Cloning and Sequencing of a *Bordetella pertussis* Serum Resistance Locus

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We have characterized a new virulence factor in *Bordetella pertussis*: serum resistance. Compared with *Escherichia coli* HB101, wild-type *B. pertussis* was relatively resistant to classical-pathway, complement-dependent killing by normal human serum. However, a mutant of *B. pertussis* (BPM2041) which is less virulent in mice and which has Tn*5 lac* inserted in a previously uncharacterized *bvg*-regulated gene was found to be at least 10-fold more susceptible to serum killing than the wild type. We have named this locus *brk*, for *Bordetella* resistance to killing. We have cloned and sequenced the *brk* locus, and it encodes two divergently transcribed open reading frames (ORFs), termed BrkA and BrkB. Both ORFs are necessary for serum resistance. Within the 300 bases which separate the two ORFs and upstream of each ORF are putative sites for BvgA binding. BrkA shows 29% identity to pertactin and has two RGD motifs in addition to a conserved proteolytic processing site and an outer membrane targeting signal. Like pertactin, BrkA is involved in adherence and invasion. Despite the similarities, a pertactin mutant was found to be not as sensitive to serum killing as the BrkA or BrkB mutants. BrkB is similar to ORFs in *E. coli* and *Mycobacterium leprae* and displays domains of homology to various transporters. On the basis of its hydropathy profile, BrkB is predicted to be a cytoplasmic membrane protein. By Southern blot, *brk* sequences were found in *Bordetella bronchiseptica* and *Bordetella parapertussis* but not in *Bordetella avium*.

Bordetella pertussis is the causative agent of whooping cough. The virulence factors of this pathogenic microorganism are positively regulated by the *bvgAS* genetic locus (43, 44, 51). Under specific environmental conditions, organisms with an intact *bvg* operon express the genes required for virulence and are virulent; organisms which do not express this virulence locus because of mutations in *bvg* are avirulent (53). In addition, certain growth conditions can reversibly suppress the expression of the virulence genes, a condition termed modulation (24, 29). Genes known to be regulated by the *bvg* locus include the toxins, such as pertussis toxin, adenylate cyclase toxin, and dermonecrotic toxin; putative attachment factors, such as filamentous hemagglutinin (FHA), fimbriae, and pertactin; and other factors, such as capsule (50, 55).

In a previous study, we isolated mutants with mutations in *bvg*-regulated genes, using the transposon Tn*5 lac* (55). This transposon possesses a promoterless β-galactosidase (β-Gal) gene which is expressed only if the transposon inserts downstream from a functional promoter, and then β-Gal expression is regulated in the same manner as the intact gene. Several thousand Tn*5 lac* insertion mutants were screened, and those which expressed β-Gal in a *bvg*-regulated manner were further characterized. Several of the mutants were deficient in known *bvg*-regulated genes, such as those encoding pertussis toxin, adenylate cyclase toxin, dermonecrotic toxin, and FHA. Other *bvg*-regulated mutants which were not deficient in expression of any of the previously described *bvg*-regulated factors were isolated. One such mutant, *B. pertussis* BPM2041, was further characterized. The Tn*5 lac* insertion in BPM2041 mapped to a *Kpn*I restriction fragment in the *B. pertussis* chromosome

distinct from that of all other known *bvg*-regulated Tn*5 lac* mutants (55). BPM2041 possesses known *bvg*-regulated virulence factors, such as pertussis toxin, adenylate cyclase toxin, dermonecrotic toxin, FHA, pertactin, and capsule (55). In addition, BPM2041 is 10 times less virulent in infant mice (56), suggesting that an important but previously unrecognized virulence factor was interrupted by the transposon. Here, we report that BPM2041 is much more susceptible than wild-type strain BP338 to killing by normal human serum via the classical (antibody-dependent) complement pathway. We have named the transposon-interrupted locus *brk*, for *Bordetella* resistance to killing.

The *brk* locus exists in all *Bordetella* species except *Bordetella avium*. We have cloned and sequenced the *brk* locus, and it contains two divergently transcribed open reading frames (ORFs), termed BrkA and BrkB. Adjacent to *brkA* is the gene for the heat shock protein-chaperonin, Cpn60. Both BrkA and BrkB, but not Cpn60, are necessary for the expression of serum resistance. BrkA has a predicted molecular mass of 103 kDa and resembles pertactin in sequence; specifically, it has two RGD motifs, an outer membrane localization signal, and a common proteolytic cleavage site. Like pertactin, BrkA also mediates adherence and invasion. However, despite the similarities, pertactin does not appear to play a significant role in serum resistance. BrkB has a predicted molecular mass of 32 kDa and appears to be a cytoplasmic membrane protein which bears some resemblance to those encoded by recently sequenced ORFs of unknown function in *Escherichia coli* and *Mycobacterium leprae*.

MATERIALS AND METHODS

Strains and plasmids. Strains and plasmids used in this study are listed in Tables 1 and 2. *B. pertussis* strains were grown on Bordet-Gengou (BG) medium (BBL, Cockeysville, Md.) containing 15% sheep blood (Cocalico Biologicals,

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
B. pertussis		
BP338	Wild type; Tohama background; Nal[r]	52
BP347	BP338 *bvgS1*::Tn5 Nal[r] Kan[r]	52
BPM2041	BP338 *brkA1*::Tn5 *lac* Nal[r] Kan[r]	55
BBC8	Wild type; W28 background; Sm[r]	27
BBC9	BBC8 *prn* Sm[r] Kan[r]	27
BBC8BrkA	BBC8 *brkA* Sm[r] Gent[r]; *brkA* inactivated with construct d in Fig. 3B	This study
BBC9BrkA	BBC9 *brkA* Sm[r] Kan[r] Gent[r]; *brkA* inactivated with construct d in Fig. 3B	This study
RF1039	BP338 *brkA* Nal[r] Gent[r]; *brkA* inactivated with construct d in Fig. 3B	This study
RF1057	BP338 *brkB* Nal[r] Gent[r]; *brkB* inactivated with construct h in Fig. 3B	This study
B. parapertussis 253	Wild type	31
B. bronchiseptica 11DH	Wild type	31
B. avium wBA70	Wild type; Nal[r] Kan[r]	49
Plasmids		
pBluescriptII SK⁻	Cloning vector; Amp[r]	Stratagene
pUW2032	Gent[r]-*oriT* cassette from pEB360 (3) in pBluescriptII SK⁺; Amp[r] Gent[r]	This study
pUW2033	Same as pUW2032 but in pBluescriptII KS⁺	This study
pRF1001	*Not*I *brk*-Tn5 *lac* fusion fragment from BPM2041	This study
pKWf12	pHC79 cosmid from BP338 library; contains entire *brk* locus	49
pRF1028	*Not*I-*Not*I upstream clone from pKWf12	This study
pRF1029	*Eco*RI-*Eco*RI downstream clone from pKWf12	This study

[a] Nal[r], nalidixic acid resistance; Kan[r], kanamycin resistance; Sm[r], streptomycin resistance; Gent[r], gentamicin resistance; Amp[r], ampicillin resistance; *prn*, pertactin gene; *brk*, *Bordetella* resistance to killing gene; *oriT*, origin of transfer (P conjugation).

Reamstown, Pa.) as previously described (53). *E. coli* HB101 and DH5α (Gibco/BRL, Gaithersburg, Md.) were grown on LB agar. When necessary, the following antibiotics at the indicated concentrations (in micrograms per milliliter) were added to the media: nalidixic acid, 30; kanamycin, 50; ampicillin, 100; gentamicin, 10; and streptomycin, 300. Plasmids were isolated by using either the midi-prep kit (Qiagen, Inc., Chatsworth, Calif.) or Magic/Wizard mini-prep kits (Promega Corporation, Madison, Wis.).

Reagents. Restriction enzymes and T4 DNA ligase were purchased from Gibco/BRL or New England Biolabs (Beverly, Mass.) and used according to the manufacturer's specifications. SeaKem and SeaPlaque (low-melting-point) agarose were purchased from FMC BioProducts (Rockland, Maine). Antibiotics and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were obtained from the Sigma Chemical Company (St. Louis, Mo.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents were purchased from Bio-Rad Laboratories (Hercules, Calif.). Tissue culture media, antibiotic supplements, and fetal bovine serum were obtained from Whittaker Bioproducts (Walkersville, Md.). DNA and protein molecular weight markers were purchased from Gibco/BRL and Pharmacia (Piscataway, N.J.), respectively.

Mapping the *brk* locus. The *brk* locus was mapped by insertion duplication mutagenesis as described elsewhere (54, 55). This procedure was also used to introduce a *brkA* mutation (see Fig. 3B, fragment d) into the W28 (BBC8) and W28prn⁻ (BBC9) strains of *B. pertussis*.

