INFECTION AND IMMUNITY, July 1995, p. 2541–2548 Vol. 63, No. 7
0019-9567/95/$04.00+0

In Vitro and In Vivo Characterization of an *ail* Mutant of *Yersinia enterocolitica*

MARIAN R. WACHTEL[1] AND VIRGINIA L. MILLER[1,2]*

Department of Microbiology and Molecular Genetics[1] and Molecular Biology Institute,[2] University of California, Los Angeles, Los Angeles, California 90095

Received 22 November 1994/Accepted 17 April 1995

Ail is a 17-kDa protein of *Yersinia enterocolitica* previously identified on the basis of its ability to confer upon *Escherichia coli* the phenotype of attachment and invasion of cultured epithelial cells. Here we report an examination of the contribution of *ail* to the pathogenicity of *Y. enterocolitica*. A low-copy-number *ail* plasmid that promoted serum resistance in *E. coli* HB101 was constructed. The serum resistance phenotype conferred by *ail* to *E. coli* was affected by the growth phase of the culture as well as by the gene copy number. In contrast, the copy number of *ail* (and the relative quantity of Ail) was found to have little effect on the amount of Ail-promoted invasion of cultured epithelial cells. An *ail* mutant of *Y. enterocolitica* was constructed and characterized in vitro. This mutant produced no detectable Ail and had a reduced ability to invade CHO cells. Serum resistance of *Y. enterocolitica* was Ail dependent and was affected by growth phase and *ail* copy number. The phenotype of the *ail* mutant was examined in vivo by using a murine model for infection. The *ail* mutant phenotype was identical to that of the wild-type strain in oral 50% lethal dose studies and early colonization of Peyer's patches as well as in kinetic studies. Western blot (immunoblot) analysis of Ail produced by bacteria growing in vivo at 48 h postinfection indicated that *ail* was expressed at this time point. Thus, our findings confirm that Ail contributes to the serum resistance and invasion phenotypes of *Y. enterocolitica* in vitro and indicate that Ail is not required to establish an infection or to cause systemic infection of BALB/c or DBA/2 mice.

Three *Yersinia* species are pathogenic for humans and cause diseases ranging from mild gastroenteritis (*Yersinia enterocolitica* and *Y. pseudotuberculosis*) to bubonic plague (*Y. pestis*). Gastrointestinal infections caused by *Yersinia* species usually occur via contaminated food or water (6, 10). After ingestion the bacteria are transported to the terminal ileum and adhere to the intestinal epithelium. Subsequently, a subset of the bacteria invade the host epithelium and proliferate in the underlying lymphoid tissue. Invasion is thought to occur through intestinal epithelial cells (58) or M cells (14, 16), which are specialized cells that play a role in antigen uptake and transport from the intestinal lumen into the underlying lymphoid tissue (37, 38, 60–62). Since invasion of M cells or intestinal epithelial cells is the first step of the infection process, a primary virulence-associated phenotype for *Y. pseudotuberculosis* and *Y. enterocolitica* is the ability to penetrate cells. Several investigators have found that the ability to invade cultured cells correlates well with the virulence of an organism (27, 35, 40, 52, 58). By using the tissue culture invasion assay as a model system, three invasion genes (*inv*, *ail*, and *yadA*) have been identified in *Y. enterocolitica* and *Y. pseudotuberculosis* (4, 22, 34, 63). Both *inv* and *ail* are encoded on the chromosome, while *yadA* is encoded on the virulence plasmid of *Yersinia* species.

The *inv* gene was first cloned from the chromosome of *Y. pseudotuberculosis* (22) and promotes the highest levels of invasion into cultured epithelial cells. The *inv* gene encodes an outer membrane protein called invasin (24). This protein was found to initiate cellular entry by binding to mammalian cell receptors belonging to the integrin family (23). The *Y. enterocolitica inv* gene has also been cloned (34) and is 77% identical to *inv* from *Y. pseudotuberculosis* (64). Invasin of *Y. enterocolitica* has been shown to be important for the initial stages of infection of BALB/c mice (43).

Analysis of invasion of tissue culture cells by a *Y. pseudotuberculosis inv* mutant indicated there was another plasmid-dependent entry mechanism (21). Plasmid-dependent entry conferred low-level invasion and thus was detectable only in an *inv* mutant background. *yadA*, a gene located on the virulence plasmid, encodes an outer membrane protein that was subsequently shown to be responsible for this low level of invasion into tissue culture cells (4, 63). More recently it was shown that *yadA* of *Y. enterocolitica* also confers a low level of invasion (45). Other phenotypes associated with YadA include adherence to collagen (12, 54) and cellular fibronectin (53, 57), the formation of fibrillae (25), reduced polymorphonuclear leukocyte phagocytosis (8), and promotion of serum resistance (1, 29, 39).

The third invasion gene, *ail*, was cloned from the chromosome of *Y. enterocolitica* (34). When produced by *Escherichia coli*, Ail promotes a variable invasion phenotype in tissue culture cells, depending on the cell line used, but attachment due to Ail is high for all cell lines tested (34). An early study of invasion of cells by *Y. enterocolitica* showed that pathogenic strains which invade eukaryotic cells have sequences homologous to *ail*, while nonpathogenic strains fail to invade eukaryotic cells and do not have *ail*-homologous sequences (27, 35).

Ail has recently been identified as a member of a family of structurally related outer membrane proteins (2, 3, 18, 32, 48, 56). One of these proteins, Rck of *Salmonella typhimurium*, confers high-level serum resistance when the structural gene is expressed from a multicopy plasmid both in *E. coli* and in

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, University of California, Los Angeles, 1602 Molecular Sciences Building, 405 Hilgard Ave., Los Angeles, CA 90095. Phone: (310) 206-3077. Fax: (310) 206-5231. Electronic mail address: virginia@microbio.lifesci.ucla.edu.

Salmonella spp. (15, 18) and confers an attachment and invasion phenotype when expressed in *E. coli* (20). Recently Ail was shown to have functional homology to Rck by the observation that both of these proteins confer serum resistance to their respective hosts and to *E. coli* (5, 18, 46). Because the complement cascade is a primary defense mechanism, resistance to serum is an important virulence phenotype for bacteria.

Though *ail* has been well characterized in vitro, its contribution to the pathogenicity of *Y. enterocolitica* has not yet been reported. The phenotypes associated with Ail, serum resistance and invasion, have been implicated in many other systems as virulence-associated phenotypes. We report here the construction of an *ail* mutant of *Y. enterocolitica* which was tested in a murine model of infection for its ability to colonize Peyer's patches, ability to spread to other tissues, and ability to cause a lethal infection.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains were stored at −80°C in Luria broth (LB) medium (Difco Laboratories, Detroit, Mich.) containing 25% (vol/vol) glycerol and were grown on LB agar plates or on M9 minimal agar plates supplemented with 0.2% glucose and 0.0001% vitamin B_1 (30). *E. coli* HB101 [F^- *hsdS20* (r_B^- m_B^-) *recA21 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*] and *E. coli* SM10λ*pir* (*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km) (26) were grown in the presence of the following concentrations of antibiotics when needed: chloramphenicol, 35 μg/ml; ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; and nalidixic acid, 20 μg/ml. Overnight cultures of bacteria were grown in LB, with shaking, for 16 to 18 h at the temperatures indicated for *Yersinia* strains and at 37°C for *E. coli*. Mid-logarithmic-phase cultures of bacteria were prepared by diluting overnight cultures 1:50 in LB and growing cells at the appropriate temperature to an optical density at 600 nm (OD_{600}) of 0.6 to 0.8. The OD_{600} values of overnight cultures were typically 3.3 to 4.9 for *E. coli* and 3.0 to 4.9 for *Y. enterocolitica*.

