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The *Salmonella dublin* Virulence Plasmid Mediates Systemic but Not Enteric Phases of Salmonellosis in Cattle

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Plasmid-bearing and plasmid-free isolates and a plasmid-cured strain of *Salmonella dublin* were compared for virulence in calves. The plasmid-bearing strains were highly virulent, causing severe enteric and systemic disease with high mortality. In contrast, the plasmid-free strains caused diarrhea but only low mortality. The infection kinetics of a wild-type and a derivative plasmid-cured strain were compared. Both strains were isolated in high numbers from intestinal sites at 3 and 6 days after oral challenge and were isolated at comparable frequencies from systemic sites at 3 days, but not at 6 days, when the wild-type strain was predominant. The strains were equally invasive in intestinal epithelia with and without Peyer's patch and elicited comparable secretory and inflammatory responses and intestinal pathology in ligated ileal loops. The effect of the virulence plasmid on growth kinetics and on the outer membrane protein profile was assessed in an in vivo growth chamber. The virulence plasmid did not influence either extracellular growth or the expression of major outer membrane proteins. These observations demonstrate that the virulence plasmid is not involved in either the enteric phase of infection or the systemic dissemination of *S. dublin* but probably mediates the persistence of *S. dublin* at systemic sites.

Salmonellosis continues to be a major economic and animal welfare problem in the cattle industry worldwide. In cattle, salmonellosis is manifested in two main clinical patterns. In young animals, *Salmonella typhimurium* is the predominant etiological agent of salmonellosis, causing acute enteritis, which results in severe dehydration and high mortality if left untreated. In contrast, *Salmonella dublin* is predominant in older animals, causing both enteric and systemic infections, including septicemia and abortion (30).

The role of the virulence plasmid in the pathogenesis of salmonellosis remains an enigma. At least 11 serotypes are known to carry virulence plasmids (27); however, not all isolates carry virulence plasmids (29). A virulence plasmid has not been detected in *Salmonella typhi*, the etiological agent of typhoid fever, a severe systemic infection in humans. The role of the virulence plasmid in pathogenesis has been studied mainly with the mouse model of salmonellosis. In mice, plasmid genes are not required for the translocation of salmonellas through the intestinal mucosa (8, 14, 15) but have been implicated in controlling the intracellular growth rate (6). Virulence plasmids are also associated with systemic disease in poultry (2) and in pigs challenged by the intravenous route (5).

Plasmid-free strains of *Salmonella* have been associated with outbreaks of enteritis in humans (11, 19). However, the precise role of the virulence plasmid in inducing enteritis has not been studied because experimental infection of both mice and chickens with salmonellas does not result in the classical *Salmonella*-induced enteritis seen in large animals and humans. As cattle succumb to both enteric and systemic phases of infection, they become good models for studying the pathogenesis of salmonellosis.

Here we report our findings on the role of the *S. dublin* virulence plasmid in mediating both the enteric and systemic phases of salmonellosis in calves.

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MATERIALS AND METHODS

Calves. Twenty-eight-day-old bull Friesian calves, fed on powdered milk since they were 2 days old, were used. Before challenge, calves were screened for the absence of salmonellas by enrichment culture of their feces in Rappaport medium.

Bacteria, inoculum preparation, and enumeration of bacteria in samples from organs. The strains used in this study are described in Table 1. Virulence plasmid carriage and the presence of the *spv* locus were confirmed previously by gel electrophoresis and colony hybridization with an 8-kb *Sal*I-*Xho*I probe encoding the *spv* genes of *S. dublin* (10). To demonstrate that the attenuation of the plasmid-cured strain was due to the absence of the virulence plasmid, the virulence plasmid was reintroduced and virulence in mice was restored (27). The inocula for orally dosing the calves were prepared as follows. Strains were grown in bactotryptose broth (9) statically for 18 h at 37°C. Immediately before dosing, approximately 1.0 ml of the inoculum was diluted in 20 ml of antiacid solution (magnesium trisilicate, 5% [wt/vol]; $MgCO_3$, 5% [wt/vol], $NaHCO_3$, 5% [wt/vol]). For assessing enteropathogenesis in loops, mid-log-phase organisms were used. Strains were grown in brain heart infusion (BHI) broth (for 16 h at 37°C with shaking) and this culture was used to inoculate a fresh 10-ml volume of BHI broth (1:100 dilution). The culture was further incubated (for 4½ h at 37°C with shaking), and the optical density at 600 nm was checked. Cells were then harvested and resuspended in the same volume of BHI broth and kept on ice until they were injected into the loops. To determine the level of intestinal invasion, the strains were prepared as described in the accompanying paper (26).

Bacteria in samples from organs were enumerated as follows. Tissue samples (1 g) were homogenized (UltraFurrax homogenizer) in 9 ml of saline and diluted accordingly. The samples were plated out in triplicate on brilliant green agar (Unipath, Basingstoke, United Kingdom) and bacteria were counted, as they were in samples from the in vivo sacs.