Serum killing assay. Overnight cultures of the bacteria were harvested in Stainer-Scholte (SS) broth without supplements to a concentration of about 10⁸ organisms per ml (optical density at 660 nm, ~0.2). A 25-μl volume of this suspension was added to 250 μl of RPMI medium (Whittaker Bioproducts) containing either 20% human serum (normal human serum) or 20% heat-inactivated human serum. The sera were stored at −20°C and thawed only once on ice prior to use. Heat-inactivated serum (30

min at 60°C) was utilized to provide a base point, since the number of organisms thus exposed did not change appreciably during the course of the experiment. Following a 60-min incubation at 37°C, complement activity was halted by diluting the organisms 10-fold in phosphate-buffered saline (PBS) containing 10 mM EDTA. Further serial dilutions were done in SS broth to determine the number of surviving organisms. In some experiments, organisms were exposed for 60 min to 20% normal human serum containing 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid] and MgCl₂. The percent survival of the organisms exposed to normal human serum, relative to that of organisms exposed to heat-inactivated serum, was calculated. Student's *t* test was used to analyze the data.

Adherence assay. The human fibroblast cell line MRC-5 (171-CCL) was obtained from the American Type Culture Collection (Rockville, Md.) and maintained in minimum essential medium supplemented with 10% fetal bovine serum, 100 U of penicillin G, and 100 μg of streptomycin per ml. Prior to use, the cells were plated to confluency in 24-well tissue culture plates (Costar, Cambridge, Mass.) in media lacking

TABLE 2. Constructs for suicide mating

Plasmid[a]	Fragment	Construct obtained from plasmid + Gent[r]-*oriT* cassette[b]
a. pRF1003	3.3-kb *Not*I-*Bam*HI	pRF1009
b. pRF1019	1-kb *Sal*I-*Bam*HI	pRF1022
c. pRF1025	1.8-kb *Sac*I-*Bam*HI	pRF1027
d. pRF1026	1.9-kb *Sal*I-*Sal*I	pRF1030
e. pRF1029	2.7-kb *Bam*HI-*Eco*RI	pRF1043
f. pRF1046	1-kb *Bam*HI-*Sst*I	pRF1046a
g. pRF1052	1.9-kb *Bam*HI-*Sma*I	pRF1053
h. pUW2141	0.7-kb *Sac*II-*Bst*YI	pUW2142

[a] a to h, fragments in Fig. 3B.

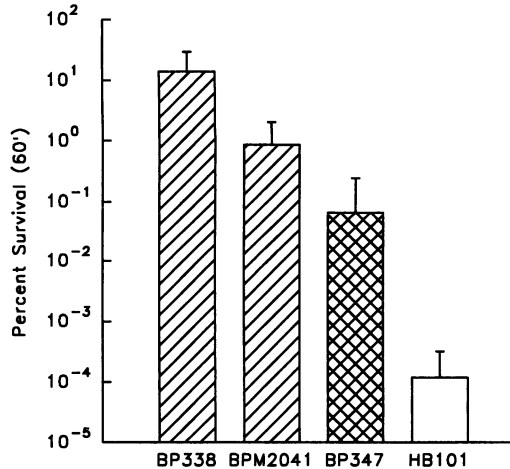[b] Gent[r], gentamicin resistance; *oriT*, origin of transfer (P conjugation).

FIG. 1. Survival of *B. pertussis* strains in human serum. *B. pertussis* BP338 (n = 30 separate experiments), BPM2041 (n = 30), and BP347 (n = 27) as well as *E. coli* HB101 (n = 8) were exposed to 20% normal human serum or 20% heat-inactivated human serum for 60 min. Percent survival was calculated as [(number of organisms surviving in normal serum)/(number of organisms surviving in heat-inactivated serum)] × 100. The mean ± standard deviation is shown for each organism.
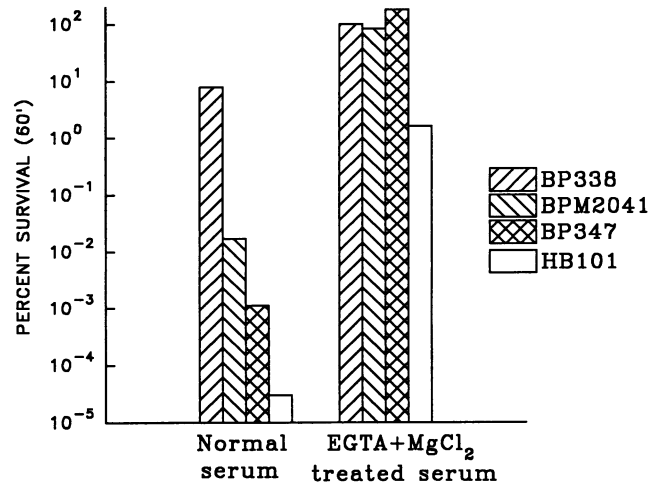

FIG. 2. Serum killing of *B. pertussis* occurs via the classical pathway of complement. Organisms were exposed to 20% normal human serum or to serum with added EGTA and $MgCl_2$ for 60 min, and percent survival was calculated as described in the legend to Fig. 1. The experiment was repeated three times, and results of one representative experiment are shown.

antibiotics. Overnight BG cultures of *B. pertussis* strains were harvested in SS broth to an optical density at 660 nm of 0.5, and 0.5 ml of each culture was labeled with 4 μCi of [^{35}S]methionine (Amersham Corporation, Arlington Heights, Ill.) for 10 min at 37°C. The organisms were washed extensively to eliminate unincorporated label and suspended in 1 ml of minimum essential medium supplemented with 10% fetal bovine serum. A 200-μl sample was removed, and the number of counts per minute per bacterium was determined. The labeled bacteria were added to the MRC-5 cells at 200 μl per well, and adherence was allowed to proceed for 3 h. The monolayers were washed five times with PBS and lysed with 200 μl of distilled water, and the radioactivity of each well was determined with in a liquid scintillation counter. The number of adherent bacteria was calculated as (total cpm per mono-layer)/(cpm per bacterium). Each experiment was performed in triplicate.

Invasion assay. Confluent monolayers of MRC-5 cells grown in 24-well tissue culture plates were infected with BP338 or BPM2041 essentially as described above, with the radiolabel-ing step omitted. After a 3-h incubation, the inoculum was removed and the monolayers were washed extensively with PBS. The last wash was replaced with medium supplemented with 100 μg of gentamicin per ml and/or 100 μg of polymyxin B per ml. After a further 3-h incubation, the antibiotic was removed. The monolayers were subsequently washed twice with PBS, lysed with distilled water, and plated on BG medium to determine the number of antibiotic-resistant (i.e., invading) bacteria. The results from 10 independent experiments are reported.

Southern blot analysis. Chromosomal DNA was isolated by the CTAB (hexadecyltrimethyl ammonium bromide) method (2). The DNA was digested with *Not*I, separated on a 0.8% agarose gel, and transferred to a positively charged nylon membrane (Boehringer Mannheim Corporation, Indianapolis, Ind.). The blot was probed with the 5' portion of the *brk* locus (see Fig. 3B, fragment a) which had been previously labeled with digoxigenin-dUTP by using the Genius kit (Boehringer).

The blot was processed with anti-digoxigenin antibodies and LumiPhos 530 (Boehringer) and exposed to X-ray film.

SDS-PAGE analysis. *B. pertussis* strains were grown on BG medium for 24 h and harvested in SS broth to an optical density at 600 nm of 0.15. A 0.5-ml volume of this suspension was pelleted and resuspended in 50 μl of lysis buffer. The samples were boiled for 5 min and loaded on a discontinuous SDS–11% polyacrylamide gel (25). Following electrophoresis, the separated proteins were visualized after being stained with Coomassie brilliant blue.

DNA sequencing and sequence analysis. Plasmids pRF1028 and pRF1029 were sequenced by Lark Sequencing Technologies, Inc. (Houston, Tex.). Sequence analysis was performed with the Genetics Computer Group package (9) and the electronic-mail servers BLAST (1) and GRAIL (47). Trans-membrane prediction analysis was done with the MEMSATPC program (22), which was obtained by anonymous ftp from ftp.embl-heidelberg.de.

Nucleotide sequence accession number. The GenBank accession number is U12276.

