

Salmonella enterica Serovar Typhimurium Response Involved in Attenuation of Pathogen Intracellular Proliferation

DAVID A. CANO,¹ MARINA MARTÍNEZ-MOYA,^{2†} M. GRACIELA PUCCIARELLI,^{2‡} EDUARDO A. GROISMAN,³ JOSEP CASADESÚS,¹ AND FRANCISCO GARCÍA-DEL PORTILLO^{2*}

Departamento de Genética, Facultad de Biología, Universidad de Sevilla, 41080 Sevilla,¹ and Centro de Biología Molecular “Severo Ochoa,” CSIC-Universidad Autónoma de Madrid, 28049 Madrid,² Spain, and Howard Hughes Medical Institute, Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110-1093³

Received 30 March 2001/Returned for modification 4 June 2001/Accepted 4 July 2001

Salmonella enterica serovar Typhimurium proliferates within cultured epithelial and macrophage cells. Intracellular bacterial proliferation is, however, restricted within normal fibroblast cells. To characterize this phenomenon in detail, we investigated the possibility that the pathogen itself might contribute to attenuating the intracellular growth rate. *S. enterica* serovar Typhimurium mutants were selected in normal rat kidney fibroblasts displaying an increased intracellular proliferation rate. These mutants harbored loss-of-function mutations in the virulence-related regulatory genes *phoQ*, *rpoS*, *slyA*, and *spvR*. Lack of a functional PhoP-PhoQ system caused the most dramatic change in the intracellular growth rate. *phoP*- and *phoQ*-null mutants exhibited an intracellular growth rate 20- to 30-fold higher than that of the wild-type strain. This result showed that the PhoP-PhoQ system exerts a master regulatory function for preventing bacterial overgrowth within fibroblasts. In addition, an overgrowing clone was isolated harboring a mutation in a previously unknown serovar Typhimurium open reading frame, named *igaA* for intracellular growth attenuator. Mutations in other serovar Typhimurium virulence genes, such as *ompR*, *dam*, *crp*, *cya*, *mviA*, *spiR* (*ssrA*), *spiA*, and *rpoE*, did not result in pathogen intracellular overgrowth. Nonetheless, lack of either SpiA or the alternate sigma factor RpoE led to a substantial decrease in intracellular bacterial viability. These results prove for the first time that specific serovar Typhimurium virulence regulators are involved in a response designed to attenuate the intracellular growth rate within a nonphagocytic host cell. This growth-attenuating response is accompanied by functions that ensure the viability of intracellular bacteria.

Salmonella enterica serovar Typhimurium causes food-borne diseases in humans and animals ranging from self-limited gastroenteritis to bacteremia and systemic infections (52). Major hallmarks of serovar Typhimurium pathogenesis include the exploitation of epithelial cell functions in inducing bacterial uptake and resistance to antimicrobial attack of phagocytic cells (reviewed in references 11, 20, and 73). These mechanisms enable serovar Typhimurium to efficiently penetrate the intestinal epithelial barrier and disseminate to deeper tissues (52, 72). In vitro and in vivo models have demonstrated that both responses are required for elicitation of disease (15, 19). Serovar Typhimurium also proliferates extensively within membrane-bound vacuoles of cultured epithelial and macrophage cells (16, 21, 25, 45). This process initiates 4 to 6 h after bacterial internalization (25) and ends at a stage at which the vacuole containing fast-growing bacilli entirely fills the preexisting host cell cytosol. In vitro models have shown that the average increase in viable intracellular bacteria ranges from 10- to 100-fold during the first 24 h of infection depending on

the cell line used (45, 65). In contrast, the increase in the intracellular growth rate estimated in vivo is in the range of 5- to 10-fold every 24 h (2, 63, 70). The discrepancy in the growth rates between the two models raises the question whether serovar Typhimurium intracellular proliferation occurs in vivo to the same extent as in cultured cell lines. Likewise, there is not yet clear evidence that a process of massive intracellular proliferation is absolutely required for a fatal outcome of the infection. Indeed, several studies have reported that massive pathogen growth is not required to cause host death. Thus, an enhanced production of immune mediators, such as tumor necrosis factor alpha and interleukin-1, may lead to fatal consequences for the host even while the host harbors nonsaturating bacterial doses (39).

The extent to which *Salmonella* intracellular proliferation is required for virulence is at present unknown. An early report claimed that intraepithelial proliferation of serovars Typhimurium and Choleraesuis was essential for virulence, since in vitro-selected mutants displaying a lower intracellular growth rate behaved as attenuated in the murine typhoid model (17, 40). However, more recent studies have shown that intraepithelial proliferation might not be relevant in vivo. Thus, confocal microscopy analysis performed on liver and spleen tissue sections of infected mice has shown that the majority of proliferating bacteria reside and multiply almost exclusively in CD18-expressing phagocytes (8, 46, 58). Interestingly, these studies did not provide evidence for infected cells carrying as many intracellular bacteria as in vitro-infected cultured cells.

* Corresponding author. Mailing address: Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología-CSIC, Campus de Cantoblanco, 28049 Madrid, Spain. Phone: (34) 91 5854923. Fax: (34) 91 5854506. E-mail: fgportillo@cnb.uam.es.

† Present address: Fraunhofer Institut für Grenzflächen- und Bioverfahrenstechnik, D-70569, Stuttgart, Germany.

‡ Present address: Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas (CSIC), Campus de Cantoblanco, 28049 Madrid, Spain.

Morbidity and mortality are severely compromised when the macrophage cell population is selectively immunodepleted in susceptible animals (31), suggesting that phagocytes are the main host cell types permissive for serovar Typhimurium intracellular growth. In addition, intracellular proliferation of *S. enterica* serovar Typhimurium seems to be significantly stimulated in tumor cells implanted in animals compared to other tissues or organs of the infected animal (44, 55). Altogether, these observations indicate that intraepithelial proliferation of serovar Typhimurium, if it exists, might be limited during the course of the infection.

The identity of the serovar Typhimurium functions required for intraepithelial proliferation is still a matter of debate. The functionality of the *Salmonella* pathogenicity island (SPI-2)-encoded type III secretion system (TTSS) was initially proposed as essential for intraepithelial growth (4). However, SPI-2 has been recently shown to be strictly required for intramacrophage survival and dispensable for intraepithelial growth (32). Other studies have reinforced the concept that SPI-2 is required to elicit enteritis and systemic disease as well as to promote intracellular bacterial growth within phagocytes located in target organs (3, 62).

A series of cell biology studies, based almost exclusively on *in vitro* models, have attempted to provide clues to how serovar Typhimurium initiates intracellular growth. It is known that bacterial proliferation within cultured epithelial cells correlates with alterations in the distribution of host lysosomal membrane glycoproteins (LGP) and the formation of filamentous structures named Sifs (25, 32). Pathogen interference with the host vacuolar trafficking machinery has also been proposed as a major strategy promoting intracellular growth (22, 48, 64). Bacterial proteins required for alteration of the lysosomal glycoprotein distribution include SifA (65), the SPI-2-encoded proteins SseF and SseG (32), and the two-component system OmpR-EnvZ (49). SpiC, a SPI-2-encoded protein, has recently been shown to prevent endosome-lysosome fusion events in macrophages (69). Whether SpiC performs a similar function in epithelial cells is at present unknown. Interestingly, serovar Typhimurium mutants lacking proteins required for Sif formation retain the ability to grow intracellularly within epithelial cells (3, 32, 65), suggesting that Sif formation is dispensable for intraepithelial bacterial proliferation.

We have recently demonstrated that virulent serovar Typhimurium strains are unable to proliferate within certain nonphagocytic cell types such as normal fibroblasts (45). Intracellular bacteria are enclosed within membrane-bound vacuoles in which they are unable to initiate growth (45). Considering that the *in vivo* model has not provided any evidence for massive pathogen proliferation within nonphagocytic cells, we have employed the fibroblast infection model to characterize the mechanisms underlying the control of serovar Typhimurium intracellular growth within nonphagocytic cells.

In an attempt to define the factors involved in preventing intracellular growth of serovar Typhimurium within fibroblast cells, we raised the hypothesis that the absence of bacterial growth could also be the result, at least in part, of a response mounted by the pathogen itself. If this prediction is correct, bacterial mutants lacking functions related to this response should overgrow. A basic positive selection procedure allowed us to isolate serovar Typhimurium mutants with an enhanced

intracellular growth rate. The study has led us to uncover a novel role for specific pathogen-encoded regulators, most of them previously characterized by their contribution to intramacrophage survival. To our knowledge, this is the first report that proves the existence of a growth self-attenuating response triggered by an intracellular bacterial pathogen within a eucaryotic cell.

MATERIALS AND METHODS

Bacterial strains. *S. enterica* serovar Typhimurium strains used in this study are listed in Table 1. Unless otherwise indicated, the reference parental strain for the infection assays was the mouse virulent strain SL1344 (36). Mutant alleles obtained from other investigators in different genetic backgrounds were transduced into SL1344 before the phenotypic analysis of intracellular proliferation within eucaryotic cells. The only exception was the series of nonpolar in-frame deletion mutants in the *spv* genes, which, due to the lack of an appropriate selection marker, were tested in comparison with the parental wild-type (wt) strain SR11 (Table 1). The bank of *MudJ* transposon-generated mutants was constructed in SL1344 according to previously described methods (37). *MudJ* and flanking regions present in overgrowing clones were passaged by P22 HT-mediated transduction to a clean SL1344 genetic background. Upon confirmation of the linkage between the phenotype and the transposon insertion, the exact location of the insertion was mapped by direct chromosomal sequencing (see below). Plasmid pEG5433 containing the wt *phoPQ* locus (29) was used to complement *phoP*- and *phoQ*-null mutants.

