INFECTION AND IMMUNITY, Dec. 1993, p. 5231–5236
0019-9567/93/125231-06$02.00/0

Vol. 61, No. 12

Expression of the Salmonella Virulence Plasmid Gene *spvB* In Cultured Macrophages and Nonphagocytic Cells

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Received 14 May 1993/Returned for modification 21 July 1993/Accepted 10 September 1993

Certain serotypes of salmonellae carry virulence plasmids that greatly enhance the pathogenicity of these bacteria in experimentally infected mice. This phenotype is largely attributable to the 8-kb *spv* regulon. However, *spv* genes are not expressed while bacteria grow in vitro. We now show that *spvB*, which is required for virulence, is expressed rapidly after *Salmonella dublin* is ingested by cultured J774 and murine peritoneal macrophages and that expression is not affected by the alkalinization of intracellular vesicles. The level of induction of *spvB* is reduced when macrophages are pretreated with gamma interferon. *spvB* is also expressed in human and canine epithelial cell lines and a human hepatoma cell line. In all cases, *spvB* expression is dependent on the *spvR* gene, just as it is in stationary-phase cultures in vitro. These data suggest that *spv* virulence genes are expressed by intracellular salmonellae in vivo in response to a signal that is common to the intracellular compartments of cells that are invaded by salmonellae.

A number of salmonella serotypes carry virulence plasmids (19). The presence of these plasmids greatly increases virulence in experimentally infected mice (6, 24), and plasmid-carrying salmonellae are isolated from naturally occurring systemic infections in animals and humans more often than from enteric infections (14, 32), indicating that plasmids play an important role in the pathogenesis of nontyphoidal salmonella bacteremia. Although different *Salmonella* serotypes each carry a distinct, characteristic virulence plasmid, the plasmids are functionally interchangeable (3, 4, 39) and share a high degree of DNA homology (25, 32, 35, 42). Most significantly, all the plasmids have a highly conserved 8-kb region that has been named the *spv* regulon (19). In experimental infections, the *spv* region is primarily responsible for the virulence phenotype of the plasmids (20, 27, 43), and the complete sequence of *spv* from several serotypes has been determined (20, 27). The regulon consists of the positive regulatory gene, *spvR*, that activates transcription of the *spvABCD* structural genes from a promoter upstream of *spvA* (10, 26). Furthermore, we have shown, using transcriptional fusions to the *lacZ* gene, that *spv* genes are not expressed during logarithmic growth in vitro but can be induced under conditions of carbon starvation (10). These results were confirmed by Valone et al., who used antibodies to detect SpvB made from the native plasmid under a number of stress conditions, including carbon starvation (40). The induction of *spv* genes during the stationary phase in salmonellae is dependent on *katF*, a gene that encodes an alternate sigma factor (11).

Despite the substantial progress that has been made in understanding the organization and regulatory control of the *spv* locus and the importance of the *spv* regulon in determining salmonella virulence, very little is known about the functions of the Spv proteins, other than SpvR. We have shown that the *Salmonella dublin* virulence plasmid increases the rate of bacterial growth in the spleen, liver, and lymph nodes of orally infected mice (23), which leads to early bacteremia and death before the infected mice can develop immunity. Similar results have been found with *S. typhimurium* (22). These observations suggest that the plasmid enhances the growth of salmonellae within tissues and, presumably, inside host cells, since salmonellae are mostly intracellular, a location at which they are protected from the bactericidal effects of gentamicin (13, 22). Thus, if the *spv* operon is important for the intracellular growth of salmonellae, the *spv* genes should be expressed inside host cells. To investigate this question, we have studied the expression of *spvB*, because we have shown that *spvB* is required for mouse virulence, whereas *spvA* (which is more abundantly expressed) is not necessary for virulence (38). Using a reporter plasmid in which *spvB* is fused with *lacZ*, we demonstrate that *spvB* is rapidly induced inside macrophages, epithelial cells, and hepatocytes.

MATERIALS AND METHODS

Bacteria and plasmids. The strain *S. dublin* Lane and the isogenic plasmid-cured strain LD842 were previously described (6). Plasmid pFF14 contains *spvR*, *spvA*, and a translational *spvB-lacZ* fusion (10). Plasmid pFF25 is identical to pFF14 but has an inactivating deletion mutation in *spvR* (10). Bacteria were grown at 37°C to the mid-log phase in Trypticase soy broth before infections.

