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Genetic Analysis of Phase Change in *Bordetella pertussis*

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Avirulent-phase derivatives of *Bordetella pertussis* (those which have simultaneously lost the ability to synthesize several virulence-associated factors) and the genetic mechanism of the phase change were studied. Increased tolerance to erythromycin was shown to be an avirulent-phase marker. By the use of efficiency of plating on erythromycin, the proportion of avirulent-phase (Vir^-) variants in a virulent-phase (Vir^+) population was determined to be between 10^{-3} and 10^{-6}, depending on the strain. We showed that the phase shift is reversible and detected a complete Vir^- to Vir^+ to Vir^- to Vir^+ cycle. In other experiments, hybridization studies with avirulent-phase mutants obtained by Tn*5* mutagenesis suggested that a single region located at a unique site in the *B. pertussis* chromosome controls the phase change. One of the avirulent Tn*5* mutants was used as a recipient in a conjugative cross with a virulent-phase donor. All recombinants which had reacquired the virulence-associated factors also lost Tn*5*, indicating the loss of Tn*5* was required to restore the Vir^+ phenotype. The Tn*5* avirulent-phase mutants behave as if the insertion interrupted the function of a transacting gene product which is required for the expression of the other virulent-phase genes. A model of the molecular basis of the phase regulation is presented.

Bordetella pertussis strains undergo a form of variation, often called a change of phase, in which virulent bacteria simultaneously lose the ability to synthesize toxins and other factors associated with pathogenicity (7, 20). These factors include pertussis toxin (15), adenylate cyclase (28), hemolysin (20), dermonecrotic toxin (4), filamentous hemagglutinin (FHA) (5), pili (1), cytochrome *d*-629 (8), and several outer membrane proteins (7, 25).

The terminology used to describe the phase shift has not been standardized. The change from a virulent state (with the potential to synthesize all of the toxins and virulence-associated factors) to the avirulent state (in which the organism is unable to express any of the virulence-associated factors) has been previously referred to as a change from phase I (with phase II as an intermediate phase) to phase III (12, 13); phase I to phase IV (16); fresh isolate to degraded state (19); or domed/hemolytic to flat/nonhemolytic (20). There are problems with all of these designations. The phase I to IV designations of Leslie and Gardner (16) are not equivalent to the phase I to III designations of Kasuga et al. (12, 13). Both classifications are based on serological differences; however, their reference sera are not generally available. A fresh isolate does not guarantee that a strain will be in the virulent phase, and laboratory passage does not mean it will become degraded. Domed colony type and hemolysis refer to only two of the many properties lost in the phase shift, and we have shown that these properties can be mutated without loss of the other phenotypic characteristics associated with the virulent phase (27). We have used a more descriptive designation, virulent phase to avirulent phase (27), as a concise term for the potential of the bacteria to cause disease when they express all of the phenotypic properties associated with virulence.

Two forms of regulation influence the expression of the virulence-associated factors of *B. pertussis*. First, the virulence genes are under a form of coordinate, reversible regulation that is influenced by the growth conditions (11, 25), including temperature or magnesium ion or nicotinic acid concentration. Under nonpermissive conditions, the synthesis of these factors does not occur; when the cells are shifted to permissive conditions, the production of these factors is rapidly resumed (11). The second form of regulation of the virulence-associated genes appears to have a hereditary component. Under permissive conditions, virulent-phase strains express the virulence-associated genes, but avirulent-phase strains do not. Their progeny inherit the same phase. The change from virulent phase to avirulent phase has been well documented (20). It had been thought that avirulent-phase strains arise from virulent-phase organisms by mutation, and thus the phase change occurred only in one direction and was essentially irreversible (19, 25). In this study, we showed that the phase change is completely reversible and studied the genetic mechanisms that control this process.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of *B. pertussis* used in this study are listed in Table 1. All of the strains used were derived either from Tohama I (virulent phase) or Tohama III (avirulent phase). It is not known whether Tohama III was obtained from Tohama I by in vitro selection for a phase variant or whether Tohama III was an independent isolate from the same patient (14). For the purposes of this study, we did not regard these two strains as isogenic. The bacteria were grown in Stainer-Scholte broth or Bordet-Gengou (BG) plates as previously described (26, 27). Continuous cultures of the strains were maintained by picking single-colony isolates onto fresh BG every 4 days. The phase of the strain was verified by scoring for the presence or absence of hemolysis. All variants selected in this study were purified by streaking for single-colony isolates at least once, and the presence of the unselected phenotypic markers of the parent was verified before characterization.

