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Differential Regulation of Enteric and Systemic Salmonellosis by *slyA*

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Mutation of *slyA*, which reduces *Salmonella typhimurium* virulence in mice, caused only minor attenuation of *S. typhimurium* virulence in orally inoculated calves. This correlated with modest reductions in intestinal invasion and enteropathogenic responses in bovine ligated ileal loops. *slyA* appears to regulate virulence genes involved in systemic, but not enteric, salmonellosis.

Salmonella serotypes can infect many different animal species, causing disease ranging in severity from mild enteritis to severe systemic salmonellosis. The nature of the disease is largely dependent on the specific serotype-host combination. The bacterial factors involved in the various stages of pathogenesis, and in different animal hosts, are not well defined, although numerous genes have been identified which are potentially involved. These genes are frequently clustered in pathogenicity islands, and these have been variously implicated in bacterial invasion (reviewed in reference 8), induction of enteropathogenic responses (20, 23), and intracellular survival (2, 4, 11, 22). In addition to these recent results, there has been great interest over many years in the characterization of a *Salmonella* toxin(s) which may have properties analogous to those of other well-characterized bacterial toxins. This has led to the identification of *slyA*, which confers hemolytic activity on *Escherichia coli* (15). *slyA* has since been shown to have properties of a regulatory gene (16) and has been implicated in the regulation of murine virulence, survival in murine macrophages, destruction of murine M cells after bacterial uptake, and resistance to oxidative stress (3, 5, 14).

A common problem in the study of the above potential virulence factors is the lack of appropriate animal models of salmonellosis. The most widely used is the murine model of typhoid fever. Following oral or parenteral inoculation of mice, net bacterial growth within the reticuloendothelial system results in severe systemic disease. There is no convenient laboratory animal model of enteric salmonellosis following oral inoculation, although it is possible to use larger animals which are susceptible to the enteric form of the disease. Calves experimentally inoculated with virulent *Salmonella typhimurium* exhibit severe diarrhea, elevated temperatures, dehydration, and anorexia and are therefore good models of *Salmonella*-induced enteritis for both cattle and other animals such as humans. In addition, the ligated-ileal-loop model allows several parameters of enteritis to be quantified in a variety of animals, including rabbits (10, 19) and calves (18, 21). Its use has enabled us to characterize the role of specific virulence factors in *Salmonella* enteropathogenesis, and the results correlate well with the oral inoculation of calves (1, 9, 13, 18, 20, 21, 23). The aim of this study was to assess the contribution of *slyA* to *Salmonella*-induced enteritis by using orally inoculated cattle and infection of bovine ligated ileal loops as models of enteritis.

Bacterial strains and experimental design. A bovine isolate of *S. typhimurium* (strain ST4/74) and an *invH*::Tn*phoA* mutant of this strain were routinely handled as previously described (21). The *slyA* plasmid insertion mutation has been described previously (14) and was transferred to *S. typhimurium* ST4/74 by P22 transduction. Transduction of the *slyA* mutation was confirmed by Southern blotting as described previously (14). The *invH* and *slyA* mutants were routinely grown in the presence of 75 μg of kanamycin ml^{-1} and 200 U of penicillin ml^{-1}, respectively, and had growth rates in vitro similar to that of the wild-type strain. In all of the in vitro assays and in the ligated-ileal-loop assay, bacterial strains were tested in triplicate and each experiment was repeated at least twice. All data is presented with the standard error of the mean.

Mutation of *slyA* results in a small reduction in *S. typhimurium* virulence for calves. Six 28-day-old Friesian bull calves with no history of enteric infection or fecal excretion of salmonellas were orally inoculated with 0.6×10^9 to 1.0×10^9 CFU of either *S. typhimurium* ST4/74 or its derivative *slyA* mutant in an antacid preparation. All of the calves excreted large numbers of salmonellas in their faeces (approximately 5.0 $\log_{10}$CFU g^{-1}) from 24 h after inoculation onward. The three calves inoculated with the wild-type strain were killed at 54, 72, and 96 h after inoculation for humane reasons, as required by the 1986 United Kingdom Animals (Scientific Procedures) Act, because they had reached the predefined clinical endpoint (anorexia, dehydration, and/or a reluctance to rise or stand). They were also producing liquid feces containing either blood, sloughed intestinal mucosa-pseudomembrane material, or both. The calves inoculated with the *slyA* mutant had pyrexic and diarrheic responses similar to those of calves inoculated with the wild-type strain, except that the onset of diarrhea was delayed by approximately 1 day.