RESULTS

The *brk* locus is associated with serum resistance. The results in Fig. 1 indicate that about 10% of the initial 10^7 wild-type *B. pertussis* organisms were able to survive for 60 min in pooled normal human serum. About 0.0001% of *E. coli* HB101 organisms survived similar treatment, suggesting that even at 10% survival, *B. pertussis* is relatively resistant to killing by human serum. The avirulent-phase (i.e., Vir⁻) mutant BP347 was killed more than 100 times more efficiently than the wild-type strain (P < 0.00002), implying that serum resistance is *bvg* regulated. The extent of survival of BPM2041 lies between those of the wild type and the Vir⁻ strain, and the survival of BPM2041 is at least 10-fold more sensitive than that of the wild type (P < 0.00003).

The serum component mediating the killing of susceptible *B. pertussis* organisms appears to be complement: both heat inactivation and the pretreatment of serum with EDTA, which removes divalent cations required for complement activation,
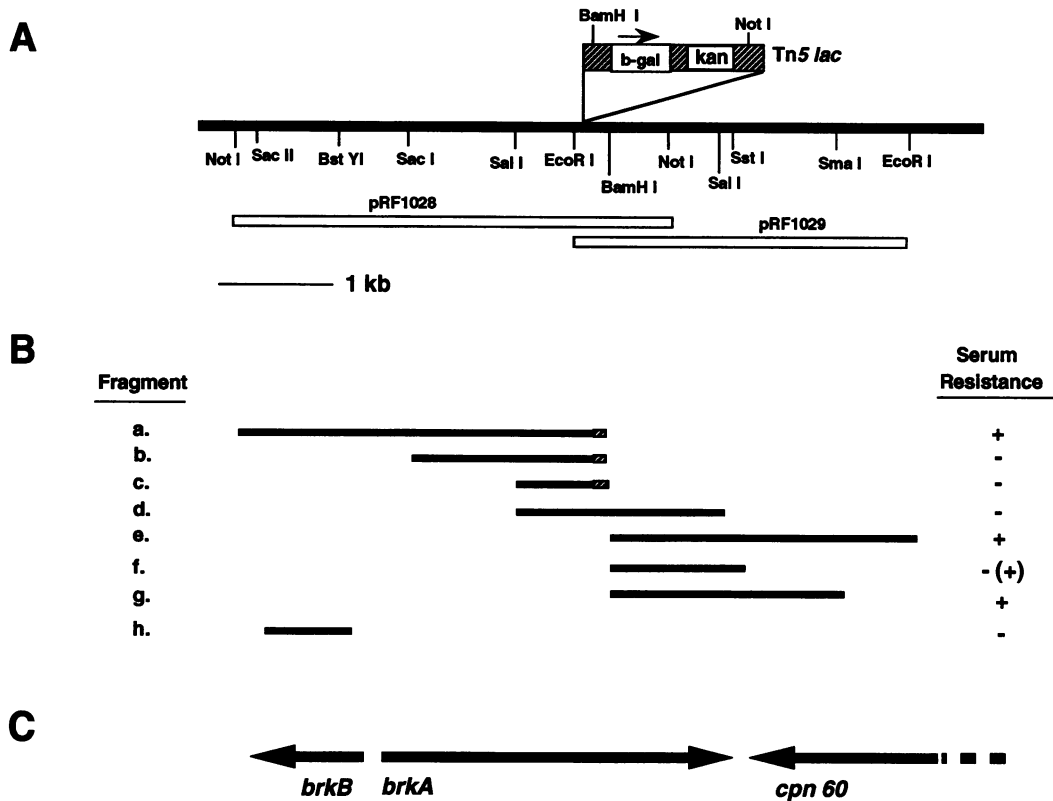
FIG. 3. Mapping of the *brk* locus. (A) Partial restriction map. Only relevant restriction sites are indicated. Tn*5 lac* (hatched box) is not drawn to scale. kan, kanamycin resistance marker; b-gal, β-Gal gene (with the arrow indicating the direction of transcription). The plasmids that were sequenced are shown. (B) Mapping of *brk* by insertion duplication mutagenesis (see text for details). At least three independent transconjugants harboring insertions and duplications of the *brk* locus in the indicated fragments were tested for serum resistance. + and −, wild-type and mutant (BPM2041) levels of resistance, respectively. (C) Organization of the ORFs in the *brk* locus. *cpn60* refers to the gene encoding the chaperonin or heat shock protein Hsp60.

result in the complete abrogation of serum killing (data not shown). To test which of the complement pathways (classical and/or alternate) was activated, organisms were exposed for 60 min to either 20% normal human serum or 20% normal human serum containing 10 mM EGTA and $MgCl_2$. The latter treatment inactivates the classical pathway by chelating Ca^{2+} (21). As indicated in Fig. 2, unlike that of *E. coli*, which is killed by both the classical and the alternate pathways, serum killing of both wild-type and mutant strains of *B. pertussis* is completely abolished under conditions which inhibit the classical pathway. Thus, the Tn*5 lac* insertion in strain BPM2041 defines a locus which affords protection from killing by the classical pathway of complement. We have called this locus *brk*.

Cloning and mapping of the *brk* locus. A partial restriction map of the *brk* locus is presented in Fig. 3A. Chromosomal DNA from the Tn*5 lac* mutant strain BPM2041 was digested with *Not*I, and the DNA was cloned in pBluescriptII SK+. Transformants were screened on plates containing X-Gal and kanamycin. A single blue colony which expressed the β-Gal and kanamycin resistance genes from Tn*5 lac* was picked and designated RF1001. Expression of β-Gal in RF1001 was probably due to a fortuitous promoter. A 3.3-kb *Not*I-*Bam*HI fragment representing the region 5′ to the Tn*5 lac* insertion site and which included the *Bam*HI site in Tn*5 lac* was subcloned to obtain pRF1003 (Fig. 3B, fragment a). Determination of the orientation of this fragment defined the direction of transcription as left to right, since the transcriptional fusion with β-Gal was used to identify this gene. pRF1003 was then used to probe a *B. pertussis* (BP338) cosmid library (49), and two positive, overlapping clones were uncovered. One of these cosmids (pKWf12) spanned the entire *brk* locus. From pKWf12, a 3.7-kb *Not*I fragment representing the upstream portion of the *brk* locus was subcloned to render pRF1028. Similarly, an overlapping 3-kb *Eco*RI fragment representing the downstream section of the *brk* locus was subcloned to obtain pRF1029 (Fig. 3A).

To map the start and the end of the *brk* locus, we employed insertion duplication mutagenesis (54). In brief, a suicide plasmid containing a gentamicin cassette, an origin of transfer for P conjugation (3), and one of the fragments of the *brk* locus depicted in Fig. 3B was engineered. The plasmid was introduced into wild-type *B. pertussis* via triparental mating (54, 55), and resistance to gentamicin was selected. Since the suicide plasmid cannot replicate in *B. pertussis*, all resulting gentamicin-resistant transconjugants are due to the insertion of the plasmid into the *B. pertussis* chromosome via homologous recombination. The chromosome thus contains two copies of the homologous region. In general, the results from insertion duplication mutagenesis can be interpreted as follows. If the cloned fragment includes either the promoter or the terminator of the gene, then no mutant phenotype is detected, since only one of the two resulting copies of the gene is disrupted by the nonhomologous region of the inserted plasmid. The other copy of the gene uses the promoter or terminator supplied by the cloned fragment. Similarly, if the cloned fragment includes both a promoter and a terminator for the gene, no mutant

phenotype is observed since there are effectively two functional copies of the gene. If, on the other hand, the cloned DNA fragment maps entirely within a gene or operon of interest, a mutant phenotype results because the gene or operon is disrupted by the plasmid.

The gentamicin-resistant transconjugants were evaluated for serum resistance. We initially ascertained that the boundaries of the *brk* locus lay between the *Not*I and *Sac*I sites at the 5′ end (Fig. 3B, fragments a and b) and between the *Sal*I and *Sma*I sites at the 3′ end (Fig. 3B, fragments d and g). A subsequent construct (Fig. 3B, fragment h) further localized the beginning of the locus to between the *Not*I and *Sac*II sites. All transconjugants with the exception of those harboring fragment f (Fig. 3B) yielded unambiguous wild-type or mutant phenotypes. We thus suspected that the terminator would actually be found close to the *Sst*I site.