Tissue cultures. Murine macrophage-like cell line J774 (ATCC TIB 67), human cervical epithelial cell line HeLa (ATCC CCL 2), human hepatoma cell line HepG2 (ATCC HB 8065), and canine kidney epithelial cell line MDCK (ATCC CCL 34) were obtained from the American Type Culture Collection, Rockville, Md., and grown in Dulbecco minimal essential medium (DMEM) supplemented with 10% fetal calf serum. Human T_{84} colon epithelial cells (a gift from

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K. Dharmsathaphorn) were grown in 50% DMEM–50% Ham's F12 medium supplemented with 2 mM glutamine and 10% fetal calf serum. Mouse peritoneal macrophages were obtained from male BALB/c:Ity^r mice (36) that had been injected intraperitoneally three days before with Brewer's modified thioglycolate broth (Difco, Detroit, Mich.). All cells were grown at 37°C in an atmosphere of 95% air–5% CO_2. Cell viability, as assessed by trypan blue exclusion, was greater than 90% in all experiments.

Opsonization. For complement opsonization, bacteria were incubated in 40% pooled normal human serum in DMEM at 37°C for 20 min. For opsonization with antibody, bacteria were incubated in heat-inactivated immune mouse serum at a dilution of 1:500 in phosphate-buffered saline (PBS) for 20 min at room temperature.

Infection protocol. To infect J774 cells, opsonized bacteria were washed three times, resuspended in DMEM, and mixed with the cells at a ratio of 10:1. The suspension was centrifuged in a 2-ml microcentrifuge tube for 5 min at room temperature to maximize contact between cells and bacteria. After 10 min of incubation at 37°C, uningested bacteria were removed by three washes with PBS–10% bovine serum albumin. Cells were resuspended in DMEM containing 12 μg of gentamicin per ml and plated on 24-well tissue culture plates. At various times after infection, the culture medium was removed, the adherent cells were lysed with 0.1% sodium dodecyl sulfate (SDS) in isotonic saline, and the number of viable intracellular bacteria was determined. An aliquot of the lysate was processed to measure β-galactosidase activity (see below). All measurements were done in triplicate. Mouse peritoneal macrophages were allowed to adhere to plastic overnight in 24-well Costar plates. Opsonized bacteria in DMEM at a ratio of 10:1 were centrifuged onto the macrophages. After 15 min, extracellular bacteria were washed away and medium containing gentamicin was added as described above.

Infections of T_{84}, HeLa, and MDCK epithelial cells were performed as previously described (9, 15). In brief, confluent monolayers in six-well tissue culture plates were infected with 10^7 to 10^8 bacteria at 37°C for 2 h. Subsequently, the monolayers were washed three times with Hanks balanced salt solution and further incubated in the presence of 50 μg of gentamicin per ml. Two hours after the addition of gentamicin, the monolayers were lysed with 0.1% SDS in isotonic saline containing 2 U of DNase per ml and processed as described for J774 cells. For time course experiments, bacteria were centrifuged onto the monolayers (400 × *g*, 5 min, 22°C) and incubated at 37°C for 15 min before washing and addition of gentamicin were done.

Assay of β-galactosidase activity. Initial attempts to measure β-galactosidase activity with the commonly used substrate *o*-nitrophenyl-β-D-galactopyranoside were unsuccessful because the enzymatic activity of uninfected J774 macrophages was too high, even at a neutral pH, reducing the sensitivity of the assay below the point at which we could detect the enzymatic activity of the intracellular salmonellae. We therefore developed an assay based on the fluorescent substrate 3-carboxyumbelliferyl-β-D-galactopyranoside (CUG) (Molecular Probes, Eugene, Oreg.). Bacteria were permeabilized with chloroform and further processed as suggested by the manufacturer. In brief, 130 μl of the bacterial suspension was mixed with 50 μl of 8 mM CUG and incubated at room temperature for 30 min. The reaction was stopped with glycine-buffered EDTA (pH 11.0), and fluorescence was determined at an excitation wavelength of 390 nm and an emission wavelength of 460 nm with a Perkin-Elmer fluorescence spectrophotometer. Enzymatic activity is expressed as fluorescence units (FU) per 10^5 viable bacteria (10^5 CFU). This is an arbitrary measure of enzymatic activity and is not corrected for the volume of the reaction mixture (130 μl). A linear relationship was obtained between the number of *spvB-lacZ*-expressing bacteria and β-galactosidase activity, as measured in FU (Fig. 1). The enzymatic activity of as few as 10^4 bacteria was reproducibly detected. Enzymatic activity in MDCK cells was measured with the fluorescent substrate fluorescein di-β-D-thiogalactopyranoside (FDG) as previously described (17). The β-galactosidase activity of uninfected cells was determined in parallel for each experiment and was subtracted as the background. Using the CUG assay for β-galactosidase, we found that enzymatic activity in LD842(pFF14) was induced 10- to 20-fold when the bacteria entered stationary phase in vitro.

