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Selection of Small-Colony Variants of *Salmonella enterica* Serovar Typhimurium in Nonphagocytic Eucaryotic Cells

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Salmonella enterica **strains are enteropathogenic bacteria that survive and proliferate within vacuolar compartments of epithelial and phagocytic cells. Recently, it has been reported that fibroblast cells are capable of restricting *S. enterica* serovar Typhimurium intracellular growth. Here, we show that prolonged residence of bacteria in the intracellular environment of fibroblasts results in the appearance of genetically stable small-colony variants (SCV). A total of 103 SCV isolates, obtained from four independent infections, were subjected to phenotypic analysis. The following phenotypes were observed: (i) δ-aminolevulinic acid auxotrophy; (ii) requirement for acetate or succinate for growth in glucose minimal medium; (iii) auxotrophy for aromatic amino acids; and (iv) reduced growth rate under aerobic conditions not linked to nutrient auxotrophy. The exact mutations responsible for the SCV phenotype in three representative isolates were mapped in the *lpd*, *hemL*, and *aroD* genes, which code for dihydrolipoamide dehydrogenase, glutamate-1-semyaldehyde aminotransferase, and 3-dehydroquinate dehydratase, respectively. The *lpd*, *hemL*, and *aroD* mutants had intracellular persistence rates in fibroblasts that were 3 to 4 logs higher than that of the parental strain and decreased susceptibility to aminoglycoside antibiotics. All three of these SCV isolates were attenuated in the BALB/c murine typhoid model. Complementation with lpd^+, hem^+, and $aroD^+$ genes restored the levels of intracellular persistence and antibiotic susceptibility to levels of the wild-type strain. However, virulence was not exhibited by any of the complemented strains. Altogether, our data demonstrate that similar to what it has been reported for SCV isolates of other pathogens, *S. enterica* SCV display enhanced intracellular persistence in eucaryotic cells and are impaired in the ability to cause overt disease. In addition, they also suggest that *S. enterica* SCV may be favored in vivo.**

The species *Salmonella enterica* comprises a series of facultative intracellular pathogens that cause gastroenteritis and systemic infections in humans and animals (17, 38). Diverse host-adapted *S. enterica* serovars are also capable of triggering asymptomatic and persistent infections at relatively high rates (12, 15). The major hallmarks of *Salmonella* pathogenesis include the capacity of the bacteria to invade nonphagocytic cells and their capacity to survive within phagocytic cells. These two virulence traits were inferred in initial studies involving infection of tissue culture cells (reviewed in references 18, 19, 20, and 38). Subsequent work with epithelial cells and macrophages focused on characterization of the vacuolar compartment in which the bacteria reside (2, 10, 21, 23, 25, 36, 42, 48–51, 53). The murine infection model has also been very valuable for identification of other virulence functions, such as those encoded in *Salmonella* pathogenicity island 2, which are critical for intracellular survival and systemic disease (38).

Despite the relevance of the murine model, infection of tissue culture cells has been very useful for determining differences in the intracellular lifestyles of *S. enterica* within distinct host cell types (9). In most cases, massive intracellular bacterial growth is observed regardless of the epithelial or macrophage cell line that is used. The situation is different in macrophages isolated from animals, which are capable of arresting bacterial growth (11). Remarkably, in vivo studies have demonstrated that *S. enterica* proliferates predominantly within liver and spleen macrophages during systemic infection of mice (44, 47). This finding indicates that in contrast to the information obtained with the in vitro infection models, macrophages are the preferred niche used by *S. enterica* to proliferate in vivo. In recent reports workers have also described the capacity of *S. enterica* to colonize other types of antigen-presenting cells, such as dendritic cells. However, both in vitro and in vivo studies have demonstrated that bacteria located within these specialized cells do not exhibit massive intracellular growth (22, 37).

Recent studies have shown that cultured melanocytes and fibroblasts restrict intracellular proliferation of *S. enterica* (13, 32, 33). Upon infection of these cell types, either *S. enterica* remains in a latent nongrowing stage (13, 33) or the mass increases but the cell division process does not culminate (32). The latter defect leads to formation of long filamentous bacteria within the infected cells (32, 33), a phenomenon also recently observed in macrophages (46). Workers in our laboratory have demonstrated that *S. enterica* serovar Typhimu-

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

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
SL1344	*hisG rpsL*, mouse virulent	29
SV4042	SL1344 *hemL-801*	This study (SCV isolate)
SV4043	SL1344 *lpd-201*	This study (SCV isolate)
SV4175	SL1344 *aroD*	This study (SCV isolate)
SV4139	SV4042(*hemL-801*)/pIZ919(*hemL*$^+$)	This study
SV4137	SV4043(*lpd-201*)/pIZ918(*lpd*$^+$)	This study
SV4601	SV4175(*aroD*)/pIZ961(*aroD*$^+$)	This study
SV4384	SL1344 *aroA551*::Tn*10*	This study
Plasmids		
pBR328	Cloning vector, Tet^r Amp^r	Used for construction of *S. enterica* serovar Typhimurium LT2 chromosomal DNA library
pBluescript II SK(+)	Cloning vector, *lac*$^+$, Amp^r	Stratagene
PNK2880	Amp^r	31
PIZ916	pBR328, *hemL*$^+$	Library clone that complemented SCV phenotype in SV4042 (*hemL*)
pIZ917	pBR328, *hemL*$^+$	Religation of 6.5-kb *Sal*I fragment from pIZ916
pIZ919	pBluescript II, *hemL*$^+$	*Nhe*I-*Sal*I fragment from pIZ917 cloned in pBluescript II SK(+)
pIZ912	pBR328, *lpd*$^+$	Library clone that complemented SCV phenotype in SV4043 (*lpd*)
pIZ913	pBR328, *lpd*$^+$	Religation of 8.5-kb *Hind*III fragment from pIZ912
pIZ915	pBR328, *lpd*$^+$	Religation of 6.0-kb *Ava*I fragment from pIZ913
PIZ918	pBluescript II, *lpd*$^+$	*Hin*dIII-*Ava*I fragment from pIZ915, converted in blunt fragment (*Ava*I site) and cloned in *Hin*dIII-*Sma*I sites of pBluescript II SK(+)
pIZ956	pBR328, *aroD*$^+$	Library clone that complemented SCV phenotype in SV4175 (*aroD*)
PIZ961	pBluescript II, *aroD*$^+$	5.5-kb *Eco*RV fragment from pIZ956 cloned in pBluescript II SK(+)

