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REGULATION OF *cra*, A REGULATORY GENE OF GLYCOLYTIC AND GLUCONEOGENIC

PATHWAYS IN *Salmonella enterica* serovar Typhimurium

BY

JAMES H. ALLEN

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE

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Abstract

The Cra protein is a global regulator of carbon and energy metabolism for glycolysis and gluconeogenesis. The live oral vaccine candidate, *Salmonella typhimurium* SR-11 Fad⁻, is unable to utilize gluconeogenic substrates as sole carbon sources and is avirulent and protective in BALB/c mice. Furthermore, the *cra* gene is interrupted in this strain of *Salmonella*. The *cra* gene was suspected to be regulated by another global regulator, acetyl phosphate. To further investigate the regulation of the *cra* gene and to determine if gluconeogenesis is linked to virulence, mutations in the gluconeogenic genes *fbp* (fructose-1,6-bisphosphatase), *maeB* (NADP-dependent malic enzyme), and *sfcA* (NAD-dependent malic enzyme) were constructed in *S. typhimurium* SR-11. A mutation in the *pta* (phosphotransacetylase) gene was also constructed to interrupt acetyl phosphate synthesis. Virulence assays in BALB/c mice were performed with these mutant strains. A *cra* promoter-*lacZ* transcriptional fusion was also inserted into the chromosome of these mutant strains to assay for the *cra* promoter activity during growth on various glycolytic and gluconeogenic substrates.

The SR-11 *fbp*⁻, SR-11 *maeB*⁻, SR-11 *sfcA*⁻ mutant strains were virulent in BALB/c mice, whereas the *pta*⁻ mutant strain was avirulent. The mutation in the *maeB* gene slightly down-regulated the *cra* promoter activity when grown on either gluconeogenic or glycolytic substrates versus the SR-11 *cradl craZ* integrant control strain. A mutation in either the *sfcA* gene or the *pta* gene significantly ($P = 0.05$) up-regulated the *cra* promoter activity for growth on both gluconeogenic and glycolytic substrates versus the control strain. This up-regulation of *cra* promoter activity, combined with their virulence in BALB/c mice, suggest that the *sfcA*-encoded NAD-dependent malic enzyme and acetyl phosphate act in concert as a repressor of the *cra* gene. It also appears that the *cra* gene may be subject to regulation by multiple regulatory proteins or multiple forms of regulation.

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Preface

The research presented in this dissertation is in the standard format.

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Introduction

General introduction. *Salmonella* species cause a variety of foodborne and waterborne illnesses ranging from localized gastroenteritis to systemic diseases such as typhoid fever in both humans and animals (1-3). The life-threatening diseases such as enteric fever, septicemia, and the focal infections, osteomyelitis and meningitis, caused by *Salmonella* involve invasion of the bacteria into the blood, the reticuloendothelial system and other organs (3).

How does *Salmonella* wreak such havoc in humans and animals? This facultative intracellular parasite, containing a 50-90 kbp virulence plasmid, has evolved an arsenal of toxins and mechanisms of pathogenesis to invade and destroy eukaryotic cells, while evading the immune system of the host cell (4-6). Fundamentally, the outer membrane of this motile gram-negative bacterium contains lipopolysaccharide (LPS) (7, 8). This endotoxin, specifically the lipid A component, is pathogenic in humans and other mammals (8, 9).

Most *Salmonella* infections result from the ingestion of contaminated water or food (2). The ingested microorganisms proceed to the intestinal tract (2). The bacteria then adhere to specialized small intestinal epithelial cells, called microfold cells (M cells), via long polar fimbriae (10). These facultative intracellular parasites produce a breach in the intestinal wall via the M cell and ultimately reach the lamina propria of the Peyer's patches (2, 10, 11). At this site, the bacteria may replicate and establish a local infection, or they may be ingested by macrophages which may disseminate these microorganisms to deeper tissues such as regional lymph nodes, the liver, and the spleen to establish a systemic infection (2). *Salmonella* possesses the ability to not only survive in and kill macrophages, but also to replicate within them and other constituents of the reticuloendothelial system (2-5, 10).

An estimated 1.4 million cases of salmonellosis, resulting in approximately 500 deaths, occur annually in the United States (12, 13). Half of the salmonellosis cases are caused by two serovars: *Salmonella enterica* serovar Typhimurium and serovar Enteritidis (12, 14). The health care costs associated with human salmonellosis caused by eating contaminated eggs and poultry is estimated at \$4 billion annually (15). Typhoid fever, a disease caused by the bacterium *Salmonella enterica* serovar Typhi, still remains a serious public health problem in developing countries with 16 million

cases of typhoid fever causing an estimated 600,000 deaths annually (16, 17). *Salmonella typhi* appears to be a human-specific pathogen, unlike *S. typhimurium*, which colonizes many animals as well as humans (18).

Poultry production also is significantly reduced by *Salmonella* bacteria (15). The U.S. poultry industry hatches approximately 7.5 billion eggs annually in incubation facilities, and nearly \$77 million is lost each year due to *Salmonella* outbreaks in poultry farms (15). Vaccination of chickens against *Salmonella* would reduce losses to the poultry industry and poultry-associated human salmonellosis.

