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Establishing a Direct Role for the *Bartonella bacilliformis* Invasion-Associated Locus B (IalB) Protein in Human Erythrocyte Parasitism

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The invasion-associated locus A and B genes (*ialAB*) of *Bartonella bacilliformis* were previously shown to confer an erythrocyte-invasive phenotype upon *Escherichia coli*, indirectly implicating their role in virulence. We report the first direct demonstration of a role for *ialB* as a virulence factor in *B. bacilliformis*. The presence of a secretory signal sequence and amino acid sequence similarity to two known outer membrane proteins involved in virulence suggested that IalB was an outer membrane protein. To develop an antiserum for protein localization, the *ialB* gene was cloned in frame into an expression vector with a six-histidine tag and under control of the *lacZ* promoter. The IalB fusion protein was purified by nickel affinity chromatography and used to raise polyclonal antibodies. IalB was initially localized to the bacterial membrane fraction. To further localize IalB, *B. bacilliformis* inner and outer membranes were fractionated by sucrose density gradient centrifugation and identified by appearance, buoyant density (ρ), and cytochrome *b* content. Inner and outer membrane proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and IalB was positively identified by Western blot. Contrary to expectations, IalB was localized to the inner membrane of the pathogen. To directly demonstrate a role for IalB in erythrocyte parasitism, the *B. bacilliformis ialB* gene was disrupted by insertional mutagenesis. The resulting *ialB* mutant strain was complemented in *trans* with a replicative plasmid encoding the full-length *ialB* gene. PCR and high-stringency DNA hybridization confirmed mutagenesis and transcomplementation events. Abrogation and restoration of *ialB* expression was verified by SDS-PAGE and immunoblotting. In vitro virulence assays showed that mutagenesis of *ialB* decreased bacterial association and invasion of human erythrocytes by 47 to 53% relative to controls. Transcomplementation of *ialB* restored erythrocyte association and invasion rates to levels observed in the parental strain. These data provide direct evidence for IalB's role in erythrocyte parasitism and represent the first demonstration of molecular Koch's postulates for a *Bartonella* species.

Bartonella bacilliformis is the only bacterium known to invade human erythrocytes. The pathogen is the causative agent of the human disease, Oroya fever, a biphasic illness whose primary-phase symptoms include a severe hemolytic anemia, where up to 100% of the circulating erythrocytes can be parasitized and 80% lysed (1, 15, 31). If untreated, this phase of the disease has a 40% fatality rate (44). Treatment with penicillin, tetracycline, or aminoglycosides is effective (43), but diagnosis can be difficult due to the slow growth and fastidious nature of the bacterium. The secondary phase of Oroya fever occurs 4 to 8 weeks following the primary hemolytic phase and is characterized by hemangiomas, nicknamed verruga peruana, on the patient's head, neck, and extremities. During the secondary phase, bacterial colonization and invasion shifts from erythrocytes to vascular endothelial cells (13, 14, 21) and results in neovascularization (13). This phase of the disease is rarely fatal but can last up to several months (43) and may cause permanent disfigurement. *B. bacilliformis* is transmitted by the phlebotamine sandfly, *Lutzomyia verrucarum*. Historically, Oroya fever has been limited to the mountainous regions of South America, presumably due to geographical restriction of its vector (19). However, recent reports of Oroya fever in coastal areas of South America suggest that the range of this pathogen is expanding (1).

Although other bacteria are known to parasitize mammalian erythrocytes (e.g., *Anaplasma* and *Haemobartonella* species), *B. bacilliformis* is unsurpassed among bacteria in its efficiency as an erythrocyte parasite. *B. bacilliformis* is able to invade nearly all circulating erythrocytes during the acute phase of infection. Erythrocytes lack the actin cytoskeleton necessary for bacterial uptake by induced endocytosis, although endocytosis can be induced under experimental conditions (35, 40). Treatment of erythrocytes with glycolysis and proton-motive-force inhibitors has no effect on *Bartonella* adhesion, suggesting that these host cells play a passive role in invasion (42). In contrast, *B. bacilliformis* plays an active role during erythrocyte invasion requiring both respiration and proton motive force (42). Taken together, these data indicate that *B. bacilliformis* is the only active participant in erythrocyte adherence and invasion. In contrast, *B. bacilliformis* entry into endothelial and epithelial cells differs significantly from its invasion of erythrocytes. Bacterium-induced rearrangement of the endothelial and epithelial cell cytoskeleton during endocytosis enhances bacterial uptake, while cytochalasin D treatment, inhibiting actin filament formation, reduces internalization by ~30% (21).

The *B. bacilliformis* invasion-associated locus A and B genes (*ialAB*) were indirectly shown to be involved in erythrocyte

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TABLE 1. Bacteria and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
B. bacilliformis		
JB584	Transformation-competent strain of *B. bacilliformis*	5
SC1	JB584 with *ialB* interrupted by pSAC100 (Km^r, *ialB* mutant)	This study
SC2	SC1 complemented in *trans* with pSAC200 (Km^r Cm^r, $ialB^+$)	This study
E. coli		
DH5α	Host strain for cloning and plasmid propagation	Gibco-BRL
M15	Host strain for fusion protein expression	41
Plasmids		
pIAL1	pUC19 containing *ialA* and *ialB* of *B. bacilliformis*	27
pIALB	pUC19 containing *ialB* of *B. bacilliformis*	27
pQE-31	Expression vector	Qiagen Inc.
pREP4	Plasmid encoding *lacI*	Qiagen Inc.
pQIALB	pQE-31 with 574-bp *Pvu*II-*Pst*I fragment encoding *ialB* minus its secretory signal sequence plus 15 nucleotides	This study
pUB1	*B. bacilliformis* suicide plasmid; Km^r	5
pSAC100	pUB1 with an internal 430-bp *Pvu*II-*Mfe*I fragment of *ialB*; Km^r	This study
pBBR1MCS	*B. bacilliformis* shuttle vector; Cm^r	18
pSAC200	Complementation plasmid; pBBR1MCS with 756-bp *Swa*I-*Bam*HI fragment containing intact *ialB*; Cm^r	This study