rium is able to attenuate intracellular growth upon infection of fibroblasts. Thus, mutations that cause loss of function in virulence-related genes, such as *phoP-phoQ*, *slyA*, *spvR*, and *rpoS*, result in exacerbated intracellular bacterial growth (13). This observation indicates that there is delicate interaction between the pathogen and the infected fibroblast, resulting in establishment of a latent state of the bacteria within the host cell. Recently, the role of fibroblasts in bacterial infections was supported by a study that revealed the presence of *Mycobacterium tuberculosis*, a bacterium that remains in a latent state in a high percentage of the human population, in this specific host cell type (28). Interestingly, it has also been shown recently that *S. enterica* serovar Pullorum persists intracellularly in vivo within splenic chicken macrophages (60). Whether adaptation to the intracellular persistence stage is accompanied by stable changes in the physiology of *S. enterica* is not known.

It has been reported that *Staphylococcus aureus* variants that display physiological changes, such as a low growth rate under aerobic conditions, arise at a high frequency in the intracellular environment of cultured endothelial cells (54). These variants, known generically as small-colony variants (SCV), are frequently isolated from cases of persistent and relapsing infection and have an enhanced capacity to persist intracellularly when they are tested in tissue culture infection models (1, 30, 40, 56, 57). A phenotypic trait shared by most *S. aureus* SCV is a defect in synthesis of either menadione or hemin (34, 58). Lack of either of these two compounds likely alters the functionality of the electron transport chain, leading to a reduced growth rate under aerobic conditions and pleiotropic effects, such as decreased alpha-toxin expression, decreased uptake of aminoglycoside antibiotics, and increased expression of clumping factor and fibronectin-binding proteins (52, 58). Different authors have claimed that *S. aureus* SCV might be selected by exposure to subinhibitory concentrations of antimicrobial agents, although SCV have also been isolated after antibiotic-free intervals (reviewed in reference 58). SCV linked to chronic and recurrent infections have been described for other pathogens, such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Burkholderia pseudomallei* (26, 27, 45).

SCV isolates of *S. enterica* have not been characterized previously. However, salmonellae are pathogens that are prone to trigger chronic and persistent infections (5, 62). Since this pathogen adapts to establish a latent, nongrowing state in the intracellular environment of fibroblasts (13, 33), we studied the fate of intracellular bacteria within this specific cell type. Our results suggest that, as observed for *S. aureus*, prolonged intracellular residence of *S. enterica* serovar Typhimurium within eucaryotic cells leads to selection of SCV.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *S. enterica* serovar Typhimurium strains and plasmids used in this study are listed in Table 1. The genomic library of *S. enterica* serovar Typhimurium strain LT2/TN1379 (*leuBCD485*) was provided by G. C. Miller (Department of Microbiology, University of Illinois, Urbana). It was constructed in vector pBR328 by *Sau*3AI partial digestion of chromosomal DNA and ligation of 8- to 12-kb fragments to the *Bam*HI-digested vector. The library was stored in P22 HT 105/1 *int201* phage (referred to below as P22 HT). Allele numbers for *S. enterica* SCV isolates described in this study were obtained from the *Salmonella* Genetic Stock Center, University of Calgary, Calgary, Alberta, Canada. All strains were grown in Luria-Bertani (LB) medium supplemented with ampicillin (50 μg ml^{-1}) or kanamycin (50 μg ml^{-1}) when appropriate.

Mapping of mutations linked to the SCV phenotype. The following two mapping strategies were used to identify the mutations present in SCV isolates: (i) Tn*10d*Tet mutagenesis and detection of insertions linked to the small-colony phenotype upon P22 HT transduction; and (ii) complementation with a pBR328-derived library of serovar Typhimurium strain LT2. Tn*10d*Tet mutagenesis was performed by transducing an F′-borne Tn*10d*Tet insertion into an LT2 derivative carrying pNK2880, a plasmid that harbors a gene encoding the altered target specificity (ATS) transposase (31). Individual Tn*10*-carrying isolates were then pooled and lysed with P22 HT. The lysate was used to select Tn*10d*Tet insertions