The calves inoculated with the *slyA* mutant were killed at the same times as those inoculated with the wild-type strain to allow direct comparison of the amounts of bacteria recovered from various intestinal and systemic sites. Viable counts were performed on triplicate samples from each site by using modified brilliant green agar. The viable-count method had a lower limit of accurate quantification of 2.0 $\log_{10}$CFU g of $tissue^{-1}$, and samples which contained numbers of bacteria below this limit were incubated in Rappaport broth (at 37°C for 18 h) and selenite brilliant green broth (at 42°C for 18 h) to enrich for *Salmonella*. Negative and positive enrichment cultures were given values of 0 and 2.0 $\log_{10}$CFU g^{-1}, respectively. There was no major difference in the bacterial recoveries at the three

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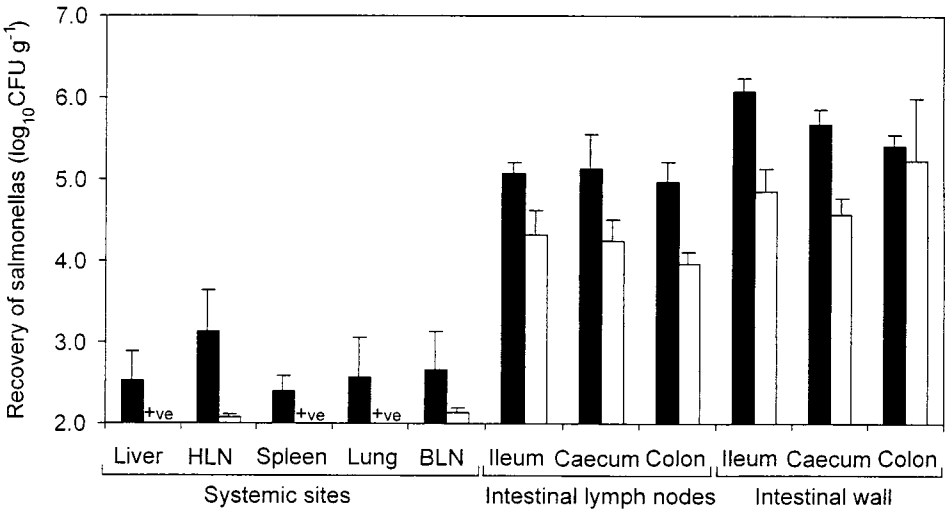

FIG. 1. Recovery of salmonellas from systemic sites, intestinal lymph nodes, and intestinal walls of calves after oral inoculation with *S. typhimurium* ST4/74. +ve indicates that salmonellas were recovered in numbers below the lower limit of accurate quantification (2.0 $\log_{10}$CFU g of tissue^{-1}). ■, wild type; □, *slyA* mutant; HLN, hepatic lymph node; BLN, bronchial lymph node.

different time points, and so, the results have been averaged for each strain (Fig. 1). The *slyA* mutation reduced the bacterial recovery from intestinal sites by approximately 1.0 $\log_{10}$CFU g^{-1}. The recovery of the *slyA* mutant from systemic tissues was also reduced, although the size of the reduction could not be accurately quantified because of the low bacterial numbers recovered. The difference in recovery between the wild type and the *slyA* mutant was significant ($P < 0.05$). The stability of the *slyA* mutation in vivo was confirmed by plating triplicate samples from two intestinal and two systemic sites on modified brilliant green agar either with or without penicillin; almost identical numbers of bacteria were obtained in each case.

The small reduction in virulence for calves is in contrast to studies with mice, in which the *slyA* mutation in *S. typhimurium* is highly attenuating, increasing the 50% lethal dose by greater than 10^3 or 10^4 following oral or intraperitoneal inoculation, respectively (14). This difference is probably due to fundamental differences in *Salmonella* pathogenesis in these two host species. In mice, infection of and net bacterial growth within the reticuloendothelial system determine the course of the disease (reviewed in reference 6). In cattle, both intestinal and systemic sites become infected, with infection of some systemic sites as early as 18 h after oral inoculation (20). However, subsequent net bacterial growth within the liver and spleen is relatively well controlled whereas the involvement of the intestines is obvious from the high bacterial numbers recovered and the severity of diarrhea. Thus, it appears that *slyA* is involved in the regulation of virulence factors influencing systemic infection of mice and only has a minor role in the enteric infection of cattle.