invasion by conferring an erythrocyte-invasive phenotype upon minimally invasive *Escherichia coli* strains (27). IalA has since been characterized as a (di)nucleoside polyphosphate hydrolase thought to be involved in reducing levels of stress-induced dinucleotides during invasion, thus aiding bacterial survival (9, 11). IalB was shown to contain a putative 22-amino-acid secretory signal sequence and to have approximately 60% amino acid similarity to the virulence determinants Ail of *Yersinia enterocolitica* and Rck of *Salmonella enterica* serovar Typhimurium. The presence of a potential secretory sequence and similarity of IalB to two outer membrane virulence determinants led to our hypothesis that IalB is exported to the bacterial surface, where it functions as an invasion factor. This study was undertaken to localize the IalB protein and directly determine its role in human erythrocyte association by *B. bacilliformis*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. bacilliformis* strains (Table 1) were cultured on heart infusion agar blood (HIAB) plates (heart infusion agar supplemented with 4% sheep erythrocytes and 2% sheep serum) in a water-saturated incubator at 30°C. When required, strains were cultured in the presence of kanamycin (25 μg/ml) and/or chloramphenicol (5 μg/ml). *E. coli* strains (Table 1) were cultured in Luria-Bertani (LB) broth at 37°C in the presence of antibiotics as needed.

Preparation and manipulation of DNA. Plasmids used or generated in this study are given in Table 1. Plasmids were propagated in *E. coli* DH5α and isolated by the methods of Birnboim and Doly (7), a Perfectprep kit (Eppendorf Scientific, Westbury, N.Y.), or a Qiagen Midiprep kit (Qiagen, Inc., Valencia, Calif.). Restriction digests and agarose gel electrophoresis were done using standard protocols (2). DNA fragments from restriction digests were purified from ethidium bromide-stained agarose gels with a GeneClean II kit (Bio 101, La Jolla, Calif.). Ligations were performed by standard protocol (2), and transformations were done by the method of Chung et al. (10). Genomic DNA was isolated using cetyltrimethylammonium bromide (CTAB) (2). Electroporation of *B. bacilliformis* was done as previously described (5).

PCR and oligonucleotide primers. PCR amplification was done in a GeneAmp 2400 thermocycler (Perkin-Elmer, Norwalk, Conn.) as previously described (5). DNA was denatured at 94°C for 5 min, amplified for 30 cycles (1 min at each of the following temperatures: 94, 59 or 65, and 72°C), and extended for 10 min at 72°C. Single-strand oligonucleotide primers for the *ialB* gene, IALBF (5′-GTA TTATGAATTACTATCGAGAATAA-3′) and IALBR (5′-ATCCGACCATAA TACTTATCTTCT-3′), and for the neomycin phosphotransferase I gene (*nptI*), NPT15′ (5′-AGCCACGTTGTGTCTCAAAATCTC-3′) and NPTI3′ (5′-CGTC CCGTCAAGTCAGCGTAATGC-3′), were used. A "junction" primer set consisting of IALBR and NPTI5′ was designed to amplify the site of homologous recombination between the chromosomal *ialB* gene and the suicide plasmid, pSAC100. Annealing sites for all primers are depicted in Fig. 2.

DNA hybridization analysis. Genomic DNA from *B. bacilliformis* and plasmid DNA were digested to completion with *Cla*I and separated on a 1.2% (wt/vol) agarose gel stained with ethidium bromide. DNA was transferred to a supported nitrocellulose membrane (pore size, 0.45 μm; Schleicher & Schuell, Keene, N.H.) by the method of Southern (37) and then baked for 1 h at 80°C. DNA probes were made by random primer extension (2) with [α-^{32}P]dCTP (New England Nuclear, Boston, Mass.). High-stringency hybridization, washes, and visualization were done as previously described (6).

SDS-PAGE. Protein concentrations were determined using a bicinchoninic acid protein kit per the manufacturer's instructions (Sigma Chemical Co., St. Louis, Mo.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done following the general procedures of Laemmli (20) with either 12.5, 15, or 15 to 20% gradient polyacrylamide (wt/vol) gels. Either 20 or 100 μg of protein was loaded per lane for gels that were Coomassie blue stained (33) and 2.5 μg was loaded per lane on gels that were silver stained (45).

Preparation of polyclonal antibodies and immunoblotting. To prepare antibodies against IalB, *E. coli* M15 (pQIALB, pREP4) was grown overnight with vigorous shaking in LB broth containing ampicillin and kanamycin. The overnight culture was used to inoculate LB broth plus antibiotics and grown to an optical density at 600 nm (OD600) of 0.7 to 0.9, and *ialB* expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 2 mM final concentration). Cultures were induced for 3 h, and the bacterial pellet was harvested by centrifugation at 4,000 × *g* for 20 min at 4°C. Bacterial pellets were solubilized in Laemmli sample buffer and proteins separated by SDS-PAGE. The IalB protein was excised from unfixed Coomassie blue-stained gels, minced, suspended in 1 ml of phosphate-buffered saline (PBS; pH 7.4), and used to generate antibody in a female New Zealand White rabbit as previously described (34).

For immunoblots, 20 to 80 μg of protein were separated by SDS-PAGE (12.5 or 15% [wt/vol] acrylamide), electrophoretically transferred to a supported nitrocellulose membrane (pore size, 0.45 μm; Schleicher & Schuell), and reacted with anti-IalB antiserum (diluted 1:1,000) as previously described (34).