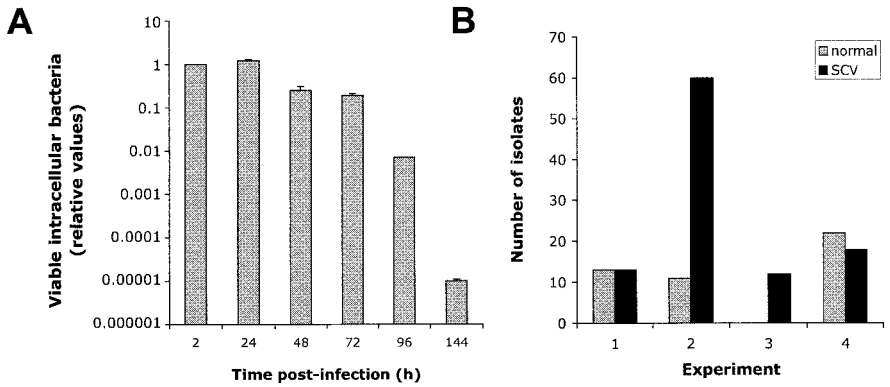

FIG. 1. Persistence of *S. enterica* serovar Typhimurium in fibroblasts favors selection of SCV isolates. (A) NRK-49F fibroblasts were infected with serovar Typhimurium strain SL1344 for 20 min. The numbers of viable intracellular bacteria surviving gentamicin treatment were determined at different times postinfection. The data are expressed relative to the number of viable bacteria quantified at 2 h postinfection. The values are medians (and standard deviations) for a representative experiment with a total of six repetitions. (B) Numbers of normal-colony-size and SCV isolates obtained in four different experiments at 6 days postinfection.

that changed the SCV phenotype to the normal-colony-size phenotype. The insertions were mapped by using the locked-in Mu*d*-P22 prophage procedure (7). The insertions linked to the SCV phenotype were also used to clone the corresponding mutant alleles and to identify the exact mutation responsible for the slow growth on plates. For complementation analysis, the pBR328-derived library of strain LT2 stored in P22 HT was used to transduce an SCV isolate. Plasmid DNA from normal-size colonies was purified and used to verify the complementation results. The inserts present in the complementing plasmid were subcloned by standard genetic procedures to determine the minimal insert size that complemented the SCV phenotype. At this stage, the flanking regions of the insert were sequenced with primers designed to amplify from vector regions, and the gene(s) present was identified.

Bacterial infection of fibroblast cells. Infection of NRK-49F fibroblasts (ATCC CRL1570) with *S. enterica* serovar Typhimuirum has been described elsewhere (13). Briefly, fibroblasts were grown in Dulbecco modified Eagle medium containing 5% fetal calf serum to 30% confluence at the time of infection. Bacteria were grown overnight in LB medium at 37°C under static conditions without shaking. The multiplicity of infection was 10 bacteria per fibroblast cell, and the infection time was 20 min. Infected cells were washed three times with phosphate-buffered saline (PBS) (pH 7.4) and incubated in fresh tissue culture medium containing 100 μg of gentamicin per ml. The antibiotic concentration was decreased to 10 μg ml^{-1} at 2 h postinfection. At different times postinfection, infected fibroblasts were lysed in 1% Triton X-100 for 5 min, and the number of intracellular viable bacteria was determined by plating serial dilutions of the cell extract onto LB agar plates. To avoid side effects of cell culture saturation in the long-term infection experiments, the infected fibroblasts were detached with a trypsin-EDTA solution at 48 to 72 h postinfection. The entire cell population was transferred to larger culture dishes containing fresh Dulbecco modified Eagle medium supplemented with 5% fetal calf serum and gentamicin (10 μg ml^{-1}). Caution was taken to avoid more than 80% confluence at any time postinfection. Cytotoxicity in the infected cell cultures was negligible at all times postinfection, as indicated by the absence of fibroblasts in the cultures permeable to 1% propidium iodine. The possibility that bacteria were present in the extracellular medium was examined by direct plating of tissue culture supernatants. This control consistently showed that the number of viable extracellular bacteria was very low at any time postinfection (≤0.1% of the number of viable intracellular bacteria). The low number of viable extracellular bacteria was considered irrelevant since three washes with PBS were performed before Triton X-100-mediated cell lysis.

Phenotypic analysis of SCV isolates. Phenotypic characterization of SCV isolates was performed by using noncitrate E (NCE) medium (8). Carbon sources, including glucose, glycerol, acetate, and succinate, were used at a concentration of 0.2% (wt/vol). δ-Aminolevulinic acid (ALA) (50 μg ml^{-1}) was used to test whether there was a deficiency in heme group synthesis. To test whether there was auxotrophy for aromatic compounds, a mixture containing 30 mM phenyalanine, 10 mM tryptophan, 10 mM tyrosine, 20 mM *para*-aminobenzoate, and 20 mM dihydroxinbenzoate was used.

Antibiotic susceptibility assays. The MIC was determined by the E test (AB Biodisk, Solna, Sweden) (61) by following the manufacturer's instructions.

Virulence assays in BALB/c mice. Bacteria were grown overnight in LB medium at 37°C under static conditions without shaking, centrifuged at 10,000 × *g* for 15 min, washed twice with sterile PBS (pH 7.4), and finally suspended in the same buffer. Serial dilutions were used to infect orally (25 μl) or intraperitoneally (200 μl) groups of five BALB/c female mice that were 7 to 8 weeks old. For the oral challenges the bacterium-containing suspension was mixed with an equal volume of 2.5% sodium bicarbonate–0.2% lactose to buffer the acidic pH of the stomach. Survival of the mice was recorded for up to 3 weeks postinfection, and 50% lethal doses were calculated by the method of Reed and Muench (43).

