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

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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 (catabolite repressor/activator) 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 Δcra 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 (fhp, 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 succCD mutant, which prevents the conversion of succinyl coenzyme A to succinate, and an ΔsdhCDA mutant, which blocks the conversion of succinate to fumarate, were both attenuated, whereas both an SR-11 ΔaspA mutant and an SR-11 ΔfrdABC 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 ΔfadD mutant and an SR-11 ΔaceA 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 (catabolite repressor/activator) 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 strain 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 dehydrogenase 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 (0.2% wt/vol), sodium citrate (0.4% wt/vol), and sodium oleate (5 mM) plus 5 mg of Brj58/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 Datyndok 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.
resistance cassettes were removed from the deletion mutants lacking the
or

\[ \text{glpX} \]

mutants. Mutant strains of SR-11 were created by deletion mutagenesis using

\[ \text{SR-11 strains} \]

\[ \text{dctA} \]

CTGACAGCAATCGCCATT TAAGCACATCGTCCAGCT

\[ \text{kgtP} \]

CGACAGCGCGACATATTC GAGATGCGGCGACGCATA

\[ \text{mdh} \]

CGATAAGACGTGAGGAGT CAGCCGATCCGGATTACG

\[ \text{sdhCDA} \]

ACGGTGACTACCGCAGTG GCTGCGTGATATGGAATTCGT

\[ \text{sucCD} \]

GTACTACTCCGCGTGAAG AGGTGGCCAACCATGTCG

\[ \text{sucAB} \]

TGGACAGACTGGACGAAC TGTGCTGCAGGTTCAACA

\[ \text{ppc} \]

CATCAACGCCTGTTCGAC ATGACGAGCTGCTGCCAG

\[ \text{gtcA} \]

GTACTCAACAAATCGAAG ACGCGGTAACGGATGACG

\[ \text{aceA} \]

CCGGTTAGCGCGAGGATTTGC CACCGACTATTCTTTGTATTACTACC

\[ \text{maeB} \]

sfcA

aspA

TCCATCATGCTCAACCAG

TACGTTATTGTGAGGAGGAGGC

CGTAAATCTATGAGCCTGCG

CAGGGATCCGCGTGATGCAT

ATTCCGAGATCGTGCTC

GCTGATGTTCCGGCGGAG CACGGGCTTCGTCACGCA

AGGCATGGCAGTCGCCG

ACGGCGATGGCAGTACC

CATCAGCTGGACGATGGC

TGACGATGGCAGTCGCCG

TGGACAGACTGGACGAAC

AGCGCAACACACTACCTA

TCATGATGATATATAAACCAACGG

CCGGTGACAGCAAGATTG

GTCACTACTCAGCGGTGAAG

CAGGTGACTACCCGAGTG

CGATAAGACGTGAGGAGT

CGACAGCGGAGATTTCC

CTGACAGCATTGCACATT

\[ \text{Plasmids} \]

\[ \text{pKD3} \]

\[ \text{pKD4} \]

\[ \text{pKD46} \]

\[ \text{pCP20} \]

\[ \text{Construction and characterization of serovar Typhimurium SR-11 deletion mutants.} \]

Mutant strains of SR-11 were created by deletion mutagenesis using either a chloramphenicol (\textit{cat}) or kanamycin (\textit{kan}) resistance cassette as described by Datsenko and Wanner (9). The resistance cassettes were removed from the deletion mutants lacking the \textit{cat} or \textit{kan} designation, as described by Datsenko and Wanner (9).

