants isolated were not determined, although Southern analysis suggested that each insertion maps to a different site in the chromosome and perhaps to as many genes (38). All were tested for virulence (Table 1), and only two mutants, BPM2041 and pertussis toxin-linked mutant BPM3171, were impaired in the ability to cause a lethal infection (Fig. 2). Both required a 10-fold greater challenge dose than the wild type to cause a lethal infection.

The mutant most severely affected in the ability to cause a lethal infection, BPM2375 (Fig. 2), does not have a mutation in a *vir*-regulated gene, as judged by differential β-galactosidase expression, but is pleiotropic for expression of all *vir*-regulated genes and appears to synthesize less of every factor tested so far (38).

Mixed challenges. Previously, we have shown that two mutants, BP348 (a mutant deficient in adenylate cyclase toxin-hemolysin production) and BP357 (a mutant deficient in pertussis toxin production), were severely diminished in the ability to cause a lethal infection (36). These results were confirmed in this study (Fig. 3); however, the adenylate cyclase toxin-hemolysin mutant (BP348) caused a lethal infection for some of the mice at a dose of 5×10^5 CFU, which is lower than the 10^7 CFU observed with BALB.K mice (36).

Mixed infections were performed with BP357 and BP348 to determine whether phenotypic complementation can occur. A mixture of equal numbers of the two mutants was used as a challenge to determine whether the pertussis toxin produced by the adenylate cyclase-hemolysin mutant (BP348) and the adenylate cyclase toxin-hemolysin produced by the pertussis toxin mutant (BP357) were sufficient to restore virulence. This mixture was not able to cause a lethal infection at a dose of approximately 10^4 or 10^5 CFU; however, the mixture caused some deaths given at a dose of approximately 10^6 CFU, a dose that was lethal with BP348 alone (Fig. 3).

Inability to cause a lethal infection was not due to inability of either of the mutants to persist (Table 2). The survivors of the challenge were sacrificed at various times postinfection, and the lungs were cultured to determine whether *Bordetella* organisms were present. BP357 was differentiated from BP348 by hemolysis. In every case, the mice were culture

TABLE 2. Persistence of *Bordetella* mutants in sublethal infections

Strain	Dose (CFU)	Days postinfection for culture result[a]	
		Positive	Negative
BP357 (pertussis toxin mutant)	1×10^5	26	31, 35, 40
BP357	4×10^4	27	33, 41, 47, 52
BP356 (pertussis toxin mutant)	2×10^4	25, 29	34, 40
BP348 (adenylate cyclase mutant)	2×10^5	26, 31, 35	46
BP348	5×10^4	27, 33, 41	
MIX (BP348 and BP357)	2×10^5	26,[b] 31,[b] 35[b]	40, 46, 50
MIX	3×10^4	28,[c] 34[c]	42, 47

[a] Survivors of a sublethal challenge were sacrificed, and the lungs were plated on Bordet-Gengou agar.
[b] Positive for both BP348 and BP357.
[c] Positive for only BP357.

TABLE 3. Fimbrial serotypes of isolates recovered from lungs of mice

Challenge strain (no. of mice)	Type of infection (day)	Dose (CFU)	Fimbrial expression[a]		
			Fimbriae 2	Fimbriae 3	Change in vitro[b]
BP338 (1)	Sublethal (40)	5.5×10^2	−	−	Yes
BP348 (3)	Sublethal	2.4×10^5	+	−	Yes
BP357 (1)	Sublethal	1.4×10^5	+	−	Yes
BP357 (1)	Sublethal	1.4×10^5	−	−	No
BP357 (2)	Sublethal (21)	2.8×10^4	−	−	No
BP357 (1)	Sublethal (35)	1.5×10^5	+	−	Yes
BPM2041 (2)	Sublethal	1.9×10^5	+	−	Yes
BPM2041 (2)	Sublethal (35)	8.5×10^2	+	−	Yes
BPM3183 (1)	Sublethal (30)	8.4×10^6	−	−	Yes
BPM3183 (2)	Sublethal (30)	8.4×10^4	±[c]	−	Yes
BPM3183 (1)	Sublethal (35)	8.4×10^4	+	−	Yes
BPM3183 (1)	Sublethal (41)	8.4×10^4	−	−	Yes
BPM2055 (1)	Sublethal (30)	1.7×10^3	±	−	Yes/no
BPM2055 (1)	Sublethal (35)	1.7×10^3	−	−	No
BPM2859 (1)	Sublethal (30)	1.3×10^3	±	−	Yes/no
BPM2859 (1)	Sublethal (35)	1.3×10^3	−	−	No
BPM177 (2)	Lethal	2.2×10^5	−	−	No
BPM245 (2)	Lethal	1.4×10^5	−	−	No
BPM1579 (1)	Lethal	1.3×10^5	−	−	No
BPM1579 (2)	Lethal	1.3×10^5	−	−	No
BPM1809 (1)	Lethal	5.0×10^4	−	−	No
BPM2119 (7)	Lethal	1.9×10^5	−	−	No
BPM1821 (1)	Lethal	2.5×10^5	−	−	No
BPM2859 (2)	Lethal	8.0×10^5	−	−	No
BPM3183 (1)	Lethal	8.4×10^6	−	−	Yes
BP357 (2)	Lethal	2.0×10^6	−	−	No

