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## **A *Salmonella enterica* Serovar Typhimurium Succinate Dehydrogenase/Fumarate Reductase Double Mutant Is Avirulent and Immunogenic in BALB/c Mice**

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## A *Salmonella enterica* Serovar Typhimurium Succinate Dehydrogenase/Fumarate Reductase Double Mutant Is Avirulent and Immunogenic in BALB/c Mice<sup>∇</sup>

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Previously we showed that the tricarboxylic acid (TCA) cycle operates as a full cycle during *Salmonella enterica* serovar Typhimurium SR-11 peroral infection of BALB/c mice (M. Tchawa Yimga et al., *Infect. Immun.* 74:1130–1140, 2006). The evidence was that a  $\Delta$ *sucCD* mutant (succinyl coenzyme A [succinyl-CoA] synthetase), which prevents the conversion of succinyl-CoA to succinate, and a  $\Delta$ *sdhCDA* mutant (succinate dehydrogenase), which blocks the conversion of succinate to fumarate, were both attenuated, whereas an SR-11  $\Delta$ *aspA* mutant (aspartase) and an SR-11  $\Delta$ *frdABCD* mutant (fumarate reductase), deficient in the ability to run the reductive branch of the TCA cycle, were fully virulent. In the present study, evidence is presented that a serovar Typhimurium SR-11  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA* double mutant is avirulent in BALB/c mice and protective against subsequent infection with the virulent serovar Typhimurium SR-11 wild-type strain via the peroral route and is highly attenuated via the intraperitoneal route. These results suggest that fumarate reductase, which normally runs in the reductive pathway in the opposite direction of succinate dehydrogenase, can replace it during infection by running in the same direction as succinate dehydrogenase in order to run a full TCA cycle in an SR-11  $\Delta$ *sdhCDA* mutant. The data also suggest that the conversion of succinate to fumarate plays a key role in serovar Typhimurium virulence. Moreover, the data raise the possibility that *S. enterica*  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA* double mutants and  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA* double mutants of other intracellular bacterial pathogens with complete TCA cycles may prove to be effective live vaccine strains for animals and humans.

Recently, it was reported that the tricarboxylic acid (TCA) cycle (Fig. 1) operates as a full cycle during infection of BALB/c mice with *Salmonella enterica* serovar Typhimurium strain SR-11 (hereafter called SR-11). Although a complete TCA cycle appeared to be required for full SR-11 virulence, the deletion of different TCA cycle genes resulted in different levels of attenuation (28). We found that an SR-11  $\Delta$ *sucAB* mutant, unable to convert  $\alpha$ -ketoglutarate to succinyl coenzyme A (succinyl-CoA) via the 2-oxoglutarate dehydrogenase complex (7, 22), was avirulent; that an SR-11  $\Delta$ *mdh* mutant, unable to convert malate to oxaloacetate via malate dehydrogenase (7, 30), was highly attenuated; that an SR-11  $\Delta$ *sucCD* mutant, unable to generate succinate from succinyl-CoA via succinyl-CoA synthetase (3, 7), was moderately attenuated; and that an SR-11  $\Delta$ *sdhCDA* mutant, unable to generate fumarate from succinate via succinate dehydrogenase (7, 23), was slightly attenuated. Mutants defective in the ability to run in the reductive branch of the TCA cycle, i.e., an SR-11  $\Delta$ *aspA* mutant (aspartase) unable to convert aspartate to fumarate (7, 27) and an SR-11  $\Delta$ *frdABCD* mutant (fumarate reductase) unable to convert fumarate to succinate (6, 7), were fully virulent (28).

It was not surprising that that the SR-11  $\Delta$ *sucAB* mutant was

avirulent, i.e., the strain was unable to make succinyl-CoA, which in addition to being converted to succinate in the TCA cycle is required for the biosynthesis of diaminopimelate, a precursor for the synthesis of lysine, methionine, and peptidoglycan (4, 5, 10, 11, 19). However, it was surprising that the SR-11  $\Delta$ *sdhCDA* mutant was less attenuated than the SR-11  $\Delta$ *mdh* mutant, since the conversion of succinate to fumarate (*sdhCDA*) precedes the conversion of malate to oxaloacetate (*mdh*) in the TCA cycle. One explanation as to why the SR-11  $\Delta$ *sdhCDA* mutant was less attenuated than the SR-11  $\Delta$ *mdh* mutant is the possibility that endogenous fumarate reductase, which is known to be able to run in the opposite direction, i.e., converting succinate to fumarate (6, 13), may be able to substitute for succinate dehydrogenase in SR-11 in vivo and thereby lessen the effect of the  $\Delta$ *sdhCDA* mutation. Indeed, in the present study, we show that an SR-11  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA* double mutant is both avirulent and immunogenic in BALB/c mice. Therefore, the data presented suggest that the conversion of succinate to fumarate plays a key role in serovar Typhimurium virulence and raises the possibility that *S. enterica*  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA* double mutants and  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA* double mutants of other intracellular bacterial pathogens with complete TCA cycles may prove to be effective live vaccine strains for animals and humans.