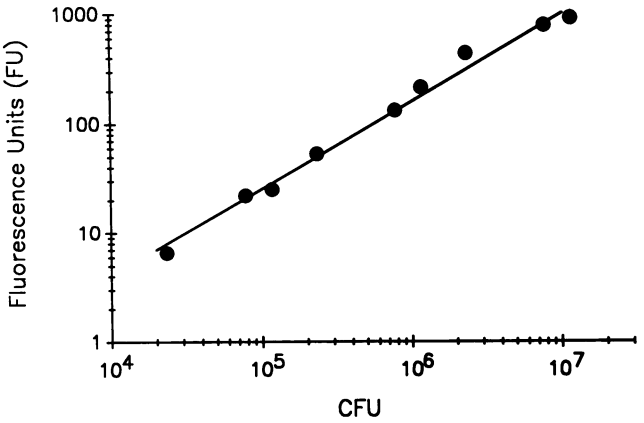

FIG. 1. Relationship between β-galactosidase activity and the number of viable stationary-phase LD842(pFF14). Enzymatic activity was measured with the fluorescent substrate CUG as described in Materials and Methods.

Cytokines. Recombinant murine gamma interferon (IFN-γ) was a gift from Genentech, Mountain View, Calif.

Data analysis. The difference between means was analyzed with Student's *t* test for small sample sizes (8).

RESULTS

Expression of the salmonella virulence plasmid gene *spvB* following bacterial phagocytosis and invasion was studied with the *spvB-lacZ* reporter construct pFF14 in *S. dublin* LD842. *spvB* expression was induced following phagocytosis of LD842(pFF14) by J774 cells (Fig. 2), whereas noningested bacteria did not show an increase in *spvB* expression (Table 1). We could detect increased gene expression 10 min after phagocytosis, and expression was maximal by 2 h, with a 10- to 20-fold increase in activity (data not shown). *spvB* expression was independent of bacterial opsonization, since comparable results were obtained with unopsonized bacteria (Fig. 2) or bacteria that were opsonized with normal human serum or immune mouse serum (data not shown).

To determine whether the expression of *spvB* inside J774 cells was dependent on *spvR*, we used plasmid pFF25, which is identical to pFF14 but has a deletion in the *spvR* gene which renders it inactive (10). No increase in β-galactosidase activity was found 2 h after phagocytosis of LD842(pFF25) by J774 cells, while *spvB* gene expression was increased more than 10-fold after phagocytosis of LD842(pFF14) (2.7 to 36.5 FU/10^5 CFU). Thus, an intact *spvR* gene is required