Genetic mating experiments. Conjugatives crosses were performed on agar surfaces by plating mixtures of donor and recipient strains onto BG containing 10 mM $MgSO_4$ as previously described (27). Antibiotics for selection of resistant organisms were incorporated into BG plates at the following concentrations: kanamycin, 50 μg/ml; nalidixic acid, 50 μg/ml; streptomycin, 400 μg/ml; rifampin, 50 μg/ml; spectinomycin, 50 μg/ml; trimethoprim-lactate (a gift from L. Elwell, Wellcome Research Laboratories, Research Tri-

TABLE 1. Origin and genotype of *B. pertussis* strains

Strain	Genotype (reference)[a]
Tohama I	Virulent phase (14, 22)
BP321	*str-3 rif-3*, derivative of Tohama I (26, 27)
BP338	*nal-1*, derivative of Tohama I (27)
BP330-2	*thi*::Tn*501* and pUW942 inserted into BP321 (26)
Tohama III	Avirulent phase (14, 22)
BP326	*str-2 rif-2*, derivative of Tohama III (27)

[a] Allelic designations: *nal*, nalidixic acid resistance; *str*, streptomycin resistance; *rif*, rifampin resistance; *thi*, thiamine biosynthesis.

angle Park, N.C.), 25 μg/ml; and mercuric chloride, 100 μM.

Erythromycin resistance determination. Erythromycin resistance was quantitated using the disk-diffusion method (2). Bacteria from 3-day-old BG cultures were harvested and adjusted to an optical density of ca. 0.3 at 580 nm in buffer (50 mM Tris, 43 mM sodium glutamate, 90 mM sodium chloride [pH 7.5]), and 0.1 ml was plated on BG. Erythromycin-containing disks (prepared in our laboratory or purchased from BBL Microbiology Systems, Cockeysville, Md.) were added, and the growth inhibition zone was measured after 3 days at 37°C.

Determination of EOP. Efficiency of plating to 50% (EOP_{50}) was determined by a modification of the procedure of Tait et al. (24). Cells were harvested from BG plates into Stainer-Scholte broth, diluted to between 100 and 200 CFU/0.1 ml, and plated on BG containing various concentrations of streptomycin. The number of colonies was determined after 4 days. These values were plotted against the concentration of streptomycin, and the concentration giving EOP_{50} was determined graphically. The EOP for erythromycin was determined by essentially the same method, except that serial dilutions of bacteria were plated on BG plates containing 0.25 μg of erythromycin per ml or on control BG plates.

Assays for biologically active factors of *B. pertussis*. Biologically active factors of *B. pertussis* were assayed as described (27) by the use of morphological response in Chinese hamster ovary cells for pertussis toxin (10), hemagglutination for FHA (27), appearance of lesions in infant mice after injection with bacteria for dermonecrotic toxin (4), and an enzymatic assay for adenylate cyclase (9).

Southern hybridizations. Restriction digestions, gel electrophoresis, transfer to nitrocellulose, and hybridization with a ^{32}P-labeled DNA probe consisting of the central *Hin*dIII fragment of Tn*5* were performed as described (27).

RESULTS

Frequency of change from virulent to avirulent phase. Dobrogosz et al. (7) have reported that avirulent-phase derivatives of *B. pertussis* displayed antibiotic sensitivity patterns different from those of virulent-phase strains. We used this observation as a means to study the genetic basis of this dramatic change. BG plates containing the antibiotic erythromycin at 0.25 μg/ml permitted the growth of an avirulent-phase strain (Tohama III) but not the virulent-phase strain (Tohama I). Tohama III cells plated on BG agar containing erythromycin had an EOP of 0.5 when compared with the plating efficiency on BG alone. In contrast, Tohama I plated on the same medium had an EOP of 2.8×10^{-6}. This result has been repeated several times with Tohama I and other derivatives, such as strains BP321 and BP338 (Table 1).

The colonies which survived the plating on erythromycin were found to be tolerant to erythromycin and fell into at least two classes. We chose to characterize two isolates from Tohama I. The first class, represented by strain BP329-1, seemed to be identical to avirulent-phase strains. This strain failed to produce hemolysin, pertussis toxin, and FHA (Table 2). The second class, represented by strain BP328-1, synthesized all of the virulent-phase determinants we assayed and had acquired only increased tolerance to erythromycin (Table 2). These latter variants occurred approximately 100 times less frequently than the former. Different classes of mutations conferring resistance to the same antibiotic have been described for other bacterial species (23).