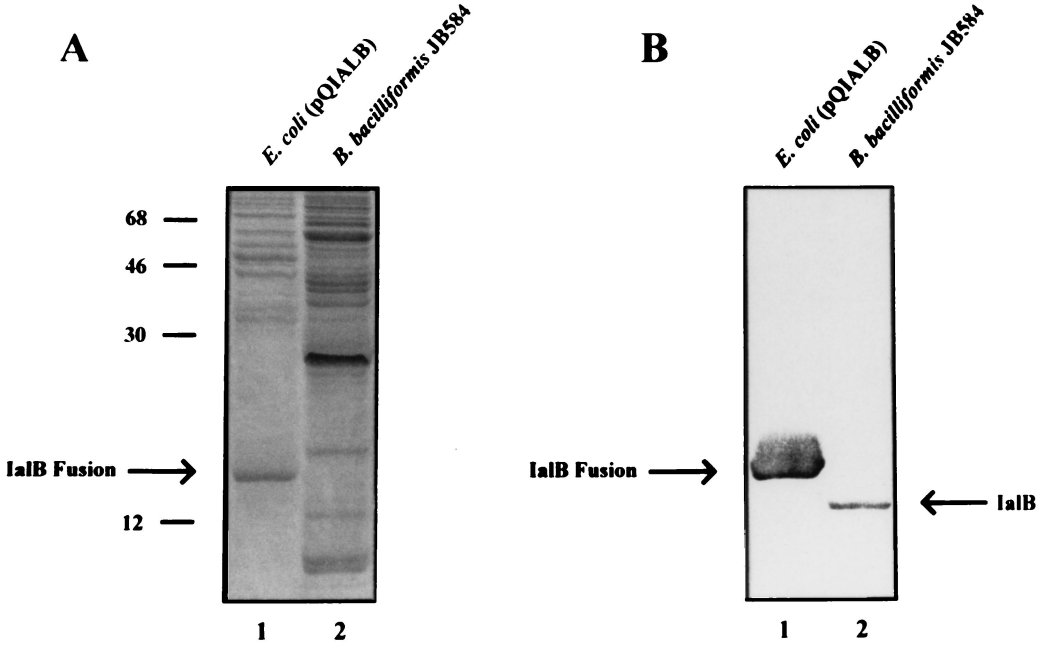

FIG. 1. Recognition of *B. bacilliformis* IalB and the IalB fusion protein by polyclonal anti-IalB antibodies. Cell lysates of IPTG-induced *E. coli* M15(pQIALB) (lane 1) and *B. bacilliformis* (lane 2) analyzed by an SDS-PAGE gel stained with Coomassie blue (A) or immunoblot reacted with polyclonal anti-IalB antibodies (B). Both *B. bacilliformis* IalB and the IalB fusion protein are specifically detected with the antiserum. Molecular mass standards in kilodaltons are indicated on the left.

Localization of IalB. Accessible outer membrane proteins of intact *B. bacilliformis* were extrinsically radioiodinated as previously described (24) and then analyzed by SDS-PAGE. Whole bacteria were extrinsically treated with various proteases (proteinase K, trypsin, subtilisin, papain, and thermolysin) to cleave any accessible, sensitive surface proteins as previously described (24), and protein profiles were analyzed by gradient SDS-PAGE. Immunofluorescent labeling of intact *B. bacilliformis* strains using anti-IalB polyclonal antibodies was done according to standard protocols (2). Twenty plates of 3-day-old *B. bacilliformis* were harvested into 1 ml of ice-cold Dulbecco's PBS, and membranes were isolated and fractionated as previously described for *B. quintana* (8). Cytochrome assays were performed using inner and outer membrane fractions (final protein concentration, 1 μg/μl) by the methods of Osborn et al. (30).

Human erythrocyte association assay. Blood was drawn from human volunteers into an acid citrate-dextrose Vacutainer tube and stored overnight at 4°C to separate plasma from the erythrocytes. After removal of the plasma, erythrocytes were washed with 10 ml of sterile saline (0.9%, wt/vol) and centrifuged at 700 × *g* for 5 min. Erythrocytes were washed a second time, counted, and resuspended in recovery broth (5) to a final concentration of 10^9 erythrocytes per ml.

Three- to four-day-old *B. bacilliformis* cultures were harvested into recovery broth and diluted to an OD_{600} of 1.0 (~1.6×10^9 CFU/ml). Approximately 5×10^8 bacteria were gently mixed with 10^8 erythrocytes (multiplicity of infection 5:1) in a total volume of 0.5 ml of recovery broth. Association reactions were incubated for 8 h at 30°C in a water-saturated environment. Erythrocytes and parasitized erythrocytes were separated from free bacteria by Percoll gradient centrifugation. Briefly, 1 ml of 70% Percoll (Sigma) containing 154 mM NaCl was centrifuged at 16,000 × *g* for 10 min to create a continuous gradient. Then, 0.1 ml of each association reaction was carefully layered onto the preformed Percoll gradient and centrifuged at 1,500 × *g* for 5 min. The erythrocyte-bacterium band was collected, washed twice with sterile saline, and pelleted by centrifugation at 1,000 × *g* for 15 s. The pellet was resuspended in 0.5 ml of heart infusion broth, serially diluted, and then plated onto HIAB plates. Plates were incubated at 30°C in a water-saturated incubator for 12 days and then counted for CFU.

Statistical analysis. Numerical data reported for human erythrocyte association assays are the means of three independent samples ± the standard errors of the mean (SEM). The statistical significance of the data was determined by use of the Student's *t* test. A *P* value of <0.05 was considered significant.

RESULTS

Expression and purification of IalB fusion protein. To obtain sufficient amounts of IalB protein to generate antibodies, the *ialB* gene (excluding the portion encoding its secretory signal sequence plus five N-terminal amino acids) was cloned in frame into the expression vector pQE-31. This vector contains a six-histidine tag and a polylinker under the control of the *lacZ* promoter. The resulting construct, pQIALB, was transformed into *E. coli* M15, and *ialB* expression was induced with IPTG. The IalB fusion protein was synthesized at high levels and localized to the insoluble fraction of *E. coli*. The insoluble fraction was treated with a strong denaturant (6 M guanidine hydrochloride), and the recombinant IalB was purified using nickel affinity chromatography. IalB was purified to apparent homogeneity when analyzed by using Coomassie blue-stained SDS-PAGE gels (data not shown). Polyclonal anti-IalB antibodies were generated and found to recognize both the IalB fusion protein synthesized in *E. coli* and wild-type IalB synthesized by *B. bacilliformis* in Western blots (Fig. 1B). On Western blots, the IalB fusion protein and IalB from *B. bacilliformis* have estimated masses of 18.6 and 17.1 kDa, respectively. From its DNA sequence, the mature *B. bacilliformis* IalB protein was predicted to be 17.5 kDa (27), in close agreement with our finding. Presumably, the larger estimated mass of the IalB fusion protein is due to the presence of the charged, six-histidine tag.

