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Role of Gluconeogenesis and the Tricarboxylic Acid Cycle in the Virulence of *Salmonella enterica* Serovar Typhimurium in BALB/c Mice

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In *Salmonella enterica* serovar Typhimurium, the Cra protein (*c*atabolite *r*epressor/*a*ctivator) regulates utilization of gluconeogenic carbon sources by activating transcription of genes in the gluconeogenic pathway, the glyoxylate bypass, the tricarboxylic acid (TCA) cycle, and electron transport and repressing genes encoding glycolytic enzymes. A serovar Typhimurium SR-11 Δ*cra* mutant was recently reported to be avirulent in BALB/c mice via the peroral route, suggesting that gluconeogenesis may be required for virulence. In the present study, specific SR-11 genes in the gluconeogenic pathway were deleted (*fbp*, *glpX*, *ppsA*, and *pckA*), and the mutants were tested for virulence in BALB/c mice. The data show that SR-11 does not require gluconeogenesis to retain full virulence and suggest that as yet unidentified sugars are utilized by SR-11 for growth during infection of BALB/c mice. The data also suggest that the TCA cycle operates as a full cycle, i.e., a *sucCD* mutant, which prevents the conversion of succinyl coenzyme A to succinate, and an Δ*sdhCDA* mutant, which blocks the conversion of succinate to fumarate, were both attenuated, whereas both an SR-11 Δ*aspA* mutant and an SR-11 Δ*frdABC* mutant, deficient in the ability to run the reductive branch of the TCA cycle, were fully virulent. Moreover, although it appears that SR-11 replenishes TCA cycle intermediates from substrates present in mouse tissues, fatty acid degradation and the glyoxylate bypass are not required, since an SR-11 Δ*fadD* mutant and an SR-11 Δ*aceA* mutant were both fully virulent.

In sensitive mice, *Salmonella enterica* serovar Typhimurium causes a systemic, often fatal disease, similar to human typhoid fever (43). After ingestion, serovar Typhimurium survives passage through the acidic environment of the stomach and reaches the terminal ileum where, within 30 min, it invades M cells in the Peyer's patches (22). Within 60 min, the M cells are destroyed and serovar Typhimurium gains access to both adjacent enterocytes and to underlying lymphoid cells in the mesenteric lymph follicles of the Peyer's patches (22, 24). Serovar Typhimurium grows logarithmically in Peyer's patches for 2 days (20) and simultaneously disseminates systemically to the liver and spleen, where it grows in macrophages (40, 43). Mice usually become ruffled and lethargic 3 to 5 days post-oral infection and usually die within 7 to 12 days. Despite much work relating to the relevant virulence mechanisms, little is known about the nutrition of serovar Typhimurium during infection.

The Cra protein (*c*atabolite *r*epressor/*a*ctivator) is a regulator of central carbon metabolism in salmonellae and *Escherichia coli*. Cra is a transcription factor that activates genes encoding key enzymes in the glyoxylate bypass, gluconeogenesis, the tricarboxylic acid (TCA) cycle, and electron transport and represses genes encoding key enzymes in the Embden-Meyerhof and Entner-Doudoroff pathways (42). It has recently been reported that a *cra* mutant of serovar Typhimurium SR-11 is totally avirulent and immunogenic in BALB/c mice when administered orally but remains fully virulent when administered intraperitoneally (1, 48). These findings suggested the possibility that while crossing the intestine, the ability to utilize gluconeogenic substrates may be required for serovar Typhimurium SR-11 virulence. In support of this view, Valentine and coworkers (49) reported that a MudJ insertion in a serovar Typhimurium NAD-linked malate oxidoreductase rendered it avirulent via the oral route in BALB/c mice. In the present study, the roles of gluconeogenesis and the TCA cycle in the virulence of serovar Typhimurium strain SR-11 were investigated systematically.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. Bacterial strains and plasmids used in this study are listed in Table 1. Luria Bertani (LB) broth (Fischer Biotech, Fair Lawn, New Jersey), LB agar, Lennox (Difco Laboratories, Detroit, Michigan), and MacConkey agar (Difco) were prepared according to package instructions. Liquid M9 minimal salts medium (31) was supplemented either with reagent grade glucose (0.2% wt/vol), glycerol (0.2% wt/vol), malic acid (0.4% wt/vol), sodium citrate (0.4% wt/vol), and sodium oleate (5 mM) plus 5 mg of Brij 58/ml, sodium pyruvate (0.4% wt/vol), sodium succinate (0.4% wt/vol), or potassium acetate (0.4% wt/vol) as 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). Bacterial strains were grown for 24 h at 37°C in liquid culture media or overnight on LB agar plates. Liquid cultures were supplemented with nalidixic acid (50 μg/ml), and agar plates were supplemented with nalidixic acid (50 μg/ml), chloramphenicol (30 μg/ml), ampicillin (100 μg/ml), or kanamycin (40 μg/ml) where appropriate.

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

Strain or plasmid	Genotype[a] and defect(s) or relevant characteristics	Source or reference
SR-11 strains		
Wild type	*gyr1816*; none	8
Fad$^-$ AX-2	*gyr1816 cra*::Tn*10 d cam*; Cra repressor/activator protein	1
Δ*glpX*	*gyr1816* Δ*glpX*::*cat*; *glpX*-encoded fructose-1,6-bisphosphatase	This study
Δ*fbp*	*gyr1816* Δ*fbp*::*cat*; *fbp*-encoded fructose-1,6-bisphosphatase	This study
Δ*glpX* Δ*fbp*	*gyr1816* Δ*glpX* Δ*fbp*::*cat*; *glpX*-and *fbp*-encoded fructose-1,6-bisphosphatases	This study
Δ*pckA*	*gyr1816* Δ*pckA*::*cat*; PEP carboxykinase	This study
Δ*ppsA*	*gyr1816* Δ*ppsA*::*cat*; PEP synthase	This study
Δ*ppsA* Δ*pckA*	*gyr1816* Δ*ppsA* Δ*pckA*::*cat*; PEP caboxykinase, PEP synthase	This study
Δ*acs*	*gyr1816* Δ*acs*; acetate synthase	This study
Δ*fadD*	*gyr1816* Δ*fadD*::*cat*; acyl-CoA synthase	This study
Δ*aspA*	*gyr1816* Δ*aspA*; aspartase	This study
Δ*sfcA*	*gyr1816* Δ*sfcA*::*kan*; malate oxidoreductase (NAD dependent)	This study
Δ*maeB*	*gyr1816* Δ*maeB*::*kan*; malate oxidoreductase (NADP dependent)	This study
Δ*sfcA* Δ*maeB*	*gyr1816* Δ*maeB* Δ*sfcA*::*kan*; both malate oxidoreductases	This study
Δ*aceA*	*gyr1816* Δ*aceA*; isocitrate lyase	This study
Δ*ppc*	*gyr1816* Δ*ppc*; PEP carboxylase	This study
Δ*sucAB*	*gyr1816* Δ*sucAB*; α-ketoglutarate dehydrogenase	This study
Δ*sucCD*	*gyr1816* Δ*sucCD*; succinyl-CoA synthetase	This study
Δ*sdhCDA*	*gyr1816* Δ*sdhCDA*; succinate dehydrogenase	This study
Δ*frdABCD*	*gyr1816* Δ*frdABCD*; fumarate reductase	This study
Δ*mdh*	*gyr1816* Δ*mdh*; malate dehydrogenase	This study
Δ*kgtP*	*gyr1816* Δ*kgtP*; α-ketoglutarate transporter	This study
Δ*dctA*	*gyr1816* Δ*dctA*; dicarboxylate transporter	This study
Δ*kgtP* Δ*dctA*	*gyr1816* Δ*kgtP* Δ*dctA*; dicarboxylate transporter, α-ketoglutarate transporter	This study
Plasmids		
pKD3	Template plasmid, contains chloramphenicol resistance cassette flanked by FLP recombinase target sites; *bla cat*	9
pKD4	Template plasmid, contains kanamycin resistance cassette flanked by FLP recombinase target sites; *bla kan*	9
pKD46	Temperature-sensitive plasmid, contains arabinose-inducible phage λ red recombinase gene for linear DNA exchange; *bla*	9
pCP20	Temperature-sensitive plasmid, contains FLP recombinase gene for removal of antibiotic resistance cassettes; *bla cat*	9