Nucleic acid purification and probe preparation. Plasmid DNA was isolated by the alkaline lysis method (28), by using Qiagen columns (Qiagen, Chatsworth, Calif.), or by using Magic (Wizard) Minipreps (Promega, Madison, Wis.). DNA restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, Mass.) and used according to the manufacturer's instructions.

Western blot (immunoblot) analysis. Bacterial cultures were grown under the specified conditions, and whole cell lysates were prepared as described previously (3). Whole cell lysates were stored at −20°C, and protein concentration was determined using the Bio-Rad Microassay (Bio-Rad Laboratories, Richmond, Calif.). Prior to separation by electrophoresis on a sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel, protein extracts were mixed with an equal volume of 2× sample buffer containing 10 M urea and boiled for 5 min. Unless otherwise indicated, 20 μg of protein was loaded in each lane of the protein gels. Western blots for Ail were probed with the monoclonal anti-Ail antibody 2B2 (a gift of James Bliska, State University of New York, Stony Brook) followed by a secondary goat anti-mouse immunoglobulin G antibody conjugated to alkaline phosphatase (Bethesda Research Laboratories, Gaithersburg, Md.). Western blots were visualized by incubating the filter with 5-bromo-4-chloro-3-indolylphosphate (XP, *p*-toluidine salt) (Sigma Chemical Co., St. Louis, Mo.) and *p*-Nitro Blue Tetrazolium chloride (United States Biochemical Corp., Cleveland, Ohio). Alternatively, Western blots were incubated with sheep anti-mouse immunoglobulin conjugated to horseradish peroxidase (Amersham Corp., Arlington Heights, Ill.) as the secondary antibody and visualized by using the Amersham ECL Western Blotting Detection System. Ail was purified from *E. coli* HB101 pVM102 as described previously (33). Protein molecular weight standards were obtained from Bio-Rad. Western blots were scanned with the Ultra Violet Products Gel Documentation System, software package SW2000 (Ultra Violet Products, Inc., San Gabriel, Calif.), or with an LKB Bromma 2222-020 Ultrascan XL enhanced laser densitometer (Pharmacia, Bromma, Sweden).

Construction of an *ail* mutant and characterization in vitro. The *ail* mutant, YE2v, was constructed as follows. The suicide plasmid pVM118 is a derivative of pJM703.1 (36) and has a 2,500-bp *Eco*RI fragment of *Y. enterocolitica* DNA containing the *ail* gene inserted in the *Eco*RI site. The *ail* gene of pVM118 contains a mini-Tn*10* insertion within the gene at position 1014 (33). This plasmid does not confer an invasive phenotype to *E. coli*, whereas the parent plasmid, pVM110, does confer an invasive phenotype to *E. coli* (data not shown). *Y. enterocolitica* 8081v^{Nal} (a gift from Rafael Martinez, University of California, Los Angeles), a nalidixic acid-resistant derivative of 8081v, was filter mated with the *E. coli* SM10λ*pir*/pVM118 as described previously (55), using heat modification of the recipient *Y. enterocolitica* strain to reduce host restriction of incoming DNA (9). Recombinant *Y. enterocolitica* strains were obtained by plating on M9 minimal medium containing nalidixic acid and tetracycline. Analysis of these strains by Southern hybridization indicated that all but one were the result of a double-crossover event. This is not the case at the *inv* or *flgM* loci, where integration of the suicide plasmid was the most frequent event (44). The *ail* mutant, YE2v, and the merodiploid, YE3v, were verified by Southern blot analysis with several restriction enzymes as described previously (35) and by Western blot analysis as described above.

Complementation of the *ail* mutant. A low-copy-number *ail*-containing plasmid, pMW1824, was constructed as follows. An 1,800-bp *Bam*HI fragment containing *mob*RP4 was subcloned from pJM703.1 (36) into the *Bam*HI site of pRS1823 (a gift from C. Masada Pepe) (41), generating the low-copy-number mobilizable vector pMW1823. Plasmid pRS1823 is a pSC101 derivative that encodes resistance to chloramphenicol. The *ail* plasmid pMW1824 was constructed by ligating the 2,500-bp *Hin*dIII fragment containing the *ail* gene from pVM102 (34) into pMW1823; the entire sequence of this fragment is known, and there are no open reading frames other than *ail*. Plasmids pMW1823 and pMW1824 were conjugated into both the wild-type and *ail* mutant *Yersinia* strains as described above.

Serum killing assays. Bacteria were grown in LB with the appropriate antibiotics under the conditions indicated, with shaking. The cell density of growing cultures was determined by optical density measurements at 600 nm. Mid-logarithmic-phase or overnight cultures of bacteria were adjusted to 10^8 cells per ml, centrifuged, and washed twice in phosphate-buffered saline (PBS) containing 5 mM $MgCl_2$. Aliquots (50 μl) of diluted bacteria containing 10^7 cells per ml were mixed with 200 μl of PBS–5 mM $MgCl_2$ and prewarmed at 37°C for 10 min. A 10-μl aliquot was sampled at time zero to determine the actual number of bacteria added to each tube. Normal human serum complement (250 μl; Sigma Chemical Co.) was added to each sample (final concentration [volume/volume] = 50%), and incubated with bacteria at 37°C for 60 min. To eliminate inhibitory growth effects from residual serum, bacteria were collected by centrifugation and resuspended in 490 μl of PBS–5 mM $MgCl_2$. Serial dilutions of the samples were prepared, and the number of CFU was determined by plating on LB agar containing the appropriate antibiotics. Bacterial strains were tested in triplicate, and the numbers were averaged for each experiment. As a control, parallel samples were incubated with serum which was heat treated to inactivate complement (56°C, 30 min). The degree of killing was expressed as follows: serum resistance index is represented by log kill, where log kill = ($\log_{10}$ CFU of bacteria per milliliter added initially) − ($\log_{10}$ CFU of bacteria per milliliter surviving incubation in normal human serum).

Tissue culture invasion assays. Chinese hamster ovary (CHO) cells were maintained and prepared for the tissue culture invasion assay as previously described (34). Bacterial strains were grown in LB containing the appropriate antibiotics. Quantitative tissue culture invasion assays were performed as described previously (34). The results are expressed as follows: percent invasion = 100 × (the number of bacteria resistant to gentamicin/the number of bacteria added initially).

Animal experiments. Six- to seven-week-old virus-free female BALB/c mice (Charles River Laboratories, Wilmington, Mass.) were used in all experiments. The mice were fed commercial rodent chow and given water ad libitum. Following shipment to this location, mice were allowed to acclimate for 1 week prior to the start of experiments. In all cases, mice infected with the same bacterial dilutions were housed together in the same cages, with a maximum of six mice per cage. Mice were infected intragastrically with 250 μl of the appropriate dilution of bacteria. Animals were sacrificed by cervical dislocation prior to dissection. The oral 50% lethal dose (LD_{50}) studies, in vivo invasion assay, and kinetics of infection studies were performed as described previously (43). All animal protocols were approved by the University of California, Los Angeles Animal Research Committee.

