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A Functional *cra* Gene Is Required for *Salmonella enterica* Serovar Typhimurium Virulence in BALB/c Mice

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A minitransposon mutant of *Salmonella enterica* serovar Typhimurium SR-11, SR-11 Fad⁻, is unable to utilize gluconeogenic substrates as carbon sources and is avirulent and immunogenic when administered perorally to BALB/c mice (M. J. Utley et al., FEMS Microbiol. Lett., 163:129–134, 1998). Here, evidence is presented that the mutation in SR-11 Fad⁻ that renders the strain avirulent is in the *cra* gene, which encodes the Cra protein, a regulator of central carbon metabolism.

Since *Salmonella enterica* serovar Typhimurium is able to utilize phosphatidylserine as the sole source of carbon, nitrogen, and phosphate for growth (11) and since phosphatidylserine is a component of eucaryotic cell membranes, it was of interest to determine whether mutants unable to utilize either the glycerophosphate portion or the fatty acid portion of the phospholipid for growth are attenuated. Several mutants unable to utilize oleate as a sole carbon source were isolated after mini-Tn*10*::*d cam* transposon mutagenesis (6), and one of them, designated serovar Typhimurium SR-11 Fad⁻ (fatty acid), was totally avirulent and immunogenic in BALB/c mice when administered perorally (21). In addition, SR-11 Fad⁻ was found in the liver and spleen in numbers 4 to 5 orders of magnitude lower than SR-11 (21). SR-11 Fad⁻ was also less virulent than SR-11 when administered to mice intraperitoneally (21). Furthermore, SR-11 Fad⁻, in addition to being unable to grow utilizing oleate as a sole carbon source, was also unable to grow utilizing citrate, isocitrate, and acetate as sole carbon sources, all of which were utilized well by SR-11 (21). SR-11 Fad⁻ grew slowly and to only one-tenth the final yield of SR-11 utilizing pyruvate as the sole carbon source in liquid culture (21). SR-11 Fad⁻ was, however, able to utilize glucose, glycerol, and a number of sugars sensitive to catabolite repression as the sole carbon sources (14). In the present study, we identify the gene made defective in SR-11 Fad⁻ by mini-Tn*10*::*d cam* transposon mutagenesis and show that the defect is responsible for SR-11 Fad⁻ avirulence in BALB/c mice.

SR-11 Fad⁻ is a *cra* (*fruR*) mutant. In order to identify the gene that is inactivated in SR-11 Fad⁻, Southern hybridization was performed using a digoxigenin (DIG)-labeled transposon-specific probe made from pJHA1 (Table 1). The transposon-specific probe was prepared with a DIG High Prime DNA Labeling and Detection Starter Kit II (Boehringer Mannheim, Indianapolis, Ind.) as described by the manufacturer. The Tn*10*::*d cam* minitransposon was located on a 4.5-kb *Pst*I SR-11 Fad⁻ DNA fragment which was inserted into the unique *Pst*I site of pBluescript II SK(+) to create pJHA7 (Table 1). The first 489 bp in the 4.5-kb SR-11 Fad⁻ insert in pJHA7 at the T3 promoter end were sequenced. The sequence was essentially identical to the last 180 bp of the serovar Typhimurium LT2 *ilvI* gene, the first 306 bp of the serovar Typhimurium LT2 *ilvH* gene, and the three intervening base pairs (GenBank accession number X55456). The first 470 bp at the opposite end of the 4.5-kb SR-11 Fad⁻ insert in pJHA7, sequenced from the T7 promoter, were 87% identical to *yabB*, a gene of unknown function in *Escherichia coli* (GenBank accession number AE000118 U00096). In both serovar Typhimurium LT2 and *E. coli*, *ilvI* and *ilvH* are immediately upstream of the *fruR* gene, which is 601 bp upstream of *yabB* in *E. coli* (GenBank accession number AE000118 U00096). Sequencing from the chloramphenicol resistance gene in pJHA7 determined that the mini-Tn*10*::*d cam* transposon was indeed inserted 45 bp upstream of the 3′ end of the LT2 *fruR* gene (reference 9 and GenBank accession number X55456). The *fruR* gene has recently been renamed *cra* (catabolite repressor/activator) (18). A diagram of the genes present in the 4.5-kb *Pst*I SR-11 Fad⁻ DNA fragment in pJHA7 and the portions sequenced is presented in Fig. 1.