\begin{table}[h]
\centering
\caption{Strains of serovar Typhimurium and plasmids used in this study}
\begin{tabular}{lll}
\textbf{Strain or plasmid} & \textbf{Genotype$^a$ and defect(s) or relevant characteristics} & \textbf{Source or reference} \\
\hline
\textit{SR-11 strains} & & \\
\textit{Wild type} & gvr1816; none & 8 \\
\textit{Fad$^{-}$ AX-2} & gvr1816 cu::TnlO d cam; Cra repressor/activator protein & 1 \\
\textit{\textit{ΔglpX}} & gvr1816 ΔglpX::cat; glpX-encoded fructose-1,6-bisphosphatase & This study \\
\textit{Δpfr} & gvr1816 Δpfr::cat; pfps-encoded fructose-1,6-bisphosphatase & This study \\
\textit{ΔglpX Δpfr} & gvr1816 ΔglpX Δpfr::cat; glpX-and pfps-encoded fructose-1,6-bisphosphatases & This study \\
\textit{Δpfr} & gvr1816 Δpfr::cat; PEP carboxykinase & This study \\
\textit{Δpfr Δpfr} & gvr1816 Δpfr Δpfr::cat; PEP carboxykinase, PEP synthase & This study \\
\textit{Δacs} & gvr1816 Δacs; acetate synthase & This study \\
\textit{ΔfadD} & gvr1816 ΔfadD::cat; acyl-CoA synthase & This study \\
\textit{ΔaspA} & gvr1816 ΔaspA: aspartate & This study \\
\textit{ΔsfcA} & gvr1816 ΔsfcA::kan; malate oxidoreductase (NAD dependent) & This study \\
\textit{ΔsfcA ΔsfcB} & gvr1816 ΔsfcA ΔsfcB::kan; both malate oxidoreductases & This study \\
\textit{ΔaceA} & gvr1816 ΔaceA; isocitrate lyase & This study \\
\textit{Δppc} & gvr1816 Δppc; PEP carboxylase & This study \\
\textit{ΔsucAB} & gvr1816 ΔsucAB; α-ketoglutarate dehydrogenase & This study \\
\textit{ΔsucCD} & gvr1816 ΔsucCD; succinyl-CoA synthetase & This study \\
\textit{ΔsdhCDA} & gvr1816 ΔsdhCDA; succinate dehydrogenase & This study \\
\textit{ΔfrdABCD} & gvr1816 ΔfrdABCD; fumarate reductase & This study \\
\textit{Δmdh} & gvr1816 Δmdh; malate dehydrogenase & This study \\
\textit{Δkgp} & gvr1816 Δkgp; α-ketoglutarate transporter & This study \\
\textit{ΔdtcA} & gvr1816 ΔdtcA; dicarboxylate transporter & This study \\
\textit{Δkgp ΔdtcA} & gvr1816 Δkgp ΔdtcA; dicarboxylate transporter, α-ketoglutarate transporter & This study \\
\textit{Plasmids} & & \\
\textit{pKD3} & Template plasmid, contains chloramphenicol resistance cassette flanked by FLP recombinase & 9 \\
\textit{pKD4} & Template plasmid, contains kanamycin resistance cassette flanked by FLP recombinase & 9 \\
\textit{pKD46} & Temperature-sensitive plasmid, contains arabinose-inducible phage λ red recombinase gene & 9 \\
\textit{pCP20} & Temperature-sensitive plasmid, contains FLP recombinase gene for removal of antibiotic resistance cassettes; \textit{bla} cat & 9 \\
\hline
\end{tabular}
\end{table}

\footnotesize

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

\begin{table}[h]
\centering
\caption{Sequences of primers used to confirm serovar Typhimurium mutants}
\begin{tabular}{lll}
\textbf{Gene} & \textbf{Forward primer sequence (5’-3’)} & \textbf{Reverse primer sequence (5’-3’)} \\
\hline
\textit{glpX} & CTGGCATTTACAGATCA & TCCGGAACGAGGAGGAGGC \\
\textit{pfps} & CTGTTGCAATGTTGCGCTCA & TGGACAGATGGCTGCG \\
\textit{sucAB} & CGTACAGCTGCTGGGCCG & TACGGCGATGGCAGTCGCCG \\
\textit{sucCD} & CTGGACAGACTGCGGAGAAC & ATGACGAGTGCCTGGAAGGATGGATC \\
\textit{sdhCDA} & CGTACAGCTGCTGGGCCG & TACGGCGATGGCAGTCGCCG \\
\textit{frdABCD} & CGTACAGCTGCTGGGCCG & TACGGCGATGGCAGTCGCCG \\
\textit{mdh} & CGTACAGCTGCTGGGCCG & TACGGCGATGGCAGTCGCCG \\
\textit{kgp} & CGTACAGCTGCTGGGCCG & TACGGCGATGGCAGTCGCCG \\
\textit{dtcA} & CGTACAGCTGCTGGGCCG & TACGGCGATGGCAGTCGCCG \\
\hline
\end{tabular}
\end{table}
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 94°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).

**RESULTS**

**Definition of fully virulent, attenuated, and avirulent (10⁶ CFU/mouse, peroral route).** In addition to infecting 4 mice orally with 10⁶ CFU of the wild-type SR-11 in each virulence experiment, 4 mice were infected orally with 10⁳ 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. Genes encoding the enzymes for each reaction are listed beside each reaction.