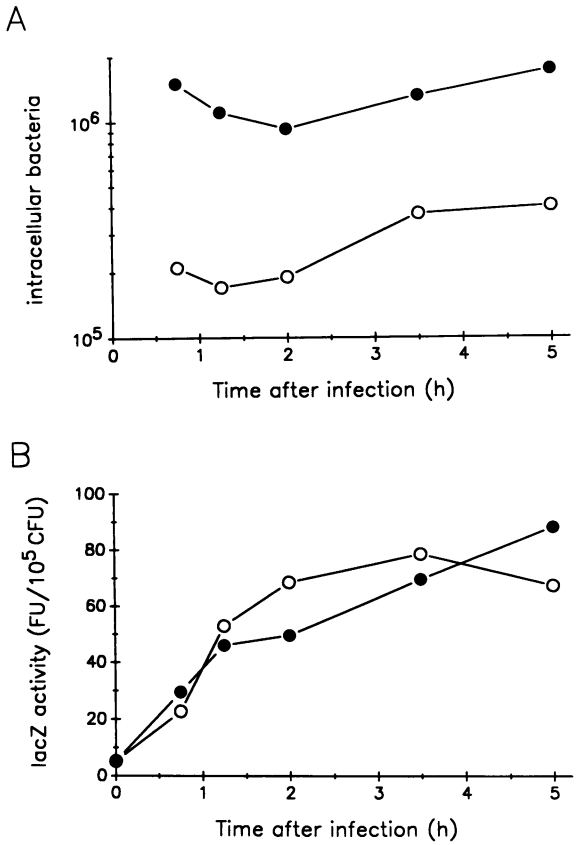

FIG. 2. Comparison of *spvB* expression by LD842(pFF14) over time in adherent J774 (●) and T_{84} (○) cells. (A) Viable intracellular bacterial counts. (B) *spvB* activity (expressed as FU/10^5 CFU). The bacteria were not opsonized in this experiment and were added to the cells while in log phase as described in Materials and Methods.

for the intracellular expression of *spvB*, and the level of induction of *spvB-lacZ* inside J774 cells was comparable to the level induced by carbon starvation in vitro.

Since the induction of the chromosomal *pagC* gene of *S. typhimurium* in J774 cells is dependent on phagosomal acidification (2), we analyzed the effect of raising the lysosomal pH on the induction of *spvB*. To raise the pH of intracellular vesicles, J774 cells were treated with 5 mM NH_4Cl or 5 μM monensin prior to infection with opsonized LD842(pFF14) (16). As shown in Table 2, alkalinization of J774 cells did not prevent efficient expression of *spvB* indicating that an acid pH is not required for *spvB* induction.

Macrophages are induced to synthesize several proteins following phagocytosis or other stimuli that activate macrophages (1). To investigate whether *spvB* expression was dependent on new protein synthesis by macrophages, we preincubated mouse peritoneal macrophages with cycloheximide for 2 h prior to adding opsonized LD842(pFF14). The expression of *spvB* inside cycloheximide-treated macrophages was not significantly different from the expression inside untreated cells (Table 2), indicating that intracellular salmonellae respond to a macrophage signal that does not require new protein synthesis. Since IFN-γ has profound effects on macrophage function (33), we investigated the effect of that cytokine on bacterial *spvB* expression. As shown in Table 2, pretreatment of J774 cells with optimal

TABLE 1. Expression of *spvB-lacZ* in macrophages and other cells[a]

Expt	Cells	Bacterial location	*spvB-lacZ* activity	
			FU/10^5 CFU	Relative increase
1		Extracellular[b]	3.1 ± 0.7	
	J774	Intracellular	49.0 ± 8.8	15.8 ± 2.8
	T_{84}	Intracellular	45.8 ± 11.8	14.8 ± 3.8
	HeLa	Intracellular	29.5 ± 13.0	9.5 ± 4.2
2		Extracellular[b]	2.7 ± 1.0	
	HepG2	Intracellular	54.2 ± 6.2	20.1
3		Extracellular[b]	631 ± 345	
	MDCK	Intracellular	5,283 ± 1,270	8.4 ± 2.0
4		Extracellular[b]	1,460 ± 761	
	HeLa	Intracellular	5,928 ± 1,125	4.8 ± 1.4

[a] Subconfluent cell cultures were infected for 2 h with 1×10^7 to 5×10^7 LD842(pFF14) cells, washed three times, and further incubated for 2 h in medium containing 50 μg of gentamicin per ml. At the end of the culture period, cells were lysed and the number of intracellular bacteria was determined. Aliquots of the lysates were assayed for *lacZ* activity with fluorescent substrates CUG for experiments 1 and 2 and FDG for experiments 3 and 4. In experiments 1 and 2, late-log-phase bacteria were used to infect the cells. In experiments 3 and 4, stationary-phase bacteria were added to the cells. Data are the mean ± standard error of the mean for four independent replicates for experiments 1 and 3 and three independent replicates for experiment 4. Experiment 2 was a single experiment; the measurement of intracellular fluorescence is the mean for triplicate wells.

[b] At the end of the 2-h infection period, the extracellular bacteria were removed, and their numbers and *lacZ* activity in the samples were determined.

doses of IFN-γ decreased *spvB* expression by about 40% compared with expression in untreated macrophages.