Nucleotide sequence accession numbers. The sequences of the *hemL* and *lpd* genes of *S. enterica* serovar Typhimurium strain SL1344 have been deposited in the EMBL database under accession numbers AJ278741/SEN278741 and AJ297525/SEN297525, respectively. Strain SV4042 (*hemL*) has an A-to-C point mutation at position 889 of the *hemL* nucleotide sequence, which results in a change from Pro to Thr. Strain SV4053 (*lpd*) has a G-to-A point mutation at position 1084 of the *lpd* nucleotide sequence that results in a change from Gly to Asp.

RESULTS

Selection of SCV of *S. enterica* serovar Typhimurium in cultured fibroblasts. The fate of serovar Typhimurium virulent strain SL1344 in NRK-49F fibroblasts, a cell line that restricts intracellular bacterial growth (13), was investigated. Cells were infected for 20 min, and the progress of the infection was monitored for 6 days. Under these experimental conditions, the viability of the intracellular bacteria decreased about 5 log units by day 6 (Fig. 1A). The main decrease in viability was observed 3 to 6 days postinfection. Consistent with previous reports (13, 33), no phase of active bacterial growth was observed at any time postinfection (Fig. 1A). A reproducible phenomenon that occurred in these long-term infection assays was the appearance of SCV when the cellular extract prepared after 6 days was plated onto LB agar plates. SCV did not appear in the population of intracellular bacteria at previous times postinfection (data not shown). The proportion of SCV in the intracellular population ranged from 45 to 100% in four independent long-term infection experiments (Fig. 1B). These results provided evidence that long-term persistence of *S. enterica* serovar Typhimurium within fibroblasts favors the selection of SCV isolates. Similar to what has been described for other pathogens, such as *S. aureus* and *E. coli* (4, 45, 54, 57),

TABLE 2. Phenotypes of *S. enterica* serovar Typhimurium SCV isolates selected in NRK-49F fibroblasts

Expt	No. of SCV isolates	No. of isolates in the following phenotypic groups of SCV isolates:			
		ALA auxotrophy	Acetate-succinate requirement to grow in NCE-glucose	Aromatic compound auxotrophy	Prototrophy (growth in NCE-glycerol)
1	13	0	13	0	0
2	60	57	0	0	3
3	12	11	0	0	1
4	18	0	0	18	0
Total	103	68	13	18	4

the *S. enterica* SCV may have acquired specific mutations that promote intracellular persistence within eucaryotic cells.

Phenotypic analysis of *S. enterica* SCV obtained from NRK-49F fibroblasts. To gain insight into the nature of the mutations responsible for the SCV phenotype, 103 SCV isolates obtained from the four independent fibroblast infection experiments (Fig. 1B) were screened for phenotypes described for SCV of other pathogens that have been isolated (34, 58). These phenotypes included auxotrophy for compounds in the heme biosynthetic pathway and differential growth on NCE minimal medium supplemented with intermediates of the Krebs cycle as alternative carbon sources. Auxotrophy for aromatic amino acids was also tested since it is known that *aro* mutants grow more slowly than the wild type in LB medium, which is limiting for these compounds. SCV isolates belonging to each of these three phenotypic groups were identified (Table 2). In addition, four SCV isolates grew in NCE minimal medium containing glycerol and therefore were considered prototrophs. Interestingly, a single SCV phenotypic class was observed in most, if not all, the SCV isolates obtained from the same experiment (Table 2). The phenotypic homogeneity in the SCV populations is consistent with strong selection for the SCV phenotype (about one SCV-linked mutation per intracellular bacterial population consisting of $\sim 10^4$ CFU). Because of the small size of the sample, the probability of selecting two different types of SCV mutations in the same experiment could be expected to be extremely low. Finally, we observed that all 103 SCV isolates examined in this screening analysis behaved like stable variants, with a rate of reversion to normal colony size of 10^{-6} to 10^{-7}, which is in the range of spontaneous mutation frequencies. Altogether, our data indicate that there are a variety of mutations that can cause the SCV phenotype in *S. enterica*. Some of the phenotypes found closely correspond to what has been found in *S. aureus*, such as a deficiency in synthesis of the heme group (4). Our study revealed that other types of mutations promote selection of *S. enterica* SCV in fibroblasts, including mutations that cause either a defect in providing catabolic compounds to the Krebs cycle or a reduced growth rate due to nutrient auxotrophy (*aro* type mutations).

Genetic characterization of *S. enterica* SCV isolates selected in cultured fibroblasts. Three SCV isolates collected in independent experiments (experiments 1, 2, and 4 [Table 2]) were designated strains SV4042, SV4053, and SV4175, propagated, and subjected to a genetic analysis to identify the spontaneous mutation responsible for the SCV phenotype. Unless indicated otherwise, the strategy involved (i) Tn*10d*Tet mutagenesis and detection of insertions linked to the SCV phenotype upon P22 HT transduction; (ii) mapping of the insertion with the locked-in Mu*d*-P22 prophage procedure (7); and (iii) complementation with a pBR328-derived library of serovar Typhimurium strain LT2.

