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A New Assay for Invasion of HeLa 229 Cells by *Bordetella pertussis*: Effects of Inhibitors, Phenotypic Modulation, and Genetic Alterations

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Invasion and intracellular survival of *Bordetella pertussis* in HeLa 229 cells was studied by a new assay that utilizes polymyxin B instead of gentamicin to rapidly kill extracellular organisms. Invasion measured by this assay was time and temperature dependent and was inhibited by the microfilament drug cytochalasin D. The invasion process was also dependent on a functional *vir* locus (also known as *bvg*), the positive regulator of virulence gene expression in *B. pertussis*. Four spontaneous Vir$^-$ phase variants of *B. pertussis* and a mutant with a transposon insertion mutation in the *vir* locus did not invade. Cells that were environmentally modulated and thus did not express virulence determinants also did not invade. Two Vir$^-$ mutants, a *vir*-directed plasmid insertion mutant and a UV-light-induced mutant, were capable of invasion, although they did not produce other known virulence factors such as pertussis toxin and hemolysin but did produce small amounts of filamentous hemagglutinin (FHA) and the 69-kilodalton outer membrane protein. None of 70 Tn*5* IS*50*$_L$::*phoA* (Tn*phoA*) insertion mutants of strain Bp18323 (including three mutants defective in FHA) tested showed any reproducible defect in invasion. A mutant carrying a site-directed deletion mutation in FHA was also capable of invasion in our assay. These data suggest that there is redundancy in the invasion functions of *B. pertussis* and that one or more of these are coordinately regulated with FHA and the 69-kilodalton outer membrane protein more tightly than with other *vir*-activated gene products.

The causative agent of whooping cough, *Bordetella pertussis*, is a noninvasive pathogen which attaches to respiratory epithelial cells and produces disease through the action of several bacterial toxins. The virulence determinants involved in causing disease include filamentous hemagglutinin (FHA), several fimbriae, a 69-kilodalton outer membrane protein (69-kDa OMP), pertussis toxin, dermonecrotic toxin, adenylate cyclase-hemolysin toxin, and tracheal cytotoxin. With the possible exceptions of the fimbriae and tracheal cytotoxin, expression of these virulence factors is positively regulated by the *vir* locus (3, 27). The *vir* locus, which is also known as *bvg* (*Bordetella* virulence gene), has been sequenced and contains three open reading frames, *bvgA*, *bvgB*, and *bvgC*, which are predicted to encode proteins of 209, 175, and 936 amino acids, respectively (2). Phase variants that do not express the *vir*-activated factors contain a single cytosine inserted in a repeated sequence of cytosines in the *vir* (*bvgC*) gene resulting in a frameshift mutation (2, 24). The *vir* locus is also responsive to environmental factors such as nicotinic acid, magnesium and sulfate ions, and low temperatures (18, 25). When grown in the presence of these factors, virulent *B. pertussis* cells (X-mode cells) undergo reversible antigenic modulation to the avirulent C mode (14). A class of mutants called *mod* has been characterized which constitutively express virulence factors in the presence of modulators and map to the *vir* region as shown by DNA subcloning (13).

Although the pathogenesis of whooping cough does not appear to require *B. pertussis* to invade host cells, the bacterium can survive inside macrophages (5) and replicate in monocytes (R. L. Friedman and P. Z. Detskey, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, D128, p. 103). Recently, Ewanowich et al. (7, 8) showed that *B. pertussis* (7) and a related species, *B. parapertussis* (8), invade and survive in a nonprofessional phagocyte, HeLa 229. Invasion of *B. pertussis* in HeLa 229 cells is mediated by microfilament-dependent endocytosis and requires *vir*-activated gene products because a mutant with a transposon mutation in *vir* (*vir-1*::Tn*5*) did not invade HeLa 229 cells. However, mutations in several known and unknown *vir*-activated determinants did not abolish invasion to the level of the *vir-1* mutant (7). Here we report the results of a series of experiments on genetic characterization of invasion by *B. pertussis*.

MATERIALS AND METHODS

Bacterial strains. *B. pertussis* strains used in this study are described in Table 1. They were grown on Bordet-Gengou agar (Difco Laboratories, Detroit, Mich.) supplemented with 1% glycerol and 20% defibrinated sheep blood (BG) or in Stainer-Scholte broth supplemented with cyclodextrin (SS) (11). Strains carrying the appropriate antibiotic markers (Table 1) were grown in media containing ampicillin (50 μg/ml), kanamycin (50 μg/ml), nalidixic acid (50 μg/ml), or tetracycline (5 μg/ml). Bacterial concentrations were estimated by optical densities at 550 nm. Viable bacterial concentrations in the inocula were confirmed for several experiments by titration on BG agar.