**Glucogenesis is not required for full SR-11 virulence (10⁶ CFU/mouse, peroral route).** During glucogenesis, 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 glucogenesis 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 Δfbp mutant, an SR-11 ΔglpX mutant, and an SR-11 ΔglpX Δfbp double mutant were constructed. As expected, the SR-11 Δfbp mutant and the SR-11 ΔglpX Δfbp 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)

<table>
<thead>
<tr>
<th>Pathway and mutant strain</th>
<th>Day 50% mortality reached for:</th>
<th>Day 100% mortality reached for:</th>
<th>Kaplan-Meier</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>Mutant</td>
<td>Wild type</td>
</tr>
<tr>
<td>Gluconeogenesis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δfbp</td>
<td>6.5</td>
<td>5.5</td>
<td>9</td>
</tr>
<tr>
<td>ΔglpX</td>
<td>6.5</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Δfbp ΔglpX</td>
<td>7</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>ΔpckA</td>
<td>7</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>ΔppsA</td>
<td>8.5</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>ΔpckA ΔppsA</td>
<td>8</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Malate to pyruvate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔmaeB</td>
<td>7.5</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>ΔsfdA</td>
<td>7.5</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>ΔsfdA ΔmaeB</td>
<td>6.5</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>TCA cycle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔsdhCDA</td>
<td>9</td>
<td>&gt;32</td>
<td>10</td>
</tr>
<tr>
<td>ΔsdhCDA ΔsdhAB</td>
<td>8</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>ΔsdhAB (10^6 CFU)</td>
<td>8</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>ΔsdhCDA composite (16 mice) &amp; Δmdh (12 mice)</td>
<td>7.5</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>Acetate, fatty acids, glyoxylate bypass</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔaceA</td>
<td>8</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Δacs</td>
<td>8</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>ΔfadD</td>
<td>8</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Anapleurotic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔpckA</td>
<td>7.5</td>
<td>7.5</td>
<td>12</td>
</tr>
<tr>
<td>ΔsdhCDA</td>
<td>7.5</td>
<td>7.5</td>
<td>13</td>
</tr>
<tr>
<td>ΔkgtP</td>
<td>5.5</td>
<td>6.5</td>
<td>13</td>
</tr>
<tr>
<td>ΔsdhCDA ΔkgtP</td>
<td>6.5</td>
<td>8.5</td>
<td>13</td>
</tr>
</tbody>
</table>

* 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 ΔglpX mutant (P = 0.98), the SR-11 Δfbp mutant (P = 0.35), and the SR-11 ΔglpX Δfbp 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.

Although the SR-11 ΔglpX Δfbp 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 ΔppsA mutant, an SR-11 ΔpckA mutant, and an SR-11 ΔppsA ΔpckA double mutant were constructed. As expected, the SR-11 ΔppsA ΔpckA 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 ΔppsA mutant (P = 0.84) and the SR-11 ΔpckA mutant (P = 0.14) were fully virulent (Table 3); however, the SR-11 ΔppsA ΔpckA 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 ΔglpX Δfbp double mutant is fully virulent and the SR-11 ΔppsA ΔpckA 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)

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Wild type</th>
<th>Δmdh</th>
<th>ΔsdhCDA</th>
<th>ΔsdhAB</th>
<th>ΔpckA ΔpckB</th>
<th>ΔpckA ΔmaeB</th>
<th>Δacsa Δacsc</th>
</tr>
</thead>
<tbody>
<tr>
<td>M9G</td>
<td>79 ± 4</td>
<td>95 ± 5</td>
<td>81 ± 4</td>
<td>80 ± 6</td>
<td>79 ± 6</td>
<td>84 ± 5</td>
<td>NG ± 2</td>
</tr>
<tr>
<td>M9GAK</td>
<td>64 ± 2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>92 ± 2</td>
</tr>
<tr>
<td>M9GDLM</td>
<td>69 ± 2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>72 ± 4</td>
</tr>
</tbody>
</table>