Infection of eucaryotic cells. HeLa cells (ATCC CCL2) and NRK-49F rat fibroblasts (ATCC no. CRL-1570) were grown and infected with the different serovar Typhimurium strains as described previously (45). Briefly, bacteria were grown overnight in Luria-Bertani (LB) medium at 37°C in static conditions, and 5 μ l of the culture (approximately 10^6 CFU) was added to eucaryotic cells cultured to up to 60 to 70% confluency in 24-well plastic plates (approximately 10^5 eucaryotic cells/well). These conditions accounted for a multiplicity of infection of 10:1 (bacterium/eucaryotic cell ratio). Cells were infected for 20 to 30 min depending on the extent of bacterial invasion and monitored by microscopic observation and estimation of cultured cells displaying membrane ruffles. Infection was maintained until 50 to 60% of cultured cells showed signs of bacterial uptake. Noninternalized bacteria were further removed by three successive washes with phosphate-buffered saline (PBS), pH 7.4. Fresh growth medium containing 100 μ g of gentamicin ml^{-1} was then added and maintained up to 2 h postinfection, the time at which the antibiotic concentration was lowered to 10 μ g ml^{-1} . At different postinfection times, infected cells were lysed with 1% Triton X-100-PBS (pH 7.4) (5 min, room temperature), and the number of viable intracellular bacteria was determined by plating of appropriate lysate dilutions in LB agar and colony counting. All samples were run at least in duplicate. The average invasion rate obtained in standard 30-min infection assays was 1 to 2% in HeLa cells and 0.2 to 0.5% in NRK-49F fibroblasts. The intracellular proliferation rate (I_{pro}) was calculated as the ratio of the number of viable intracellular bacteria present at 24 h to those present at 2 h postinfection.

Selection of serovar Typhimurium mutants with increased intracellular growth rate in fibroblasts. Selection of overgrowing bacterial mutants was addressed with NRK-49F fibroblasts, since these cells, in contrast to HeLa epithelial cells, are nonpermissive for bacterial intracellular proliferation (45). These fibroblasts do not show signs of cytotoxicity at any time postinfection as tested by dye exclusion assays (data not shown). To select for serovar Typhimurium isolates with high I_{pro} values within NRK-49F fibroblasts, 10 pools, each representing 5,000 kanamycin-resistant clones of serovar Typhimurium, were first obtained by *MudJ* mutagenesis (37). These pools were used separately to infect NRK-49F fibroblast cultures in three to four successive infection cycles. Each of these cycles consisted of 30 min of incubation of NRK-49F fibroblasts with bacteria, 1.5 h in growth medium containing 100 μ g of gentamicin ml^{-1} , and up to 72 h of maintenance of the infection in growth medium containing 10 μ g of gentamicin ml^{-1} . The rationale for infecting up to 72 h was the fact that the viability of intracellular wt serovar Typhimurium within NRK-49F fibroblasts decreases more than 90% at this postinfection time (45), thus rendering the enrichment procedure more effective. In all cases, the inoculum for the intermediate infection cycles was prepared by direct inoculation of the 1% Triton X-100-PBS (pH 7.4) detergent lysate obtained at 72 h into fresh LB medium and further overnight incubation at 37°C. In parallel, the I_{pro} value of the pool was calculated in every infection cycle to validate the enrichment for overgrowing mutants within each pool. Upon the last infection cycle, the 1% Triton X-100-PBS (pH 7.4) lysate was plated, and mutants belonging to the pools that showed

TABLE 1. *S. enterica* serovar Typhimurium strains used in this study

Strain	Genotype	Source or reference
14028s	Wild type	15
MS5996s	14028s <i>phoQ5996::Tn10</i>	14
EG5799	14028s <i>spiR(ssrA)5799::MudJ</i>	51
SL2823	14028s <i>soxRS::Amp^r</i>	13
SL2918	14028s <i>fnr::Tn10</i>	Stephen J. Libby
SL2236	14028s <i>slyA::pRR10ΔtrfA Pen^r</i>	42
12023spvA	14028s <i>spvA::Km^r</i>	35
ST173	LT2 <i>lrp-1::Tn5</i>	Michael P. Spector
SFS78	UK1 <i>aldB2::MudJ mviA(rssB)::Cam^r</i>	Michael P. Spector
SL1344	<i>hisG46 rpsL</i> ; virulent strain	36
SV4056	SL1344 <i>phoP7953::Tn10</i>	23
SV4365	SL1344 <i>phoQ5996::Tn10</i>	This work
EG5170	SL1344 <i>phoP5170::MudJ</i>	E. A. Groisman
GVB311	SL1344 <i>rpoE::Km^r</i>	38
SV4232	SL1344 <i>rpoE::Km^r phoP7953::Tn10</i>	This work
SV4237	SL1344 <i>spiR(ssrA)5799::MudJ</i>	This work
SV4241	SL1344 <i>spiR5799::MudJ phoP7953::Tn10</i>	This work
SV4386	SL1344 <i>spiA::K1XX</i>	This work
SV4387	SL1344 <i>spiA::K1XX phoP7953::Tn10</i>	This work
CJD359	SL1344 <i>ompR1009::Tn10</i>	6
SV4235	SL1344 <i>ompR1009::Tn10</i>	This work
SV4236	SL1344 <i>ompR1009::Tn10 phoP5170::MudJ</i>	This work
SV1610	SL1344 <i>dam228::MudJ</i>	23
SV4089	SL1344 <i>dam228::MudJ phoP7953::Tn10</i>	23
SMS521	SL1344 <i>crp773::Tn10</i>	Michael Spector
SV4197	SL1344 <i>phoQ201 zzz::MudJ</i>	This work
SV4198	SL1344 <i>slyA21::MudJ</i>	This work
SV4210	SL1344 <i>rpoS1151::MudJ</i>	This work
SV4215	SL1344 <i>igaA188 zzz::MudJ</i>	This work
SV4431	SL1344 <i>zzz::MudJ</i>	P22 (SV4215) × SL1344
SV4221	SL1344 <i>spvA101::MudJ</i>	This work
SV4222	SL1344 <i>rpoS1152::MudJ</i>	This work
SV4224	SL1344 <i>phoQ201</i>	This work
SV4234	SL1344 <i>spvA101::MudJ</i>	This work
SV4254	SL1344 <i>igaA188 zhf-6311::Tn10dTet</i>	This work
SV4255	SL1344 <i>zhf-6311::Tn10dTet</i> , nonmucoid	P22 (SV4254) × SL1344
SV4312	SL1344 <i>spvA103::MudJ</i>	This work
SV4313	SL1344 <i>spvA104::MudJ</i>	This work
SV4314	SL1344 <i>spvR105::MudJ</i>	This work
ST97	SL1344 <i>relA21::Tn10</i>	60
SV4285	SL1344 <i>relA21::Tn10 phoP5170::MudJ</i>	This work
SV4253	SL1344 <i>lrp-1::Tn5</i>	This work
SV4286	SL1344 <i>lrp-1::Tn5 phoP7953::Tn10</i>	This work
SMS593	SL1344 <i>oxyR::Tn10</i>	61
SV4287	SL1344 <i>oxyR::Tn10 phoP5170::MudJ</i>	This work
SV4233	SL1344 <i>mviA(rssB)::Cam^r</i>	This work
SV4288	SL1344 <i>mviA(rssB)::Cam^r phoP7953::Tn10</i>	This work
SV4261	SL1344 <i>soxRS::Amp^r</i>	This work
SV4262	SL1344 <i>fnr::Tn10</i>	This work
SMS438	SL1344 <i>rpoS::Ω-Amp^r</i>	53
SV4240	SL1344 <i>rpoS::Ω-Amp^r phoP7953::Tn10</i>	This work
SV4250	SL1344 <i>slyA::pRR10ΔtrfA Pen^r</i>	This work
SV4251	SL1344 <i>spvA::pRR10ΔtrfA Pen^r phoP7953::Tn10</i>	This work
SV4257	SL1344 <i>spvA::Km^r</i>	This work
SV4258	SL1344 <i>spvA::Km^r phoP7953::Tn10</i>	This work
χ3306	SR11, wild type	76
UF082	χ3306 Δ <i>spvR</i>	76
UF104	χ3306 Δ <i>spvA</i>	76
UF051	χ3306 Δ <i>spvB</i>	76

higher I_{pro} values were tested individually in NRK-49F fibroblasts. Analysis of individual clones was made only with pools 1, 2, 3, 4, and 6, in which the I_{pro} value after the last infection cycle was at least 2 orders of magnitude higher than that obtained in wt strain infections. Mapping of the MudJ insertions showed that mutations in genes such as *spvA* and *rpoS* were selected in different pools. Mutations in *phoQ*, *slyA*, *spvR*, and *igaA* genes were selected once, but in different pools.

Mapping of mutations linked to intracellular overgrowth phenotype. Direct chromosomal sequencing was used to determine the exact location of MudJ insertions in serovar Typhimurium isolates displaying an increased I_{pro} . Chromosomal DNA was prepared as follows: bacteria grown overnight in 1.5 ml of LB medium were spun down and suspended in the same volume of 10 mM Tris-HCl (pH 8.0)–25 nM EDTA (pH 8.0). An 0.55-ml volume of lysozyme solution (10 mg ml⁻¹ in 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) was added, and the

mix was incubated for 20 min at 37°C. Proteinase K (100 µg ml⁻¹) was added, and the mix was incubated for 1 h at 55°C. After three to four phenol and chloroform-isoamyl alcohol (24:1) extractions, DNA was precipitated with ammonium acetate and absolute ethanol and finally suspended in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA (pH 8.0). DNA was further sheared with a 23-gauge needle and cleaned in a Sephadex G-50 column. The oligonucleotide used as a primer specific for the left arm of the *MudJ* transposon was 5' CGAATAATC CAATGTCTCC 3'. Conditions for chromosomal sequencing were (i) incubation of 2 µg of chromosomal DNA at 58°C for 30 min; (ii) addition of 13 pmol of primer oligonucleotide, 16 µl of BigDye Terminator mix, 1 µl of ThermoFidelity I (Fidelity Systems, Gaithersburg, Md.), and H₂O up to 40 µl; (iii) PCR (95°C) for 5 min (95°C for 30 s, 53°C for 20 s, and 60°C for 4 min) (55 cycles), 60°C for 5 min, and holding at 4°C; and (iv) DNA precipitation with Centrisep spin columns (catalog no. 401763; Applied Biosystems–Perkin-Elmer, Madrid, Spain). To define the spontaneous mutation present in strains SV4197-SV4224 (*phoQ*) and SV4215-SV4254 (*igaA*), the *phoPQ* and *igaA* genes were PCR amplified from chromosomal DNA extracted from wt (SL1344) and mutant serovar Typhimurium strains. The pairs of primers used to amplify the *phoPQ* genes were *pho-1D* (5' GAGTTGACCGTGGCAAGCG 3') and *pho-1R* (5' GCGTTCA AGAAAGTCGGCC 3'). To amplify the *igaA* gene, the oligonucleotides *yF-1Dbis* (5' CATTGTGCGACATACCG 3') and *yF-1Rbis* (5' TATGCATGGGG AACTCC 3') were used. The PCR products were sequenced over the full length of the genes using oligonucleotides corresponding to internal sequences as primers. All the partial sequences were confirmed from products obtained in two independent PCR amplifications. The point mutation in the overgrowing SV4197-SV4224 (*phoQ201*) strains consisted of a C→T nucleotide change that caused an R (arginine)→W (tryptophan) amino acid change at position 313 of the PhoQ protein. In the SV4215-SV4254 (*igaA188*) strains, the point mutation consisted of a G→A nucleotide change that caused an R (arginine)→H (histidine) amino acid change at position 188 of the IgaA protein.