Host cells. HeLa 229 cells (American Type Culture Collection, Rockville, Md.) were grown in a 5% CO_2 atmosphere in Dulbecco modified Eagle medium supplemented

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Phenotype or genotype[a]	Reference or source
Bordetella pertussis		
Bp338	Nal^r mutant of Tohama I	28
Bp8001	Spontaneous nonhemolytic derivative of Bp338 selected on BG containing 0.25 μg of erythromycin per ml	This study
Bp347	Bp338 *vir-1*::Tn*5* Km^r Sm^r	28
Bp348	Bp338 *hyl-1*::Tn*5* Km^r Sm^r	28
Bp353	Bp338 *fha-1*::Tn*5* Km^r Sm^r	28
Bp357	Bp338 *ptx-2*::Tn*5* Km^r Sm^r	28
Bp101	*fhaBΔ101* in Bp536, which is an Sm^r mutant of Bp338	22
Bp326	Spontaneous Vir$^-$ mutant of Tohama I, Sm^r Rif^r. Mutation due to cytosine insertion in *bvgC*	24
Bp18323	Mouse challenge strain for vaccine potency test	ATCC 9797
Bp9002	Spontaneous nonhemolytic derivative of Bp18323, selected on BG containing 0.1 μg of erythromycin per ml	This study
SK16	Bp18323 *vag-16*::Tn*phoA* FHA$^-$ Km^r Sm^r	13; Finn et al., unpublished data
SK39	Bp18323 *vag-39*::Tn*phoA* PT$^-$ Km^r Sm^r	13; Finn et al., unpublished data
SK75	Bp18323 *vag-75*::Tn*phoA* FHA$^-$ Km^r Sm^r	13; Finn et al., unpublished data
SK91	Bp18323 *vag-91*::Tn*phoA* FHA$^-$ Km^r Sm^r	13; Finn et al., unpublished data
SK101	Bp18323 *mod-1 xyz*::Tn*phoA* (PhoA$^-$) Km^r Sm^r	13; Finn et al., unpublished data
SK301	Bp18323 *vag-34*::Tn*phoA vir*::pVI7-2 Ap^r Km^r Sm^r	This study
Bp1392-1	Hemolytic UV-exposed colony from clinical isolate Bp1392	6
Bp1394	Nal^r mutant of Bp1392-2, which is a UV-induced nonhemolytic derivative of clinical isolate Bp1392	4; this study
Bp1397	Spontaneous nonhemolytic derivative of Bp1392-1, selected on BG containing 0.1 μg of erythromycin per ml	This study
Plasmids		
pGB304	*vir* insert in pLAFR2 (IncPI *cos* Tc^r)	13
pVI7-2	0.93-kilobase *SalI-SalI* fragment of *vir* inserted in pJM703.1 (*oriR6K mobRP4* Ap^r)	13

[a] Nal^r, Nalidixic acid resistant; Km, kanamycin; Sm, streptomycin; Rif, rifampin; PT, pertussis toxin; Ap, ampicillin; Tc, tetracycline.

with 10% fetal calf serum. Cell concentrations were determined by counting trypan blue-excluding cells in a hemacytometer. For a typical invasion assay, 25-cm^2 flasks were seeded with 10^6 HeLa 229 cells 24 h before infection.

Invasion assay. The nonconfluent monolayers containing 2×10^6 HeLa 229 cells were inoculated with 100 CFU of *B. pertussis* per cell in Dulbecco modified Eagle medium containing 2% fetal calf serum. The infected cells were incubated at 37°C and 5% CO_2 for the times specified and washed three times with phosphate-buffered saline containing $MgCl_2$ and $CaCl_2$. The extracellular *B. pertussis* cells were then killed by an antibiotic which does not penetrate host cells. We incubated cells in 100 μg of polymyxin B sulfate (Sigma Chemical Co., St. Louis, Mo.) per ml at 37°C and 5% CO_2 for 1 h. This procedure reduced the number of viable extracellular *B. pertussis* cells from 1×10^8 to 2×10^2 without injury to HeLa 229 cells as determined by trypan blue exclusion. In our hands, equivalent killing with 50 μg of gentamicin (Sigma) per ml required incubation for 3 h. The polymyxin B-treated monolayers were then washed three times with phosphate-buffered saline containing $MgCl_2$ and $CaCl_2$. The cells were detached by gentle shaking with approximately 25 5-mm glass beads in 1 ml of phosphate-buffered saline. The entire cell suspension was then placed in a 25-ml Erlenmeyer flask containing approximately 45 glass beads, and the intracellular bacteria were released by sonication in a bath sonicator for 5 min at 8°C. The number of viable intracellular *B. pertussis* cells was determined by titration on BG agar.