[a] All mutants were constructed using either the chloramphenicol (*cat*) or kanamycin (*kan*) resistance cassette as described by Datsenko and Wanner (9). The resistance cassettes were removed from the deletion mutants lacking the ::*cat* or ::*kan* designation, as described by Datsenko and Wanner (9).

Construction and characterization of serovar Typhimurium SR-11 deletion mutants. Mutant strains of SR-11 were created by deletion mutagenesis using either a chloramphenicol cassette or a kanamycin cassette as described by Datsenko and Wanner (9). All constructs were verified by PCR and sequencing. The specific primers used to construct the deletions and to confirm the allelic exchange were designed for each of the mutants by referring to the complete genome of *Salmonella enterica* serovar Typhimurium LT2 (28) and are listed in Table 2. In instances where the chloramphenicol cassette or the kanamycin cassette might cause downstream polar effects, the cassettes were removed as described by Datsenko and Wanner (9). For sequencing, PCR products were

TABLE 2. Sequences of primers used to confirm serovar Typhimurium mutants

Gene	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
glpX	CGTATCATGCTCAACCAG	TCCGGATCTTTACGATCA
fbp	TACGGTTATTGGTAGGGAGAGC	GTTGTGCATGTTATGTTCGCCG
pckA	CGTAAATCTATGAGCCTTGTCG	TGGCACCAGCGAGCGTGGCG
ppsA	CAGGGAATGCCGTGCTATGC	TGACGCTGGAAGAGGTGATC
Acs	ATTGCGGATCGTTGCCTG	ACGCCAGGATCGGCGAGA
fadD	GCTGATGTTCCGGCGGAG	CACGGGCTTCGTCACGCA
aspA	TCCAGCTGAAGCCTACTA	CCAGCACAACTTCACGTA
sfcA	TCAGTGATGAATATTAAACCAACAGG	TGCACAATTTTAGCCGCATCTTCCG
maeB	CCGGTTAGCGCGAGGATTTGC	CACCGACTATTCTTTGTATTACTACC
aceA	GTACTCAACAAATCGAAG	ACGCGGTAACGGATGACG
ppc	CATCAACGCCTGTTCGAC	ATGACGAGCTGCTGCCAG
sucAB	TGGACAGACTGGACGAAC	TGTGCTGCAGGTTCAACA
sucCD	GTACTACTCCGCGTGAAG	AGGTGGCCAACCATGTCG
sdhCDA	CAGGTAGATTCACCTCTG	GTGAGCGTTGGTGGTGGA
frdABCD	ACGGTGACTACCGCAGTG	GCTGCGTGATATGGAATTCGT
mdh	CGATAAGACGTGAGGAGT	CAGCCGATCCGGATTACG
kgtP	CGACAGCGCGACATATTC	GAGATGCGGCGACGCATA
dctA	CTGACAGCAATCGCCATT	TAAGCACATCGTCCAGCT

purified with a QIAGEN Qiaquick PCR purification kit (QIAGEN, Maryland) 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 dye terminator cycle sequencing quick start master mix (Beckman Coulter, Fullerton, California). The thermal cycling program contained 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 completion of the cycle sequencing, samples were purified by ethanol precipitation and separated by polyacrylamide gel electrophoresis on a CEQ 8000 genetic analysis system (Beckman Coulter).

Growth of mutants on various carbon sources. Wild-type serovar Typhimurium SR-11 and its deletion mutants were grown overnight in LB at 37°C with shaking to about 10^9 CFU/ml. Cultures were washed twice with M9 minimal salts medium (31) lacking a carbon source and were resuspended in the same medium at about 10^9 CFU/ml. The washed cultures were diluted to about 10^5 CFU/ml in M9 minimal salts medium supplemented with one of the carbon sources listed above. Cultures were grown for 24 h at 37°C with shaking. Growth was assessed visually by the presence or absence of turbidity.

The generation times of selected attenuated and nonattenuated mutants in M9 minimal salts medium containing reagent grade glucose (0.2% wt/vol) were determined. Inocula were prepared as follows. Overnight cultures (10 ml) in LB were started from single colonies on Luria agar plates and were incubated at 37°C with shaking in 125-ml tissue culture bottles. The next morning, the LB cultures were washed twice in M9 minimal medium (no carbon source), and 10 μl of the washed cultures was transferred to M9 minimal glucose (0.2% wt/vol) medium and then incubated overnight at 37°C with shaking in 125-ml tissue culture bottles. The next morning, each culture was diluted to an A_{600} reading of about 0.08 into fresh M9 medium (10 ml) containing glucose (0.2% wt/vol) and supplements, as required (see text). The cultures were incubated at 37°C with shaking in 125-ml tissue culture bottles. Growth was monitored spectrophotometrically (A_{600}) using a Pharmacia Biotech Ultrospec 2000 UV/visible spectrophotometer. Generation times were determined from three independent experiments with each strain from semilogarithmic plots of the growth data ($\log_{10} A_{600}$ versus time).

Virulence assays. Virulence assays were carried out as described previously (1). Four-week-old, 13- to 15-g female BALB/c mice (Charles River Laboratories, Wilmington, MA) were housed no more than 4 per cage, with pine shavings as bedding. Prior to infection, the mice were starved for food and water for 4 h. Following starvation, 50 μl of 10% sodium bicarbonate was administered orally to each mouse to neutralize gastric acidity, and 30 min later, 10^8 CFU of a specific strain in 20 μl of phosphate-buffered saline (pH 7.4) containing 0.1% gelatin was administered orally to each of 4 mice. For virulence assays via the intraperitoneal route, 10^3 CFU of a specific strain in 100 μl of phosphate-buffered saline (pH 7.4) containing 0.1% gelatin were injected by sterile syringe into each of 4 mice. The number of CFU administered to mice was measured by diluting and then plating bacterial suspensions onto MacConkey agar containing nalidixic acid (50 μg/ml). After infection, food and water were returned and the mice were inspected four times daily for signs of illness and death. The virulence data presented here for each strain are a composite of the results from at least two independent experiments in which 4 mice were infected with a specific mutant and 4 additional mice were infected with the SR-11 wild-type strain. Where noted in the figures, data from a composite of three independent experiments are presented.

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. Where indicated in the text, the generations times derived from triplicate samples of specific SR-11 mutants were compared to the generation time derived from triplicate samples of the SR-11 wild-type by Student's t test. Generation times were considered to be different if the P value was less than 0.05.