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### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth media.** The bacterial strains and plasmids used in this study are listed in Table 1. LB broth, Lennox (Difco Laboratories, Detroit, MI); LB agar, Lennox (Difco); and MacConkey agar (Difco) were prepared according to package instructions. Liquid M9 minimal

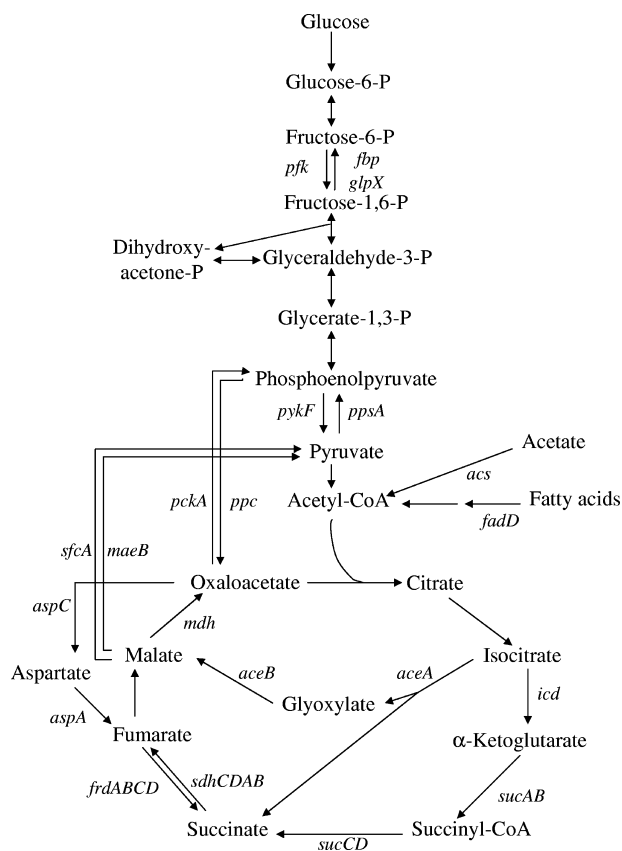


FIG. 1. Embden-Meyerhoff pathway, gluconeogenic pathway, and TCA cycle. Arrows indicate the physiological directions of the reactions. Genes encoding the enzymes for each reaction are listed beside each reaction.

salts medium (20) was supplemented either with reagent-grade glucose (0.2%, wt/wt) or sodium succinate (0.4%, wt/wt) as the sole sources of carbon and energy. When necessary, the pH was adjusted to 7.2. SOC medium was prepared as described by Datsenko and Wanner (9). Agar plates were supplemented with nalidixic acid (50 µg/ml) and/or chloramphenicol (30 µg/ml), as appropriate.

**Construction and characterization of SR-11 strains.** The SR-11  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA::cat* mutant was constructed from the SR-11  $\Delta$ *frdABCD* mutant by deletion mutagenesis using a chloramphenicol cassette as described by Datsenko and Wanner (9). The primers used previously to create the SR-11  $\Delta$ *sdhCDA::cat* mutant (28) were used to construct the double mutant. The “restored” SR-11  $\Delta$ *frdABCD* mutant (wild-type *sdhCDA*) was constructed by replacing the *sdhCDA::cat* sequence in the SR-11  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA::cat* mutant with the SR-11 wild-type sequence using the method described by Datsenko and Wanner (9). A “restored” SR-11  $\Delta$ *frdABCD* mutant was selected after plating transformants on M9 minimal agar plates containing sodium succinate (0.4%, wt/wt) as the sole carbon and energy source. As expected, the “restored” SR-11  $\Delta$ *frdABCD* mutant was chloramphenicol sensitive and grew at the same rate as the original SR-11  $\Delta$ *frdABCD* mutant in M9 minimal medium containing succinate (0.6%, wt/wt) as the sole carbon and energy source. The primers used to amplify the wild-type SR-11 *sdhCDA* sequence used for transformation were as follows: forward, 5'-GCGTACAGGTAGATTACCTCTG-3' (5' end of the primer begins 68 base pairs upstream of the ATG start codon of the *sdhC* gene); reverse, 5'-GGAAGTCCACATTTCCATGTC-3' (5' end of the primer begins 753 base pairs downstream of the ATG start codon of the *sdhA* gene). Constructs were verified by PCR and sequencing. The primers used for sequencing were the forward and reverse primers listed previously. The primers were designed by referring to the complete genome of *Salmonella enterica* serovar Typhimurium LT2 (18). When appropriate, the chloramphenicol cassette was removed from the strains as described by Datsenko and Wanner (9). For sequencing, PCR products were purified with a Qiaquick PCR purification kit (Qiagen, MD) by following the manufacturer's instructions. Each sequencing mixture contained between 50 and 150 ng of PCR product, 1.6 pmol of primer, and 8.0 µl of Quick Start dye terminator cycle sequencing master mix (Beckman Coulter, Fullerton, CA). The thermal cycling program consisted of 30 cycles of denaturation at 96°C for 20 s, annealing at 50°C for 20 s, and elongation at 60°C for 4 min. After the completion of the cycle sequencing, the samples were purified by ethanol precipitation and separated by polyacrylamide gel electrophoresis on a CEQ 8000 genetic analysis system (Beckman Coulter).