Identification of *vir*-activated factors. Hemolysis was determined by unaided examination of *B. pertussis* colonies grown for at least 72 h on BG agar plates. Plates containing nonhemolytic colonies were further incubated for 24 h at 4°C to enhance small hemolytic zones that may have been overlooked. FHA was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15) of extracts of *B. pertussis* cells grown on BG agar. Extracts were subjected to electrophoresis in a diallyltartardiamide-cross-linked 9% acrylamide gel followed by staining with Coomassie brillant blue. Full-length FHA of approximately 200 kDa was identified in the extracts by comigration with purified FHA (kindly provided by L. Winberry and R. Walker). Pertussis toxin was determined in cell extracts and culture fluid of cells by an enzyme-linked immunosorbent assay in which microdilution plates were sensitized with 100 μg of fetuin per ml (Spiro method; GIBCO Laboratories, Grand Island, N.Y.) and pertussis toxin bound to fetuin was detected with the monoclonal antibody Cx-4 (kindly provided by J. Kenimer), which is directed against the S1 subunit.

RESULTS

Characterization of *B. pertussis* invasion of HeLa 229 cells. Invasion of HeLa 229 cells by *B. pertussis* was complete after 2 h at 37°C (Fig. 1). In keeping with the invasion time used by Ewanowich et al. (7, 8), our invasion assays were routinely allowed to continue for 5 h as well. Invasion was temperature dependent, but attachment was temperature independent. Incubation of *B. pertussis* with HeLa 229 cells at 0°C abolished invasion but did not reduce the number of bacteria that were attached after 5 h. The attached population was operationally defined by the number of bacteria that could not be washed away (Table 2).

Ewanowich et al. (7) showed that invasion by *B. pertussis* was abolished when HeLa 229 cells were treated by the microfilament-disrupting agent cytochalasin D. We found

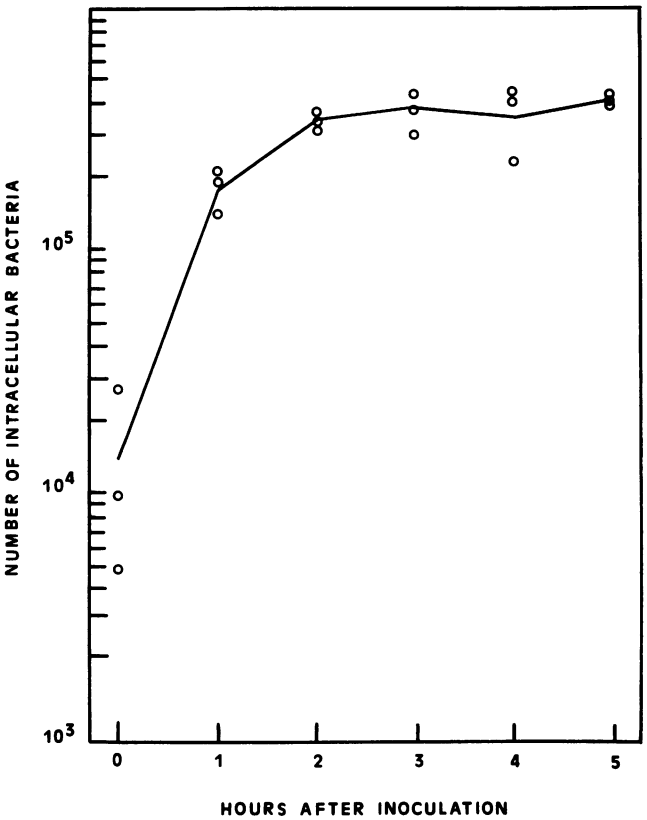

FIG. 1. Time course of HeLa 229 cell invasion by Bp18323. Bacteria were grown for 24 h in SS medium. Invasion in triplicate flasks was determined for each time point (○), and the mean was plotted.

similarily that when HeLa 229 cells were pretreated for 1.5 h with 10 μg of cytochalasin D (Sigma) per ml, the HeLa 229 cells were still attached but were no longer spread out and invasion by strain Bp18323 was reduced to 0.62% (4×10^3 CFU) of the untreated control (6.5×10^5 CFU).