Immunofluorescence. Serovar Typhimurium-infected cells were fixed with 3% paraformaldehyde for 10 min at room temperature. Labeling for immunofluorescence was performed with the following primary antibodies: monoclonal mouse immunoglobulin G (IgG) antibody MLK33 (gift of J. M. Schlauch, University of Illinois), which recognizes serovar Typhimurium lipopolysaccharide (LPS), and polyclonal rabbit antibody C38 (gift of I. Sandoval, Centro de Biología Molecular "Severo Ochoa," Madrid, Spain), which recognizes rat LGPs. The secondary antibodies included goat anti-mouse IgG conjugated to Texas Red, goat anti-mouse IgG conjugated to fluorescein isothiocyanate, and goat anti-rabbit IgG conjugated to Texas Red. DAPI (4,6-diamidino-2-phenylindole; 1 µg ml⁻¹) was added for 1 min to stain nuclei. The staining was performed as described previously (45).

Nucleotide sequence accession number. The *phoPQ* nucleotide sequence of serovar Typhimurium strain SL1344 was deposited in the EMBL database under the accession no. AJ272210/STY272210. The *igaA* gene is under the accession no. AJ301649/SEN301649.

RESULTS

Isolation of *S. enterica* serovar Typhimurium mutants with increased ability to proliferate within fibroblasts. *S. enterica* serovar Typhimurium persists in a resting or slow-growing state within NRK-49F fibroblasts (45). To investigate whether this reduced proliferation was a consequence of a bacterial self-attenuation growth response, we undertook a genetic strategy to identify the hypothetical functions involved. The rationale was that mutations causing loss of function in hypothetical growth-attenuating genes should result in bacterial strains with increased proliferation. A bank of at least 50,000 independent transposon *MudJ*-generated mutants derived from the virulent serovar Typhimurium strain SL1344 was used (36, 37). Upon three to four cycles of infection of NRK-49F cells, several clones showing enhanced *I*_{pro} rates were chosen for further characterization. When tested individually, all these isolates displayed invasion rates similar to that of the wt strain (0.2 to 0.5% [data not shown]). However, the ratio of viable intracellular bacteria at 24 h to that at 2 h (*I*_{pro} value) obtained for these clones was substantially higher than the ratio ob-

TABLE 2. *S. enterica* serovar Typhimurium mutants selected for high *I*_{pro} in NRK-49F cells

Strain	Selected mutation ^a	<i>I</i> _{pro} ^b
SL1344	None, wild type	1.7 ± 0.4
SV4197 ^c	Point mutation in <i>phoQ</i> (R313W); zzz:: <i>MudJ</i>	41.0 ± 6.0
SV4198	<i>slyA21</i> :: <i>MudJ</i>	12.0 ± 0.7
SV4210	<i>rpoS1151</i> :: <i>MudJ</i>	11.5 ± 4.3
SV4215 ^c	Point mutation in <i>igaA</i> (R188H); zzz:: <i>MudJ</i>	15.2 ± 3.2
SV4431 ^d	<i>igaA</i> ⁺ zzz:: <i>MudJ</i> (insertion from SV4215)	3.7 ± 1.1
SV4221	<i>spvA101</i> :: <i>MudJ</i>	14.5 ± 2.8
SV4222	<i>rpoS1152</i> :: <i>MudJ</i>	10.2 ± 2.7
SV4224 ^c	<i>phoQ201</i> (R313W)	32.5 ± 7.5
SV4234	<i>spvA102</i> :: <i>MudJ</i>	22.0 ± 2.3
SV4254 ^c	<i>igaA</i> (R188H) <i>zhf-6311</i> ::Tn10dTet	17.7 ± 4.2
SV4255 ^d	<i>igaA</i> ⁺ <i>zhf-6311</i> ::Tn10dTet (nonmucoic derivative of SV4254)	3.1 ± 0.4
SV4312	<i>spvA103</i> :: <i>MudJ</i>	18.4 ± 5.1
SV4313	<i>spvA104</i> :: <i>MudJ</i>	11.4 ± 1.2
SV4314	<i>spvR105</i> :: <i>MudJ</i>	20.6 ± 4.7

^a *MudJ* insertions were mapped by direct chromosomal sequencing.

^b Means ± standard deviations of triplicate samples from representative experiments. The assay was repeated a minimum of three times for each strain.

^c Mutation identified in PCR-amplified *phoPQ* or *igaA* loci.

^d Control strains that link *igaA* to negative control of intracellular growth (see text).

tained for the wt strain (Table 2). The phenotypic analysis allowed us to differentiate two major groups of overgrowing bacterial mutants. One class was represented by isolate SV4197, which showed an *I*_{pro} value up to 24-fold higher than that exhibited by the wt strain (Table 2). The second class included nine isolates (SV4198, SV4210, SV4215, SV4221, SV4222, SV4234, SV4312, SV4313, and SV4314) with *I*_{pro} values 6- to 13-fold higher than that of the wt (Table 2). Linkage of the *MudJ* transposon insertion to the overgrowth phenotype was demonstrated in all isolates, except SV4197 and SV4215 (see below). Because insertion mutations usually cause loss of function, the success of these hunts for mutants indicated that specific serovar Typhimurium factors exerting negative control on bacterial intracellular growth do exist.

The PhoP-PhoQ two-component system is a negative regulator of bacterial growth within fibroblasts. Standard genetic analysis showed that the mutation harbored by the isolate SV4197, which displayed the highest *I*_{pro} value, was not linked to the *MudJ* insertion. However, this strain had distinct phenotypic traits: inability to grow on N-minimal plates containing low levels of Mg²⁺ (8 µM) and formation of dark colonies on green plates. These phenotypes are characteristic of *phoP* or *phoQ* mutants (26; our unpublished observations). Moreover, the high-*I*_{pro} phenotype of SV4197 was 90% cotransducible with *purB*, which is closely linked to the *phoPQ* locus (29). To avoid any undesirable effect of the *MudJ* insertion present in SV4197 and not linked to control of intracellular growth rate, the spontaneous mutation was passaged using the *purB* marker to a wt genetic background to construct strain SV4224. This strain retained all phenotypes related to lack of a functional PhoP-PhoQ system, including the inability to grow on N-minimal plates with low magnesium and to induce an *mgtC*::*MudJ* transcriptional fusion in micromolar concentrations of Mg²⁺ (data not shown). To ascertain the type of mutation harbored

by strains SV4197 and SV4224, the promoter and entire coding region of the *phoPQ* genes from SL1344 (wt), SV4197, and SV4224 were PCR amplified and sequenced (see Materials and Methods). As expected, the sequence analysis showed that SV4197 and its derivative SV4224 harbored the same mutation consisting of a spontaneous nucleotide change in the *phoQ* gene that resulted in a substitution of an R (arginine) for a W (tryptophan) at position 313 of the PhoQ protein. The two-component regulatory system PhoP-PhoQ has been shown elsewhere to play a central regulatory function in *Salmonella* virulence, mediating, among other processes, the survival of intracellular bacteria within phagocytic cells (28, 52). Our results unveil a novel, unexpected function for the PhoP-PhoQ system: the attenuation of serovar Typhimurium proliferation within normal cultured fibroblasts.

To confirm the involvement of the PhoP-PhoQ system in this novel pathogen intracellular response, previously reported *phoP*- and *phoQ*-null mutant alleles were transduced into the SL1344 background (strains SV4056 [*phoP7956::Tn10*] and SV4365 [*phoQ5996::Tn10*], respectively), and the resulting strains were tested for proliferation within fibroblasts. The absence of either PhoP or PhoQ protein caused a dramatic increase in the ability of intracellular bacteria to initiate growth (Fig. 1A). Increased proliferation was also observed in other nonpermissive cell lines, such as 3T3 mouse fibroblasts (data not shown), but not in HeLa cells (Fig. 1A). These results suggest that the self-attenuating growth response triggered by intracellular bacteria might occur only in specific nonphagocytic cell lines. Two additional observations confirmed that the PhoP-PhoQ system exerts negative control on bacterial growth within NRK-49F fibroblasts. First, the high intracellular growth rate shown by the *phoP* mutant in NRK-49F fibroblasts was abolished by complementation with plasmid pEG5433 containing the wt *phoPQ* genes (29) (Fig. 1A). Second, high intracellular bacterial growth rates were obtained with distinct *phoP* mutant alleles irrespective of the genetic background (data not shown). Microscopic analysis of infected NRK-49F fibroblasts confirmed the kinetics data and showed that the absence of either PhoP or PhoQ regulatory protein caused exacerbated intracellular bacterial proliferation (Fig. 1B). Despite the marked difference in the intracellular growth rate between HeLa epithelial cells and NRK-49F fibroblasts (45), the phenomenon of massive release of vesicles containing LPS occurs to the same extent in the two cell types (Fig. 1B) (24). Nonetheless, the vast amount of LPS observed in the cytosol of wt-infected NRK-49F fibroblasts might also derive in part from bacteria that are degraded by the fibroblast. Indeed, our previous electron microscopy analysis demonstrated the occurrence of bacterial degradation within NRK-49F fibroblasts (45). We then reasoned whether the capacity of *phoP* or *phoQ* mutants to proliferate extensively within NRK-49F fibroblasts was associated with avoidance of bacterial degradation by altering the intracellular trafficking route. Confocal immunofluorescence microscopy demonstrated that intracellular wt and *phoP* bacteria are enclosed in compartments containing LGPs irrespective of the extent of bacterial growth (Fig. 2). Moreover, the accessibility of fluid endocytic tracers as dextran sulfate to the phagosome remains blocked in wt- or *phoP* or *phoQ* mutant-infected fibroblasts (data not shown). These observations agree essentially with the data previously obtained in the