The in vivo expression assay was performed as described previously (42), with the following modifications. Animals were sacrificed after 48 h, and all visible Peyer's patches were excised from each mouse. The follicles were weighed and suspended in 3 ml of PBS. Homogenates were prepared immediately in a Stomacher apparatus (Tekmar Corp., Cincinnati, Ohio) and filtered through a 5.0-μm-pore-size filter unit (Millex-SV; Millipore, Bedford, Mass.) to eliminate eukaryotic tissue. A 10-μl aliquot of the filtered homogenate was taken and diluted for viable counts to determine the number of bacteria. The remaining bacteria were harvested by centrifugation, resuspended in 20 μl of 10 mM Tris (pH 8.0), and added to 20 μl of 2× sample buffer containing 10 M urea. Samples were boiled for 5 min prior to loading on an SDS–12.5% polyacrylamide gel. Western blot analysis was performed on the bacteria recovered in vivo as described above as well as on aliquots of bacteria grown in vitro for infection of the mice.

RESULTS

Copy number of *ail* in *E. coli* affects serum resistance but not tissue culture invasion. The *ail* gene of *Y. enterocolitica* has been shown to confer an invasive phenotype to a previously noninvasive strain of *E. coli* (34). The ability to penetrate cultured cell lines is known to correlate with virulence (27, 35, 40, 52, 58); however, the contribution of *ail* to the pathogenic-

TABLE 1. Serum resistance phenotype and tissue culture invasion phenotype promoted by *ail* in *E. coli*

Strain	Plasmid copy no.[a]	% Invasion[b]	Serum resistance index[c]	Difference in log kill[d]	Relative amt of Ail[e]
Mid-log					
HB101/pMW1823	Low	0.015 ± 0.004	6.06 ± 0.70	0.07	0
HB101/pMW1824	Low	0.698 ± 0.085	5.99 ± 0.71		1.51
HB101/pBR322	Medium	0.004 ± 0.004	6.79 ± 0.05	2.20	0
HB101/pVM102	Medium	0.674 ± 0.103	4.59 ± 0.14		2.31
Stationary					
HB101/pMW1823	Low	0.039 ± 0.006	5.83 ± 0.27	1.76	0
HB101/pMW1824	Low	3.462 ± 0.384	4.07 ± 0.17		1.88
HB101/pBR322	Medium	0.014 ± 0.001	6.04 ± 0.31	4.02	0
HB101/pVM102	Medium	1.838 ± 0.303	2.02 ± 0.17		4.12

[a] The copy number of the low-copy-number plasmids (pMW1823 and pMW1824) is ~5; the copy number of the medium-copy-number plasmids (pBR322 and pVM102) is ~15 to 20 (50).

[b] Bacteria were grown and assayed for their invasion phenotype as described in Materials and Methods. Data show results of a single experiment done in duplicate with CHO cells; similar results were obtained in five experiments.

[c] Bacteria were grown and assayed for resistance to serum as described in Materials and Methods. Serum resistance index is represented by the log kill. Log_{10} kill = (log_{10} CFU/ml added initially) − (log_{10} CFU/ml surviving incubation in normal human serum). A high value for log kill represents a strain more sensitive to the bactericidal effects of serum, while a low value represents a more resistant strain. Values represent the average of a single experiment done in triplicate; similar results were obtained from nine experiments. The ranges represent the largest possible standard deviation for one experiment.

[d] Difference in log kill = (serum resistance index cloning vector) − (serum resistance index *ail*-containing vector). A larger difference in log kill represents a strain more resistant to the bactericidal effects of serum, while a smaller difference in log kill represents a more sensitive strain.

[e] Bacterial cultures were grown overnight (stationary phase) or to mid-logarithmic phase as indicated. Whole cell lysates were prepared, and the proteins were separated by SDS–12.5% polyacrylamide gel electrophoresis; equal amounts of protein were loaded from all samples. After transfer of the proteins to nitrocellulose, the filter was incubated with the anti-Ail monoclonal antibody 2B2 followed by a sheep anti-mouse immunoglobulin antibody conjugated to horseradish peroxidase as described in Materials and Methods. The relative amount of Ail present was determined after densitometry; the amount present in the Ail standard was set at 1.

ity of *Y. enterocolitica* has not been examined. Thus, the goal of this study was to investigate the role of *ail* in the infection process in an animal model. For these studies, we constructed a plasmid, pMW1824, carrying *ail* on a low-copy-number, pSC101-based vector to be used for complementation of an *ail* mutant in animal experiments.

The *ail* plasmid pMW1824 was tested in *E. coli* to verify the *ail*-associated phenotypes: serum resistance and invasion of tissue culture cells. In our initial study of serum resistance, we used cells grown to mid-logarithmic phase, since actively growing cultures are thought to be homogeneous and yield more reproducible results. For *E. coli* HB101 grown to mid-logarithmic phase, there was no significant difference in the log kill promoted by *ail* from pMW1824 compared with the vector pMW1823 (Table 1). In contrast, Bliska and Falkow (5) observed approximately a 6-log difference in *ail*-dependent serum resistance in stationary-phase cultures of *E. coli* HB101 carrying *ail* cloned into pBR322 (designated pVM102) (34). To determine if growth phase of the culture or *ail* copy number affected *ail*-dependent serum resistance, we examined stationary-phase cultures as well as HB101 carrying pVM102. We found that stationary-phase cultures of *E. coli* HB101/pMW1824 were more resistant to serum than mid-log-phase cultures (log kill of 4.07, compared with 5.99; Table 1). However, stationary-phase cultures of *E. coli* HB101/pMW1824 were less serum resistant than stationary-phase cultures of *E. coli* HB101/pVM102 (log kill of 4.07, compared with 2.02; Table 1), which carries *ail* in higher copy number. Thus, the *ail*-dependent serum resistance phenotype in *E. coli* was influenced by both the growth phase of the culture and the gene copy number.

To examine this in more detail, *E. coli* HB101 carrying pBR322 or pVM102 was tested in serum killing assays during various stages of growth. Serum resistance conferred by HB101/pVM102 was found to increase steadily throughout the entire growth phase (OD_{600} = 0.3 through 1.5; Table 2), or showed uniform levels of serum resistance throughout the growth phase (OD_{600} = 0.3 through 1.5; mean difference in log kill = 1.34 ± 0.24), depending on the assay or lot of serum. However, it was only after the strains had grown for 16 to 18 h (OD_{600} = 3.62 or 4.39) that Ail promoted high levels of serum resistance relative to the control strain (difference in log kill > 5 logs) (Table 2); this result was consistent regardless of which lot of serum was used.

Western blot analysis was performed on the cultures used for these serum killing assays to determine if there was a correlation between the relative amount of Ail present and levels of serum resistance. The amount of Ail synthesized by HB101/pVM102 increased slightly until the OD_{600} reached 1.04 and then remained constant in all cultures, including the overnight culture (Table 2). These results did not correlate with the serum resistance phenotype of this strain when tested throughout the growth phase. This finding suggests that the amount of Ail is not solely responsible for the magnitude of serum resistance. Similar results were observed when mid-log- and stationary-phase cultures of HB101 pMW1824 were compared (Table 1).