Infection kinetics following oral challenge. To assess the role of the virulence plasmid in the systemic distribution of *S. dublin*, two groups of four calves each were orally challenged with approximately 6×10^8 CFU of strain SD2229 or SDM173c. Two animals from each group were killed at 3 and 6 days postchallenge. The number and distribution of test strains in the mucosae of the large and small intestines and in the liver, spleen, and lungs and their associated lymph nodes were assessed. Following oral challenge, pyrexia, diarrhea, and anorexia were monitored. Diarrhea was scored by using a cumulative daily scoring scheme based on the consistency and the contents of the feces. Scores for consistency were as follows: 0, normal; +1, semisolid; +2, liquid; and +3, watery. Scores for the fecal contents were as follows: +1, fresh blood; +2, sloughed mucosa. Anorexia was defined as the consumption of less than half of the normal amount of food per day. Animals with a diarrhea score of 20 or animals which consumed no food for 2 days or less than half of their food for 4 days were killed to minimize suffering.

Quantification of enteropathogenesis. Ileal loops were prepared as follows. Calves were anesthetized for the duration of the experiment with pentobarbital (Sagatal; 0.44 ml/kg of body weight). The abdominal wall was opened and the distal ileum was exteriorized. The lumen of the distal ileum proximal to the

TABLE 1. *Salmonella* strains used in this study

Strain	Virulence plasmid status	Origin	Reference
SD2229	+	Clinical isolate	27
SD3246	+	Clinical isolate	
SD82/76	+	Clinical isolate	
SD82/46	−	Clinical isolate	
SD82/158	−	Clinical isolate	
SDM173c	−	Plasmid-cured derivative of SD2229	27
SDM173(pM120)	+	Plasmid-restored derivative of SDM173c	27
ST4/74	+	Clinical isolate	
ST4/74 *invH 201*::Tn*phoA*	+	Transposon insertion in *invH* of ST4/74	26

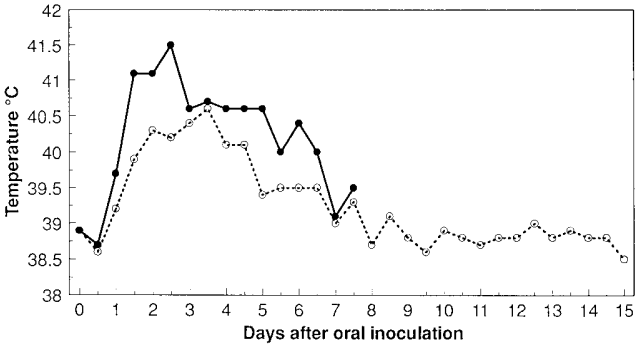

FIG. 1. Mean daily temperatures of four calves challenged with 10^{10} CFU of either SD2229 (●) or SDM173c (○) (two calves per strain).

continuous Peyer's patch was flushed with saline, and 10-cm loops with 1-cm spacers were ligated with surgical silk. The loops were inoculated with 1 ml of the test strains or with 1 ml of BHI broth as a negative control. The loops were replaced within the abdominal cavity and the wound was repaired. A solution of 500 ml of glucose (5% [wt/vol]) in saline was administered intravenously by drip.

Twelve hours after challenge, the loops were sampled and enteropathogenesis was assessed with respect to fluid accumulation, neutrophil infiltration, and histological changes in tissue architecture. The animals were killed by barbiturate overdose. Fluid secretion was measured as a ratio of volume of fluid accumulated to loop length. Neutrophils were labelled with ^{111}In (see below) and infiltration was measured as a ratio of ^{111}In activity in test loops to that in control loops inoculated with sterile BHI broth. Samples of intestinal mucosa from ligated loops were taken for histological analysis. The loops were injected antemortem with neutral buffered formalin (10% [vol/vol] formaldehyde in phosphate-buffered saline [PBS]). Full-thickness biopsy samples were taken from loops and immersed in neutral buffered formalin overnight before being processed the following day.

^{111}In labelling of neutrophils. Approximately 4 h after loop inoculation, a 60-ml sample of blood was taken, from which neutrophils were isolated by the method described by Carlson and Kaneko (3). The neutrophils were finally resuspended in 2 ml of Ca^{2+}-free Tyrodes buffer and labelled with indium-111 oxinate (Mallinckrodt, Northampton, United Kingdom) as described previously (23).

Determination of level of intestinal invasion. The technique used for determination of the level of intestinal invasion is described in the accompanying paper (26).

In vivo growth kinetics in intraperitoneal sacs. Dialysis sacs were constructed and implanted as described previously (7). Briefly, dialysis sacs (12 to 14K cutoff; Fisons) containing 40 ml of dextran sulfate solution (6% [wt/vol] in PBS) were implanted into the peritoneal cavity through a flank laparotomy involving an 8-cm vertical incision in the sublumbar fossa while the animal was under local anesthesia (Wylotox) plus sedation (Rompun; Bayer, United Kingdom). After implantation the wound was repaired around a sampling tube which was closed by a three-way tap. A similar operation was performed on the contralateral side. Forty-eight hours after dialysis sac implantation, the sacs were inoculated with approximately 10^3 CFU. At 3-h intervals for a total of 24 h, the sac contents were sampled (100 μl) and viable counts were determined as described above. Twenty-four hours after inoculation, the sacs were emptied and the cells were harvested for outer membrane protein (OMP) analysis.