Generating an *ialB* mutant and a transcomplemented strain of *B. bacilliformis*. A 426-bp, *Pvu*II-*Mfe*I internal fragment of the *ialB* gene was cloned into pUB1 to create the suicide vector, pSAC100. The pMB1 origin of pSAC100 is not func-

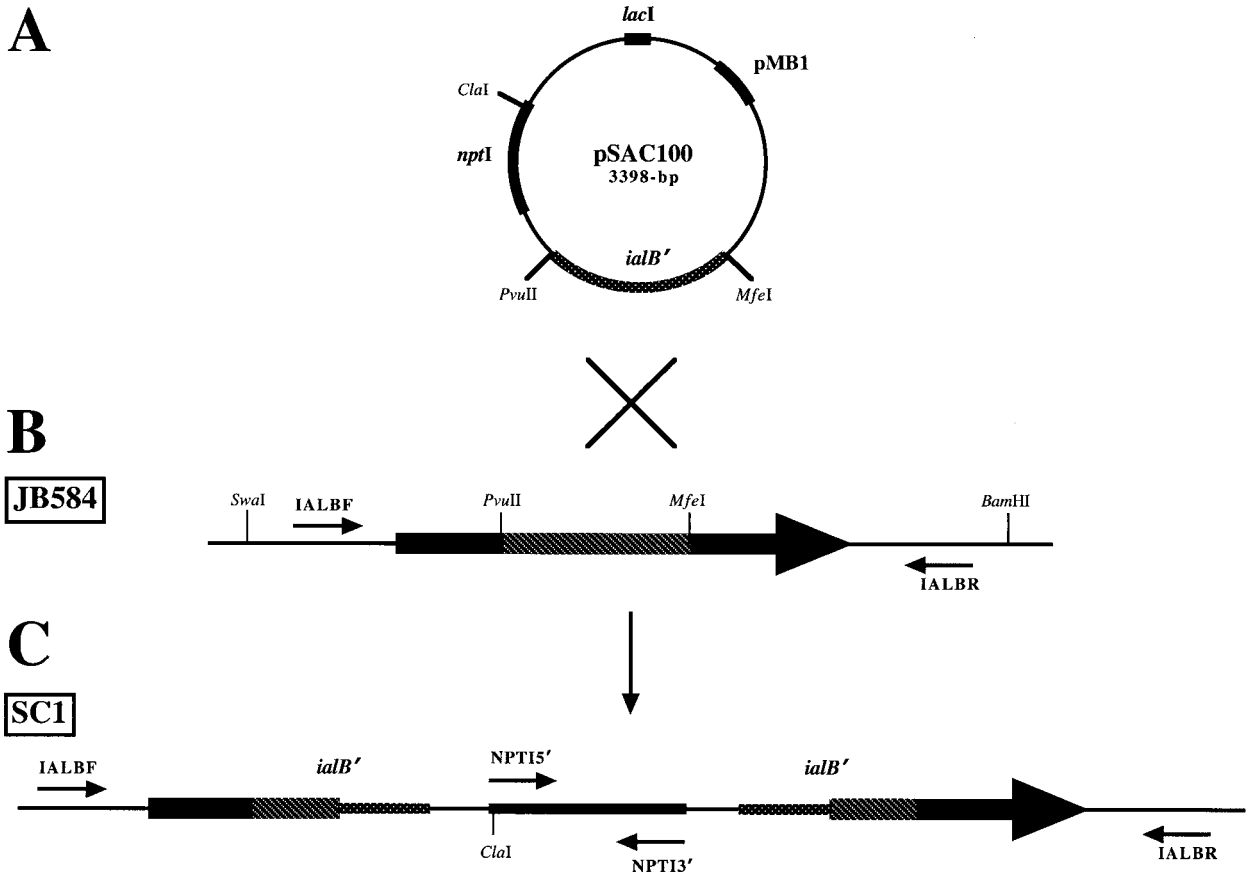

FIG. 2. Schematic representation depicting site-directed mutagenesis of the *B. bacilliformis ialB* gene. (A) The suicide plasmid, pSAC100, was created by cloning a 426-bp *Pvu*II-*Mfe*I internal fragment of the *B. bacilliformis ialB* gene (*ialB′*) into pUB1. (B) The JB584 strain of *B. bacilliformis* encodes the wild-type *ialB* gene. (C) Electroporation of JB584 with pSAC100, followed by recombination between the suicide plasmid, pSAC100, and the chromosomal *ialB* gene, results in insertional disruption and generation of the *B. bacilliformis ialB* mutant strain, SC1. Primer sites for *ialB* (IALBF and IALBR) and *nptI* (NPTI5′ and NPTI3′) are indicated by small arrows. (The figure is not drawn to scale.)

tional in *B. bacilliformis* (5); therefore, expression of the *nptI* gene, conferring kanamycin resistance, would only occur following recombination of the suicide plasmid into the chromosome. Cloning an internal fragment of the *ialB* gene ensured that homologous recombination between pSAC100 and the chromosome would not result in reconstitution of a full-length gene.

The JB584 strain of *B. bacilliformis* was electroporated with pSAC100. Kanamycin-resistant colonies were isolated, cultured, and initially characterized by PCR. The *ialB* gene, the *nptI* gene, or the junction where pSAC100 recombined with the chromosomal *ialB* gene were PCR amplified as depicted in Fig. 2. The *nptI* gene primer set (NPTI5′ and NPTI3′) amplified a 983-bp segment of the *nptI* gene in the kanamycin-resistant strain, SC1, but not the parental strain, JB584 (Fig. 3A, lanes 3 and 2, respectively), showing that kanamycin resistance in SC1 was due to *nptI* and not to selection of spontaneous kanamycin-resistant mutants. The *ialB* gene primer set (IALBF and IALBR) was expected to produce a 4,097-bp product from the site of homologous recombination or a 688-bp product from an intact *ialB* gene. Upon analysis, an amplicon of ~4,000-bp was obtained from the kanamycin-resistant strain, SC1, indicating that pSAC100 had recombined with the chromosomal *ialB* (Fig. 3A, lane 6). No PCR product would be amplified from unintegrated pSAC100 since the *ialB* primers are complementary to chromosomal sequences flanking the *ialB* gene and absent in pSAC100. As expected, a 688-bp amplicon was obtained from the intact *ialB* gene in JB584 (Fig. 3A, lane 5).

The junction primer set (NPTI5′ and IALBR) produced an amplicon of approximately 1,700-bp from SC1 and no product from the parental strain, JB584 (Fig. 3A, lanes 11 and 8, respectively). As expected, no amplicon was obtained when pSAC100 DNA was added to JB584 genomic DNA and then amplified with the junction primer set (Fig. 3A, lane 10). From these data we concluded that homologous recombination had occurred between pSAC100 and the chromosomal *ialB* gene, creating an *ialB* mutant strain, SC1.

