INFECTION AND IMMUNITY, May 1997, p. 1786–1792 Vol. 65, No. 5
0019-9567/97/$04.00+0

The *spv* Genes on the *Salmonella dublin* Virulence Plasmid Are Required for Severe Enteritis and Systemic Infection in the Natural Host

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Received 10 December 1996/Returned for modification 16 January 1997/Accepted 16 February 1997

The pathogenic role of the *spv* (*Salmonella* plasmid virulence) genes of *Salmonella dublin* was determined in the natural, bovine host. Since the lack of overt signs of enteritis or enterocolitis due to *Salmonella* infections in mice has limited the development of a convenient experimental system to study enteric disease, we used calves to study the contribution of the *spv* genes to *S. dublin*-induced salmonellosis. Since the SpvR transcriptional regulator is required for expression of the *spvABCD* operon, we constructed an *spvR* knockout mutation in a calf-virulent strain of *S. dublin*. Calves were infected with the wild-type strain, an *spvR* mutant, and an *spvR* mutant containing a complementing plasmid. Calves that were infected with the wild type or the complemented *spvR* mutant rapidly developed severe diarrhea and became moribund. Calves that were infected with the *spvR* mutant showed little or no clinical signs of systemic salmonellosis and developed only mild diarrhea. The survival and growth of the wild-type strain and the *spvR* mutant were determined by using blood-derived bovine monocytes. Wild-type *S. dublin* survived and grew inside cells, while the *spvR* mutant did not proliferate. These results suggest that the *spv* genes of *S. dublin* promote enhanced intracellular proliferation in intestinal tissues and at extraintestinal sites in the natural host.

Members of the genus *Salmonella* infect a wide variety of vertebrate hosts, including reptiles, birds, and mammals. Natural infection involves ingestion of organisms and colonization of the gastrointestinal tract. Although enteritis is a common feature of salmonellosis, involvement of systemic tissues with or without intestinal disease is an important complication of infection. The manifestations of *Salmonella* infection depend on both host and bacterial factors. The lack of overt signs of enteritis or enterocolitis in mice, the major animal model for salmonellosis, has limited the development of a convenient experimental system with which to study enteric disease. Although a large number of nontyphoid *Salmonella* serovars have been associated with enteritis in humans and large-animal hosts such as cattle and swine (45), certain serovars are particularly associated with systemic infections, and these strains are capable of producing lethal infections in experimental models using mice or chickens. A common feature of these serovars is the presence of the plasmid-encoded *spv* genes, which enhance the virulence of these strains in systemic models of disease (23, 24, 29). The *spv* genes from different serovars are highly homologous, and they are found in nearly all natural isolates of *Salmonella* that are host adapted to animals (*Salmonella dublin*, *S. choleraesuis*, *S. gallinarum-pullorum*, and *S. abortusovis*) but not in *S. typhi*. Plasmid-encoded *spv* genes are also found in the common broad-host-range serovars *S. typhimurium* and *S. enteritidis*, but only a variable proportion of isolates carry these virulence plasmids.

The *spv* locus consists of five genes, designated *spvRABCD*, with *spvABCD* organized as an operon (36). *spvR* encodes a MetR/LysR-type transcriptional activator and is transcribed separately from the *spvABCD* structural genes. SpvR binds to both the *spvR* and *spvA* promoters and is required for expression of the *spvABCD* genes (17, 22, 36). Transcription from both the *spvR* and *spvA* promoters is induced during stationary phase under control of the alternative sigma factor RpoS (11, 17, 18, 35). The *spv* genes are strongly expressed by *S. typhimurium* and *S. dublin* following uptake of the bacteria into eukaryotic cells (19, 48). The intracellular induction of the *spv* locus is controlled by both SpvR and RpoS (12, 19).

The *spv* genes were initially defined on the basis of their ability to enhance the virulence of *Salmonella* serovars capable of producing lethal disease in mice (23, 24, 29). In general, the *spv* genes decrease the 50% lethal doses of strains for mice 10- to 10^6-fold, depending on the serovar and route of infection. In studies using the murine model, the *spv* genes have been shown not to affect colonization of the bowel or invasion through the intestinal mucosa, and they are not required for *Salmonella* to infect extraintestinal tissues such as the liver and spleen (27, 32, 42, 46). However, the *spv* genes significantly increase the growth rate of organisms within these tissues, probably in an intracellular compartment (20, 21, 29, 32). Attempts to demonstrate *spv*-mediated differences in growth within murine macrophages in vitro have been unsuccessful (27, 49).