After Tn*10d*Tet mutagenesis in SV4042 and further transduction, an insertion mapping between centisomes 3 and 4, *zaf-6309*::Tn*10d*Tet, was selected as an element that exhibited 30% linkage to the SCV phenotype. Complementation analysis with the LT2 genomic library and subsequent subcloning provided a 2-kb fragment that restored normal colony size (plasmid pIZ919 [Table 1]). Sequencing of this insert revealed the presence of a single open reading frame corresponding to the *hemL* gene, which encodes glutamate-1-semialdehyde aminotransferase. This enzyme catalyzes the last step in the biosynthetic pathway of ALA, a precursor of the heme group (16). Cloning and complete sequencing of the *hemL* allele harbored by SV4042 showed that there was a point mutation that caused a nonconservative amino acid change. Normal size colony was obtained upon complementation with a *hemL* wild-type gene, confirming that no additional mutation responsible for the SCV phenotype was present in SV4042. The SCV-linked insertion obtained in SV4053 after transposon mutagenesis, *zad-6310*::Tn*10d*Tet, was mapped at centisome 3, between the *nadC* and *proA* loci. Complementation of normal colony size and subcloning yielded a minimal 2.5-kb insert (plasmid pIZ918 [Table 1]), which harbored a fragment of the *aceF* gene and the complete *lpd* gene encoding lipoamide dehydrogenase or the E3 component of pyruvate and 2-oxoglutarate dehydrogenases (41). The Lpd protein has also been shown to be the L component of the enzymatic complex known as the glycine cleavage system (41). As in SV4042, cloning and sequencing of the *lpd* allele harbored by the SV4053 isolate showed that there was a point mutation that caused a nonconservative amino acid change. Complementation analysis with an *lpd*$^+$ gene restored wild-type colony size, eliminating the possibility that there were additional mutations in SV4053 responsible for the SCV phenotype.

Genetic analysis of SV4175 was simpler since this SCV isolate was a representative of the Aro$^-$ phenotypic class (Table 2, experiment 4). Complementation of the auxotrophy with the LT2 plasmid library, followed by subcloning, revealed that there was a minimal 5.5-kb insert that restored both prototrophy and large colony size (plasmid pIZ961 [Table 1]). Sequencing of an insert boundary revealed the presence of an open reading frame homologous to *ydiF*, an *E. coli* gene whose function is not known. In both *E. coli* and *S. enterica*, *ydiF* lies near the *aroD* gene, which in turn is 14 kb away from *aroH*. Since the insert that restored normal colony size was only 5.5 kb long, we concluded that the mutation carried by the SV4175 SCV isolate mapped in *aroD*, the gene that encodes 3-dehydroquinate dehydratase. This enzyme is involved in the biosynthesis of chorismate, a common precursor of aromatic amino acids (35).

***S. enterica* serovar Typhimurium SCV show increased intracellular persistence within fibroblasts.** It has been shown that *S. aureus* SCV display increased intracellular persistence within cultured eucaryotic cells (4, 57). To assess whether the *S. enterica* SCV selected in our study behaved in a similar

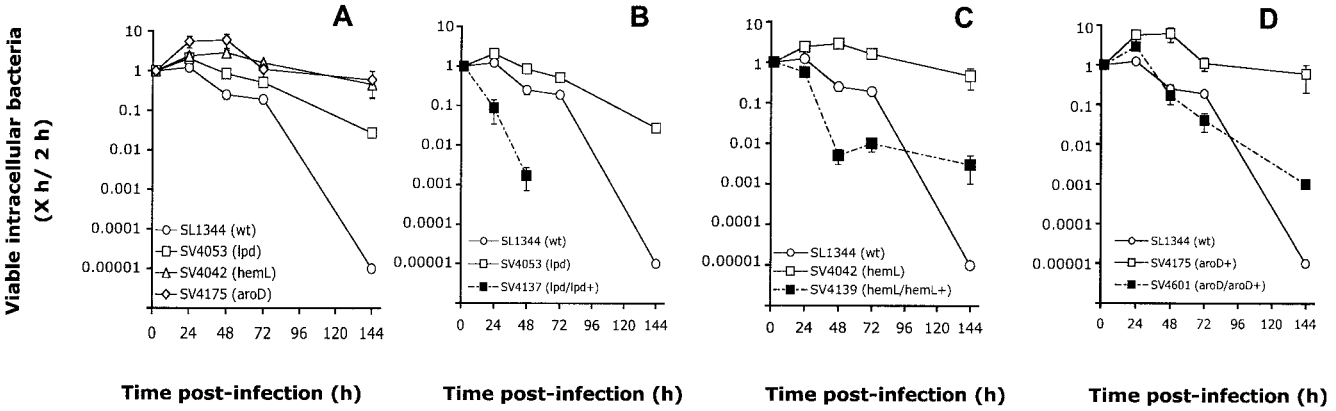

FIG. 2. Intracellular persistence of representative *S. enterica* serovar Typhimurium SCV isolates. (A) NRK-49F fibroblasts were infected with wild-type strain SL1344 (○) and the SCV derivatives SV5053 (*lpd*) (□), SV4042 (*hemL*) (△), and SV4175 (*aroD*) (◇). The numbers of viable intracellular bacteria were calculated for different times postinfection and are expressed relative to the value obtained at 2 h postinfection. (B to D) Behavior of the SV4137 (*lpd*/*lpd*$^+$), SV4139 (*hemL*/*hemL*$^+$), and SV4601 (*aroD*/*aroD*$^+$) complemented strains (■) compared to the behavior of the SL1344 wild-type strain (○) and the corresponding SCV isolates (□). Note that complementation with the wild-type genes caused a marked decrease in the intracellular persistence.