Animal models. Although *S. typhimurium* can cause disease in numerous non-human animals, many animal carriers of *S. typhimurium* do not show any signs of disease (19). The rhesus monkey is the animal that most resembles humans in its response to a *S. typhimurium* infection (19). The guinea pig model also appears to closely mimic the disease in humans (19). Yet, these animal models are not used by most investigators (19). Most investigators use mice are routinely used as the animal model for reasons of cost and convenience (19). More importantly, the disease caused by *S. typhimurium* in mice is not the gastroenteritis observed utilizing the other animal models (19). The disease caused by *S. typhimurium* in mice mimics typhoid fever in humans and is well accepted as a model for human typhoid (20). Whatever the reason for the difference between mice and primates, humans and other primates usually control the infection within a week, whereas in mice the bacteria spread to the liver and spleen before the animal can mount an effective protective response (21).

Acid tolerance response. After ingestion of *Salmonella*, the acid tolerance response allows the microorganisms to survive passage through the acidic environment of the stomach, a prerequisite for infection (22, 23). *S. typhimurium* can survive well *in vitro* at pH values down to about pH 4, but below that rapid death of the bacteria is observed (23). However, cells given a chance to adapt (growth for one generation at pH 6), can survive at pH values as low as pH 3 for prolonged periods. The acid tolerance response appears to be regulated by the Fur protein (22, 23). Fur is required for the acid-induced activation of *atr* genes and the expression of iron uptake systems under iron-limiting conditions (22). Fur appears to be sensing and responding to pH as well as iron (23).

More recently, another acid tolerance response has been identified (23). This response is controlled by the PhoP-PhoQ two-component regulatory system, which regulates the expression of many of the virulence genes in *S. typhimurium* (23). The acid tolerance response seems to be co-regulated with the modulation of genes in helping the bacteria survive and invade once they reach the intestine. This is the first time such a clear connection between the acid tolerance response and the general virulence response has been found (23).

Two-component regulatory systems. Two-component regulatory systems (phosphorylation-dependent signal transduction systems) act like switches (24). One component, referred to as the sensor (such as the periplasmic sensor PhoQ), is a histidine kinase protein (24, 25). This protein binds ATP and is autophosphorylated at a conserved histidine residue. The second component of the switch formed by two-component regulatory systems is known as the response regulator (such as PhoP) (24, 25). The response regulator protein contains a domain that is transiently phosphorylated on a conserved aspartyl residue (24). Phosphoryl groups are transferred from the histidine residue of the sensor to the conserved aspartyl residue of the response regulator, which activates the response regulator protein (25). The activated response regulator protein is involved in the regulation of transcription of genes (25).

The expression of many of the virulence genes in *S. typhimurium* is regulated by PhoP-PhoQ in response to changes in pH and the concentration of divalent cations such as Mg^{2+} and Ca^{2+} (25). Limiting concentrations of extracellular divalent cations activate the system, resulting in the net phosphorylation of PhoP by PhoQ. Phosphorylated PhoP interacts with the promoters of PhoP-PhoQ regulated genes and activates or represses transcription of these genes (24, 25).

Examples of PhoP-PhoQ gene regulation include the activation of *mgtA*, a gene encoding a high affinity Mg^{2+} transporter and activation of the *hlyA* and *sirA* genes, which are also involved the regulation of *Salmonella* virulence genes (25, 26). PhoP-PhoQ is thought to repress *Salmonella* pathogenicity island 1 (SPI-1; large segments of DNA found in *S. typhimurium* but not in the nonvirulent *E. coli* strain K12) invasion genes while activating expression of *pags* (PhoP activated genes) (22, 26). These *pags* include the *mgtCB* operon, which also encodes a high affinity Mg^{2+}

uptake system, and genes on SPI2 (22). PhoP-PhoQ has also been implicated in modulating another putative two-component signal transduction system: PmrA-PmrB. PmrA binds to and activates promoters of certain *pags*, including several whose products alter LPS *in vitro*. Members of the PmrA regulon, including *pmrCAB* itself, are activated by PhoP-PhoQ (22).

Adherence and invasion. After passage and survival through the mouse stomach, *S. typhimurium* reaches the ileum, a section of small intestine attached to the cecum (21-23). Short-chain fatty acids in the distal ileum provide the signal for productive infection by *Salmonella* (27). The rising concentration of acetate and the low concentration of oxygen in the distal ileum provide the signal for invasion gene expression (27, 28).

S. typhimurium has a preference for the Peyer's patches in mice (21). Peyer's patches belong to a group of organized lymphoid tissues known collectively as the mucosal-associated lymphoid tissue which defend vulnerable membrane surfaces (11). The mucosal-associated lymphoid tissue carries an extremely large population of antibody-producing plasma cells. Lymphoid cells are found in three regions of the vulnerable mucus membrane which lines the gastrointestinal tract. The outer mucosal layer contains intraepithelial lymphocytes: T-cells which express unusual T-cell receptors and exhibit limited diversity for antigens (11). Below the outer mucosal layer is the lamina propria, which contains large numbers of B cells, plasma cells, activated helper-T cells, and macrophages in loose clusters. Below the lamina propria, still within the submucosal layer, are nodules consisting of 30-40 organized lymphoid follicles, called the Peyer's patches (11).

