Citrobacter rodentium espB Is Necessary for Signal Transduction and for Infection of Laboratory Mice

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Citrobacter rodentium is the causative agent of transmissible murine colonic hyperplasia and contains a locus of enterocyte effacement (LEE) similar to that found in enteropathogenic *Escherichia coli* (EPEC). EPEC *espB* is necessary for intimate attachment and signal transduction between EPEC and cultured cell monolayers. Mice challenged with wild-type *C. rodentium* develop a mucosal immunoglobulin A response to EspB. In this study, *C. rodentium espB* has been cloned and its nucleotide sequence has been determined. *C. rodentium espB* was found to have 90% identity to EPEC *espB*. A nonpolar insertion mutation in *C. rodentium espB* was constructed and used to replace the chromosomal wild-type allele. The *C. rodentium espB* mutant exhibited reduced cell association and had no detectable fluorescent actin staining activity on cultured cell monolayers. The *C. rodentium espB* mutant also failed to colonize laboratory mice following experimental inoculation. The *espB* mutation could be complemented with a plasmid-encoded copy of the gene, which restored both cell association and fluorescent actin staining activity, as well as the ability to colonize laboratory mice. These studies indicate that *espB* is necessary for signal transduction and for colonization of laboratory mice by *C. rodentium.*

Citrobacter rodentium (formerly *C. freundii* biotype 4280) and enteropathogenic *Escherichia coli* (EPEC) infections are characterized by epithelial cell hyperproliferation similar to that seen in human proliferative bowel disorders including Crohn's disease and ulcerative colitis (3, 35). Individuals with these diseases are known to suffer an increased risk and early onset of colorectal cancer (27). In mice experimentally infected with *C. rodentium*, hyperplasia is detectable as early as 4 days postinfection, with maximal hyperplasia occurring 2 to 3 weeks later. Prior to the development of maximal hyperplasia, attaching and effacing (AE) lesions are present in the descending colon (17). These lesions are characterized by dissolution of the brush border, cupping of the adherent bacteria by the epithelial cell plasma membrane, and cytoskeletal rearrangements in the underlying enterocyte cytoplasm that lead to disruption of the terminal web (17). These histologic changes are indistinguishable from AE lesions produced by EPEC (31). In adult mice, colonic hyperplasia eventually regresses, with the colon returning to normal 6 to 8 weeks postinoculation. Suckling mice experience secondary inflammation of the colon that is associated with retarded growth, soft feces, and greater than 50% mortality (4).

EPEC causes diarrhea in infants and in young children (10). Initially, adherence to epithelial cells by EPEC occurs via a plasmid-encoded bundle-forming pilus (16). This first step is followed by the triggering of epithelial signal transduction pathways that leads to the reorganization of filamentous actin (24). Signaling from EPEC to epithelial cells results in tyrosine phosphorylation of substrates that colocalize with the accumulated actin underneath adherent bacteria (33). The major phosphorylation substrate detected in EPEC-infected cells is the bacterial translocated intimin receptor (Tir), which is targeted to the host cell membrane, where it becomes the receptor for the EPEC adhesin intimin (21). EPEC also induces other signaling cascades including fluxes of inositol phosphates (15), changes in membrane potential (41), and activation of protein kinase C (8), phospholipase-Cγ (20), and NF-κB (36). The end result of these cascades is the formation of pedestal-like AE lesions composed largely of filamentous actin under the cell associated bacteria (31).

Both *C. rodentium* and EPEC contain a 35-kb pathogenicity island known as the locus for enterocyte effacement (LEE) (28). The nucleotide sequence of the entire LEE in EPEC has been determined and has been shown to contain 41 potential open reading frames (ORFs) (12). These include *eaeA* (intimin) (28); *tir* (21); the *esc* genes (12), a group of genes which encode a type III secretion system; as well as *espA* (22), *espB* (11, 14), and *espD* (25). Several studies have confirmed that EspB is targeted to the host cytoplasm (23, 42, 46). EspB has also been shown to be required for translocation of Tir to the host cell membrane (21). Both EspA and EspD are required for EspB translocation, and both have been implicated as components of a surface organelle involved in the delivery of EspB to the cytoplasm (23, 42, 43). The LEE has been found in enterohemorrhagic *Escherichia coli* (EHEC) O157:H7, rabbit EPEC strains including RDEC-1, and pathogenic strains of *E. coli* previously designated *Hafnia alvei* (13). While all of these bacterial species contain a version of the LEE, none have been shown to colonize the intestinal tract of laboratory rodents or to be associated with mucosal hyperplasia in rodents. In a previous study, a *C. rodentium eaeA* mutant strain was constructed. This strain does not colonize and does not cause AE lesion formation (38).