To determine if *ail*-dependent tissue culture invasion by *E. coli* was also affected by Ail quantity and growth phase, invasion assays of CHO cells were performed with *E. coli* carrying *ail* in low (pMW1824) and medium (pVM102) copy number, grown to mid-logarithmic and stationary phases. Although there was a slight effect of growth phase on the Ail-associated invasion phenotype, *ail* copy number had no effect on the invasion phenotype (Table 1).

Construction and in vitro characterization of an *ail* mutant of *Y. enterocolitica*. To examine the contribution of *ail* to the pathogenicity of *Y. enterocolitica*, an *ail* mutant was constructed. Filter matings of the *E. coli* donor SM10λ*pir*/pVM118 with the *Y. enterocolitica* 8081v^{Nal} recipient were performed. Plasmid pVM118 contains chromosomal DNA from *Y. enterocolitica*, including *ail* with a mini-Tn*10* element within the gene. Tetracycline-resistant exconjugants were isolated and evaluated by Southern blot analysis at the *ail* locus, using several restriction enzymes (data not shown). Several anomalies occurred during the construction of the *ail* mutant. First, the double crossover of the suicide plasmid, resulting in the *ail* mutant, was found at a higher frequency than the merodiploid

TABLE 2. Serum resistance promoted in *E. coli* HB101 carrying pBR322 or pVM102 (*ail*) during various stages of growth

Strain	OD_{600}	Serum resistance index[a]	Difference in log kill[b]	Relative amt of Ail[c]
HB101/pBR322	0.30	6.54 ± 0.07		
	0.63	6.70 ± 0.03		
	0.83	6.72 ± 0.05		
	1.20	6.76 ± 0.03		
	1.52	6.76 ± 0.04		
	4.39 (overnight)	6.28 ± 0.12		
HB101/pVM102 (*ail*)	0.32	4.44 ± 0.09	2.10	1
	0.60	4.05 ± 0.14	2.65	1.44
	0.84	3.75 ± 0.18	2.97	1.41
	1.04	3.50 ± 0.04	3.26	1.72
	1.52	2.77 ± 0.03	3.99	1.74
	3.62 (overnight)	0.83 ± 0.03	5.45	1.62

[a] Bacteria were grown and assayed for resistance to serum as described in Materials and Methods. Serum resistance index is represented by the log kill. Log_{10} kill = (log_{10} CFU/ml added initially) − (log_{10} CFU/ml surviving incubation in normal human serum). A high value for log kill represents a strain more sensitive to the bactericidal effects of serum, while a low value represents a more resistant strain. Values represent the average of a single experiment done in triplicate; similar results were obtained from four experiments.

[b] Difference in log kill = (serum resistance index cloning vector) − (serum resistance index *ail*-containing vector). A larger difference in log kill represents a strain more resistant to the bactericidal effects of serum, while a smaller difference in log kill represents a more sensitive strain.

[c] Bacterial cultures were grown at 37°C to various stages of the growth phase and prepared for Western analysis as described in Materials and Methods. Equal amounts (5 μg) of total cellular protein were separated on an SDS–12.5% polyacrylamide gel, transferred to nitrocellulose, and probed with the anti-Ail monoclonal antibody 2B2. The blot was then probed with a sheep anti-mouse immunoglobulin antibody conjugated to horseradish peroxidase and visualized as described in Materials and Methods. The relative amount of Ail in each sample was determined by densitometry; the value for the HB101/pVM102, OD_{600} = 0.32 sample was set at 1.

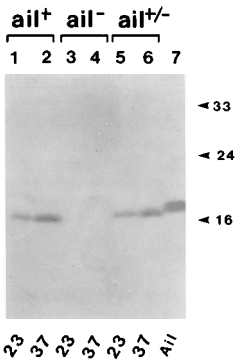

FIG. 1. Western blot analysis of the *ail* mutant. Bacteria were grown for 18 h in LB containing the appropriate antibiotics. Whole cell lysates were prepared, and the proteins separated by SDS–12.5% polyacrylamide gel electrophoresis as described in Materials and Methods. The Western blot was probed with the anti-Ail monoclonal antibody 2B2 (a gift of J. Bliska), followed by a goat anti-mouse immunoglobulin G antibody conjugated to alkaline phosphatase. The molecular masses of the protein standards are indicated in kilodaltons at the right. Lane 1, $8081v^{Nal}$ (wild type), 23°C; lane 2, $8081v^{Nal}$, 37°C; lane 3, YE2v (*ail*), 23°C; lane 4, YE2v, 37°C; lane 5, YE3v (*ail* merodiploid), 23°C; lane 6, YE3v, 37°C; lane 7, purified Ail.

strain; in fact, only one merodiploid strain was obtained. Second, the *ail* mutant strain, YE2v, contains an uncharacterized chromosomal DNA rearrangement. Both anomalies occurred repeatedly after several independent attempts to construct an *ail* mutant, though three different recipient *Yersinia* strains were used. An *ail* gene disrupted with a kanamycin resistance cassette was also used instead of *ail*::mini-Tn*10* to create the *ail* mutant, with the same result. Southern blot analysis of YE2v chromosomal DNA digested with *Cla*I, *Ava*I, or *Hin*dIII yielded the expected size fragments and demonstrated that the *ail* gene and the region 1.2 kb downstream and 10 kb upstream of the mini-Tn*10* insertion were the same as in the parental strain. However, when *Eco*RI was used to analyze the chromosomal DNA, the resulting *ail* fragment was larger than the 20-kb fragment found in the wild-type strain. The *Eco*RI pattern was uninterpretable, suggesting that a rearrangement has occurred somewhere beyond the 11.2-kb region defined by analysis with *Cla*I, *Ava*I, and *Hin*dIII (data not shown). The merodiploid, YE3v, also has this rearrangement. The reason why the region of the chromosome near *ail* rearranges with such high frequency is unknown.

Western blot analysis of the *ail* mutant indicated that YE2v did not produce Ail and that the merodiploid strain, YE3v, retained the ability to produce the protein (Fig. 1). Expression of *ail* is maximal at 37°C (46), and YE3v expressed higher levels of Ail at this temperature, as did the wild-type strain $8081v^{Nal}$ (Fig. 1). The tissue culture invasion phenotype of the *ail* mutant was determined in CHO cells and was reduced almost threefold relative to the wild-type strain (Table 3); this result is comparable to what was previously reported for an *ail* mutant (46). Invasion of the *ail* mutant into CHO cells was unaffected by the presence of the vector, pMW1823. However, YE2c expressing *ail* from pMW1824 had invasion levels comparable to those of the wild type carrying pMW1823 or pMW1824. The *ail* merodiploid strain, YE3c, also had an invasion phenotype comparable to that of the wild-type strain (data not shown).