The level of tolerance to erythromycin was quantitated using the disk-diffusion growth inhibition assay (Fig. 1). The virulent-phase bacteria (Tohama I) were more sensitive than all of the avirulent-phase strains, including Tohama III, the derivatives of Tohama I obtained by erythromycin selection (strain BP329-1), and a spontaneous avirulent derivative (strain BP319-10), as shown by the larger inhibition zone for the virulent-phase strain. The erythromycin-selected virulent-phase derivative, strain BP328-1, seemed to have intermediate resistance. Using the 2-μg erythromycin disk, we arbitrarily called strains with an inhibition zone of 20 mm or less erythromycin resistant; between 25 and 30 mm, intermediate; and greater than 30 mm, erythromycin sensitive. It should be noted that this designation of resistance to erythromycin is only relative. Interpretation of standard antibiotic sensitivity tests would score even the avirulent-phase strains as very sensitive to erythromycin (17).

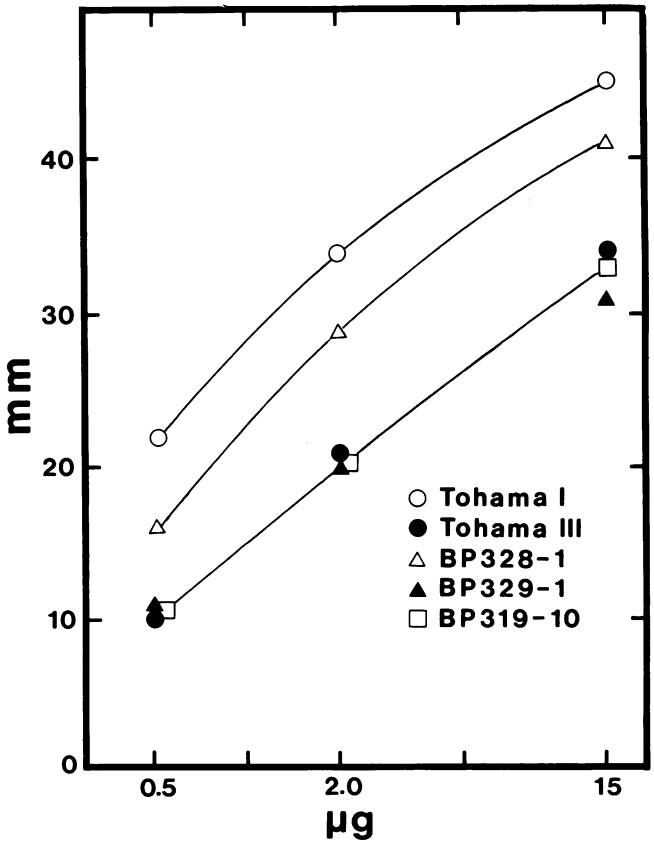

FIG. 1. Quantitation of erythromycin resistance by the disk-diffusion assay. The growth inhibition zone size in millimeters is plotted on the ordinate versus the micrograms of erythromycin in each disk plotted on the abscissa.