In this study we have attempted to better understand the role played by AE lesion formation in the induction of hyperplasia by *C. rodentium*. Whether AE activity is sufficient to induce mucosal hyperplasia or whether *C. rodentium* has other specialized virulence determinants required for the induction

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TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Description	Source or reference
Strains		
DBS100	*C. rodentium* ATCC 51459	37
DBS255	DBS100 *eaeA* mutant; Kan^r	38
DBS506	DBS100 *espB* merodiploid; Kan^r	This study
DBS578	DBS100 *espB* mutant; Kan^r	This study
DBS586	DBS578 *espB* merodiploid; Kan^r	This study
DBS587	DBS578 *espB* revertant; Kan^s	This study
JPN15	Plasmid-cured derivative of EPEC E2348/69	26
DH5α	F$^-$ φ80d*lacZ*ΔM15 *recA hsdR supE thi gyrA* Δ(*lacZYAargF*)	BRL^a
SM10 (λ*pir*)	*thi thr leu tonA lacY supE recA*::RP4-2-Tc^r::Mu Km^r λ*pir* RK6	30
Plasmids		
pDBS2	Cosmid clone containing DBS100 DNA with *espB* homology; Amp^r	38
pUC18K	pUC18 derivative with 850-bp nonpolar *aphA*-3 cassette; Amp^r Kan^r	29
pBAZ391	pFSV-1 derivative with *nptI-sacB-sacR* cartridge; Cm^r Kan^r	This study
pBAZ463	1.0-kb *Hin*dIII subclone of pDBS2; Amp^r	This study
pBAZ481	1.1-kb *Hin*dIII subclone of pDBS2; Amp^r	This study
pBAZ503	pSK(−) containing a 3-kb *Pvu*II *espB* nonpolar mutant cassette; Amp^r Kan^r	This study
pBAZ504	*Pvu*II fragment from pBAZ503 cloned into pBAZ391; Cm^r Kan^r	This study
pJVN106	1,926-bp *Nae*I-*Bgl*II *espB* clone; Amp^r	This study
pJVN109	1,972-bp *Xho*I-*Bgl*II fragment of pJVN106 cloned into pFSV-1; Cm^r	This study
pJVN111	1,972-bp *Xho*I-*Bgl*II fragment of pJVN106 cloned into pACYC184; Cm^r	This study
Primers		
BAZ104	5′ GGCGCGAGTGATGTCGC 3′	39
BAZ105	5′ AGCGAGCCGCTTGCCCC 3′	39
ESPB1	5′ TGAGACAGTTGGCACATTGC 3′	This study
ESPB2	5′ TGGTGGTACAACTCTTCGAGC 3′	This study
aphA-3out	5′ CGAAAACTGGGAAGAAGACAC 3′	This study

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of mucosal hyperplasia is still not clear. *C. rodentium espB* was selected for study because EspB is required for AE lesion formation in EPEC and is the only protein secreted by EPEC that has been shown to be targeted to the host cell cytoplasm.

MATERIALS AND METHODS

Strains and plasmids. Bacteria were stored at −80°C in Luria-Bertani (LB) broth containing 50% (vol/vol) glycerol. The bacteria were grown at 37°C in LB broth or on LB agar. For maintenance of recombinant plasmids, strains were grown in medium supplemented with appropriate antibiotics (ampicillin at a final concentration of 100 μg/ml, kanamycin at 40 μg/ml, chloramphenicol at 30 μg/ml, naladixic acid at 20 μg/ml, and tetracycline at 20 μg/ml). Colonies were assayed for β-galactosidase activity on LB agar supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) as a chromogenic substrate and isopropyl-β-D-thiogalactoside (IPTG) as an inducer, both at final concentrations of 32 μg/ml. The strains and plasmids used in this study are described in Table 1.

Animals. Three-week-old outbred Swiss Webster mice (Taconic Laboratories, Germantown, N.Y.), were housed in microisolator caging within a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care. The mice were maintained on pelleted rodent chow and water ad libitum. All experiments were approved by the Massachusetts Institute of Technology Institutional Animal Care and Use Committee. Mice were orally inoculated with either 100 μl of an overnight culture of bacteria (approximately 5 × 10^8 CFU) or 100 μl of sterile LB broth. At predetermined time points, feces were collected from individual animals, weighed, and plated in sterile phosphate-buffered saline, and plated on MacConkey lactose agar with or without the appropriate antibiotics. Overall colonization levels were measured by determining the number of CFU of *C. rodentium* per gram of feces at 3, 5, and 7 days postinoculation. At 10 days postinoculation, the animals were euthanized. The entire colon was collected aseptically and visually inspected for evidence of hyperplasia. The weight of the colon was determined, and the colon was then homogenized as described previously (37). Appropriate dilutions of the tissue homogenate were plated on differential media with and without antibiotic selection to determine the number of CFU of *C. rodentium* per gram of tissue. The lower limit of detection was 1 CFU/mg of tissue.

Recombinant DNA methods. Chromosomal DNA was isolated as described previously (39). Plasmid DNA was isolated using Qiagen tips as recommended by the manufacturer (Qiagen Inc., Chatsworth, Calif.). DNA ligation, restriction endonuclease digestion, and gel electrophoresis were performed by standard methods (34). Enzymes were purchased from New England Biolabs, Inc. (Beverly, Mass.). Plasmid DNA was introduced into *E. coli* and *C. rodentium* by high-voltage electroporation with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.).

