Characterization of a Two-Gene Locus from *Bartonella bacilliformis* Associated with the Ability To Invade Human Erythrocytes

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Bartonella bacilliformis, the agent of human Oroya fever, invades erythrocytes and causes a severe hemolytic anemia. The ability of two minimally invasive strains of *Escherichia coli* (DH5 α and HB101) to invade human erythrocytes was enhanced 6- to 39-fold by transformation with pIAL1, a plasmid containing a 1,469-bp *Bam*HI fragment from the *B. bacilliformis* chromosome. Invasiveness was confirmed by gentamicin protection and transmission electron microscopy. DNA hybridization analysis confirmed the presence of the locus in *B. bacilliformis* KC583 and KC584 and its absence in *E. coli* chromosomal DNA. Sequencing of the DNA insert of pIAL1 revealed tandem open reading frames of 510 and 558 bp, designated *ialA* and *ialB*, respectively. Invasion assays with *E. coli* containing only an *ialA* or *ialB* recombinant suggest that both genes are necessary for invasiveness. The *ialA* gene is predicted to code for a polypeptide of 170 amino acids (20.1 kDa), and *ialB* is predicted to code for a polypeptide of 186 amino acids (19.9 kDa). In vitro transcription and translation of pIAL1 produced insert-specific protein bands with masses of approximately 21 and 20 kDa when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Expression of *ialA* and *ialB* in *E. coli* maxicells produced proteins with masses of approximately 21 kDa (IalA) and 18 kDa (IalB). Maxicell and computer analyses suggest that IalB contains an N-terminal secretory signal sequence which is posttranslationally cleaved. Searches of various DNA and protein databases revealed that IalA contains an N-terminal region of 35 amino acids with a high degree of homology to an NTPase consensus domain. There is 63.6% sequence conservation between the IalB protein and the invasion-associated protein Ail of *Yersinia enterocolitica*.

Bartonella bacilliformis is an arthropod-borne intracellular parasite of human erythrocytes and endothelial cells (40). Bartonellosis is manifested by two distinct phases: a primary stage characterized by a severe hemolytic anemia, known as Oroya fever (Carrion's disease), followed 4 to 8 weeks later by a secondary phase which presents with hemangiomas on the face, neck, and extremities (20, 48). The insect vectors of bartonellae are nocturnal sand flies of the genera *Phlebotomus* and *Lutzomyia* (20, 23, 57). Because of the limited range of the vector, bartonellosis is restricted to the western side of the Andes Mountains in South America. In areas of endemicity, 60% or more of the indigenous population are seropositive for the bacterium (27), and 5 to 10% of the population are active carriers (29). The primary phase of the disease was fatal in approximately 40% of the cases prior to the availability of modern antibiotics (9). Bartonellosis is currently treated with penicillin, chloramphenicol, or tetracycline (20, 22, 57), but delayed diagnosis resulted in the deaths of 14 Peruvian villagers (88% fatality rate for untreated cases) in 1987 (22).

During the primary (hematic) phase of bartonellosis, upwards of 80% of the circulating erythrocytes may be parasitized and lysed (24, 40). The resultant decrease in erythrocytes (from 5 million/mm³ to less than 1 million/mm³) is accompanied by malaise, fever, and enlargement of the organs in the reticuloendothelial system (20). The acute hematic phase is striking because it leads to a type of acquired immune deficiency in which the afflicted person is susceptible to infectious diseases such as salmonellosis, shigellosis, toxoplasmosis, and tuberculosis (9, 20). Following resolution of the primary phase, the secondary clinical syndrome (verruca peruana) may occur for weeks or even months. The hemangiomas occurring in the

secondary phase are thought to be caused by proliferation of vascular endothelial cells, possibly induced by an angiogenic factor produced by the pathogen (18). This phase usually is not fatal and typically is self-resolving.

The virulence factors and pathogenic mechanisms of *B. bacilliformis* are poorly understood. No other bacterial pathogen demonstrates such a strong propensity to invade human erythrocytes. Most intracellular bacterial pathogens use actin-dependent endocytosis by the host cell during invasion (15). However, because erythrocytes lack the complex actin-based cytoplasmic structure of endocytotic cells, the method by which *B. bacilliformis* invades erythrocytes is still unclear. Many previous studies have focused on describing the invasion of *B. bacilliformis* into endocytotic endothelial (19) or epithelial (31, 33, 38, 39) cells. In endothelial cell invasion, *B. bacilliformis* appears to enter into host cells via small, membrane-bound inclusions (19). Raji et al. have partially characterized a 6.2-kb *Bam*HI-*Bam*HI DNA fragment and a 2.0-kb *Bgl*II-*Bam*HI DNA fragment which are implicated in the invasion of *B. bacilliformis* into epithelial cells (38, 39). More recently, they have characterized epithelial and endothelial invasion and suggested that the process allows bartonellae entry into epithelial cells by a microfilament-dependent process similar to phagocytosis, although they have not yet identified specific genes or protein products which cause such an effect (31). This invasive process can be blocked by inhibiting actin synthesis with cytochalasin D (6). Previous investigators have also described a number of other potential virulence factors for the bacterium, including flagellar motility (5, 45), bacterial adhesion to erythrocytic surface glycolipids via bartonella proteins with sizes of 45 and 80 kDa (2, 56), and secretion of a 67-kDa deformation factor which causes the erythrocyte cytoplasmic membrane to invaginate even in the absence of bacteria (33, 58). In this study, we describe the cloning and characterization of two closely linked genes which together confer the ability to invade

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human erythrocytes to minimally invasive strains of *Escherichia coli*, DH5 α and HB101.

MATERIALS AND METHODS

Bacterial strains and culture media. *B. bacilliformis* KC583 and KC584 were purchased from the American Type Culture Collection, Rockville, Md. *B. bacilliformis* was grown on heart infusion agar (Difco Laboratories, Detroit, Mich.) supplemented with 5% defibrinated sheep erythrocytes and 5% filter-sterilized sheep serum by volume (Colorado Serum Co., Denver, Colo.) at 30°C in a water-saturated atmosphere. *B. bacilliformis* cells were harvested after 5 days of growth for all experiments. *E. coli* DH5 α or HB101 was used in invasion assays and cloning experiments. *E. coli* HB101 was used for maxicell analyses. *E. coli* strains were typically grown overnight in Luria-Bertani broth (LB) at 37°C with constant shaking. LB medium was supplemented with 100 μ g of ampicillin per ml when needed.