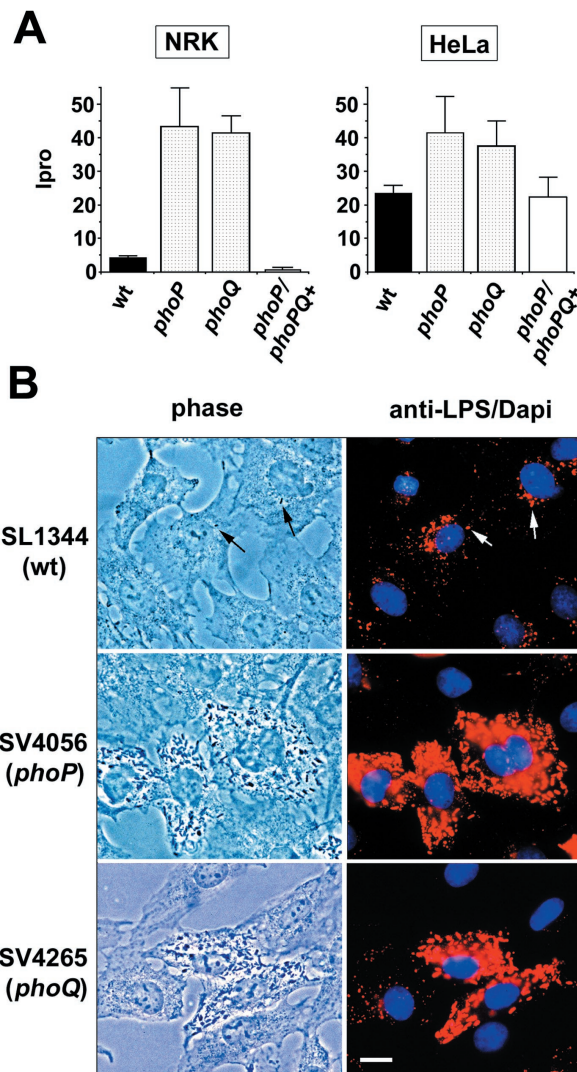


FIG. 1. The PhoP-PhoQ two-component regulatory system is required to attenuate intracellular growth within fibroblasts. (A) Intracellular growth rates (I_{pro} values) of wt (SL1344), *phoP7953::Tn10* (SV4056), and *phoQ5996::Tn10* (SV4365) serovar Typhimurium strains in NRK-49F fibroblasts and HeLa epithelial cells. Strains harboring the pEG5433 plasmid containing the *phoPQ* locus are marked as *phoP/phoPQ+*. Data are representative of a minimum of five experiments. (B) Absence of attenuating growth response in intracellular *phoP* and *phoQ* mutants. NRK-49F fibroblast cells were fixed at 24 h postinfection and processed for immunofluorescence microscopy as described elsewhere (45). Primary antibody was monoclonal mouse IgG antibody MLK33 anti-serovar Typhimurium LPS. Secondary antibody was goat anti-mouse IgG conjugated to Texas Red. DAPI ($1 \mu\text{g ml}^{-1}$) was added to stain nuclei. Arrows indicate two intracellular wt bacteria that do not exhibit massive proliferation. Bar, $10 \mu\text{m}$.

epithelial cell infection model (22). Altogether, these results prove that the PhoP-PhoQ two-component regulatory system is required to attenuate intracellular bacterial growth within fibroblast cells. However, this role is apparently not exerted by modulating processes such as the secretion of LPS vesicles (24), the targeting of bacteria to LGP-containing compartments (22, 25), or the disconnection of the phagosome with the endocytic route (22).

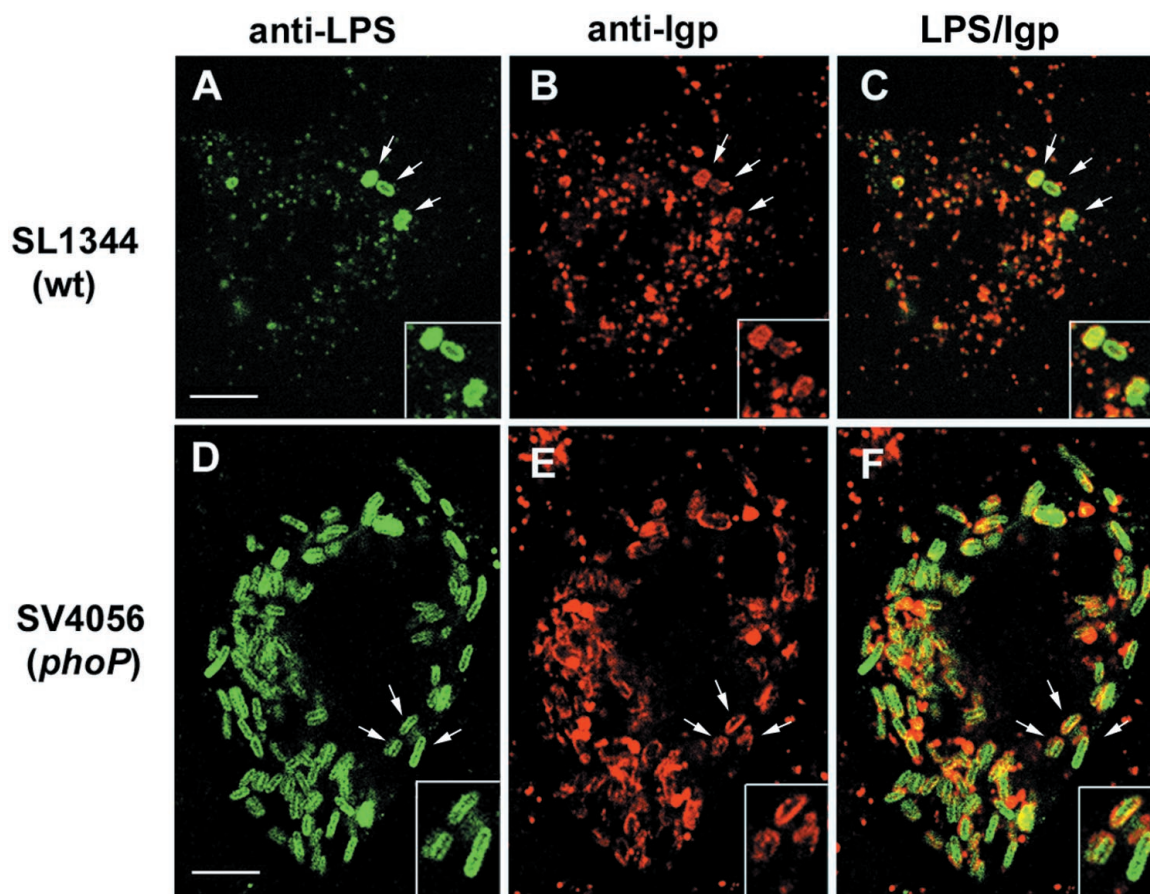


FIG. 2. wt and *phoP* bacteria are equally targeted to LGP-containing vacuoles of NRK-49F fibroblasts. Infected NRK-49F cells were fixed at 24 h postinfection and processed for confocal immunofluorescence microscopy as described elsewhere (45). Primary antibodies were mouse monoclonal antibody MLK33 anti-serovar Typhimurium LPS and rabbit polyclonal C38 antibody anti-rat LGP. Secondary antibodies were anti-mouse IgG conjugated to fluorescein isothiocyanate and anti-rabbit IgG conjugated to Texas Red. Arrows show examples of colocalization of bacteria with the late endosome-lysosomal marker. Bar, 5 μ m.

The virulence transcriptional regulators RpoS, SlyA, and SpvR mediate attenuation of intracellular bacterial growth in fibroblasts. Transductional analysis showed that mutants belonging to the phenotypic class II (I_{pro} values 6- to 13-fold above those of the wt [Table 2]), except strain SV4215, harbored independent MudJ insertions 100% linked to overgrowth phenotype. The only exception was represented by strain SV4215 (Table 2; also see below). Sequencing the chromosomal regions flanking the MudJ in these mutants showed that the MudJ was inserted in the *slyA*, *rpoS*, *spvA*, and *spvR* genes (Table 2). Four distinct insertions were mapped in the *spvA* gene (strains SV4221, SV4234, SV4312, and SV4313), two were mapped in the *rpoS* gene (strains SV4210 and SV4220), and one was mapped in the *slyA* and *spvR* genes (strains SV4198 and SV4314, respectively) (Table 2). All these genes, except *spvA*, have been shown previously to be essential for *Salmonella* responses linked to resistance to macrophage attack (*slyA* and *rpoS*) and intracellular proliferation in vivo (*spvR*) (5, 12, 50, 74). SlyA, the alternative sigma factor RpoS, and SpvR are all transcriptional regulators (9). To confirm the involvement of these factors in attenuation of bacterial growth rate in fibroblasts, the I_{pro} value in fibroblasts was calculated for SL1344 derivatives carrying previously characterized *slyA*

or *rpoS* mutant alleles (35, 42, 53). All mutant strains behaved like the isolates selected for overgrowth in NRK-49F fibroblasts (Table 3). Moreover, a series of in-frame nonpolar deletion mutations in the *spvR*, *spvA*, and *spvB* genes was used to define in more detail the involvement of the proteins encoded

TABLE 3. Intracellular growth rates of *S. enterica* serovar Typhimurium mutants harboring defined *slyA*, *rpoS*, and *spvA* mutations

Strain	Genotype ^a	I_{pro}^b	
		Expt 1	Expt 2
SL1344	Wild type	1.0 \pm 0.05	3.4 \pm 0.6
SV4056	<i>phoP7953::Tn10</i>	26.0 \pm 2.4	32.0 \pm 4.9
SMS438	<i>rpoS::Ω-Amp^r</i>	11.2 \pm 1.9	
SV4240	<i>rpoS::Ω-Amp^r phoP7953::Tn10</i>	23.0 \pm 1.1	
SV4250	<i>slyA::pRR10ΔtrfA Pen^r</i>		12.0 \pm 2.3
SV4251	<i>slyA::pRR10ΔtrfA Pen^r phoP7953::Tn10</i>		26.0 \pm 5.5
SV4257	<i>spvA::Km^r</i>		25.5 \pm 1.7
SV4258	<i>spvA::Km^r phoP7953::Tn10</i>		33.0 \pm 3.6

^a All mutant strains are isogenic with strain SL1344.

^b Shown are means \pm standard deviations of two representative experiments. The assay was repeated a minimum of three times for each strain.