**Growth on glucose and sodium succinate in M9 minimal medium.** To test the rates of growth of wild-type SR-11 and SR-11  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA::cat* in M9 minimal medium containing glucose as the sole carbon and energy source (20), overnight cultures grown in LB broth were washed twice in M9 minimal medium (no carbon source), 100 µl of the washed cultures was transferred to 10 ml of M9 minimal medium containing reagent-grade glucose (0.4%, wt/wt) as the sole carbon and energy source, and the cultures were incubated at 37°C with shaking in 125-ml tissue culture bottles overnight. The next morning, overnight cultures were diluted to an  $A_{600}$  of about 0.1 in 20 ml of fresh M9 minimal medium containing reagent-grade glucose (0.4%, wt/wt) as the sole carbon and energy source, and the cultures were incubated at 37°C with shaking in 125-ml tissue culture bottles.  $A_{600}$  measurements were taken at 60-min intervals. The same protocol used for growth on glucose was used for testing the rates of growth of the original SR-11  $\Delta$ *frdABCD* mutant and the “restored” SR-11  $\Delta$ *frdABCD*

TABLE 1. Strains and plasmids of serovar Typhimurium used in this study

Strain or plasmid	Relevant characteristic(s)	Defect(s)	Source or reference
<b>Strains</b>			
Wild-type SR-11	<i>gyr-1816</i>	None	8
SR-11 $\Delta$ <i>frdABCD</i>	<i>gyr-1816</i> $\Delta$ <i>frdABCD</i>	Fumarate reductase	28
SR-11 $\Delta$ <i>sdhCDA</i>	<i>gyr-1816</i> $\Delta$ <i>sdhCDA</i>	Succinate dehydrogenase	28
SR-11 $\Delta$ <i>frdABCD</i> $\Delta$ <i>sdhCDA::cat</i>	<i>gyr-1816</i> $\Delta$ <i>frdABCD</i> $\Delta$ <i>sdhCDA::cat</i>	Fumarate reductase and succinate dehydrogenase	This study
“Restored” SR-11 $\Delta$ <i>frdABCD</i>	<i>gyr-1816</i> $\Delta$ <i>frdABCD</i>	Fumarate reductase	This study
<b>Plasmids</b>			
pKD3	Template plasmid containing chloramphenicol resistance cassette flanked by FLP recombinase target sites; <i>bla cat</i>		9
pKD46	Temperature-sensitive plasmid containing arabinose-inducible phage $\lambda$ red recombinase gene for linear DNA exchange; <i>bla</i>		9
pCP20	Temperature-sensitive plasmid containing FLP recombinase gene for removal of antibiotic resistance cassettes; <i>bla cat</i>		9

mutant on M9 minimal medium containing sodium succinate (0.6%, wt/wt) as the sole carbon and energy source. In all growth experiments, growth was monitored spectrophotometrically ( $A_{600}$ ) using a Pharmacia Biotech Ultraspec 2000 UV/visible-spectrum spectrophotometer.

**Virulence assays.** Virulence assays were carried out as described previously (1). Briefly, 4-week-old, 13- to 15-g female BALB/c mice (Charles River Laboratories, Wilmington, MA) were housed at no more than four per cage, with pine shavings as bedding. Prior to infection, the mice were starved for food and water for 4 h. Following starvation, 50  $\mu$ l of 10% sodium bicarbonate was administered orally to each mouse in order to neutralize gastric acidity, and 30 min later,  $10^8$  CFU of a specific strain in 20  $\mu$ l of phosphate-buffered saline (pH 7.4) containing 0.1% gelatin was administered orally to each of four mice. For virulence assays via the intraperitoneal (i.p.) route,  $10^5$  CFU or  $10^4$  CFU of a specific strain in 100  $\mu$ l of phosphate-buffered saline (pH 7.4) containing 0.1% gelatin was injected by sterile syringe into each of four mice. The number of CFU administered to mice was measured by diluting and then plating bacterial suspensions onto MacConkey agar containing nalidixic acid (50  $\mu$ g/ml). After infection, food and water were restored to the mice and the mice were inspected four times daily for obvious signs of illness (ruffled fur, crusted and closed eyes, loss of appetite, crouched posture and shivering) and death.