The epithelial cells of mucous membranes play an important role in promoting the immune response by delivering small samples of foreign antigen from the digestive tract to the underlying mucosal-associated lymphoid tissue (11). This antigen transport is carried out by specialized M cells, which contain a deep invagination in the basolateral membrane filled with a cluster of B cells, T cells, and macrophages (10, 11). Antigens transported across the mucous membrane by M cells activate B cells. B cells differentiate into plasma cells which secrete the IgA class of antibodies. These antibodies are then transported across the epithelial cells and released as secretory IgA into the lumen where they can interact with antigens present in the lumen (11). It is the M cell that is the major site of

invasion for *S. typhimurium* in mice (11, 21, 29). Other intestinal cells, called enterocytes, are the minor site of invasion for *Salmonella* in the murine model (28, 29).

S. typhimurium produces a number of adhesins for entry via the M cell (30). These include type 1 fimbriae (encoded by *fim* genes), plasmid-encoded fimbriae (*pef* genes), long polar fimbriae (*lpf* genes), and thin aggregative fimbriae (curli; encoded by *agf* genes). The binding specificity of type 1 fimbriae is unknown (30). The *pef* genes are located on the 90 kbp plasmid, called the virulence plasmid (pSLT), which is found in all virulent strains of *S. typhimurium*. These plasmid-encoded fimbriae mediate binding of the bacteria to the microvilli of enterocytes (30). The long polar fimbriae mediate attachment to the Peyer's patches. The thin aggregative fimbriae called curli may aid in attachment to the microvilli of enterocytes, but they also cause the bacteria to become attached to each other (30). The gene *rck* encodes a surface protein which acts as an adhesin and may be involved in invasion of tissue culture cells. The surface protein also increases the resistance of *S. typhimurium* to killing by complement (30).

Shortly after coming into close contact with the host cell, *Salmonella* induces profound changes in the brush border of the intestinal epithelium, characterized by the denaturation of microvilli at the point of contact between the bacteria and the host cell membrane (31). Interactions of *S. typhimurium* with M cells also leads to membrane ruffling (31). *Salmonella* forces the host cells to engulf it (32). Membrane ruffling and internalization of the bacteria within a membrane-bound vesicle are accompanied by extensive actin-mediated cytoskeletal and cell surface rearrangements in the vicinity of the invading bacteria (10, 32).

Once internalized, *S. typhimurium* destroys the M-cell and enters macrophages in the mesenteric lymph follicles (10, 33, 34). Macrophage survival is now important for *Salmonella* because it enables the evasion of the immune system (2, 10, 22). *Salmonella*-infected macrophages may disseminate to deeper tissues such as regional lymph nodes, liver, and spleen to establish a systemic infection (2, 10, 22). *Salmonella* possesses the ability to not only survive in and kill macrophages, but also to replicate within them and other constituents of the reticuloendothelial system (2-5, 10, 22). For example, *S. typhimurium* can enter and survive within human B and T cells, which could play a role in

the dissemination of infection (31). Furthermore, *Salmonella* can gain access to non-phagocytic cells of the liver and spleen, which may constitute a "safe site" for replication during the early phases of systemic infection (31). Overall, the main sites of replication during systemic infections in mice are the liver and spleen (35). *In vivo* studies have shown that *S. typhimurium* resides in CD18-positive leukocytes in the liver and replicates in red pulp and scavenger receptor-expressing marginal zone macrophages in the spleen (35).

Upon reaching the liver and spleen, the bacteria must continue to survive and replicate in order to cause disease in mice (22). Several genes whose products alter LPS structure and promote polymyxin resistance *in vitro* may aide *S. typhimurium* resist killing by PMN- and macrophage-produced cationic peptides at systemic sites. Also, genes on the *Salmonella* virulence plasmid are required for bacterial replication in these tissues (22). As the bacteria proliferate, they produce lipid A, promoting inflammatory cytokine and inducible nitric oxide responses (iNOS) that may kill the murine host (9, 22).

Virulence gene clusters. Many clusters of virulence genes are found in *S. typhimurium* including three *Salmonella* pathogenicity islands (SPI-1, 2, 3), two lysogenic phages (Gifsy-1, 2), and a 90 kb virulence plasmid (pSLT) (22, 36). In addition to the genes which code for a type III secretion system and its secreted proteins, SPI-1 contains genes which code for chaperones that control folding of proteins, and regulatory proteins (PhoP-PhoQ, HilA, InvF, SirA). SPI-2 carries genes for a second type III secretion system along with the genes encoding the proteins secreted by the second type III secretion system (22). Genes encoding chaperones that control protein folding are also found on SPI-2 (36). SPI-2 is involved in inhibition of phagosome-lysosome fusion and is important during the systemic phase of disease (36). SPI-3 carries genes encoding a high affinity Mg^{2+} transporter and proteins of unknown function (26, 36). SPI-3 may aid in the survival and growth of *Salmonella* in the Mg^{2+} -poor vacuole of the invaded cell (36).