RESULTS

Definition of fully virulent, attenuated, and avirulent (10^8 CFU/mouse, peroral route). In addition to infecting 4 mice orally with 10^8 CFU of the wild-type SR-11 in each virulence experiment, 4 mice were infected orally with 10^8 CFU of the SR-11 *cra* mutant (Table 1). All of the mice infected with the SR-11 *cra* mutant in all experiments remained healthy throughout the duration of the experiment, which is our definition of avirulent. The term fully virulent is used when the survival curve of mice infected with a particular mutant is not statistically different from that of mice infected with the wild-type SR-11 strain. The term attenuated is used when the survival curve of mice infected with a particular mutant 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 terms slightly attenuated, attenuated, or highly attenuated, which will become clear after viewing the figures.

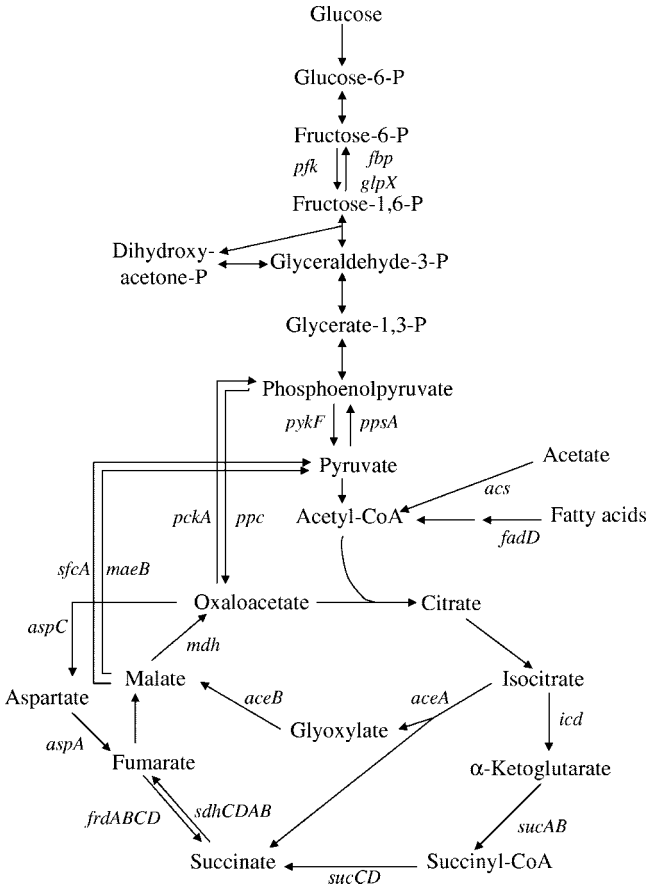

FIG. 1. Embden Meyerhoff pathway, gluconeogenic pathway, and TCA cycle. Arrows indicate the physiological directions of the reactions. Genes encoding the enzymes for each reaction are listed beside each reaction.

Gluconeogenesis is not required for full SR-11 virulence (10^8 CFU/mouse, peroral route). During gluconeogenesis, the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate is catalyzed irreversibly by fructose-1,6-bisphosphatase, encoded by *fbp* (44). An *fbp* mutant is therefore unable to carry out gluconeogenesis above fructose-1,6-bisphosphate but is able to carry out glycolytic reactions normally (Fig. 1). A second fructose-1,6-bisphosphatase has been characterized in *E. coli*, encoded by *glpX* (11); however, GlpX has been shown to be functional only when overexpressed (11). A *glpX* homolog is present in serovar Typhimurium (28). An SR-11 Δ*fbp* mutant, an SR-11 Δ*glpX* mutant, and an SR-11 Δ*glpX* Δ*fbp* double mutant were constructed. As expected, the SR-11 Δ*fbp* mutant and the SR-11 Δ*glpX* Δ*fbp* double mutant grew with glucose as the sole carbon source but, in contrast to wild-type SR-11,

TABLE 3. Virulence of SR-11 mutants: oral infection (10^8/mouse)

Pathway and mutant strain	Day 50% mortality reached for:		Day 100% mortality reached for:		Kaplan-Meier *P* value[a]
	Wild type	Mutant	Wild type	Mutant	
Gluconeogenesis					
Δ*fbp*	6.5	5.5	9	8	0.35
Δ*glpX*	6.5	6	9	10	0.98
Δ*fbp* Δ*glpX*	7	8	9	16	0.50
Δ*pckA*	7	8	11	11	0.14
Δ*ppsA*	8.5	8	12	13	0.84
Δ*pckA* Δ*ppsA*	8	10	11	18	**0.02**
Malate to pyruvate					
Δ*maeB*	7.5	6	13	9	0.079
Δ*sfcA*	7.5	8	13	12	0.77
Δ*sfcA* Δ*maeB*	6.5	11	9	19	**0.0001**
TCA cycle					
Δ*sucAB*	9	>32	10	>32	**0.0001**
Δ*sucCD*	8	18	14	>32	**0.0068**
Δ*sdhCDA*	8	11	14	>22	0.15
Δ*sdhCDA* (10^6 CFU)	9	13	14	>22	**0.0051**
Δ*sdhCDA* composite (16 mice)	8.5	12	14	>22	**0.0026**
Δ*mdh* (12 mice)	7.5	20	14	>34	**<0.0001**
TCA cycle (reductive branch)					
Δ*aspA*	7	8	11	12	0.20
Δ*frdABCD*	8	7	14	15	0.98
Acetate, fatty acids, glyoxylate bypass					
Δ*aceA*	8	8	9	11	0.49
Δ*acs*	8	8	9	17	0.69
Δ*fadD*	8	9	9	11	0.65
Anapleurotic					
Δ*ppc*	7.5	7.5	14	12	0.93
Δ*dctA*	7.5	7.5	13	11	0.71
Δ*kgtP*	5.5	6.5	13	9	0.26
Δ*dctA* Δ*kgtP*	6.5	8.5	13	9	0.98

[a] *P* values denoting statistical differences between the survival curves of mice infected with the SR-11 wild type and a specific mutant are shown in boldface type.

failed to grow on glycerol and gluconeogenic substrates (acetate, citrate, malate, succinate, oleate, and pyruvate) as sole carbon and energy sources (not shown). However, the SR-11 Δ*glpX* mutant ($P = 0.98$), the SR-11 Δ*fbp* mutant ($P = 0.35$), and the SR-11 Δ*glpX* Δ*fbp* double mutant ($P = 0.50$) were each as virulent as the wild-type SR-11 (Table 3), suggesting that as yet unidentified sugars that feed into the glycolytic/gluconeogenic pathway at or above fructose-6-phosphate are utilized for growth during infection.

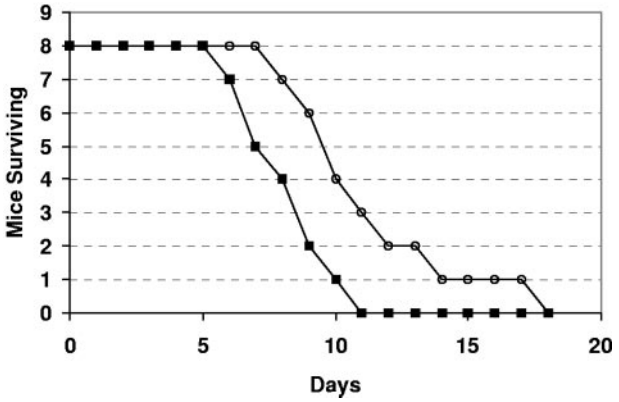

FIG. 2. Survival of BALB/c mice infected orally with 10^8 CFU of either wild-type SR-11 (■) or the SR-11 Δ*ppsA* Δ*pckA* double mutant (○).