***espB* probe.** PCR primers for *espB* were designed by using the *espB* sequence of EPEC E2348/69 (11, 22). The *espB* probe was generated with primers BAZ104 and BAZ105 and chromosomal DNA from EPEC JPN15 as previously described (39).

Cloning *espB*. pDBS2 cosmid DNA (38) was isolated and digested with *Hin*dIII. Digested DNA fragments were ligated with either pBluescript SK(−) or pBluescript KS(−) (Stratagene, La Jolla, Calif.) that had been linearized with *Hin*dIII. The ligation mixture was used to transform DH5α, and bacterial clones containing recombinant plasmids were recognized as white colonies when plated on LB agar with ampicillin supplemented with X-Gal and IPTG. Plasmid DNA was isolated from a number of these clones, and Southern blot analysis was performed.

Southern blot analysis. Digested genomic and plasmid DNA fragments were separated by electrophoresis on a 0.75% agarose gel. DNA fragments were transferred to a nylon membrane (Hybond-N+; Amersham Corp., Arlington Heights, Ill.) by the capillary method as described previously (34). Membranes were UV cross-linked (Stratalinker; Stratagene). The PCR-amplified *espB* fragment was directly labeled with an ECL chemiluminescence detection kit (Amersham) for use as the probe. Membranes were hybridized with probe overnight and washed at high stringency (twice for 20 min in 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at 55°C, followed by twice for 5 min in 20× SSC at room temperature). After addition of detection reagents, positive signal was detected by exposure to radiographic film (Hyperfilm-ECL; Amersham).

Nucleotide sequence determination. The nucleotide sequence of the two *Hin*dIII fragments containing *espB* homology was determined. Initial DNA sequences from clones containing these inserts were determined by the dideoxy-chain termination method with Sequenase version 2.0 (U.S. Biochemicals, Naperville, Ill.). The remaining sequences were determined at the Massachusetts Institute of Technology Biopolymer Laboratory with a DyeDeoxy Terminator cycle sequencing kit and a model 373A DNA sequencer (Applied Biosystems, Foster City, Calif.).

Construction of a nonpolar insertional *espB* mutant. The two *Hin*dIII fragments containing *espB* homology, pBAZ463 and pBAZ481, were used to construct a mutant *espB* allele. To avoid the polar effects associated with insertional mutations, the *aphA*-3 nonpolar cassette developed by Ménard et al. (29) was
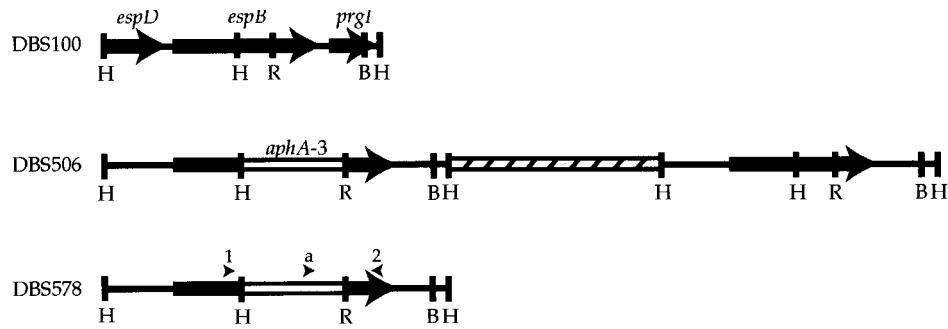
FIG. 1. Physical maps of the *espB* region of the *C. rodentium* strains used in this study. Heavy lines represent *C. rodentium* DNA, the open box represents *aphA*-3, and the hatched box represents plasmid pBAZ391 (not to scale). The arrows represent the ORFs of *espD*, *espB*, and the *prgI* homolog. Arrowheads represent the binding sites of ESPB1 (arrowhead 1), ESPB2 (arrowhead 2), and *aphA*-3out (arrowhead a) used in sequencing. Abbreviations: B, *Bgl*II; R, *Eco*RV; and H, *Hin*dIII.

used to disrupt the gene. The *aphA*-3 cassette contains a kanamycin resistance gene preceded by translation stop codons in all three reading frames and is immediately followed by a consensus ribosomal binding site and a start codon. A 283-bp *Eco*RV deletion was made in the 5′ end of the *espB* fragment in pBAZ481 so that when the 850-bp *aphA*-3 cassette was cloned upstream into the *Sac*I-*Pst*I sites, the 3′ end of *espB* would be expressed by the downstream start codon of the nonpolar cassette. A 2.2-kb *Sca*I-*Sac*I fragment containing the 5′ portion of *espB* from pBAZ463 was cloned upstream, generating the *espB* nonpolar insertion mutation designated pBAZ503. The construct was confirmed to contain a nonpolar insertion of *aphA*-3 by sequencing. A 3-kb *Pvu*II fragment containing the *espB* nonpolar insertion mutation from pBAZ503 was cloned into pBAZ391, a pFSV-1 derivative containing the *nptI-sacB-sacR* cassette, to generate the suicide vector pBAZ504. pBAZ504 was transformed into SM10(λ*pir*) for maintenance.