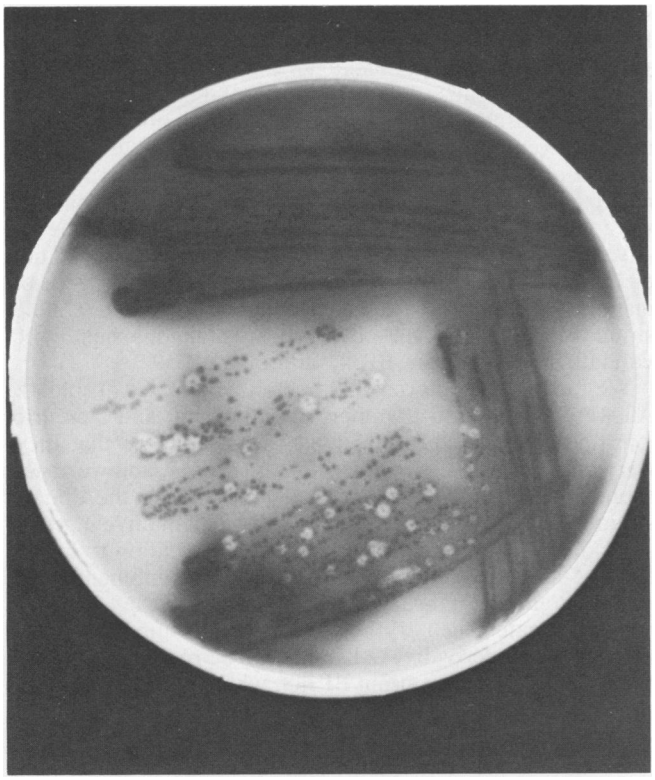

FIG. 2. Hemolytic phase revertants on BG plates. A single colony of avirulent-phase strain BP369-3 was streaked for single colonies on a fresh BG plate and incubated for 5 days at 37°C. The spontaneous revertants are surrounded by the bright halo of hemolysis.

Spontaneous revertants to virulent phase. The *B. pertussis* phase change, like that in *Salmonella* species (29), seems to be reversible. We have occasionally observed avirulent-phase bacteria revert to virulent phase, as determined by the reappearance of hemolysis (Fig. 2). The properties of two hemolytic derivatives, strains BP368-2 and BP368-3, of strain BP326 (a Tohama III derivative) are described in Table 2. They had regained the ability to hemagglutinate sheep erythrocytes and synthesize pertussis toxin and were sensitive to erythromycin. We were unable to precisely quantitate the frequency of this change from avirulent to virulent phase because we do not have a method for direct selection for virulent-phase bacteria and could only score the presence of hemolytic colonies within the population of nonhemolytic cells.

The appearance of these revertants may be influenced by an undefined factor in the growth medium. Occasionally BG plates appeared to be inhibitory to growth of avirulent-phase strains (but not virulent-phase strains), as determined by the reduced ability of these strains to form single-colony isolates, even though growth occurred in the heavily inoculated portions of the plate. This could favor the growth of virulent-phase variants in the population. We transferred the *B. pertussis* strains every 4 days, and only rarely (as infrequently as once in 25 times) did a plate with any revertants occur. However, we detected revertants eight times, and four of those times more than one avirulent strain had reverted. One would not expect an infrequent, random event to occur in clustered outbreaks as often as we observed. A common factor was the use of BG plates prepared at the same time. The BG plates used to propagate the bacteria contained 15% sheep blood, and fresh blood was obtained every week. Since the blood was the only variable component of this medium, perhaps some component of blood or the immune

TABLE 2. Properties of *B. pertussis* strains[a]

Strain	Phenotype						
	Hly	Fha	Ptx	Adc	Dnt	Ery	Origin
Tohama I	+	+	+	+	+	S	
Tohama III	−	−	−	−	−	R	
BP338	+	+	+	+	+	S	
BP326	−	−	−	−	−	R	
BP321	+	+	+	ND	ND	S	
BP330-2	+	+	+	ND	ND	S	
Spontaneous phase revertants							
BP319-10	−	−	−	ND	ND	R	From Tohama I
BP368-2	+	+	+	ND	ND	S	From BP326
BP368-3	+	+	+	ND	ND	S	From BP326
Erythromycin-selected strains							
BP328-1	+	+	+	ND	ND	I	From Tohama I
BP329-1	−	−	−	ND	ND	R	From Tohama I
BP369-2	−	−	−	ND	ND	R	From BP368-2
BP369-3	−	−	−	ND	ND	R	From BP368-3
Tn*5* insertion mutants							
BP347 (*vir-1*::Tn*5*)	−	−	−	−	−	R	Tn*5* in BP338 (27)
BP359 (*vir-2*::Tn*5*)	−	−	−	ND	ND	R	Tn*5* in BP338

[a] Phenotypes or genotypes are: Hly, hemolysin; Fha, filamentous hemagglutinin; Ptx, pertussis toxin; Dnt, dermonecrotic toxin; Ery, erythromycin susceptibility; *vir*, virulent phase. +, Demonstrable activity; −, negative; R, resistance; S, sensitivity; I, intermediate resistance; ND, not determined.

status of the animal may favor the growth of virulent-phase organisms.

To determine whether virulent-phase derivatives could revert back to the avirulent phase, strains BP368-2 and BP368-3 were plated on BG containing erythromycin. Only a small percentage of the bacteria survived, and most appeared to be nonhemolytic avirulent-phase strains. The EOP of strain BP368-3 on BG containing 0.25 μg of erythromycin per ml was 9.6×10^{-4}, much higher than that obtained for Tohama I. Two of these erythromycin-resistant derivatives, strains BP369-2 and BP369-3, were further characterized (Table 2). They were found to be deficient for synthesis of the virulence-associated factors and seemed to be identical to strain BP326. One of these strains, BP369-3, reverted back to virulent phase (Fig. 2). Thus, the cycle from avirulent phase to virulent phase and back appears to be completely reversible. Erythromycin resistance and sensitivity changed with the other virulence markers, supporting the role of erythromycin resistance as a phase marker.

