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 dihydrodipicolinate dehydrogenase, glutamate-1-semialdehyde aminotransferase, and 3-dehydroquininate 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-
Plasmids that cell type (28). Interestingly, it has also been shown recently, a bacterium that remains in a latent state in the pathogen and the infected observation indicates that there is delicate interaction between result in exacerbated intracellular bacterial growth (13). This supported by a study that revealed the presence of Mycobac-}

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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td>SL1344</td>
<td>hisG rpsL, mouse virulent</td>
<td>29</td>
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<tr>
<td>SV4042</td>
<td>SL1344 hemL-80I</td>
<td>This study (SCV isolate)</td>
</tr>
<tr>
<td>SV4043</td>
<td>SL1344 lpd-20I</td>
<td>This study (SCV isolate)</td>
</tr>
<tr>
<td>SV4175</td>
<td>SL1344 aroD</td>
<td>This study (SCV isolate)</td>
</tr>
<tr>
<td>SV4139</td>
<td>SV4042(hemL-80I)/pIZ919(hemL+)</td>
<td>This study</td>
</tr>
<tr>
<td>SV4137</td>
<td>SV4043(lpd-20I)/pIZ918(lpd+)</td>
<td>This study</td>
</tr>
<tr>
<td>SV4601</td>
<td>SV4175(aD)/pIZ961(aD+)</td>
<td>This study</td>
</tr>
<tr>
<td>SV4384</td>
<td>SL1344 aroS51::Tn10</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Plasmids**

- pBR328: Cloning vector, Tet Amp
- pBluescript II SK(+) Cloning vector, lac+, Amp
- PNIK2880: Amp
- PIZ916: pBR328, hemL+
- PIZ917: pBR328, hemL+
- PIZ918: pBluescript II, hemL+
- PIZ919: pBR328, lpd+
- PIZ920: pBR328, lpd+
- PIZ921: pBluescript II, lpd+
- PIZ956: pBR328, aroD+
- PIZ961: pBluescript II, aroD+

- Used for construction of *S. enterica* serovar Typhimurium LT2 chromosomal DNA library
- Stratagene
- 31
- Library clone that complemented SCV phenotype in SV4042 (hemL)
- Religation of 6.5-kb SalI fragment from pIZ916
- NheI-SalI fragment from pIZ917 cloned in pBluescript II SK(+) (]
- Library clone that complemented SCV phenotype in SV4043 (lpd)
- Religation of 8.5-kb HindIII fragment from pIZ912
- Religation of 6.0-kb AviI fragment from pIZ913
- HindIII-AviI fragment from pIZ915, converted in blunt fragment (AviI site)
- and cloned in HindIII-SmaI sites of pBluescript II SK(+)
- Library clone that complemented SCV phenotype in SV4175 (aroD+)
- 5.5-kb EcoRV fragment from pIZ956 cloned in pBluescript II SK(+) (]

**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 Sac3AI partial digestion of chromosomal DNA and ligation of 8- to 12-kb fragments to the BamHI-digested vector. The library was stored in P22 HT 105/1 int207 plage (referred to below as P22 HT). Allele numbers for *S. enterica* SCV isolates described in this study were obtained from the Salmoella 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) Tn10/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. Tn10/Tet mutagenesis was performed by transducing an F⁺ borne Tn10/Tet insertion into an LT2 derivative carrying pNK2880, a plasmid that harbors a gene encoding the altered target specificity (ATS) transposase (31). Individual Tn10-carrying isolates were then pooled and lysed with P22 HT. The lysate was used to select Tn10/Tet insertions
that changed the SCV phenotype to the normal-colony-size phenotype. The insertions were mapped by using the locked-in MuAl-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 insert present in the complementing plasmid was 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 Typhimurium has been described elsewhere (13). Briefly, fibroblasts were grown in Dulbecco modified Eagle medium containing 5% fetal calf serum to 30% confluence. 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 sucinate, were used at a concentration of 0.2% (wt/vol). l-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 phenylalanine, 10 mM tryptophan, 10 mM tyrosine, 20 mM para-aminobenzoate, and 20 mM dihydroxybenzoate 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 infection, 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),

**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.
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 four independent experiments (Fig. 1B). In addition, four SCV isolates selected in our study behaved in a similar manner to what has been found in *S. aureus* (57). To assess whether the SCV phenotype was present in SV4053 after transposon mutagenesis, *zac-6310::Tn10dTet*, 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 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) Tn10dTet mutagenesis and detection of insertions linked to the SCV phenotype upon P22 HT transduction; (ii) mapping of the insertion with the locked-in Mud-P22 prophage procedure (7); and (iii) complementation with a pBR328-derived library of serovar Typhimurium strain LT2.

After Tn10dTet mutagenesis in SV4042 and further transduction, an insertion mapping between centisomes 3 and 4, *zaf-6309::Tn10dTet*, 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, *zac-6310::Tn10dTet*, 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-dehydroquininate 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

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<p>| Table 2. Phenotypes of <em>S. enterica</em> serovar Typhimurium SCV isolates selected in NRK-49F fibroblasts |</p>
<table>
<thead>
<tr>
<th>Expt</th>
<th>No. of SCV isolates</th>
<th>ALA auxotrophy</th>
<th>Acetate-succinate requirement to grow in NCE-glucose</th>
<th>Aromatic compound auxotrophy</th>
<th>Prototrophy (growth in NCE-glycerol)</th>
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<tbody>
<tr>
<td>1</td>
<td>13</td>
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<td>13</td>
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<td>0</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>57</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>11</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>4</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>103</td>
<td>68</td>
<td>13</td>
<td>18</td>
<td>4</td>
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</table>
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 Tn10lOtTet 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<sup>+</sup>, lpd<sup>+</sup>, and aroD<sup>+</sup> 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, quinolones, chloramphenicol, and trimethoprim-sulfamethoxazole, were examined.

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

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (µg/ml)</th>
</tr>
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<tr>
<td></td>
<td>SL1344 (wild type)</td>
</tr>
<tr>
<td>Amoxicillin-clavulanic acid</td>
<td>0.75</td>
</tr>
<tr>
<td>Cefotaxime</td>
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</tr>
<tr>
<td>Amikacin</td>
<td>1.5</td>
</tr>
<tr>
<td>Gentamicin</td>
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</tr>
<tr>
<td>Nalidixic acid</td>
<td>6.0</td>
</tr>
<tr>
<td>Ofloxacine</td>
<td>0.094</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1.5</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>0.125</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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).
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 quinolones 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 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 slightly 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\(^4\) cells. 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\(^{-4}\), and 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 intracellular 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

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Oral Median lethal dose(^{a})</th>
<th>Intrapertoneal Median lethal dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL1344 (wild type)</td>
<td>(6 \times 10^4)</td>
<td>10</td>
</tr>
<tr>
<td>SV4042 (hemL)</td>
<td>(4 \times 10^4)</td>
<td>(1.5 \times 10^3)</td>
</tr>
<tr>
<td>SV4053 (lpd)</td>
<td>(&gt;5 \times 10^8)</td>
<td>(&gt;1 \times 10^8)</td>
</tr>
<tr>
<td>SV4175 (aroD)</td>
<td>(&gt;5.5 \times 10^9)</td>
<td>(&gt;1.1 \times 10^8)</td>
</tr>
</tbody>
</table>

\(^{a}\) Median lethal doses were calculated by the method of Reed and Muench (43).
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 are 2- to 10-fold higher than those for the wild type (Table 3), the MICs for S. aureus SCV are ≥20-fold higher than 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 aroA 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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