The suicide plasmid pBAZ504 was mated into *C. rodentium* as previously described (38). Since plasmids with the R6K replicon cannot replicate in the absence of the *pir*-encoded π protein (30), kanamycin-resistant exconjugates represented merodiploid strains into which the plasmid had been integrated by homologous recombination. A physical representation of the strains used in this study is shown in Fig. 1. Total bacterial DNA from an individual clone, designated DBS506, was screened by Southern blot analysis to confirm homologous recombination of the suicide plasmid into the *C. rodentium* chromosome (see Fig. 2). The suicide vector used, pBAZ504, contained a copy of *sacB*, expression of which has been shown to be lethal for some gram-negative organisms grown in the presence of 5% sucrose (29). However, no growth disadvantage of the strain was seen under these conditions. Therefore, to isolate an *espB* mutant, the merodiploid DBS506 was screened for homologous recombination resulting in loss of the wild-type *espB* allele and adjacent vector sequences. Single colonies were patched onto LB agar containing either chloramphenicol or kanamycin to identify isogenic *espB* mutant strains. One exconjugate, designated BAZ578, was selected for further characterization. The DNA sequence of the mutant *espB* allele was sequenced with primers ESPB1, ESPB2, and *aphA*-3out.

trans **complementation and replacement of the mutant allele.** The 1,020-bp *Nae*I-*Hin*dIII fragment of pBAZ463, containing the 5′ end and upstream sequences of *espB*, and the 906-bp *Bgl*II-*Hin*dIII fragment from pBAZ481, containing the 3′ end of *espB*, were cloned into pSP72 digested with *Pvu*II and *Bgl*II to give pJVN106. The 1,972-bp *Xho*I-*Bgl*II fragment of pJVN106 was cloned into pACYC184 digested with *Bam*HI and *Sal*I to generate pJVN111. In addition to complementing the *espB* mutation in *trans*, the marked deletion in the mutant strain was replaced with the wild-type allele of *espB*. To accomplish this, the 1,972-bp *Xho*I-*Bgl*II fragment of pJVN106 pJVN106 was treated with the Klenow fragment of DNA polymerase I and ligated to pFSV-1 (7) which had been linearized with *Eco*RV, generating pJVN109. The suicide plasmid pJVN109 was transformed into *espB* mutant DBS578. Chloramphenicol-resistant transformants represented merodiploid strains into which the plasmid had been integrated by homologous recombination. Using the same strategy employed to generate an isogenic *espB* mutant, a strain carrying the *espB* wild-type allele was isolated and confirmed by Southern blot analysis (see Fig. 2).

SDS-PAGE analysis of secreted proteins. A modification of the method of Kenny et al. was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18). Briefly, *C. rodentium* strains were grown overnight, washed in salt-free medium, and diluted 1:100 into Dulbecco's modified Eagle's medium (DMEM) containing 0.1 M HEPES (pH 7.4). The bacteria were incubated standing at 37°C until the optical density of the culture at 600 nm reached 0.5 and were then removed by centrifugation at 16,000 × *g* for 5 min. Supernatant proteins were precipitated by the addition of 10% trichloroacetic acid for 60 min on ice, pelleted by centrifugation at 4°C at 16,000 × *g* for 30 min, and resuspended in 10 mM Tris-HCl (pH 8.0). Protein samples were diluted in Laemmli loading buffer, resolved by SDS-PAGE on a 12% polyacrylamide gel, and visualized by silver staining.

Cell association assay. A modification of the procedure described for RDEC-1 by Abe et al. was used (1). Briefly, HEp-2 cells (10⁴/well) were seeded in 24-well plates and grown overnight at 37°C in 5% CO_2 in DMEM. Monolayers were infected with bacteria at a multiplicity of infection of 70 and incubated at 37°C in 5% CO_2 for 3 h. The monolayers were washed six times with phosphate-buffered saline and stained with Diff-Quik (Dade Behring Inc. Newark, Del.). Fifty individual HEp-2 cells were scored for the number of cell-associated bacteria. Data were analyzed with Statview 5.0 and are reported as the mean ± standard deviation. Differences between groups were compared by using a two-tailed *t* test.

Fluorescent actin staining assay. The fluorescent actin staining (FAS) assay was a modification of the method of Knutton et al. (24) and was performed as previously described (14). Briefly, HEp-2 cells were seeded at 5 × 10⁴ CFU on circular coverslips in 24-well plates and grown overnight at 37°C in 5% CO_2 in DMEM. Bacteria were added at a multiplicity of infection of 70 and were incubated for 6 h at 37°C in 5% CO_2. Midway through the incubation period, the cell culture medium was replaced with fresh medium. Monolayers were washed six times with phosphate-buffered saline, fixed with paraformaldehyde, and extracted with ice-cold acetone. Double fluorescence labeling was performed. The presence of *C. rodentium* was detected by an anti-*C. rodentium* rabbit polyclonal antibody and Cascade blue-conjugated goat anti-rabbit immunoglobulin G (Molecular Probes, Eugene, Oreg.). Actin was labeled with Texas red-phalloidin (Molecular Probes). Coverslips were mounted and examined on a Nikon Labophot epifluorescence microscope fitted with 400- and 580-nm dichroic filters. Single labeling with the anti-*C. rodentium* antibody revealed no crossover when visualized with the 580-nm filter.