DNA isolation and manipulations. Chromosomal DNA was isolated from *B. bacilliformis* by the methods of Ausubel et al. (3). A *Bam*HI genomic DNA library of *B. bacilliformis* KC583 was prepared in pUC19 (59) by a standard protocol (42). Cloning of individual DNA fragments was accomplished by purifying DNA from ethidium-bromide-stained agarose gels with a GeneClean kit as recommended by the manufacturer (Bio 101, La Jolla, Calif.), followed by ligation into pUC19 by a standard protocol (42). Transformation of *E. coli* DH5 α or HB101 with pUC19 recombinants was accomplished by the method of Chung et al. (8). Large-scale isolations of purified plasmids were accomplished through the use of a Qiagen (Chatsworth, Calif.) midi-preparation kit, per the manufacturer's instructions.

Invasion assays. Gentamicin protection assays were done by a modification of the method described by Isberg et al. (25, 26). *E. coli* DH5 α or HB101 containing plasmids was grown overnight in LB plus 100 μ g of ampicillin per ml at 37°C with constant shaking. The overnight culture (1 ml) was used to inoculate 20 ml of LB-ampicillin and grown for 2 h at 37°C with shaking. The cultures were centrifuged at 4°C at 4,340 $\times$ g for 5 min, the supernatant was discarded, and the bacterial pellet was resuspended in 1.5 ml of Dulbecco's modified Eagle medium (DMEM; Sigma Chemical Co., St. Louis, Mo.) and placed on ice. Bacterial concentrations were ascertained by using a Petroff-Hauser counter. Recently outdated human erythrocytes (American Red Cross) were prepared by washing 0.1 ml of erythrocytes in 0.95 ml of sterile phosphate-buffered saline–10 mM glucose–5 mM inosine (PBS-GI [pH 7.4]). Erythrocytes were gently pipetted and centrifuged for 20 s at 1,310 $\times$ g in a microcentrifuge, and the supernatant was discarded. The erythrocyte pellet was then resuspended in 0.95 ml of DMEM at 25°C. Erythrocyte concentrations were determined by hemocytometer counts.

Invasion assays were carried out in 1.5-ml microcentrifuge tubes. Sufficient erythrocytes were added to approximately 2.5×10^8 bacteria to yield a multiplicity of infection (MOI) of 5:1. Additional experiments were done at MOIs of 50:1 and 500:1 by keeping the number of *E. coli* HB101 cells constant (2.5×10^8 bacteria) and decreasing the number of erythrocytes. Total volumes were then adjusted to 0.5 ml by the addition of DMEM. The tubes were placed in a 37°C water bath for 1 h, centrifuged for 2 min at 1,310 $\times$ g to closely associate the bacteria and erythrocytes, and then incubated for 2 h at 37°C. Samples were then centrifuged for 2 min at 1,310 $\times$ g, the supernatants were discarded, and the pellets were then resuspended in 0.5 ml of PBS-GI. Samples were centrifuged again for 2 min at 1,310 $\times$ g and then resuspended in 0.5 ml of DMEM containing 200 μ g of gentamicin (Sigma) per ml. The tubes were immediately returned to a 37°C water bath, incubated for 1 h, and centrifuged for 2 min at 1,310 $\times$ g. The supernatants were removed, and the pellets were resuspended for a second time in 0.5 ml of fresh DMEM containing 200 μ g of gentamicin per ml for 1 h at 37°C. Samples were then washed twice in PBS-GI, and the final pellets were resuspended in 0.1 ml of deionized water to lyse the erythrocytes. The lysates were plated onto LB plates containing 100 μ g of ampicillin per ml and grown overnight at 37°C. Colonies were counted the following morning.

The effective killing dose of gentamicin in invasion assays was empirically determined as 150 μ g/ml by subjecting samples of 2.5×10^8 bacteria to increasing concentrations of the antibiotic for 2 h until killing was clearly established (data not shown). In addition, the sensitivity of all strains to gentamicin was verified by attempting growth in DMEM or LB containing only 15 μ g of gentamicin per ml (1/10th the effective killing concentration) by incubation for 24 h at 37°C with shaking.

Invasion data for native *B. bacilliformis* KC584 were obtained (46) by using a similar gentamicin protection assay, which has been previously described (45).

DNA hybridization analysis. Total DNA from *E. coli* DH5 α and *B. bacilliformis* KC583 and KC584 and pIAL1 plasmid DNA were isolated, digested to completion with *Bam*HI, and then separated on an ethidium bromide-stained 1% agarose (wt/vol) gel. The gel was then blotted to nitrocellulose (0.45- μ m-pore-size filter; Schleicher & Schuell, Keene, N.H.) by the method of Southern (50) and baked for 1 h at 80°C. The *Bam*HI insert of pIAL1 was purified from agarose gels with GeneClean (Bio 101) and labeled by random primer extension (16) with [α -³²P]dCTP (New England Nuclear, Boston, Mass.) and the Klenow fragment of *E. coli* polymerase I (Gibco-BRL, Gaithersburg, Md.). The blot was probed overnight at 50°C with the ³²P-labeled pIAL1 insert and washed at high stringency (approximately 7% DNA mismatch), as previously described (36).

FIG. 1. Detection of pIAL1 DNA insert in *B. bacilliformis* chromosome by DNA hybridization. (A) Ethidium bromide-stained agarose gel (1% [wt/vol]) containing λ HindIII DNA size standard (lane 1), *Bam*HI-digested chromosomal DNA of *B. bacilliformis* (*B. b.*) KC583 (lane 2), *Bam*HI-digested chromosomal DNA of *B. bacilliformis* KC584 (lane 3), *Bam*HI-digested chromosomal DNA of *E. coli* DH5 α (lane 4), no sample (lane 5), and *Bam*HI-digested pIAL1 (lane 6). (B) Corresponding autoradiograph following DNA hybridization with ³²P-labeled pIAL1 DNA insert. The arrow points to the comigrating 1.5-kb *Bam*HI fragment producing the hybridization signals. Lanes: same as for panel A.