Characterization of Tn*5* mutants multiply deficient in virulence-associated traits. Two independent Tn*5* insertion mutants derived from a virulent-phase strain (BP338) were isolated. Strain BP347 was characterized in a previous study (27). Strain BP359 was independently isolated as a nonhemolytic mutant. When further characterized, strain BP359, like strain BP347, was unable to hemagglutinate erythrocytes or produce pertussis toxin and was resistant to erythromycin (Table 2). Both mutants appear to be identical to avirulent-phase bacteria.

Physical characterization of the Tn*5* insertion site. We have previously shown that a single Tn*5* insertion occurred in the avirulent-phase mutant BP347 (27). Physical characterization of the independently derived Tn*5*-induced avirulent mutant BP359 described above was also performed. Southern hybridization with a Tn*5*-specific probe was performed on restriction digests of total chromosomal DNA derived from strain BP326 and the two Tn*5*-induced avirulent mutants (Fig. 3). No sequences homologous to the Tn*5* probe were detected in strain BP326. Bands of identical electrophoretic mobility were obtained for the two Tn*5* mutants when their DNA was digested with an enzyme which does not cleave within the Tn*5* sequence (21), such as *Eco*RI (Fig. 3, lanes c and d) or *Cla*I (Fig. 3, lanes f and g). This suggests that both Tn*5* insertions occurred within the same *Eco*RI and *Cla*I fragment in the *B. pertussis* chromosome. Hybridization to DNA cleaved with *Sma*I, a restriction enzyme which cuts within Tn*5*, gave two bands of different electrophoretic mobility for each mutant. For strain BP347, the two fragments were ca. 16 and 15 kilobases, and for strain BP359, they were ca. 17 and 14 kilobases (Fig. 3). It appears that the sum of the molecular masses of the two fragments is the same for both mutants (ca. 31 kilobases). This is the result one would predict if an additional *Sma*I site from Tn*5* were introduced within the same chromosomal *Sma*I fragment by two independent but not identical insertions. This difference could be due to either a different site of insertion or a difference in the orientation of transposon Tn*5*, or both, since the *Sma*I site maps about 500 base pairs from the middle of Tn*5* (21). The chromosomal distance separating the site of the two insertions could not be greater than 3 kilobases.

Genetic studies of the Tn*5*-induced avirulent mutants. To demonstrate that the avirulent-phase phenotype of strain BP347 was due to the Tn*5* insertion and not the result of a spontaneous mutation, we utilized the fact that transposon Tn*5* encodes kanamycin resistance and that this resistance should map to the site of the insertion. We mated strain BP347 with a genetic donor, strain BP330-2 (a rifampin- and streptomycin-resistant, nalidixic acid-sensitive, virulent-phase strain). This donor strain contains a conjugative plasmid integrated into the chromosome that mobilizes chromosomal genes in genetic transfer experiments (26). After 5 or 24 h of mating, the cells were plated on BG containing nalidixic acid to counterselect the donor. Recombinants were identified by scoring for hemolytic colonies (Table 3). After 5 h of mating, two hemolytic strains were observed out of 704 recipients, a frequency of 2.8×10^{-3}. After 24 h of mating, this frequency had increased to 6×10^{-2}. Unlike strain BP326, we never observed spontaneous hemolytic variants of either strain BP347 or strain BP359.

We further characterized 29 hemolytic isolates (Table 4). All were sensitive to rifampin, trimethoprim, and mercuric ions and did not require thiamine. This indicates that they were derived from the recipient, strain BP347, and were not spontaneous nalidixic acid-resistant mutants of the donor, strain BP330-2. These derivatives were all kanamycin sensitive, indicating that they had lost Tn*5*. In addition to losing the Tn*5*-specific marker, the hemolytic recombinants had also reacquired erythromycin sensitivity and the ability to hemagglutinate sheep erythrocytes and thus appeared to be virulent-phase derivatives. The hemolytic recombinants appear to be identical to strain BP338, which is what would be expected if all of the phenotypic changes in strain BP347 were the result of a single Tn*5* insertion in the BP338