Sequence analysis of the *brk* locus. Plasmids pRF1028 and pRF1029, containing the upstream and downstream portions of the *brk* locus, respectively, were sequenced. The DNA sequence and the deduced amino acid sequences of BrkA and BrkB are presented in Fig. 4. The 6-kb DNA sequence, which has a GC content of 68%, was examined for ORFs by using the FRAMES and CODONPREFERENCE programs of the Genetics Computer Group (9) and by using the GRAIL (47) electronic-mail server. Three highly predicted ORFs were discovered, and their orientations are illustrated in Fig. 3C. The largest ORF spans the site of the transposon insertion and encodes a protein of 1,010 amino acids with a predicted molecular mass of 103 kDa. The second ORF is divergently transcribed off the complementary strand, and it encodes a protein of 296 amino acids having a molecular mass of 32 kDa. Downstream of the large ORF, and transcribed in the opposite direction off the complementary strand, is the third ORF. This ORF encodes a protein of 547 amino acids with a calculated molecular mass of 57.5 kDa. The first two ORFs, termed BrkA (103 kDa) and BrkB (32 kDa), were found to be essential for the expression of serum resistance since independent knockout mutations of either BrkA (Fig. 3B, fragments b through d, and BPM2041) or BrkB (Fig. 3B, fragment h) rendered a serum-sensitive phenotype. On the basis of the mapping studies (Fig. 3B, fragments e and g), the third ORF was determined to be unnecessary for the expression of serum resistance. Interestingly, the *Sst*I site at position 4249 lies between the BrkA stop codon (positions 4240 to 4242) and the putative terminator (positions 4258 to 4287). Thus, the intermediate phenotype associated with transconjugants containing fragment f (Fig. 3B) might be due to message instability.

No obvious −10 and −35 promoter sequences or Shine-Dalgarno sequences were detected. The intergenic region between *brkA* and *brkB* did, however, contain putative BvgA binding sites (41). A single copy of the sequence TTTCCT was found upstream of *brkA*, whereas two copies were found upstream of *brkB*. The Tn*5 lac* insertion site was mapped to position 3232 in *brkA*.

The database was searched for homologs of BrkA and BrkB by using BLAST (nonredundant protein database) and FASTA (SwissProt release 25). The best match for BrkA was the *Bordetella* outer membrane protein pertactin (4). BrkA is 100 amino acids larger than pertactin. With FASTA, BrkA was found to be 29% identical to pertactin in their 910 overlapping amino acids. The best match for BrkB was a 23.5% identity to 272 overlapping amino acids in the 290-amino-acid hypothetical *E. coli* protein ORF o290 identified by the *E. coli* genome project (37). The third ORF (57.5 kDa) showed remarkable identity (data not shown) to the GroEL class of heat shock proteins and chaperonins (14).

Comparison of BrkA with pertactin. (i) Sequence. A comparison of the salient features of BrkA and pertactin (4) is presented in Fig. 5. The most striking identity is found in the C-terminal end. The two proteins are 54.5% identical over their last 300 amino acids and share a C terminal outer membrane localization motif (alternating hydrophobic residues in the last nine C-terminal amino acids ending with [preferentially] phenylalanine or tryptophan) (45). Of particular note is the conservation of the pertactin proteolytic processing sites (10) in BrkA. In addition, each protein has two RGD (arginine-glycine-aspartic acid) sequences; the RGD pattern is associated with cell adhesion (42). The spacing of the RGD's, however, is not conserved between the two proteins. Whereas the positioning and alignment of the first RGD are fairly close between the two proteins, the positioning of the second RGD is not. In pertactin, the second RGD is found downstream of the processing site and appears to be absent from the mature protein (10). In BrkA, however, the second RGD is located approximately halfway between the first RGD and the processing site. There are other notable differences between the two proteins. BrkA, unlike pertactin, does not have a discernible signal sequence, nor does it possess the $(GGXP)_5$ and $(PQP)_5$ repeats. However, as uncovered in a search of the PROSITE database using GRAIL (47), BrkA does have two potential glycosaminoglycan attachment sites (SGXG) (12), which are absent in pertactin.

(ii) Role of BrkA in adherence and invasion. In a previous study, the original BrkA mutant, BPM2041, was shown to have a twofold reduction in the capacity to invade HeLa cells (8). We have extended these results to the human lung fibroblast cell line MRC-5. The numbers of bacteria (means ± standard deviations) adhering to MRC-5 cells were $4.1 \times 10^5 \pm 1.1 \times 10^5$ for the wild type (BP338) and $1.7 \times 10^5 \pm 2.2 \times 10^4$ for BPM2041. Similarly, in separate experiments, the numbers of bacteria invading MRC-5 cells were $3.4 \times 10^5 \pm 2.2 \times 10^5$ for BP338 and $1.8 \times 10^5 \pm 1.6 \times 10^5$ for BPM2041. Thus, in MRC-5 cells, both adherence ($P < 0.02$) and invasion ($P < 0.004$) were decreased in BPM2041 by approximately 50%, suggesting that, like pertactin, BrkA can indeed function as an adhesin and/or invasin.

(iii) Role of pertactin in serum resistance. Given the functional similarities as well as sequence similarities between BrkA and pertactin, we decided to examine what role, if any, pertactin played in serum resistance. Since we did not have a pertactin mutant of BP338 (Tohama background), we used the *B. pertussis* W28 pertactin mutant BBC9 and its parent strain, BBC8 (27). BrkA mutants of each strain were made by using construct d in Fig. 3B, and the resistance to serum killing was assessed. As shown in Fig. 6, while the pertactin mutant is reduced in its ability to survive serum killing, this result is not significantly different from that for the parent strain ($P < 0.13$). Disruption of the *brkA* gene in the parental BBC8 resulted in an approximately 20-fold reduction in serum resistance ($P < 0.05$), not unlike what is seen with BP338 BrkA mutants (Fig. 1). The pertactin-*brkA* double mutant also displayed a 20-fold reduction in serum killing compared with that of BBC9 ($P < 0.02$), thus belying a significant role for pertactin in serum resistance.

BrkB is predicted to be a cytoplasmic membrane protein. An examination of the hydrophobicity profile of BrkB revealed the presence of six hydrophobic domains. This, coupled with the lack of a signal sequence and an outer membrane localization motif, suggested that BrkB may be a cytoplasmic membrane protein. This thesis was supported by the MEMSAT membrane prediction program (22), which takes into account both structural and topological constraints (48). By this anal-

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  1   gcggccgcctgccgcctTCAGGCGGGGGCGGCCCGGCGCAGGCTGCCGAAGCGCTCGGCGAACTGCCGGGTGAAGACGGCGCCGAAGAAGAAAATCTGCG
                     * A  P  A  A  R  R  L  S  G  F  R  E  A  F  Q  R  T  F  V  A  G  F  F  F  I  Q

101   CTGAATAATAGATCCACAGCAGCAGCGCGATCAGCGAACCCGCCGCCCCGTACGCCGATACCGCCGCGCCGCGTCCCAGGTACAGGCCGATGCCCCATTT
      A  S  Y  Y  I  W  L  L  L  A  I  L  S  G  A  A  G  Y  A  S  V  A  A  G  R  G  L  Y  L  G  I  G  W  K

201   GCCGGCCAGGAACAGCGCCGCGGTCACGATCGCGCCGGGTATCACGTCGAGCCAGGGAATGCGCTTGCTGGGCAGCAGCTTGTAGACCACGGCGAACAGC
      G  A  L  F  L  A  A  T  V  I  A  G  P  I  V  D  L  W  P  I  R  K  S  P  L  L  K  Y  V  V  A  F  L

301   GCGGTCACCACCGCGAACGAAAAGAGGTTCGACAGCCAGTCGGCCGCCATCGCGAAGGCCGATGTGCTCCATAGATCGCCGTAGTATCCCTTGGCCGCGC
      A  T  V  V  A  F  S  E  L  N  S  L  W  D  A  A  M  A  F  A  S  T  S  W  L  D  G  Y  Y  G  K  A  A

401   CCAGCGCCGCGTTGAGGGTCAGCGAAAGCAGCAGGAACAGCGCCAGCACCAGCACCAGGCCGAATGACAGCATGCGGCTGCGCACCAGCCCCTGCAAACC
      G  L  A  A  N  L  T  L  S  L  L  L  F  L  A  L  V  L  V  L  G  F  S  L  M  R  S  R  V  L  G  Q  L  G

501   GCTCTTGTCTTCCTTGACGTCCCACAGTTCGTCCAGGCTGGCCTTGAGTTCGGCGAAGGCCGTGGTGGCGCTGAACACCAGCACGCATATCGACAGCAGC
      S  K  D  E  K  V  D  W  L  E  D  L  S  A  K  L  E  A  F  A  T  T  A  S  F  V  L  V  C  I  S  L  L

601   GCGGCCAGCCAGCCGCTGCCCGATTCGTGCGCGCTGGCCAGCACCGTCTGGATCACCTCGGCGCCGCGCTCGCCGGTGAGGTCGCGCACCTGGCTGAACA
      A  A  L  W  G  S  G  S  E  H  A  S  A  L  V  T  Q  I  V  E  A  G  R  E  G  T  L  D  R  V  Q  S  F