The blot was subsequently exposed for 1 h to X-ray film (X-Omat XAR-5; Eastman Kodak Co., Rochester, N.Y.) to visualize hybridized DNA fragments.

Nucleotide sequencing and transcript analysis. The nucleotide sequence for the *Bam*HI insert of pIAL1 was obtained from both DNA strands by the methods of Sanger et al. (44), using a model 373A automated nucleic acid sequencer (Applied Biosystems, Foster City, Calif.). In addition to M13 universal forward and reverse primers, internal sequencing primers were synthesized by using a model 394 DNA synthesizer (Applied Biosystems). Computer analyses were performed by using PCGENE 6.8 (Intelligentics, Mountain View, Calif.) and the National Center for Biotechnology Information's sequence-homology search program, BLAST (1). The transcriptional start sites of *ialA* and *ialB* were also determined. Total RNA was isolated from bartonellae by the diethyl pyrocarbonate method described by Summers (52). Primers complementary to approximately the +70 region of the sense strand for each gene (see Fig. 3) were synthesized. Three picomoles of each 15-mer was mixed with 15 μ g of purified RNA in 3 μ l of hybridization buffer (100 mM KCl, 50 mM Tris-HCl [pH 8.3]), and the mixture was then denatured for 5 min at 90°C. The primers were allowed to anneal to the RNA for 10 min at 42°C, and the mixture was placed on ice for 15 min. Then, 1.8 μ l of 5 $\times$ reverse transcriptase buffer (250 mM Tris-HCl [pH 8.3]; 200 mM KCl; 15 mM MgCl₂; 10 mM dithiothreitol; 1 mM [each] dATP, dGTP, and dTTP), 1 μ l of [α -³²P]dCTP (New England Nuclear), and 1.5 μ l (300 U) of RNase H-free reverse transcriptase (Promega, Madison, Wis.) were added and incubated for 60 min at 37°C to obtain cDNA. The reactions were stopped by adding one volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). The mixture was heated for 2 min at 90°C. A 4- μ l aliquot was then run adjacent to a sequencing reaction mixture obtained by using a Sequenase 2.0 kit (U.S. Biochemical, Cleveland, Ohio) and α -³⁵S-dATP (New England Nuclear) with the same primers and pIAL1 DNA.

Gene expression. In vitro transcription and translation of pUC19 and pIAL1 purified by using the kit were performed by using a prokaryotic DNA transcription-translation kit per the manufacturer's instructions (Amersham Co., Arlington Heights, Ill.). Proteins were labeled with a [³⁵S]methionine-cysteine mix (Express; New England Nuclear), denatured by resuspension in Laemmli sample buffer (LSB) (30), boiled for 10 min, and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12.5% acrylamide [wt/vol]). In vivo expression of genes from pIAL1, as well as from pIALA and pIALB (containing only recombinant *ialA* and *ialB*, respectively; see Fig. 2), was accomplished in *E. coli* HB101 by the methods of Sancar et al. (43), using a 50-s UV irradiation of 195 μ W/cm² at a 15-cm exposure distance. The ³⁵S-labeled maxicells were either pelleted and resuspended in 40 μ l of LSB or pelleted and immediately subjected to protease accessibility analysis prior to LSB denaturation and SDS-PAGE.

Extrinsic protease accessibility of the plasmid-encoded gene products was ascertained by subjecting the fresh *E. coli* maxicells to trypsin or proteinase K treatment (26). Labeled maxicells were washed in 0.5 ml of fresh Dulbecco's PBS (14), briefly microcentrifuged, and then resuspended in 0.5 ml of Dulbecco's

FIG. 2. Partial restriction endonuclease map of pIAL1, pIALA, and pIALB inserts. Positions of the *ialA* and *ialB* ORFs are indicated by the boldface arrows. The arrowheads indicate the direction of the *lacZ'* promoter in the cloning vector, pUC19. Subclones pIALA and pIALB are indicated below the respective restriction sites from pIAL1 which were used in their construction. Dotted lines indicate portions of the pUC19 vector used to construct the pIALA and pIALB subclones from pIAL1 as described in Results.

PBS. Trypsin (Sigma) was added to a final concentration of 25 $\mu\text{g/ml}$, and the sample was incubated at 37°C for 30 min. Phenylmethylsulfonyl fluoride (PMSF; Sigma) in 95% ethanol was added to a final concentration of 25 $\mu\text{g/ml}$, mixed, and then centrifuged for 6 min at 1,440 $\times g$. The supernatant was discarded, and then the pellet was washed in PBS containing PMSF (25 $\mu\text{g/ml}$), briefly microcentrifuged, and resuspended in 1 volume of LSB containing 50 μg of PMSF per ml. A similar treatment was performed with 0.4 mg of proteinase K (Sigma) per ml for 40 min. Samples were boiled for 10 min prior to analysis by SDS-PAGE. Ten micrograms of a five-protein mixture of molecular mass standards (Sigma) was also subjected to identical trypsin or proteinase K treatment and analyzed by SDS-PAGE to verify protease activity.

Association assay. The ability of *E. coli* DH5 α to adhere to erythrocytes was compared with that of *E. coli* DH5 α containing the plasmids pUC19, pIAL1, pIALA, and pIALB (see Fig. 2) by previously described methods (5, 45). Briefly, 1.5-ml density-gradient separation tubes were prepared by centrifuging 1.0 ml of 70% Percoll (Sigma) containing 0.154 M NaCl for 15 min at 10,000 $\times g$ at 4°C. Following a 3-h invasion incubation, 100 μl of each invasion assay mixture was layered onto individual gradients and centrifuged for 5 min at 1,500 $\times g$ at 4°C. The density gradient separated the invasion assay mixture into three clearly defined layers: the top layer contained free bacteria, the middle band contained erythrocytes with adherent bacteria, and the unbound erythrocytes pelleted to the bottom of the tube. The middle band was carefully removed with a sterile 1.0-ml syringe with an 18-gauge needle and gently washed twice in PBS-GI. After brief microcentrifugation, the final pellet was resuspended in 35 μl of PBS-GI, spread onto a glass slide, and stained with a modified Giemsa stain (Stat-Stain; VWR Scientific, West Chester, Pa.). Association was determined by scoring 1,000 erythrocytes for bound bacteria. Results are the means $\pm$ standard errors of the means for three independent samples.