Mutation of *slyA* does not significantly reduce the enteropathogenic responses induced by *S. typhimurium*. The magnitudes of fluid secretion into the intestinal lumen (secretory response) and of the polymorphonuclear leukocyte (PMN) influx (inflammatory response) into the intestinal mucosa and lumen following infection with *S. typhimurium* were assessed in bovine ligated ileal loops. The surgical techniques used and the preparation of bacterial inocula have been described in detail elsewhere (18). Briefly, bacteria were incubated in mid-ileal loops for 12 h, during which time the PMNs from each calf were isolated, labeled with ^{111}In, and reinjected. After 12 h, the secretory response (volume of fluid within a loop/length of loop expressed in milliliters per centimeter) and the influx of PMNs into the test loops versus the negative-control loops were recorded. The results from four representative calves are shown in Fig. 2. The inocula were in the range of 1.0×10^9 to 2.3×10^9 CFU $loop^{-1}$. In all calves, the secretory responses of negative-control loops were less than 0.05 ml cm^{-1}. The *slyA* mutation caused either no reduction or only small reductions in both secretory and inflammatory responses which, in the majority of the calves, were not statistically significant. Similar results were obtained when either logarithmic- or stationary-phase inocula were used. The reduction associated with the *slyA* mutation was small in comparison to that associated with mutation of the *invH* gene. These modest effects on enteropathogenesis in ligated ileal loops support the results from oral inoculation of calves and together indicate that *slyA* does not have a major role in the pathogenesis of enteric salmonellosis.

Mutation of *S. typhimurium slyA* results in a small reduction in bacterial invasion. The effect of the *slyA* mutation on bacterial invasion was assessed in bovine ligated ileal loops and cultured Int 407 cells. The conditions of the invasion assays, both of which involve incubation of infected cells or mucosa with gentamicin to kill extracellular bacteria, have been previously described in detail (21). For the cultured-cell assay, the following modifications were made to minimize any cell lysis: the durations of incubation during the initial infection and then with gentamicin were reduced to 1 h each, the ratio of infection was reduced to five bacteria per cell, and medium without phenol red was used to allow lactate dehydrogenase (LDH) release by the infected epithelial cells to be measured by a colorimetric assay, the CytoTox 96 assay (Promega, Southampton, United Kingdom). No damage to the Int 407 cells was detected by either microscopic observations or by assaying for the release of LDH. Wild-type *S. typhimurium* was recovered from the Int 407 cells at 5.61 ± 0.03 $\log_{10}$CFU ml^{-1}, the *slyA* mutant was recovered at 5.46 ± 0.02 $\log_{10}$CFU ml^{-1}, and the *invH* mutant was recovered at 4.39 ± 0.06 $\log_{10}$CFU ml^{-1}. The reduction associated with the *slyA* mutation was significant ($0.05 > P > 0.02$) but was relatively small, for example, compared with the reduction associated with the *invH* mutation ($P < 0.001$). In the ligated-ileal-loop invasion assay, loops were constructed in the bovine distal ileum, which