Although the SR-11 Δ*glpX* Δ*fbp* double mutant cannot complete gluconeogenesis above fructose-1,6-bisphosphate, it can still make all the intermediates from pyruvate to fructose-1,6-bisphosphate from gluconeogenic substrates (Fig. 1). Gluconeogenesis can be prevented above pyruvate by blocking the production of phosphoenolpyruvate (PEP) from both oxaloacetate via phosphoenolpyruvate carboxykinase, encoded by the *pckA* gene (29), and from pyruvate via phosphoenolpyruvate synthase, encoded by the *ppsA* gene (33) (Fig. 1). An SR-11 Δ*ppsA* mutant, an SR-11 Δ*pckA* mutant, and an SR-11 Δ*ppsA* Δ*pckA* double mutant were constructed. As expected, the SR-11 Δ*ppsA* Δ*pckA* double mutant grew with glucose as the sole carbon and energy source (Table 4) but was unable to utilize gluconeogenic substrates for growth (not shown). The SR-11 Δ*ppsA* mutant ($P = 0.84$) and the SR-11 Δ*pckA* mutant ($P = 0.14$) were fully virulent (Table 3); however, the SR-11 Δ*ppsA* Δ*pckA* mutant ($P = 0.02$) was found to be slightly attenuated, i.e., death was delayed by about 2 days (Fig. 2). Since the SR-11 Δ*glpX* Δ*fbp* double mutant is fully virulent and the SR-11 Δ*ppsA* Δ*pckA* double mutant appears to be only slightly attenuated, gluconeogenesis plays, at most, a minor role in SR-11 virulence, i.e., the avirulence of the SR-11 *cra* mutant can't be explained by its inability to perform glucone-

TABLE 4. Generation times of serovar Typhimurium SR-11 mutants growing aerobically in M9 minimal medium containing glucose (0.2% wt/vol)

Growth medium	Generation time (min) ± SD ($n = 3$) for:							
	Wild type	Δ*mdh*	Δ*sdhCDA*	Δ*sucCD*	Δ*ppsA* Δ*pckA*	Δ*sfcA* Δ*maeB*	Δ*sucAB*	Δ*ppc*
M9G[a]	79 ± 4	95 ± 5	81 ± 4	80 ± 6	79 ± 6	84 ± 5	NG[d]	NG
M9GAK[b]	64 ± 2	ND[e]	ND	ND	ND	ND	ND	92 ± 2
M9GDLM[c]	69 ± 2	ND	ND	ND	ND	ND	72 ± 4	ND

[a] M9G, M9 minimal medium containing glucose (0.2% wt/vol).
[b] M9GAK, M9G supplemented with 0.01% aspartate and 0.01% α-kletoglutarate.
[c] M9GDLM, M9G containing diaminopimelate (40 μg/ml), lysine (50 μg/ml), and methionine (50 μg/ml).
[d] NG, no growth.
[e] ND, not done.

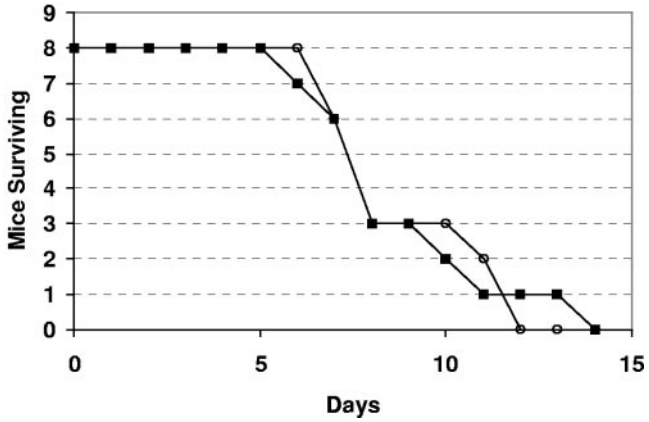

FIG. 3. Survival of BALB/c mice infected orally with 10^8 CFU of either wild-type SR-11 (■) or the SR-11 Δ*ppc* mutant (○).

ogenesis. Moreover, since the SR-11 Δ*ppsA* Δ*pckA* double mutant is unable to make any gluconeogenic intermediates above pyruvate, it appears that the majority of metabolic intermediates from fructose-1,6-bisphosphate to PEP required for full virulence can be produced glycolytically.

Phosphoenolpyruvate carboxylase is not required for full SR-11 virulence (10^8 CFU/mouse, peroral route). Phosphoenolpyruvate carboxylase (Ppc), which converts PEP to oxaloacetate (41), replenishes the TCA cycle when precursor metabolites are withdrawn for biosynthesis under conditions where a high level of sugar is available as the sole carbon and energy source (47). Since SR-11 appeared to obtain the majority of its PEP glycolytically in vivo, it was of interest to examine the role of Ppc in SR-11 virulence. An SR-11Δ*ppc* mutant was constructed and, as reported previously for serovar Typhimurium (47), was unable to grow using either glucose or glycerol as a sole carbon source unless cofactor amounts (0.01%) of both aspartate and α-ketoglutarate were added to the medium. Therefore, the SR-11 Δ*ppc* mutant does not appear to utilize the glyoxylate bypass as an alternative anapleurotic pathway for replenishment of oxoloacetate, as has been reported for a *ppc* mutant of *E. coli* strain BW25113 (37). As expected, the SR-11 Δ*ppc* mutant grew normally on either acetate or citrate as a sole carbon source (not shown). Despite its growth defect on sugars, the SR-11 Δ*ppc* mutant ($P = 0.93$) was fully virulent (Fig. 3), suggesting that anapleurotic formation of oxaloacetate from PEP is not required during SR-11 infection.

The reductive branch of the TCA cycle is not required for full SR-11 virulence (10^8 CFU/mouse, peroral route). During anaerobic growth in the absence of an alternative electron acceptor, the TCA cycle does not function as a full cycle but as an oxidative branch which runs from citrate to α-ketoglutarate and a reductive branch which runs backwards from oxaloacetate to succinyl coenzyme A (succinyl-CoA) (Fig. 1) (7, 39). Under these conditions, both branches serve biosynthetic functions and are not used for energy generation (7). The reductive branch runs in part via reversal of the direction of the TCA cycle using the enzymes of the TCA cycle and in part via aspartate aminotransferase, encoded by *aspC* (14), which generates aspartate from oxaloacetate and via aspartase, encoded by *aspA* (46), which generates fumarate from aspartate (7). Succinate is then generated from fumarate via fumarate reductase (7), encoded by the *frdABCD* genes (6), and succinyl-CoA is generated from succinate via succinyl-CoA synthetase (7), encoded by the *sucCD* genes (3). Succinyl-CoA is derived from succinate via succinyl-CoA synthetase rather than from α-ketoglutarate via α-ketoglutarate dehydrogenase (*sucAB*) because anaerobically α-ketoglutarate dehydrogenase synthesis is severely repressed relative to other TCA cycle enzymes (7, 35). Succinate dehydrogenase (*sdhCDAB*) is also severely repressed under anaerobic conditions in the absence of an alternative electron acceptor (21, 36).

To determine whether SR-11 virulence depends on the reductive branch of the TCA cycle, an SR-11 Δ*aspA* mutant and an SR-11 Δ*frdABCD* mutant were constructed and tested for virulence. Both the SR-11 Δ*aspA* mutant ($P = 0.20$) and the SR-11 Δ*frdABCD* mutant ($P = 0.98$) were fully virulent (Table 3), suggesting that the TCA cycle does not function in the branched mode during SR-11 infection. Furthermore, these data suggest that extracellular aspartate is not being used to replenish fumarate in the TCA cycle during SR-11 infection.