**Definition of fully virulent, attenuated, and avirulent.** A set of four mice were infected orally with  $10^8$  CFU of a specific SR-11 deletion mutant, and an additional four mice were infected orally with  $10^8$  CFU of wild-type SR-11 in each virulence experiment. The data presented here are composites of at least two identical but independent experiments (eight mice). When mice infected with a specific mutant remain healthy, i.e., show no signs of illness throughout the 30-day duration of the experiment, the mutant is considered to be avirulent. The term “fully virulent” is used when mice infected with a particular mutant show the same signs of illness as mice infected with the wild-type SR-11 strain and the survival curve is not statistically different from that of mice infected with the wild-type SR-11 strain. The term “attenuated” is used when the mice infected with a particular mutant show signs of illness but the survival curve is statistically different from that of mice infected with the wild-type SR-11 strain and death is delayed. The extent to which death is delayed is denoted by the term “slightly attenuated,” “attenuated,” or “highly attenuated,” conditions which are clarified by results shown in the figures.

**Protection assays.** Thirty days after peroral infection with SR-11  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA::cat*, which is sufficient time for mice to mount an intestinal mucosal immunological response and a systemic immunological response, BALB/c mice were challenged perorally with  $10^8$  CFU of wild-type SR-11, as described above. In each experiment, an age-matched set of sham-vaccinated BALB/c mice were also challenged with  $10^8$  CFU of wild-type SR-11. Mice were then observed each day in the morning and 8 hours later for obvious signs of illness (ruffled fur, crusted and closed eyes, loss of appetite, crouched posture and shivering) and death.

**CFU in Peyer's patches, liver, and spleen.** Mice were euthanized by CO<sub>2</sub> asphyxiation. Peyer's patches (four per mouse) were removed from the ileum and terminal ileum as described by Curtiss and Kelly (8) and homogenized. Homogenates were diluted and plated on MacConkey agar containing nalidixic acid (50  $\mu$ g per ml) to determine the number of CFU and were tested for protein concentration by the Bradford assay (3a). Data are presented as log<sub>10</sub> number of CFU/mg of protein. Livers and spleens were removed and homogenized in Luria broth. Homogenates (0.1 g per ml, wt/wt) were diluted and plated on MacConkey agar containing nalidixic acid (50  $\mu$ g per ml) to determine the number of CFU per organ. Plates were incubated at 37°C for at least 18 h prior to counting the CFU. Data are presented as log<sub>10</sub> number of CFU/organ.

**Statistics.** Mouse survival curves were compared for differences using the Kaplan-Meier method (MedCalc Software, Belgium). Survival curves were considered to be different if the *P* value was less than 0.05.

## RESULTS

**An SR-11  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA::cat* double mutant is avirulent ( $10^8$  CFU/mouse, peroral route).** BALB/c mice were infected orally with  $10^8$  CFU (i.e., about  $10^3$  times the wild-type 50% lethal dose [LD<sub>50</sub>]) (8) of either wild-type SR-11, SR-11  $\Delta$ *sdhCDA*, SR-11  $\Delta$ *frdABCD*, or SR-11  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA::cat*. As reported previously, the SR-11  $\Delta$ *sdhCDA* mutant was slightly attenuated (*P* = 0.0068) (Fig. 2A) and the SR-11  $\Delta$ *frdABCD* mutant was fully virulent (*P* = 0.94) (Fig. 2A). In