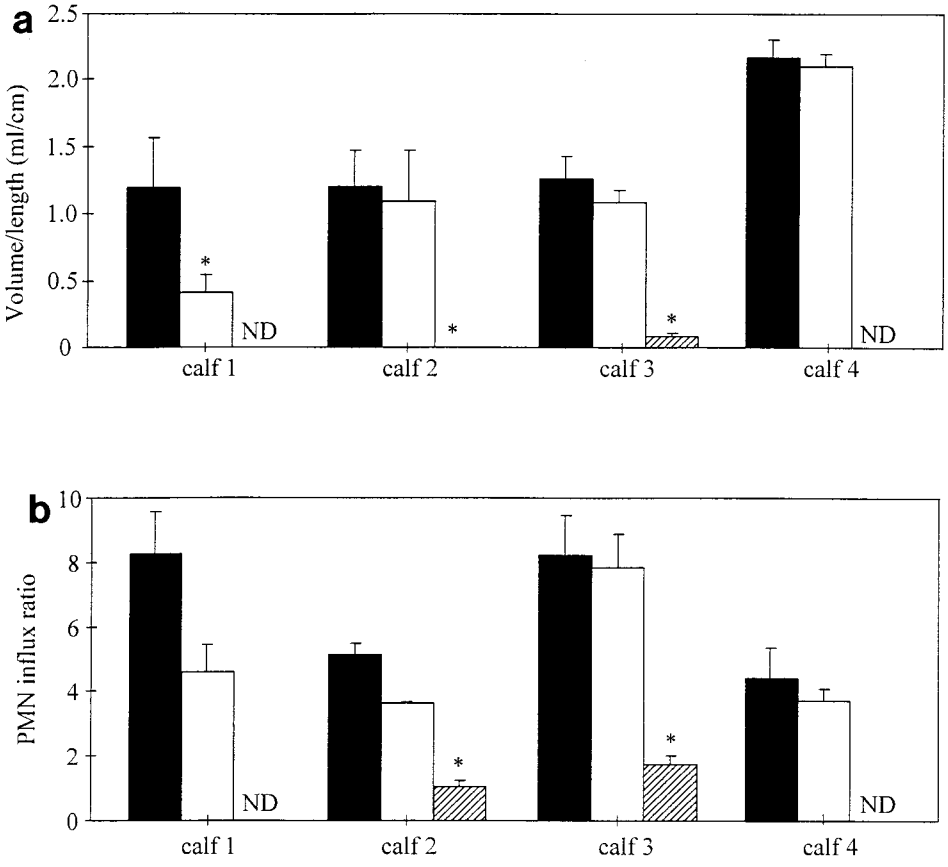

FIG. 2. Secretory (a) and inflammatory (b) responses induced 12 h after infection of bovine ligated ileal loops with *S. typhimurium* ST4/74. The PMN influx ratio is defined as the influx of PMNs into the test loop (as measured by gamma emission of ^{111}In-labeled PMNs) compared to the mean PMN influx in the negative-control loops. Logarithmic-phase inocula were used in calves 1, 2, and 3, and stationary-phase inocula were used in calf 4. The asterisks indicate significant differences between the wild-type and derivative mutant strains. ND, not done; ■, wild type; □, *slyA* mutant; ▨, *invH* mutant.

contains a continuous strip of Peyer's patches, allowing mucosa both with and without Peyer's patches to be sampled from each loop. Inocula were in the range of 1.6×10^9 to 2.0×10^9 CFU loop^{-1}. The recovery of *S. typhimurium* from biopsies of uniform size was measured 3 h after infection of bovine ligated ileal loops, including incubation in situ with gentamicin during the last hour. For mucosa without Peyer's patches, wild-type *S. typhimurium* was recovered at 6.83 ± 0.02 $\log_{10}$CFU ml^{-1}, the *slyA* mutant was recovered at 6.62 ± 0.03 $\log_{10}$CFU ml^{-1}, and the *invH* mutant was recovered at 5.98 ± 0.09 $\log_{10}$CFU ml^{-1}. The reduction associated with the *slyA* mutation was again small but significant ($0.05 > P > 0.02$). Similar results were obtained from mucosa with Peyer's patches.

The small reduction in invasion associated with the *slyA* mutation, both in vitro and in bovine ileal loops, appears to contradict the results from other studies (5, 14). However, the reduction is relatively small and its detection may depend on the specific experimental conditions used. In particular, Daniels et al. (5) quantified bacterial invasion at 150 min after infection of murine ligated ileal loops (90 min of infection, 60 min of incubation with gentamicin). Between 120 and 180 min after infection, portions of the dome were completely denuded in loops infected with the wild-type strain but not in those infected with the *slyA* mutant. Therefore, comparisons of bacterial recovery were not made in tissues in equivalent states. Furthermore, the experimental design, in which excised tissue was incubated in Luria-Bertani broth containing gentamicin for 1 h and then thoroughly washed, is likely to cause considerable artifactual mucosal damage and cell exfoliation (and hence loss of associated bacteria), thus making the recovery data unreliable.