Investigation of the role of the *spv* genes in nonmurine hosts has been limited. The virulence plasmid of *S. gallinarum* is required for the syndrome of fowl typhoid, a disseminated infection not characterized by enteritis (3). In swine, the plasmid of *S. choleraesuis* enhanced systemic disease when the strains were given by intravenous inoculation, but oral infection was not tested (16). The effect of the *S. dublin* virulence plasmid has been tested in calves, using both naturally occurring plasmid-free strains as well as a plasmid-cured derivative (53). This study showed that the mortality of calves infected with the plasmid-cured strain was less than that of animals given a comparable dose of wild-type organisms. In addition,

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TABLE 1. Strains and plasmids used

Strain or plasmid	Genotype	Source or reference
Salmonella		
SL2260	Wild type, virulent, virulence plasmid pSD2260	Pat Blanchard
SL2297	SL2260 plasmid cured	This study
SL2303	*S. dublin* Lane pSD6	14
SL2403	SL2260 *spvR*::Tn*5*::*oriT*	This study
SL2495	SL2403 pSB2300	This study
E. coli DH5α	*endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 recA1 pyrA*(Nal^r) *relA1* Δ(*lacIAA-argF*) *U169 deoR*[ϕ80*dlac*Δ(*lacZ*)*M15*]	Lab stock
Plasmids		
pSC101	Wild-type plasmid with a single *Eco*RI site, Tet^r	15
pFF18	2.3-kb *Sal*I/*Eco*RI fragment contained *spvR* in pUC18	17
pSB2300	2.3-kb *Sal*I/*Eco*RI *spvR* fragment from pFF18 cloned in the *Eco*RI site of pSC101	This study

the majority of the calves infected with the plasmid-cured derivative as well as the wild-type parent strain developed diarrhea, although the severity of the diarrhea was not reported. In each of these studies, the effect of the entire virulence plasmid, rather than the specific function of the *spv* locus, was investigated.

Our hypothesis was that definition of the role of the *spv* genes of *S. dublin* for infection of the natural host, cattle, would reveal a clearer understanding of the pathogenesis unattainable in the mouse model. These studies demonstrate that expression of the *spv* locus is required for production of severe enteritis as well as lethal disease in young calves. Furthermore, the use of primary bovine macrophages reveals a difference in the intracellular survival between wild-type *S. dublin* and an *spv* mutant.

MATERIALS AND METHODS

Bacterial strains, growth media, and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Wild-type SL2260 was obtained from Patricia Blanchard, California Veterinary Diagnostic Laboratory, University of California, Davis, Tulare, Calif. Bacteria were grown in LB medium, and antibiotics were added as needed: penicillin, 200 μg ml^{-1}; tetracycline, 10 μg ml^{-1}; and kanamycin, 50 μg ml^{-1}.

Strain construction and cloning. The virulence plasmid in *S. dublin* SL2260 was eliminated by methods previously described by our laboratory (14). Loss of the virulence plasmid was confirmed by Southern analysis and probing with the 14-kb *Sal*I fragment from pCR4 containing the entire *spv* operon (37, 50). To construct an *spvR* mutant of SL2260, a P22 lysate was made on *S. dublin* Lane pSDL15-1 harboring a Tn*5*-*oriT* insertion in the *spvR* gene (4) as described previously (11). The resulting lysate was used to transduce SL2260 to kanamycin resistance, and resulting transductants were purified on EBU medium containing kanamycin to screen for phage-free colonies (41). Verification of the interruption of the *spvR* gene on the virulence plasmid in SL2260 was by Southern hybridization, and this strain was designated SL2403. Chromosomal DNA preparations were performed as previously described (39), and Southern hybridization and detection were performed with an Amersham ECL kit as instructed by the manufacturer.

Cloning of the *spvR* gene in pSC101 for complementation. To complement the *spvR* mutation in SL2403, the 2.3-kb *Sal*I/*Eco*RI fragment from pFF18 (17) was cloned into the *Eco*RI site of pSC101 (15). pFF18 was restricted with *Sal*I/*Eco*RI and treated with Klenow enzyme, and the 2.3-kb fragment containing the functional *spvR* gene was purified by gel electrophoresis and electroelution. pSC101 was restricted with *Eco*RI, the ends were made blunt by treatment with Klenow enzyme and ligated with the *spvR* fragment, and the plasmid was transformed into DH5α; the transformants were selected on LB agar containing tetracycline. Cloning of the *spvR* fragment into pSC101 was verified by restriction digests. Plasmid DNA was prepared from a clone containing pSC101-*spvR* (pSB2300) and electroporated into SL2403 by methods described previously (31), and transformants were selected on tetracycline- and kanamycin-containing LB agar.