Nucleotide sequence accession number. The nucleotide sequence of the *C. rodentium espB* gene has been submitted to GenBank and has been assigned accession no. AF177537.

RESULTS

***C. rodentium* espB clones and nucleotide sequence.** Seven *Hin*dIII fragments were cloned from the *C. rodentium* LEE region contained on the pDBS2 cosmid. Two of these inserts were found to have *espB* homology by Southern blot analysis. pBAZ463 contained a 1-kb insert with homology to 596 bp of the 5′ end of *espB*. pBAZ481 contained a 1.1-kb insert with homology to 376 bp of the 3′ end of *espB*. Characterizing this contig, *C. rodentium espB* was found to be 966 nucleotides in length and was predicted to yield a protein product with a molecular mass of 33 kDa. No hydrophobic leader sequence was apparent, indicative of a type III secretion mechanism. The gene was found to be preceded by consensus sequences for −35 and −10 promoter regions and by a strong ribosomal binding site. DNA sequence alignment of *C. rodentium espB* with that of EPEC E2348/69 showed 90% identity. Comparison of the *C. rodentium* and EPEC EspB hypothetical proteins showed them to have 85% identity and 90% similarity. An unusual sequence of 20 amino acids, 17 of which are either serine or threonine, appeared near the amino terminus of the predicted protein; this sequence was conserved between EPEC and *C. rodentium* (11).
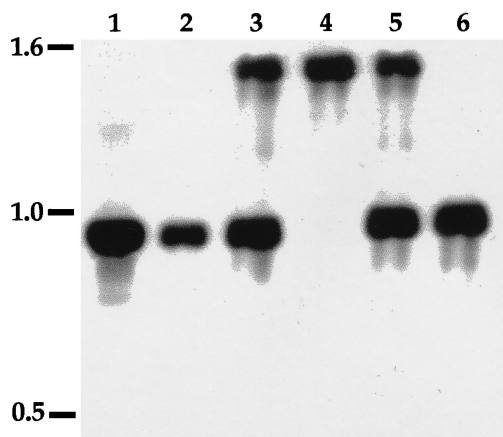
FIG. 2. Southern analysis of *C. rodentium* wild-type and mutant strains, probed with the 0.9-kb *Bgl*II-*Hin*dIII fragment of pBAZ481 containing the 3′ end of the *espB* gene. DNA was digested with *Bgl*II and *Hin*dIII. Lanes: 1, pBAZ481; 2, wild-type DBS100; 3, *espB* merodiploid DBS506; 4, *espB* mutant DBS578; 5, *espB* merodiploid DBS586; 6, *espB*⁺ DBS587. Numbers on the left are sizes in kilobases.

The nucleotide sequence of the DNA 5′ of *C. rodentium* *espB* had homology to the EPEC *espD* gene (2). The DNA 3′ of *C. rodentium* *espB* contained a 222-bp ORF. The predicted protein product of this ORF was a 74-residue polypeptide with significant homology to the PrgI protein of *Salmonella typhimurium*. In *S. typhimurium*, PrgI is involved in the secretion of epithelial-cell signaling proteins (32).

Construction of a nonpolar insertional *C. rodentium* *espB* mutant. To test the hypothesis that *espB* is required for cell association and signal transduction by *C. rodentium*, an isogenic nonpolar insertional *espB* mutant was constructed. The merodiploid strain DBS506 was passaged in LB broth, and clones were tested for Kanʳ and Cmˢ. Of the 2,000 clones tested, 1 such clone was isolated (0.05%) and was designated DBS578. DBS578 was confirmed by Southern blot analysis to have lost vector and wild-type allele sequences (Fig. 2).

Complementation of the *C. rodentium* *espB* mutant. DBS578 was complemented in *trans* with pJVN111. In addition, the marked deletion in DBS578 was replaced with the wild-type allele of *espB*. To accomplish this, the merodiploid strain DBS586 was passaged in LB broth and clones were screened for Kanˢ. Of the 900 clones that were screened, 1 was found to be both Kanˢ and Cmˢ (0.1%). This clone was designated DBS587 and was confirmed by Southern blot analysis to have the wild-type allele of *espB* in place of the mutant allele (Fig. 2).