OMP analysis. The outer membranes of strains SD2229 and SDM173c grown in vivo were prepared by Sarkosyl extraction as described by Achtman et al. (1). Briefly, 10^{10} cells were harvested by centrifugation (2,500 × *g* for 30 min at 4°C) and the pellet was resuspended in 10 ml of distilled H_2O at 4°C. The suspension was sonicated for 20 min with a cup-horn sonicator (model W385; Ultrasonics) at 50% cycle, 2-s pulse, output 6. The sonicate was centrifuged (2,400 × *g* for 20 min at 4°C) to remove undisrupted cells, and the supernatant was further centrifuged (11,000 × *g* for 60 min at 20°C). The pellet was resuspended in 100 μl of distilled H_2O and stored at −20°C prior to extraction. Fifty microliters of the outer membrane preparation was extracted with 8 volumes of Sarkosyl (sodium lauryl sarcosinate, 1.67% [vol/vol] in 11 mM Tris-HCl [pH 7.6]) for 20 min at

TABLE 2. Clinical outcome for calves following oral challenge with plasmid-containing and plasmid-free strains of *S. dublin*

Strain	Status	Dose (CFU)	No. of survivors/no. in group	No. of animals with symptom/no. in group	
				Diarrhea	Anorexia
SD3246	Plasmid$^+$	10^8	3/3	0/3	0/3
		10^9	0/2	2/2	2/2
SD2229	Plasmid$^+$	10^9	3/3	1/3	2/3
		10^{10}	0/5	5/5	5/5
SD82/76	Plasmid$^+$	10^{10}	2/2	0/2	0/2
SD82/46	Plasmid$^-$	10^{10}	2/2	0/2	0/2
SD82/158	Plasmid$^-$	10^{10}	2/2	2/2	0/2
SDM173c	Plasmid-cured	10^{10}	6/7	6/7	0/7

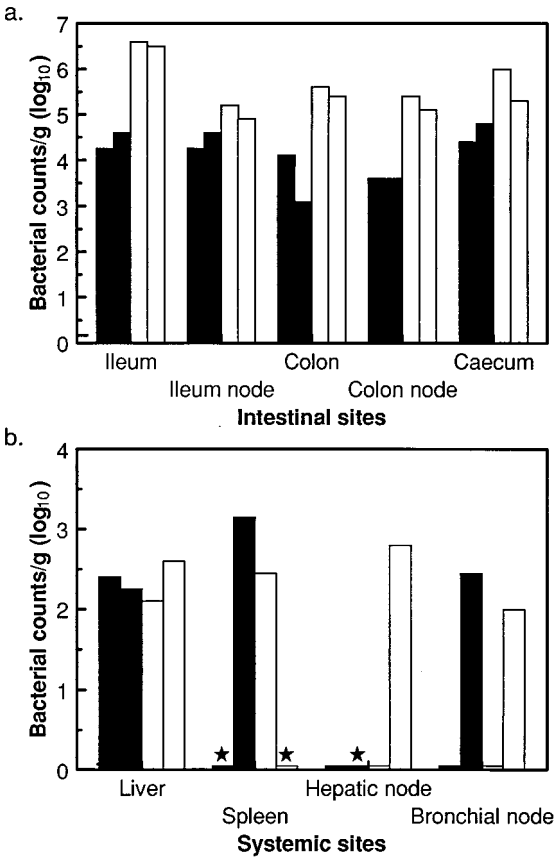

FIG. 2. *Salmonella* counts from intestinal (a) and systemic (b) sites in four calves, 3 days following oral challenge with either SD2229 (■) or SDM173c (□). ★, sample positive on enrichment culture.

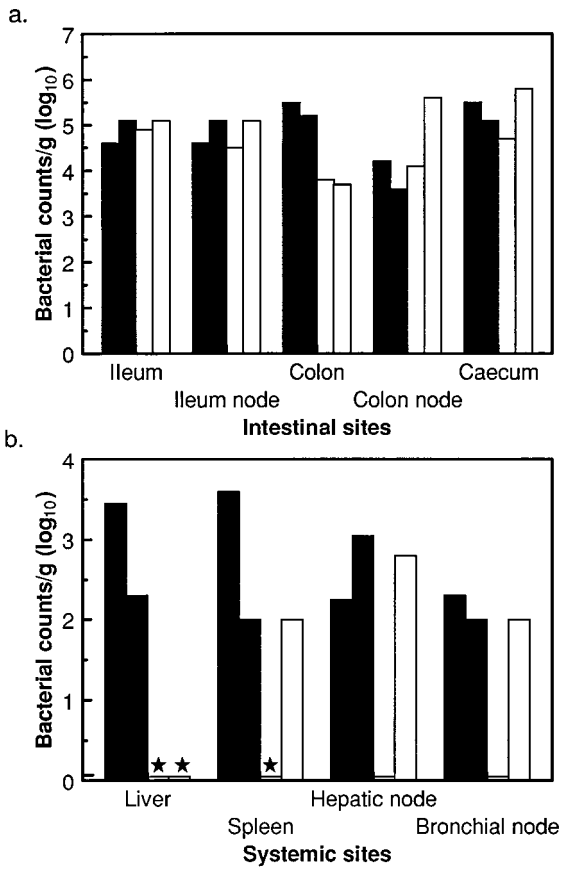

FIG. 3. *Salmonella* counts from intestinal (a) and systemic (b) sites in four calves, 6 days following oral challenge with either SD2229 (■) or SDM173c (□). ★, sample positive on enrichment culture.