Mouse infection studies. Female BALB/c mice were injected intraperitoneally with 2×10^4 to 5×10^4 CFU of an overnight culture of SL2260, SL2297, SL2403, or SL2495 grown in the appropriate antibiotics. Mice that received wild-type SL2260 or SL2495 died within 3 to 5 days or were euthanized if they became moribund. Mice that received SL2297 or SL2403 had no signs of salmonellosis, yet analysis of the livers and spleens revealed infection with the *Salmonella* strains used.

Calf infection studies. Twenty-one-day-old Friesian-Holstein calves were purchased from a local dairy (Dublin, Tex.) and maintained on antibiotic-free milk replacer. Fecal and blood samples of all calves were cultured prior to experimental infection and were negative for *Salmonella*. Overnight cultures of *S. dublin* were grown in LB broth with the appropriate antibiotics in Fernbach flasks. The cell density of *S. dublin* grown in this manner reaches approximately 2×10^9 to 5×10^9 cells per ml. Calves were infected orally with 2×10^{11} to 5×10^{11} bacteria either in LB or in a solution of 5% magnesium trisilicate, 5% sodium bicarbonate, and 5% magnesium carbonate to reduce stomach acid. This dose was chosen to ensure reproducible disease in all animals infected with the wild type. Calves were weighed, blood and fecal samples were collected for culture, and clinical signs were noted daily. Calves that became moribund were euthanized. The extent of diarrhea was scored by using the arbitrary scale described in reference 44 as follows: 0, normal feces with distinct conformation; 1, normal feces without distinct conformation; 2, soft feces with loss of distinct conformation; 3, loose feces with reduced solid matter; 4, running or watery feces with significantly reduced or little solid matter, with occasional blood stains and/or shreds of fibrinous membranes. Behavioral signs such as anorexia or depression were also noted. Animals surviving for 10 days were euthanized. Necropsy was performed, and tissues were prepared for histopathology and cultured for bacteria.

Necropsy, culture methods, and histopathology. Samples of feces were collected in sterile plastic bags, and samples of jugular venous blood were inoculated into blood culture medium (Difco). Fecal samples were transferred to XLT4 agar (Difco) plates and to tetrathionate broth enrichment (BBL) tubes via sterile cotton-tipped swabs. The plates and tubes were incubated overnight at 37°C. The tetrathionate samples were then subsequently transferred to XLT4 agar plates and incubated overnight at 37°C. Blood cultures were incubated initially for 24 h at 37°C before plating on XLT4 agar.

Tissue samples of liver, lung, spleen, mesenteric lymph node, Peyer's patch, kidney, ileum, jejunum, cecum, and colon were collected at necropsy and submitted for culture. Organ tissues (liver, lung, spleen, mesenteric lymph node, and kidney) were seared with a hot spatula to reduce surface contamination, cut with a sterile scalpel, and swabbed. Gut tissue and Peyer's patches were swabbed directly on the mucosal surface. Swabs were then treated as described above. The tissues listed above were collected in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, coded for blinded examination, and viewed by light microscopy.

Macrophage culture and infection. Macrophage infection assays were done as previously described by our groups (5–10, 19, 39, 47). Briefly, bovine venous blood was collected in Alsever's solution anticoagulant and diluted 1:1 with phosphate-buffered saline–citrate, and the mononuclear cells were collected by centrifugation onto Percoll. Cells were washed three times and resuspended in RPMI 1640 supplemented with 4% autologous bovine serum. Cells were allowed to adhere overnight in Teflon flasks, and the nonadherent population was removed by washing. Monocytes were allowed to differentiate into macrophages over 10 days in RPMI 1640 supplemented with 12% autologous serum. For infection with *S. dublin*, macrophages were harvested from the Teflon flasks, counted, seeded at a density of 10^5 cells per well into microtiter plates, centrifuged, and allowed to adhere overnight. Macrophage wells were then infected with *S. dublin* grown under conditions that have been shown not to express the *inv* locus, which is associated with macrophage cytotoxicity (13, 43). Macrophages were infected at a multiplicity of infection of 5 and centrifuged to optimize contact between bacteria and macrophages. After 30 min, extracellular bacteria were removed by washing and residual extracellular organisms were killed by the addition of gentamicin at 20 μg/ml. At each time point, triplicate wells were harvested by lysis of the macrophages in 1% deoxycholate, and the number of viable bacteria was determined by serial dilution and plate count.