***C. rodentium* *espB* encodes a 37-kDa secreted protein.** EPEC grown in cell culture medium secretes five polypeptides, including proteins of 100 kDa (EspC), 39 kDa (EspD), 37 kDa (EspB), and 24 kDa (EspA) (19). *C. rodentium* showed a similar secretion profile except for the absence of a protein with an apparent molecular mass similar to that of EspC (40) (Fig. 3). It has been shown that EspC is not encoded by a LEE gene and is not required for induction of host signal transduction pathways by EPEC (40). DBS100 secreted a protein similar in mobility to EPEC EspB. The mutant strain DBS578 did not secrete EspB as judged by its secretion profile (Fig. 3). The 24-kDa and 42-kDa proteins were still secreted by the *espB* mutant DBS578. *trans* complementation of the *espB* mutation in DBS578 with pJVN111 resulted in expression of EspB, although the amount of secreted protein appeared to be reduced. DBS587, the chromosomal revertant derived from DBS578, also expressed EspB. These findings confirm that no

unlinked mutations are present in DBS578 that affect EspB secretion.

***espB* is required for cell association of *C. rodentium* with cell monolayers.** To investigate the role of *C. rodentium* EspB in cell association, the *espB* mutant strain was compared to wild-type *C. rodentium* in a cell association assay. The cell association of *espB* DBS578 was significantly reduced compared to that of wild-type DBS100 ($p < 0.05$) and was comparable to that seen with *eaeA* DBS255 (Fig. 4). The reduction in cell association seen with DBS578 could be complemented in *trans* or could be restored by replacing the mutant allele with a wild-type copy of the *espB* gene (Fig. 4). These data indicate that in *C. rodentium*, *espB* is required for cell association with cultured cell monolayers.

***espB* is required for FAS activity by *C. rodentium*.** To determine whether *C. rodentium* *espB* is required for FAS activity, double fluorescence microscopy was performed. DBS100 was able to cause the accumulation of cytoskeletal actin into pedestals beneath cell-associated bacteria (Fig. 5). Both the percentage of cell-associated bacteria exhibiting actin rearrangement and the intensity of actin staining beneath cell-associated DBS100 were lower than that seen with JPN15, an EPEC strain cured of the plasmid that encodes the *bfp* locus (26). Cytoskeletal actin accumulation beneath cell-associated bacteria was not seen with the *espB* mutant DBS578. Complemen-
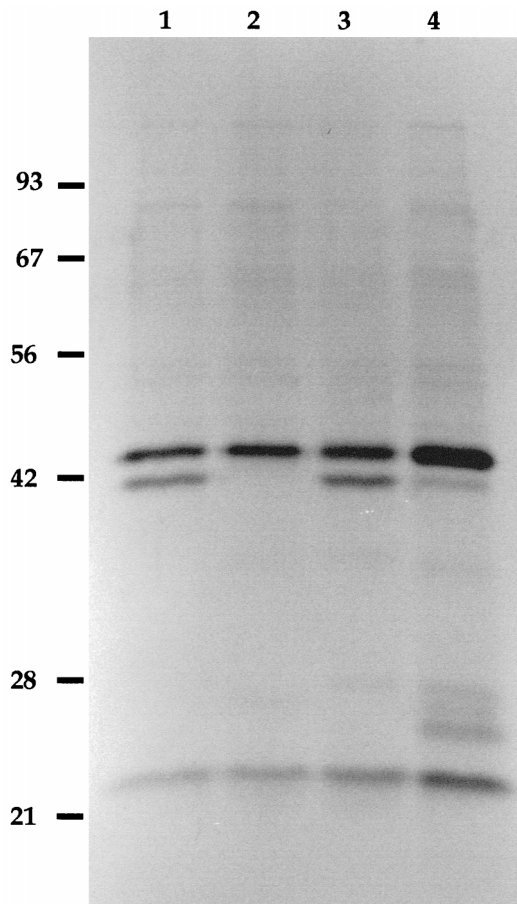

FIG. 3. Secreted proteins of isogenic *C. rodentium* mutant strains. Secreted proteins were separated by SDS-PAGE on a 12% polyacrylamide gel and stained with silver. Lanes: 1, wild-type DBS100; 2, *espB* mutant DBS578; 3, *espB*⁺ DBS587; 4, transcomplemented DBS578/pJVN111 strain. Numbers on the left are molecular masses in kilodaltons.
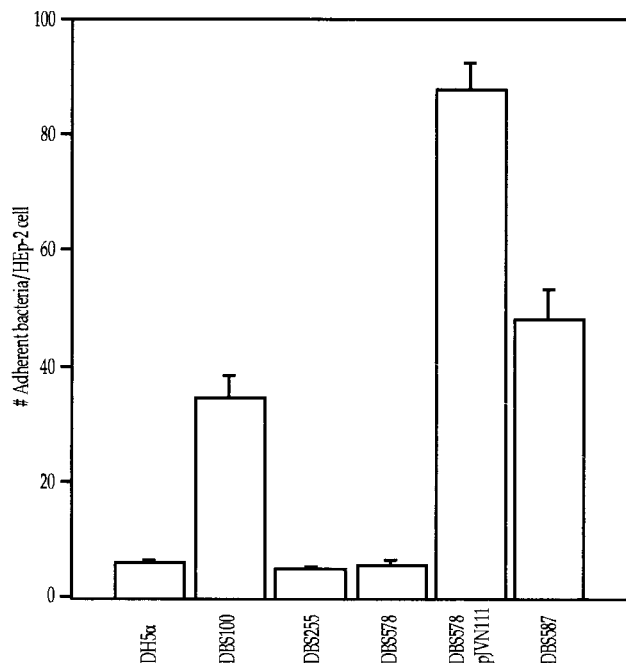
FIG. 4. EspB is required for cell association by *C. rodentium*. The number of adherent bacteria on 50 HEp-2 cells was counted. Cell association of wild-type DBS100 was not significantly different from that of *espB*$^+$ DBS587 ($P = 0.05$). The *espB* mutant DBS578, like *eaeA* mutant DBS255, had reduced cell adherence ($P < 0.001$). The *trans*-complemented DBS578/pJVN111 strain had increased cell association compared to wild-type DBS100 ($P < 0.001$).