So far only a few genes carried on the lysogenic phages Gifsy-1 and Gifsy-2 have been characterized (26, 36). Gifsy-2 carries a *sodCI* gene encoding superoxide dismutase (26, 37, 38). This enzyme, which converts toxic superoxide to less toxic peroxide, may help the bacteria survive the

macrophage oxidative burst (26, 36). Gifsy-2 also carries a gene (*gtgE*) that encodes the protein *gtgE*, which is necessary for full virulence in *S. typhimurium* (38). Gifsy-1 and Gifsy-2 may contribute to survival during the systemic phase of infection (36).

The virulence plasmid (pSLT) carries a gene for fimbrial adhesion (*pef*), an adhesion/serum resistance gene (*rck*), and genes coding for Spv proteins. The SpvB protein is a toxin that ADP-ribosylates actin (36, 39). Other *spv* genes encoded within pSLT are required for bacterial replication in the reticuloendothelial system (39). *S. typhimurium* carries the conjugal transfer gene *traT* making it self-transmissible (39). The virulence plasmid is important in all phases of infection (36).

Type III secretion system required for initial invasion. A type III secretion system is an essential basic virulence determinant utilizing a conserved mechanism of protein secretion (34). However, the secreted proteins themselves are highly divergent. *Salmonella* is the only genus known to possess two type III secretion systems (34). These two type III systems appear to play different roles during pathogenesis: the first being required for initial penetration of the intestinal mucosa and the second necessary for subsequent systemic stages of infection (22, 34).

The *inv* genes, along with other genes on SPI-1 (*spa*, *prg*, and *org*), encode the first type III secretion system (26, 28). The *inv* genes on SPI-1 are responsible for the membrane ruffling associated with invasion of cells by *S. typhimurium* (26). SPI-1 also carries the genes that encode the proteins that are injected into the eukaryotic cell by the type III secretion system (26). These genes include *sptP*, which encodes a tyrosine phosphatase that mimics signal transduction enzymes of eukaryotic cells and may play a role in altering the response of mucosal cells to outside stimuli (26). SopE and SipA are additional proteins injected into the mucosal cells via the type III secretion system (26, 34). These proteins are thought to be responsible for the ruffling response (26). SopE can activate host cell G proteins such as Cdc42 and Rac, which control actin polymerization. Activation of these proteins by SopE initiates the actin rearrangements involved in the ruffling response. Ruffling is a very localized phenomenon; localization of the host cell's response is thought to be the role of SipA (26). SipA binds actin directly and inhibits depolymerization, thus increasing the amount of

polymerized actin in its vicinity. SipA is confined to the immediate area in which the bacteria are found, accounting for the localized nature of the ruffling phenomenon (26).

Survival of *Salmonella* in macrophages. Macrophages are phagocytic cells capable of ingesting and digesting exogenous antigens such as whole bacteria (40). *S. typhimurium* enters host macrophages and can induce either an almost immediate cell death or establish an intracellular niche within the phagocytic vacuole (41). Rapid cell death depends on the SipB effector on *Salmonella* pathogenicity island SPI-1 and the host protein caspase-1 (35,41). Caspase-1 is a member of the pro-apoptotic caspase family of proteases, and the process was originally thought to be apoptotic (35, 41). Recent studies suggests that it is an unusual form of necrosis (35). Caspase-1-dependent cell death leads to the activation of the potent pro-inflammatory cytokines interleukin (IL)-1 β and IL-18 to produce bioactive cytokines (41). Animal studies indicate that the activation of these cytokines is necessary for infection and efficient colonization of the murine gastrointestinal tract (41). *Salmonella* that reside in the phagocytic vacuole do not cause this early cell death and are capable of triggering macrophage death at a much later time point (41). This late-phase cell death is dependent upon genes encoded on the *Salmonella* pathogenicity island SPI-2 and the transcription factor OmpR (41, 42).

After uptake, *Salmonella* resides within a unique organelle, the *Salmonella*-containing vacuole (SCV; *Salmonella*-containing phagosome) in which it eventually replicates (43). Studies of the *S. typhimurium* vacuole in macrophages have been hampered by its heterogeneous behavior (35). Infection of primary macrophages or a macrophage cell line by a culture of *S. typhimurium* results in variable numbers of bacteria in each host cell (35). For example, twelve hours after bacterial uptake, some macrophages contain clusters of numerous bacteria, while in others only a few dispersed bacteria are visible (35). This probably reflects the simultaneous processes of growth and killing that occur in murine macrophages (35).

There are also conflicting results regarding the fusion of the SCV with lysosomal compartments (35). Two groups concluded from studies involving primary macrophages that SCVs fuse with lysosomal compartments (35). Conflicting evidence suggests that *Salmonella* can inhibit phagosome-lysosome (SCV-lysosome) fusion in both primary macrophages and macrophage-like cell

lines (35, 44). Despite this, a consensus has emerged over the last few years that is generally consistent with the current understanding of SCVs (35). According to this, the majority of SCVs acidify but fail to acquire lysosomal hydrolytic enzymes or reactive oxygen intermediates that would normally accumulate in a phagolysosome (35). There is evidence that both the PhoP-PhoQ two-component regulatory system and SPI-2 type III secretion system play important roles in this process, which leads to the establishment of a compartment which is conducive to bacterial replication (35). Replicating bacteria remain in membrane-bound vacuoles, and this requires a continuous supply of membrane to enclose dividing bacterial cells. The SPI-2 effector protein SifA, and the assembly of an actin meshwork, play major roles in this aspect of intracellular growth (35).