Serovar Typhimurium LJ2443, a known *cra* mutant (3), is unable to grow on minimal agar plates containing gluconeogenic substrates as sole carbon sources (3, 18). Its phenotype with respect to utilization of different carbon sources was compared to SR-11 Fad⁻. Both mutants were essentially identical with respect to growth on M9 minimal agar plates containing either sodium oleate (5 mM), sodium citrate (0.2% [wt/wt]), potassium acetate (0.4% [wt/wt]), sodium pyruvate (0.4% [wt/wt]), sodium succinate (0.6% [wt/wt]), potassium fumarate (0.4% [wt/wt]), glucose (0.2% [wt/wt]), or glycerol (0.2% [wt/wt]), i.e., they failed to grow on the gluconeogenic substrates as sole carbon sources in 48 h at 37°C but grew as well as their wild-type parents on glucose and glycerol. The wild-type SR-11 *cra* gene was cloned into pBR322 to create pJHA8 (Table 1). Both SR-11 Fad⁻ and LJ2443, when complemented with

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype and/or phenotype	Source or reference
S. enterica serovar Typhimurium strains (plasmid)		
SR-11	*gyr1816*	4
SR-11 Fad$^-$	*gyr1816 cra*::Tn*10 d cam*	21
SR-11 Fad$^-$ AX-2	*gyr1816 cra*::Tn*10 d cam*	This study
SR-11 Fad$^-$(pBR322)	*gyr1816 cra*::Tn*10 d cam*, *bla tet*	This study
SR-11 Fad$^-$(pJHA8)	*gyr1816 cra*::Tn*10 d cam*, *cra*$^+$ *tet*	This study
LJ2443	*fruR51*::Tn*10*	3
UK-1	Virulent avian strain	5
UK-1 Fad$^-$ AX-1	*cra*::Tn*10 d cam*	This study
E. coli S-17 λ *pir*	Host for pLD55 and pMJN10	V. de Lorenzo
Plasmids		
pBR322	*bla tet*	2
pJHA1	1.4-kb *cam* gene from Tn*10*::*d cam* in *Bam*HI site of pBR322	This study
pJHA7	4.3-kb *Pst*I fragment containing *cra*::Tn*10 d cam* in pBR322, *cam tet*	This study
pJHA8	1.4-kb *cra*$^+$ gene in *Pst*I site of pBR322, *tet*	This study
pLD55	Suicide vector for allelic exchange, *bla tetAR*	15
pMJN10	4.3-kb *Pst*I fragment containing *cra*::Tn*10 d cam* in pLD55, *cat bla tetAR*	This study

pJHA8, regained the ability to grow on M9 agar plates containing the aforementioned gluconeogenic substrates as sole carbon sources. pBR322 did not functionally complement the *cra* mutation in either SR-11 or LJ2443. These experiments established conclusively that serovar Typhimurium SR-11 Fad$^-$ is a *cra* mutant.

SR-11 Fad$^-$ (pJHA8) is virulent. It was possible that inactivation of the *cra* gene with the mini-Tn*10*::d *cam* insertion caused a downstream effect that resulted in avirulence. We therefore complemented SR-11 Fad$^-$ with pJHA8, which contains the wild-type SR-11 *cra* gene as the only nonvector gene in pBR322, and tested its virulence.

Groups of four BALB/c mice were perorally infected with either 3.1×10^8 CFU per mouse of SR-11 Fad$^-$ (pBR322) or 4.3×10^8 CFU per mouse of SR-11 Fad$^-$ (pJHA8). By 8 days postinfection, three of the four mice infected with SR-11 Fad$^-$ (pJHA8) had died. The fourth mouse infected with SR-11 Fad$^-$ (pJHA8) appeared to be very sick for several days (ruffled fur, loss of appetite, huddled) but recovered. In contrast, the four mice infected with SR-11 Fad$^-$ (pBR322) remained alive and healthy. Since complementing SR-11 Fad$^-$ with the wild-type *cra* gene results in renewed virulence, it is the defect in the *cra* gene caused by the mini-Tn*10*::*d cam* insertion that renders the strain avirulent.