701   GTTCCGAGCGCACGGCCTCTTCGCCAAAGAAGGCGCCGGCCACCGCGATCACCAGGATCAGCATGGGCGCCAGCGAGAACACCATGTACAGCGACAGCGC
      L  E  S  R  V  A  E  E  G  F  F  A  G  A  V  A  I  V  L  I  L  M  P  A  L  S  F  V  M  Y  L  S  L  A

801   CGCCCCCTTGCTGGAAGCGCGATGGCGCGACCATTCGCGGGCCGAATCGGTCAGCAGCGCGCCGATGTCGCGGAGGTGGGGCAAGCGGATCTGGCGTGGC
      A  G  K  S  S  A  R  H  R  S  W  E  R  A  S  D  T  L  L  A  G  I  D  R  L  H  P  L  R  I  Q  R  P

901   AAGCGCATGgcctcgggttggatgggacagcttggtggcggaagtatcgaccgtggccggccgcggttcgaggaggtcttccggacttcatgacgtcgtg
      L  R  M
      ←—— BrkB.
1001  cgcaattctcatᵃ⁹⁹ᵃᵃᵃgcacggctattᵈᵃ⁹⁹ᵃᵃᵃgctcttgttgggcctcggacttcatccggcattcattatgacgtgggctttggatactgcgcaa
              tccttt          tccttt

1101  atccccgcatggctcgtgcctgatcccgccgggacgcagtcgttcctctcgcgtcaccggcgtcgtacggcaggcgggtcggtttgttcaacttctcttt

1201  tggtggcacATGTATCTCGATAGATTCCGTCAATGTCCGTCTTCCTTGCAGATCCCGCGTTCCGCGTGGCGCCTGCATGCGCTGGCCGCAGCTCTGGCGC
               M  Y  L  D  R  F  R  Q  C  P  S  S  L  Q  I  P  R  S  A  W  R  L  H  A  L  A  A  A  L  A  L
               BrkA —→

1301  TGGCCGGCATGGCCCGGCTGGCGCCCGCGGCGGCGCAGGCGCCGCAGCCGCCCGTGGCCGGCGCGCCGCATGCGCAGGACGCCGGGCAGGAAGGAGAGTT
      A  G  M  A  R  L  A  P  A  A  A  Q  A  P  Q  P  P  V  A  G  A  P  H  A  Q  D  A  G  Q  E  G  E  F

1401  CGACCACCGGGACAACACGCTCATTGCAGTCTTTGACGACGGCGTCGGCATCAATCTCGACGACGATCCCGACGAGCTCGGCGAGACGGCGCCCCCCACG
      D  H  R  D  N  T  L  I  A  V  F  D  D  G  V  G  I  N  L  D  D  D  P  D  E  L  G  E  T  A  P  P  T

1501  CTCAAGGACATCCACATCTCGGTGGAGCACAAGAACCCGATGAGCAAGCCGGCCATCGGGGTGCGTGTCAGCGGCGCCGGCCGCGCGCTGACGCTGGCCG
      L  K  D  I  H  I  S  V  E  H  K  N  P  M  S  K  P  A  I  G  V  R  V  [S  G  A  G]  R  A  L  T  L  A  G

1601  GCTCGACCATCGATGCCACCGAGGGCGGCATTCCCGCAGTGGTACGGCGCGGCGGCACGCTGGAGCTGGATGGCGTCACCGTGGCGGGCGGGGAAGGGAT
      S  T  I  D  A  T  E  G  G  I  P  A  V  V  R  R  G  G  T  L  E  L  D  G  V  T  V  A  G  G  E  G  M

1701  GGAGCCGATGACGGTCTCTGACGCCGGCAGCCGCCTGAGCGTGCGCGGCGGCGTGCTGGGCGGCGAAGCGCCGGGCGTCGGCCTGGTCCGGGCCGCGCAA
      E  P  M  T  V  S  D  A  G  S  R  L  S  V  R  G  G  V  L  G  G  E  A  P  G  V  G  L  V  R  A  A  Q

1801  GGCGGCCAGGCGAGCATCATCGACGCGACGCTGCAGAGCATCCTCGGGCCCGCGCTCATTGCCGACGGCGGCTCCATTTCCGTCGCCGGCGGTTCGATCG
      G  G  Q  A  S  I  I  D  A  T  L  Q  S  I  L  G  P  A  L  I  A  D  G  G  S  I  S  V  A  G  G  S  I  D

1901  ACATGGACATGGGCCCGGGATTCCCGCCGCCGCCTCCACCGCTTCCCGGGGCGCCGCTGGCCGCGCATCCGCCGCTCGATCGCGTTGCCGCGGTGCACGC
      M  D  M  G  P  G  F  P  P  P  P  P  P  L  P  G  A  P  L  A  A  H  P  P  L  D  R  V  A  A  V  H  A

2001  CGGCCAGGACGGCAAGGTGACACTGCGGGAGGTGGCGCTGCGGGCTCACGGGCCGCAGGCGACGGGCGTCTATGCGTATATGCCTGGCAGCGAAATCACC
      G  Q  D  G  K  V  T  L  R  E  V  A  L  R  A  H  G  P  Q  A  T  G  V  Y  A  Y  M  P  G  S  E  I  T

2101  CTGCAGGGAGGCACAGTCAGCGTGCAGGGCGATGACGGGGCCGGCGTGGTCGCCGGCGCGGGCCTGCTCGACGCCTTGCCGCCCGGCGGCACGGTGCGGC
      L  Q  G  G  T  V  S  V  Q  G  D  D  G  A  G  V  V  A  G  A  G  L  L  D  A  L  P  P  G  G  T  V  R  L

2201  TGGACGGAACCACGGTGTCGACCGATGGCGCCAACACCGATGCCGTGCTGGTTCGCGGCGACGCGGCGCGCGCCGAGGTCGTCAACACCGTGCTGCGCAC
      D  G  T  T  V  S  T  D  G  A  N  T  D  A  V  L  V  [R  G  D]  A  A  R  A  E  V  V  N  T  V  L  R  T

2301  CGCCAAGAGCCTGGCCGCCGGCGTATCGGCCCAGCATGGAGGCCGCGTCACGCTGCGGCAGACCCGCATCGAGACCGCGGGCGCGGGGGCCGAGGGCATC
      A  K  S  L  A  A  G  V  S  A  Q  H  G  G  R  V  T  L  R  Q  T  R  I  E  T  A  G  A  G  E  I
```

FIG. 4. DNA and predicted amino acid sequences of the *brk* locus. DNA coding (uppercase) and noncoding (lowercase) sequences are shown. Only the DNA sequence is numbered. Stop codons (asterisks), putative BvgA binding sites (tttcct) (boldface and boxed), a rho-independent terminator at the end of *brkA* suggested by a stem-loop structure (dashed underlines), the Tn5 *lac* insertion site (arrow), putative cell adhesion sites (RGD) and glycosaminoglycan attachment sites (SGXG) in BrkA (boxed), potential proteolytic processing sites (boldface and underlined), and the outer membrane localization signal (dashed box) are indicated.

```
2401  TCCGTGCTGGGCTTCGAGCCGCAGTCCGGCTCCGGCCCGGCCAGCGTCGACATGCAGGGCGGCAGCATCACCACGACCGGCAACCGCGCCGCCGGCATCG
      S   V   L   G   F   E   P   Q  |S   G   S   G| P   A   S   V   D   M   Q   G   G   S   I   T   T   T   G   N   R   A   A   G   I   A

2501  CGCTCACCCACGGCAGCGCCCGCCTGGAAGGCGTGGCGGTGCGCGCCGAGGGCAGCGGCTCGAGCGCCGCGCAGCTGGCCAACGGCACGCTGGTCGTCAG
      L   T   H   G   S   A   R   L   E   G   V   A   V   R   A   E   G   S   G   S   S   A   A   Q   L   A   N   G   T   L   V   V   S

2601  CGCAGGGTCGCTGGCCTCGGCCCAGTCCGGCGCGATCAGCGTGACCGACACGCCGCTGAAGCTGATGCCGGGCGCCCTGGCCAGCAGCACGGTCTCGGTC
      A   G   S   L   A   S   A   Q   S   G   A   I   S   V   T   D   T   P   L   K   L   M   P   G   A   L   A   S   S   T   V   S   V

2701  CGGTTGACCGACGGCGCCACGGCGCAAGGCGGCAATGGCGTGTTCCTCCAGCAGCATTCCACCATTCCGGTGGCGGTTGCCCTCGAGAGCGGCGCCCTGG
      R   L   T   D   G   A   T   A   Q   G   G   N   G   V   F   L   Q   Q   H   S   T   I   P   V   A   V   A   L   E   S   G   A   L   A