room temperature. The insoluble outer membrane was pelleted by centrifugation (11,000 × *g* for 90 min at 20°C) and resuspended in 50 μl of sample buffer (2% [vol/vol] sodium dodecyl sulfate [SDS], 2% [vol/vol] mercaptoethanol, and 1% [vol/vol] glycerol in 62.5 mM Tris-HCl [pH 6.8]) and boiled for 5 min prior to analysis by SDS-polyacrylamide gel electrophoresis (PAGE). OMPs were separated on the basis of molecular weight by using a modification of the method of Laemmli (13). Electrophoresis was carried out at 30 mA per gel at a constant current in stacking and resolving gels containing 4 and 10% acrylamide, respectively. Protein (30 μg) was loaded into each lane. After electrophoresis, protein bands were visualized by immersing gels in Coomassie blue R-250 staining solution (0.25% [wt/vol] in 50% [vol/vol] methanol containing 10% acetic acid) for 4 h, and excess stain was removed with destain solution (5% [vol/vol] methanol, 7.5% [vol/vol] acetic acid).

The resolved proteins were transferred to nitrocellulose by electrophoresis (300 mA, 4°C, overnight; Powerlid; Hoefer). Filters were washed in PBST (PBS containing 0.05% [vol/vol] Tween 20), blocked with horse serum (50% [vol/vol] in PBST–0.5% [vol/vol] Tween 20) for 2 h at room temperature, and washed again with PBST. The filters were then probed for 2 h at room temperature with bovine serum (10% [vol/vol] in PBST–0.5% [vol/vol] Tween 20) from convalescent animals challenged with *S. typhimurium* and *S. dublin* and were washed with PBST. The filters were then incubated in anti-bovine immunoglobulin G (whole molecule)–horseradish peroxidase (Sigma) (diluted 1/2,000 in 5% [vol/vol] horse serum in PBST–0.5% [vol/vol] Tween 20) for 2 h at room temperature and washed with PBST. Protein bands were visualized with diaminobenzidine with nickel chloride enhancement (0.05% [wt/vol] diaminobenzidine, 0.03% [wt/vol] $NiCl_2$, and 0.3% [vol/vol] H_2O_2 in PBS) for approximately 5 min, and the reaction was stopped by washing in distilled H_2O.

Statistical analysis. The data from the invasion assays and secretory and inflammatory responses were analyzed with Minitab Inc. (State College, Pa.) statistical software. A one-way analysis of variance was carried out. In the event that a significant difference was found, strains were compared by using the Student *t* test. The variance used in the *t* test was obtained from the residual mean square calculated in the analysis of variance.

RESULTS

Virulence of wild-type and plasmid-free strains of *S. dublin* in cattle. Three wild-type plasmid-bearing strains (SD3246, SD2229, and SD82/76), two wild-type naturally plasmid-free strains (SD82/46 and SD82/158), and a plasmid-cured derivative of SD2229 (SDM173c) were used to infect calves. The outcome of infection is shown in Table 2. Not all plasmid-bearing strains were equally virulent. Strain SD3246 proved to be the most virulent, causing diarrhea, anorexia, and mortality in both an-