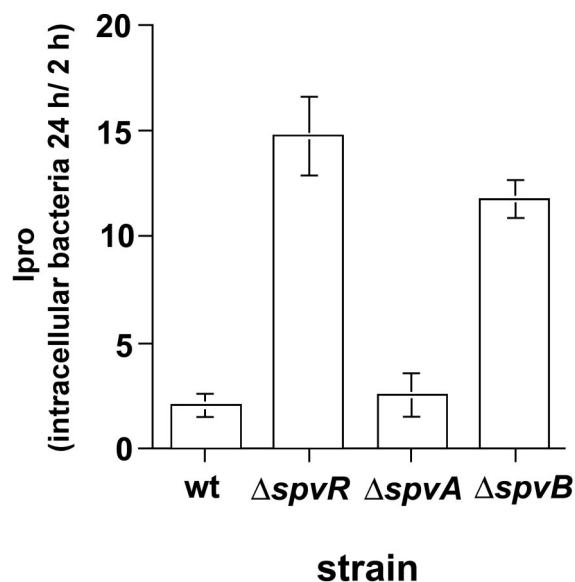


FIG. 3. SpvR and SpvB proteins are involved in attenuation of intracellular growth within NRK-49F fibroblasts. Shown are the I_{pro} values at 24 h postinfection for wt (SR11 and χ 3306) and the in-frame nonpolar deletion mutant derivatives UF082 ($\Delta spvR$), UF104 ($\Delta spvA$), and UF051 ($\Delta spvB$). Bars represent means \pm standard deviations of a representative experiment from a total of three repetitions.

by these genes in the growth-attenuating response. The $\Delta spvR$ and $\Delta spvB$ mutants, but not the $\Delta spvA$ mutant, displayed a higher I_{pro} than did the wt (Fig. 3). These results are consistent with the role in virulence previously assigned to SpvB. Furthermore, they indicate that SpvB is essential to repress bacterial growth within fibroblasts and suggest that the phenotype of the *spvA::MudJ* mutations selected in the overgrowing mutants SV4221, SV4234, SV4312, and SV4313 is likely due to polar effects on the expression of downstream genes. Altogether, these experiments validate our selection method and demonstrate that the virulence-related regulators SlyA, RpoS, and SpvR, as well as the SpvB protein, play a role in the attenuation of bacterial growth within fibroblasts. It is relevant that, unlike the epithelial or macrophage infection models, the fibroblast infection system provides a discernible phenotype for serovar Typhimurium *spv* mutants.

As the I_{pro} values of *slyA*, *rpoS*, and *spvR* regulatory mutants were lower than those of *phoP* or *phoQ* mutants, we investigated whether the function of these proteins as negative regulators of bacterial intracellular growth was dependent on the PhoP-PhoQ system. If such an association existed, double mutants carrying a nonfunctional PhoP-PhoQ system and a deficiency in any of the other regulators would display the same intracellular growth phenotype, e.g., similar I_{pro} values. The intracellular growth rates of *phoP slyA*, *phoP rpoS*, and *phoP spvR* double mutants were then determined (Table 3). No additive effect on I_{pro} value was observed for any of the double mutants. These results suggest that the role of SlyA, RpoS, and SpvR regulators related to intracellular growth attenuation might be under the control of the PhoP-PhoQ two-component system.

A novel gene, *igaA*, participates in the growth attenuation response within fibroblasts. Strain SV4215, which belongs to class II of the overgrowth mutants with intermediate I_{pro} values in NRK-49F fibroblasts, failed to show genetic linkage between the overgrowth phenotype and the MudJ insertion: transduction of the MudJ insertion to the wt resulted in transductants with wt I_{pro} values (strain SV4431 [Table 2]). Interestingly, the SV4215 mutant was mucoid on plates. To determine whether the mucoidy phenotype was linked to the ability to overgrow in fibroblasts, strain SV4215 was mutagenized with Tn10dTet. Six pools, each of 5,000 Tet^r colonies, were then prepared and lysed with P22. These lysates were used to transduce the wt, selecting Tet^r resistance. Mucoid colonies were then sought among the Tet^r transductants. One Tet^r mucoid transductant, propagated as strain SV4254, proved to retain the high I_{pro} value of the parental strain SV4215 (Table 2), indicating that mucoidy and loss of attenuation of intracellular growth were linked. To rule out the possibility that the cotransducible Tn10dTet insertion (hereafter *zhf-6311::Tn10dTet*) had an effect on intracellular growth, a nonmucoid strain carrying the *zhf-6311::Tn10dTet* insertion (SV4255) was also used to infect NRK-49F fibroblasts. This strain showed an I_{pro} value similar to that of the wt (Table 2), confirming that the *zhf-6311::Tn10dTet* insertion had no effect on intracellular proliferation. The cotransduction frequency between mucoidy and the *zhf-6311::Tn10dTet* insertion was around 0.35, suggesting that the locus controlling capsule production and the *zhf-6311::Tn10dTet* insertion were 12 to 14 kb apart. Mapping with locked-in P22 prophages (1) indicated that *zhf-6311::Tn10dTet* was located at centisome 75 on the chromosome of serovar Typhimurium, near *cysG*. Three-factor crosses indicated that the locus under study, named *igaA* for intracellular growth attenuator, mapped near *mrcA*. The gene order was *aroB-mcrA-igaA*. An open reading frame homologous to *yrfF* of *Escherichia coli* (80% identity, 89% homology) and to *umoB* of *Proteus mirabilis* (39% identity, 57% homology) exists at this position. While the biological function of YrF has not been investigated in *E. coli*, the UmoB protein has been shown previously to modulate expression of *flhDC*, which controls the swarming phenotype in *P. mirabilis* (7). Sequencing of PCR products of the *igaA* region from SL1344 (wt), SV4215, and SV4254 strains showed that the YrF-UmoB-related open reading frame of strains SV4215 and SV4254 harbored a point mutation causing an amino acid substitution of R (arginine) for H (histidine) at position 188 of the putative IgaA protein. Attempts to construct *igaA*-null mutants were unsuccessful (data not shown), suggesting that *igaA* may be an essential gene. If such is the case, the *igaA188* mutation must not be null. As in the tests performed with SlyA, RpoS, and SpvR regulators, the possibility of a functional relationship between IgaA and PhoP-PhoQ was tested. A *phoP igaA* double mutant displayed an I_{pro} value similar to that of a *phoP* mutant (data not shown), suggesting that the IgaA function might belong to the same proliferation control pathway as the PhoP-PhoQ system. This view is supported by the observation that, unlike SV4255 (*zhf-6311::Tn10dTet*), the strain SV4254 (*igaA188 zhf-6311::Tn10dTet*) is attenuated for virulence in the murine typhoid model (unpublished observations). Altogether, these observations provide evidence that the IgaA function is involved in

TABLE 4. Role of *S. enterica* serovar Typhimurium virulence regulators in attenuation of intracellular growth within NRK-49F cells

Strain	Mutated gene(s) ^a	I _{pro} ^b	
		Expt 1	Expt 2
SL1344	None (wild type)	1.0 ± 0.05	4.4 ± 0.35
SV4056	<i>phoP</i>	26.0 ± 2.4	45.0 ± 3.5
GVB311	<i>rpoE</i>	0.3 ± 0.1	
SV4232	<i>rpoE phoP</i>	2.8 ± 1.1	
SV4237	<i>spiR</i>	1.6 ± 0.25	
SV4241	<i>spiR phoP</i>	25.9 ± 0.9	
SV4386	<i>spiA</i>	0.38 ± 0.13	
SV4387	<i>spiA phoP</i>	3.5 ± 0.8	
SV4235	<i>ompR</i>		4.8 ± 0.9
SV4236	<i>ompR phoP</i>		35.0 ± 3.5
SV1610	<i>dam</i>		4.5 ± 0.9
SV4089	<i>dam phoP</i>		36.2 ± 5.0
SMS521	<i>crp</i>	1.05 ± 0.2	
SV4284	<i>crp phoP</i>	15.9 ± 2.3	
ST97	<i>relA</i>	1.1 ± 0.3	
SV4285	<i>relA phoP</i>	34.6 ± 2.5	
SV4253	<i>lrp</i>	1.2 ± 0.2	
SV4286	<i>lrp phoP</i>	26.6 ± 0.4	
SMS593	<i>oxyR</i>	2.3 ± 0.4	
SV4287	<i>oxyR phoP</i>	32.8 ± 8.3	
SV4233	<i>mviA</i>	2.6 ± 0.4	
SV4288	<i>mviA phoP</i>	26.5 ± 10	
SV4261	<i>soxS</i>		4.5 ± 1.3
SV4262	<i>fnr</i>		4.4 ± 0.3

^a All mutant strains are isogenic with strain SL1344.

^b Shown are means ± standard deviations of two representative experiments. The assay was repeated a minimum of three times for each strain.

negative control of bacterial proliferation within NRK-49F fibroblasts.

Specificity of the response involved in attenuating pathogen growth rate. Although 40 to 50 genes have been shown elsewhere to be required for *Salmonella* pathogenesis (30), our selection procedure produced only a very small subset of virulence proteins. To determine whether additional regulators were involved in the attenuation of the bacterial growth rate within fibroblasts, we tested mutants affected in the following regulatory proteins: (i) RpoE, an alternative sigma factor (38); (ii) SpiR (SsrA) and SpiA, a regulator and a type III structural protein, respectively, both encoded in SPI-2 (34, 51); (iii) OmpR, a transcriptional regulator controlling outer membrane protein content (43); (iv) Dam, a DNA-adenine methylase required for systemic disease (23, 33); (v) the global regulators Crp, RelA, and Lrp (18); (vi) OxyR and SoxRS, related to oxidative stress (13, 67); (vii) MviA, a regulator required for eliciting systemic disease in mice (66); and (viii) Fnr, which controls switching to anaerobic metabolism. Unlike the mutations in the *phoP*, *phoQ*, *slyA*, *rpoS*, *spvR*, and *igaA* genes, none of the other virulence-related mutations resulted in an I_{pro} value higher than that of the wt (Table 4). Moreover, when combined with *phoP*-null mutant alleles, no alteration in the capacity of intracellular bacteria to attenuate growth was detected (Table 4). These results suggest that the growth-attenuating response is under the control of a specific subset of virulence-related regulatory proteins. Even though none of these mutants displayed an overgrowth phenotype, two of them, the *spiA* and *rpoE* mutants, were defective for mainte-

nance of viability in the intracellular environment of fibroblasts, with I_{pro} values in the range of 30% of that of the wt (Table 4). The combination of the mutations producing survival defects (*spiA* and *rpoE*) with *phoP* mutant alleles also caused a marked decrease in the intracellular growth rate. Nonetheless, the I_{pro} value of the *spiA phoP* or *rpoE phoP* double mutants was still 10-fold higher than that showed by single *spiA* or *rpoE* mutants (Table 4). These results may indicate that not all the population of intracellular bacteria is affected to the same extent by the fibroblast defense mechanisms. Interestingly, the low I_{pro} value of the *spiA* mutant was not reproduced by the *spiR* mutant, which suggests that the *spiR* mutant might retain a residual activity of the SPI-2-encoded TTSS in the intracellular environment of fibroblast cells.