TABLE 2. Virulence of *S. dublin* strains in mice[a]

Strain	Phenotype	No. dead
SL2260	Wild type, plasmid containing	3
SL2297	Plasmid cured	0
SL2403	*spvR* mutant	0
SL2495	SL2403 with pSC101-*spvR*	3

[a] Six-week-old BALB/c mice were infected i.p. with 10^4 bacteria. Each group contained three mice.

RESULTS

Strain construction and mouse virulence. A clinical isolate of *S. dublin* (now called SL2260) was obtained from a calf that died of *S. dublin* septicemia. All strains used in these studies are isogenic derivatives of this isolate. We constructed an *spvR* mutation in SL2260 by P22 transduction of an *spvR*::Tn*5*-*oriT* insertion from a construct previously made in our laboratory (4). The virulence plasmid in SL2260 was cured by methods previously described by our laboratory (4, 14) and confirmed by Southern hybridization (data not shown). To complement the *spvR* mutation in SL2403 in *trans*, the intact *spvR* gene was cloned into the *Eco*RI site of pSC101 (15). We have used pSC101 as a stable cloning vector for *Salmonella* to complement other mutations (39).

Wild-type SL2260, virulence plasmid-cured SL2297, *spvR* mutant SL2403, and SL2495 (containing the complementing plasmid) were tested for virulence in BALB/c mice. Mice received 10^4 organisms by the intraperitoneal route of infection. These results are shown in Table 2. Both wild-type SL2260 and SL2495 were virulent in mice, but the strains lacking the virulence plasmid (SL2297) or harboring an *spvR* mutation (SL2403) did not kill the mice even at this relatively high dose. SL2403 and SL2297 could be isolated from the livers and spleens of these mice (data not shown). These results confirm our earlier report that the *spv* region is required for the pathogenesis of *S. dublin* (38) and demonstrate that the *spvR* mutant can be complemented by the cloned *spvR* region on pSC101. These isogenic strains were used to infect calves in the experiments described below.

***spvR* is required for the pathogenesis of *S. dublin*.** *Salmonella dublin* is a host-adapted serovar that causes a systemic and often fatal disease in young animals and a chronic infection in older animals, providing the source for continued herd infection (33). The calf model of *S. dublin* infection has been used to determine virulence of wild-type *S. dublin* (52) and several genetically defined mutants of *S. dublin*, including *aroA* (34, 44) and virulence plasmid-cured (53) strains. Wallis et al. (53) reported that the virulence plasmid of *S. dublin* is required for fatal systemic infection rather than the enteric phase of the disease. To determine the role of the *spvR* gene, we chose to compare the virulence of the wild type and an *spvR* mutant in calves. Friesian-Holstein bull calves (14 to 21 days old) were purchased from a local dairy (Dublin, Tex.) and maintained on antibiotic-free milk replacer for 2 weeks. Fecal and blood samples of all calves were cultured to exclude the possibility of prior infection with *Salmonella*.

Two groups of three calves were orally infected with either wild-type SL2260 or the *spvR* mutant SL2403 at a final dose of 10^{11} CFU. The calves infected with SL2260 rapidly developed clinical signs of salmonellosis manifested by severe diarrhea, anorexia, and lethargy. Calves infected with SL2403 did not show any of these signs; they ate well and were alert and active. The clinical severity of enteric disease in the two groups is shown in Fig. 1. Interestingly, there was no significant difference in the mean daily body temperature between calves infected with the wild type and those infected with the *spvR* mutant throughout the course of the experiment (data not shown). Calves infected with SL2403 did have mild diarrhea, but none of these animals developed the severe, debilitating diarrhea seen in all of the animals infected with the wild-type strain. Within 7 days, all of the calves that were infected with wild-type organisms required euthanasia.