Invasion by *vir* mutants. To determine whether *vir*-regulated factors are required for invasion of HeLa 229 cells, we examined Vir$^-$ strains. The phenotype designation Vir$^-$ is used to describe strains that do not express known virulence determinants; with the exception of two strains which we will describe in detail, these Vir$^-$ strains were tested for and did not produce FHA, pertussis toxin, and hemolysin. The Vir$^-$ strains that we tested included mutants with insertions in the *vir* locus, spontaneous phase variants, and C-mode cells modulated by environmental factors. Similar to the findings of Ewanowich et al. (7), the strain with a transposon mutation in *vir*, Bp347, did not invade HeLa 229 cells (Table 3). Spontaneous phase variants of strains Bp338, Bp18323, and Bp1392-1 (Bp8001, Bp9002, and Bp1397, respectively) and a phase variant whose mutation was due to a C insertion in the C repetitive sequence of *vir* (Bp326 [24]) also did not invade HeLa 229 cells. The *vir* plasmid pGB304 (13) restored the hemolysis and invasion phenotypes in Bp347, Bp8001, and Bp9002 (Table 3). Restoration of invasion was much less than 100% for the following reason. The tetracycline promoter in pGB304 is weakly expressed in *B. pertussis*, and we were unable to use a high enough tetracycline concentration to completely eliminate growth of plasmid-free segregants, which occurred at rates of 40 to 90%. Therefore, inocula of Bp347(pGB304), Bp8001(pGB304), and Bp9002(pGB304) unfortunately included plasmid-free segregants which did not invade.

Two of the Vir$^-$ mutants that we tested retained their ability to invade HeLa 229 cells. One of these (SK301) carries a plasmid insertion in the *vir* locus (S. Knapp and J. J. Mekalanos, unpublished data), and the second (Bp1394) was a nonhemolytic colony which arose from a UV-irradiated population. These two strains had several characteristics in common. They were both nonhemolytic, and the levels of pertussis toxin were 0.3% (SK301) and 4.3% (Bp1394) of wild-type levels as measured by an enzyme-linked immunosorbent assay. These levels of pertussis toxin were similar to those obtained for spontaneous phase variants such as Bp9002. However, unlike the other Vir$^-$ mutants, SK301 and Bp1394 produced reduced amounts of full-length FHA (Fig. 2) and the 69-kDa OMP as measured by Western blot (immunoblot) with the monoclonal antibody BPE3 (kindly provided by M. J. Brennan; data not shown) and retained full

TABLE 2. Effect of temperature on attachment and ingestion of Bp18323[a]

Temp (°C) of infection[b]	Time of infection (h)	Polymyxin B treatment	CFU
0	0	−	4.6×10^5
0	5	−	1.0×10^7
37	0	−	1.0×10^6
37	5	−	6.2×10^7
0	0	+	$<1 \times 10^3$
0	5	+	$<1 \times 10^3$
37	0	+	$<1 \times 10^3$
37	5	+	5×10^5

[a] Bp18323 was grown for 24 h in SS medium.
[b] 0°C incubation was achieved by placing the culture flasks on ice.

TABLE 3. Ability of *B. pertussis* phase variants, *vir* mutants, and *vir*-complemented strains to invade HeLa 229 cells

Strain[a]	% Invasion[b]
Bp338	100[c]
Bp8001	0.53
Bp8001(pGB304)	25[d]
Bp347	0.44
Bp347(pGB304)	49[e]
Bp326	<0.23
Bp18323	100[f]
Bp9002	0.22
Bp9002(pGB304)	36[g]
SK301	33
SK101	72
Bp1392-1	100[h]
Bp1394	120
Bp1397	1.3

[a] Bacterial strains were grown for 24 h in SS medium. Tetracycline was added to 5 μg/ml for strains containing the plasmid GB304. SK301 was grown in medium containing 50 μg of ampicillin per ml.
[b] Quantitated as percentage of bacteria surviving polymyxin B treatment relative to the parental strains.
[c] 3.4×10^6 CFU.
[d] 44% of the colonies were nonhemolytic.
[e] 18% of the colonies were nonhemolytic.
[f] 1.8×10^6 CFU.
[g] 100% of the colonies were nonhemolytic.
[h] 2.4×10^6 CFU.