Role of the TCA cycle in SR-11 virulence (10^8 CFU/mouse, peroral route). Since it appeared that the branched operation of the TCA cycle was not required for full SR-11 virulence, it seemed that a complete TCA cycle might be necessary. Therefore, several TCA cycle mutants were constructed, and as described below, growth phenotypes were as in previously published reports (7, 18, 50). With the exception of the SR-11 Δ*sucAB* mutant, all the TCA cycle mutants grew with glucose as a sole carbon and energy source (Table 4). The SR-11 Δ*sucAB* mutant, unable to convert α-ketoglutarate to succinyl-CoA (Fig. 1) and therefore unable to grow on either acetate or citrate as a sole carbon and energy source (not shown), was totally avirulent ($P = 0.0001$) (Fig. 4A). In addition, the SR-11 Δ*sucCD* mutant, unable to convert succinyl-CoA to succinate and therefore unable to grow on citrate or acetate as a sole carbon source (not shown), was attenuated ($P = 0.0068$) (Fig. 4B). Furthermore, the SR-11 Δ*mdh* mutant, unable to convert malate to oxaloacetate via malate dehydrogenase (51) and therefore unable to grow on either citrate or malate as a sole carbon and energy source (not shown), was highly attenuated ($P < 0.0001$) (Fig. 4C). The SR-11 Δ*sdhCDA* mutant, unable to convert succinate to fumarate and therefore unable to grow on acetate or succinate as a sole carbon and energy source (not shown), was fully virulent statistically ($P = 0.15$) but appeared attenuated visually (Fig. 4D). Therefore, the SR-11 Δ*sdhCDA* mutant was tested for virulence at a 100-fold-lower level (10^6 CFU/mouse) and was found to be attenuated ($P = 0.0051$) (Table 3). Moreover, comparison of the SR-11 wild-type survival curve and the SR-11 Δ*sdhCDA* mutant survival curve generated from the combined data of the 10^8 CFU and 10^6 CFU virulence assays (16 mice) (Fig. 4E) also showed that the SR-11 Δ*sdhCDA* mutant was attenuated ($P = 0.0026$). Since expression of all the TCA cycle genes tested are necessary for full virulence, it appears that the complete TCA cycle runs during SR-11 infection and is necessary for full virulence.

Role of malate oxidoreductase in SR-11 virulence (10^8 CFU/mouse, peroral route). Pyruvate can be generated from PEP by the PEP-dependent, sugar-transporting phosphotransferase system (PTS), from PEP via pyruvate kinase glycolytically, or

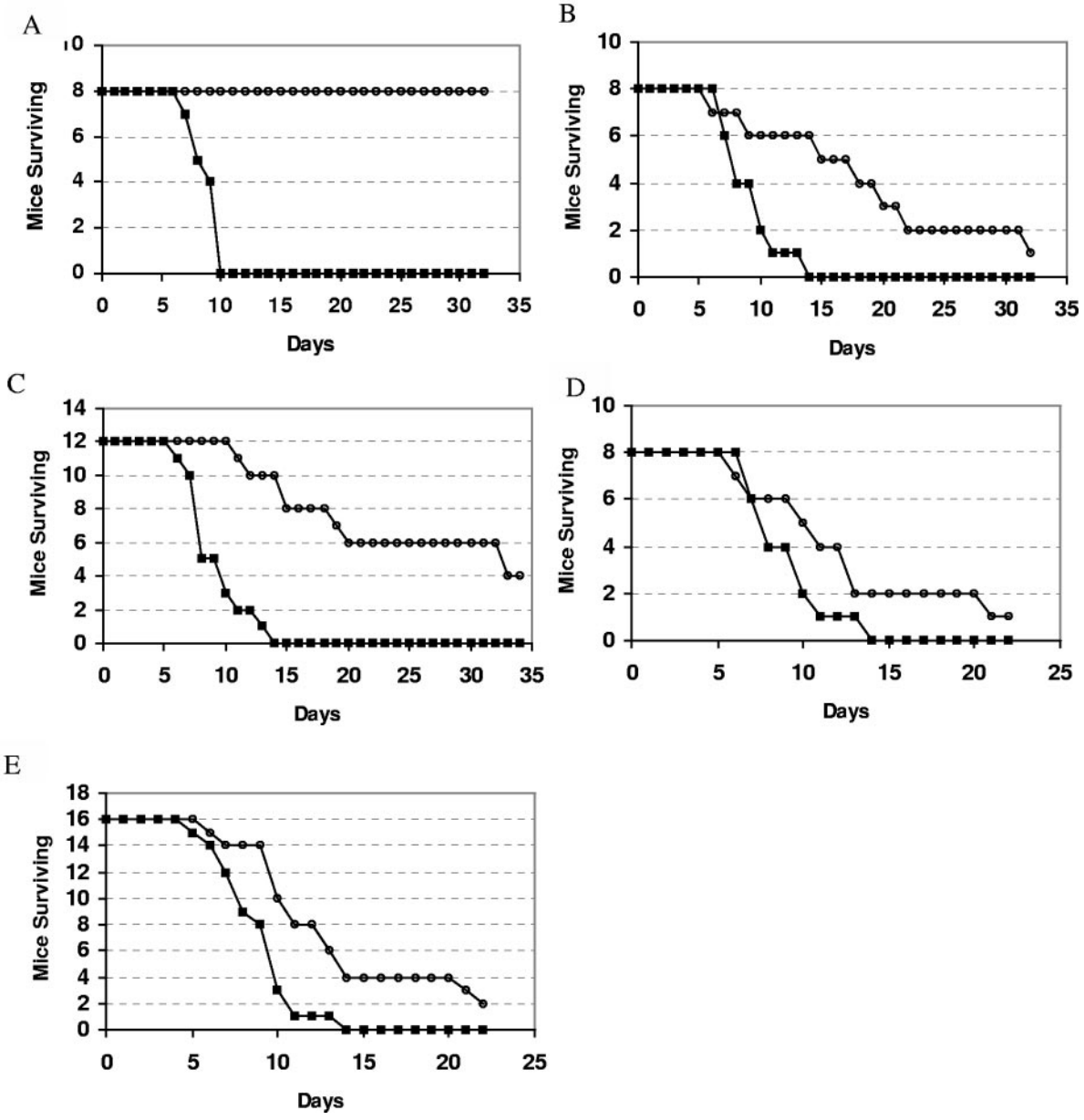

FIG. 4. Survival of BALB/c mice infected orally with 10^8 CFU of either wild-type SR-11 (■) or the SR-11 Δ*sucAB* mutant (○) (A), 10^8 CFU of either wild-type SR-11 (■) or the SR-11 Δ*sucCD* mutant (○) (B), 10^8 CFU of either wild-type SR-11 (■) or the SR-11 Δ*mdh* mutant (○) (C), or 10^8 CFU of either wild-type SR-11 (■) or the SR-11 Δ*sdhCDA* mutant (○) (D). (E) Composite of mice infected with 10^8 CFU and 10^6 CFU of either wild-type SR-11 (■) or the SR-11 Δ*sdhCDA* mutant (○).

from malate via malate oxidoreductase ("malic enzyme") (Fig. 1). Serovar Typhimurium contains an NAD-dependent malate oxidoreductase, encoded by the *sfcA* gene (26) and an NADP-dependent malate oxidoreductase, putatively encoded by the *maeB* gene (2). It has been reported that a MudJ insertion in the *sfcA* gene rendered serovar Typhimurium avirulent after oral infection in BALB/c mice (49). We were unable to confirm that an SR-11 Δ*sfcA* mutant was avirulent. In fact, in our hands, both the SR-11 Δ*sfcA* mutant ($P = 0.77$) and the SR-11 Δ*maeB* mutant ($P = 0.079$) were fully virulent (Table 3). However, although not avirulent, the SR-11 Δ*sfcA* Δ*maeB* double mutant was attenuated ($P = 0.0001$) (Fig. 5). These data suggest that a major source of pyruvate during SR-11 infection is from malate. Moreover, since it appears that pyruvate generated from malate during SR-11 infection is not used to any great extent for gluconeogenesis, it is likely that it is used, at least in part, to generate acetyl-CoA, which, in addition to being necessary for citrate formation for the TCA cycle, is the precursor of fatty acids for phospholipid biosynthesis.