***slyA* mutants induce typical lesions in intestinal mucosa.** The severity and nature of the damage at 3 h, 2 h, and 15 min after infection of bovine ligated ileal loops were assessed by electron microscopy. Loops for electron microscopy were fixed in situ by injecting 5 ml of 0.1 M phosphate-buffered 3% glutaraldehyde (pH 7.3) and prepared for microscopy as described previously (17). Biopsies from a total of six calves were examined, two calves for each time point. At 3 h after infection, there was extensive damage to the ileal mucosa, including enterocyte exfoliation, blunting of absorptive and dome villi, an influx of PMNs, and a lack of differentiation of M cells. There was relatively little difference between loops infected with the wild-type strain and those infected with the *slyA* mutant, although in one calf there was a larger influx of PMNs and in the other calf there were more-severe lesions associated with the wild-type strain. At 2 h after infection, the damage was relatively less severe and the reduction in damage associated with the *slyA* mutation, although still small, was more reproducible (Fig. 3). At 15 min after infection, there was relatively little damage induced by either strain, although both the absorptive villi and dome villi were slightly stunted. The dome villi above crypt level appeared abnormal, with extrusion of M cells and PMNs into the intestinal lumen and formation of lamellipodia from peripheral microfolds of M cells. There was no denuding of any area of the dome villus epithelial mono-