2801  CTCGCGGCGATATCGTCGCCGACGGCAACAAGCCCCTCGATGCCGGGATCTCCCTCAGCGTGGCCAGCGGCGCCGCCTGGCACGGCGCCACCCAGGTGCT
      L  |R   G   D| I   V   A   D   G   N   K   P   L   D   A   G   I   S   L   S   V   A   S   G   A   A   W   H   G   A   T   Q   V   L

2901  CCAGTCGGCCACGCTGGGCAAGGGCGGAACCTGGGTCGTGAACGCCGACTCCCGGGTGCAGGACATGTCGATGCGCGGCGGGCGGGTCGAATTCCAGGCG
      Q   S   A   T   L   G   K   G   G   T   W   V   V   N   A   D   S   R   V   Q   D   M   S   M   R   G   G   R   V   E   F   Q   A

3001  CCCGCGCCCGAGGCCTCTTACAAGACCCTGACCCTGCAAACCCTGGACGGCAACGGCGTGTTCGTGCTGAACACCAACGTCGCCGCCGGGCAGAACGACC
      P   A   P   E   A   S   Y   K   T   L   T   L   Q   T   L   D   G   N   G   V   F   V   L   N   T   N   V   A   A   G   Q   N   D   Q

3101  AGTTGCGGGTCACCGGCCGCGCCGATGGCCAGCACCGCGTGCTGGTGCGCAATGCCGGAGGCGAGGCCGACAGCCGGGGCGCCCGCCTGGGCCTGGTGCA
      L   R   V   T   G   R   A   D   G   Q   H   R   V   L   V   R   N   A   G   G   E   A   D   S   R   G   A   R   L   G   L   V   H
                                                                                    ↓
3201  TACCCAGGGGCAGGGCAACGCCACCTTCCGGCTGGCCAACGTCGGCAAGGCGGTTGACCTGGGCACGTGGCGCTACAGCCTGGCGGAGGATCCGAAGACG
      T   Q   G   Q   G   N   A   T   F   R   L   A   N   V   G   K   A   V   D   L   G   T   W   R   Y   S   L   A   E   D   P   K   T

3301  CATGTCTGGAGCTTGCAGCGCGCGGGCCAGGCCCTGTCGGGGGCGGCCAATGCCGCCGTGAACGCGGCGGATCTTTCCAGCATCGCCCTGGCCGAGTCCA
      H   V   W   S   L   Q   R   A   G   Q   A   L   S   G   A̲ ̲A̲ ̲N̲ ̲A̲ ̲A̲ ̲V̲ ̲N̲  A   A   D   L   S   S   I   A   L   A̲ ̲E̲ ̲S̲ ̲N̲

3401  ACGCGCTGGACAAGCGCCTGGGCGAGCTGCGCCTGCGCGCCGACGCCGGCGGGCCATGGGCGCGTACGTTCAGCGAGCGCCAGCAGATCAGCAACCGCCA
      A̲ ̲L̲ ̲D̲ ̲K̲ ̲R̲ ̲L̲  G   E   L   R   L   R   A   D   A   G   G   P   W   A   R   T   F   S   E   R   Q   Q   I   S   N   R   H

3501  CGCCCGCGCCTACGACCAGACGGTCAGCGGGCTGGAGATCGGCCTGGACCGTGGCTGGAGCGCGTCGGGCGGGCGCTGGTACGCCGGCGGCCTGCTCGGC
      A   R   A   Y   D   Q   T   V   S   G   L   E   I   G   L   D   R   G   W   S   A   S   G   G   R   W   Y   A   G   G   L   L   G

3601  TACACCTATGCCGACCGCACCTATCCCGGCGACGGTGGCGGCAAGGTCAAGGGCCTGCACGTCGGCGGCTACGCCGCCTATGTCGGCGATGGCGGCTACT
      Y   T   Y   A   D   R   T   Y   P   G   D   G   G   G   K   V   K   G   L   H   V   G   G   Y   A   A   Y   V   G   D   G   G   Y   Y

3701  ATCTCGACACCGTGCTGCGGCTGGGCCGCTACGATCAGCAATACAACATTGCCGGCACCGATGGCGGCCGCGTCACCGCCGACTACCGCACAAGCGGCGC
      L   D   T   V   L   R   L   G   R   Y   D   Q   Q   Y   N   I   A   G   T   D   G   G   R   V   T   A   D   Y   R   T   S   G   A

3801  CGCATGGTCGCTCGAAGGCGGGCGCCGGTTCGAGCTGCCCAACGACTGGTTCGCCGAACCGCAGGCCGAGGTCATGCTGTGGCGCACGTCAGGCAAGCGC
      A   W   S   L   E   G   G   R   R   F   E   L   P   N   D   W   F   A   E   P   Q   A   E   V   M   L   W   R   T   S   G   K   R

3901  TATCGCGCCAGCAATGGCCTGCGCGTCAAGGTGGACGCCAACACCGCCACGCTGGGCCGCCTGGGCTTGCGCTTCGGCCGCCGCATCGCCCTGGCCGGCG
      Y   R   A   S   N   G   L   R   V   K   V   D   A   N   T   A   T   L   G   R   L   G   L   R   F   G   R   R   I   A   L   A   G   G

4001  GCAACATCGTGCAGCCCTACGCCAGGCTCGGCTGGACGCAGGAGTTCAAAAGCACGGGCGATGTGCGCACCAATGGCATTGGCCATGCCGGCGCAGGCCG
      N   I   V   Q   P   Y   A   R   L   G   W   T   Q   E   F   K   S   T   G   D   V   R   T   N   G   I   G   H   A   G   A   G   R

4101  CCACGGCCGCGTGGAACTGGGCGCGGGCGTCGACGCCGCGTTGGGCAAGGGGCACAACCTCTATGCTTCGTACGAGTACGCGGCGGGCGACCGGATCAAC
      H   G   R   V   E   L   G   A   G   V   D   A   A   L   G   K   G   H   N   L   Y   A   S   Y   E   Y   A   A   G   D   R   I   N