Genes required for the survival and replication of *Salmonella* in macrophages. The interior of a macrophage can be a very inhospitable environment for a bacterium. However, *S. typhimurium* has acquired an arsenal of genes involved in the survival and replication inside macrophages (35). Many of the genes' functions are still unknown. Genes encoded within SPI-2 involved with the SPI-2 type III secretion system and their function are as follows: *ssaC* (subunit forming the outer membrane secretin porin), *ssaV* (secretion of SPI-2 proteins), *sseA* (required for survival and replication), *sseBCD* (translocation of SPI-2 effectors), *sseFG* (interaction of phagosomal membranes after translocation; contributes to Sif formation in epithelial cells), *sseJ* (regulates the dynamics of the SCV membrane), *ssrAB* (two-component system regulating SPI-2 gene expression), *spiC* (inhibition of trafficking; actin polymerization), and NC (the gene has not been characterized but is associated with SPI-2 secretion and involves inhibition of the oxidative burst) (35, 45-48).

Survival and replication of *Salmonella* in macrophages requires other genes associated with the SPI-2 type III secretion system, which are encoded outside of SPI-2 (35). The genes and their function are as follows: *ompR-envZ* (two-component system regulating the acid-induced *ssrAB* virulence operon expression), *sifA* (contributes to Sif formation in epithelial cells and maintains the SCV membrane), *srfK* (unknown but regulated by *ssrAB*), and *sseJ* (required for translocation of effector proteins) (35, 48-50).

Many genes, required for the survival and replication of *Salmonella* in macrophages, are controlled by the PhoP-PhoQ regulon (35). These include *mgtC* (magnesium acquisition), NC (the gene has not been characterized but it is involved in the inhibition of SCV-late endosomal interactions) and *pags* (PhoP activated genes; LPS modification and resistance to antimicrobial peptides) (35).

Other genes, neither associated with the SPI-2 type III secretion system nor controlled by the PhoP-PhoQ regulon, are required for the survival and replication of *Salmonella* in macrophages (9, 22, 35, 36, 42, 45-55). The genes and their function are as follows: *spvB* (ADP-ribosylation of actin), *sodA* and *sspJ* (resistance to oxidative stress), *slyA* (regulates resistance to oxidative stress), *prc* (periplasmic protease), *htrA* (stress-induced serine protease), *rpoE* (stress response regulator), *smpB* (unknown function), *feoB* (ABC ferrous iron transporter), *mntH* (proton-dependent manganese transporter), and *sitABCD* (putative ABC iron and/or manganese transporter), *lon* (ATP-dependent protease), *zwf* (glucose-6-phosphate dehydrogenase, the product of the first enzymatic step in the pentose pathway; resistance to reactive oxygen and nitrogen intermediates via NADPH), *sodCI* (superoxide dismutase; intercepts reactive oxygen species), *sodCII* (intercepts reactive oxygen species), *htrA* and *surA* (intramacrophage survival; protein folding and/or degradation in the periplasm), *fkpA* (peptidylprolyl isomerase; invasion and survival) *waaN* (biosynthesis of lipid A; induces cytokine and inducible nitric oxide synthase (iNOS) responses) *virK* and *somA* (remodeling of the outer bacterial membrane), and *msgA* (unknown function) (9, 22, 35, 36, 42, 51-55).

Regulation of virulence genes by two-component signal transduction systems. Several two-component signal transduction systems regulate virulence genes *in vitro* (22). PhoP-PhoQ and PmrA-PrmB are two which have already been discussed in detail (see Acid tolerance response, Two-component regulatory systems, and Genes required for the survival and replication of *Salmonella* in macrophages). Evidence indicates that PhoR-PhoB, which regulates genes in response to inorganic phosphate (P_i) concentration, can repress SPI-1 invasion genes (22). PhoR-PhoB may also activate SPI-2 genes because their expression is induced by low P_i levels *in vitro*. OmpR-EnvZ, a two-component regulatory system that modulates gene expression in response to osmotic conditions and pH, may also regulate virulence genes because disrupting this regulatory pathway decreases SPI-1

gene expression (22, 50). Furthermore, recent evidence indicates that OmpR may play a role in regulation of SPI-2 genes (10, 22). Two other two-component signal transduction systems, SsrA-SsrB and BarA-SirA, respond to unknown environmental cues (22). SsrA-SsrB regulates SPI-2 gene expression of the SPI-2 type III secretion system as well as its translocated effectors; SsrA-SsrB is in turn regulated by the OmpR-EnvZ two-component system (22, 50, 56). BarA-SirA is necessary for full expression of invasion genes on SPI-1 and SPI-4 (22, 27). Another two-component system, CsrA-CsrB, alters RNA stability which may modulate invasion gene expression (22). Finally, the superoxide radical response regulon, SoxR-SoxS, can upregulate the expression of the *zwf* gene, which confers resistance to reactive oxygen and nitrogen intermediates via NADPH (53).