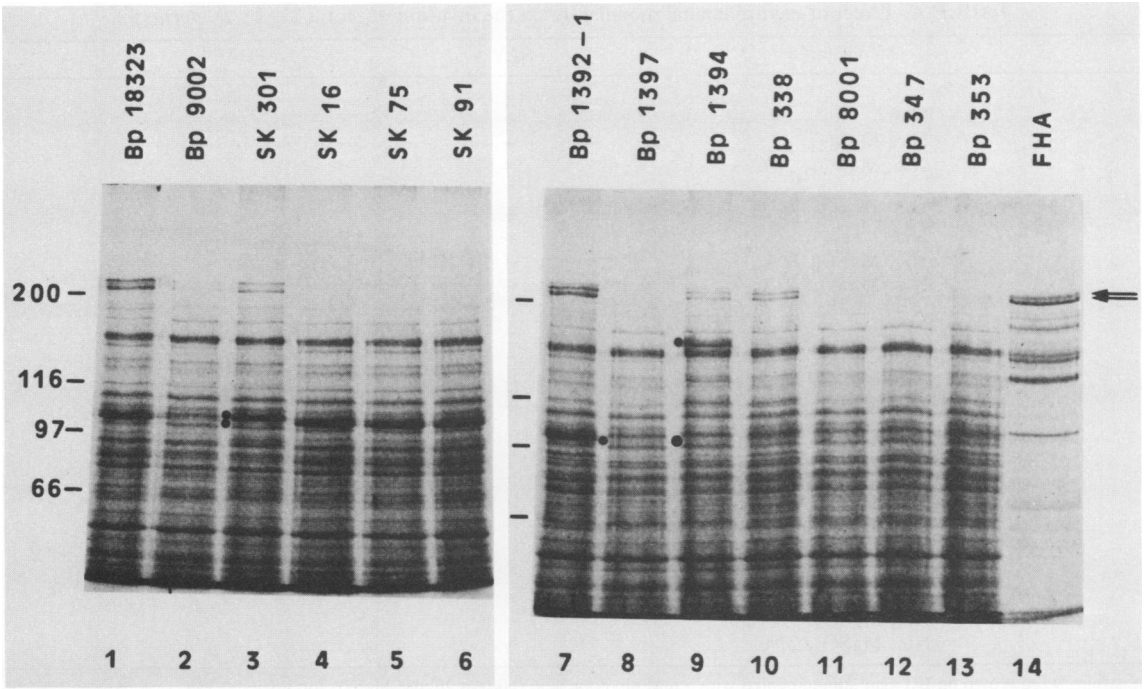

FIG. 2. Sodium dodecyl sulfate-9% polyacrylamide gel electrophoretic analysis of proteins in *B. pertussis* cells. *B. pertussis* strains were grown in BG agar or BG agar containing 50 μg of ampicillin per ml (SK301). The numbers on the left represent molecular mass standards in kilodaltons. Lane 14 contains 4 μg of purified FHA. The arrows on the right denote the two bands of approximately 200 kDa which we interpret as being full-length FHA.

invasion capacity in our assay (Table 3). The *vir* plasmid pGB304, when introduced into Bp1394 and SK301, restored hemolysin, pertussis toxin, and FHA to a level approaching that of the Vir^+ strains Bp1392-1 and Bp18323. We have not yet tested for the presence of the 69-kDa OMP in these transconjugants. The Vir^- mutants Bp1394 and SK301 have in common other features besides those already described. Examination of their total protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed the following. BP1394 and SK301 failed to express a 102-kDa protein (Fig. 2, lower circles in lanes 3 and 9) which was also absent in the invasion-negative spontaneous phase variants Bp1397 and Bp9002 but was present in invasion-positive FHA mutants such as SK16, SK75, and SK91 (Fig. 2). A second observation was the expression of a 170-kDa protein in Bp1394 (Fig. 2, upper circle in lane 9) and a 110-kDa protein in SK301 (Fig. 2, upper circle in lane 3) which were absent in the Vir^+ parental strains and the spontaneous phase variants (Fig. 2). The origins of these polypeptides and their relationship to FHA or other *vir*-regulated gene products are not known. These results suggest that, unlike other Vir^- mutants such as Bp347 and spontaneous phase variants, SK301, and Bp1394 are defective in some *vir*-activated gene products but not others which include invasion properties.

Invasion by antigenically modulated *B. pertussis*. Consistent with the hypothesis that *vir*-regulated factors are required for invasion of HeLa 229 cells, we found that C-mode *B. pertussis* (Bp18323, Bp338, and Bp1392-1) grown in the presence of modulators did not invade HeLa 229 cells (Table 4). In our hands, the suppression of *vir*-regulated functions such as hemolysin and FHA often, but not always, required the presence of more than one modulator. However, the suppression of the invasion phenotype in strains Bp18323, Bp338, and Bp1392-1 consistently correlated with the absence of other modulation-sensitive virulence determinants. Invasion by Bp1394 and SK301, the two strains which did not express pertussis toxin and hemolysin but retained the invasion phenotype, was also sensitive to modulation signals (Table 4).

Role of *vir*-regulated factors in invasion. We wished to determine which of the known virulence factors of *B. pertussis* might contribute to the invasion process. Hemolysin, adenylate cyclase, pertussis toxin, and FHA did not play a role in invasion because mutants lacking these proteins retained the ability to invade HeLa 229 cells. The Tn*5* mutant Bp348 lacks hemolysin and adenylate cyclase enzymatic activity (28) and invaded HeLa 229 cells at an efficiency of 96% (8.0×10^5 CFU) relative to the parental strain Bp338. The role of pertussis toxin was determined by testing the Tn*5* mutant Bp357, which produces a small amount of biologically active toxin (16), and the Tn*5* $IS50_L$::*phoA* (Tn*phoA*) fusion strain SK39, which produces no functional toxin (T. M. Finn, R. Shahin, S. Knapp, and J. J. Mekalanos, unpublished data). Bp357 and SK39 invaded at relative efficiencies of 269% (1.4×10^6 CFU) and 141% (1.03×10^6), respectively.