present in mice infected with DBS578/pJVN111, and no hyperplasia was evident in the colons of LB-, DBS255-, or DBS578-inoculated mice (data not shown).

DISCUSSION

In this study, we describe the cloning and the characterization of *espB* from *C. rodentium*. Previous characterization of the LEE in EPEC, EHEC, *C. rodentium* and the *E. coli* strains previously designated *H. alvei* has shown that this pathogenicity island contains homologs of *eaeA*, *espB*, and the type III secretion system encoded by the *esc* genes. Homologous genes are not present in nonpathogenic *E. coli* strains, in other spe-
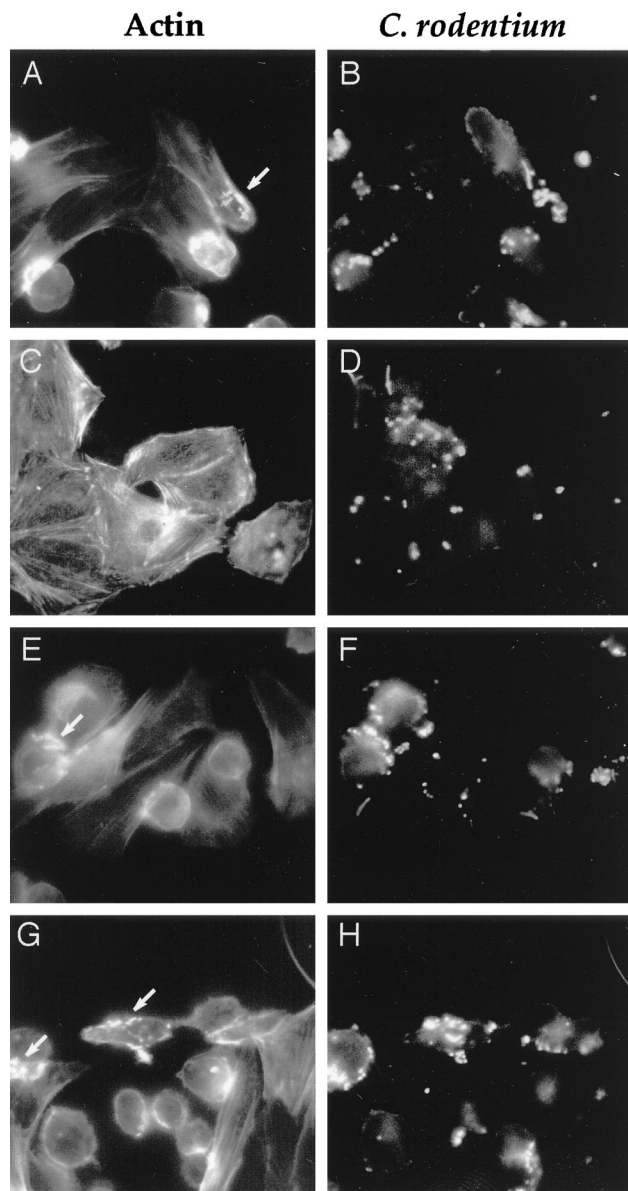
tation of the chromosomal *espB* mutation in *trans* or by replacement with the wild-type allele resulted in restored FAS activity (Fig. 5).

***espB* is a colonization factor for *C. rodentium*.** When mice were challenged with the *espB* mutant DBS578, no kanamycin-resistant bacteria were recovered from stool after 3 days. As expected, the *eaeA* mutant DBS255 was also absent from the feces of mice by 3 days postinoculation. The expected levels of DBS100 CFU were recovered from mice inoculated with the wild-type strain, while no *C. rodentium* was recovered from any of the control mice receiving sterile broth. To confirm that it was the *espB* mutation that was responsible for the loss of colonization, attempts were made to complement the *espB* mutation in *trans*. While DBS578/pJVN111 CFU were recovered from infected mice at each time point, the number of CFU recovered was 2 log units lower than that of DBS100. At each time point, 100 DBS578/pJVN111 colonies from each mouse were scored for Cm^r. On average, 10% of the colonies were found to be Cm^s, suggesting that pJVN111 was segregating in vivo. When mice were challenged with *espB*$^+$ DBS587, fecal counts equivalent to DBS100 counts were seen on day 7 postinoculation.