Ail is known to promote serum resistance in *Y. enterocolitica* (5, 46); therefore, we examined the serum resistance phenotype of the *ail* mutant and the mutant complemented with the low-copy-number *ail* plasmid pMW1824. The *Yersinia* strains used for these experiments were cured of the virulence plasmid, a phenomenon that occurs during in vitro growth at 37°C. Thus, in this set of experiments, we have eliminated the serum protection afforded by YadA (1, 29, 39). The elimination of the virulence plasmid enables serum resistance to be assayed with bacteria grown at 37°C, body temperature for natural hosts of

TABLE 3. Serum resistance phenotype promoted by *ail* in *Y. enterocolitica*

Strain and relevant phenotype	Serum resistance index[a]		% Invasion[b]
	Mid-log	Stationary	
8081c (Ail^+)	3.19 ± 0.06	1.52 ± 0.10	83.12 ± 16.11
YE2c (Ail)	6.10 ± 0.13	6.50 ± 0.83	32.02 ± 3.72
8081c/pMW1823 (Ail^+)	3.33 ± 0.16	1.17 ± 0.10	126.27 ± 18.78
8081c/pMW1824 (Ail^+)	0.09 ± 0.22	0.16 ± 0.05	122.54 ± 7.97
YE2c/pMW1823 (Ail)	5.83 ± 0.50	5.38 ± 0.72	25.13 ± 1.12
YE2c/pMW1824 (Ail^+)	0.02 ± 0.12	0.35 ± 0.04	128.61 ± 9.95

[a] Bacteria were grown and assayed for resistance to serum as described in Materials and Methods. Serum resistance index is represented by the log kill. Log_{10} kill = (log_{10} CFU/ml added initially) − (log_{10} CFU/ml surviving incubation in normal human serum). A high value for log kill represents a strain more sensitive to the bactericidal effects of serum, while a low value represents a more resistant strain. Values represent the average of a single experiment done in triplicate; similar results were obtained from at least three experiments. The ranges represent the largest possible standard deviation for one experiment.

[b] Bacteria were grown overnight at 37°C and assayed for invasion into CHO cells as described in Materials and Methods. Data show results of a single experiment done in duplicate; similar results were obtained from four experiments.

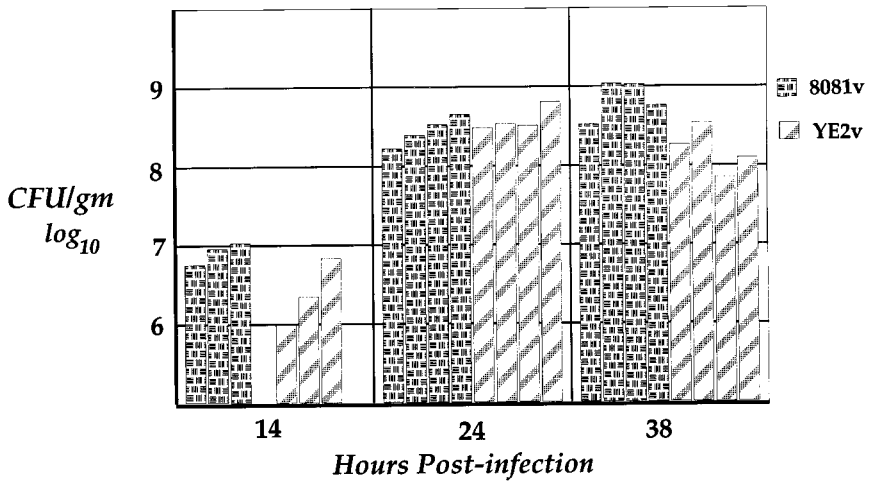

FIG. 2. Early colonization of the Peyer's patches by the *ail* mutant. BALB/c mice were infected intragastrically with approximately 10^8 CFU of the wild-type *Y. enterocolitica* strain, 8081v^{Nal}, or the *ail* mutant, YE2v. Peyer's patches were dissected at 14, 24, and 38 h postinfection. Each bar represents the number of bacteria recovered from a single mouse.

Yersinia species and the optimal condition for expression of *ail* (46).

The serum resistance phenotype of the wild-type strain 8081c was growth phase dependent, as seen in *E. coli* (Table 3). In contrast, the *ail* mutant, YE2c, was serum sensitive; compared with the wild-type phenotype, the serum resistance phenotype of the *ail* mutant was decreased by 3 logs at midlogarithmic phase and 5 logs at stationary phase (Table 3). The merodiploid strain, YE3c, had the same serum resistance phenotype as 8081c (data not shown). This finding suggests that the serum-sensitive phenotype of YE2c is due to the disruption of *ail* rather than to the chromosomal rearrangement, since YE2c and YE3c both have the rearrangement. To further ensure that the observed serum resistance effects were due to properties of Ail and not due to the rearrangement, the serum resistance phenotype was tested when *ail* was supplied in *trans* on the low-copy-number plasmid pMW1824. The *ail* plasmid pMW1824 increased the level of serum resistance of 8081c and restored levels of serum resistance similar to those of the *ail* mutant (Table 3). However, the vector pMW1823 contained in either the wild-type strain 8081c or YE2c produced no significant change in serum resistance levels relative to the parental strains at either stage of growth. The increased levels of serum resistance displayed by the wild-type strain carrying pMW1824 presumably resulted from the presence of several copies of *ail* in this strain and suggest that in *Y. enterocolitica*, as in *E. coli*, the serum resistance phenotype promoted by *ail* is copy number dependent. Similar results were obtained when *ail* from a non-American strain of *Y. enterocolitica* (serotype O9) was supplied in *trans* (data not shown).

Phenotype of the *ail* mutant in a murine model of infection. The use of tissue culture invasion models has been useful for the identification of putative invasion genes. However, a definitive role for the products of these genes can be determined only in the context of the natural infection cycle for a given pathogen. Therefore, we conducted a study to determine the contribution of *ail* to the pathogenicity of *Y. enterocolitica*, using a murine model for infection. The oral LD_{50} of YE2v was determined and found to be identical to that of the wild-type strain 8081v in BALB/c mice (3.43×10^6 CFU for YE2v and 5.47×10^6 CFU for 8081v). In addition, on average, the mean days of death were similar for these two strains (8.9 for YE2v and 8.4 for 8081v). Similar results were obtained with DBA/2 mice.

Because *ail* has been shown to confer adherence and invasion in vitro (34), the ability of YE2v to cross the intestinal epithelium and reach the Peyer's patches early after infection was tested. In this experiment, bacteria were grown at 23°C, fed intragastrically to mice, and quantitated from Peyer's patches at 14, 24, and 38 h postinfection. Throughout the time points tested, similar numbers of bacteria were recovered from the Peyer's patches of mice infected with the wild-type strain 8081v and mice infected with the *ail* mutant YE2v (Fig. 2). This experiment was repeated several times with similar results. Similar results were also obtained if the bacteria were grown at 37°C prior to infection (data not shown).

During the normal course of infection, *Y. enterocolitica* reaches the mesenteric lymph nodes from the Peyer's patches and then is able to establish a systemic infection by penetrating deeper tissues, such as the liver and spleen (14, 16). It was possible that Ail was not involved in the early stages of infection but was involved in later steps of the disease process. If this were the case, then the tissue distribution of YE2v over time might differ from that of the wild type, as was observed for the *inv* mutant (43). Therefore, a kinetics of infection experiment was conducted to determine the number of bacteria in the Peyer's patches and the deeper tissues throughout a 2-week period. Mice were infected with an oral LD_{50} dose of bacteria, and the Peyer's patches, mesenteric lymph nodes, liver, and spleen were harvested on days 1, 2, 4, 7, 10, and 14. No detectable difference was observed between 8081v and YE2v in either the tissue distribution of bacteria over time or the number of recoverable bacteria at each site (data not shown). Similar results were obtained when other doses of bacteria were used or when DBA/2 mice were used instead of BALB/c mice (data not shown).