DISCUSSION

In this study, we present evidence that a specific subset of *S. enterica* serovar Typhimurium virulence determinants prevent pathogen overgrowth with cultured normal fibroblasts. This was unexpected, considering that virulence functions are generally associated with promotion of survival and proliferation in the intracellular environment of the host cell. Interestingly, recent data obtained with animal models support the idea that massive intracellular proliferation of serovar Typhimurium occurs predominantly within CD18-expressing phagocytes and not in epithelial cells (31, 46, 58). These reports raise the interesting question of whether, during the infection, the invasion of nonphagocytic cells by *S. enterica* might be followed by adaptation of the pathogen to a slow-growth or resting state. In this respect, a very recent report by Hernández-Pando et al. has provided evidence by in situ PCR that nonphagocytic cells carry *Mycobacterium tuberculosis* that remains latent in the lungs of asymptomatic individuals (35a). In this study, it was shown that fibroblasts are the host cell type that exhibits a stronger signal for mycobacterial DNA (35a).

To evaluate the significance of the lack of *S. enterica* growth within nonphagocytic cells, we reasoned whether the lifestyle of this pathogen within cultured fibroblasts could somehow reflect the growth kinetics of *S. enterica* in animal tissues. In addition, fibroblasts must be considered a potential host cell type for *S. enterica* since they are efficiently invaded in vitro by this pathogen and located in anatomical sites close to the infection loci. Though no study has yet proved that *S. enterica* infects fibroblasts upon penetration of the intestinal epithelium, it is known that fibroblasts exist in the lamina propria (56, 57). These so-called intestinal subepithelial myofibroblasts modulate the mobility, differentiation, and proliferation of epithelial and parenchymal cells by secreting a large number of cytokines, chemokines, growth factors, and other immunomodulatory molecules (56, 57). A nonproliferative intracellular infection of these subepithelial fibroblasts by *S. enterica* would provide the bacteria with the opportunity to orchestrate the physiology of the intestinal epithelium while remaining masked from recognition by immune cells. As in the case of *M. tuberculosis* (35a), a latent *S. enterica* infection of cells with minimal antigen presentation capacity, such as fibroblasts, would perhaps enable the pathogen to persist for a long time. In accord with these postulates, we have obtained preliminary data with the calf ligated loop assay that suggest that the PhoP-PhoQ

system might mediate intracellular growth attenuation at the level of the lamina propria (P. Watson and F. Garcia-del Portillo, unpublished observations).

In principle, two non-mutually exclusive processes could explain the attenuation of serovar Typhimurium growth within fibroblasts. On one side, normal fibroblasts, though not considered professional phagocytes, could mount an effective antibacterial response. In this context, Eriksson et al. have proposed that *S. enterica* triggers nitric oxide (NO) release by macrophages and that mutants which do not stimulate host NO release display an intracellular growth advantage (10). We have observed that NO levels of NRK-49F fibroblast cells increase two- to threefold in response to serovar Typhimurium infection. However, blockage of NO production does not result in an increase of the intracellular growth rate (data not shown). Therefore, unlike the macrophage model, attenuation of intracellular bacterial growth in fibroblasts does not seem to rely, at least entirely, on host NO production.

An alternative hypothesis to explain the absence of massive intracellular proliferation of serovar Typhimurium within fibroblasts is that intracellular bacteria mount a specific response to restrain themselves from massive growth. Regardless of the relative contribution of the fibroblast to attenuation of bacterial growth, a bacterial product(s) can arrest intracellular proliferation. A simple positive selection procedure provided us with overgrowing serovar Typhimurium mutants. The genetic characterization of these isolates revealed that functions previously known to be involved in intramacrophage survival and in vivo intracellular proliferation, such as PhoP-PhoQ, SlyA, SpvR, and RpoS, are essential to down-regulating the I_{pro} in cultured fibroblasts. To our knowledge, these data represent the first example of attenuation of bacterial overgrowth in the intracellular environment of a nonphagocytic eucaryotic cell that is directed by pathogen factors.

The detailed characterization of the phenotype displayed by individual overgrowing clones permitted us to differentiate two major classes. In the first class, exemplified by *phoP* and *phoQ* mutants, a substantial derepression of the intracellular growth rate occurred at 24 h, reaching values up to 20- to 30-fold higher than those for the wt virulent strain. This observation was unexpected, considering that the lack of the PhoP-PhoQ two-component system had been always linked to major intramacrophage survival defects and marked attenuation in the murine typhoid model (30, 52). Our data demonstrate that the absence of either of these two regulatory proteins results in enhanced serovar Typhimurium intracellular proliferation within normal fibroblast cells. The point mutant allele *phoQ201* (R313W) isolated in our screening was associated with loss of function for the PhoQ protein, since the overgrowing clone carrying this mutant allele was defective for growth in low- Mg^{2+} minimal medium and activation of different *pag* genes (unpublished observations). The *phoQ201* mutation maps in the cytosolic transmitter domain of the PhoQ sensor protein. Interestingly, this point mutation does not affect synthesis or stability of the protein, as indicated by Western blotting analysis performed with *phoQ*-null mutants carrying the *phoQ201* allele in a plasmid (data not shown). This result suggests that the PhoQ (R313W) protein, though probably able to receive the extracellular signal, is unable to transmit it to the PhoP transcriptional regulator. How the intracellular growth-atten-

uating function of the PhoP-PhoQ system is related to *Salmonella* pathogenesis is at present unknown. One could speculate that, besides its central role in promoting intramacrophage survival, the PhoP-PhoQ system might also prolong residence of intracellular bacteria that have invaded nonprofessional phagocytic cells. Indeed, it has been known for decades that a small percentage of *Salmonella* infections occurring in livestock and humans end with persistence of bacteria in host tissues, a phenomenon poorly characterized at a cellular or molecular level. Monitoring the *phoP* or *phoQ* mutants in intestinal and systemic sites upon oral administration could provide insights into this putative relationship.

Our study also identified other virulence-related regulatory proteins of serovar Typhimurium involved in the negative regulation of intracellular growth rate. Thus, knockout mutants defective in the transcriptional regulator SlyA, RpoS, or SpvR displayed 6- to 13-fold-higher intracellular growth rates than those of the wt strain. These transcriptional regulators have been shown to regulate *Salmonella* virulence. SlyA, like the PhoP-PhoQ system, is essential for *Salmonella* to withstand the macrophage antimicrobial attack. RpoS has been shown previously to be required for systemic disease (5). SpvR, which positively regulates the plasmid-encoded *spvABCD* operon (27), is also needed for systemic disease (41). Interestingly, neither *rpoS* nor *spvR* mutants display any intracellular survival defect in cultured macrophages. This lack of an in vitro phenotype agrees with the fact that RpoS positively regulates *spvR* expression (59). The interconnection between the PhoP-PhoQ system and the SlyA, RpoS, and SpvR regulators is at present undefined, although it has been proposed previously that the PhoP-PhoQ system may control in a positive manner the expression of the *spvABCD* operon (47). Whether this control is exerted via the SpvR regulator is unknown. Regulation of expression of the *spvR* gene is very complex, and besides RpoS, other proteins that exert control over *spvR* include H-NS, Crp, and Lrp as negative regulators and integration host factor (IHF) as a positive regulator. No overgrowing mutant was selected with a defect in the positive regulator IHF, which could indicate that loss of RpoS, but not of IHF, ensures a significant repression of the *spvR* gene in the intracellular environment of fibroblasts. Deletion of the *spvR* or *spvB* genes enhances the I_{pro} , which suggests that SpvB plays an important role in attenuating growth rate. Though the biological function of Spv proteins remains undefined, the SpvB protein exhibits mono(ADP-ribosyl)transferase activity toward host actin (54, 68). Although further work is required to determine the exact biological function of Spv proteins, our fibroblast model offers an opportunity to address this question. The fact that double mutants lacking *phoP* and *rpoS* or *slyA* or *spvR* did not display higher proliferation rates than did the single mutants suggests that SlyA, RpoS, and SpvR might be under the control of the PhoP-PhoQ system to attenuate growth within fibroblasts.

An additional mutant having a defect in a previously unknown protein of *S. enterica* was also selected as an overgrowing clone. This protein, which we named IgaA for intracellular growth attenuator, is homologous to the *E. coli* YrF and *P. mirabilis* UmoB proteins. Whereas the biological function of YrF is currently unknown, UmoB has been shown elsewhere to act as a positive regulator of FlhDC, the master regulator of flagella and swarming (7). More recently, FlhDC has been

shown to repress cell division during *P. mirabilis* swarming, suggesting that UmoB could repress cell division via FlhDC. This biological function, if maintained in *S. enterica*, could sustain a putative negative control of cell division and growth exerted by IgaA in intracellular bacteria. Unlike the *phoQ201* (R313W) point mutant allele, the *igaA188* (R188H) mutant allele must confer a partial loss of function, since IgaA is an essential protein in a wt genetic background in laboratory conditions. The analysis of IgaA secondary structure predicts that it might be a membrane protein with at least four transmembrane domains. As the *igaA188* point mutant overexpresses capsule material, there is likely an interconnection between IgaA function and structural (*cps*) or regulatory (*rcsA* and *rcsCB*) genes involved in capsule synthesis. It would be also interesting to link IgaA function with the PhoP-PhoQ two-component regulatory system.

Unlike the strains defective in the other regulators, the *rpoE* and *spiA* mutants exhibited a survival defect within fibroblasts, retaining only 30% viability as shown in comparison with the wt strain. Our data are consistent with the survival defect previously reported for *rpoE* mutants in epithelial cells and macrophages (38) and the loss of viability of *spiA* in macrophages. Likewise, our observations imply that fibroblasts might harbor antimicrobial activities analogous to some extent to the variety of macrophage defenses. Interestingly, in contrast to the *spiA* mutant, which does not have a functional SPI-2 TTSS, the *spiR* SPI-2 regulatory mutant showed the same survival rate as did the wt strain. This difference suggests that this *spiR* mutant may have residual activity of the SPI-2 TTSS, which might be sufficient to maintain viability. Similar differences in intracellular viability rates have been found in cultured macrophages (E. A. Groisman, unpublished observations).