Strain stability. Stability of the DH5 α (pIAL1) clone under the invasion assay conditions was determined by subjecting the strain to three sequential invasion assays and subsequently verifying the presence of the IAL1 insert by digestion with *Bam*HI and separation on an ethidium bromide-stained 1% agarose (wt/vol) gel. Stability of *E. coli* strains containing pUC19 and the pIAL1, pIALA, and pIALB recombinants was verified under ampicillin selection by at least five sequential passages of individual clones on LB-ampicillin (100 $\mu\text{g/ml}$) plates, followed by identification of the DNA insert by restriction endonuclease digestion and electrophoretic separation on agarose gels.

A growth assay was carried out with all strains of *E. coli* by using the same protocol as the invasion assay but stopping prior to the gentamicin treatment step. Aliquots (10 μl) were removed from three independent samples immediately before and after the 3-h invasion period, serially diluted in PBS-GI, resuspended in 90 μl of distilled H₂O to lyse any invaded erythrocytes, and plated onto selective media. The samples were incubated overnight at 37°C, and colonies were counted the following morning.

Transmission electron microscopy. *E. coli* DH5 α containing pIAL1 or pUC19 was allowed to invade human erythrocytes for 3 h as described above for the invasion assay. Samples were then centrifuged for 2 min at 1,310 $\times g$ in a microcentrifuge at room temperature. The pellet was resuspended in 2% glutaraldehyde in PBS (pH 7.4) for 2 to 12 h at 4°C. Samples were then rinsed in PBS, postfixed in 1% (wt/vol) osmium tetroxide, rinsed in distilled water, and preembedded in agar. The samples were then dehydrated via an alcohol series (50 to 100% ethanol at 10 min per step, 25°C) and subjected to a 5-min treatment with 50% (vol/vol) ethanol-propylene oxide and a final 5-min treatment with 100% propylene oxide. The samples were embedded in Polybed 812 (Polyscience, Warrington, Pa.) (24 h of polymerization at 60°C) and divided by ultramicrotome sectioning. Sections were recovered onto 300-mesh copper-palladium grids and stained with uranyl acetate and lead citrate. Transmission electron microscopy was performed with a model 7100 microscope (Hitachi, Mountain View, Calif.) at 75 kV.

Statistical analysis. Numerical data are reported (except as noted) as the means for three independent samples $\pm$ standard errors of the means. Invasion assays were conducted at least four times (depending on the plasmid and/or *E. coli* strain [DH5 α or HB101]), with consistent patterns of invasiveness. Statistical significance of the data was ascertained by use of Student's *t* test. A value of *P* of <0.05 was considered significant.

Nucleotide sequence accession number. The GenBank accession number for the nucleic acid sequence reported in Fig. 3 is L25276.

RESULTS

Isolation of invasive clones. To determine which gene(s) might contribute to the ability of *B. bacilliformis* to invade nonendocytotic host cells such as erythrocytes, a modified gentamicin invasion assay was designed. A pUC19 genomic library of random *Bam*HI fragments from *B. bacilliformis* KC583 was constructed in *E. coli* DH5 α and tested for clones which were able to invade human erythrocytes in vitro. Bacteria were combined with erythrocytes for 3 h and then treated twice with gentamicin for 1 h. Since gentamicin cannot effectively enter eukaryotic cells, bacteria which had successfully invaded erythrocytes escaped the antibiotic, whereas extracellular bacteria were killed. Initial screening of the *Bam*HI chromosomal library of *B. bacilliformis* KC583 (approximately 750 clones) identified three *E. coli* clones which had escaped the gentamicin treatment. In comparison to the control strain DH5 α (pUC19), one clone exhibited a 2-fold increase in invasiveness (data not shown), and two clones demonstrated an increase in invasiveness of approximately 7- to 10-fold. Further analysis revealed that the two most invasive *E. coli* clones contained the same recombinant, subsequently named pIAL1 (invasion-associated locus clone 1).

DNA hybridization analysis. DNA hybridizations at high stringency were done to confirm that the pIAL1 insert originated from *B. bacilliformis* (Fig. 1). Southern blots probed with the ³²P-labeled pIAL1 insert produced hybridization signals in the *Bam*HI-digested chromosomal DNA of two strains of *B. bacilliformis* (strains KC583 and KC584) that comigrated with the pIAL1 *Bam*HI insert (Fig. 1B, lanes 2 and 3). No signal was observed in *Bam*HI-digested chromosomal DNA from *E. coli* DH5 α or in the 2.7-kb pUC19 DNA band (Fig. 1B, lanes 4 and 6, respectively).

Nucleotide sequence and analysis. The *B. bacilliformis*-derived *Bam*HI insert of pIAL1 was mapped with a number of restriction endonucleases (Fig. 2). Subsequent nucleotide sequencing revealed that the 1,469-bp fragment contained two tandem open reading frames (ORFs) separated by an intergenic region of 116 nucleotides (Fig. 2 and 3) and had a 36.4-mol% G+C content, in close agreement with the 39-

FIG. 3. Nucleotide sequence of 1,469-bp *Bam*HI insert of pIAL1, containing *ialA*, *ialB*, and flanking regions. The deduced amino acid sequence is shown above the first base of its respective triplet codon. The residues comprising the predicted secretory signal sequence of *IalB* are shown in italics, with the putative signal peptidase cleavage site indicated by a diamond. Putative -35 and -10 promoter hexamers and the potential RBS are shown for each ORF. Nucleotides within ORFs are given in uppercase letters. The transcriptional start sites determined by primer extension analysis are marked by downward-pointing arrowheads. Closed, single-sided arrows underline the sequences complementary to the 15-mer oligodeoxynucleotides used in the primer extension analysis. The single-sided arrows above the sequence indicate inverted repeats which may serve as ρ -dependent termination signals. The stop codon (taa) following each ORF is marked with an asterisk.

mol% G+C content of *B. bacilliformis* KC583 (7). Both ORFs are oriented in opposition to the *lacZ'* promoter on the pUC19 cloning vector, and both have potential endogenous promoter sites. The first and second ORFs were designated *ialA* and *ialB* for invasion-associated loci A and B, respectively. Recombinants containing either *ialA* or *ialB* were constructed and named pIALA or pIALB, respectively (Fig. 2). pIALA was produced by enzymatic removal of the 604-bp *Pst*I fragment of pIAL1 and religation, while pIALB was produced by enzymatic removal of the 710-bp *Sma*I-*Swa*I fragment of pIAL1 and religation (Fig. 2).