Because we did not observe a phenotype for the *ail* mutant in the early in vivo invasion assay, we wanted to determine if *ail* is actually expressed during this phase of the infection. *ail* is known to be maximally expressed at 37°C and poorly expressed at room temperature (46); however, it is not known if *ail* is expressed in vivo. To test this, mice were infected intragastrically with 100 times the oral LD_{50}, and Peyer's patches were dissected at 48 h postinfection. Homogenates were collected

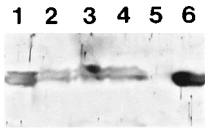

FIG. 3. Early in vivo expression of Ail. BALB/c mice were infected with 2×10^8 CFU of wild-type bacteria for 48 h. Peyer's patches were harvested, and whole cell lysates were prepared immediately from bacteria as described in Materials and Methods. Expression of *ail* was detected by probing with a polyclonal antibody directed against Ail (generated in this laboratory) followed by a donkey anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Amersham Corp.). The Western blot was visualized by using the Amersham ECL Western Blotting Detection System. Lane 1, aliquot of bacteria grown at 23°C prior to mouse infection, 2.4×10^8 CFU; lanes 2 to 4, Ail detected from in vivo-grown bacteria harvested from three different mice (9.40×10^5, 1.65×10^6, and 2.59×10^6 CFU, respectively); lane 5, no Ail detected from the homogenates of a mouse mock infected with PBS; lane 6, purified Ail.

from the infected Peyer's patches, and total cellular protein was retrieved from the recovered bacteria. The bacteria were collected directly from mice and were not grown in vitro prior to Western analysis for detection of Ail. Ail was detected in all mice examined at 48 h postinfection (Fig. 3). No Ail was detected from homogenates from a mouse infected with PBS as a control in this experiment. Comparison of the relative amount of Ail per lane (as determined by densitometry) with the number of bacteria used in each sample indicated that *Y. enterocolitica* growing in vivo produce approximately 30 times the amount of Ail produced by in vitro-cultured bacteria. This analysis assumes that the proportions of viable bacteria are similar for the two conditions. Nevertheless, this result clearly indicates that Ail is synthesized in vivo during the first 2 days of infection in amounts comparable to that synthesized in vitro (Fig. 3).

DISCUSSION

The study reported here was conducted to assess the role of Ail in the virulence of *Y. enterocolitica*. Several observations suggested that Ail may be a virulence factor. First, DNA homologous to *ail* is found only in clinical isolates of *Y. enterocolitica* associated with disease (35). Second, two phenotypes associated with Ail, invasion of tissue culture cells (34) and resistance to serum (5, 46), contribute to the virulence of other pathogens. Third, a protein closely related to Ail from *S. typhimurium*, PagC, has been shown to be a virulence factor (31). Thus, to test the hypothesis that Ail is a virulence factor, we constructed an *ail* mutant of *Y. enterocolitica* to test in a murine model of infection.

In a natural infection, *Y. enterocolitica* is ingested from contaminated food or water (6, 10). After ingestion, bacteria pass through the stomach and localize to the terminal ileum. Subsequently, they cross the intestinal epithelium to reach the Peyer's patches, where they multiply and occasionally spread to the mesenteric lymph nodes. Further spread to the liver and spleen is uncommon except in those who are immunocompromised. Early stages of an oral infection of mice are similar to what has been observed in humans (7).

The disease process can be divided into three distinct phases: (i) adhesion, invasion, and translocation across the intestinal epithelium of the terminal ileum, (ii) proliferation in local lymph nodes, followed by (iii) dissemination to deeper tissues to establish a systemic infection (14, 43). The effect of an *ail* mutation on each of these phases was examined. To determine if Ail is involved in the initial process of adhesion and translocation across the intestinal epithelium, the ability of the *ail* mutant to colonize the Peyer's patches early after intragastric inoculation was assessed. The *ail* mutant, YE2v, retained the ability to invade the intestinal epithelium and reached the Peyer's patches as well as the wild-type strain, 8081v. There is some evidence that Ail, YadA, or other unidentified factors may promote inefficient invasion into Peyer's patches, since *inv* mutants are able to traverse the intestinal epithelium of some mice, albeit with delayed kinetics (43). It is difficult to say if Ail contributes to this less efficient invasion process; if so, it could not be detected in a wild-type *inv yadA* background.

The absence of Ail did not affect proliferation in local lymph nodes or dissemination to deeper tissues, as no effect was observed for either the LD_{50}, tissue distribution of the bacteria or the number of CFU per gram of the various tissues when the *ail* mutant was compared with the wild-type strain. Together, these different animal infection experiments could demonstrate no effect of the *ail* mutation on the pathogenesis of a *Y. enterocolitica* infection in mice. While it is possible that *ail* does not contribute to the virulence of *Y. enterocolitica*, several other interpretations of this result are possible. Limitations of our model system may mask a phenotype for Ail. Therefore, testing in another animal model might demonstrate a role for Ail. Just as no role for *Y. enterocolitica* enterotoxin Yst was detected in mice (51) or gnotobiotic piglets (49), Yst did affect the ability of *Y. enterocolitica* to produce diarrhea, weight loss, and death in young rabbits (11). In addition, the strains of mice tested may be too sensitive to a *Y. enterocolitica* infection for a subtle effect to be detected, or the mice may lack a host defense, normally found in humans or other animals, to which *Y. enterocolitica* is sensitive. For example, both Ail and YadA contribute to *Y. enterocolitica*'s resistance to human serum (1, 5, 29, 39, 46), but this factor may not be pertinent to this animal model, since mouse serum is not bactericidal for *Y. enterocolitica* (17, 59).

In the process of characterizing our *ail* mutant and complementing plasmids used in this study, we extended the observations of others (5, 46) regarding the Ail-associated serum resistance phenotype. The Ail-dependent serum resistance phenotype in both *E. coli* and *Y. enterocolitica* was affected by both growth phase and copy number. The serum resistance levels in both organisms were significantly higher for bacteria grown to stationary phase than for bacteria grown to mid-logarithmic phase. Once a threshold level of Ail was reached in *E. coli*, the quantity of Ail did not change despite dramatic changes in the amount of serum resistance when the culture entered stationary phase. In contrast, there was no significant difference in the levels of invasion promoted by Ail under different conditions of growth or differences in gene copy number.

One interpretation of these results is that Ail interacts with another surface component of *Y. enterocolitica* and *E. coli* to confer serum resistance. Thus, early in the growth phase of the organism, the second component may not be present on the cell surface or may be present at levels insufficient for full expression of the serum resistance phenotype. Alternatively, the presence of some other cell surface components could partially mask Ail during early stages of the growth phase; loss of this component as the cells enter stationary phase would expose Ail. Growth phase regulation of serum resistance factors is not unprecedented. Some strains of *Leishmania* resist lysis by serum during stationary phase but are killed while in log phase (13). The mechanism of serum resistance in this case appears to be due to spontaneous release of the C5b-9 membrane attack complex (MAC) into the surrounding media or loose association of the MAC which results in failure to lyse the parasite (47). The mechanism of serum resistance promoted by Ail is unknown at present. However, Rck, a related

protein from *S. typhimurium*, prevents the formation of a fully polymerized tubular membrane attack complex (19). Another possible interpretation of the observed growth phase effect on the Ail-dependent serum resistance phenotype is that overnight cultures of bacteria may contain a significant number of nonviable cells which could nevertheless deplete the amount of complement. If this is the case, then assays done with mid-logarithmic-phase cultures may more accurately reflect the level of serum protection conferred by Ail, and this level is considerably less than previously reported (5, 46).