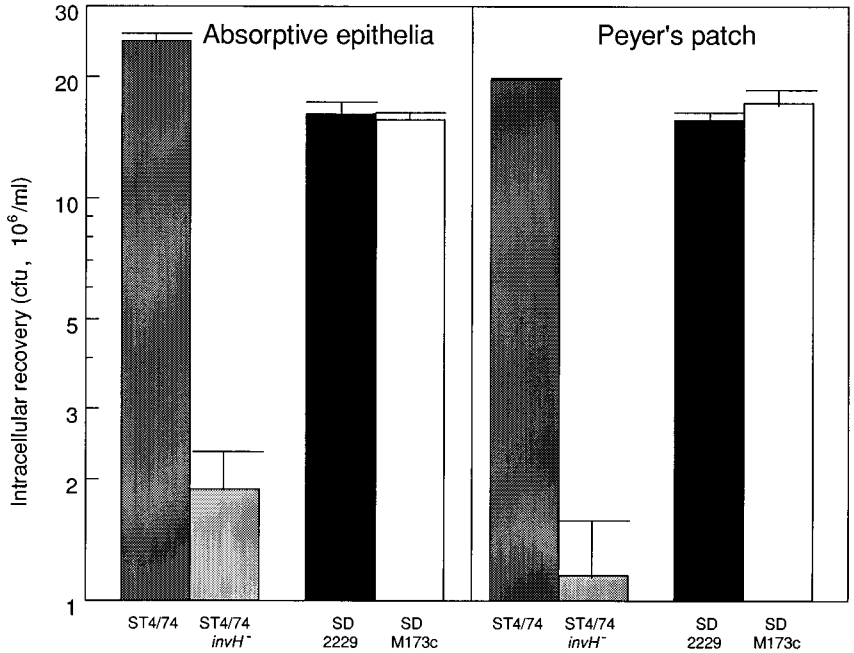

FIG. 4. Role of virulence plasmid in intestinal invasion. Data are geometric means + standard errors (error bars) for 18 biopsy samples from six loops per strain of *S. dublin* in two calves. The assay was controlled with wild-type and *invH* mutant *S. typhimurium* strains, with one loop of each strain per calf.

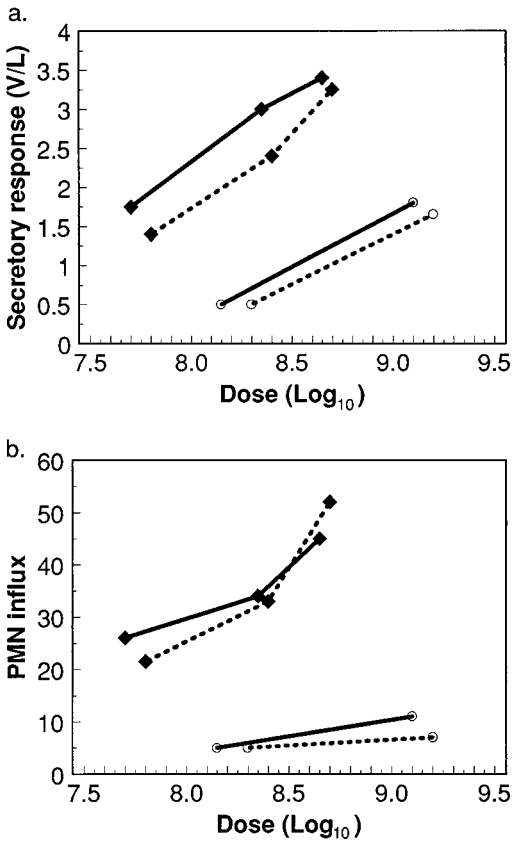

FIG. 5. Quantification of secretory (a) and inflammatory (b) responses induced by SD2229 (——) and SDM173c (– – –) in ligated ileal loops. Dose response was measured in adjacent loops in two calves. ◆, calf 1; ○, calf 2. V/L, ratio of volume of fluid accumulated to loop length (milliliters per centimeter); PMN, polymorphonuclear leukocyte. Dose is in CFU.

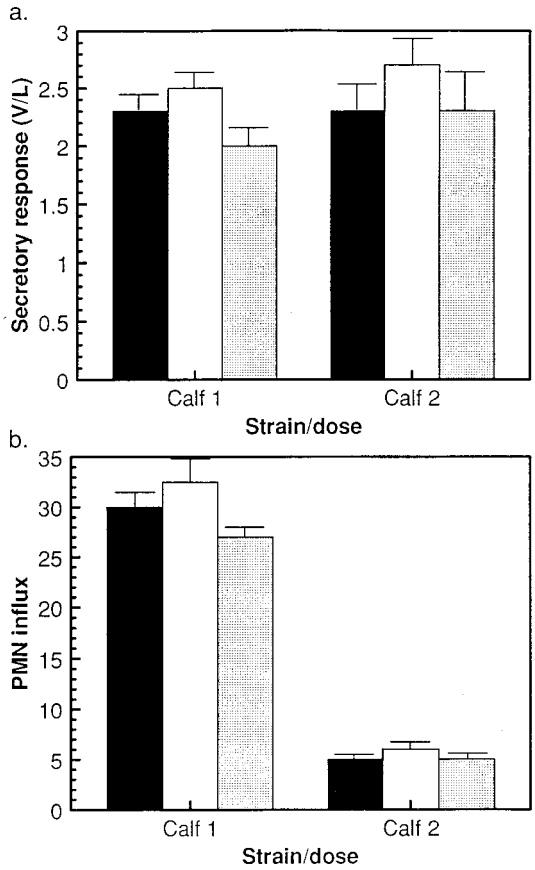

FIG. 6. Quantification of secretory (a) and inflammatory (b) responses induced by SD2229 (■), SDM173c (□), and SDM173cpM120 (▨). Data are means + standard errors (error bars) for four loops per strain. V/L, ratio of volume of fluid accumulated to loop length (milliliters per centimeter); PMN, polymorphonuclear leukocyte.

imals challenged with 10^9 CFU. Strain SD82/76 was avirulent in this model at a dose of 10^{10} CFU, indicating that factors in addition to the virulence plasmid are required for virulence in cattle. Ten of 11 animals challenged with the naturally plasmid-free strain or the cured strain survived challenge and did not become anorexic; diarrhea occurred in 8 of the 11 animals.