A serovar Typhimurium UK-1 *cra* mutant constructed by allelic exchange is avirulent. It was of interest to determine whether a *cra* mutant of a second serovar Typhimurium strain was also avirulent. Therefore, a *cra* mutant of the virulent strain serovar Typhimurium UK-1 (23), designated UK-1 Fad$^-$ AX-1 (allelic exchange), was constructed by allelic exchange of the wild-type *cra* gene with the mutant *cra* gene using pMJN10 (see Table 1). pMJN10 contains the mutant *cra* gene and flanking sequences as a 4.5-kb *Pst*I DNA fragment (Fig. 1) in pLD55, an allelic exchange vector (15). That the only *cra* gene that UK-1 Fad$^-$ AX-1 contained was the defective *cra* gene was shown by four lines of evidence. First, UK-1 Fad$^-$ AX-1 was unable to grow on any of the gluconeogenic substrates. Second, complementing UK-1 Fad$^-$ AX-1 with the wild-type *cra* gene returned its ability to grow on the gluconeogenic substrates. Third, when *Pst*I-cut UK-1 Fad$^-$ AX-1 DNA was probed with either the wild-type *cra* gene or the chloramphenicol resistance gene in Southern hybridization experiments, a single 4.5-kb band was detected, as expected if the 3.1-kb *Pst*I DNA fragment containing the wild-type *cra* gene was replaced with the defective *cra* gene (not shown). Fourth, when UK-1 and UK-1 AX-1 DNA were amplified by PCR using the forward and reverse primers used to amplify the SR-11 wild-type *cra* gene (see Fig. 1 and the legend to Fig. 2), the amplified fragments were as expected (Fig. 2), i.e., in UK-1 a 1.4-kb fragment containing the wild-type *cra* gene and in UK-1 Fad$^-$ AX-1 a 2.8-kb fragment (the 1.4-kb *cra* fragment containing the inserted 1.4-kb chloramphenicol resistance gene).

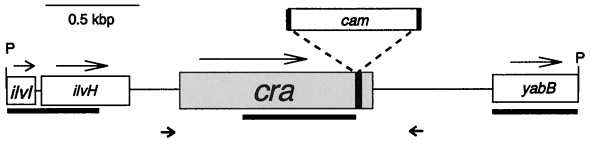

FIG. 1. Genes present in the SR-11 Fad$^-$ 4.5-kb *Pst*I DNA fragment that contains the chloramphenicol resistance gene (*cam*). Heavy lines below the genes denote regions in the fragment that were sequenced. Sequencing reactions were performed with the BigDye Terminator Cycle Sequencing Kit, and reactions were analyzed on the ABI PRISM 310 (PE Applied Biosystems, Forest City, Calif.). Nucleotide sequences were analyzed using Clone Manager and Align Plus 3 programs (Sci-Ed Software, Durham, N.C.). Arrows above the genes denote the direction of transcription. Arrows below the genes denote the positions of the forward and reverse primers used for PCR amplification of the wild-type and defective *cra* genes. P, *Pst*I.

To determine whether UK-1 Fad$^-$ AX-1 is avirulent, four BALB/c mice were infected perorally with UK-1 (2.6×10^8 CFU per mouse), and four mice were identically infected with UK-1 Fad$^-$ AX-2 (1.8×10^8 CFU per mouse). By day 8 postinfection, all four UK-1 infected mice had died, whereas the four mice infected with UK-1 AX-1 were healthy and active and had never appeared to be sick. The UK-1 Fad$^-$ AX-1-infected mice were still healthy and active when the experiment was terminated at 3 weeks postinfection. Therefore, avirulence caused by a defect in *cra* is not restricted to SR-11.

SR-11 Fad$^-$ AX-2 was also constructed by allelic exchange of the wild-type *cra* gene with the mutant *cra* gene using pMJN10 (see Table 1). SR-11 Fad$^-$ AX-2 had the phenotype of a *cra* mutant with respect to utilization of carbon sources. Moreover, SR-11 Fad$^-$ AX-2 regained the wild-type phenotype, when complemented with the wild-type *cra* gene, and