The glyoxylate bypass is not required for full SR-11 pathogenesis (10^8 CFU/mouse, per-oral route). The glyoxylate bypass (Fig. 1), required for utilization of two major gluconeogenic carbon sources, acetate and fatty acids (7), has been reported to not be required for serovar Typhimurium strain

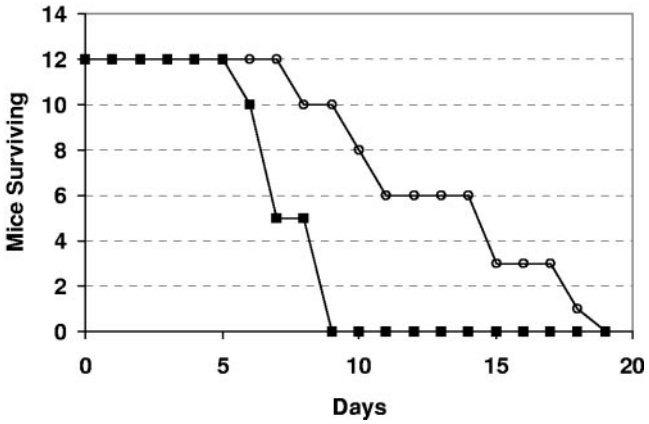

FIG. 5. Survival of BALB/c mice infected orally with 10^8 CFU of either wild-type SR-11 (■) or the SR-11 Δ*sfcA* Δ*maeB* double mutant (○).

14028s acute virulence (13). To determine whether the glyoxylate bypass contributes to SR-11 virulence, an SR-11 Δ*aceA* mutant was constructed which is unable to convert isocitrate to glyoxylate and succinate (Fig. 1) (27). As expected, the SR-11 Δ*aceA* mutant was unable to utilize either acetate or oleate as a sole source of carbon and energy (not shown) and, in agreement with Fang et al. (13), was found to be fully virulent ($P = 0.49$) (Table 3). Therefore, the glyoxylate bypass is not required for SR-11 virulence. Furthermore, an SR-11 Δ*fadD* mutant ($P = 0.65$), unable to degrade fatty acids to acetyl-CoA (Fig. 1), and an SR-11 Δ*acs* mutant ($P = 0.69$), unable to convert acetate to acetyl-CoA (Fig. 1), were unable to grow with oleate and acetate as a sole carbon and energy source, respectively (not shown), but were fully virulent (Table 3). These data suggest that neither external acetate nor fatty acids present in mouse tissues are required as sources of acetyl-CoA for running the TCA cycle during SR-11 infection.

Growth of attenuated mutants aerobically in glucose minimal medium. The evidence to this point suggested that cyclic operation of the TCA cycle is required for full SR-11 virulence in BALB/c mice. However, it might be argued that the attenuated TCA cycle mutants have general growth defects that would be observed even if the TCA cycle were not running as a full cycle. It was therefore of interest to examine growth rates of the attenuated SR-11 TCA cycle mutants during aerobic growth with glucose (0.2% wt/vol) as the sole source of carbon and energy (Table 4), a condition in which the TCA cycle operates in the oxidative and reductive branched mode (7, 32), requiring neither succinate dehydrogenase nor α-ketoglutarate dehydrogenase (5, 7). Under these conditions, the generation time of the attenuated SR-11 Δ*mdh* mutant (Table 4) was significantly greater than that of the wild-type SR-11 strain ($P < 0.02$), suggesting that malate dehydrogenase is indeed necessary for running the reductive branch of the TCA cycle as well as the full TCA cycle. Therefore, malate dehydrogenase would most likely be required for full virulence whether or not the TCA cycle was running as a full cycle in vivo. In contrast, the generation times of the attenuated SR-11 Δ*sdhCDA* mutant (Table 4) and the attenuated SR-11 Δ*sucCD* mutant (Table 4) were essentially the same as that of the wild-type SR-11 ($P > 0.10$ in each case), suggesting that the attenuation observed in vivo is in fact due to the inability of these mutants to run a full TCA cycle.

The remaining attenuated SR-11 mutants were also grown on glucose aerobically, and generation times were determined. The generation times of the slightly attenuated SR-11 Δ*ppsA* Δ*pckA* double mutant and the generation time of the attenuated SR-11 Δ*sfcA* Δ*maeB* double mutant were the same as that of the SR-11 wild-type strain ($P > 0.10$ in each case) (Table 4). These data suggest that when growing on glucose aerobically, i.e., under conditions in which gluoneogenesis driven by TCA cycle intermediates is not necessary, these mutants can achieve maximum SR-11 growth rates. It therefore appears that attenuation of the SR-11 Δ*ppsA* Δ*pckA* and SR-11 Δ*sfcA* Δ*maeB* mutants in BALB/c mice is due to defects in their inability to send TCA cycle intermediates out of the TCA cycle under in vivo conditions and not due to nonspecific growth defects.

The avirulent SR-11 Δ*sucAB* mutant does not grow on glucose aerobically unless diaminopimelic acid, lysine, and methionine are added to the growth medium (5). With these supplements, however, the SR-11 Δ*sucAB* mutant and the SR-11 wild-type strain grew with identical generation times ($P > 0.10$) (Table 4). These data suggest that the SR-11 Δ*sucAB* is probably avirulent due to the unavailability of one of the supplements in vivo, most likely diaminopimelate, which is required for succinyl-CoA synthesis. Finally, the SR-11 Δ*ppc* mutant is unable to convert PEP to oxaloacetate and, therefore, does not grow on glucose aerobically unless cofactor amounts (0.01%) of both aspartate and α-ketoglutarate are added to the medium (47). Surprisingly, when the SR-11 Δ*ppc* mutant was grown in the presence of the supplements, its generation time was still 44% greater than the wild-type SR-11 ($P < 0.001$) (Table 4), yet it was fully virulent in BALB/c mice (Table 3). It will be of great interest to determine whether the SR-11 Δ*ppc* mutant and the wild-type SR-11 strain grow equally well in Peyer's patches and in macrophages in vivo.