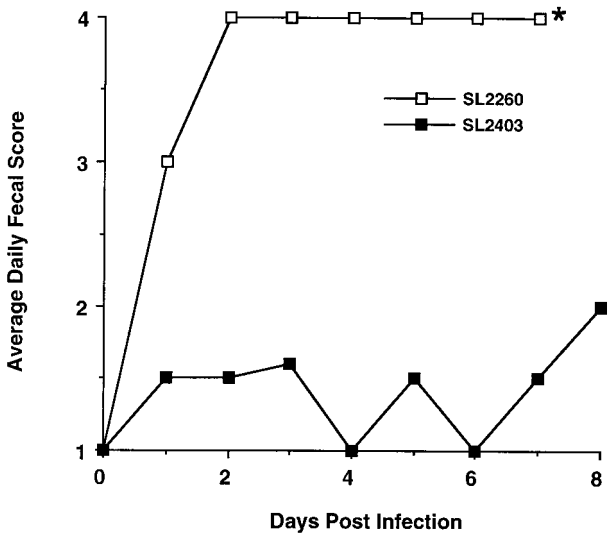

FIG. 1. Average daily fecal scores of calves infected with either wild-type SL2260 or *spvR* mutant SL2403. Fecal scores, a measure of the severity of diarrhea, were determined each day for calves according to the scale described in Materials and Methods. Calves that were infected with the wild type rapidly developed severe diarrhea, while calves infected with the *spvR* mutant did not. *, by day 7 postinfection, calves infected with the wild type had died or were euthanatized.

Culture results (Table 3) show that *S. dublin* grew in fecal samples from all animals in both groups by day 4 after inoculation. Calves infected with the *spvR* mutant had slightly fewer positive blood cultures. The finding that all animals infected with the *spvR* mutant eventually became blood culture positive on days 6 and 7 shows that this strain is able to invade from the intestinal tract and cause significant bacteremia, a result also seen in the mouse model. In summary, the results of this experiment suggest that the *spv* genes are involved in the pathogenesis of both severe enteric and systemic infections of calves.

TABLE 3. Summary of culture results for calves infected with SL2260 and SL2403

Day postinfection	No. of calves[a] with:			
	Positive fecal culture		Positive blood culture	
	SL2260	SL2403	SL2269	SL2403
0	0	0	0	0
1	1	1	0	0
2	2	1	3	2
3	3	2	2	1
4	3	3	3	1
5	3	3	3	3
6	3	3	3	3
7	Died	3	Died	0

[a] Each group contained three calves.

TABLE 4. Summary of culture results for calves infected with SL2260, SL2403, and SL2495[a]

Day postinfection	No. of calves/total								
	Positive fecal culture			Positive blood culture			With diarrhea		
	SL2260	SL2403	SL2495	SL2260	SL2403	SL2495	SL2260	SL2403	SL2495
0	0/3	0/3	0/4	0/3	0/3	0/4	0/3	0/3	0/4
1	3/3	3/3	4/4	3/3	0/3	3/4	3/3	1/3	2/4
2	3/3	3/3	4/4	3/3	0/3	3/4	3/3	1/3	4/4

[a] Daily blood and fecal samples were taken for culture. Within 2 days postinfection, calves that had been infected with wild-type SL2260 or the complemented *spvR* mutant SL2495 developed severe diarrhea and had *S. dublin* in their blood and feces. Calves that were infected with SL2403, the *spvR* mutant, had detectable levels of *Salmonella* in fecal cultures but had only sporadic diarrhea and did not develop positive blood cultures over this period. By the third day, calves in the SL2260 group began to require euthanasia.

Complementation of the *spvR* mutation in vivo in calves. Having established that expression of the *spv* operon is required for virulence in calves, we complemented the defect by supplying *spvR* in *trans* on a stable, low-copy-number plasmid. To ensure a more uniform survival of the infecting strains in the acidic environment of the abomasa of the calves maintained on milk replacer (51), we chose to infect with the bacteria suspended in a 5% solution of magnesium trisilicate, sodium bicarbonate, and magnesium carbonate (53). Three groups of calves were infected with either the wild type (SL2260), an *spvR* mutant (SL2403), or the complemented *spvR* mutant (SL2495). Wild-type-infected calves became febrile and lethargic within 24 h, developing grayish yellow, fetid feces which accumulated in the perineal region. These signs worsened as the wild-type-infected calves developed a more profound, bloody mucoid diarrhea and became more febrile and dehydrated, with all of the calves dying by the fifth day after infection. Analysis of blood and fecal culture data showed that they rapidly developed enteric and systemic infections (Table 4). Three of the four calves that were infected with SL2495, the complemented *spvR* mutant, became sick and died within 8 days. These animals rapidly developed severe diarrhea and bacteremia, indicating a systemic infection. Although all calves received the same inoculum of bacteria, one of the four calves survived, although this single animal was weak and did not eat well. Calves that were infected with the *spvR* mutant did not become sick and showed no signs of serious salmonellosis for the duration of the experiment, although one calf died of a non-*Salmonella* pneumonia. As in the first experiment, some animals infected with the *spvR* mutant developed a transient, mild diarrhea. There was no significant difference in the daily body temperature among the calves. The fact that the complemented *spvR* mutant caused disease and mortality at approximately the same rate as the wild type demonstrates in vivo complementation for virulence.