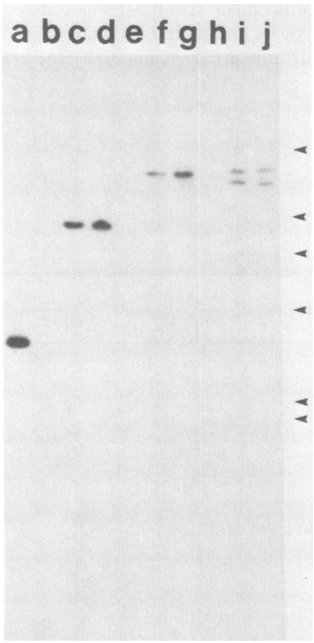

FIG. 3. Detection of Tn*5*-specific sequences by Southern hybridization. Restriction enzyme digests of chromosomal DNA fragments were separated by electrophoresis in a 0.7% agarose gel buffered with Tris-acetate and hybridized with a ^{32}P-labeled *Hin*dIII fragment internal to Tn*5*. Lane a, the *Hin*dIII Tn*5* probe; lane b, BP326 *Eco*RI digest; lane c, BP347 *Eco*RI digest; lane d, BP359 *Eco*RI digest; lane e, BP326 *Cla*I digest; lane f, BP347 *Cla*I digest; lane g, BP359 *Cla*I digest; lane h, BP326 *Sma*I digest; lane i, BP347 *Sma*I digest; and lane j, BP359 *Sma*I digest. The arrows indicate the mobility of 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.

TABLE 3. Genetic transfer of phase determinants and rifampin resistance[a]

Time of mating (h)	Selected markers[b]	Recombination frequency[c]
5	Nal and Hly	2.8×10^{-3}
24	Nal and Hly	6.0×10^{-2}
5	Nal and Rif	$<1.7 \times 10^{-7}$
24	Nal and Rif	2.6×10^{-5}
24	Nal, Rif, and Hly	1.8×10^{-6}

[a] Plate matings were performed using strain BP330-2 (Str^r, Rif^r, virulent phase) as the donor and strain BP347 (Nal^r, avirulent phase) as the recipient. Nal selection was used to counterselect the donors.

[b] For phenotypic designations, see footnote *a*, Tables 1 and 2.

[c] Frequencies denote the number of bacteria expressing the marker scored or selected per nalidixic acid-resistant recipient. The spontaneous mutation frequency for strain BP347 to rifampin resistance was less than 1.6×10^{-9}, and for strain BP330-2 to nalidixic acid resistance, it was less than 1.7×10^{-9}. Spontaneous hemolytic variants of strain BP347 have never been observed.

chromosome. The correlation between loss of kanamycin resistance and reacquisition of the virulent-phase characteristics supports the hypothesis that the Tn*5* insertion in strain BP347 is closely linked to a region essential for the expression of the virulent-phase genes. We were unable to perform the reciprocal cross to demonstrate cotransfer of kanamycin resistance and avirulent phase because strain BP347 is not a genetic donor.

The hemolysin marker was shown to be poorly linked to another marker, rifampin resistance. In other matings, rifampin resistance was selected. The rifampin marker was transferred at a much lower frequency than the hemolysin marker (Table 3). Cotransfer of rifampin and hemolysis occurred in 6 of 84 rifampin-resistant isolates, whereas no rifampin-resistant isolates were seen when the hemolytic phenotype alone was scored. Six rifampin-resistant hemolytic recombinants were characterized. They had lost kanamycin resistance and reacquired the other virulent phase markers and were identical to the other hemolytic recombinants except for the rifampin marker (Table 4). These results support the conclusion that the virulent-phase marker maps to a more proximal position relative to the origin of transfer than rifampin (assuming unidirectional gene transfer) and that there is a very close linkage of the site of the Tn*5* insertion and the virulent-phase locus, but not of rifampin resistance and either Tn*5* or the virulent-phase locus.