We then proceeded to create a transcomplemented strain using SC1 as the parental strain. The pIALB plasmid was digested with *Swa*I and *Bam*HI, the 756-bp fragment containing the intact *ialB* gene isolated, and cloned into the broad-host-range plasmid, pBBR1MCS to produce the shuttle plasmid, pSAC200. pSAC200 was subsequently electroporated into SC1, and transformants were selected on HIAB plates supplemented with both kanamycin and chloramphenicol. Potential

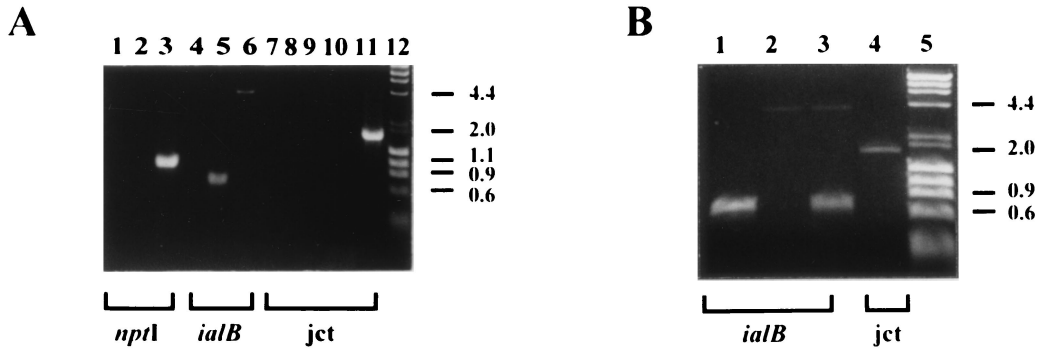

FIG. 3. Electrophoretic analysis of PCR products derived from *ialB* mutant strain, SC1, and transcomplemented strain, SC2. PCR products were generated by amplification of genomic DNA from parent and recombinant strains using three amplimer sets (*nptI* [NPTI3′ and NPTI5′], *ialB* [IALBF and IALBR], and junction [jct] [NPTI5′ and IALBR]). Brackets below the gel indicate the amplimer set used in each reaction. Amplimer sets and template DNA for PCR used in this analysis are as follows. (A) Lane 1, NPTI3′ and NPTI5′, no template; lane 2, NPTI3′ and NPTI5′, JB584; lane 3, NPTI3′ and NPTI5′, SC1; lane 4, IALBF and IALBR, no template; lane 5, IALBF and IALBR, JB584; lane 6, IALBF and IALBR, SC1; lane 7, NPTI5′ and IALBR, no template; lane 8, NPTI5′ and IALBR, JB584; lane 9, NPTI5′ and IALBR, pSAC100; lane 10, NPTI5′ and IALBR, JB584 and pSAC100; lane 11, NPTI5′ and IALBR, SC1; lane 12, lambda DNA/*Hin*dIII and ϕX174 DNA/*Hae*III markers. (B) Lane 1, IALBF, and IALBR, JB584; lane 2, IALBF and IALBR, SC1; lane 3, IALBF and IALBR, SC2; lane 4, NPTI5′ and IALBR, SC2; lane 5, lambda DNA/*Hin*dIII and ϕX174 DNA/*Hae*III markers. PCR products were analyzed by ethidium bromide-stained agarose (1.2%, wt/vol) gel electrophoresis. Size standards in kilobase pairs are indicated on the right.

transcomplemented strains were isolated, cultured, and characterized by PCR.

The *ialB* gene primer set (IALBF and IALBR) was used to screen for potential transcomplemented strains. One strain, SC2, produced amplicons of 4,097 and 688 bp representing the interrupted *ialB* gene on the bacterial chromosome and the intact *ialB* gene on pSAC200, respectively (Fig. 3B, lane 3). PCR amplification of SC2 DNA using the junction primer set (NPT15′ and IALBR) resulted in a product of approximately 1,700 bp (Fig. 3B, lane 4), indicating that the original site of integration was intact.

To determine whether expression of the *ialB* gene had been disrupted in SC1 and transcomplemented in SC2, cell lysates of the bacteria were analyzed by SDS-PAGE and Western blot (Fig. 4). A 17.1-kDa band was present in both JB584 and SC2 lysates but absent in SC1 lysates. This protein was positively identified as IalB by Western blots (Fig. 4B). We consistently observed more IalB in cell lysates of SC2 relative to JB584, by both SDS-PAGE and Western blots. Presumably, increased synthesis in SC2 is due to the multiple copies of pSAC200 encoding *ialB* (Fig. 5B).

Genotypes of the mutant and transcomplemented strains were corroborated using DNA hybridization (Fig. 5). Restriction endonuclease digestion of pIALB with *Kpn*I and *Hin*dIII yielded a 744-bp fragment containing *ialB* that was used to probe Southern blots of *Cla*I-digested genomic DNA from each strain (Fig. 5A). Hybridization of the probe with JB584 DNA showed a single, distinct band of ~23 kbp (Fig. 5B, lane 2), while hybridization with the *ialB* mutant strain, SC1, gave two bands of ~23 and ~3.7 kbp (Fig. 5B, lane 3). The two hybridization products in SC1 are due to the presence of a *Cla*I restriction enzyme site in the integrated suicide plasmid (Fig. 2). Each band contains a portion of the *ialB* gene. The insertionally mutagenized *ialB* gene of the transcomplemented strain SC2 gives the expected two-band pattern like SC1, plus an additional hybridization band of ~5.4 kbp from *ialB* on pSAC200 (Fig. 5B, lane 4).

No overt phenotypic differences between the parental, *ialB* mutant, and transcomplemented strains were apparent.