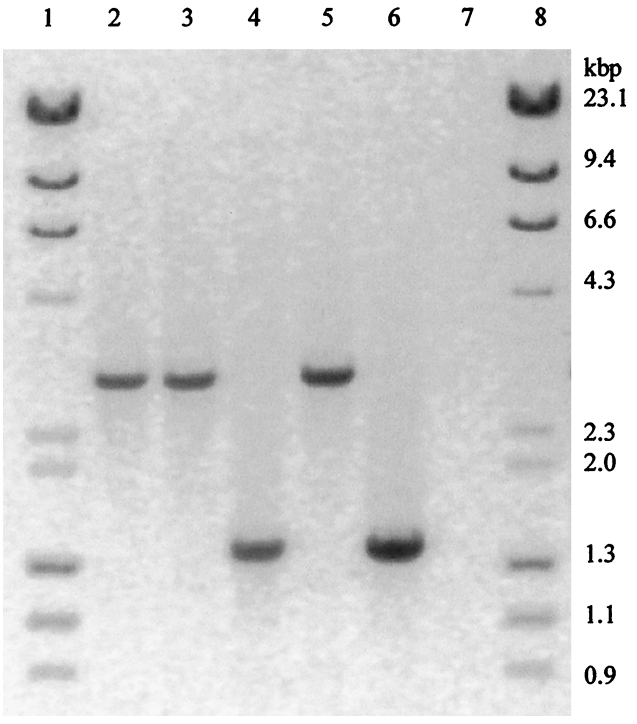

FIG. 2. PCR amplification of the defective *cra* genes of serovar Typhimurium SR-11 Fad$^-$, SR-11 Fad$^-$ AX-2, and UK-1 Fad$^-$ AX-1. Genomic DNA preparations were performed by standard techniques (13) or by following the manufacturer's instructions. Genomic DNA preparations of SR-11, SR-11 Fad$^-$, SR-11 Fad$^-$ AX-2, UK-1, and UK-1 AX-1 were amplified by PCR using 5′-ATCGACTGCAGTGCGAAATCCGTGGTAACCCGG-3′ as the forward primer and 5′-TAGCTCTGCAGCCTGTTTAACGTGTGCGGTGCC-3′ as the reverse primer. PCR reaction mixtures contained 2.0 mM magnesium chloride and 5% dimethyl sulfoxide and were subjected to 25 cycles of 94°C for 30 s, followed by 65°C for 30 s, followed by 72°C for 2.5 min. Lane 1, kilobase ladder; lane 2, SR-11 Fad$^-$ AX-2; lane 3, SR-11 Fad$^-$; lane 4, SR-11; lane 5, UK-1 Fad$^-$ AX-1; lane 6, UK-1; lane 7, no DNA added; lane 8, kilobase ladder.

contained the chloramphenicol resistance gene on a 4.5-kb *Pst*I fragment, and the expected 2.8-kb PCR product was amplified from SR-11 Fad$^-$ AX-2 using the forward and reverse primers specific to the SR-11 wild-type *cra* gene (Fig. 2). SR-11 Fad$^-$ AX-2 was also found to be avirulent. Four BALB/c mice were infected perorally with SR-11 (2.1×10^8 CFU per mouse), and five mice were identically infected with SR-11 Fad$^-$ AX-2 (2.8×10^8 CFU per mouse). By day 8 postinfection, all four SR-11 infected mice had died, whereas the five mice infected with SR-11 Fad$^-$ AX-2 were healthy and active and had never appeared to be sick. The SR-11 Fad$^-$ AX-2-infected mice were still healthy and active when the experiment was terminated at 3 weeks postinfection.

Behavior of SR-11 and SR-11 Fad$^-$ in infection models. Serovar Typhimurium utilizes nutrients present in intestinal mucus for growth in the intestine (12, 14). Mammalian intestinal mucus contains several sugars that SR-11 and SR-11 Fad$^-$ could metabolize via glycolytic pathways, including *N*-acetylglucosamine, *N*-acetylgalactosamine, galactose, fucose, sialic acid, ribose, arabinose, mannose, gluconate, and the hexuronates glucoronate and galacturonate (1, 17, 20). Since serovar Typhimurium SR-11 Fad$^-$ is not defective in its ability to utilize sugars for growth (21), it is unlikely that it is defective in the ability to grow in the mouse intestine. In support of this view, in a previous study (8), when SR-11 and SR-11 Fad$^-$ were inoculated (4.0×10^4 CFU/ml) into mouse intestinal mucus in vitro, they grew equally well, reaching levels of about 6.0×10^8 CFU/ml in 24 h at 37°C. For this reason, we turned our attention to the ability of SR-11 Fad$^-$ to adhere to and invade intestinal epithelial cells and to survive within macrophages.