Although FHA is coregulated with invasion in strains SK301 and Bp1394 (Fig. 2; Table 3), we found no evidence that FHA is involved in invasion. The FHA mutants that we tested included the Tn*5* mutant Bp353, the Tn*phoA* fusion strains SK16, SK75, and SK91, and a mutant which carries a deletion in the structural gene for FHA (Bp101 [22]). Bp353 produces a small amount of biologically active FHA (25, 26); SK16 and SK91 produce a small amount of immunologically reactive FHA, whereas SK75 does not produce any FHA as determined either by an enzyme-linked immunosorbent assay (T. Cabezon and C. Locht, personal communications) or by hemagglutination of goose erythrocytes (Finn et al., unpublished data); Bp101 does not produce full-length FHA as determined by Western blot and hemagglutination of

TABLE 4. Effect of environmental modulators on the invasion of HeLa 229 by *B. pertussis*

Strain	Modulator(s)[a]	HLY[b]	FHA[c]	% Invasion[d]
Bp18323		+	+	100[e]
Bp18323	Nic	+	+	103
Bp18323	Nic, $MgSO_4$	+	+	105
Bp18323	Nic, $MgSO_4$, 27°C	−	−	1.4
Bp338		+	+	100[f]
Bp338	Nic	+	+	143
Bp338	Nic, $MgSO_4$	−	−	14
Bp338	Nic, $MgSO_4$, 27°C	−	−	0.51
Bp1392-1		+	+	100[g]
Bp1392-1	Nic	−	−	19
Bp1392-1	Nic, $MgSO_4$	−	−	2.3
Bp1392-1	Nic, $MgSO_4$, 27°C	−	−	0.49
Bp1394		−	±	100[h]
Bp1394	Nic	−	−	5.0
Bp1394	Nic, $MgSO_4$	−	−	23
Bp1394	Nic, $MgSO_4$, 27°C	−	−	5.7
SK301		−	±	100[i]
SK301	Nic	−	−	<6.2
SK301	Nic, $MgSO_4$	−	−	<0.6
SK301	Nic, $MgSO_4$, 27°C	−	−	0.7

[a] Bacterial strains were grown on BG agar supplemented with 5 mM nicotinic acid (Nic) or 20 mM $MgSO_4$ for 3 days at 35°C or 4 days at 27°C. Bacterial cells were suspended in SS medium, and cell concentrations were determined by optical density at 550 nm. Nicotinic acid and $MgSO_4$ were added to the tissue culture medium during the 5-h invasion when appropriate, although invasion experiments were done at 37°C.

[b] Hemolysin (HLY) activity in inocula was determined by methods described in Materials and Methods.

[c] FHA in inocula was determined by methods described in Materials and Methods.

[d] Percentage of bacteria surviving polymyxin B treatment relative to the unmodulated culture. Colonies were enumerated on BG agar without modulators, and all colonies were hemolytic except for Bp1394.

[e] 6.6×10^5 CFU.

[f] 9.8×10^5 CFU.

[g] 7.5×10^5 CFU.

[h] 2.8×10^5 CFU.

[i] 1.5×10^6 CFU.

sheep erythrocytes (22). The invasion efficiencies of Bp353, SK16, SK75, SK91, and Bp101 were 136% (7.1×10^5 intracellular CFU), 50% (4.8×10^5 CFU), 85% (6.4×10^5 CFU), 103% (7.5×10^5 CFU), and 89% (5.0×10^5 CFU), respectively, when compared with their parental strains.

We also screened a set of fusion strains in which the transposon Tn*phoA* (17) was used to generate fusions between the gene for *Escherichia coli* alkaline phosphatase and Bp18323 genes encoding secreted proteins (13). Of the 70 fusion strains tested, 48 had invasion efficiencies between 60 and 140% (Fig. 3). Mutants which showed low or high levels of invasion always retested within the normal range of values for Bp18323 (Fig. 3). The Vir^- mutant Bp347 gave invasion values of 0.086, 0.17, 0.3, 0.44, 1.0, 1.4, 1.5, 1.6, 1.7, 2.5, 2.5, and 6.9% in 12 separate experiments. With the exception of a single experiment (6.9%), the percent invasion values of Bp347 were 10- to 250-fold lower than the lowest value obtained for the Tn*phoA* mutants (22%).