* M9G, M9 minimal medium containing glucose (0.2% wt/vol).
* M9GAK, M9G supplemented with 0.01% aspartate and 0.01% α-ketoglutarate.
* M9GDLM, M9G containing diaminopimelate (40 μg/ml), lysine (50 μg/ml), and methionine (50 μg/ml).
* NG, no growth.
* ND, not done.
metabolites are withdrawn for biosynthesis under conditions in which oxaloacetate (41), replenishes the TCA cycle when precursor phosphoenolpyruvate carboxylase (Ppc), which converts PEP to oxaloacetate from PEP is withdrawn for biosynthesis under conditions, aspartate aminotransferase, 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 sacCD genes (3). Succinyl-CoA is derived from succinate via succinyl-CoA synthetase rather than from α-ketoglutarate via α-ketoglutarate dehydrogenase (sacAB) 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 ∆aspA mutant and an SR-11 ∆frdABCD mutant were constructed and tested for virulence. Both the SR-11 ∆aspA mutant (P = 0.20) and the SR-11 ∆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 (10⁶ 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 ∆asuCAB mutant, all the TCA cycle mutants grew with glucose as a sole carbon and energy source (Table 4). The SR-11 ∆asuCAB 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 ∆asuCD 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 ∆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 ∆sdcDCA 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 ∆sdcDCA mutant was tested for virulence at a 100-fold-lower level (10⁴ 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 ∆sdcDCA mutant survival curve generated from the combined data of the 10⁶ and 10⁴ CFU virulence assays (16 mice) (Fig. 4E) also showed that the SR-11 ∆sdcDCA mutant was attenuated (P = 0.0026). Since expression of all the TCA cycle genes tested is 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 (10⁶ 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. 3. Survival of BALB/c mice infected orally with 10⁶ CFU of either wild-type SR-11 (■) or the SR-11 ∆ppc mutant (○).
Serovar Typhimurium contains an NAD-dependent malate oxidoreductase, encoded by the \textit{sfcA} gene (26) and an NADP-dependent malate oxidoreductase, putatively encoded by the \textit{maeB} gene (2). It has been reported that a MudJ insertion in the \textit{sfcA} gene rendered serovar Typhimurium avirulent after oral infection in BALB/c mice (49). We were unable to confirm that an SR-11 \textit{sfcA} mutant was avirulent. In fact, in our hands, both the SR-11 \textit{ΔsfcA} mutant (\(P = 0.77\)) and the SR-11 \textit{ΔmaeB} mutant (\(P = 0.079\)) were fully virulent (Table 3). However, although not avirulent, the SR-11 \textit{ΔsfcA ΔmaeB} 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...
14028s acute virulence (13). To determine whether the glyoxylate bypass contributes to SR-11 virulence, an SR-11 ΔaceA mutant was constructed which is unable to convert isocitrate to glyoxylate and succinate (Fig. 1) (27). As expected, the SR-11 ΔaceA 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 ΔmdhD mutant (P = 0.65), unable to degrade fatty acids to acetyl-CoA (Fig. 1), and an SR-11 Δacs 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 Δmdh 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 ΔidhCD mutant (Table 4) and the attenuated SR-11 ΔaceCD 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 ΔppsA ΔpckA double mutant and the generation time of the attenuated SR-11 ΔsfcA ΔmaeB 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 gluconeogenesis 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 ΔppsA ΔpckA and SR-11 ΔsfcA ΔmaeB 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 ΔwcaAB 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 ΔwcaAB 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 ΔwcaAB 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 Δppc 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 Δppc 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 Δppc 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 (10^9 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 Δppc 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 under in vivo conditions serves to remove 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 precursor (10). KgtP is a proton-driven transporter for α-ketoglutarate, which is constitutively expressed (45). SR-11 ΔkgtP, SR-11 ΔdctA, and SR-11 ΔkgtP ΔdctA mutants were constructed, and each strain was tested for virulence. The SR-11 ΔkgtP mutant (P = 0.26), the SR-11 ΔdctA mutant (P = 0.71), and the SR-11 ΔkgtP ΔdctA double

FIG. 5. Survival of BALB/c mice infected orally with 10^6 CFU of either wild-type SR-11 (■) or the SR-11 ΔsfcA ΔmaeB double mutant (○).
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 Δmdh, SR-11 ΔsfcA ΔmaeB, SR-11 ΔsdhCDA, and SR-11 ΔsucCD) or avirulent perorally (SR-11 ΔsucAB) were tested for virulence via the intraperitoneal (i.p.) route. The SR-11 ΔsucAB mutant, which was avirulent via the oral route was also avirulent i.p. ($P = 0.0001$) (Fig. 6A). The SR-11 ΔsfcA ΔmaeB mutant ($P = 0.0011$), the SR-11 ΔsdhCDA mutant ($P = 0.0014$), and the SR-11 ΔsucCD 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 Δmdh 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 complemented each of the attenuated and avirulent mutants. In contrast, the SR-11 ΔfrdABCD ΔsdhCDA double mutant is least attenuated (Fig. 4E). One explanation as to why the SR-11 ΔsdhCDA 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 ΔsdhCDA mutation. It will therefore be of interest to test the virulence of an SR-11 ΔfrdABCD ΔsdhCDA double mutant. In contrast, the SR-11 ΔsuccAB 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 Δmdh 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 ΔacrA 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 phosphatase to remove Cra from its target promoters. When growth takes place on gluconeogenic substrates, i.e., not in vivo, SR-11 grows on low levels of sugars, the aerobic C4-dicarboxylate transport (AceA) is required for virulence but not for acute lethal infection in the chicken in vivo. However, the SR-11 ΔacrA mutant, which encodes a fumarate reductase deficient strain of Salmonella enterica serovar Typhimurium LT2, expresses the pcrA gene and virulence of Salmonella enterica serovar Typhimurium K-12 relevant to genetic and allosteric regulation and homology of E. coli phosphonopyruvate carboxykinase with those of the pig aspartate aminotransferase (AceA) is required for virulence of Salmonella enterica serovar Typhimurium LT-2. J. Bacteriol. 215:711–7156.


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