[a] Two or three bacterial colonies isolated from mouse lungs were tested for fimbrial expression by agglutination.
[b] Indicates whether the culture isolated from the lungs changed from the strain grown in vitro.
[c] ±, Both fimbria-positive and -negative cultures were obtained.

positive for *Bordetella* organisms for some time postinfection (Table 2). However, adenylate cyclase toxin-hemolysin mutant BP348 seemed to persist longer (beyond 41 days) and in greater numbers (data not shown) than pertussis toxin mutant BP357, which was never cultured past day 27. However, in mixed infections with BP348, BP357 seemed to persist longer.

Role of fimbriae. Previously, we have shown that expression of fimbriae can be switched on and off in *B. pertussis* (38), similar to the metastable pilus expression in *Escherichia coli* (23) and *Neisseria gonorrheae* (28). Infant mice were challenged by intranasal infection with BP338-5, a nonfimbriated derivative of BP338, at a dose of 5×10^3 CFU; and both died (Fig. 1). This is equivalent to the lethal dose for the fimbria 2-positive strain. We tested the stability of the fimbria-negative phenotype after passage through an animal. The lungs of one of the mice was cultured, and a pure culture of *B. pertussis* was obtained. Sixteen independent single-colony isolates from the lung were serotyped, and all were found to be negative for fimbriae 2. These data suggest that fimbriae do not appear to be required to cause a lethal infection in mice and passage through an animal does not necessarily alter the serotype of a strain.

Most of the mutants tested negative for fimbrial production before the animal challenges were performed (Table 1). In this study, we characterized the fimbrial serotypes of some of the bacterial isolates recovered from the lungs of the mice (Table 3). Most of the isolates from a lethal infection were negative for fimbrial production; however, isolates recovered from the lungs of mice sacrificed several weeks postinfection were often positive for fimbrial production.

A different pertussis toxin mutant, BP356 (35), caused a lethal infection at a dose of 2×10^5 CFU and thus appeared to be more virulent than BP357 (Fig. 3). This difference could be due to an allelic difference, since neither BP357 nor BP356 is totally devoid of pertussis toxin, but in fact, they are deficient only in production of the S3 subunit (17, 18, 37), and both mutants appear to be defective in toxin secretion (18). Alternatively, the serotype of BP357 has been reported to be 1.3, whereas that of BP356 has been reported to be 1.2.3., and we have confirmed this result (Table 1), which suggests that BP357 is afimbriated and BP356 makes fimbriae 2. This finding led us to examine whether the difference between the lethal doses was due to the presence of fimbriae or an allelic difference in the Tn*5* insertion. We recovered a stable, fimbriated derivative of BP357 from an infected animal (Table 3) and used this strain to challenge infant mice (Fig. 3). The fimbriated derivative was not more virulent than the nonfimbriated derivative of BP357, suggesting that the difference in the lethal dose between BP356 and BP357 is probably due to differences in the fusion protein generated by the Tn*5* insertion and not fimbrial production, since transcription and translation continue into the Tn*5* DNA until appropriate stop signals are encountered.

Characterization of the infection. We cultured the lungs of most of the animals that died from the bacterial challenge, and in every case, large numbers of bacteria were present. Transmission from the infected infants to the mother was never observed, as determined by culturing of the lungs of the mothers at the completion of the study.

DISCUSSION

We used intranasal challenge of infant mice to study how *B. pertussis* causes disease. Although Halperin et al. (12) have reported that this method is less reproducible than

aerosol challenge, we are encouraged by the facts that we seldom observed challenge doses at which some animals lived and some died, and when we did it was usually with a severely deficient mutant or at a doses approaching the 50% lethal dose (LD_{50}).