The DctA and KgtP transporters are not required for replenishing TCA cycle intermediates during SR-11 infection (10^8 CFU/mouse, peroral route). Taken together, the evidence described above suggests that cyclic operation of the TCA cycle and formation of pyruvate from malate are important for full virulence of SR-11 in BALB/c mice. Since gluconeogenesis is not required for full virulence, it appears that SR-11 can use sugars for growth in vivo. Yet the SR-11 Δ*ppc* mutant is fully virulent despite being unable to replenish the TCA cycle when grown on sugars as sole carbon and energy sources (47). The fact that Ppc is not required to replenish TCA intermediates and that malate appears to be removed from the TCA cycle to serve as a major source of pyruvate suggests the possibility that TCA cycle intermediates can be replenished during infection independently of Ppc. Therefore, we wanted to test whether uptake of TCA cycle intermediates was important for SR-11 virulence. DctA is a sodium-dependent dicarboxylate transporter, responsible for the uptake of fumarate, succinate, and malate under aerobic conditions as well as for the uptake of orotate, a pyrimidine precusor (10). KgtP is a proton-driven transporter for α-ketoglutarate, which is constitutively expressed (45). SR-11 Δ*kgtP*, SR-11 Δ*dctA*, and SR-11 Δ*kgtP* Δ*dctA* mutants were constructed, and each strain was tested for virulence. The SR-11 Δ*kgtP* mutant ($P = 0.26$), the SR-11 Δ*dctA* mutant ($P = 0.71$), and the SR-11 Δ*kgtP* Δ*dctA* double

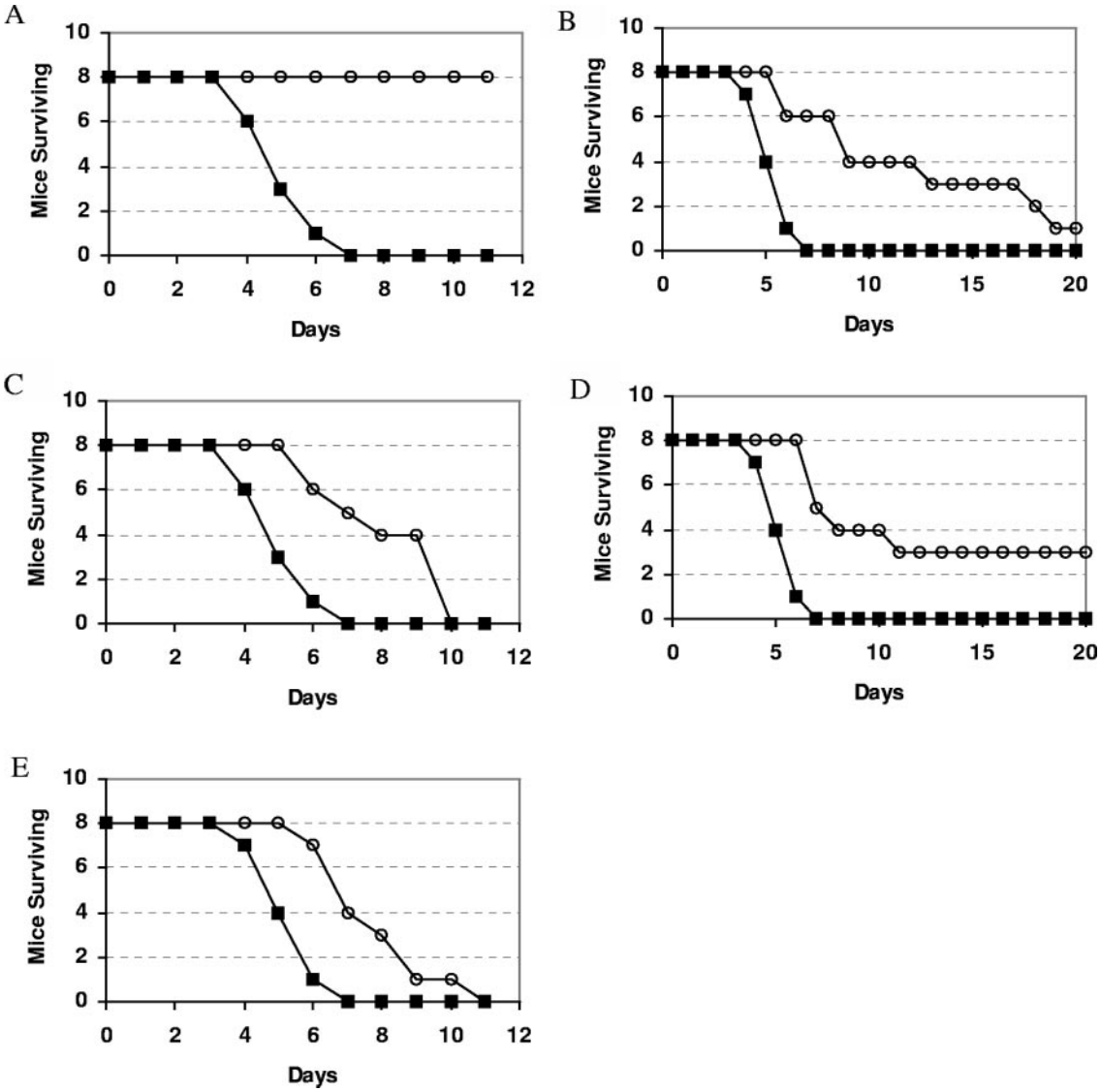

FIG. 6. Survival of BALB/c mice infected intraperitoneally with 10^3 CFU of either wild-type SR-11 (■) or the SR-11 Δ*sucAB* mutant (○) (A), 10^3 CFU of either wild-type SR-11 (■) or the SR-11 Δ*sfcA* Δ*maeB* double mutant (○) (B), 10^3 CFU of either wild-type SR-11 (■) or the SR-11 Δ*sdhCDA* mutant (○) (C), 10^3 CFU of either wild-type SR-11 (■) or the SR-11 Δ*sucCD* mutant (○) (D), or 10^3 CFU of either wild-type SR-11 (■) or the SR-11 Δ*mdh* mutant (○) (E).

mutant ($P = 0.98$) were each fully virulent (Table 3). Therefore, although we have not as yet identified the intermediates that replenish the TCA cycle, it appears that neither DctA nor KgtP is required for uptake of TCA cycle intermediates during SR-11 infection of BALB/c mice. Note that neither the anaerobic systems for dicarboxylate uptake (16, 34) nor the citrate transport system (52) were tested.

Virulence of SR-11 mutants via the intraperitoneal route (10^3 CFU/mouse). Since SR-11 growth presumably takes place in lymphoid cells of the Peyer's patches (20, 22) and in macrophages in the liver and spleen when mice are infected via the peroral route (24, 40), the SR-11 mutants that were at least moderately attenuated (SR-11 Δ*mdh*, SR-11 Δ*sfcA* Δ*maeB*, SR-11 Δ*sdhCDA*, and SR-11 Δ*sucCD*) or avirulent perorally (SR-11 Δ*sucAB*) were tested for virulence via the intraperitoneal (i.p.) route. The SR-11 Δ*sucAB* mutant, which was avirulent via the oral route was also avirulent i.p. ($P = 0.0001$) (Fig. 6A). The SR-11 Δ*sfcA* Δ*maeB* mutant ($P = 0.0011$), the SR-11 Δ*sdhCDA* mutant ($P = 0.0014$), and the SR-11 Δ*sucCD* mutant ($P = 0.0003$) were attenuated i.p. to about the same extent as via the peroral route (Fig. 6B to D); however, the SR-11 Δ*mdh* mutant ($P = 0.0014$) appeared to be less attenuated via the i.p. route than perorally (compare Fig. 4C and 6E), suggesting that sources of replenishment of oxaloacetate for SR-11 during growth in macrophages in the liver and spleen may be more plentiful than for growth of SR-11 in the Peyer's patches.