Localization of IalB in the bacterium. As expected, SDS-PAGE analysis of total membranes showed that IalB was present in the membrane fraction of JB584 and SC2 but not the mutant strain, SC1, and its identity as IalB was verified by Western blot (data not shown). Extrinsic radioiodination of intact JB584 and SC1 showed no difference in protein profiles

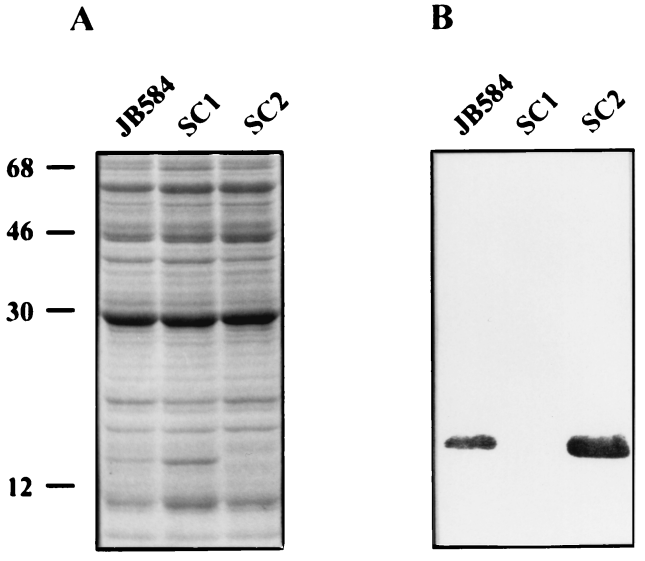

FIG. 4. Abrogation and complementation of *ialB* expression in *B. bacilliformis* strains. (A) Cell lysate proteins (80 μg/lane) separated by SDS-PAGE (12.5%, wt/vol) and stained with Coomassie blue. Lane 1, JB584; lane 2, SC1; lane 3, SC2. (B) Corresponding immunoblot reacted with polyclonal anti-IalB antibodies showing IalB is present in the parental *B. bacilliformis* strain, JB584 (lane 1), and the transcomplemented mutant strain, SC2 (lane 3), but is absent in the *ialB* mutant strain, SC1 (lane 2). Molecular mass standards in kilodaltons are indicated on the left.

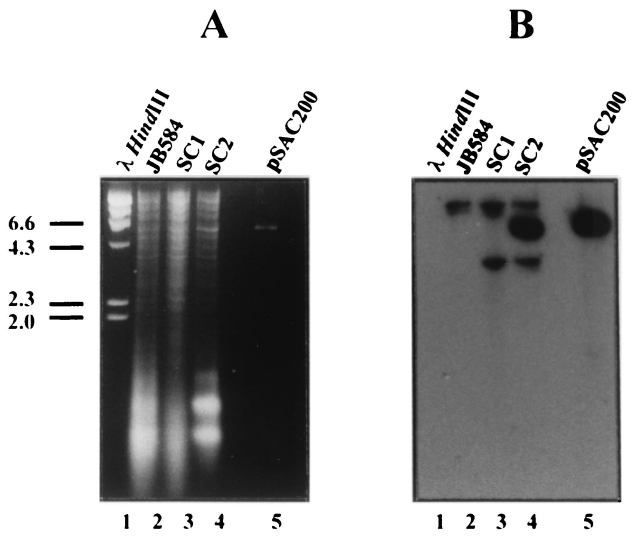

FIG. 5. Detection of the wild-type and mutated *ialB* genes in *B. bacilliformis* strains using DNA hybridization. (A) Ethidium bromide-stained agarose gel (1.2%, wt/vol) of *Cla*I-digested genomic DNA from the parental *B. bacilliformis* strain, JB584 (lane 2), the *ialB* mutant strain, SC1 (lane 3), and the transcomplemented strain, SC2 (lane 4). The shuttle plasmid used in transcomplementation, pSAC200, digested with *Cla*I is shown in lane 5, and DNA size standards (Lambda DNA/*Hin*dIII markers) are provided in lane 1. (B) Corresponding Southern blot hybridized with the *ialB* probe. Lane 1, DNA size standards; lane 2, single hybridization band of ~23 kbp from the parental *B. bacilliformis* strain, JB584; lane 3, two-band hybridization pattern from the disrupted *ialB* gene in the *ialB* mutant strain, SC1; lane 4, two-band hybridization pattern from the disrupted *ialB* gene, as well as the ~5.4-kbp hybridization band from pSAC200 in the transcomplemented strain, SC2; lane 5, single hybridization band from pSAC200. Size standards in kilobase pairs are indicated on the left.

when analyzed by SDS-PAGE (data not shown). Whole JB584 bacteria extrinsically treated with several proteases showed no alteration in the migration of IalB on gradient SDS-PAGE gels (data not shown). No difference in immunofluorescence was seen when whole JB584 and SC1 bacteria were surface labeled using anti-IalB polyclonal antibodies (data not shown). Radioiodination, proteolysis, and immunofluorescence data suggested that IalB is an inner membrane protein.

To conclusively localize IalB to the inner membrane, crude lysates were subjected to sucrose density gradient centrifugation as we previously described for *B. quintana* (8). Inner and outer membrane bands were collected from gradients and identified on the basis of their appearance. Outer membrane fractions typically showed a white flocculent appearance, while inner membrane fractions were typically tea colored (28). The average buoyant densities (ρ) were determined from three membrane preparations and calculated to be 1.08 g/cm^3 for the inner membrane and 1.22 g/cm^3 for the outer membrane. These values are very similar to the buoyant densities for the outer and inner membranes of *E. coli* (28) and *Salmonella* spp. (30) and are nearly identical to those we obtained from *B. quintana* membrane fractions (8). Outer membrane fractions analyzed by SDS-PAGE on a 15 to 20% gradient gel and stained with silver gave a protein profile similar to that previously reported for *B. bacilliformis* (24). In addition, the outer, but not the inner, membrane fractions contained the 42-kDa flagellin protein (34) and three bacteriophage proteins with molecular masses of 32, 34, and 36 kDa (4). The identity of the inner membrane fraction was unequivocally established by the presence of cytochrome *b*. Difference spectra for the inner and outer membrane fractions were obtained between 499 and 600 nm. The inner, but not the outer, membrane fraction had an absorbance peak at 558 nm, which is characteristic of cytochrome *b*. Once the identity of the inner and outer membrane fractions was established, their respective protein profiles were analyzed using SDS-PAGE. Contrary to our hypothesis that IalB was an outer membrane protein, the protein was found in the inner membrane fractions of both JB584 and SC2 (Fig. 6, lanes 3 and 7). The identity of IalB was confirmed by Western blot (Fig. 6B).

Role of IalB in erythrocyte adhesion and invasion. Following the 8-h association assays, Percoll gradient centrifugation was used to separate erythrocytes from free bacteria. Since both adherent and invaded bacteria were complexed with erythrocytes, CFU counts from these assays include bacteria that are adhering to, or have invaded, erythrocytes.