ACKNOWLEDGMENTS

We thank Cyndy Pepe for the low-copy-number plasmid pRS1823, Rafael Martinez for the wild-type 8081v^{Nal} strain, Jim Bliska for the monoclonal anti-Ail antibody 2B2, and Karen Beer for making the anti-Ail polyclonal antibody. We thank Jefferey F. Miller, Susan Valone, Susan Kinder, Deborah Schmiel, Glenn Young, and Yiping Han for critical reading of the manuscript. We also thank Susan Valone for technical help with the serum killing assays and M. John Pickett for the use of his laboratory space.

This work was supported in part by a Sigma Xi Grants-in-Aid award to M.R.W. and by National Institutes of Health grant AI27342 to V.L.M., who is a Pew Scholar in the Biomedical Sciences.

REFERENCES

1. **Balligand, G., Y. Laroche, and G. Cornelis.** 1985. Genetic analysis of virulence plasmid from a serogroup 9 *Yersinia enterocolitica* strain: role of outer membrane protein P1 in resistance to human serum and autoagglutination. Infect. Immun. **48:**782–786.
2. **Barondess, J. J., and J. Beckwith.** 1990. A bacterial virulence determinant encoded by the lysogenic coliphage lambda. Nature (London) **346:**871–874.
3. **Beer, K. B., and V. L. Miller.** 1992. Amino acid substitutions in naturally occurring variants of Ail result in altered invasion activity. J. Bacteriol. **174:**1360–1369.
4. **Bliska, J. B., M. C. Compass, and S. Falkow.** 1993. The *Yersinia pseudotuberculosis* adhesin YadA mediates intimate bacterial attachment to and entry into HEp-2 cells. Infect. Immun. **61:**3914–3921.
5. **Bliska, J. B., and S. Falkow.** 1992. Bacterial resistance to complement killing mediated by the Ail protein of *Yersinia enterocolitica*. Proc. Natl. Acad. Sci. USA **89:**3561–3565.
6. **Bottone, E. J.** 1977. *Yersinia enterocolitica*: a panoramic view of a charismatic organism. Crit. Rev. Microbiol. **5:**211–241.
7. **Carter, P. B.** 1975. *Yersinia* enteritis, animal model: oral *Yersinia enterocolitica* infection of mice. Am. J. Pathol. **81:**703–706.
8. **China, B., B. T. N'guyen, M. DeBruyere, and G. R. Cornelis.** 1994. Role of YadA in resistance of *Yersinia enterocolitica* to phagocytosis by human polymorphonuclear leukocytes. Infect. Immun. **62:**1275–1281.
9. **Cornelis, G., and C. Colson.** 1975. Restriction of DNA in *Yersinia enterocolitica* detected by recipient ability for a derepressed R factor from *Escherichia coli*. J. Gen. Microbiol. **87:**285–291.
10. **Cover, T. L., and R. C. Aber.** 1989. *Yersinia enterocolitica*. N. Engl. J. Med. **321:**16–24.
11. **Delor, I., and G. R. Cornelis.** 1992. Role of *Yersinia enterocolitica* Yst toxin in experimental infection of young rabbits. Infect. Immun. **60:**4269–4277.
12. **Emödy, L., J. Heessemann, H. Wolf-Watz, M. Skurnik, G. Kapperud, P. O'Toole, and T. Wadström.** 1989. Binding to collagen by *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*: evidence for *yopA*-mediated and chromosomally encoded mechanisms. J. Bacteriol. **171:**6674–6679.
13. **Franke, E. D., P. B. McGreevy, S. P. Katz, and D. L. Sacks.** 1985. Growth cycle-dependent generation of complement-resistant *Leishmania* promastigotes. J. Immunol. **134:**2713–2718.
14. **Grutzkau, A., C. Hanski, H. Hahn, and E. O. Riecken.** 1990. Involvement of M cells in the bacterial invasion of Peyer's patches: a common mechanism shared by *Yersinia enterocolitica* and other enteroinvasive bacteria. Gut **31:** 1011–1015.
15. **Hackett, J., P. Wyk, P. Reeves, and V. Mathan.** 1987. Mediation of serum resistance in *Salmonella typhimurium* by an 11-kilodalton polypeptide encoded by the cryptic plasmid. J. Infect. Dis. **155:**540–549.
16. **Hanski, C., U. Kutschka, H. P. Schmoranzer, M. Naumann, A. Stallmach, H. Hahn, H. Menge, and E. O. Riecken.** 1989. Immunohistochemical and electron microscope study of interaction of *Yersinia enterocolitica* serotype O8 with intestinal mucosa during experimental enteritis. Infect. Immun. **57:**673–678.
17. **Hanski, C., M. Naumann, A. Grutzkau, G. Pluschke, B. Friedrich, H. Hahn, and E. O. Riecken.** 1991. Humoral and cellular defense against intestinal murine infection with *Yersinia enterocolitica*. Infect. Immun. **59:**1106–1111.
18. **Heffernan, E. J., J. Harwood, J. Fierer, and D. Guiney.** 1992. The *Salmonella typhimurium* virulence plasmid complement resistance gene *rck* is homologous to a family of virulence-related outer membrane proteins, including *pagC* and *ail*. J. Bacteriol. **174:**84–91.
19. **Heffernan, E. J., S. Reed, J. Hackett, J. Fierer, C. Roudier, and D. Guiney.** 1992. Mechanism of resistance to complement-mediated killing of bacteria encoded by the *Salmonella typhimurium* virulence plasmid gene *rck*. J. Clin. Invest. **90:**953–964.
20. **Heffernan, E. J., L. Wu, J. Louie, S. Okamoto, J. Fierer, and D. Guiney.** 1994. Specificity of the complement resistance and cell association phenotypes encoded by the outer membrane protein genes *rck* from *Salmonella typhimurium* and *ail* from *Yersinia enterocolitica*. Infect. Immun. **62:**5183–5186.
21. **Isberg, R. R.** 1989. Determinants for thermoinducible cell binding and plasmid-encoded penetration detected in the absence of the *Yersinia pseudotuberculosis* invasin protein. Infect. Immun. **57:**1998–2005.
22. **Isberg, R. R., and S. Falkow.** 1985. A single genetic locus encoded by *Yersinia pseudotuberculosis* permits invasion of cultured animal cells by *Escherichia coli* K12. Nature (Lond.) **317:**262–264.
23. **Isberg, R. R., and J. M. Leong.** 1990. Multiple β1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell **60:**861–871.
24. **Isberg, R. R., D. L. Voorhis, and S. Falkow.** 1987. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell **50:**769–778.
25. **Kapperud, G., E. Namork, M. Skurnik, and T. Nesbakken.** 1987. Plasmid-mediated surface fibrillae of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*: relationship to the outer membrane protein YOP1 and possible importance for pathogenesis. Infect. Immun. **55:**2247–2254.
26. **Kolter, R., M. Inuzuka, and D. R. Helinski.** 1978. Transcomplementation-dependent replication of a low molecular weight origin fragment from plasmid RK6. Cell **15:**1199–1208.
27. **Lee, W. H., P. P. McGrath, P. H. Carter, and E. L. Eide.** 1977. The ability of some *Yersinia enterocolitica* strains to invade HeLa cells. Can. J. Microbiol. **23:**1714–1722.
28. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Martinez, R. J.** 1989. Thermoregulation-dependent expression of *Yersinia enterocolitica* protein 1 imparts serum resistance to *Escherichia coli* K-12. J. Bacteriol. **171:**3732–3739.
30. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 431. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Miller, S. I., A. M. Kukral, and J. J. Mekalanos.** 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. USA **86:**5054–5058.
32. **Miller, V. L.** 1992. *Yersinia* invasion genes and their products. ASM News **58:**26–33.
33. **Miller, V. L., J. B. Bliska, and S. Falkow.** 1990. Nucleotide sequence of the *Yersinia enterocolitica ail* gene and characterization of the Ail protein product. J. Bacteriol. **172:**1062–1069.
34. **Miller, V. L., and S. Falkow.** 1988. Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of epithelial cells. Infect. Immun. **56:**1242–1248.
35. **Miller, V. L., J. J. Farmer III, W. E. Hill, and S. Falkow.** 1989. The *ail* locus is found uniquely in *Yersinia enterocolitica* serotypes commonly associated with disease. Infect. Immun. **57:**121–131.
36. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2775–2583.
37. **Owen, R. L.** 1977. Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. Gastroenterology **72:**440–451.
38. **Owen, R. L., N. F. Pierce, R. T. Apple, and W. C. Cray, Jr.** 1986. M cell transport of *Vibrio cholerae* from the intestinal lumen into Peyer's patches: a mechanism for antigen sampling and for microbial transepithelial migration. J. Infect. Dis. **153:**1108–1118.
39. **Pai, C. H., and L. DeStephano.** 1982. Serum resistance associated with virulence in *Yersinia enterocolitica*. Infect. Immun. **35:**605–611.
40. **Pedersen, K. B., S. Winblad, and V. Bitsch.** 1979. Studies on the interaction between different O-serotypes of *Yersinia enterocolitica* and HeLa cells. Acta Pathol. Microbiol. Scand. Sect. B **87:**141–145.
41. **Pepe, C. M.** 1993. Ph.D. thesis. University of California, Los Angeles.
42. **Pepe, J. C., J. L. Badger, and V. L. Miller.** 1994. Growth phase and low pH affect the thermal regulation of the *Yersinia enterocolitica inv* gene. Mol. Microbiol. **11:**123–135.
43. **Pepe, J. C., and V. L. Miller.** 1993. *Yersinia enterocolitica* invasin: a primary role in the initiation of infection. Proc. Natl. Acad. Sci. USA **90:**6473–6477.
44. **Pepe, J. C., J. W. A. Olson, and V. L. Miller.** Unpublished data.
45. **Pepe, J. C., M. R. Wachtel, E. A. Wagar, and V. L. Miller.** Unpublished data.
46. **Pierson, D. E., and S. Falkow.** 1993. The *ail* gene of *Yersinia enterocolitica* has a role in the ability of the organism to survive serum killing. Infect. Immun. **61:**1846–1852.