The temperature responses in animals infected with SD2229 or its plasmid-cured derivative SDM173c are shown in Fig. 1. Strain SD2229 caused a higher mean rate of daily fever than strain SDM173c over the 8 days on which the strains could be compared.

Effect of virulence plasmid on infection kinetics in calves. The distribution of salmonellas within tissues following oral challenge with 6×10^8 CFU of strains SD2229 and SDM173c was determined. The gut-associated and systemic counts at 3 and 6 days are depicted in Fig. 2 and 3, respectively. Both strains were recovered at lower numbers from the systemic sites than from the intestinal sites. Accurately enumerating bacteria at such concentrations is problematic, and therefore enrichment culture was carried out to aid in the detection of salmonellas. Considerable interanimal variation was seen, which may reflect either inherent variation in host resistance to infection or the limitations of the assay for detecting salmonellas at low numbers.

Both strains were primarily associated with the distal ileum and colon rather than the upper small intestine data not shown. Strain SDM173c was reproducibly reisolated from intestinal sites at higher numbers than was strain SD2229 at day 3 but not at day 6, when both strains were isolated at similar numbers. The systemic distribution of wild-type and plasmid-cured strains showed different patterns. Three days after infection, the strains were isolated with similar frequencies and at similar numbers. By 6 days, the wild-type strain appeared predominant, as it was isolated at quantifiable numbers from all eight systemic sites tested; the cured strain was recovered at similar numbers only from three of eight sites.

Role of virulence plasmid in intestinal invasion. The observation that SDM173c was detected at higher numbers at intestinal sites than was SD2229 at 3 days following oral challenge suggested that the plasmid-cured strain was possibly more invasive than the wild-type strain. The intestinal invasiveness of these strains was therefore assessed. The strains were detected at comparable numbers in epithelia with and without Peyer's patch mucosa (Fig. 4). The invasion assay was controlled with a wild-type strain and a less invasive *invH* mutant of *S. typhimurium* (26). The *invH* mutant was recovered from mucosae with and without Peyer's patch at significantly lower numbers than was the wild-type strain ($P<0.01$), indicating that the assay is able to quantify differences in the magnitude of intestinal invasion.

Role of virulence plasmid in enteropathogenesis. Induction of fluid secretion, neutrophil influx, and tissue damage in ligated loops in the middle ileum was used to assess the enteropathogenicity of strains SD2229 and SDM173c.