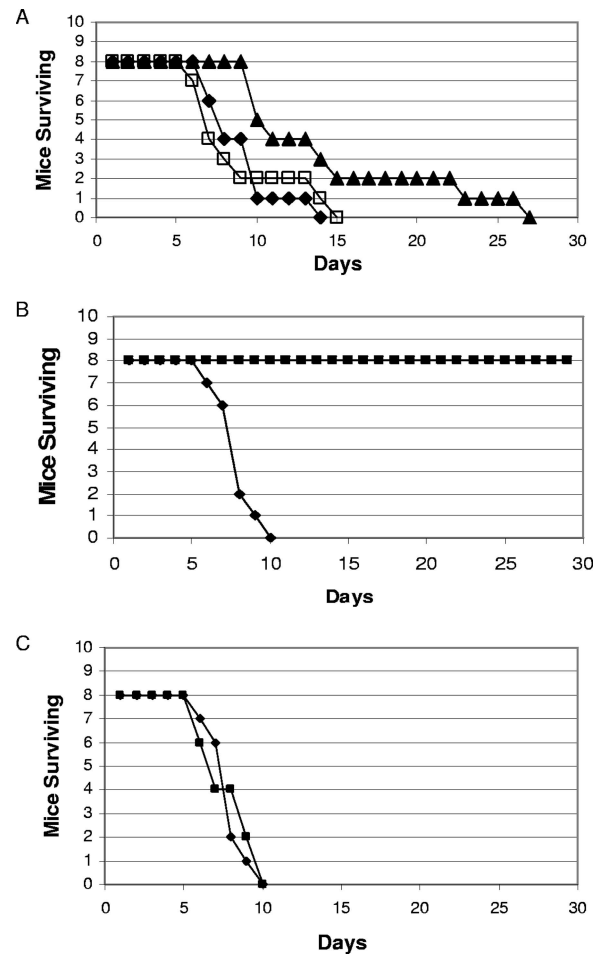


FIG. 2. Survival of BALB/c mice infected orally with  $10^8$  CFU of wild-type SR-11 ( $\blacklozenge$ ), SR-11  $\Delta$ *frdABCD* ( $\square$ ), or SR-11  $\Delta$ *sdhCDA* ( $\blacktriangle$ ) (A); either wild-type SR-11 ( $\blacklozenge$ ) or SR-11  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA::cat* ( $\blacksquare$ ) (B); and either wild-type SR-11 ( $\blacklozenge$ ) or the “restored” SR-11  $\Delta$ *frdABCD* mutant ( $\blacksquare$ ) (C).

contrast, the SR-11  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA::cat* double mutant was avirulent (*P* < 0.0001) (Fig. 2B). The SR-11  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA::cat* double mutant has a chloramphenicol cassette replacing the wild-type *sdhCDA* genes (Table 1). When the chloramphenicol cassette was removed as described by Datsenko and Wanner (9), the strain remained totally avirulent (data not shown), proving that the chloramphenicol cassette was not responsible for the avirulence of the SR-11  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA::cat* double mutant.

The SR-11  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA::cat* double mutant was constructed by deleting *sdhCDA* from the SR-11  $\Delta$ *frdABCD* mutant. To prove conclusively that the  $\Delta$ *sdhCDA* deletion was responsible for the avirulence of the SR-11  $\Delta$ *frdABCD*  $\Delta$ *sdhCDA::cat* double mutant, the wild-type *sdhCDA* genes were reinserted into the double mutant to regenerate the SR-11  $\Delta$ *frdABCD* mutant (see Materials and Methods). The “restored” SR-11  $\Delta$ *frdABCD* mutant grew with succinate as the sole source of carbon and energy at the same rate as the original SR-11  $\Delta$ *frdABCD* mutant (not shown) and was fully virulent (*P* = 0.89) (Fig. 2C). Collectively, these data suggest that in BALB/c mice, fumarate reductase can take over for

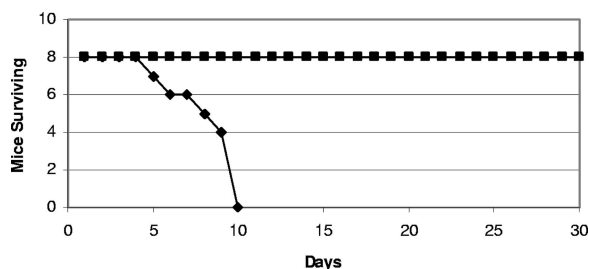


FIG. 3. Survival of BALB/c mice initially sham infected orally and infected 30 days later with  $10^8$  CFU of wild-type SR-11 (◆) or initially infected orally with  $10^8$  CFU of SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  and infected 30 days later with  $10^8$  CFU of wild-type SR-11 (■).

succinate dehydrogenase in the SR-11  $\Delta sdhCDA$  mutant to convert succinate to fumarate and thereby run a full TCA cycle with only a minor reduction in virulence. In addition, the data suggest that the conversion of succinate to fumarate is required for SR-11 virulence in BALB/c mice.

**The SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  double mutant is immunogenic.** Not all avirulent serovar Typhimurium mutants protect BALB/c mice against subsequent infection with the wild-type strain. For example, the SR-11  $\Delta sucAB$  mutant is avirulent (28), but when mice were orally challenged with  $10^8$  CFU of the wild-type SR-11 strain 30 days after being fed  $10^8$  CFU of the SR-11  $\Delta sucAB$  mutant, all of the mice died within 12 days (M. Tchawa Yinga, unpublished data). In contrast, when BALB/c mice originally fed  $10^8$  CFU of the SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  mutant were orally challenged with  $10^8$  CFU of the wild-type SR-11 strain 30 days later, they never appeared ill and remained healthy throughout the ensuing 30 days postchallenge (Fig. 3). However, sham-infected mice orally challenged with  $10^8$  CFU of the wild-type SR-11 strain 30 days after sham infection all died within 10 days (Fig. 3). Therefore, the SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  double mutant was fully protective against a challenge with wild-type SR-11 ( $P = 0.0001$ ).