The results for the streptomycin marker were unusual (Table 4). Strain BP347 was derived from the streptomycin-sensitive strain, BP338, by Tn*5* mutagenesis. The resistance to streptomycin acquired by strain BP347 was an unexpected finding since transposon Tn*5* does not confer streptomycin resistance to *E. coli*, nor has this aminoglycoside been reported to be a substrate for the type II neomycin phosphotransferase of Tn*5* (6). On further examination, we found that all of the *B. pertussis* Tn*5* insertion mutants we have characterized in this study and another study (27) have acquired streptomycin resistance. This resistance was independent of the virulent-phase phenotype, indicating that the resistance was associated only with the acquisition of transposon Tn*5*. The streptomycin resistance associated with Tn*5* was intermediate to the chromosomal streptomycin resistance. The EOP_{50} for the strain used to generate the Tn*5* mutants, BP338, on BG was ca. 2 μg/ml, whereas the Tn*5* insertion mutants had an EOP_{50} of ca. 100 to 200 μg/ml. A mutant selected for chromosomal streptomycin resistance, strain BP321 (the strain from which strain BP330-2 was derived), was much more resistant to streptomycin; it had an EOP_{50} of greater than 800 μg/ml, the highest concentration tested. The streptomycin resistance for the Tn*5* mutants was not due to transposon Tn*7*, a second transposon present on the plasmid used to construct the Tn*5* mutants, since these strains were not resistant to trimethoprim or spectinomycin (two markers encoded by Tn*7* in addition to streptomycin). Also, colony blots with a Tn*7* DNA probe, plasmid pRKTV5, the plasmid used to make the Tn*5* delivery vehicle (27), detected no Tn*7* sequences (data not shown). We have not characterized the nature of the Tn*5*-encoded streptomycin resistance further, except to document that it was associated with the acquisition of Tn*5* and was lost from cells which have lost Tn*5*.

DISCUSSION

Erythromycin tolerance has been shown to be a phase marker for *B. pertussis*. The biochemical nature of this resistance has not been determined, but the extensive alterations in the surface properties between the virulent- and avirulent-phase strains that have been described (7, 20, 25) could be responsible for the difference in susceptibility to this antibiotic.

Phase change in *B. pertussis* does not appear to involve the accumulation of multiple point mutations in response to in vitro selection for variants which outgrow the fastidious virulent-phase bacteria (19), since of all spontaneous variants and the Tn*5*-induced avirulent mutants had an identical phenotype. The reversibility of the phase change indicates that the mechanism does not involve the loss of genetic information, as could occur if curing of a plasmid or prophage containing the structural genes was involved. In addition, coordinate expression of the virulence-associated genes does not seem to occur because they are encoded in a polycistronic operon, based on results obtained in a previous study (27). Tn*5* has been shown to cause polar mutations (3), and the frequency of the loss of a phenotype will be ordered

TABLE 4. Characterization of strain BP347 hemolytic recombinants

Strain	Phenotype[a]									
	Nal	Kan	Hly	Ery	Fha	Str	Rif	Thi	Tmp	Mer
BP330-2 (donor)	S	R	+	S	+	R	R	−	R	R
BP347 (recipient)	R	R	−	R	−	I	S	+	S	S
Transconjugants[b] (29 of 29)	R	S	+	S	+	S	S	+	S	S

[a] Phenotypes are abbreviated and scored as described in footnote *a*, Tables 1 and 2. Other markers are: Kan, kanamycin resistance; Tmp, trimethoprim resistance; and Mer, mercuric ion resistance.

[b] Hemolytic recombinants from the mating described in Table 3 were first purified by streaking for single colony isolates.

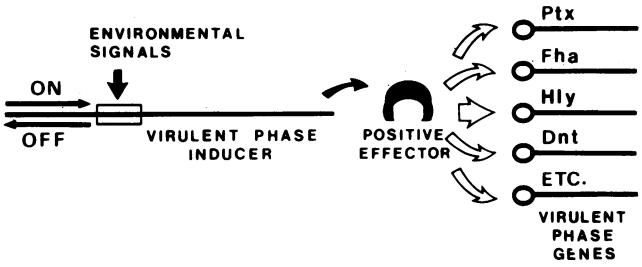

FIG. 4. Model for regulation of the virulence-associated genes by a positive effector. The lines represent the DNA coding regions for the regulatory gene and the virulence-associated genes. The circles represent the site of action for the common regulatory element. The *trans*-acting positive effector could be a protein encoded by the virulent-phase inducer gene, or this gene could encode an activity which synthesized a product required for the expression of the virulence-associated genes, analogous to cyclic AMP. Environmental signals, such as temperature, Mg^{2+} ions, or nicotinic acid, could modulate the expression of the positive effector. The molecular mechanism of ON to OFF switch of phase variation is unknown, but it is diagrammed here as an invertible sequence as a working hypothesis.