4201  ATTCCGTGGTCGTTCCACGCCGGCTACCGCTACAGCTTCTGAgcgagctccaatgaaaaacccccgccaggcgaacctggcgggggtttttttttgggaagg
      I   P   W   S  :F   H   A   G   Y   R   Y   S   F: *                ─────────────     ─────────────
```

FIG. 4—*Continued.*

ysis, BrkB is strongly predicted to have six membrane-spanning (spanner) regions, with both the carboxy terminus and the amino terminus being found on the cytoplasmic side of the membrane (Fig. 7A). In addition, potential sites for phosphorylation are present on the putative cytoplasmic side, with the predicted periplasmic side having sites for glycosaminoglycan attachment (12) and phosphorylation. Whether these sites are important for biological function is unknown. Although the best match from the database showed only 23.5% identity to BrkB, *E. coli* ORF o290 has a remarkably similar hydrophobicity plot. The next match in the database search (18.9% over 249 amino acids) was to an *M. leprae* 313-amino-acid ORF (GenBank no. U00022) recently identified by the *M. leprae* genome project. As with the *E. coli* o290 ORF, there was a

striking similarity in the hydrophobicity profiles. In addition to the match with the aforementioned ORFs, domains of identity to the transmembrane regions of several different proteins (Fig. 7B) which function to transport ions, electrons, and other small molecules were detected. The N-terminal half of the protein had a further match (Fig. 7B) to the heptad repeat, putative dimerization domain of the tumor suppressor adenamatous polyposis coli protein (11).

SDS-PAGE analysis. To visualize the presence of the Brk proteins, SDS-PAGE was performed. The Coomassie blue-stained-protein profile for the wild-type strain was compared with profiles for BrkA and BrkB knockout mutants. We were looking for proteins present in the wild-type strain but absent in the respective mutants. As illustrated in Fig. 8, there is an
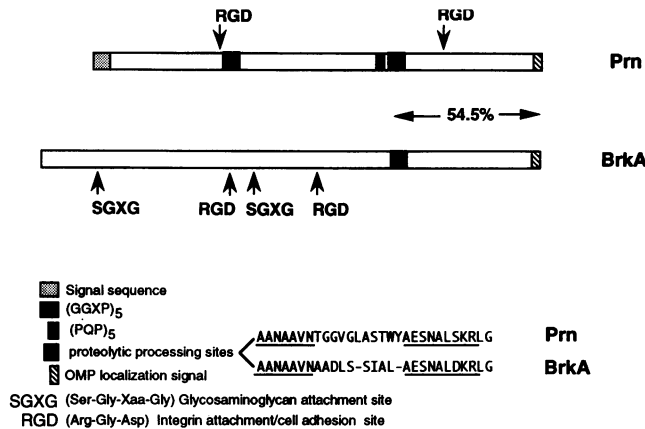
FIG. 5. Sequence comparison of BrkA and pertactin (Prn). The overall sequence identity of 29% rises to 54.5% at the C terminus. X and Xaa refer to any amino acid.

approximately 73-kDa protein present in both the wild-type strain (lane A) and the BrkB mutant (lane E). This size is not compatible with the predicted molecular mass of 103 kDa for BrkA but is consistent with the predicted molecular mass of the processed protein. The 73-kDa protein is noticeably absent in both brkA mutants (Fig. 8, lanes C and D) and in the phase-variant mutant, BP347 (lane B), suggesting that it may be BrkA. A similar analysis did not reveal a candidate for BrkB.

Presence of the brk locus in other Bordetella species. We next sought to determine whether the brk locus is present in other Bordetella species. A Southern blot containing NotI-digested chromosomal DNA from B. pertussis, B. parapertussis, B. bronchiseptica, and B. avium was probed with a labeled fragment of DNA (see Fig. 3B, fragment a) which did not include the 3' sequences which closely resembled pertactin. From Fig.
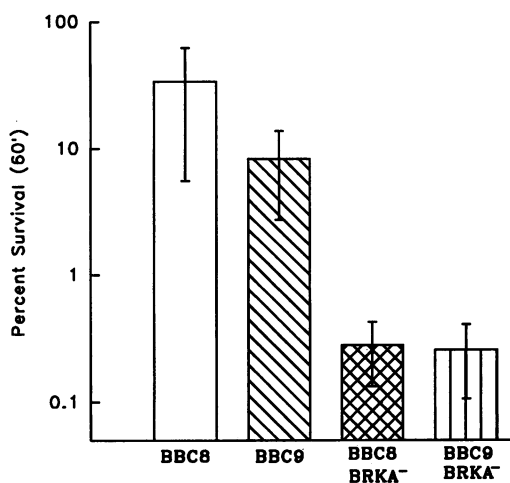
FIG. 6. Comparison of pertactin-negative and BrkA-negative mutants for serum resistance. BrkA mutants BBC8BrkA and BBC9BrkA were constructed with the W28 strains BBC8 (wild type) and BBC9 (pertactin mutant), respectively, using insertion duplication mutagenesis with fragment d (Fig. 3B). Means ± standard deviations are reported. $n = 5$ for BBC8 and BBC9; $n = 3$ for each of the BrkA⁻ strains.

9, it is evident that the brk locus is present in all Bordetella species except B. avium.

DISCUSSION

We have shown that serum resistance encoded by the brk locus is a newly described virulence factor for B. pertussis. Like other B. pertussis virulence factors, the brk locus is bvg regulated, with putative BvgA binding sites present upstream of both brkA and brkB (Fig. 4). Most significantly, however, the original Brk mutant, BPM2041, is less virulent in infant mice (56), thus underlining the importance of the brk locus in pathogenesis. It is reasonable to imagine that the ability to resist complement killing may be of consequence in B. pertussis infections in that it would serve to effectively enhance colonization. Although we cannot discount the possibility that resistance to serum killing may mimic other mucosal host defense systems, such as the defensin-like tracheal antimicrobial peptide (6), both antibody (23) and complement (35) have been shown to be present in the respiratory tract and as such provide a tenable threat to B. pertussis. Other primarily mucosal pathogens, such as Neisseria gonorrhea (32) and Streptococcus pyogenes (16, 17), also have mechanisms to avoid complement killing or opsonization, respectively.

We have cloned, mapped, and sequenced the Bordetella locus for resistance to killing (brk) and have shown that it has two highly predicted ORFs, termed BrkA and BrkB. Both are essential for serum resistance, since independent knockout mutations resulted in the loss of wild-type levels of resistance. A third ORF, which is not essential for serum resistance, is about 100 bases downstream of brkA and resembles the heat shock protein-chaperonin Cpn60. The juxtapositioning of heat shock genes and virulence genes has been demonstrated in Vibrio cholerae (33). In V. cholerae, several virulence genes, including the tcpC gene, which is involved in serum resistance, are regulated by toxR (7, 34). The toxR gene is adjacent to htpG, the gene encoding the heat shock protein Hsp90. toxR and htpG are divergently transcribed, and their expression is modulated by temperature (33). At 22°C, toxR is expressed, whereas at 37°C htpG is expressed. It was suggested that the competition between sigma-32 and sigma-70 RNA polymerases for the promoter site dictates which gene is expressed (33). In B. pertussis, the cpn60 gene is downstream of brkA, and the two genes in this case share a common 3' terminator. Contrary to the role of the Hsp90 gene in V. cholerae, our mapping studies suggest that the Cpn60 gene in B. pertussis plays a neglible role, if any, in serum resistance.

The best match to BrkA in the database was found to be the Bordetella outer membrane protein pertactin. The two proteins were particularly similar over their last 300 amino acids. In fact, the proteolytic processing sites in pertactin, which produce N-terminal proteins of either 58.4 or 60.4 kDa (10), are conserved in BrkA. If indeed BrkA is also processed at these sites, then the predicted molecular mass for BrkA would be about 73 kDa instead of 103 kDa. Our SDS-PAGE result which demonstrates an approximately 73-kDa protein present in the wild-type strain but absent in BrkA mutants supports this hypothesis.

Ligands bearing the RGD motif bind to integrin receptors on host cells (42). Including BrkA, B. pertussis now has at least three virulence factors with RGD motifs. The first RGD, in pertactin, has been shown to bind to both HeLa and CHO cells (27), and the RGD in FHA mediates attachment to the complement receptor CR3 on monocytes (40). Carbohydrates such as galactose and sulfated saccharides such as heparin also play a large role in the adherence of FHA to host cells (28).
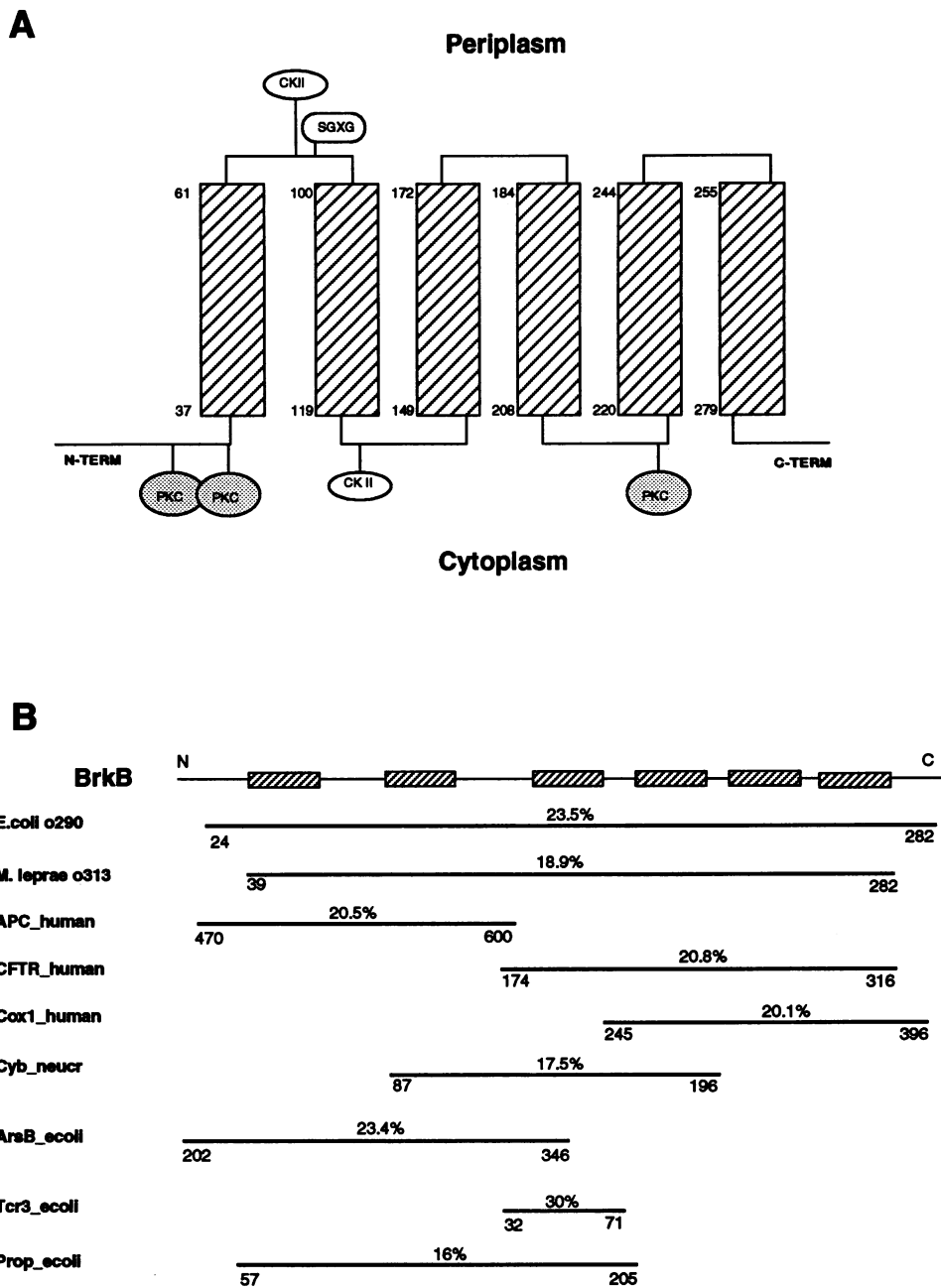
A

B