Regulation of virulence genes by alternative sigma factors. The alternative sigma factor, RpoS, which controls many genes expressed in stationary phase and for stress protection, also regulates virulence genes (57, 58). RpoS is necessary for sustaining a log-phase acid tolerance response, induction of a stationary-phase acid survival system, production of thin aggregative fimbriae, and expression of *spv* genes during stationary phase (22). The *rpoE* gene, which encodes the extracytoplasmic stress response sigma factor sigmaE (RpoE), is critically important for the virulence of *S. typhimurium* (59). RpoE regulates many genes required for survival in macrophages and proteins involved in protein folding and/or degradation in the periplasm (53, 54, 59).

Other mechanisms of regulation for virulence genes. The DNA adenine methylase enzyme (*dam*), which modifies GATC sequences, has pleiotropic effects on the expression of many different virulence genes (22, 60, 61). To begin with, Dam methylation regulates the expression of plasmid-encoded fimbriae (*pef*) encoded by the pSLT virulence plasmid (22, 36, 61). The conjugative transfer of this virulence plasmid, which requires the *finP*-encoded F-type pili, is regulated by the levels of Dam methylation; the transfer of the pSLT virulence plasmid is elevated by low levels of Dam methylation (61). A *dam*⁻ mutation in *S. typhimurium* revealed reduced secretions of invasion effectors encoded in SPI-1 genes (22, 62). In addition, a reduction in the relative amount of peptidoglycan-associated lipoprotein, OmpA (a highly immunogenic, non-specific, outer membrane porin, and murein lipoprotein bound to peptidoglycan was observed in actively growing Dam⁻ mutants, indicating

increased cell envelope instability (62). The Dam⁻ mutant was unable to proliferate in target organs *in vivo* but persisted in low numbers (63). Use of the ileal loop assay revealed that Dam⁻ mutants were less cytotoxic to M-cells and failed to invade enterocytes (63). In the tissue culture model, lack of DNA adenine methylation reduced the ability of the bacteria to invade non-phagocytic cells (63). Collectively, these results indicate that DNA methylation plays several roles in regulating virulence genes in *S. typhimurium* (22, 62, 63).

Other regulators specifically modulate the expression of virulence genes whose products are thought to have related functions (22). For example, the regulators RtsA, HilA, InvF, HilC, HilD, and HilE directly or indirectly modulate the expression of genes encoding the SPI-2 type III secretion system and its secreted effectors (22, 64-66). FimZ and AgfD are two other regulators believed to modulate specific virulence genes of related function and are required for *in vitro* expression of type I fimbriae and thin aggregative fimbriae (22).

Many regulators control the expression of multiple virulence genes (22, 67). The global regulator CRP (catabolite repressor protein) regulates a variety of genes in *Salmonella* in response to the levels of cAMP (67). CRP negatively regulates the *spv* operon on the *Salmonella* virulence plasmid, while positively regulating various fimbrial operons (67). The *fliZ* gene positively regulates the expression of class II flagellar genes and induces SPI-1 invasion gene expression (22).

Growth requirements and central fueling pathways of *S. typhimurium*. *E. coli*, studied more extensively than *S. typhimurium*, requires the synthesis of twelve precursor metabolites for growth: glucose-6-phosphate, fructose-6-phosphate, ribose-5-phosphate, erythrose-4-phosphate, triose-3-phosphate (glyceraldehyde-3-phosphate), 3-phosphoglycerate (from glycerate-1,3-bisphosphate), phospho(enol)pyruvate, pyruvate, acetyl coenzyme A (acetyl-CoA), α -ketoglutarate, succinyl-CoA, and oxaloacetate (68, 69). Since *S. typhimurium* contains homologues for the enzymes in the pathways that synthesize these precursor metabolites, it is assumed that *S. typhimurium* grows essentially the same (except where noted) as *E. coli* (70, 71).

These twelve precursor metabolites, except acetyl-CoA, are synthesized by a series of central fueling pathways that are collectively called central metabolism (72, 73). Central metabolism includes

the Embden-Meyerhof-Parnas (EMP) pathway, which converts glucose-6-phosphate to pyruvate; the tricarboxylic acid (TCA) cycle, which oxidizes acetyl-CoA to CO₂; and the pentose phosphate cycle, which oxidizes glucose-6-phosphate to CO₂ (Figure 1) (72-74). Acetyl-CoA is synthesized by a linker reaction between the EMP pathway and the TCA cycle (72). The Entner-Doudoroff pathway is also utilized by *S. typhimurium* to metabolize gluconate (Figure 1) (72, 73). Regardless of the fueling reactions that *S. typhimurium* employs, these twelve precursor metabolites are synthesized and are the metabolic link between fueling and biosynthesis (68).