To find out whether the induction of *spvB* was specific for macrophages, we infected three epithelial cell lines, T_{84}, HeLa, and MDCK, and HepG2 hepatoma cells with LD842(pFF14). As a control, adherent J774 macrophages were infected with the same bacteria. We found increased *spvB* expression by intracellular bacteria in all three epithelial cell lines and in the hepatoma cells (Table 1). As shown in Fig. 2, the time courses of *spvB* induction were identical in T_{84} and J774 cells. There were only minor changes in bacterial colony counts during the 2-h incubation and no difference between the J774 and T_{84} cells.

DISCUSSION

We demonstrated that *spvB* was rapidly induced after phagocytosis by J774 and mouse peritoneal macrophages and that alkalinization of the lysosomes did not prevent or reduce *spvB* expression. In these regards, the intracellular regulation of *spv* differs from the behavior of *pag*, another salmonella virulence gene that is induced in macrophages (2). Pag protein expression, which is regulated by *phoP* (18, 31), is not induced until several hours after phagocytosis by J774 cells and is abrogated by alkalinization of lysosomes with NH_4Cl (2). Of note, although NH_4Cl and monensin may have multiple effects on macrophages, the fact that *pagC* expression was decreased by those treatments and that *spvB* expression was not still indicates that the two genes respond to different conditions. In addition, Pag proteins are not expressed inside epithelial cells (2), whereas we found that *spvB* was induced in these cells. Thus, there are clear differences in the time course of intracellular expression, pH

TABLE 2. Modulation of *spvB* expression by *S. dublin* in macrophages[a]

Macrophages	Treatment (no. of expts)	*spvB-lacZ* expression[b] (% of control)
J774	None (15)	100
	NH_4Cl[c] (3)	119 ± 18[d]
	Monensin[c] (3)	113 ± 4[d]
	IFN-γ[e] (15)	57 ± 6[f]
Murine peritoneal	None (2)	100
	Cycloheximide[c] (2)	147 ± 21[d]

[a] Macrophages were infected with LD842(pFF14) for 15 min and plated on 24-well plates in the presence of gentamicin as described in Materials and Methods. After 2 h in culture, the cells were lysed and the number of viable intracellular bacteria and β-galactosidase activity were measured.

[b] *spvB* expression was determined as the ratio of β-galactosidase activity to bacterial counts and is expressed as a percentage of the value for bacteria in untreated macrophages for each experiment. Data are means ± standard errors of the means for the percentages in several independent experiments.

[c] NH_4Cl (5 mM), monensin (5 μM), and cycloheximide (10 to 50 μg/ml) were added to the macrophages 2 h before infection and were removed just before infection.

[d] No significant difference ($P > 0.1$) compared with expression in untreated macrophages.

[e] IFN-γ (50 to 500 U/ml) was added 12 to 16 h before infection and was removed immediately before infection.

[f] Significant difference ($P < 0.01$) compared with expression in untreated J774 cells.

dependence, and host cell specificity between the *spvB* and *pagC* systems, suggesting that these two virulence genes are induced in response to different stimuli inside host cells. Also implied is that salmonellae have developed an orchestrated response to changing conditions inside macrophages. It is likely that the sequential expression of both *spv* and *pag* is necessary for optimal intracellular growth of salmonellae.

We found evidence that macrophages were not passive partners in the induction of *spvB* expression, since *spvB* expression was significantly reduced in IFN-γ-treated macrophages. This result was not due to decreased viability, since IFN-γ did not enhance the killing of salmonellae in treated macrophages, as has been reported by van Dissel et al. (41). However, it is not clear whether the reduction in *spvB* expression has physiologic significance or whether bacteria make less *spvB* in activated macrophages because of less stimulation or because activated macrophages actively prevent *spvB* expression. In any case, our findings underline the notion of complex, reciprocal interactions between host cells and ingested salmonellae.

In addition to being expressed inside macrophages, *spvB* was also induced after invasion of epithelial and hepatic cells. The expression of *spvB* in epithelial cells is somewhat surprising, since previous work on salmonella pathogenesis did not provide evidence that the presence of the virulence plasmid has an effect on clinical invasiveness during the first days after an oral salmonella infection (21, 23, 34). Moreover, the plasmid does not enhance the growth of *S. dublin* in cultured MDCK epithelial cells (14a). Nevertheless, the finding of *spvB* expression inside epithelial cells may be useful for defining the intracellular signals which lead to *spvB* induction. Salmonellae are located inside vacuoles in both macrophages and epithelial cells (15). This fact suggests that the signal for *spvB* expression is common to the environment of all such vacuoles or that *spvR* responds to multiple signals. Garcia-del Portillo et al. recently demonstrated that salmonellae in MDCK cells are in vacuoles that have limiting concentrations of Fe^{2+}, Mg^{2+}, mannose, and lysine and a low O_2 tension (17).