DISCUSSION

The observation that *B. pertussis* invades nonprofessional phagocytes is seemingly inconsistent with the pathogenesis of whooping cough. However, it is possible that if invasion does occur in vivo, its role may be to establish a quiescent carrier state in an intracellular environment, safe from the host immune system. To achieve this, *B. pertussis* must gain entrance into the normally nonphagocytic host cell. This first step in invasion, attachment and entry, is mediated by microfilament-dependent endocytosis (7, 8). Once inside, the intracellular environment is extremely hostile (20), and to survive, *B. pertussis* needs a mechanism to escape lysosomal attack. Several different mechanisms have evolved for bacterial survival in host cells after endocytosis (20). For example, *Rickettsia tsutsugamushi* escapes from phagosomes and replicates free in the cytoplasm of the host (9). *Coxiella burnetii* is resistant to lysosomal enzymes and survives in phagolysosomes (1); *Chlamydia psittaci* remains in phagosomes and has evolved mechanisms to prevent phagolysosomal fusion (10). The intracellular survival mechanism used by *B. pertussis* is not known. Ewanowich et al. (7, 8) showed by electron microscopy that intracellular *B. pertussis* and *B. parapertussis* retained the phagocytic membrane around them, therefore ruling out the first of the three mechanisms described.

In our assay for invasion, we are unable to distinguish attachment and entry from subsequent survival in the invaded cell because we score for live intracellular bacteria. That invasion requires *vir*-activated gene factors was demonstrated by the observation that the *vir* mutant Bp347 (7) (Table 3), spontaneous phase IV variants, and C-mode *B. pertussis* (Tables 3 and 4) did not invade. Because many of the *vir*-activated factors are involved in bacterial adherence (27), it is likely that one or more of these adherence factors also mediate attachment and entry into host cells.

Although there is some evidence that FHA is involved in the invasion phenotype (7), we cannot conclude that the

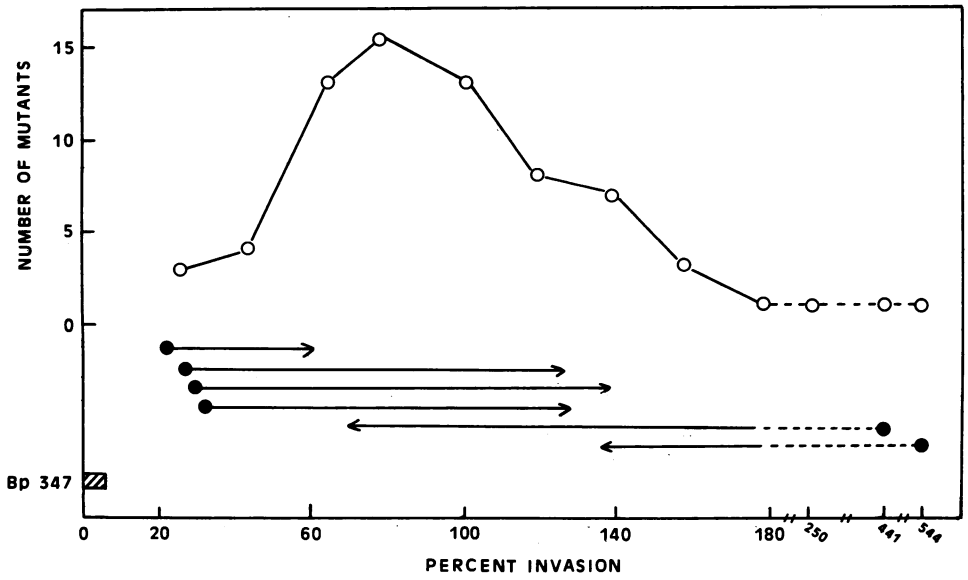

FIG. 3. Invasion of HeLa 229 cells by Tn*phoA* mutants. Percent invasion was measured relative to the parental strain Bp18323. Values between 21 and 40%, 41 and 60%, 61 and 80%, 81 and 100%, 101 and 120%, 121 and 140%, 141 and 160%, and 161 and 180% were averaged, and the number of fusion strains in each group was plotted (○). Six mutants were retested (●), and the arrows indicate the new values. The hatched bar represents the range of percent invasion values obtained for the *vir-1* mutant Bp347 in 10 separate experiments.

small amount of FHA produced by SK301 and Bp1394 is solely responsible for the invasive properties of these two Vir$^-$ strains. Three different FHA$^-$ strains (SK16, SK75, and SK91) and one site-specific FHA$^-$ mutant (Bp101) were all found to be invasive in our assay, although they produced far less detectable FHA than strains SK301 and Bp1394. Another likely candidate for a *B. pertussis* molecule which can mediate attachment and entry is the 69-kDa OMP. Like FHA, the 69-kDa OMP has two sites containing the amino acid sequence arginine-glycine-aspartic acid (4, 22) which have been implicated in attachment and invasion of protozoan parasites (21). We were also aware that there were no 69-kDa OMP mutants among the 70 Tn*phoA* fusion strains that we tested in our invasion assay (Finn et al., unpublished data). Therefore, we tested for the presence of the 69-kDa OMP in SK301 and Bp1394 and found that reduced amounts were present in both strains. Whether the presence of this protein is responsible for the invasion-positive phenotype in these strains remains to be determined.