DISCUSSION

While the virulence mechanisms of serovar Typhimurium have been studied intensively, little is known about the central

metabolic pathways used during infection. As a first step, we used systematic mutational analysis to evaluate the involvement of central metabolic pathways in SR-11 virulence in BALB/c mice. While we believe that this approach has merit, there are several reasons that the data must be interpreted with caution. First, the fact that a specific mutation has no effect on virulence does not rule out the possibility that the disrupted metabolic pathway normally plays a role in virulence, i.e., it is possible that an alternative pathway can compensate for the defect. Second, it is possible that virulence gene expression may be regulated by specific metabolic signals, and thus, while attenuation of a specific mutant might be attributed to interruption of a metabolic pathway, attenuation might be primarily caused by repression of virulence factor synthesis. Third, virulence is a highly complicated process involving many different types of host-bacterium interactions, any one of which could be affected by a specific metabolic defect. Thus, a specific metabolic defect might have important consequences at one stage of infection but not at another stage. The virulence assay employed here cannot address this possibility. Fourth, we have not complemented each of the attenuated and avirulent mutants with the wild-type gene or genes that each is missing to be sure that full virulence has been restored. Nevertheless, despite these caveats, and recognizing that far more work will be required to fully understand serovar Typhimurium central metabolism in vivo, including studies of central carbon metabolism in Peyer's patches and in macrophages in vivo, we offer the following possibilities to explain our data.

Gluconeogenesis (Δ*fbp* Δ*glpX* mutant and Δ*ppsA* Δ*pckA* mutant) and the anapleurotic replenishment of oxaloacetate in the TCA cycle from PEP (Δ*ppc* mutant) appear not to be needed for full SR-11 virulence, but formation of pyruvate from malate, which would require replenishment of TCA cycle intermediates, does appear to be important (Δ*sfcA* Δ*maeB* mutant). It is also clear that cyclic operation of the TCA cycle (Δ*sucAB*, Δ*sucCD*, Δ*sdhCDA* mutants) is important for full virulence. Thus, the data are consistent with the hypothesis that during infection of BALB/c mice, SR-11 grows in a mixed mode using glycolytic sugars and either amino acids or intermediates of the TCA cycle to replenish the TCA cycle, thereby allowing formation of pyruvate from malate.

Apparently, the majority of PEP required for full SR-11 virulence can be generated by glycolysis, i.e., blocking PEP production via the gluconeogenic pathway did not have a major effect on virulence (Fig. 2). Furthermore, as stated above, the fact that the SR-11 Δ*ppc* mutant is fully virulent (Fig. 3) suggests that replenishment of oxaloacetate from PEP is not required. Three nonexclusive interpretations of these results are possible. First, the PEP generated by glycolysis is consumed in other ways, e.g., during transport of sugars by the PTS system, during synthesis of vitamins and cofactors, and perhaps during synthesis of the aromatic amino acids, etc., and is therefore not available to replenish oxaloacetate. Second, sugar concentrations are insufficient to produce enough PEP to drive cyclic operation of the TCA cycle. Third, uptake of TCA cycle intermediates or substrates that feed into the TCA cycle obviate the need for the anapleurotic reaction catalyzed by Ppc.

We have not as yet identified anapleurotic reactions that replenish the TCA cycle. However, since the SR-11 Δ*dctA* Δ*kgtP* double mutant is fully virulent (Table 4), it appears that SR-11 does not transport extracellular α-ketoglutarate, fumarate, succinate, and malate aerobically for this purpose. It is still possible, however, that SR-11 transports dicarboxylates anaerobically during infection, since serovar Typhimurium contains genes for anaerobic dicarboxylate transport (16, 34) that were not tested here. It is also possible that other untested anapleurotic reactions contribute to replenishing TCA cycle intermediates. For example, serovar Typhimurium can take up extracellular citrate via the TctABC transporter (52), which could enter directly into the TCA cycle or be converted to oxaloacetate via a putative citrate lyase (28).

Although a complete TCA cycle appears to be required for full SR-11 virulence, deleting different TCA cycle genes resulted in different levels of attenuation. Thus, the SR-11 Δ*sucAB* mutant is avirulent (Fig. 4A), the SR-11 Δ*mdh* mutant is highly attenuated (Fig. 4C), the SR-11 Δ*sfcA* Δ*maeB* mutant and the SR-11 Δ*sucCD* mutants are moderately attenuated (Fig. 4B and 5), and the SR-11 Δ*sdhCDA* mutant is least attenuated (Fig. 4E). One explanation as to why the SR-11 Δ*sdhCDA* mutant is less attenuated than the other TCA cycle attenuated mutants is the possibility that endogenous fumarate reductase present in SR-11 cells may be able to substitute for succinate dehydrogenase (6, 19) and thereby lessen the effect of the Δ*sdhCDA* mutation. It will therefore be of interest to test the virulence of an SR-11 Δ*frdABCD* Δ*sdhCDA* double mutant. In contrast, the SR-11 Δ*sucAB* mutant is presumably avirulent because the strain is unable to make succinyl-CoA during infection. Succinyl-CoA is required for biosynthesis of diaminopimelate, a precursor for the synthesis of lysine, methionine, and peptidoglycan (4, 5, 15, 17, 30).

Although the data presented here implicate the utilization of sugars for full SR-11 virulence, at the present time we don't know which sugars are metabolized during SR-11 infection, although it has recently been suggested that serovar Typhimurium utilizes gluconate, a non-PTS sugar, for growth in macrophages in vitro (12). PTS sugars all utilize PtsI, which is phosphorylated by PEP (38). Recently, a serovar Typhimurium *ptsI* mutant was shown to be attenuated via the i.p. route (23); however, *ptsI* mutants are not only unable to transport PTS sugars, they are unable to utilize a number of non-PTS sugars and TCA cycle intermediates for growth (38). Therefore, to identify sugars that contribute to virulence, it will be necessary to knock out the ability to utilize individual sugars and test their virulence.

Serovar Typhimurium grows both in lymphoid cells and macrophages in the Peyer's patches and in macrophages after it crosses the intestine and disseminates systemically (22, 24, 40, 43). Therefore, the data obtained from infection via the oral route presumably reflect a composite of growth in lymphoid cells and macrophages, whereas the data obtained by infection via the i.p. route probably reflect growth in macrophages. In general, those mutants that were either avirulent or attenuated via the oral route remained as such via the i.p. route; however, the SR-11 Δ*mdh* mutant was clearly more attenuated orally than i.p. (compare Fig. 4C and 6E). It will be of great interest to determine the growth rates of the wild-type SR-11 and the attenuated SR-11 mutants in Peyer's patches and in macrophages in vivo.

Finally, it is clear that the inability to perform gluconeogen-

esis is not the reason the SR-11 Δ*cra* mutant is avirulent. Cra is thought to bind to DNA and exert its regulatory effects only when growth takes place on gluconeogenic substrates, i.e., not in the presence of sugars as carbon sources (42). Yet the data presented here suggest that SR-11 utilizes sugars for growth in vivo. However, if in vivo SR-11 grows on low levels of sugars, Cra could still play a role during infection. Fructose-1,6-bisphosphate is the effector that removes Cra from its target promoters when growth takes place in the presence of high sugar levels (42). Low levels of sugars would presumably not generate a high enough concentration of fructose-1,6-bisphosphate to remove Cra from its target promoters. As a consequence, despite sugars being used for SR-11 growth in vivo, Cra could still positively regulate both the TCA cycle and electron transport systems (42) and perhaps serve to positively regulate virulence gene expression, along with other known regulators (25). Further research is required to examine these possibilities.

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