**The SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  double mutant is found in numbers equal to those of wild-type SR-11 in Peyer's patches.** After ingestion, serovar Typhimurium survives passage through the acidic environment of the stomach and reaches the terminal ileum, where it invades M cells in the Peyer's patches (15). Serovar Typhimurium then gains access to both adjacent enterocytes and underlying lymphoid cells in the mesenteric lymph follicles of the Peyer's patches (15, 16). Since growth initially takes place in the Peyer's patches (14) and the SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  double mutant was avirulent via the peroral route, experiments were performed to determine whether it had a defect in growth or survival in Peyer's patches. As shown in Fig. 4, the numbers of CFU of the SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  double mutant in Peyer's patches were essentially identical to those of the wild-type SR-11 strain throughout the 6-day duration of the experiment. The same experiment was performed using the SR-11  $\Delta sucCD$  mutant, which is moderately attenuated; i.e., the death of BALB/c mice fed the SR-11  $\Delta sucCD$  mutant is delayed 10 days relative to that of BALB/c mice fed wild-type SR-11 (28). The numbers of CFU of the SR-11  $\Delta sucCD$  mutant in Peyer's patches were essentially identical to those of the wild-type

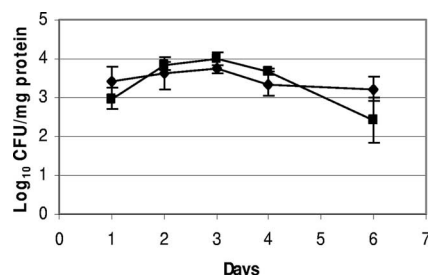


FIG. 4. Recovery of wild-type SR-11 (◆) from Peyer's patches of BALB/c mice orally infected with  $10^8$  CFU of the strain, and recovery of SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  from Peyer's patches of BALB/c mice orally infected with  $10^8$  CFU of the strain (■). Each data point for days 1 to 4 represents the  $\log_{10}$  number of CFU per mg of Peyer's patch protein (mean  $\pm$  the standard error) for four animals. The day 6 data points are derived from two animals.

SR-11 strain throughout the 6-day duration of the experiment (not shown).

**The SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  double mutant does not grow well in the liver and spleen relative to the growth of wild-type SR-11.** As serovar Typhimurium grows in Peyer's patches, it simultaneously disseminates systemically in macrophages to the liver and spleen, where it continues to grow (25, 26). Experiments were therefore performed to determine the rate of appearance of SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  in the liver and spleen relative to that of wild-type SR-11. As shown in Fig. 5A and B, the SR-11 wild-type strain appeared in the liver and spleen a day before SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  and by day 6 was, in numbers, about 5 orders of magnitude higher than in the liver (Fig. 5A) and about 6 orders of magnitude higher than in the spleen (Fig. 5B). The same experiment was performed using the moderately attenuated SR-11  $\Delta sucCD$  mutant (28). The numbers of the SR-11  $\Delta sucCD$  mutant in the liver and spleen were essentially identical to those of the wild-type SR-11 strain throughout the 6-day duration of the experiment (not shown). While these data tell us nothing about why the SR-11  $\Delta sucCD$  mutant is moderately attenuated, they do suggest that the moderate attenuation has nothing to do with early systemic dissemination.

**The SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  double mutant is highly attenuated via the i.p. route.** As stated above, when mice are infected via the oral route, serovar Typhimurium initially enters M cells, then enters lymphoid cells of the Peyer's patches (14, 15), and finally resides and grows in macrophages in the liver and spleen (16, 25). In contrast, infection via the i.p. route results exclusively in systemic exposure in macrophages. An i.p. infection with as few as 40 to 50 CFU of wild-type SR-11 can be fatal to BALB/c mice (8, 29). Of the eight BALB/c mice infected i.p. with  $10^3$  CFU of the SR-11  $\Delta frdABCD \Delta sdhCDA$  double mutant (about 25 times the wild-type  $LD_{50}$  [8]), six mice survived the 30 days of the experiment (Fig. 6A), whereas of eight BALB/c mice infected with  $10^4$  CFU of the SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  double mutant (about 250 times the wild-type  $LD_{50}$  [8]), only three survived the entire 30 days (Fig. 6B). However, of 16 BALB/c mice infected i.p. with the SR-11 wild-type strain (8 mice with  $10^3$  CFU and 8 mice with  $10^4$  CFU), all 16 were dead within 5 days postinfection (Fig. 6A and B). Of the surviving mice infected with the SR-11  $\Delta frdABCD$