The precursor metabolites, along with inorganic ions, are required for the biosynthesis of cellular components (75, 76). For example, glucose-6-phosphate yields the sugar backbone of nucleotides, and fructose-6-phosphate is converted to amino sugars (69). Ribose-5-phosphate is the precursor metabolite for purine and pyrimidine nucleotides as well as the heptose component of LPS (67, 74). Erythrose-4-phosphate is utilized for the biosynthesis of aromatic amino acids (69, 76). Triose-3-phosphate (glyceraldehyde-3-phosphate) is the precursor metabolite for the biosynthesis of nicotinamide coenzymes and phospholipids (76). The biosynthesis of the amino acids serine, glycine, cysteine, and tryptophan use 3-phosphoglycerate as their precursor metabolite (69, 76). Phospho(enol)pyruvate is the building block for vitamins and cofactors as well as aromatic amino acids. Pyruvate is utilized for the biosynthesis of the amino acids alanine, valine, leucine, and isoleucine (69, 76). Acetyl-CoA is the precursor metabolite for fatty acids and the outer membrane component murein. The amino acids glutamate, glutamine, arginine, and proline are all synthesized from α -ketoglutarate (67, 74). The precursor metabolite succinyl-CoA is used to form heme (76). Finally, the oxaloacetate is the precursor metabolite for the biosynthesis of the amino acids aspartate, asparagine, threonine, methionine, and isoleucine (68, 69).

There is tremendous flexibility in the way the central fueling pathways operate (72). For example, formation of the precursor metabolites during aerobic growth of *S. typhimurium* on a limiting source of glucose utilizes a major anapleurotic reaction (74, 77-79). Anapleurotic reactions are the interconnecting, reversing, and bypassing reactions which replenish the pools of precursor metabolites drained by biosynthesis (68). This anapleurotic reaction, catalyzed by the *ppc*-encoded enzyme

phospho(enol)pyruvate carboxylase, forms oxaloacetate by carboxylation of phospho(enol)pyruvate (Figure 1) (77). Components of the TCA cycle function almost exclusively to provide three precursor metabolites, not as an energy-generating cycle (77).

Aerobic growth of *S. typhimurium* on a gluconeogenic substrate such as malate is extensively different from growth on a glycolytic substrate such as glucose: TCA components are shunted off into the EMP pathway to form the required precursor metabolites (77). Two routes lead from malate to pyruvate in the EMP pathway; the *maeB*- and *sfcA*-encoded malic oxidoreductase enzymes catalyze these redundant reactions (Figure 1) (71, 77, 80, 81). Oxaloacetate is converted to phospho(enol)pyruvate and also shunted off into the EMP pathway (Figure 1) (77). A reversal of flow in the EMP pathway, called gluconeogenesis, occurs (72, 73, 77, 82). Gluconeogenesis leads to the pentose phosphate cycle and both produce the remaining precursor metabolites (72, 77). The total cost of fueling reactions operating to produce precursor metabolites is much greater for a gluconeogenic substrate, such as malate, than for a glycolytic substrate such as glucose (83). Therefore, growth on gluconeogenic substrates, such as malate, is slower than growth on glycolytic substrates such as glucose (77).

Levels of nutrients and oxygen at specific locations in the *S. typhimurium* infection process. The level of nutrients are high and the oxygen level is very low in the lumen and mucus of the ileum (28, 33, 84). Inside the M cell, or epithelial cell vacuole, the nutrient and oxygen levels are both low (84). Inside macrophages, the level of nutrients is low and the oxygen level is moderate (35, 84). The spleen contains high levels of nutrients and oxygen (28, 85). *S. typhimurium* replicates in the CD18-positive leukocytes in the liver which are bathed by hepatic blood vessels (35, 85). It is assumed that both the levels of nutrients and oxygen are moderate to high. The levels of oxygen appear to be from microaerophilic to aerobic at all the specific locations of the *S. typhimurium* infection process (28, 33, 35, 85).

Glycolysis and the utilization of glycolytic substrates. Glycolysis, also called the Embden-Meyerhof-Parnas (EMP) pathway, is the metabolic pathway utilized to break down glucose to pyruvate (73, 87-89). Four important events occur during glycolysis; {1} substrate-level phosphorylation which

is the synthesis of adenosine triphosphate (ATP) by the donation of a high-energy phosphate to adenosine diphosphate (ADP) from a reaction coupled with the exergonic breakdown of a high-energy substrate molecule, {2} the breaking of the six-carbon glucose molecule into two three-carbon pyruvate molecules, and {3} the transfer of two electrons to the coenzyme nicotinamide adenine dinucleotide (NAD^+) to form $\text{NADH} + \text{H}^+$ (87, 89).

The usual route for glucose uptake and phosphorylation is the phospho(enol)pyruvate phosphotransferase system (PTS) (73). The PTS transports and simultaneously phosphorylates glucose in a process called group translocation (90). The PTS in *S. typhimurium* contains general PTS proteins and proteins specific for glucose transport (91). The general PTS proteins, enzyme I (EI) and histidine protein (Hpr) are the soluble cytoplasmic proteins that participate in the phosphorylation of all PTS carbohydrates (91). EI and Hpr in *S. typhimurium* are encoded by the genes *ptsH* and *ptsI* respectively (90-92). The EII^{Glc} enzyme is glucose-specific, membrane-bound, encoded by the gene *ptsG*, and consists of two domains: IIC-IIB (90, 91, 93). The final component, the *crr*-encoded enzyme IIA^{Glc} , is soluble (91, 92, 94). All of the PTS enzymes and proteins participate, in sequence, in the transfer of a phosphoryl group to glucose (91). Glucose phosphorylation is coupled to glucose translocation across the cytoplasmic membrane, the energy for these processes being provided by the glycolytic intermediate phospho(enol)pyruvate (91).