manner, isolates SV4042 (*hemL*), SV4053 (*lpd*), and SV4175 (*aroD*) were used individually to infect NRK-49F fibroblasts. All three SCV isolates exhibited intracellular persistence rates higher than the intracellular persistence rate of the wild type (Fig. 2A). The differences in intracellular viability between the wild type and the SCV isolates increased progressively after 3 days postinfection (Fig. 2A). On day 6, both the *hemL* and *aroD* mutants exhibited a viability rate that was close to 5 log units higher than that of the wild type, whereas the viability rate of the *lpd* mutant was about 4 log units higher (Fig. 2A). None of the mutants overgrew the gentamicin-containing extracellular medium since direct plating on this medium yielded low numbers of bacteria (≤0.1% of the viable intracellular bacteria) at all times postinfection. In addition, the differences in the intracellular persistence rates exhibited by the SCV isolates selected in fibroblasts were reproduced with SCV strains reconstructed with the SCV-linked Tn*10d*Tet insertions and SCV strains carrying knocked-out *hemL*, *lpd*, or *aroD* alleles (data not shown). Complementation of the point mutations selected in fibroblasts with *hemL*$^+$, *lpd*$^+$, and *aroD*$^+$ genes resulted in decreased intracellular persistence rates that were even lower than the rate of the wild-type strain (Fig. 2B to D). Altogether, these data confirmed that there was clear-cut linkage between the SCV phenotype and an enhanced capacity of *S. enterica* serovar Typhimurium to persist within fibroblast cells.

Antibiotic susceptibility analysis of the *S. enterica* serovar Typhimurium SCV isolates. SCV isolates of *S. aureus*, *P. aeruginosa*, and *E. coli* have been shown to share defects in the electron transport respiratory chain, which explains the low growth rates of these variants under aerobic conditions (4, 45, 57). It has been proposed that these defects in the respiratory chain reduce the membrane potential and impair energy-dependent processes, such as transport of molecules across the membrane. Uptake of antibiotics such as aminoglycosides, which are dependent on a normal energized state of the membrane, is notably reduced. Accordingly, some workers have reported diminished aminoglycoside susceptibility of SCV of *S. aureus* and *P. aeruginosa* recovered from cystic fibrosis patients (27, 30, 58). To determine whether the *S. enterica* serovar Typhimurium SCV isolates behave in a similar manner, their susceptibilities to distinct classes of antibiotics, including β-lactam antibiotics, aminoglycosides, quinonoles, chloramphenicol, and trimethoprim-sulfamethoxazole, were examined. The MICs of gentamicin and amikacin for the SCV isolates were increased and were approximately 10-fold higher for the *hemL*

TABLE 3. Antibiotic susceptibilities of representative *S. enterica* serovar Typhimurium SCV isolates

Antibiotic	MIC (μg/ml)						
	SL1344 (wild type)	SV4042 (*hemL*)	SV4053 (*lpd*)	SV4175 (*aroD*)	SV4139 (SV4042 *hemL*$^+$)	SV4137 (SV4053 *lpd*$^+$)	SV4601 (SV4175 *aroD*$^+$)
Amoxicillin-clavulanic acid	0.75	0.38	0.38	0.75	NA[a]	NA	NA
Cefotaxime	0.064	0.032	0.032	0.064	NA	NA	NA
Amikacin	1.5	16	3	3	1	1	1
Gentamicin	0.75	8	3	1.5	0.75	0.75	0.75
Nalidixic acid	6	1.5	4	2	3	3	4
Ofloxacin	0.094	0.094	0.094	0.094	0.094	0.047	0.064
Chloramphenicol	1.5	1	0.75	2	1	1	1.5
Trimethoprim-sulfamethoxazole	0.125	0.125	0.125	0.125	0.125	0.125	0.125

[a] NA, not applicable since the strain harbors the corresponding complementing gene cloned in the pBluescript II SK(+) plasmid, which carries an ampicillin resistance determinant (Table 1).

TABLE 4. Virulence attributes of representative *S. enterica* serovar Typhimurium SCV isolates in the murine typhoid model

Strain	Median lethal dose[a]	
	Oral	Intraperitoneal
SL1344 (wild type)	6×10^4	10
SV4042 (*hemL*)	4×10^7	1.5×10^3
SV4053 (*lpd*)	$>5 \times 10^9$	$>10^4$
SV4175 (*aroD*)	$>5.5 \times 10^8$	$>1.1 \times 10^5$

[a] Median lethal doses were calculated by the method of Reed and Muench (43).

mutant (Table 3). A fourfold increase in the MIC of gentamicin was also noted for the *lpd* mutant (Table 3). No major differences were observed between the wild type and the SCV in terms of susceptibility to β-lactam antibiotics, chloramphenicol, trimethoprim-sulfamethoxazole, and the quinonoles nalidixic acid and ofloxacin (Table 3). These results suggest that *S. enterica* SCV isolates are less sensitive to aminoglycosides, an expected finding considering that their mutations may potentially lead to defects in respiratory metabolism. Complementation of the *hemL*, *lpd*, and *aroD* mutants with the corresponding wild-type genes increased antibiotic susceptibility (Table 3), confirming that there is a direct relationship between the point mutations responsible for the SCV phenotype and the observed changes in sensitivity to aminoglycoside antibiotics.