Association assays were carried out at least four times, with each experiment containing two to five independent samples. While the number of CFU varied between experiments, the data trends remained consistent. For the association assays conducted with the *ialB* mutant strain, SC1, and the parental strain, JB584, SC1 adherence and invasion decreased 47 to 53% compared to JB584. In a representative experiment, SC1 showed a significant decrease ($P < 0.05$) of 53% in adherence and invasion compared to JB584 (mean CFU of 91,750 ± 14,655 versus 196,300 ± 12,537, respectively) (Fig. 7A). Association assays conducted with JB584 and the complemented strain, SC2, showed statistically insignificant differences in adherence and invasion, although the range of values varied more than that observed in assays with JB584 and SC1. This increased scatter in SC2 values may be due to multiple plasmid copies of the *ialB* gene in SC2. In a representative experiment, the *trans*-complemented strain, SC2, showed no significant change ($P = 0.7825$) in association assays when compared to JB584 (mean CFU of 10,833 ± 1,906 versus 11,775 ± 2,575, respectively) (Fig. 7B).

DISCUSSION

This study is the first demonstration of molecular Koch's postulates (12) for a *Bartonella* species. Insertional mutagenesis of *ialB*, creating the *B. bartonella* mutant strain, SC1, resulted in a 47 to 53% decrease in human erythrocyte adherence and invasion compared to the parental strain, JB584. Transcomplementation of *ialB*, creating the SC2 strain, restored erythrocyte adherence and invasion to parental levels. These data clearly establish IalB as a virulence determinant for *B. bacilliformis* erythrocyte parasitism.

Mitchell and Minnick originally isolated and characterized the two-gene locus, *ialAB*, reporting that both *ialA* and *ialB* were necessary to confer an invasive phenotype upon *E. coli* (27). However, the results of the present study demonstrate that *ialB* has a significant effect on *B. bacilliformis* erythrocyte parasitism. In vivo experiments with the rat pathogen, *B. tribicorum*, support our findings that *ialB* is a virulence factor. Specifically, an *ialB* mutant strain of *B. tribicorum* failed to

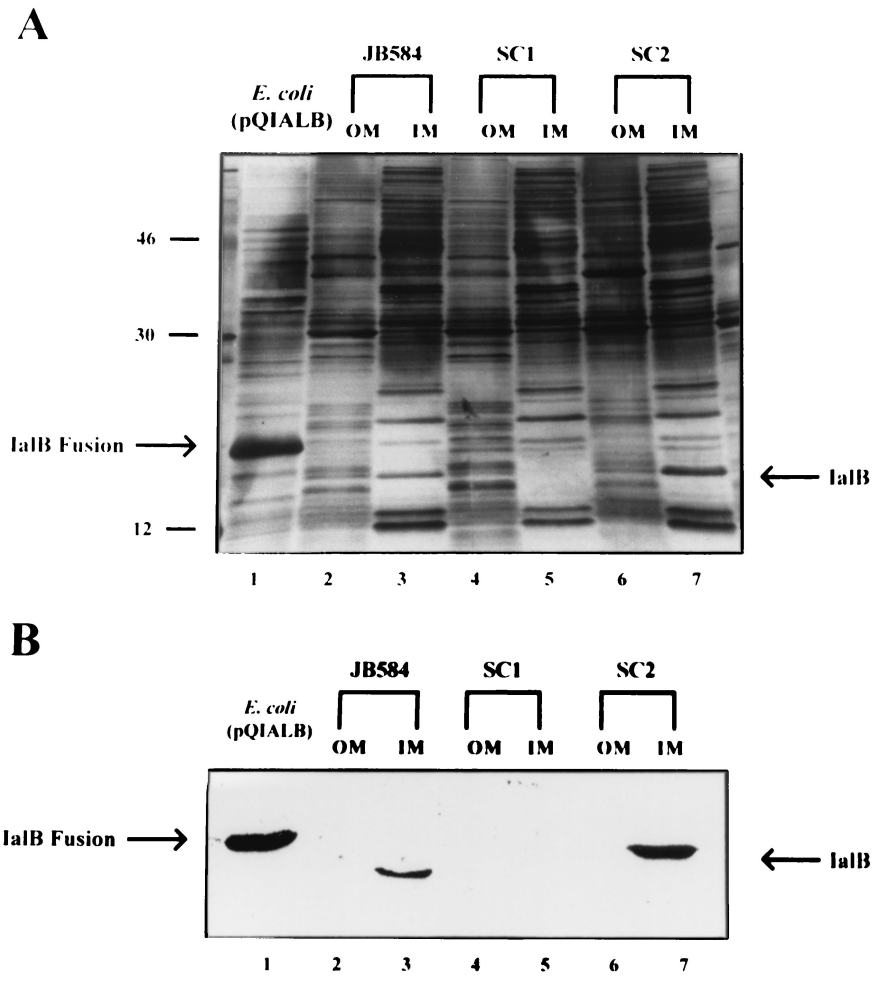

FIG. 6. Localization of IalB to the *B. bacilliformis* inner membrane. (A) Proteins (2.5 μg/lane) were separated by SDS-PAGE (15 to 20% [wt/vol] gradient), and the gel was silver stained. IalB was found in the inner membrane fractions of JB584 (lane 3) and SC2 (lane 7), the parental and transcomplemented *B. bacilliformis* strains, respectively. IalB was absent from all outer membrane fractions and the inner membrane fraction of SC1, the *ialB* mutant strain. (B) Corresponding immunoblot reacted with polyclonal anti-IalB antibodies. IalB localized to the inner membrane fractions of JB584 and SC1 (lanes 3 and 7, respectively). IPTG-induced *E. coli* M15(pQIALB) cell lysate is provided as a control in lane 1.

develop bacteremia and to invade rat erythrocytes in vivo (C. Gille, C. Lanz, and C. Dehio, Abstr. 1st Int. Conf. *Bartonella* Emerging Pathogens, abstr. 28, 1999).

ialA and *ialB* homologues are present in the three most prevalent, human pathogenic species of *Bartonella: B. henselae, B. quintana*, and *B. bacilliformis* (26). *B. henselae* and *B. quintana* cause cat-scratch disease and trench fever, respectively. All three species share phenotypic similarities: they are transmitted by arthropod vector, are intracellular parasites, and have an absolute growth requirement for hemin. All three species invade or attach to erythrocytes during the course of infection (17, 22, 23) and can cause neovascularization of infected tissue (25). Erythrocyte parasitism and neovascularization may provide the blood and heme required for these pathogenic bacteria. Given the phenotypic similarities of *B. bacilliformis, B. quintana*, and *B. henselae*, IalA and IalB may share similar functions contributing to the virulence of all three species.