At necropsy, calves infected with wild-type *S. dublin* were found to have severe intestinal and extraintestinal disease. The bowel lumen was distended, and the ileum exhibited an acute fibrinohemorrhagic enteritis. Histologic examination of the ileal mucosa revealed extensive exfoliation with blunting and destruction of the villous architecture (Fig. 2B). A dense inflammatory infiltrate consisting of large numbers of neutrophils as well as mononuclear cells was seen. Occasionally, thin layers of fibrinocellular exudate were attached to the blunted villi. Fibrin thrombi were observed in submucosal capillaries of the ileum associated with edema. The livers of these calves infected with the wild-type strain had randomly distributed small foci of necrosis and associated reactive microgranuloma formation of the classical paratyphoid nodule type (Fig. 2C). The intestines from calves infected with the *spvR* mutant appeared normal, with no histopathology (Fig. 2A). Animals infected with the complemented *spvR* strain SL2495 had fluid-distended bowels indicative of catarrhal enteritis, with less fibrinohemorrhagic change than in wild-type-infected calves. Histology showed moderate inflammatory infiltrates with better preservation of the villous architecture. Cultures of tissues at necropsy showed widespread involvement with *S. dublin* in the intestines, Peyer's patches, mesenteric nodes, and systemic organs (spleen, liver, lungs, and kidneys) of wild-type-infected animals. Positive cultures were more sporadic for the *spvR* mutant, with the majority of animals from both experiments having negative spleen and liver cultures, while most host tissues were positive for the *spvR*-complemented strain. The mortality and major necropsy findings are summarized in Table 5. Although the clinical disease was less rapid for the complemented strain than for the wild-type strain, and the necropsy findings were not as severe, this strain was clearly capable of producing significant enteritis and ultimately fatal disease, indicating functional complementation of the *spvR* mutation.

The *spv* genes are required for in vitro growth in bovine blood-derived monocytes. Both plasmid-cured and *spvR* mutants of *S. dublin* grow equally well in broth or defined medium and in cultured murine macrophages. Our laboratory and others have speculated that the role of the *spv* genes on the virulence plasmid is to facilitate bacterial survival and replication in the tissues of the host, primarily inside macrophages (23–27, 32).

To test this hypothesis, we compared the survival and growth of the wild type, the *spvR* mutant, and the complemented *spvR* mutant in monocyte-derived bovine macrophages. Venous blood was collected, and monocytes were purified and allowed to differentiate into macrophages over 5 to 8 days in RPMI 1640 supplemented with autologous serum. Macrophages were infected with bacteria and at each time point, triplicate wells were harvested by lysis of the macrophages, and the number of viable bacteria was determined by serial dilution and plate count. These results are shown in Fig. 3. Wild-type SL2260 survived and grew in these cells, increasing in number over 6 h, while the *spvR* mutant did not proliferate. The complemented *spvR* mutant showed intermediate growth in these cells but grew significantly more than the *spvR* mutant. These results demonstrate a difference in the abilities of spv^+ and *spv* bacteria to proliferate inside bovine macrophages that closely parallels the difference in virulence seen in the calf infection experiments.

DISCUSSION

Use of the bovine host of *S. dublin* to study the role of the virulence plasmid *spv* genes provides a unique opportunity to determine the molecular mechanisms of natural infection and host adaptation. These studies in calves demonstrate that the