***S. enterica* serovar Typhimurium SCV isolates are attenuated in the BALB/c mouse virulence model.** Previous reports provided evidence that there is reduced production of virulence factors in SCV. For instance, *S. aureus* SCV isolates have decreased alpha-toxin production and low coagulase activity (58, 59), which may prevent overt disease and host cell damage. Likewise, SCV of *P. aeruginosa* have been shown to be less virulent in normal and moderately leukopenic mice (24). The *hemL*, *lpd*, and *aroD S. enterica* SCV isolates showed marked attenuation in BALB/c mice when they were tested by using the oral or intraperitoneal route (Table 4). The data strongly suggest that conversion of *S. enterica* to SCV notably reduces the capacity of this pathogen to cause disease. Interestingly, no apparent reversion to a virulent, normal-colony-size form was observed in the animals since bacteria isolated from organs retained the SCV phenotype on plates (data not shown). We also tested whether complementation of the SCV phenotype with the wild-type *hemL*$^+$, *lpd*$^+$, and *aroD*$^+$ genes could result in the reappearance of virulence. Among the complemented strains, only the *lpd*/*lpd*$^+$ strain displayed a slighter lower median lethal dose, although the lethal dose was only on the order of 1 log lower than the lethal dose for the *lpd* SCV mutant (data not shown). In contrast, both the *hemL*/*hemL*$^+$ and *aroD*/*aroD*$^+$ strains remained as attenuated as the parental SCV isolates. The latter results led us to examine whether the complementing plasmid was unstable in vivo. Liver and spleen extracts were prepared from mice that survived after 20 days of bacterial challenge with the *hemL*/*hemL*$^+$ and *aroD*/*aroD*$^+$ strains. Interestingly, SCV appeared in some cases, whereas plasmid-bearing bacteria were not detected in any organ extract at this time postinfection (data not shown). These results were consistent with rapid loss of the complementing plasmid during infection.

DISCUSSION

To our knowledge, this study is the first one in which *S. enterica* serotype Typhimurium SCV were isolated and characterized. Phenotypic screening performed with 103 *S. enterica* SCV isolates showed that at least some of them resemble SCV of other bacterial pathogens, such as *S. aureus*. Thus, *S. enterica* SCV isolates with mutations in genes related to respiratory metabolism were identified. Examples include mutants requiring ALA and mutants requiring intermediates of the Krebs cycle for normal growth (*hem* and *lpd* phenotypic groups, respectively). Interestingly, a defect in the *hemB* gene was the first characterized mutation leading to an *S. aureus* SCV phenotype (57). A third phenotypic class, auxotrophy for aromatic compounds, was also observed. Strikingly, most if not all SCV isolated from each of the long-term fibroblast infections belonged to the same phenotypic group. This result was in accord with the relatively low number of intracellular bacteria that were analyzed. In fact, the size of the sample of viable intracellular bacteria recovered from fibroblasts was on the order of 10^3 to 10^4cells. Despite this relatively low number of bacteria, we successfully detected at least one mutation linked to the SCV phenotype in each experiment. A detailed genetic analysis of each of the SCV isolates recovered from a single experiment should provide clues about whether the isolates are derivatives of a single clone. Regardless of whether all mutants of a phenotypic class bear the same mutation, our results demonstrate that the frequency of formation of *S. enterica* SCV in fibroblasts is on the order of 10^{-3} to 10^{-4}, in close agreement with the study of Vesga et al., in which these authors estimated that the rate of formation of *S. aureus* SCV in cultured endothelial cells was 10^{-3} (54). However, one difference between the two pathogens is that emergence of *S. aureus* SCV in endothelial cells occurs after 72 h of infection, whereas *S. enterica* SCV are selected in cultured fibroblasts after 6 days of infection. The reasons for this difference are not known, but factors such as the host cell type and/or bacterial type may certainly contribute to it.

An interesting aspect of our study is the diversity of the mutations that apparently account for an increased capacity of *S. enterica* to persist intracellularly within fibroblasts. In the *hem* and *lpd* mutants the respiratory metabolism capacity may have been altered, probably leading to decreased generation of oxygen-reactive subproducts. In this sense, *S. enterica* SCV isolates would be less prone to self-intoxication in the intravacuolar environment with these oxygen-derived compounds. Further analysis of the cytochrome content and measurement of the respiratory rates of these mutants should certainly provide new clues concerning this hypothesis. Our study also showed that mutations that cause a defect in synthesis of aromatic amino acids, as in the case of *aroD*, favor intracellular bacterial persistence in fibroblasts. Precedents for linkage between *aro* mutations and *S. enterica* persistence exist for *aroA purA* double mutants, which persist for many months in organs of BALB/c mice (62). We observed that infection of fibroblasts with a well-characterized *S. enterica aroA* mutant resulted in an intracellular persistence rate comparable to that of the *aroD*

mutant (data not shown). Interestingly, both *aroA* and *aroD* mutants are defective for synthesis of chorismate, a precursor of not only aromatic amino acids but also folic acid, ubiquinone, and enterobactin. Lack of enterobactin might result in iron starvation and subsequent impairment of cytochrome synthesis. The fact that all SCV isolates selected in our study are potentially defective in respiratory metabolism led us to postulate that switching to nonrespiratory metabolism may be a major mechanism used by *S. enterica* to increase intracellular persistence within eucaryotic cells.