Homologues of *ialA* and *ialB* have been found in other gram-negative pathogenic bacteria. *Brucella melitensis* is a facultative intracellular pathogen and the causative agent of ovine brucellosis. The ability of *B. melitensis* to cause disease is tied to its ability to adapt and survive in a range of environments. *B. melitensis*' adaptive responses to heat, oxidative, and acid stress were recently characterized (39). Protein levels, in response to these stresses, were analyzed by two-dimensional PAGE. In response to heat shock (a temperature shift from 37 to 42°C), an appreciable reduction in synthesis was observed for a protein with homology to the IalB protein of *B. bacilliformis*. No change in synthesis was seen for the IalB homologue in response to either oxidative or acid stress. *Brucella* and *Bartonella* are closely related α-proteobacteria, and their phylogenetic relationship is underscored by the ability of both genera to interact with eukaryotic cells in a parasitic or mutualistic association. In light of these similarities, it is interesting that these two species may share a virulence factor associated with eukaryotic cell invasion. We are currently examining the effect of environmental cues on *ialB* expression, as the transfer of *B.*

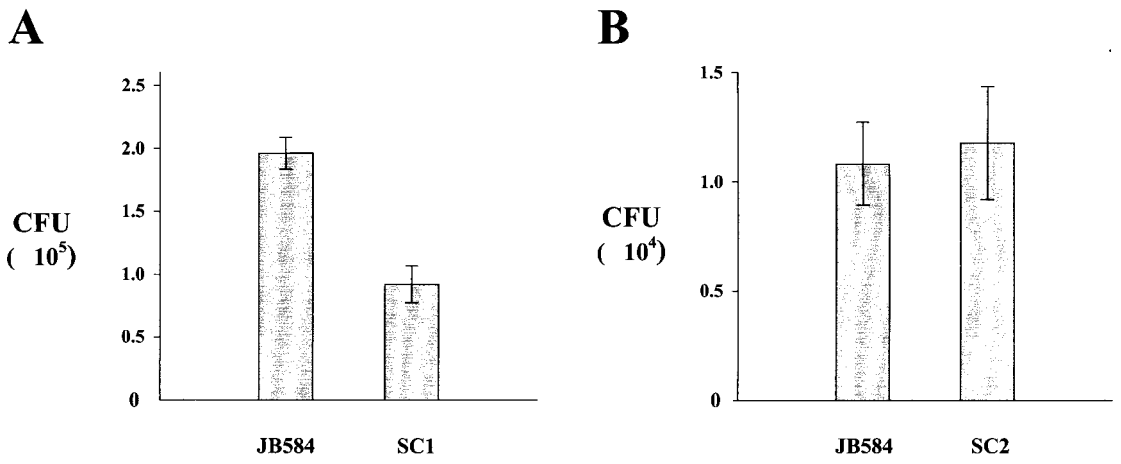

FIG. 7. Effect of *ialB* mutagenesis and transcomplementation on human erythrocyte adherence and invasion on *B. bacilliformis* strains. (A) The *ialB* mutant strain, SC1, shows a 53% decrease in erythrocyte adherence and invasion compared to the parental strain, JB584. The *n* values for JB584 and SC1 are 5 and 4, respectively. (B) Transcomplementation of *ialB* in SC2 restores erythrocyte adherence and invasion to parental strain levels. The *n* values for JB584 and SC2 are 3 and 2, respectively. Experimental data presented graphically in panels A and B are the mean ± the SEM from two separate but representative experiments.

bacilliformis from sandfly to human would be associated with significant changes in temperature, iron availability, pH, and oxidative stress. These environmental cues could serve as signals for expression of virulence factors necessary for human infection.

In another study, differential fluorescence induction was used to identify *E. coli* K1 genes expressed under environmental conditions favoring bacterial invasion of human brain microvascular endothelial cells (HBMEC) (3). One gene identified in that study was an IalA homologue (38% homology). Site-directed mutagenesis of this *E. coli* gene reduced HBMEC invasion twofold, and transcomplementation restored the invasive phenotype to wild-type levels. IalA and IalB homologues are being identified in a number of bacterial species, all of which invade eukaryotic cells. Additionally, experimental evidence for the role of these proteins in virulence is accumulating.

We originally hypothesized that IalB is exported to the bacterial surface, where it functions as an invasion factor. Contrary to our hypothesis, IalB was localized to the inner membrane in this study. Our original hypothesis was, in part, based on the reported ~60% amino acid sequence similarity of IalB to Ail and Rck (27). However, although these proteins have significant amino acid similarity, their amino acid identity is actually quite low (~11%). The IalB protein also lacks a terminal phenylalanine amino acid residue characteristic of most outer membrane proteins (38), including Ail and Rck.

Localization of IalB to the cytoplasmic membrane necessitated rethinking of its function as a virulence factor. Virulence-related activities for inner membrane proteins include transport of virulence factors, uptake of nutrients, response to environmental stresses, chemotaxis, cell motility, and intracellular survival, to name a few. These various functions fall into one of two general categories: transport or signal transduction. For example, the *virB* operon of *Brucella suis* and *Brucella abortus* was found to be essential for virulence and intracellular survival of these mammalian pathogens. The *virB* operon encodes homologues to a type IV secretory system including putative inner membrane proteins (29, 36). An intriguing example of a signal-transducing, inner membrane protein is found in *Pseudomonas aeruginosa*. Normally, the sigma factor responsible for expression of a mucoid phenotype is sequestered at the cytoplasmic membrane by an inner membrane protein. Release of this sigma factor into the cytosol, presumably in response to some signal, results in the expression of mucoidy (32). Phosphorylation is another mechanism by which an inner membrane protein could facilitate signal transduction. The *etk* gene of *E. coli* encodes an inner membrane protein capable of autophosphorylation (16). Interestingly, while all *E. coli* strains possess the *etk* gene, it is only expressed by a subset of pathogenic strains.

With these examples as precedents for cytoplasmic membrane proteins serving as virulence factors, we are currently investigating whether IalB functions as a transporter or signal transduction protein. To date, database searches for proteins with homology to IalB have not suggested any function. This lack of homology to known proteins may reflect IalB's unique and unusual role in erythrocyte parasitism by *B. bacilliformis*.

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