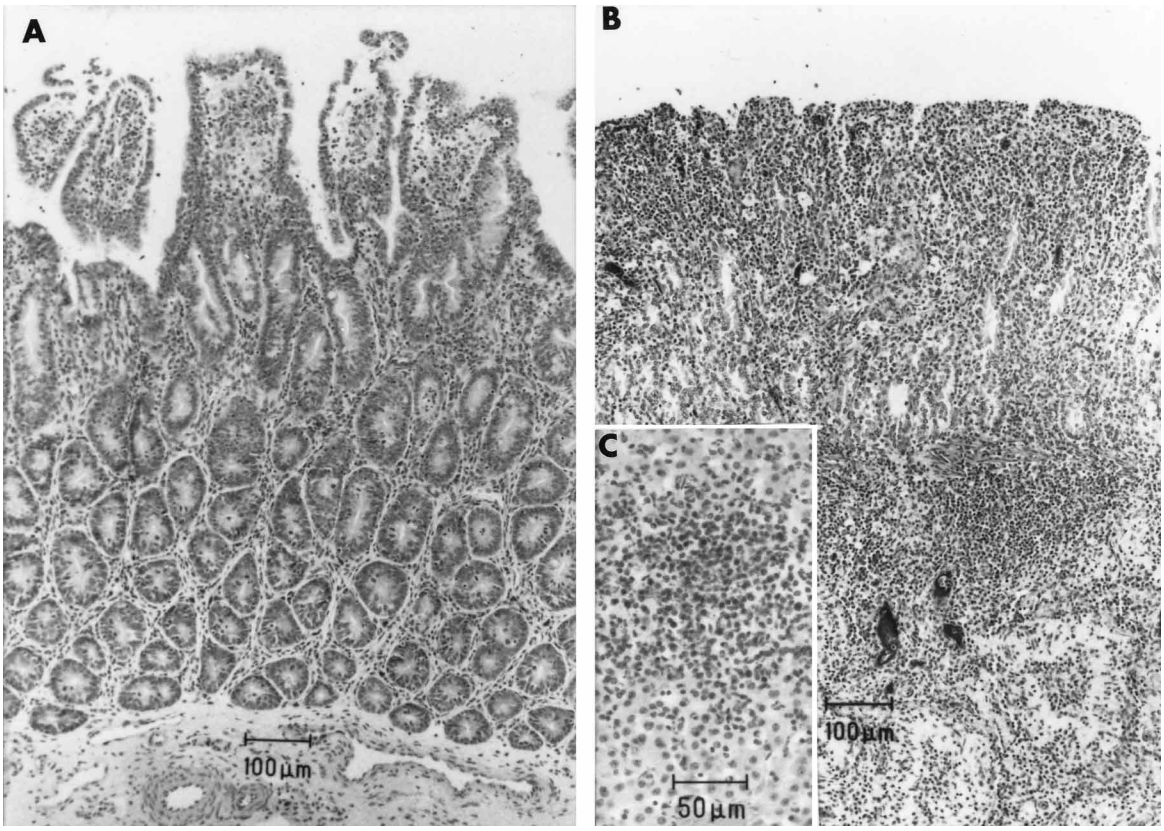

FIG. 2. Histopathology of ileal sections from calves infected with either SL2403 or SL2260. (A) Histologically normal ileum of a calf infected with *S. dublin spvR* mutant SL2403. (B) Acute erosive ileitis of a calf infected with *S. dublin* wild-type SL2260, demonstrating severe exfoliation and blunting of villi massively infiltrated with neutrophils and mononuclear cells. (C) Reactive hepatic microgranuloma (paratyphoid nodule) from a calf infected with SL2260.

S. dublin virulence plasmid *spv* genes affect both enteric and systemic disease processes. Previous experimental approaches have shown that the *spv* genes enhance the ability of nontyphoid *Salmonella* strains to cause progressive extraintestinal infection, but the role of these genes in intestinal disease was not clear (23, 24, 29). We used a specific mutation in *spvR* to demonstrate that the *spv* locus, rather than other genes on the *S. dublin* virulence plasmid, were essential for the virulence effect in calves. In addition, we were able to complement the *spvR* mutant by using a cloned *spvR* gene. This finding proves that the defect in *spvR* is responsible for the attenuation of the mutant.

Extensive studies of mice have established that the *spv* genes increase the proliferation of *Salmonella* in a variety of tissues (27, 32, 46). Although organisms are found in the bowel wall and Peyer's patches after oral inoculation, mice develop neither clinical evidence of intestinal disease nor the pathologic findings of inflammatory enteritis. This deficiency of the mouse model has severely limited the identification of *Salmonella* genes that have a role in enteric virulence. In contrast, large domestic animals such as cattle frequently develop diarrhea as a manifestation of salmonellosis, and calves can exhibit severe enteritis with dehydration as a complication of infection by particularly virulent strains such as *S. dublin* (52). Our findings underscore the value of testing defined mutants for virulence in the natural host, since the mouse studies predicted only the systemic virulence phenotype of the *spv* genes, not the effect on enteric disease.