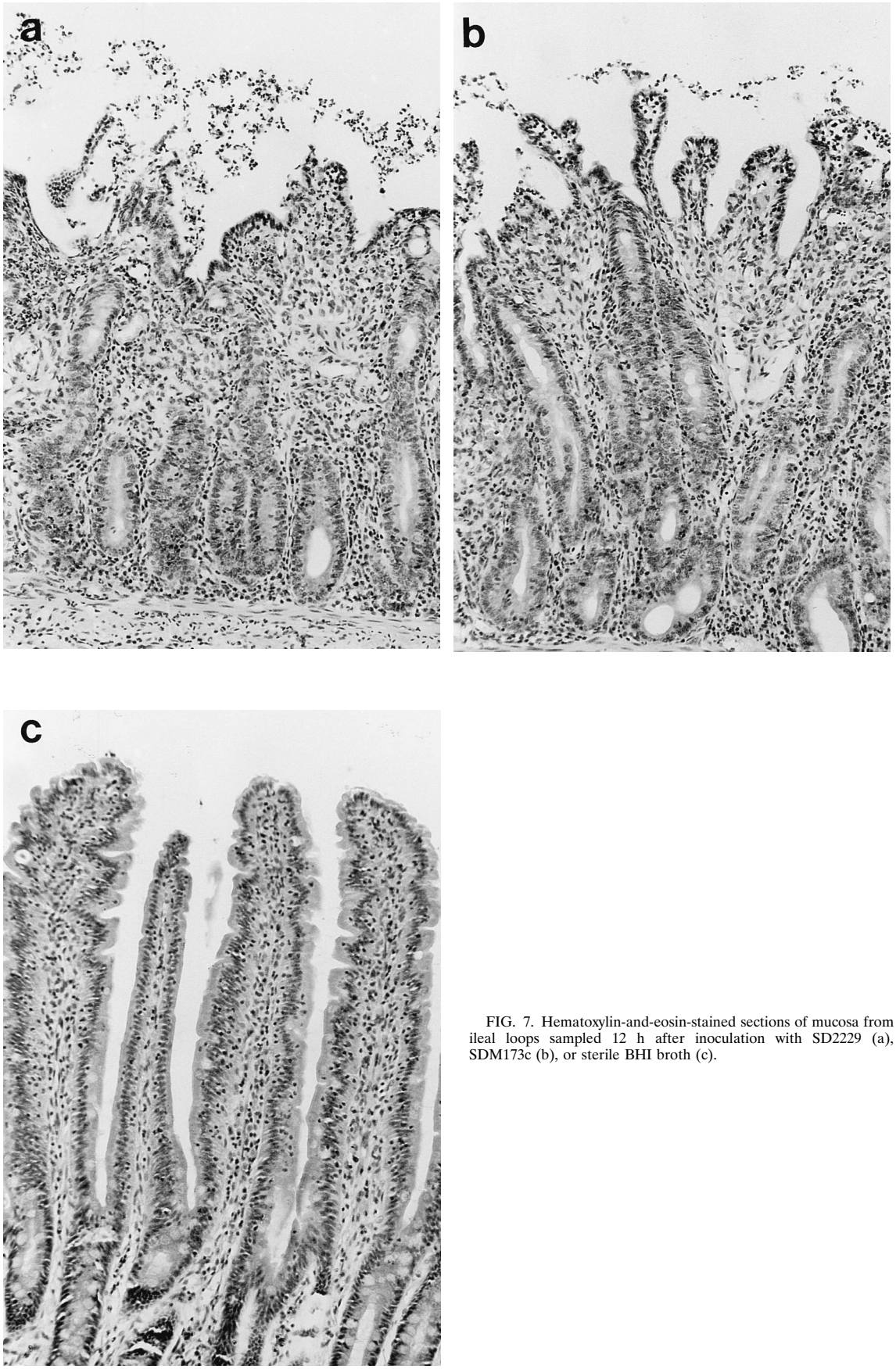

FIG. 7. Hematoxylin-and-eosin-stained sections of mucosa from ileal loops sampled 12 h after inoculation with SD2229 (a), SDM173c (b), or sterile BHI broth (c).

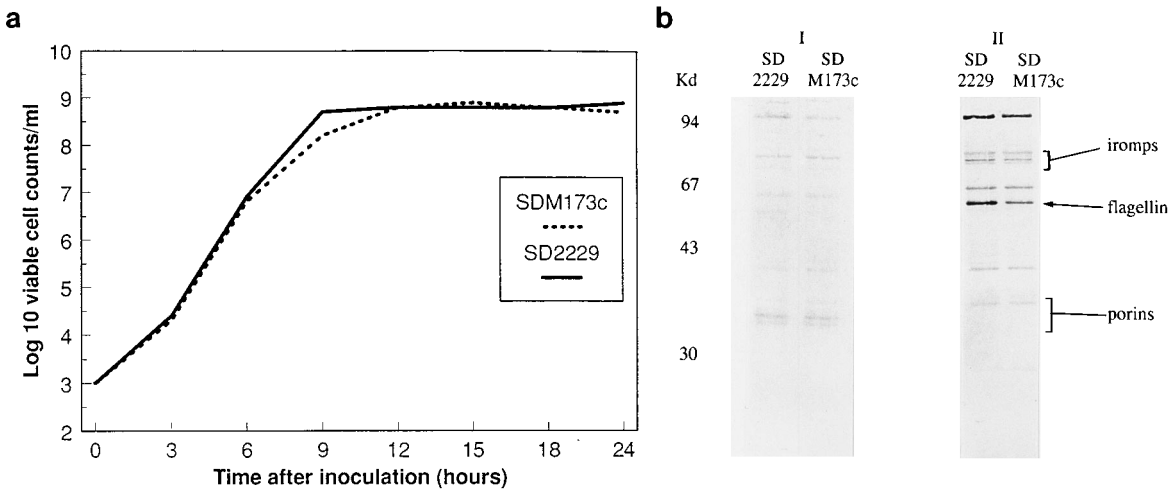

FIG. 8. (a) Growth kinetics of SD2229 and SDM173c in dialysis sacs implanted in peritoneal cavities of calves. (b) OMP profiles of SD2229 and SDM173c grown in vivo and visualized by Coomassie blue staining (I) and Western blotting with hyperimmune bovine serum (II). The relative positions of the iron-regulated OMPs (iromps), flagellin, and porins are indicated.

The dose responses to these strains in two calves were assessed (Fig. 5), and considerable interanimal variation was observed. In calf 1, potent secretory and inflammatory responses were induced by both strains, and the magnitudes of the responses were related to the dose. In calf 2, both strains evoked secretory and inflammatory responses; less potent responses were recorded for calf 2 than for calf 1, and no inflammatory dose response was seen (Fig. 5b). In both animals, strain SD2229 evoked slightly greater secretory and inflammatory responses at comparable doses than those evoked by strain SDM173c. Both strains were therefore tested in quadruplicate in two other animals at a dose of approximately 5×10^8 CFU per loop to confirm whether these differences were significant (Fig. 6). Interanimal variation was again observed; the magnitudes of the inflammatory responses differed significantly. In each animal, however, strains SD2229, SDM173c, and SDM173c(pM120) induced comparable secretory and inflammatory responses ($P \geq 0.1$).

The strains induced comparable pathologies in the intestinal mucosa. This was typified by exfoliation of villus tip enterocytes with associated villus blunting, mucosal ulceration, and infiltration by inflammatory cells (Fig. 7).