47. **Puentes, S. M., R. P. DaSilva, D. L. Sacks, C. H. Hammer, and K. A. Joiner.** 1990. Serum resistance of metacyclic stage *Leishmania major* promastigotes is due to release of C5b-9. J. Immunol. **145:**4311–4316.
48. **Pulkkinen, W. S., and S. I. Miller.** 1991. A *Salmonella typhimurium* virulence protein is similar to a *Yersinia enterocolitica* invasion protein and a bacteriophage lambda outer membrane protein. J. Bacteriol. **173:**86–93.
49. **Robins-Browne, R. M., S. Tzipori, G. Gonis, J. Hayes, M. Withers, and J. Kaya Prpic.** 1985. The pathogenesis of *Yersinia enterocolitica* infection in gnotobiotic piglets. J. Med. Microbiol. **19:**297–308.
50. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., vol. 1, p. 1.4. Cold Spring Harbor Laboratory, Plainview, N.Y.
51. **Schiemann, D. A.** 1981. An enterotoxin-negative strain of *Yersinia enterocolitica* serotype O:3 is capable of producing diarrhea in mice. Infect. Immun. **32:**571–574.
52. **Schiemann, D. A., and J. A. Devenish.** 1982. Relationship of HeLa cell infectivity to biochemical, serological, and virulence characteristics of *Yersinia enterocolitica*. Infect. Immun. **35:**497–506.
53. **Schulze-Koops, H., H. Burkhardt, J. Heesemann, T. Kirsch, B. Swoboda, C. Bull, S. Goodman, and F. Emmrich.** 1993. Outer membrane protein YadA of enteropathogenic *Yersiniae* mediates specific binding to cellular but not plasma fibronectin. Infect. Immun. **61:**2513–2519.
54. **Schulze-Koops, H., H. Burkhardt, J. Heesemann, K. von der Mark, and F. Emmrich.** 1992. Plasmid-encoded outer membrane protein YadA mediates specific binding of enteropathogenic yersiniae to various types of collagen. Infect. Immun. **60:**2153–2159.
55. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
56. **Stoorvogel, J., M. J. A. W. M. van Bussel, J. Tommassen, and J. A. M. van de Klundert.** 1991. Molecular characterization of an *Enterobacter cloacae* outer membrane protein (OmpX). J. Bacteriol. **173:**156–160.
57. **Tertti, R., M. Skurnik, T. Vartio, and P. Kuusela.** 1992. Adhesion protein YadA of *Yersinia* species mediates binding of bacteria to fibronectin. Infect. Immun. **60:**3021–3024.
58. **Une, T.** 1977. Studies on the pathogenicity of *Yersinia enterocolitica*. I. Experimental infection in rabbits. Microbiol. Immunol. **21:**349–363.
59. **Wachtel, M. R., and V. L. Miller.** Unpublished data.
60. **Walker, R. I., E. A. Schmauder-Chock, and J. L. Parker.** 1988. Selective association and transport of *Campylobacter jejuni* through M cells of rabbit Peyer's patches. Can. J. Microbiol. **34:**1142–1147.
61. **Wassef, J. S., D. F. Keren, and J. L. Mailloux.** 1989. Role of M cells in initial antigen uptake and in ulcer formation in the rabbit intestinal loop model of shigellosis. Infect. Immun. **57:**858–863.
62. **Wolf, J. L., D. H. Rubin, R. Finberg, R. S. Kauffman, A. H. Sharpe, J. S. Trier, and B. N. Fields.** 1981. Intestinal M cells: a pathway for entry of reovirus into the host. Science **212:**471–472.
63. **Yang, Y., and R. R. Isberg.** 1993. Cellular internalization in the absence of invasin expression is promoted by the *Yersinia pseudotuberculosis yadA* product. Infect. Immun. **61:**3907–3913.
64. **Young, V. B., V. L. Miller, S. Falkow, and G. K. Schoolnik.** 1990. Sequence, localization, and function of the invasin protein of *Yersinia enterocolitica*. Mol. Microbiol. **4:**1119–1128.