TABLE 5. Summary of calf infection experiments

Expt	No. dead/total	Necropsy finding
1		
SL2260	3/3	Acute catarrhal enteritis with regional fibrinohemorrhagic ileitis
SL2403	0/3	Normal bowel appearance
2		
SL2260	3/3	Acute catarrhal enteritis with regional fibrinohemorrhagic ileitis
SL2495	3/4	Acute catarrhal enteritis
SL2403	0/3	Normal bowel appearance

The mechanism of action of the *spv* genes has not been fully elucidated. Based on studies of mice, the *spv* genes appear to enhance the growth rate of the organism within an intracellular compartment (30). In coinfection experiments between isogenic spv^+ and virulence plasmid-cured *spv* strains, the spv^+ organisms do not increase the growth of *spv* bacteria, suggesting that the *spv* genes do not have a global or even local effect on the host response (28). Instead, spv^+ bacteria outgrow the virulence plasmid-cured *spv* organisms. Although macrophages in the liver and spleen are postulated to be major targets for the intracellular growth of virulent *Salmonella*, studies of mouse macrophage infection in vitro have failed to demonstrate a difference in macrophage survival between spv^+ and *spv* strains (27, 49). Due to these problems in the mouse system, we used primary bovine monocyte-derived macrophages to study the role of the *spv* genes in cells from the natural host.

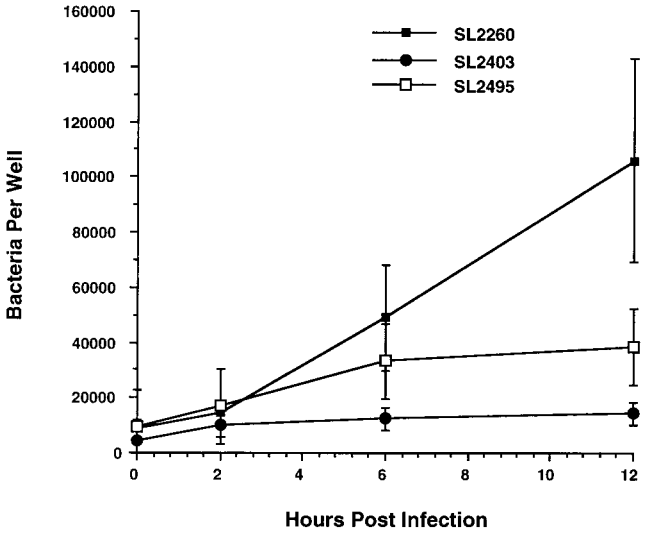

FIG. 3. Survival of *S. dublin* strains in bovine blood-derived monocytes. Blood-derived monocytes were prepared, allowed to differentiate for 7 days, and then infected with wild-type SL2260, *spvR* mutant SL2403, and complemented *spvR* mutant SL2495. Bacterial counts were averaged from triplicate wells for each time point, the standard deviation was calculated, and the data were plotted.

We were able to demonstrate a reproducible difference in macrophage survival between wild-type *S. dublin* and the *spvR* derivative. There are a number of possible reasons for the differences between the results in mouse and bovine cells. The in vitro macrophage survival assay requires a subtle balance between intracellular growth and killing, and the population of bacteria is heterogeneous (1, 2, 7). If bacteria grow too rapidly and/or exert a cytotoxic effect, the macrophage will die and the bacteria will be killed by the extracellular gentamicin (40). We postulate that primary bovine macrophages are able to partially restrict the cytotoxicity of wild-type *S. dublin*, allowing the differences in survival of the mutants to be measured in this assay.

On the basis of our results in the bovine host, we propose that the *spv* genes enhance proliferation of *S. dublin* in both intestinal tissue and extraintestinal sites. The macrophage is likely to be a major target cell for *S. dublin* in all organs. In the intestine, Peyer's patch lymphoid tissue is rich in macrophages. However, the *spv* locus may also enhance bacterial growth in other cell types, since induction of *spv* gene expression occurs inside intestinal epithelial cells as well as macrophages (19). The severe mucosal destruction seen in Fig. 2B suggests widespread involvement of the epithelium by the spv^+ strain.

While the *spv* genes clearly affect the severe enteritis produced by *S. dublin* in young calves, *spv*-containing virulence plasmids are not required by *Salmonella* serovars in general to produce intestinal disease. Many *Salmonella* strains isolated from human and animal cases of gastroenteritis do not contain virulence plasmids. However, our results suggest that in serovars that carry virulence plasmids, the *spv* genes can enhance the severity of the enteric infection and can produce lethal disease.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (AI-32178 and DK-35108 to D.G.G.) and the U.S. Department of Agriculture (93-37204 to D.G.G.).

We are grateful to Patricia Blanchard for providing *S. dublin* strains. We especially thank Roberta Pugh and Doris Hunter for preparing the bovine monocytes and Joel Shields for taking care of the calves.

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Editor: P. E. Orndorff