An interesting characteristic of the response described in this study is that it relies on the function of a specific subset of virulence regulators. Thus, *S. enterica* virulence regulators, such as the OmpR-EnvZ two-component system, the Dam methylase, Crp, Cya, and MviA, among others, are dispensable for attenuation of the intracellular growth rate within fibroblasts. These differences illustrate the tremendous variety of intracellular responses that pathogens can trigger depending on distinct environmental cues present in specific host cell types.

A final question that arises from our observations is how to explain the lack of virulence shown in animal models by the class I and class II overgrowing mutants (*phoQ*, *slyA*, *spvR*, *rpoS*, and *igaA*). Whereas decreased bacterial survival and/or proliferation in cultured macrophages is primarily associated with attenuation (e.g., *phoPQ* and *slyA* mutants [71, 75]), massive proliferation within nonphagocytic cells could also result in a suicide strategy for these bacteria. Thus, lysis of an infected nonphagocytic cell due to intracellular bacterial overgrowth may lead to bacterial release into extracellular locations. These extracellular bacteria can be subsequently ingested by nearby activated phagocytic cells, in which these bacterial mutants rapidly lose viability. Thus, a combination of growth attenuation (via PhoP-PhoQ, SlyA, RpoS, and SpvR) and viability maintenance (via RpoE and SPI-2 TTSS) might provide the pathogen with the wherewithal to adapt to a long-persistence state within the infected nonphagocytic cell.

ACKNOWLEDGMENTS

We greatly appreciate the generosity of Paul Gulig, Stephen J. Libby, Ferric C. Fang, Mark Roberts, Gordon Dougan, Michael P. Spector, and David Holden for sending strains. We also thank James M. Slauch and I. Sandoval for providing antibodies; Nuria Gómez-López for her assistance in the PCR, *phoPQ* and *igaA* locus sequencing, and direct chromosomal sequencing of MudJ insertion sites; Amparo Haro for the confocal microscopy analysis; and Felix Solomon for strain construction.

This work was supported by grants from the Ministerio de Educación y Cultura of Spain (PM97-0148-C02), Comunidad de Madrid (08.2/0045.1/2000), European Union (QLK2-1999-00310), and North Atlantic Treaty Organization (NATO) (CRG971613). E.A.G. is an Associate Investigator of the Howard Hughes Medical Institute.

REFERENCES

- Benson, N. R., and B. S. Goldman. 1992. Rapid mapping in *Salmonella typhimurium* with Mud-P22 prophages. *J. Bacteriol.* **174**:1673–1681.
- Bowe, F., C. J. Lipps, R. M. Tsois, E. Groisman, F. Heffron, and J. G. Kusters. 1998. At least four percent of the *Salmonella typhimurium* genome is required for fatal infection of mice. *Infect. Immun.* **66**:3372–3377.
- Brumell, J. H., C. M. Rosenberger, G. T. Gotto, S. L. Marcus, and B. B. Finlay. 2001. SifA permits survival and replication of *Salmonella typhimurium* in murine macrophages. *Cell. Microbiol.* **3**:75–84.
- Cirillo, D. M., R. H. Valdivia, D. M. Monack, and S. Falkow. 1998. Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. *Mol. Microbiol.* **30**:175–188.
- Coynault, C., V. Robbe-Saule, and F. Norel. 1996. Virulence and vaccine potential of *Salmonella typhimurium* mutants deficient in the expression of the RpoS (sigma S) regulon. *Mol. Microbiol.* **22**:149–160.
- Dorman, C. J., S. Chatfield, C. F. Higgins, C. Hayward, and G. Dougan. 1989. Characterization of porin and *ompR* mutants of a virulent strain of *Salmonella typhimurium*: *ompR* mutants are attenuated in vivo. *Infect. Immun.* **57**:2136–2140.
- Dufour, A., R. B. Furness, and C. Hughes. 1998. Novel genes that upregulate the *Proteus mirabilis* *flhDC* master operon controlling flagellar biogenesis and swarming. *Mol. Microbiol.* **29**:741–751.
- Dunlap, N. E., W. H. Benjamin, A. K. Berry, J. H. Eldridge, and D. E. Briles. 1992. A 'safe-site' for *Salmonella typhimurium* is within splenic polymorphonuclear cells. *Microb. Pathog.* **13**:181–190.
- El-Gedaily, A., G. Paesold, and M. Krause. 1997. Expression profile and subcellular location of the plasmid-encoded virulence (Spv) proteins in wild-type *Salmonella dublin*. *Infect. Immun.* **65**:3406–3411.
- Eriksson, S., J. Bjorkman, S. Borg, A. Syk, S. Pettersson, D. I. Andersson, and M. Rhen. 2000. *Salmonella typhimurium* mutants that downregulate phagocyte nitric oxide production. *Cell. Microbiol.* **2**:239–250.
- Ernst, R. K., T. Guina, and S. I. Miller. 1999. How intracellular bacteria survive: surface modifications that promote resistance to host innate immune responses. *J. Infect. Dis.* **179**:S326–S330.
- Fang, F. C., S. J. Libby, N. A. Buchmeier, P. C. Loewen, J. Switala, J. Harwood, and D. G. Guiney. 1992. The alternative sigma factor *katF* (*rpoS*) regulates *Salmonella* virulence. *Proc. Natl. Acad. Sci. USA* **89**:11978–11982.
- Fang, F. C., A. Vazquez-Torres, and Y. Xu. 1997. The transcriptional regulator SoxS is required for resistance of *Salmonella typhimurium* to paraquat but not for virulence in mice. *Infect. Immun.* **65**:5371–5375.
- Fields, P. I., E. A. Groisman, and F. Heffron. 1989. A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. *Science* **243**:1059–1062.
- Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron. 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc. Natl. Acad. Sci. USA* **83**:5189–5193.
- Finlay, B. B., and J. H. Brumell. 2000. *Salmonella* interactions with host cells: in vitro to in vivo. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **355**:623–631.
- Finlay, B. B., S. Chatfield, K. Y. Leung, G. Dougan, and S. Falkow. 1991. Characterization of a *Salmonella choleraesuis* mutant that cannot multiply within epithelial cells. *Can. J. Microbiol.* **37**:568–572.
- Foster, J. W., and M. P. Spector. 1995. How *Salmonella* survive against the odds. *Annu. Rev. Microbiol.* **49**:145–174.
- Galan, J. E., and R. Curtiss. 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. *Proc. Natl. Acad. Sci. USA* **86**:6383–6387.
- Galan, J. E., and D. Zhou. 2000. Striking a balance: modulation of the actin cytoskeleton by *Salmonella*. *Proc. Natl. Acad. Sci. USA* **97**:8754–8761.
- García-del Portillo, F., and B. B. Finlay. 1994. Invasion and intracellular proliferation of *Salmonella* within non-phagocytic cells. *Microbiologia* **10**:229–238.
- García-del Portillo, F., and B. B. Finlay. 1995. Targeting of *Salmonella typhimurium* to vesicles containing lysosomal membrane glycoproteins by-