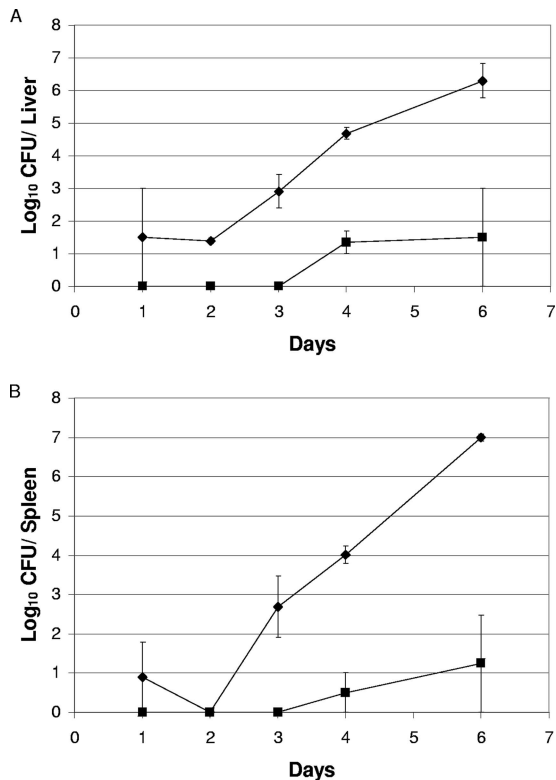


FIG. 5. Recovery of wild-type SR-11 (◆) from the livers (A) and spleens (B) of BALB/c mice orally infected with  $10^8$  CFU of the strain and recovery of SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  (■) from the livers (A) and spleens (B) of BALB/c mice orally infected with  $10^8$  CFU of the strain. Each data point represents the log<sub>10</sub> mean number of CFU per organ (mean  $\pm$  the standard error) for four animals.

$\Delta sdhCDA::cat$  double mutant, all had ruffled fur for about 3 weeks, but by the end of the experiment, they looked completely healthy. Therefore, although not completely avirulent via the i.p. route, the SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  double mutant was highly attenuated when mice were inoculated with either  $10^3$  CFU ( $P = 0.0001$ ) or  $10^4$  CFU ( $P < 0.0001$ ).

**SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  grows normally in M9 minimal medium containing glucose.** The fact that the SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  double mutant is avirulent supports the previous report (28) suggesting that full SR-11 virulence requires the TCA cycle to operate as a complete cycle. It might be argued, however, that the SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  double mutant has a general growth defect that would be observed even if the TCA cycle were not running as a complete cycle. It was therefore of interest to examine the growth rates of wild-type SR-11 and the SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  double mutant during aerobic growth with glucose (0.4%, wt/wt) as the sole source of carbon and energy, a condition in which the TCA cycle operates in the oxidative- and reductive-branch mode (7, 21), requiring neither succinate dehydrogenase nor  $\alpha$ -ketoglutarate dehydrogenase (5, 7). Each strain grew identically, with generation times of about 70 min (not shown). Therefore, the avirulence of the SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  double mutant does not appear to be due to a general growth defect but does appear to be due to the inability of the mutant to run a full TCA cycle.

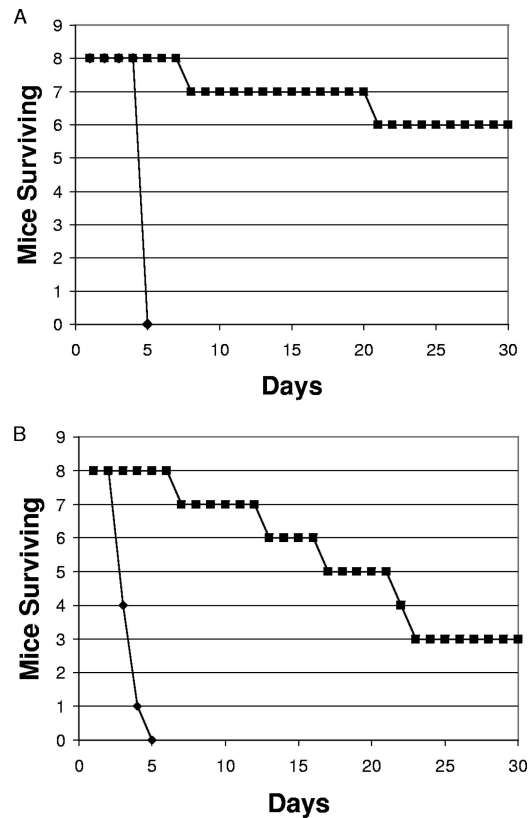


FIG. 6. Survival of BALB/c mice infected intraperitoneally with  $10^3$  CFU (A) or  $10^4$  CFU (B) of wild-type SR-11 (◆) or SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  (■).