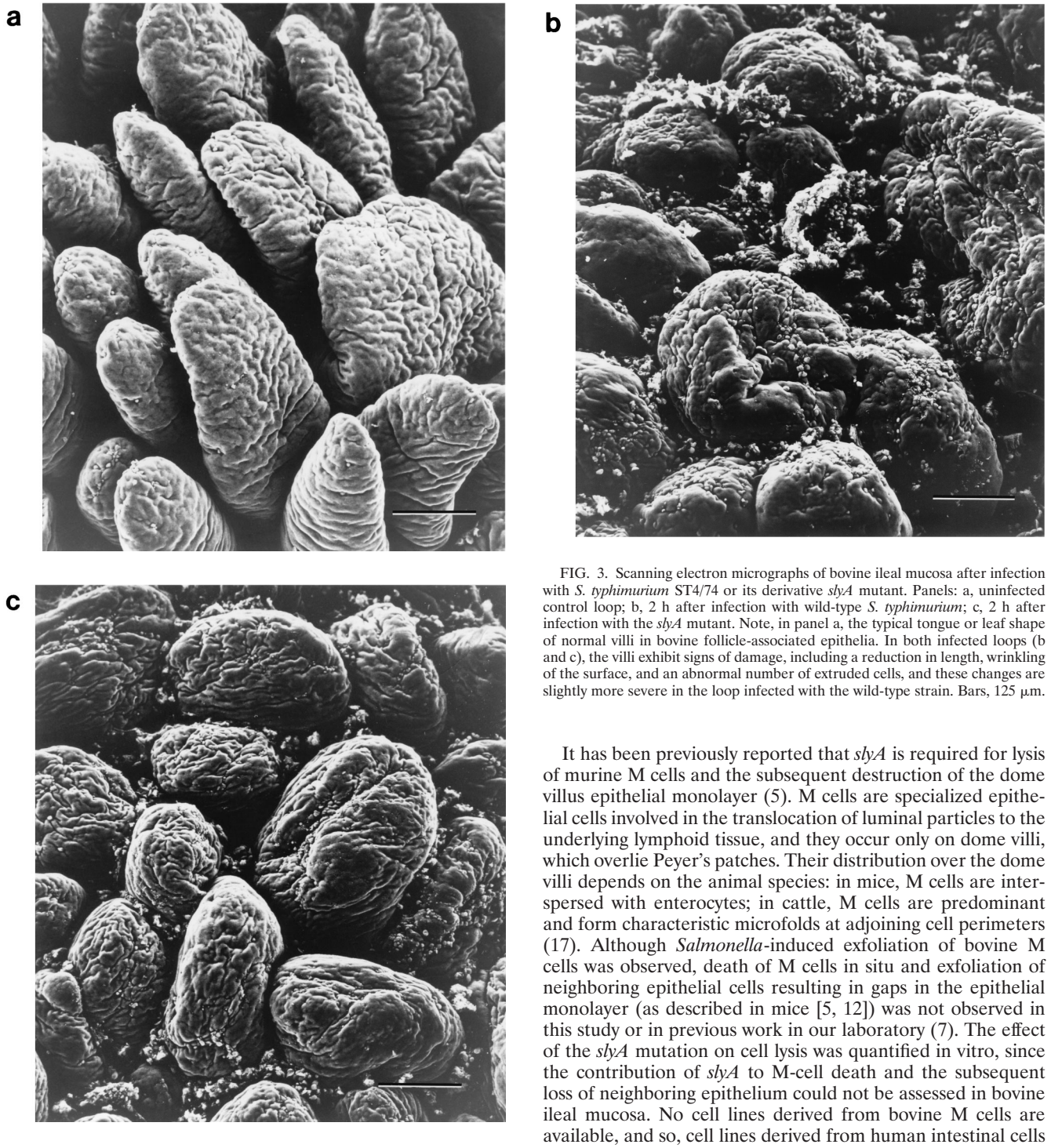

FIG. 3. Scanning electron micrographs of bovine ileal mucosa after infection with *S. typhimurium* ST4/74 or its derivative *slyA* mutant. Panels: a, uninfected control loop; b, 2 h after infection with wild-type *S. typhimurium*; c, 2 h after infection with the *slyA* mutant. Note, in panel a, the typical tongue or leaf shape of normal villi in bovine follicle-associated epithelia. In both infected loops (b and c), the villi exhibit signs of damage, including a reduction in length, wrinkling of the surface, and an abnormal number of extruded cells, and these changes are slightly more severe in the loop infected with the wild-type strain. Bars, 125 μm.

layer. In the absorptive villi, there were discrete areas of enterocyte ruffling and exfoliation. Similar changes during *Salmonella* infection of bovine ligated ileal loops have been reported previously (7). It was not possible to accurately quantify any differences between the wild type and the *slyA* mutant by scanning electron microscopy because the differences were relatively small and there was some variation within and between loops. However, in general, loops infected with the *slyA* mutant appeared to contain either lesions similar to those of loops infected with the wild-type strain or fewer and less severe lesions.

It has been previously reported that *slyA* is required for lysis of murine M cells and the subsequent destruction of the dome villus epithelial monolayer (5). M cells are specialized epithelial cells involved in the translocation of luminal particles to the underlying lymphoid tissue, and they occur only on dome villi, which overlie Peyer's patches. Their distribution over the dome villi depends on the animal species: in mice, M cells are interspersed with enterocytes; in cattle, M cells are predominant and form characteristic microfolds at adjoining cell perimeters (17). Although *Salmonella*-induced exfoliation of bovine M cells was observed, death of M cells in situ and exfoliation of neighboring epithelial cells resulting in gaps in the epithelial monolayer (as described in mice [5, 12]) was not observed in this study or in previous work in our laboratory (7). The effect of the *slyA* mutation on cell lysis was quantified in vitro, since the contribution of *slyA* to M-cell death and the subsequent loss of neighboring epithelium could not be assessed in bovine ileal mucosa. No cell lines derived from bovine M cells are available, and so, cell lines derived from human intestinal cells (Int 407) and bovine kidney cells (MDBK) were used. The effect of the *slyA* mutation on *Salmonella*-induced lysis was assessed by measuring the release of intracellular LDH into the culture supernatants by using the CytoTox 96 assay. The assay conditions were identical to those of the invasion assay, except that monolayers were incubated for up to 6 h after infection without the addition of gentamicin. There was no release of LDH at either 2 or 4 h after infection, compared to uninfected cells. At 6 h after infection, wild-type *S. typhimurium* induced the release of 19.8% ± 1.1% and 17.9% ± 1.3% LDH and the *slyA* mutant induced the release of 11.0%

± 0.7% and 13.0% ± 1.0% from Int 407 and MDBK cells, respectively. The reduction associated with the *slyA* mutation was significant ($0.02 > P > 0.001$). The *invH* mutant induced the release of 4.6% ± 0.1% and 1.8% ± 0.1% LDH from Int 407 and MDBK cells, respectively, which was similar to the background level of LDH release in uninfected cells. The small but significant reduction in cell lysis associated with the *slyA* mutation is consistent with its postulated role in influencing M-cell lysis. However, it also demonstrates that *slyA*-regulated cell lysis is not restricted to M cells and that the reduction is relatively small, for example, compared to that caused by mutation of *invH*, which disrupts the function of *Salmonella* pathogenicity island 1. The small reduction in cell lysis, together with the other relatively modest effects on the interaction of *S. typhimurium* with bovine intestines, probably accounts, at least in part, for the partial attenuation of the *slyA* mutant in cattle. The decreased ability of the *slyA* mutant to survive in macrophages (14) may reduce its spread to and/or colonization of systemic tissues, and this may also contribute to its partial attenuation in cattle.

The different effects of mutation of *slyA* on *Salmonella* virulence in mice and cattle imply a role for *slyA* in the regulation of virulence genes influencing systemic, but not enteric, pathogenesis. The identification of genes regulated by *slyA* is the next key step in understanding the role of this gene in pathogenesis.

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