Our working hypothesis is that virulence is multifactorial and interaction of several factors must occur to cause a lethal infection. In human whooping cough, the bacteria attach to tissues in the respiratory tract, grow, evade the host defenses, and finally produce enough toxin(s) to damage the host. We used lethal infection of mice as a convenient model, although some of the aspects of this system differ from human disease. Standfast and Dolby (25) demonstrated that mice die when the bacterial count reaches 10^8 CFU in the lungs but recover if the bacterial count can be kept below this point. We reasoned that this is rather a stringent test of virulence.

This study confirmed previous studies that demonstrated that both pertussis toxin and adenylate cyclase toxin-hemolysin are required to cause a lethal infection. Interestingly, a challenge with a mixture of the two mutants did not cause a lethal infection at a lower challenge dose, although both mutants were recovered from the lungs of the same animal when it was sacrificed. For phenotypic complementation to occur, both toxins would have to be secreted. Some studies suggest that adenylate cyclase toxin-hemolysin acts as a surface-associated contact toxin that is not secreted (39), and secreted pertussis toxin has been shown to bind to the surface of the bacteria and serve as an adhesin (29). Both of these processes may limit the amount of toxin free to diffuse in an animal. Another explanation for the lack of phenotypic complementation comes from the recent demonstration of an intracellular state for *B. pertussis*. Ewanowich et al. (9) have suggested that virulent-phase but not avirulent-phase *Bordetella* organisms can invade and survive inside human epithelial cells, and this process may play a role in persistence of the bacterial infection. Phenotypic complementation may not occur because the bacteria may reside in different compartments, that is, inside different cells. In addition, Ewanowich et al. have shown that pertussis toxin mutant BP357 is deficient in invasion; however, adenylate cyclase toxin-hemolysin mutants invade more efficiently than do wild-type cells. This could explain the finding (Table 2) that adenylate cyclase toxin-hemolysin mutants persisted longer and in greater numbers than did pertussis toxin mutants.

It is interesting that *B. parapertussis* did not cause a lethal infection at doses comparable to those of *B. pertussis*, although this organism is capable of causing clinically apparent disease. *B. parapertussis* does not produce pertussis toxin, although it has a silent gene for this toxin (2) and is capable of expressing this toxin if a copy of the functional pertussis toxin gene is introduced into the cell. Failure to produce pertussis toxin might make *B. parapertussis* seem to be equivalent to *B. pertussis* mutants like BP357, which are also unable to cause a lethal infection in this model. However, *B. parapertussis* is very different from *B. pertussis* in its growth characteristics, and these studies should be expanded and repeated with more strains.

Genetic analysis of *B. pertussis* is in its infancy, and we are only now learning some of its rules. It is becoming increasingly clear that many of the virulence factors are encoded in polycistronic operons (10, 17) and with long open reading frames (22), suggesting that the exception for *E. coli* may be the rule for *B. pertussis*. This has very important implications when transposon mutagenesis is used. Rather than isolating mutants that are genetically and phenotypically ablations of a single gene and protein, we obtained mutants whose phenotypes are complicated. Decreased synthesis of a perfectly good toxin could occur when insertions downstream from a structural gene influence the stability of the mRNA or insertions occur in genes required for secretion. Decreased toxin production could have profound effects in a mouse challenge. Alternatively, some unknown factor may map downstream from a known factor and one could mistakenly attribute loss of virulence to loss of the factor known to be missing. Characterizing the mutants you possess is useful, if only to serve as an impetus for further studies. An example is mutant BPM3171. The insertion in BPM3171 maps 2.7 kilobases downstream from the *Bam*HI site (17) in the pertussis toxin structural gene. This mutant produced a positive reaction for pertussis toxin, the characteristic clustering appearance in the Chinese hamster ovary cell assay (13), suggesting that this mutant is not negative for pertussis toxin. However, it requires a 10-fold greater challenge than the parental strain to cause a lethal infection. Another mutant, TOX5105, with an insertion downstream from the structural gene for pertussis toxin was shown to produce less pertussis toxin activity (3). Studies are in progress to determine whether the pertussis toxin operon extends father than previously thought and what is encoded at the insertion site.