To confirm these results, colonic colonization was also determined. No *espB* mutant DBS578 and *eaeA* mutant DBS255 CFU were isolated from total mouse colon, whereas equal numbers of wild-type DBS100 and *espB*$^+$ DBS587 CFU were recovered (Table 2). DBS578/pJVN111 CFU counts were 2 log units lower than those for DBS100 and DBS587. Some mortality was seen in mice fed either wild-type DBS100 or *espB*$^+$ DBS587 but not in mice infected with *espB* mutant DBS578, DBS578/pJVN111, or *eaeA* mutant DBS255. In addition, marked hyperplasia was present in the descending colons of all mice infected with DBS100 and DBS587, mild hyperplasia was

FIG. 5. EspB is required for FAS activity. Photomicrographs of FAS assay mixtures after a 6-h incubation show filamentous actin (A, C, E, and G) and *C. rodentium* (B, D, F, and H). Wild-type DBS100, *trans* complemented DBS578/pJVN111, and *espB*$^+$ DBS587 were positive for FAS activity (arrows in panels A, E, and G), while no FAS activity was seen with *espB* mutant DBS578 (C). Magnification, ×40.

TABLE 2. *espB* is required for colonization of mice by
C. rodentium[a]

Inoculum	Log CFU/g of stool-filled colon[b]	n[c]
Sterile LB	0.00 ± 0.00	9
DBS100	8.95 ± 0.09	10[d]
DBS255	0.00 ± 0.00	6
DBS578	0.00 ± 0.00	10
DBS578/pJVN111	6.88 ± 0.14[e]	6
DBS587	9.03 ± 0.11	5[d]

[a] The experiment measuring the number of CFU in stool-filled colon on day 10 postinoculation was done twice, and the data presented in this table are a composite of the results of the two experiments.

[b] Mean $\pm$ standard deviation; the lower limit of detection was 1 CFU/g.

[c] Number of mice used.

[d] Prior to day 10, two mice infected with DBS100 and one mouse infected with DBS587 died.

[e] Chloramphenicol-resistant counts only.

cies of *Citrobacter* (39), or in avirulent strains of *H. alvei* (28). Sequences homologous to *espB* have previously been reported to be present on pDBS2 (39), a cosmid containing both *eaeA* and ORFU (38). The physical organization of these genes in the chromosome of *C. rodentium*, human EPEC, and RDEC-1 is conserved (1, 11, 22).

The *C. rodentium espB* mutant strain did not secrete a 37-kDa protein. Secretion of this protein could be restored by *trans* complementation or by replacing the mutant allele with a wild-type copy of the *espB* gene. In the *trans* complemented strain, a number of additional protein bands appeared in the 25- to 28-kDa range. It has been previously shown that the secreted 37-kDa EspB protein in EPEC has 28-, 25-, and 16-kDa breakdown products (44). This may indicate that when *espB* is present in multiple copies that the EspB protein is unstable.

C. rodentium carrying an *espB* mutation was found to have significantly reduced cell association and no detectable FAS activity. These activities could be restored by *trans* complementation or by replacement of the mutant allele with a wild-type copy of the *espB* gene. These data demonstrate that signal transduction mediated by EspB is required for cell association and for FAS activity by *C. rodentium* on cultured cell monolayers. The *trans* complemented strain had significantly increased cell association compared to the wild type; the reasons for this remain unclear.

Our *espB* mutant also failed to colonize mice. An *eaeA* mutant has been previously reported to have the same phenotype (38), suggesting that AE lesion formation is required for colonization by *C. rodentium*. This distinguishes *C. rodentium* from the AE lesion-forming *E. coli* strains, which appear to utilize initial adhesins (6, 16, 26, 45). In human volunteer studies conducted with EPEC strain E2348/69 and an isogenic *eaeA* mutant, no overall difference was seen in the peak excretion levels of the organisms in stool, although there was significantly less severe diarrhea in subjects who had been fed the *eaeA* mutant (9). This demonstrates that intimate attachment is not required for colonization by EPEC. In other human volunteer studies, the initial adherence factor, bundle-forming pili, was shown to be required for full virulence but not for colonization (5, 26). We speculate that AE lesion formation may be the primary mechanism for mucosal attachment by *C. rodentium*. Indeed, in the absence of the initial plasmid-encoded adhesin AF/R1, AE lesion-proficient RDEC-1 colonizes only the colon and not the small bowel (44), further supporting the notion that the default site of LEE-mediated attachment is

the colon. In all whole-animal studies to date, as in cultured cell systems, *espB* is required for AE lesion formation.

The factors responsible for inducing mucosal hyperproliferation have not been defined. It is possible that formation of AE lesions in the colon of mice is sufficient to cause transmissible colonic hyperplasia. However, a comprehensive search for virulence determinants in *C. rodentium* has not been undertaken. The characterization of additional genes within and outside the LEE will help define the molecular pathogenesis of transmissible colonic hyperplasia. We believe that studies with laboratory mice infected with *C. rodentium* will continue to provide insights into cytokinetics and cancer risk and will provide a useful model system with which to study AE activity.

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