In vivo extracellular growth kinetics and OMP profile. The wild-type and plasmid-cured strains of *S. dublin* grew rapidly in the in vivo growth chamber. The virulence plasmid did not influence the growth kinetics over 24 h (Fig. 8a). Similarly, the virulence plasmid did not influence the expression of the major OMPs as detectable by Coomassie blue-stained SDS-PAGE gels and Western blotting (immunoblotting) with hyperimmune bovine serum (Fig. 8b). Comparable levels of expression of iron-regulated OMPs, flagellin, porins, and two undefined 100- and 66-kDa OMPs were detected in outer membrane preparations from wild-type and plasmid-cured strains.

DISCUSSION

An understanding of the role of the virulence plasmid in the pathogenesis of salmonellosis is based almost entirely on studies using the murine model. Because mice fail to develop enteritis following challenge with virulent salmonellas, the role of the virulence plasmid in this important phase of infection has not been studied. The precise mechanisms by which salmonellas induce intestinal fluid secretion remain unclear, and the subject has recently been reviewed (18). *Salmonella* enterotoxins have been identified (12, 17, 24); however, their role in enteropathogenesis remains unclear (23, 25). Chopra and colleagues (4) reported the cloning of a chromosomally encoded gene related to that for cholera toxin, whereas Yang and Tan (31) reported the cloning of a gene from plasmids of *S. typhimurium* strains showing homology to that of *Escherichia coli* which encodes a heat-labile enterotoxin. Furthermore, low levels of homology between the predicted amino acid sequences of the *ace* enterotoxin of *Vibrio cholerae* and the product of the *spvB* gene of the virulence plasmid have been reported (20). The latter two observations implicate a role for the virulence plasmid in inducing enteritis.

This paper demonstrates that both naturally occurring plasmid-free strains and a plasmid-cured strain of *S. dublin* induced enteritis in orally challenged calves. However, as determining the degree of enteritis in diarrheic animals is difficult, a wild-type strain of *S. dublin* and its plasmid-cured derivative of known virulence in mice (28) were tested in ligated ileal loops with a quantitative assay for assessing enteropathogenesis. In this assay, the strains elicited comparable secretory and inflammatory responses and pathological changes in intestinal mucosae. These observations clearly demonstrate that the genes encoded by the virulence plasmid are not primarily associated with enteropathogenesis.

The infection kinetics studies and the intestinal invasion assay with the wild-type strain and plasmid-cured derivative support the observations of other groups studying mice, i.e., that the plasmid does not affect either penetration and proliferation within the intestinal mucosa or translocation of salmonellas through the intestinal mucosa, despite the fact that the routes of invasion in mice and cattle are apparently different (26). We have shown that the wild-type and plasmid-cured strains were detected with similar frequencies at both intestinal and systemic sites 3 days after challenge. By 6 days, the wild-type strain appeared to be predominant at systemic sites. Although our observations were made for a limited number of animals, they suggest that the virulence plasmid affects the persistence of bacteria at systemic sites. This observation is consistent with the increased mortality and pyrexia observed in calves orally infected with the wild-type strain rather than the

plasmid-cured strain, which implicates the plasmid in mediating systemic salmonellosis in cattle. Bacterial persistence within a host is determined by two key parameters, bacterial growth and death rates. Enumerating bacteria at two time points does not enable us to identify which mechanism mediates increased persistence. Recently, it was reported that the virulence plasmid increases the intracellular growth rate but did not influence the intra- or extracellular location of salmonellae in mice (6). However, the extracellular growth rate was determined at only one time point, 48 h, and therefore was not definite. Our observations are consistent with the notion that the plasmid increases the growth rate, but why this is evident only at systemic sites and not intestinal sites is not clear. One explanation is that multiplication of strains within the intestinal lumen, possibly unregulated by the virulence plasmid, results in continuous seeding of the intestinal sites, which would mask the difference in the abilities of these strains to persist in vivo. Alternatively, it may reflect differences in the abilities of reticuloendothelial cells at these various sites to control *Salmonella* growth.

We assessed the role of the plasmid in mediating growth over 24 h in an in vivo growth chamber, a model which facilitates the study of extracellular growth; no effect on growth rate could be detected.

The analysis of bacterial surface components is useful for the identification of virulence determinants, as it is these components which directly interact with the host during the disease process. The expression of such components is determined by bacterial genotype and the microenvironment surrounding the cell. The virulence plasmid is not involved in the regulation of lipopolysaccharide expression (22), but its role in regulating the expression of OMPs in vivo has not previously been studied. The virulence plasmid did not affect major OMP expression in this model. Clearly, free Fe^{2+} was not available within the peritoneal cavity, as indicated by the expression of the 81-, 78-, and 74-kDa iron-regulated OMPs. The virulence plasmid proteins SpvA, -B, and -C have been reported to be expressed under conditions of iron limitation and in the stationary phase but only in small amounts in vitro (21). Reagents to specifically detect the expression of SpvA, -B, and -C in vivo were not available at the time of analysis; however, clearly the virulence plasmid does not regulate expression of major OMPs in the extracellular environment.

It has been widely speculated that the virulence plasmid mediates survival within macrophages; however, to date there is no evidence to support this (16). Thus, the precise niche within the host where the virulence plasmid modifies pathogenesis remains unclear.

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