## DISCUSSION

In a previous report, we showed that an SR-11  $\Delta frdABCD$  mutant, unable to make fumarate reductase, was completely virulent in BALB/c mice and that an SR-11  $\Delta sdhCDA$  mutant, unable to make succinate dehydrogenase, was only slightly attenuated (28). In the present study, evidence is presented that an SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  double mutant is avirulent. Fumarate reductase and succinate dehydrogenase are physiologically reversible isoenzymes which are induced under anaerobic and aerobic conditions, respectively (6, 13). Our data suggest that fumarate reductase, which normally runs in the reductive pathway in the opposite direction of succinate dehydrogenase for branched TCA cycle operation, takes over for succinate dehydrogenase in the SR-11  $\Delta sdhCDA$  mutant during infection to run a full TCA cycle with only a slight reduction in virulence (Fig. 2A). In addition, it appears that the conversion of succinate to fumarate is key to the virulence of serovar Typhimurium.

At the present time, we do not know why blocking the conversion of succinate to fumarate results in avirulence but blocking the conversion of succinyl-CoA to succinate, which precedes the conversion of succinate to fumarate in the TCA cycle (Fig. 1), results in only moderate attenuation (28). We do know, however, that an SR-11  $\Delta mdh$  mutant, which is unable to make malate dehydrogenase and therefore cannot convert malate to oxaloacetate (30), is highly attenuated (28) and that

an SR-11  $\Delta sfcA \Delta maeB$  mutant, unable to make the “malic” enzymes for the conversion of malate to pyruvate (2, 17), is attenuated (28). Thus, a continuous supply of malate is required not only for the generation of oxaloacetate but also for the generation of pyruvate (Fig. 1). Removing malate from the TCA cycle to make pyruvate requires that malate be replenished to keep a full TCA cycle operative. Since replenishment appears to be independent of the conversion of phosphoenolpyruvate to oxaloacetate (see Fig. 1), i.e., an SR-11  $\Delta ppc$  mutant has been shown to be fully virulent (28), it may be that malate replenishment comes via succinate present in mouse tissue or via ornithine and arginine present in mouse tissue that may be converted to succinate (24). If so, and if neither fumarate nor malate in tissue is available to replenish malate in the SR-11 TCA cycle, succinate conversion to fumarate and then to malate would be key to SR-11 virulence. This scenario also explains why the conversion of succinyl-CoA to succinate is not as key to virulence as the conversion of succinate to fumarate; i.e., in an SR-11  $\Delta sucCD$  mutant, the mouse tissue succinate would still allow some replenishment of malate in the TCA cycle. If this hypothesis is true, it would be expected that an SR-11  $\Delta fumA \Delta fumB \Delta fumC$  triple mutant, unable to convert fumarate to malate either aerobically or anaerobically (12), and an SR-11  $\Delta sfcA \Delta maeB \Delta mdh$  triple mutant, unable to generate either pyruvate or oxalacetate from malate (Fig. 1), would both be avirulent. Experiments designed to test this hypothesis are currently under way.

When fed to BALB/c mice orally, the SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  double mutant appears to have no growth defect in Peyer's patches (Fig. 4) but is delayed in reaching the liver and spleen and does not accumulate in these organs to nearly the same extent as does wild-type SR-11 (Fig. 5A and B). In fact, the data presented here for the SR-11  $\Delta frdABCD \Delta sdhCDA$  double mutant are reminiscent of those reported for an SR-11  $\Delta cya \Delta crp$  mutant that was unable to make both adenylyl cyclase and the cAMP receptor protein (8); i.e., in both cases, the strains are avirulent via the peroral route, have no apparent defect in invading or persisting in Peyer's patches, are impaired in their ability to reach and grow in the liver and spleen, are severely attenuated but not avirulent via the i.p. route of infection, and are immunogenic.

While the reason that the SR-11  $\Delta frdABCD \Delta sdhCDA::cat$  double mutant is avirulent is not fully explained by our experiments, it is clear that this strain effectively protects BALB/c mice against subsequent infection with wild-type SR-11. In this context, it will be of great interest to determine whether  $\Delta frdABCD \Delta sdhCDA$  mutants of intracellular bacterial pathogens other than *S. enterica* serovar Typhimurium that cannot convert succinate to fumarate are avirulent and whether they can also protect animals against infection by their virulent parents. If so, it may be that *S. enterica*  $\Delta frdABCD \Delta sdhCDA$  double mutants and  $\Delta frdABCD \Delta sdhCDA$  double mutants of other intracellular bacterial pathogens with complete TCA cycles can serve as effective live vaccine strains for both animals and humans. Moreover, serovar Typhimurium  $\Delta frdABCD \Delta sdhCDA$  double mutants might be effective as vehicles for genes that express virulence antigens of other pathogens to induce protective immunity against those pathogens.

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