The *ialA* gene extends from the AUG translational start codon at nucleotide 198 to the UAA stop codon at nucleotide 708 (Fig. 3). Analysis of the region 5' to the start codon revealed a putative promoter region containing -35 (TTAAAA)

Organism	Protein	Sequence
<i>B. bacilliformis</i>	IalA	47-WQFPGGGIDEGEPLDAARRELYEETGMRSVNLIK
<i>E. coli</i>	MutT	33-LEPPGGKIEMGTPEQAVVRELOEVEVGITPQHPSSL
<i>X. laevis</i>	antiFGF	72-WKPPGGLSDQGDIGATAVREVLVEETGTHSEFKSL
Vaccinia virus	Prot. D10	121-AIYPGGIPKRGVNEPECLSRREIKBEVNIDNSFVFI
consensus		G E * R E E

FIG. 4. Alignment of *B. bacilliformis* protein IalA with 35-amino-acid residues containing NTPase consensus region of proteins from *E. coli*, *Xenopus laevis*, and vaccinia virus (28). Positions of N-terminal residues relative to mature proteins are noted prior to each polypeptide sequence, and consensus amino acid residues are noted in boldface type beneath the sequences. Solid lines denote identities between residues, and double dots indicate similar residues between two neighboring sequences. The symbol * denotes alanine or a bulky aliphatic residue, whereas the & symbol indicates a bulky hydrophobic residue. Sequence accession numbers are GenBank L25276 for *B. bacilliformis*, SWISSPROT P08337 for *E. coli*, SWISSPROT P13420 for *X. laevis*, and SWISSPROT P21012 for vaccinia virus.

and -10 (TATGTC) hexamer sequences, which are separated by 17 bases and are similar to the consensus sequences from *E. coli* (32). In addition, a polypurine-rich sequence for the putative ribosomal binding site (RBS) (AGAAG) (21, 51) was found 7 nucleotides upstream of the start codon. Mapping of the putative promoter by primer extension analysis (data not shown) from total RNA of bartonellae revealed an apparent transcriptional start site at nucleotide 171 of the *Bam*HI insert, 14 bases upstream of the RBS and 16 bases downstream of the -10 hexamer (Fig. 3). A 6-nucleotide inverted repeat was found 22 nucleotides downstream of the stop codon and may serve as a ρ -dependent transcription terminator (41).

Computer analysis using the search algorithm BLAST (1) revealed no significant homologies between *ialA* and any nucleic acid sequences in the available databases. However, the predicted protein, IalA (170 amino acids, predicted M_r of 20,100), contained a region with a high degree of homology to a 35-residue consensus sequence for a conserved motif of a putative NTPase catalytic core (Fig. 4) (28). The NTPase-homologous region of IalA also exhibited a predicted secondary structure (loop- α -helix-loop) similar to the residues comprising the NTPase consensus domain (28). IalA had little homology to regions flanking the NTPase consensus region in any of the aligned proteins (data not shown). Further computer analyses indicated that IalA contains alternating hydrophobic and hydrophilic regions and may be a globular protein with an isoelectric point of 5.49.

The *ialB* gene spans 558 bases, from an AUG start codon at nucleotide 827 of the *Bam*HI fragment to a UAA stop codon at nucleotide 1385 (Fig. 3). Examination of the region 5' to the start codon revealed a potential promoter region containing -35 (TTGTGA) and -10 (TATTAT) hexamer sequences similar to the *E. coli* consensus hexamers (32) and separated by 33 bases. The potential promoter region of *ialB* overlaps the putative transcription-termination region of *ialA*. A polypurine-rich sequence which may serve as the RBS (AGGA) was found 12 bp upstream of the start codon (21, 51). Primer extension analysis (data not shown) indicated an apparent transcriptional start site at nucleotide 777 of the *Bam*HI insert, 33 bases upstream of the RBS and 8 bp downstream of the -10 promoter hexamer (Fig. 3). The stop codon (UAA) is followed 12 bases downstream by a 7-nucleotide inverted repeat which may act as a ρ -dependent transcriptional terminator (41).

Searches of the available databases by using the BLAST program did not reveal any significant DNA homologies to *ialB*; however, the encoded protein, IalB (186 amino acids, predicted M_r of 19,900), contained 63.6% sequence conservation (Fig. 5) and an M_r similar to that of the Ail protein of

FIG. 7. Invasiveness of transformed *E. coli* DH5 α (A) and HB101 (B) into human erythrocytes in gentamicin protection assays. *E. coli* containing pUC19, pIAL1, pIALA, or pIALB was allowed to invade erythrocytes as described in Materials and Methods. The pIAL1(-) data in were obtained from mock assays with *E. coli* (pIAL1) in the absence of erythrocytes to verify the efficacy of the gentamicin killing in the invasion assay. The values are means $\pm$ standard errors of the means for three independent samples of a representative experiment.

(pUC19) or DH5 α (pIAL1), DH5 α containing pIALA or pIALB alone did not exhibit significant invasive ability and resulted in a mean of 15 ± 8 or 91 ± 15 CFU, respectively (Fig. 7A). The invasive ability for DH5 α (pIAL1) ranged from 6- to 39-fold over that for DH5 α (pUC19) for seven separate experiments, all repeated in triplicate.