A mutant deficient in a previously untested phenotype, dermonecrotic toxin production, was as virulent as the wild-type strain. This toxin has been reported to be localized to the cytoplasm of the bacteria (5). The mechanism of action of this toxin in promoting dramatic skin lesions has been attributed to a toxic effect on endothelial cells that leads to vasoconstriction (7, 8). These findings, taken together, might suggest that this toxin would play no role in the disease, because it is not secreted efficiently by the bacteria and endothelial cells are not likely to be exposed to the toxin, since the bacteria never seem to invade the bloodstream (34). Further evaluation of the role of this toxin in the disease process remains to be performed.

It was unexpected that so many mutants in *vir*-regulated genes seem to be unaffected in the ability to cause disease. Perhaps virulence is multifactorial, but there is redundancy in the system, and the bacteria may have several factors that perform similar functions. There is some evidence that this is true for bacterial attachment. FHA (29, 30, 32), pertussis toxin (29, 30), and two serologically distinct fimbriae (11) have all been implicated as possible attachment factors. The nature of bacterial adherence in *B. pertussis* is unresolved. Textbook descriptions of human pertussis contend that the bacteria are localized exclusively to ciliated cells; however, the older literature suggests that lungs of humans become infected with *B. pertussis*, particularly in fatal cases (34). In the infant mouse model, bacterial attachment to cilia is not a predominate feature, while lethal pneumonia is (34).

We feel that the infant mouse model provides a sensitive assay for the rare but potentially fatal pneumonia seen in human infants and may not be the ideal model to study attachment to cilia and the initial stages of the disease. The pioneering studies of Tuomanen et al. demonstrated that FHA and pertussis toxin play an important role in attachment to human ciliated cells (29, 30). In other studies, she has shown that *B. pertussis* attaches inefficiently to mouse cilia (31). The inability to attach efficiently to mouse cilia might explain why FHA mutants are as virulent as wild-type strains. In fact, lack of efficient localization to the cilia may lead to more severe disease. In a rabbit model of acute pathologic lung changes, bacteria were introduced into the

trachea, the animals were sacrificed 4 h later, and the lungs were examined. The FHA mutants failed to attach, entered the lungs, and produced more severe lesions than did the wild type (30), suggesting that efficient attachment could halt entry into the lungs and subsequent development of lethal pneumonia. In support of this hypothesis, the two pertussis toxin mutants have been shown to have very different efficiencies of attachment to human ciliated cells (29). Attachment of BP357 to ciliated cells was as efficient as or more efficient than that of the wild type; however, BP356 attached only if exogenous pertussis toxin was added, and then in a dose-dependent manner (29). In this study, these two mutants were found to have very different lethal doses (Fig. 3). The more virulent of the two, BP356, is the mutant that attaches less efficiently to cilia. This also supports the suggestion that there is an inverse association between attachment to cilia and fatal infection. A frightening prospect is that a vaccine that only prevents attachment might lead to a more serious infection.

Fimbriae have been implicated as potential adhesins; however, their role in the disease remains unclear. We have shown that fimbrial expression is variable (38) and strains can switch fimbrial expression off and on, like type 1 pilus expression in *E. coli* (23). In this study, we showed that expression of fimbriae at the time of challenge does not appear to change the dose required to cause a lethal infection for either the wild-type strain or the pertussis toxin mutant, and most of the bacteria recovered after a lethal infection did not express fimbriae. In contrast, several mutants did not express either of the fimbrial types before challenge; however, some of the bacteria recovered from an animal that had been sacrificed did express fimbriae. This suggests that temporal selection favoring survival of fimbriated bacteria may occur later in infection. Perhaps fimbriae play some role in persistence but not in lethal infection. However, not all isolates from a sublethal infection were fimbriated and the role of the fimbriae needs to be examined further.

In summary, our studies identified only pertussis toxin and the adenylate cyclase toxin-hemolysin as required factors for lethal infection. The identities of the factors defined by mutants BPM2041 and BPM3171 remain to be determined. The rather disappointing results of the Swedish vaccine trials (1) with defined component vaccines composed of pertussis toxoid alone or pertussis toxoid in combination with FHA suggest that more antigens are needed to confer protection. Our studies have implicated adenylate cyclase toxin-hemolysin as an important antigen to consider for inclusion in a component vaccine. In addition, these studies suggest that whooping cough is more than simple intoxication by pertussis toxin. More animal studies using other models, in addition to the infant mouse model, are required to unravel the molecular basis of this important disease.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-23695 from the National Institute of Allergy and Infectious Diseases and by a Pew Foundation Fellowship to A.A.W.

Monoclonal antibodies were provided by Michael Brennan.

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