with respect to its position in the operon; for example, the last gene in a operon would be missing every time another gene in the operon was mutated. We have shown that the genes for the virulence-associated factors (FHA, hemolysin, and pertussis toxin) can be mutated independently with Tn*5* and that the loss of one virulence-associated gene product does not lead to the loss of others (27). We have also shown that the virulence-associated genes were not physically linked. The Tn*5* insertion for each mutant mapped within different restriction fragments in the *B. pertussis* chromosome, sometimes on fragments greater than 20 kilobases in size (27). This lack of hierarchy for the virulence-associated genes, coupled with the observation that they seem to be physically separated, leads us to reject the hypothesis that all of these genes occur in a polycistronic operon and that regulation at a single promoter could control their expression.

Our data support the involvement of a *trans*-acting gene product, which acts as a positive inducer for the virulence-associated genes. Mutants BP347 and BP359, described in this study, each have a single insertion in a site in the *B. pertussis* chromosome within a few kilobases of each other that affects the expression of all of the virulence-associated genes. We have shown that after mating, all BP347 recombinants selected as having been restored to the virulent phase had lost Tn*5*, supporting the hypothesis that the Tn*5* insertion occurred in a region required for the expression of the virulence-associated genes. Since the Tn*5* mutants had a negative phenotype and were not constitutive for the virulence genes, it appears that an inducer and not a repressor was inactivated by the Tn*5* insertion. The model we favor for the mechanism of phase variation in *B. pertussis* is depicted in Fig. 4. The putative *trans*-acting gene product would not be produced when the bacteria were in the avirulent phase or when this region had been mutated. In addition, regulation of the production of this same gene product could be responsible for the reversible regulation of the virulence-associated genes in response to environmental conditions (such as temperature, magnesium ion concentrations, or nicotinic acid concentrations). The mechanism of action of the putative *trans*-acting regulatory element is unknown.

A different event must be responsible for the reversible expression of the regulatory element at the genetic level. One of the best-characterized forms of gene regulation involving a reversible transition from "on" to "off" states involves DNA rearrangements. In *Salmonella* species, phase variation involving the choice of synthesis of one of two possible antigenic types of flagella is controlled by a DNA rearrangement which involves an inversion of a DNA sequence containing the promotor (29). Pilus expression in *Neisseria gonorrhoeae* also is accompanied by DNA rearrangements (18). We are currently using the Tn*5* insertion mutants to determine the nature of this regulation and whether DNA rearrangements have occurred.

Questions arise as to why *B. pertussis* would have a reversible genetic mechanism to turn on and off the synthesis of the factors that seem to be essential for its existence and the significance of the phase change, if any, to the bacteria. The phase transition does not seem to be an in vitro artifact. Kasuga et al. (14) have isolated avirulent-phase organisms when patients were periodically cultured for *B. pertussis* during the course of an infection. Most patients at the early stages of the disease appeared to be colonized with only phase I organisms (virulent phase by our nomenclature), but as the disease progressed, a greater proportion of cells in other phases (avirulent phase) were isolated. Kasuga et al. (14) concluded that only virulent-phase isolates can initiate infection but that the other phases can appear once the infection has been established.

The phase change could be a defense mechanism to escape immune detection, like *Salmonella* flagellar phase variation (29), where the change of antigenic type helps the bacteria evade the immune system. The *B. pertussis* phase change has been shown to involve many antigenic changes (12, 16) and would be an extreme example of antigenic variation, where instead of substitution of antigenic types, a loss of all of the antigens associated with the virulent state occurs. Since avirulent-phase organisms do not seem to be able to initiate new infections, failure to escape from the host defenses does not appear to be a good long-term survival strategy.

The phase switch in *B. pertussis* could be a survival strategy for the organism, allowing the bacteria to exist in some nonhuman, and as yet unidentified, environment. Alternatively, avirulent bacteria could persist without causing disease in an infected individual, in much the same way that normal flora persist in a human host. Such individuals could be asymptomatic carriers and serve as the reservoir for the bacteria until a phase switch back to the virulent state occurs and the bacteria become able to initiate new infections in susceptible individuals. Understanding the phase shift may help answer many of the questions about whooping cough.

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ADDENDUM IN PROOF

Putnoky et al. have shown that Tn*5* confers streptomycin resistance in *Rhizobium meliloti* but not *Escherichia coli* (P. Putnoky, G. B. Kiss, I. Ott, and A. Kondorosi, Mol. Gen. Genet. **191**:288–294, 1983).

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