HB101 (pIAL1) also invaded human erythrocytes at a significantly ($P < 0.05$) higher level than that of the control, HB101 (pUC19) (mean CFU of 636 ± 34 versus 74 ± 16 , respectively) (Fig. 7B). As with DH5 α , the subclone HB101 (pIALA) did not exhibit significant ($P > 0.05$) invasive ability (mean CFU, 4 ± 1) compared with HB101 (pUC19). HB101 (pIALB) did exhibit a slight, but not significant, increase in invasiveness (mean CFU, 243 ± 10) compared with HB101 (pUC19). The invasive ability of HB101 (pIAL1) ranged from 6- to 24-fold higher than that of HB101 (pUC19) for four separate experiments, all repeated in triplicate.

Because previous invasion assays have shown an inverse relationship of MOI to invasive CFU (12), an experiment to examine the effect of varying the ratio of bacteria to erythrocytes was performed with *E. coli* HB101 (Fig. 8). The results indicate that there were a significant ($P < 0.05$) decrease in invasiveness as the MOI increased from 5 bacteria:1 erythrocyte to 50:1 and a smaller but significant ($P < 0.05$) decrease as the MOI increased to 500:1 (mean CFU, 500 ± 22 versus 154 ± 6 versus 112 ± 9 , respectively).

Association assay. To determine if the invasiveness was simply caused by an increase in the adherence of transformed *E. coli* to erythrocytes followed by the normal, low-level invasive ability of the parent strain, an association assay was performed as previously described (45). Following a 3-h incubation of *E. coli* DH5 α with human erythrocytes at an MOI of 5:1, the samples were separated by Percoll density-gradient centrifugation, plated onto glass slides, stained, and analyzed microscopically. As shown in Fig. 9, there were no significant ($P > 0.05$) differences in the mean erythrocyte-bacterium associations between DH5 α (379 ± 38 CFU), DH5 α (pUC19) (389 ± 24 CFU), DH5 α (pIAL1) (280 ± 38 CFU), and DH5 α (pIALB)

(398 ± 23 CFU). However, DH5 α (pIALA) (111 ± 38 CFU) had a significantly ($P < 0.05$) reduced ability to adhere to erythrocytes compared with the other strains.

Strain stability. The stability of the DH5 α (pIAL1) clone under the conditions of the invasion assay was determined by maintenance of both the invasive phenotype and pIAL1 through three sequential rounds of invasion and gentamicin selection (data not shown). Furthermore, the stability of all recombinant DNA was determined by maintenance of each plasmid through five sequential passages of the strains in LB containing ampicillin (100 μ g/ml) (data not shown).

FIG. 8. Effect of MOI on invasion of erythrocytes by *E. coli* containing pUC19 (■) or pIAL1 (▨). Transformed *E. coli* DH5 α was allowed to invade erythrocytes (as described in Materials and Methods) at MOIs of 5:1, 50:1, and 500:1 (bacteria to erythrocytes). Values represent the means $\pm$ standard errors of the means for three independent samples.

FIG. 9. Association of transformed *E. coli* DH5α with human erythrocytes as measured microscopically following a 3-h association-invasion period and separation by Percoll density-gradient centrifugation. Data are shown for both untransformed *E. coli* DH5α and *E. coli* DH5α containing pUC19, pIAL1, pIALA, or pIALB. Values were obtained by scoring the number of erythrocytes with associated bacteria per 1,000 erythrocytes (RBCs). Values are the means $\pm$ standard errors of the means for three independent samples.

In order to exclude the possibility that the observed increase in invasiveness was actually due to an enhanced growth phenotype of the *E. coli* strains containing the recombinants relative to DH5α (pUC19), a growth assay was carried out. Aliquots from six independent samples were removed before and after a 3-h invasion assay incubation period (prior to gentamicin treatment) and the resulting CFUs were counted on selective media. As shown in Fig. 10, there were no significant ($P < 0.05$) differences between the fold increase of DH5α (2.7 ± 0.2 CFU), DH5α (pUC19) (3.5 ± 0.5 CFU), DH5α (pIAL1) (3.5 ± 0.4 CFU), or DH5α (pIALB) (4.4 ± 0.7 CFU). However,

FIG. 10. Growth of transformed *E. coli* DH5α during an invasion period with human erythrocytes. Data are shown for both untransformed *E. coli* DH5α and *E. coli* DH5α containing pUC19, pIAL1, pIALA, or pIALB. Fold increase represents the increase in CFU for samples taken at the beginning and end of a 3-h invasion period, as described in Materials and Methods. Values represent the means $\pm$ standard errors of the means for six independent samples.

DH5α (pIALA) (16.1 ± 2.3 CFU) did exhibit a significant ($P < 0.05$) increase in its growth phenotype compared with all other strains.

Transmission electron microscopy. To corroborate the gentamicin protection assays, we confirmed the results of our invasion assays and controls by transmission electron microscopy. Following a 3 h-invasion, samples were fixed, embedded, sectioned, and stained. Inspection of the resulting thin sections revealed that DH5α (pIAL1) did indeed penetrate human erythrocytes (Fig. 11A to E). As seen in Fig. 11A, and at a fivefold higher magnification of the same bacterium (Fig. 11B), the membranes of the bacteria and erythrocytes appear to come into very close apposition. Figure 11C shows two bacteria which simultaneously invaded a single erythrocyte; an arrow points to the complementary deformation of the erythrocytic membrane which occurred around one of the invading DH5α (pIAL1) cells. Following invasion, the bacteria occasionally left an apparent trail where they appeared to have entered the erythrocyte (Fig. 11D). There does not appear to be a well-defined vacuolar membrane surrounding a fully enclosed bacterium (Fig. 11E). A small zone of weak staining was observed around intracellular bacteria (Fig. 11E). DH5α containing only the cloning vector DH5α (pUC19) was not observed to invade erythrocytes (Fig. 11F).

DISCUSSION

B. bacilliformis is responsible for one of the most severe hemolytic anemias known to occur in humans. During the primary phase of the disease, bacteria invade nearly all circulating erythrocytes, multiply, and lyse the host cells before starting a new cycle of parasitemia. Many intracellular bacterial pathogens can enter target cells by parasitizing the actin-dependent endocytotic mechanism of the host cell (15). However, erythrocytes lack the actin-based cytoskeletal structure found in most mammalian host cells and are essentially non-endocytotic. Therefore, *B. bacilliformis* must use an unusual mechanism(s) to complete its life cycle. We conducted this study in an attempt to identify a gene(s) which might be responsible for the invasion of human erythrocytes by bartonellae. Because there are no genetic systems available for the direct manipulation of rickettsial genomes, we necessarily relied on indirect observations of gene expression in an alternative host cell, *E. coli*.