Valone et al. recently showed that SpvA, SpvB, and SpvC are synthesized when *S. dublin* is exposed to an acidic pH (5.0 to 5.5) in vitro (40). However, we doubt that this is the signal in vivo, since *spvB* was expressed inside macrophages even when the cells were pretreated with NH_4Cl or monensin to prevent phagosomal acidification. Moreover, it has been shown that *S. typhimurium* in J774 cells resides in an intracellular compartment with a pH that is above 5.6 (2). In addition, Garcia-del Portillo et al. have shown that inside MDCK cells, the pH of the salmonella vacuole is approximately 6.0 (17). Taken together, these findings make it unlikely that an acidic pH is an important signal for the induction of *spvB* expression in vivo. The stimulus for *spvB* expression inside cells is not known and may vary from cell to cell.

The rapid induction of *spvB* in response to phagocytosis suggests that SpvB may be important for bacterial survival inside macrophages. However, several laboratories have used different strategies for finding salmonella macrophage survival genes, and although over 30 genes have been identified in *S. typhimurium* as being involved in macrophage survival, none have been shown unequivocally to be on the virulence plasmid (5, 12, 29). These screening methods, in essence, define genes which are required for salmonella survival in macrophage cell lines or in tissue macrophages (12, 29) by finding mutants that cannot survive in macrophages. *spv* genes are not detected by these screening methods because they do not fall into this category. In vitro studies by Riikonen et al. (37) showed that the loss of the virulence plasmid had no effect on the macrophage survival of *S. typhimurium*. The conclusion that *spvB* is not required for macrophage survival is supported by our previous work showing that the cured strain, LD842, multiples in the liver and spleen of infected mice, although the parent strain, *S. dublin* Lane, multiplies faster (23). That observation was recently confirmed with *S. typhimurium* by Gulig and Doyle, who also showed that the effect of *spv* in vivo was in fact to promote growth in vivo, not to prevent killing (22). Thus, the predominance of evidence suggests that salmonellae survive in macrophages even without a virulence plasmid and that the *spv* operon provides to intracellular salmonellae a small, but biologically crucial, growth advantage which, probably for technical reasons, is difficult to demonstrate in vitro with isolated macrophages. The enhanced rate of bacterial growth (attributable to the *spv* operon) is enough to convert a self-limited infection into a highly lethal infection.

In these studies, we found that *S. dublin* invaded a human hepatoma cell line, and Conlan and North recently showed that salmonellae multiply within hepatocytes as well as within Kupffer cells in experimentally infected mice (7). Since *spvB* is induced in the hepatoma cell line, it also may be induced in normal hepatocytes and so affect the rate of bacterial growth in these cells.

The signal that is responsible for *spvB* gene expression inside mammalian cells has not yet been identified. Several in vitro observations suggest that nutrient deprivation, the stimulus for in vitro expression, can also be an important signal inside cells. For example, carbon starvation induces *spvB* expression (10), and the expression of Spv proteins in response to iron deprivation has been documented (40). We have also found that *spvB* is induced in *S. dublin* in response to the limitation of nicotinic acid and nitrogen (12a). The availability of nutrients inside macrophage phagosomes has not been studied in detail, but adenine is not present in a

sufficient supply to allow salmonella *purA* mutants to survive in macrophages (30). If the limitation of critical nutrients is indeed the signal for SpvB expression, one would expect the expression of SpvB to be dependent on regulatory factors known to be important in regulating bacterial responses to starvation. One of these factors is the alternative sigma factor KatF (28), and we have shown that KatF is required for *spvB* expression by salmonellae in vitro (11). It will be important to determine whether KatF is also required for the expression of *spvB* inside macrophages and epithelial cells.

ACKNOWLEDGMENTS

This work was supported in part by grants from the V.A. Medical Research Service, the Universitywide AIDS Taskforce (R91SD057), and the MRC of Canada and by Program Project PHS DK35108 from NIH. F.F. was supported by Physician-Scientist award DK01408. L.E. is a Research Fellow of the Crohn's and Colitis Foundation of America. We thank Sharon Okamoto for excellent technical assistance.

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