S. enterica serovar Typhimurium passes through the epithelium by penetrating and destroying the M cells of the Peyer's patches (10). Both SR-11 and SR-11 Fad$^-$ invaded BALB/c mouse M cells equally well after infection of ileal loops, made as described previously (10), and both were found in enclosed vacuoles within the M cells. In addition, differences were not found in the ability of SR-11 and SR-11 Fad$^-$ to adhere to and invade human T-84 intestinal epithelial monolayers using procedures described previously (13), i.e., 2.41 ± 0.74% (mean ± the standard deviation [SD] of triplicate samples) of the SR-11 inoculum (20 CFU per T84 cell) and 2.06 ± 0.50% of the SR-11 Fad$^-$ inoculum (20 CFU per T84 cell) became cell associated in 60 min. In the same time, 0.094 ± 0.009% of the SR-11 inoculum and 0.089 ± 0.016% of the SR-11 Fad$^-$ inoculum were internalized. Collectively, these results make it unlikely that SR-11 Fad$^-$ is avirulent because it is defective in its ability to adhere to and invade M cells and/or intestinal epithelial cells.

The ability to survive in macrophages in vitro has been correlated with *S. enterica* pathogenicity in mice (7, 16). Resident peritoneal macrophages were isolated from BALB/c mice (7), and survival of SR-11 and SR-11 Fad$^-$ in macrophages was determined as described previously (7). Both SR-11 and SR-11 Fad$^-$ survived equally well in resident peritoneal macrophages. That is, after 60 min 0.057 ± 0.0075% (mean ± the SD of triplicate samples) of the SR-11 inoculum and 0.056 ± 0.018% of the SR-11 Fad$^-$ inoculum survived and, in each case, both strains remained at the 1-h survival level for the next 23 h. While these data rule out the possibility that SR-11 Fad$^-$ is inherently inferior to SR-11 in surviving in BALB/c peritoneal macrophages, they do not rule out the possibility that SR-11 Fad$^-$ is avirulent because its ability to survive and grow in macrophages in vivo is decreased relative to that of SR-11. Serovar Typhimurium may, in fact, utilize gluconeogenic substrates for survival and growth in macrophages in vivo (e.g., succinate, pyruvate, etc.). If so, SR-11 Fad$^-$ would be at a distinct disadvantage. In the present experiments, the BALB/c resident peritoneal macrophages were continuously bathed in RPMI 1640 medium. RPMI 1640 medium contains 2 g of glucose per liter which SR-11 and SR-11 Fad$^-$ metabolize equally well.

In summary, a functional *cra* gene is necessary for serovar Typhimurium virulence in BALB/c mice. The Cra protein is a regulator of central carbon metabolism (18). When interacting with the genes it regulates, i.e., in the presence of gluconeogenic substrates (18), the Cra protein positively regulates transcription of those genes encoding biosynthetic and oxidative enzymes (e.g., key enzymes in the tricarboxylic acid cycle, the glyoxylate bypass, the gluconeogenic pathway, and electron transport) and negatively regulates transcription of genes encoding glycolytic enzymes, e.g., key enzymes in the Embden-Meyerhof and Entner-Doudoroff pathways (18). Since in a *cra* mutant the genes involved in glycolytic pathways are highly expressed, whereas the expression of genes involved in the gluconeogenic pathway and the glyoxylate bypass is reduced, it is possible that gluconeogenesis is required for serovar Typhimurium virulence. In support of this view, it has recently been reported that a Mud*J* insertion in a putative malate oxidoreductase gene, involved in gluconeogenesis, renders serovar Typhimurium avirulent and immunogenic (22). Since SR-11 Fad$^-$ grows as well as SR-11 in intestinal mucus (8),

enters M cells as well as SR-11 in vivo, and adheres to epithelial cells as well as SR-11 in vitro, it is unlikely that SR-11 Fad$^-$ is avirulent because it is either defective in its ability to grow in the intestine or defective in its ability to adhere to and enter intestinal epithelial cells and M cells in vivo. If, however, the only available carbon sources for *S. enterica* serovar Typhimurium survival and growth in M cells and/or in macrophages in vivo are the gluconeogenic substrates, since SR-11 Fad$^-$ would be unable to grow under these conditions, it would not be surprising if this were the reason for its avirulence in mice. In situ experiments will be required to test this hypothesis.

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