We report on the isolation and cloning of a 1,469-bp *Bam*HI fragment from *B. bacilliformis* which confers a statistically significant ($P < 0.05$) invasive phenotype upon two minimally invasive strains of *E. coli*, DH5α and HB101. Both gentamicin protection assays (Fig. 7) and transmission electron microscopy (Fig. 11) confirmed the ability of the pIAL1-containing *E. coli* to invade human erythrocytes. DNA hybridization confirmed the *B. bacilliformis* origin of the pIAL1 DNA insert (Fig. 1), and subsequent nucleic acid sequencing revealed that the insert contained two genes, which we designated *ialA* and *ialB* (Fig. 2 and 3). Both genes are necessary for *E. coli* to demonstrate the invasive phenotype, since clones containing only *ialA* (pIALA) or *ialB* (pIALB) (Fig. 2) were not able to confer significant invasiveness ($P > 0.05$) (Fig. 7). HB101 (pIALB) exhibited a slight, but not significant, increase in invasiveness.

Although pIAL1 conferred a 6- to 39-fold increase in the invasiveness of *E. coli*, depending upon experimental conditions and the host strain, only $[(6 \pm 2) \times 10^{-4}]$ % of the

FIG. 11. Transmission electron micrographs of human erythrocyte invasion by *E. coli* DH5 α (pIAL1). (A) Bacterium beginning invasion of erythrocyte (bar, 1.0 μ m). (B) Higher magnification (fivefold) of panel A, showing close association between bacterium and erythrocyte (bar, 0.25 μ m). (C) Two bacteria simultaneously invading a single erythrocyte. The arrow highlights the complementary invaginations of the erythrocytic membrane to the invading bacteria (bar, 1.0 μ m). (D) Bacterium fully enclosed following invasion. The arrow points to the presumed path of invasion (bar, 1.0 μ m). (E) Higher magnification of fully enclosed DH5 α (pIAL1). Note the lack of a defined vacuole membrane (arrow) (bar, 0.25 μ m). (F) Control micrograph demonstrating typical interaction between human erythrocytes and DH5 α (pUC19) (bar, 1.0 μ m).

original inoculum was invasive. This rate of invasion is low, but several factors suggest that it is biologically relevant. The invasiveness that pIAL1 confers on *E. coli* differs by less than one log from the (5×10^{-3})% level of erythrocyte invasiveness for *B. bacilliformis* determined by this laboratory with the same assay system (46). A similar decrease in invasiveness of 0.5 to 1.5 logs has been described during alternative host expression of invasion-related genes from other pathogenic bacteria, in-

cluding enteropathogenic *E. coli* (49), *Y. enterocolitica* (35), and *Salmonella typhimurium* (17).

In addition to the invasion assays, four other observations lend support to the invasive phenotype of *E. coli* transformed with pIAL1. First, the transmission electron micrographs (Fig. 11) clearly show entry of DH5 α (pIAL1) into erythrocytes. Figures 11A and B demonstrate the complementary invagination of the erythrocyte as a bacterium begins to enter an eryth-

rocyte, and Fig. 11C illustrates the continuing invagination as the bacterium penetrates more deeply. Following entry, *E. coli* is completely enclosed within the erythrocyte (Fig. 11D) and appears to be free within the cytosol without an apparent vacuole membrane (Fig. 11E). Similarly, the lack of a defined vacuole membrane during *B. bacilliformis* invasion of human erythrocytes was also observed by Cuadro and Takano (10). In addition, they made an analogous observation of a clear space surrounding the infecting bacterium, which they attributed to specimen shrinkage during preparation for electron microscopy.

A second supporting observation is the requirement of both genes for effective invasion. *E. coli* transformed with pIALA or pIALB was unable to confer a significant invasive phenotype (Fig. 7). Despite the occasional increase in invasiveness exhibited by *E. coli* HB101 (pIALB) (Fig. 7B), it is apparent that the combination of two genes confers a significantly ($P < 0.05$) greater invasiveness on the host bacterium. The data suggest that the increased invasiveness results from the expression of both genes in the pIAL1 construct, not simply from introducing bartonellae or vector DNA into *E. coli*.

Third, the inverse relationship between MOI and invasiveness (Fig. 8) would probably not occur for a noninvasive phenotype. If the invasiveness was simply due to the normally low level of invasive ability of *E. coli*, an increased MOI would have resulted in a direct increase in CFU. We observed a completely opposite effect; namely, as the MOI increased, the number of CFU decreased. This inverse relationship between MOI and invasiveness has also been reported by Douce et al. (12), whose experiments involving *S. typhimurium* indicated that high numbers of invading bacteria may damage the host cells and allow gentamicin to enter. A decrease in CFU at a high MOI may have occurred because of bacterially induced damage to the erythrocytes and subsequent exposure of the bacteria to gentamicin.

The fourth set of observations which support an invasive phenotype are the results of the stability assays. Phenotypic stability of pIAL1-containing strains was verified by two independent methods. The growth assay (Fig. 10) indicated that there were no significant ($P > 0.05$) differences in CFU increases during the 3-h invasion assay incubation between DH5 α (pIAL1) and the control strains DH5 α and DH5 α (pUC19) (fold increases of 3.5 ± 0.4 , 2.7 ± 0.2 , and 3.5 ± 0.5 , respectively). Therefore, we believe that the 6- to 39-fold increase in invasiveness of pIAL1-containing strains was not an artifact resulting from an enhanced growth rate during the invasion assay.