The analysis of antibiotic sensitivity showed that, as reported previously for SCV isolates of *S. aureus* and *P. aeruginosa* (3, 4, 34, 39, 45, 57, 58), the *S. enterica* SCV isolates have reduced aminoglycoside sensitivity. This phenotype could indicate that selection of *S. enterica* SCV within cultured fibroblast cells was merely caused by prolonged exposure of intracellular bacteria to gentamicin, an antibiotic maintained in the tissue culture medium. Several observations argue against this possibility. First, selection of *S. aureus* SCV by exposure to sublethal concentrations of gentamicin has been reported to take place in as little as 30 min (34). In contrast, recovery of *S. enterica* SCV isolates from infected fibroblasts occurs at an appreciable frequency only at very late infection times (6 days postinfection). Second, acquisition of gentamicin resistance concomitant with the SCV phenotype seems to be a reversible phenomenon in *S. aureus* since antibiotic-resistant SCV revert rapidly to sensitive forms upon incubation in antibiotic-free media (34). High-frequency reversion to normal colony size and antibiotic-sensitive forms was not observed in any of the *S. enterica* SCV isolates described in this study, which suggests that there may be intrinsic differences between SCV selection under antibiotic exposure conditions and intracellular persistence within fibroblast cells. Third, the differences in the reversibility of the phenotype are accompanied by distinct levels of antibiotic resistance. Whereas the MICs of gentamicin for *S. enterica* SCV are 2- to 10-fold higher than those for the wild type (Table 3), the MICs for *S. aureus* SCV are ≥20-fold higher that those for the parental strain (34). Fourth, despite the increased MICs of gentamicin for the three *S. enterica* SCV isolates analyzed (1.5, 3, and 8 μg ml^{-1} for the *aroD*, *lpd*, and *hemL* mutants, respectively [Table 3]), none of these variants overgrew the tissue culture medium containing 10 μg of gentamicin per ml during the fibroblast infection experiment. Finally, it has been demonstrated previously by using a quantitative enzyme-linked immunosorbent assay that unlike phagocytic cells, NRK-49F fibroblasts do not accumulate gentamicin intracellularly (33). Altogether, these findings led us to consider selection of *S. enterica* SCV isolates in the intracellular environment of fibroblasts a unique event unrelated to the eventual exposure of intracellular bacteria to gentamicin.

Aside from the same behavior in terms of persistence within fibroblasts cells and antibiotic susceptibility, the three *S. enterica* SCV clones characterized at a genetic level (*hemL*, *lpd*, and *aroD*) were highly attenuated in the BALB/c mouse typhoid model. *S. enterica aroA* mutants, which persist efficiently in NRK-49F fibroblasts, were the first genetically defined strains in which marked attenuation was observed in the mouse model (29). In a subsequent study the workers also reported attenuation of a *hemA* mutant of serovar Typhimurium (6), which, in agreement with the results of the present study, reinforces the contribution of the biosynthetic pathway involved in synthesis of the heme group to *S. enterica* pathogenesis. We found evidence that when administrated by the oral route, the *hemL*, *lpd*, and *aroD* SCV isolates are capable of reaching the liver and spleen (data not shown). These results are consistent with normal passage through the intestinal epithelium and subsequent failure of the bacteria to survive or proliferate within phagocytic cells present in target organs. Additional work is required to assess whether these SCV isolates also persist intracellularly in vivo. Globally, our data are consistent with previous observations made with *S. aureus* and *P. aeruginosa* that show that there is direct linkage between the SCV phenotype and an inability to cause overt disease (24, 57, 58). Interestingly, we did not find evidence of conversion to the normal-colony-size type during infection with SCV isolates. Furthermore, our attempts to complement the attenuation phenotype displayed by the *S. enterica* SCV isolates with plasmids carrying the wild-type genes $hemL^+$, lpd^+, and $aroD^+$ were unsuccessful. The fact that the complementing plasmid was lost rapidly during infection may explain these results. In addition, plasmid-bearing bacteria are impaired in terms of the ability to survive intracellularly (Fig. 2), which suggests that the complemented bacteria may be rapidly counterselected in vivo. Overall, these results confirm that there is a strict relationship between the SCV phenotype and virulence attenuation in *S. enterica* and selection occurring against normal-colony-size phenotype once the bacteria have switched to an SCV.

A final consideration in our work was the possibility that similar to *S. aureus*, natural SCV of *S. enterica* could be responsible for triggering chronic and persistent infections. So far, virtually nothing is known about the mechanisms of *S. enterica* persistence. However, as previously noted, an *S. enterica* serovar Typhimurium *aroA purA* double mutant, which is an SCV, persists in BALB/c mice (62). Although the basis of this phenomenon has not been defined, it supports the hypothesis that there is probably linkage between the SCV phenotype and the in vivo persistence of *S. enterica*. This hypothesis does not contradict the results of epidemiological studies showing that bacteria released into the environment by asymptomatic human and animal carriers retain pathogenic potential. Thus, normal-colony-size bacteria may coexist in vivo with SCV, as has been observed in long-term infection of fibroblasts (Table 2, experiments 1, 2, and 4). Under these conditions, an effective host immune response elicited by the SCV (e.g., by an *aro* mutant [29]) may limit the ability of normal-colony-size bacteria to cause disease. Reactivation of disease due to non-SCV bacteria would occur only during periods when immunity is reduced. Alternatively, reactivation of the disease may be associated with the appearance of second mutations that suppress the SCV phenotype. Such a concept has been postulated for the reactivation of infection in the case of chronic infections caused by an *E. coli hemB* mutant (45). Finally, it should be noted that in the specific case of serovar Typhi, a host-adapted serovar that triggers chronic and asymptomatic infections in humans, many clinical isolates are auxotrophs for aromatic amino acids (55). Altogether, these observations indicate that a more profound molecular analysis of *S. enterica* SCV isolates is necessary, which may provide clues about whether switching to a stable SCV phenotype has consequences for *Salmonella* pathogenesis. The recent discovery

that polynucleotide phosphorylase is a global regulator of virulence and persistence in *S. enterica* (14) supports the hypothesis that there is a delicate balance between these two processes. Additional work is required to confirm whether the emergence of stable SCV isolates is a consequence of the interplay between such bacterial regulatory networks and the host immune defenses.

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