Unlike the *vir* gene products expressed by spontaneous phase variants, those expressed by SK301 and Bp1394 were still capable of partially transactivating a subset of *vir*-activated genes. Strain SK301 is a plasmid insertion mutant in the *vir* gene of strain Bp18323. This strain was constructed by cloning the *Sal*I fragment D (24) of *vir* into the suicide vector pJM703.1 to give plasmid pVI7-2, which was transferred into Bp18323 by conjugation. Because this plasmid cannot replicate in *B. pertussis*, transfer of pVI7-2 into *B. pertussis* results in integration of the plasmid into the *vir* gene by homologous recombination between the *Sal*I-D sequences of *vir*. SK301 is such a *vir*::pVI7-2 mutant and is predicted to be disrupted in sequences downstream of the *Sal*I D fragment of *vir*. Examination of the nucleotide sequence of the *vir* locus indicates that it encodes three polypeptides: BvgA, BvgB, and BvgC (2). Deletion of sequences downstream of the *Sal*I D fragment is predicted to truncate 85 amino acid residues from the C terminus of the last polypeptide BvgC (total size, 936 amino acids), resulting in an 851-amino-acid polypeptide. Therefore, it is possible that this C-terminal sequence is not required for the positive regulation of invasion, although it is required for the regulation of other *vir*-activated genes. It is clear, however, that further truncation of BvgC eliminates expression of the invasion phenotype because strain Bp326 did not invade. This spontaneous Vir$^-$ phase variant is known to carry a frameshift mutation in a repetitive cytosine sequence that should result in the loss of an additional 49 residues from the C terminus of BvgC (resulting in an 802-amino-acid polypeptide) in comparison with the mutation carried by strain SK301. Because Bp1394 was UV irradiated, it is possible that the mutation which alters the *vir* locus in Bp1394 occurs at a site other than the repetitive cytosine sequence and thus may have a phenotype similar to that of SK301.

It can also be predicted from the nucleotide sequence of the *vir* locus that the BvgA and BvgB gene products may still be made in SK301 because these genes are located upstream of the *bvgC* gene in the operon. BvgA, when overproduced, has been shown to be sufficient for activation of the FHA promoter in *E. coli* (23). Given that SK301 and Bp1394 are the only two Vir$^-$ mutants that produce some FHA and 69-kDa OMP and are still invasive, we propose that BvgA may directly activate promoters for the 69-kDa OMP and an invasion gene of *B. pertussis*.

Since the intracellular environment may be rich in modulation signals, it is of interest to determine whether C-mode proteins which are expressed in avirulent *B. pertussis* cells in the presence of environmental modulators such as magnesium sulfate and nicotinic acid (14) play a role in the intracellular survival of *B. pertussis*. This could be achieved by determining the intracellular survival of *mod* mutants which constitutively express virulence determinants (X-mode proteins) and do not express C-mode proteins in the presence of modulators. Unfortunately, we do not have any *mod* mutants which are constitutive for X-mode protein expression in the presence of three or more modulators. SK101 is a *mod-1* mutant which constitutively expresses X-mode proteins in the presence of either $MgSO_4$ or nicotinic acid (13). SK101 invades and survives in HeLa 229 cells

to the same extent as the parental strain Bp18323 (Table 3). The intracellular environment probably contains more than one modulating factor, and therefore we cannot assume that C-mode proteins were not present in intracellular SK101 cells.

The data presented in Fig. 3 show that there is some variability in the invasion assay, and it is important to retest strains that fall outside the normal range of values. However, the invasion values of Vir^- strains, such as Bp347, were consistently well below the values of Vir^+ strains. Therefore, we believe that our assay can be used to identify true invasion-defective mutants. A simple explanation for our failure to isolate a Tn*phoA* fusion strain which had a null phenotype in invasion is the necessity to screen a larger number of strains. Of the 70 $PhoA^+$ Tn*phoA* mutants screened so far, only one pertussis toxin mutant was found (SK39; insertion in the gene encoding subunit S1), although one would predict that insertions in all five of the pertussis toxin subunit genes should give $PhoA^+$ fusions. Thus, perhaps it is necessary to isolate 5- or 10-fold more Tn*phoA* mutants to saturate the *B. pertussis* genome with Tn*phoA* fusions. An alternative explanation is the presence of redundancy in the factors required for invasion. There is evidence that such redundant systems occur in *Yersinia pseudotuberculosis* (12) and *Yersinia enterocolitica* (19). If the second explanation is correct, it would be difficult to isolate an invasion-defective mutant by this method. Further genetic, biochemical, and immunological analyses will be needed to clarify the mechanisms and significance of the invasion properties of *B. pertussis*.

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