The gentamicin-based protection assay may not yield high numbers of CFU for erythrocyte-invading pathogens for several reasons. First, unlike our use of erythrocyte host cells, previous work has relied almost exclusively on adherent monolayers of host cells. Prior to application of the gentamicin, three to six washes generally remove the vast majority of non-invading or nonadherent bacteria. In contrast, our method requires centrifugation of both erythrocytes and bacteria to prevent loss of invaded erythrocytes, which results in nearly all of the bacteria remaining in the 0.5-ml reaction mixture volume. Therefore, we were forced to use an empirically determined level of gentamicin (200 μ g/ml), which is much higher than that typically used in monolayer-based assays (≤ 50 μ g/ml) (13). This high level of gentamicin may cause some leakage into erythrocytes and thus decrease the final CFU values (an effect recently reported by Drevets et al. [13]). We believe that the requirement for high gentamicin levels was due to the observed decrease in pH during the course of an invasion experiment (presumably caused by bacterial metabolism). Dre-

vets et al. reported that a decrease in pH from 6.0 to 5.5 resulted in gentamicin becoming bacteriostatic for *Listeria monocytogenes* (13). This bacteriostatic conversion of gentamicin, in addition to the low-level native invasiveness of *E. coli*, may be responsible for the few control bacteria which escaped gentamicin treatment (Fig. 7). Although this might also have allowed some pIAL1-containing bacteria to escape the gentamicin, the results of the growth assay (Fig. 10) indicated that the number of escapees due to gentamicin bacteriostasis would be similar for both DH5 α (pIAL1) and DH5 α (pUC19).

A second major difference between this study's invasion assay and that used by other investigators is the use of a nonendocytotic host cell. Most pathogenic bacteria appear to parasitize the actin-based cytoskeletal structure of their host cells, which are invariably capable of endocytosis. Endocytosis does not normally occur in mature erythrocytes, as they lack extensive actin networks. Therefore, any genes introduced from *B. bacilliformis* must be able to cause penetration of the erythrocyte by the nonmotile host *E. coli* (DH5 α or HB101) or induce endocytosis by unknown methods. In either case, it does not seem unreasonable to assume that the bartonella mechanism may be inefficient when expressed in *E. coli*, as even the closely related enteropathogenic *E. coli* strains rely upon host-cell endocytosis for entry (11).

Lastly, we note that the gentamicin protection assay can be difficult to use for exact quantitation of invasiveness. This is perhaps best summed up by Douce et al. (12), who observed that "the pattern of comparative strain behavior, but not absolute invasion values, was highly reproducible on different days and in different hands." Despite the caveats listed above, we believe that the assay method provides reproducible and statistically significant results.

The exact mechanisms by which the predicted IalA and IalB proteins function remain unclear. One possible function of the *ialA* and/or *ialB* gene is the expression of protein(s) responsible for close adherence to the erythrocyte, followed by an unknown mechanism which results in internalization of the bacterium. However, as shown in Fig. 9, it seems unlikely that pIALA or pIALB significantly contributes to any increased ability in *E. coli* DH5 α adherence. Interestingly, *ialB* may partially offset the decreased association apparently caused by *ialA*, as the combination of the *ialA* and *ialB* genes in pIAL1 results in association at a level intermediate between that of the two strains containing pIALA or pIALB (Fig. 9). The decreased erythrocyte association observed with DH5 α (pIALA) (Fig. 9) may be related to this strain's aberrantly high growth rate under conditions of the invasion assay. Relative to all other strains tested, DH5 α (pIALA) produced approximately sixfold more CFU during the 3-h incubation period (Fig. 10). Reasons for the enhanced growth rate of the strain are unclear and are presently under investigation.

A second possibility is that IalA and/or IalB might induce erythrocyte endocytosis. Tsang et al. (53) have described entrapment of proteins, viruses, bacteria, and DNA during a form of erythrocytic endocytosis. Although erythrocytes lack the normal actin-based structures employed by most cells during endocytosis, Schrier et al. (47) have described experiments in which endocytosis was induced in sealed human erythrocyte ghosts by activation of a membrane-associated Ca,Mg-ATPase. Data suggest that energy for the process was derived from Mg-ATP and that activation of the Ca,Mg-ATPase was accompanied by a Ca^{2+} efflux. In light of ATPase-mediated erythrocytic endocytosis, the observation that IalA contains a 35-residue sequence homologous to an NTPase catalytic core consensus motif (28) warrants further investigation (Fig. 4).

IalB physically resembles Ail, a protein encoded by the

650-bp *ail* gene of *Y. enterocolitica* (35). Comparison of the IalB and Ail protein sequences revealed 63.6% sequence conservation (Fig. 5). The *ail* gene encodes a nascent protein with a size of 17 kDa which contains a 23-residue N-terminal signal sequence. The protein is subsequently exported to the cell surface and modified to yield a mature protein with a size of 15 kDa (34). IalB also contains a (predicted) signal sequence, with an apparent N-terminal peptidase cleavage resulting in reduction of the nascent 20-kDa protein (Fig. 6A, lane 2) to approximately 18 kDa (Fig. 6B, lane 3). The *ail* (attachment invasion locus) gene was originally described as transforming minimally invasive *E. coli* into a strongly adherent and invasive bacterium (34). Miller et al. (34) initially reported that Ail may act in a manner similar to afimbrial adhesins but that it does not appear to form pilus-like structures on the bacterial surface. More recently, it has been demonstrated that *ail* may protect *Y. enterocolitica* against serum killing (37) but that its adherence function is not completely understood and may be much weaker than originally believed (4, 54). Although the exact mechanism by which Ail functions remains unclear, a similar invasive effect appears to arise from expression of pIAL1-encoded genes in *E. coli*.

Previous research has identified a number of determinants which are potentially involved in the pathogenesis of *B. bacilliformis*. Motility (5, 45, 56), deformation of erythrocytes (5, 33, 58), and other putative adherence and invasive factors (2, 31, 38, 39) all mediate the pathogenic properties of this bacterium. Other researchers have described epithelial cell invasion by *E. coli* containing a cloned 6.2-kb *Bam*HI-*Bam*HI *B. bacilliformis* DNA fragment (38) and, more recently, a 2.0-kb *Bam*HI-*Bgl*II fragment (39). Unfortunately, until more data become available for these clones, it is difficult to integrate the available information into a cohesive model for invasion. Although we cannot yet assign a definitive role for either *ialA* or *ialB*, it is apparent that they may facilitate invasion in the absence of the previously described bartonella virulence factors. The role of the protein product from each gene in bartonellae is currently under investigation through the use of monospecific antibodies and mutagenesis. In addition, genes which flank *ialA* and *ialB* are being cloned and characterized.

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