- passes compartments with mannose 6-phosphate receptors. *J. Cell Biol.* **129**:81–97.
23. Garcia-Del Portillo, F., M. G. Pucciarelli, and J. Casadesus. 1999. DNA adenine methylase mutants of *Salmonella typhimurium* show defects in protein secretion, cell invasion, and M cell cytotoxicity. *Proc. Natl. Acad. Sci. USA* **96**:11578–11583.
 24. Garcia-del Portillo, F., M. A. Stein, and B. B. Finlay. 1997. Release of lipopolysaccharide from intracellular compartments containing *Salmonella typhimurium* to vesicles of the host epithelial cell. *Infect. Immun.* **65**:24–34.
 25. Garcia-del Portillo, F., M. B. Zwick, K. Y. Leung, and B. B. Finlay. 1993. *Salmonella* induces the formation of filamentous structures containing lysosomal membrane glycoproteins in epithelial cells. *Proc. Natl. Acad. Sci. USA* **90**:10544–10548.
 26. Garcia-Vescovi, E., F. C. Soncini, and E. A. Groisman. 1996. Mg²⁺ as an extracellular signal: environmental regulation of *Salmonella* virulence. *Cell* **84**:165–174.
 27. Grob, P., D. Kahn, and D. G. Guiney. 1997. Mutational characterization of promoter regions recognized by the *Salmonella dublin* virulence plasmid regulatory protein SpvR. *J. Bacteriol.* **179**:5398–5406.
 28. Groisman, E. A. 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. *J. Bacteriol.* **183**:1835–1842.
 29. Groisman, E. A., E. Chiao, C. J. Lipps, and F. Heffron. 1989. *Salmonella typhimurium* *phoP* virulence gene is a transcriptional regulator. *Proc. Natl. Acad. Sci. USA* **86**:7077–7081.
 30. Groisman, E. A., and H. Ochman. 1997. How *Salmonella* became a pathogen. *Trends Microbiol.* **5**:343–349.
 31. Gulig, P. A., T. J. Doyle, J. A. Hughes, and H. Matsui. 1998. Analysis of host cells associated with the Spv-mediated increased intracellular growth rate of *Salmonella typhimurium* in mice. *Infect. Immun.* **66**:2471–2485.
 32. Guy, R. L., L. A. Gonias, and M. A. Stein. 2000. Aggregation of host endosomes by *Salmonella* requires SPI2 translocation of SseFG and involves SpvR and the *fms-aroE* intragenic region. *Mol. Microbiol.* **37**:1417–1435.
 33. Heithoff, D. M., R. L. Sinsheimer, D. A. Low, and M. J. Mahan. 1999. An essential role for DNA adenine methylation in bacterial virulence. *Science* **284**:967–970.
 34. Hensel, M., T. Nikolaus, and C. Egelseer. 1999. Molecular and functional analysis indicates a mosaic structure of *Salmonella* pathogenicity island 2. *Mol. Microbiol.* **31**:489–498.
 35. Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden. 1995. Simultaneous identification of bacterial virulence genes by negative selection. *Science* **269**:400–403.
 - 35a. Hernández-Pando, R., M. Jeyanathan, G. Mengistu, D. Aguilar, H. Orozco, M. Harboe, G. A. W. Rook, and G. Bjune. 2000. Persistence of DNA from *Mycobacterium tuberculosis* in superficially normal lung tissue during latent infection. *Lancet* **356**:2133–2137.
 36. Hoiseith, S. K., and B. A. Stocker. 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* **291**:238–239.
 37. Hughes, K. T., and J. R. Roth. 1988. Transitory *cis* complementation: a method for providing transposition functions to defective transposons. *Genetics* **119**:9–12.
 38. Humphreys, S., A. Stevenson, A. Bacon, A. B. Weinhardt, and M. Roberts. 1999. The alternative sigma factor, σ^E , is critically important for the virulence of *Salmonella typhimurium*. *Infect. Immun.* **67**:1560–1568.
 39. Khan, S. A., P. Everest, S. Servos, N. Foxwell, U. Zahring, H. Brade, E. T. Rietschel, G. Dougan, I. G. Charles, and D. J. Maskell. 1998. A lethal role for lipid A in *Salmonella* infections. *Mol. Microbiol.* **29**:571–579.
 40. Leung, K. Y., and B. B. Finlay. 1991. Intracellular replication is essential for the virulence of *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA* **88**:11470–11474.
 41. Libby, S. J., L. G. Adams, T. A. Ficht, C. Allen, H. A. Whitford, N. A. Buchmeier, S. Bossie, and D. G. Guiney. 1997. The *spv* genes on the *Salmonella dublin* virulence plasmid are required for severe enteritis and systemic infection in the natural host. *Infect. Immun.* **65**:1786–1792.
 42. Libby, S. J., W. Goebel, A. Ludwig, N. Buchmeier, F. Bowe, F. C. Fang, D. G. Guiney, J. G. Songer, and F. Heffron. 1994. A cytolysin encoded by *Salmonella* is required for survival within macrophages. *Proc. Natl. Acad. Sci. USA* **91**:489–493.
 43. Lindgren, S. W., I. Stojiljkovic, and F. Heffron. 1996. Macrophage killing is an essential virulence mechanism of *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA* **93**:4197–4201.
 44. Low, K. B., M. Ittensohn, T. Le, J. Platt, S. Sodi, M. Amoss, O. Ash, E. Carmichael, A. Chakraborty, J. Fischer, S. L. Lin, X. Luo, S. I. Miller, L. Zheng, I. King, J. M. Pawelek, and D. Bermudes. 1999. Lipid A mutant *Salmonella* with suppressed virulence and TNF α induction retain tumor-targeting in vivo. *Nat. Biotechnol.* **17**:37–41.
 45. Martinez-Moya, M., M. A. de Pedro, H. Schwarz, and F. Garcia-del Portillo. 1998. Inhibition of *Salmonella* intracellular proliferation by non-phagocytic eucaryotic cells. *Res. Microbiol.* **149**:309–318.
 46. Matsui, H., M. Eguchi, and Y. Kikuchi. 2000. Use of confocal microscopy to detect *Salmonella typhimurium* within host cells associated with Spv-mediated intracellular proliferation. *Microb. Pathog.* **29**:53–59.
 47. Matsui, H., T. Kawakami, S. Ishikawa, H. Danbara, and P. A. Gulig. 2000. Constitutively expressed *phoP* inhibits mouse-virulence of *Salmonella typhimurium* in an Spv-dependent manner. *Microbiol. Immunol.* **44**:447–454.
 48. Meresse, S., O. Steele-Mortimer, B. B. Finlay, and J. P. Gorvel. 1999. The rab7 GTPase controls the maturation of *Salmonella typhimurium*-containing vacuoles in HeLa cells. *EMBO J.* **18**:4394–4403.
 49. Mills, S. D., S. R. Ruschkowski, M. A. Stein, and B. B. Finlay. 1998. Trafficking of porin-deficient *Salmonella typhimurium* mutants inside HeLa cells: *ompR* and *envZ* mutants are defective for the formation of *Salmonella*-induced filaments. *Infect. Immun.* **66**:1806–1811.
 50. Nickerson, C. A., and R. Curtiss III. 1997. Role of sigma factor RpoS in initial stages of *Salmonella typhimurium* infection. *Infect. Immun.* **65**:1814–1823.
 51. Ochman, H., F. C. Soncini, F. Solomon, and E. A. Groisman. 1996. Identification of a pathogenicity island required for *Salmonella* survival in host cells. *Proc. Natl. Acad. Sci. USA* **93**:7800–7804.
 52. Ohl, M. E., and S. I. Miller. 2001. *Salmonella*: a model for bacterial pathogenesis. *Annu. Rev. Med.* **52**:259–274.
 53. O'Neal, C. R., W. M. Gabriel, A. K. Turk, S. J. Libby, F. C. Fang, and M. P. Spector. 1994. RpoS is necessary for both the positive and negative regulation of starvation survival genes during phosphate, carbon, and nitrogen starvation in *Salmonella typhimurium*. *J. Bacteriol.* **176**:4610–4616.
 54. Otto, H., D. Tezcan-Merdol, R. Girisch, F. Haag, M. Rhen, and F. Koch-Nolte. 2000. The *spvB* gene-product of the *Salmonella enterica* virulence plasmid is a mono(ADP-ribosyl)transferase. *Mol. Microbiol.* **37**:1106–1115.
 55. Pawelek, J. M., K. B. Low, and D. Bermudes. 1997. Tumor-targeted *Salmonella* as a novel anticancer vector. *Cancer Res.* **57**:4537–4544.
 56. Powell, D. W., R. C. Mifflin, J. D. Valentich, S. E. Crowe, J. I. Saada, and A. B. West. 1999. Myofibroblasts. I. Paracrine cells important in health and disease. *Am. J. Physiol.* **277**:C1–C19.
 57. Powell, D. W., R. C. Mifflin, J. D. Valentich, S. E. Crowe, J. I. Saada, and A. B. West. 1999. Myofibroblasts. II. Intestinal subepithelial myofibroblasts. *Am. J. Physiol.* **277**:C183–C201.
 58. Richter-Dahlfors, A., A. M. Buchan, and B. B. Finlay. 1997. Murine salmonellosis studied by confocal microscopy: *Salmonella typhimurium* resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo. *J. Exp. Med.* **186**:569–580.
 59. Robbe-Saule, V., F. Schaeffer, L. Kowarz, and F. Norel. 1997. Relationships between H-NS, sigma S, SpvR and growth phase in the control of *spvR*, the regulatory gene of the *Salmonella* plasmid virulence operon. *Mol. Gen. Genet.* **256**:333–347.
 60. Rudd, K. E., B. R. Bochner, M. Cashel, and J. R. Roth. 1985. Mutations in the *spoT* gene of *Salmonella typhimurium*: effects on *his* operon expression. *J. Bacteriol.* **163**:534–542.
 61. Seymour, R. L., P. V. Mishra, M. A. Khan, and M. P. Spector. 1996. Essential roles of core starvation-stress response loci in carbon-starvation-inducible cross-resistance and hydrogen peroxide-inducible adaptive resistance to oxidative challenge in *Salmonella typhimurium*. *Mol. Microbiol.* **20**:497–505.
 62. Shea, J. E., C. R. Beuzon, C. Gleeson, R. Mundy, and D. W. Holden. 1999. Influence of the *Salmonella typhimurium* pathogenicity island 2 type III secretion system on bacterial growth in the mouse. *Infect. Immun.* **67**:213–219.
 63. Stanley, T. L., C. D. Ellermeier, and J. M. Schlauch. 2000. Tissue-specific gene expression identifies a gene in the lysogenic phage Gifsy-1 that affects *Salmonella enterica* serovar Typhimurium survival in Peyer's patches. *J. Bacteriol.* **182**:4406–4413.
 64. Steele-Mortimer, O., S. Meresse, J. P. Gorvel, B. H. Toh, and B. B. Finlay. 1999. Biogenesis of *Salmonella typhimurium*-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway. *Cell. Microbiol.* **1**:33–49.
 65. Stein, M. A., K. Y. Leung, M. Zwick, F. Garcia-del Portillo, and B. B. Finlay. 1996. Identification of a *Salmonella* virulence gene required for formation of filamentous structures containing lysosomal membrane glycoproteins within epithelial cells. *Mol. Microbiol.* **20**:151–164.
 66. Swords, W. E., A. Giddings, and W. H. Benjamin. 1997. Bacterial phenotypes mediated by *mviA* and their relationship to the mouse virulence of *Salmonella typhimurium*. *Microb. Pathog.* **22**:353–362.
 67. Taylor, P. D., C. J. Inchley, and M. P. Gallagher. 1998. The *Salmonella typhimurium* AhpC polypeptide is not essential for virulence in BALB/c mice but is recognized as an antigen during infection. *Infect. Immun.* **66**:3208–3217.
 68. Tezcan-Merdol, D., T. Nyman, U. Lindberg, F. Haag, F. Koch-Nolte, and M. Rhen. 2001. Actin is ADP-ribosylated by the *Salmonella enterica* virulence-associated protein SpvB. *Mol. Microbiol.* **39**:606–619.
 69. Uchiya, K., M. A. Barbieri, K. Funato, A. H. Shah, P. D. Stahl, and E. A. Groisman. 1999. A *Salmonella* virulence protein that inhibits cellular trafficking. *EMBO J.* **18**:3924–3933.
 70. Valentine, P. J., B. P. Devore, and F. Heffron. 1998. Identification of three highly attenuated *Salmonella typhimurium* mutants that are more immunogenic and protective in mice than a prototypical *aroA* mutant. *Infect. Immun.* **66**:3378–3383.

71. **VanCott, J. L., S. N. Chatfield, M. Roberts, D. M. Hone, E. L. Hohmann, D. W. Pascual, M. Yamamoto, H. Kiyono, and J. R. McGhee.** 1998. Regulation of host immune responses by modification of *Salmonella* virulence genes. *Nat. Med.* **4**:1247–1252.
72. **Vazquez-Torres, A., and F. C. Fang.** 2000. Cellular routes of invasion by enteropathogens. *Curr. Opin. Microbiol.* **3**:54–59.
73. **Vazquez-Torres, A., and F. C. Fang.** 2001. Oxygen-dependent anti-*Salmonella* activity of macrophages. *Trends Microbiol.* **9**:29–33.
74. **Watson, P. R., S. M. Paulin, A. P. Bland, S. J. Libby, P. W. Jones, and T. S. Wallis.** 1999. Differential regulation of enteric and systemic salmonellosis by *slyA*. *Infect. Immun.* **67**:4950–4954.
75. **Wick, M. J., C. V. Harding, N. J. Twosten, S. J. Normark, and J. D. Pfeifer.** 1995. The *phoP* locus influences processing and presentation of *Salmonella typhimurium* antigens by activated macrophages. *Mol. Microbiol.* **16**:465–476.
76. **Wilson, J. A., and P. A. Gulig.** 1998. Regulation of the *spvR* gene of the *Salmonella typhimurium* virulence plasmid during exponential-phase growth in intracellular salts medium and at stationary phase in L broth. *Microbiology* **144**:1823–1833.

Editor: B. B. Finlay