FIG. 7. (A) Cytoplasmic membrane protein model for BrkB. The predicted transmembrane domains (hatched boxes) as well as the topological orientation (i.e., cytoplasmic versus periplasmic) of BrkB are shown. The sequential number of the amino acids falling within the transmembrane spanners is indicated to the left of each box. Potential sites for protein kinase C phosphorylation (PKC) (57) and potential sites for casein kinase II phosphorylation (CKII) (36) are indicated. SGXG is a putative glycosaminoglycan attachment site (12). N-term and C-term, N terminus and C terminus, respectively. (B) Line drawing of sequence comparisons of BrkB with other proteins in the database. The location of each match relative to BrkB is indicated by a line. The percent identity (FASTA) of each protein to BrkB is shown above the line; the amino acid numbers of the matched proteins are indicated below the line. *E. coli* o290 (37) and *M. leprae* o313 (GenBank no. U00022) are ORFs of unknown function. The rest of the proteins are identified by their names in the SwissProt database. APC_human, human tumor suppressor familial adenamatous polyposis coli protein; CFTR_human, cystic fibrosis transmembrane conductance regulator; Cox1_human, cytochrome oxidase polypeptide I; Cyb_neucr, *Neurospora crassa* cytochrome *b*; ArsB_ecoli, *E. coli* arsenical pump membrane protein; Tcr3_ecoli, tetracycline resistance efflux protein; Prop_ecoli, proline-betaine transporter.

BrkA has two putative glycosaminoglycan attachment sites which could conceivably mimic the FHA-heparin interaction. Our data showed that the adherence of a BrkA-negative strain to the lung fibroblast cell line MRC-5 is about 50% of the level of the wild-type strain in a 3-h assay, suggesting that BrkA plays at least a minor role in adherence. Whether this is mediated by either the RGD motif or the glycosaminoglycan attachment sites or both awaits further investigation.
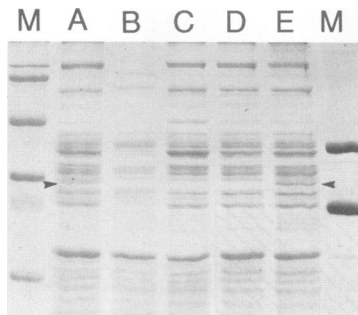
FIG. 8. SDS-PAGE analysis of Brk mutants. Total protein from *B. pertussis* strains was isolated and separated by SDS-PAGE. Lanes A to E, BP338, BP347, BPM2041, RF1039 (a BrkA⁻ mutant), and RF1057 (a BrkB⁻ mutant), respectively; lanes M, molecular weight markers. Markers on the left, from top to bottom, are myosin (212 kDa), α2-macroglobulin (170 kDa), β-Gal (116 kDa), and transferrin (76 kDa). Markers on the right are phosphorylase *b* (94 kDa) and bovine serum albumin (67 kDa). Arrowheads point to a 73-kDa protein.

The presence of the RGD motif in pertactin has been associated with invasion of CHO cells (26). In comparison with wild-type *B. pertussis*, BrkA mutants are compromised by a factor of 2 in their ability to invade both HeLa (8) and MRC-5 cells, implying that BrkA also has some role in invasion and intracellular survival. Despite the similarities in sequence and in functions such as attachment and invasion, BrkA and pertactin differ in their abilities to confer serum resistance (Fig. 6).

The second protein of the *brk* locus, BrkB, resembles proteins encoded by similar-size ORFs in *E. coli* (37) and *M. leprae* (GenBank no. U00022) both in the extent of amino acid identity and in their predicted hydropathy profiles. The functions of these ORFs have yet to be assigned. BrkB is predicted to be a cytoplasmic membrane protein with domains of homology to different transporters or pumps. The amino terminus of BrkB also displays some identity to one of the heptad repeat domains of the tumor suppressor adenomatous polyposis coli
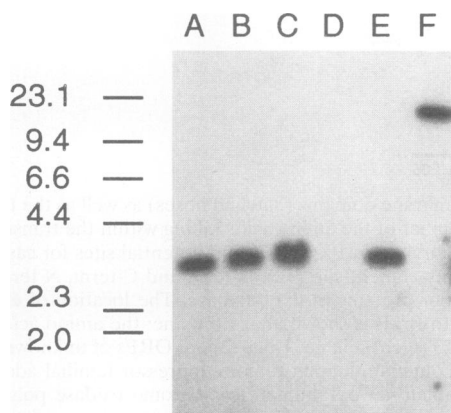

FIG. 9. Southern blot analysis of different *Bordetella* spp. for the presence of *brk*. Chromosomal DNAs were isolated, digested with *Not*I, and separated on a 0.8% agarose gel. The blot was probed with DNA from the *brk* locus that was 5′ to the transposon insertion site (Fig. 3B, fragment a). Lanes A to F, *B. pertussis* (BP338), *B. parapertussis* (253), *B. bronchiseptica* (11DH), *B. avium* (wBA70), *B. pertussis* (BP347), and *B. pertussis* (BPM2041), respectively. DNA markers (in kilobases) are indicated on the left.

protein (11). Indeed, there is a perfect heptad repeat in the first transmembrane-spanning domain of BrkB. The presence of apolar amino acids in the first and fourth positions allows a protein to adopt an α-helical coiled-coil structure (5) with another heptad repeat domain. Intramembrane helix interactions are not uncommon in integral membrane proteins (5), and it is possible that in addition to the intramembrane interactions, BrkB may form a homodimer, thus conforming to the two-by-six structure (where six is the number of transmembrane spanners) which is a hallmark of many other transport proteins (15).

Possible mechanisms of serum resistance can be offered on the basis of the predicted amino acid sequences of BrkA and BrkB. The outer membrane localization signal (45) on BrkA implies that it is targeted to the surface of *B. pertussis*. As a surface structure, it may act to sterically hinder or block the binding of lytic antibody to a bactericidal target, or it could elicit blocking antibody, which would perform a similar function (18–20). Alternatively, the putative glycosaminoglycan attachment site on BrkA may influence serum resistance. The serum protein vitronectin has a heparin (glycosaminoglycan) binding site which acts to inhibit the polymerization of the complement component C9 (30, 38). It is possible that BrkA could utilize its putative heparin binding site to hinder C9 polymerization. In this regard, inhibition of C9 polymerization is believed to be the mechanism by which the Rck protein mediates serum resistance in *Salmonella typhimurium* (13).

We have proposed that BrkB is a cytoplasmic membrane protein. If so, how might a cytoplasmic membrane protein act to confer serum resistance? One possibility is that BrkB might aid in the secretion of BrkA. The outer membrane localization signal on BrkA predicts its targeting to the bacterial surface. BrkA, however, lacks an apparent signal sequence, suggesting that the conventional *sec*-mediated machinery (39) is not operative in this case. Thus, the serum sensitivity of the BrkB mutants may be explained by the failure of BrkA to be expressed on the surface, although BrkA does appear to be present in its processed form in BrkB mutants (Fig. 8).

Alternatively, BrkB might serve to counteract the damage caused by complement attack. As argued by Taylor (46), it is the damage to the cytoplasmic membrane and not necessarily the outer membrane which leads to the complement-mediated death of gram-negative bacteria. Perturbations of the cytoplasmic membrane resulting from complement attack can alter membrane potential, inhibit oxygen consumption, and cause a leakage of small molecules and ions (46). It is tempting to speculate that the similarity of BrkB to other pumps may suggest that it acts as a transporter or channel that functions to maintain homeostasis following complement attack. Whether BrkB acts to transport BrkA or whether it acts as a transporter of small molecules is the focus of future studies.

Finally, since the *brk* locus also exists in *B. parapertussis* and *B. bronchiseptica*, and since possible homologs of BrkB have been detected in *E. coli* and *M. leprae*, it would be of interest to explore the role of *brk* in these organisms.

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