Another enzyme, the *glk*-encoded glucokinase, plays a minor role in glucose metabolism (73, 95, 96). Under anabolic stress the enzyme may be required to supplement the levels of glucose-6-phosphate (95). Both the PTS and glucokinase yield the first glycolytic intermediate: glucose-6-phosphate (Figure 1) (73, 91, 95).

The interconversion of glucose-6-phosphate to fructose-6-phosphate by the enzyme glucosephosphate isomerase (*pgi*) is the next step in glycolysis (Figure 1) (73, 97, 98). Fructose-6-phosphate is then phosphorylated to form fructose-1,6-bisphosphate by a pair of isozymes, 6-phosphofructokinase-I (*pfkA*) and 6-phosphofructokinase-II (*pfkB*) (73, 88, 99-102). These reactions are irreversible and provide one of the key control points for glycolysis (82). The main phosphofructokinase is PFK-1 since it is 9-fold more active than PFK-II (73, 97, 99). PFK-1 is

activated allosterically by nucleoside diphosphates (ADP and GDP) and inhibited by phospho(enol)pyruvate (73, 100). PFK-1 is somewhat sensitive to inhibition by fructose-1,6-bisphosphate and ATP (100).

S. typhimurium contains genes for both the fructose bisphosphate aldolase Class II enzyme (*fba*) and the fructose bisphosphate aldolase Class I enzyme (*fbaB*) (73, 92, 93, 103, 104). These enzymes split fructose-1,6-bisphosphate into dihydroxy-acetone-phosphate and glyceraldehyde-3-phosphate (Figure 1) (73, 103, 104). The fructose bisphosphate aldolase Class II enzyme requires divalent metal ions such as Zn^{2+} , Co^{2+} , Fe^{2+} , Ni^{2+} , or Mn^{2+} (73, 103).

In the subsequent reactions of glycolysis, triose phosphate isomerase (*tpiA*) interconverts dihydroxy-acetone-phosphate and glyceraldehyde-3-phosphate (Figure 1) (73, 92, 105). Next, the glyceraldehyde-3-phosphate dehydrogenase-A complex (*gapA*) converts glyceraldehyde-3-phosphate to glycerate-1,3-bisphosphate (73, 92, 106, 107). The reaction adds a phosphate group while NAD^+ is reduced to NADH (87, 106, 107). A subsequent sequence of reactions occurs for the conversion of glycerate-1,3-bisphosphate to phospho(enol)pyruvate (73). The sequence of reactions include the enzymes phosphoglycerate kinase (*pgk*), phosphoglycerate mutase 1 (*gpmA*), phosphoglycerate mutase 2 (*gpmB*), phosphoglyceromutase (*pmgI*), and enolase (*eno*) (73, 92, 93, 108-114). ATP is produced in this sequence of reactions by substrate-level phosphorylation (89).

The last step in glycolysis is the conversion of phospho(enol)pyruvate to pyruvate by the isozymes, pyruvate kinase I (*pykF*) and pyruvate kinase II (*pykA*) (73, 115-118). ATP is produced in these reactions by substrate-level phosphorylation (89). These reactions are irreversible and provide a second key control point for glycolysis (Figure 1) (82). Both pyruvate kinases require divalent and monovalent cations, usually Mg^{2+} and K^+ (117). Pyruvate kinase I is activated by low Ca^{2+} and Mg^{2+} concentrations and is strongly activated by fructose-1,6-bisphosphate (73, 115). Inhibitors of pyruvate kinase I include succinyl-CoA, ATP, and high concentrations of Ca^{2+} (115). Pyruvate kinase II is activated by AMP and several sugar phosphates (73, 117).

Phospho(enol)pyruvate can also bypass the conversion to pyruvate and take an alternate route: the enzyme phospho(enol)pyruvate carboxylase (*ppc*) converts it to oxaloacetate (Figure 1) (73, 92,

119). Phospho(enol)pyruvate carboxylase is a regulatory enzyme which replenishes oxaloacetate in the TCA cycle; oxaloacetate is the precursor of other intermediates of the TCA cycle and of major families of biosynthetic products (73, 119). Phospho(enol)pyruvate carboxylase is allosterically activated by acetyl-CoA, fructose-1,6-bisphosphate, guanosine triphosphate (GTP), and fatty acids (119). L-aspartate and malate allosterically inhibit phospho(enol)pyruvate carboxylase (119).

For the purposes of this study, a glycolytic substrate is considered any carbohydrate such as glucose, fructose, and gluconate which enters the glycolytic (EMP) pathway.

Gluconeogenesis and the utilization of gluconeogenic substrates. The EMP pathway is an amphibolic system and functions in both glycolysis and gluconeogenesis (82). Most of the reactions are reversible, with the notable exception of the 6-phosphofructokinase (*pfkAB*) and the pyruvate kinase (*pykAF*) catalyzed reactions which are glycolytic (Figure 1) (73, 82). These two steps in the Embden-Meyerhof pathway are replaced in the gluconeogenic pathway by the *fbp*-encoded enzyme fructose-1,6-bisphosphatase and the *pps*-encoded enzyme phospho(enol)pyruvate synthetase (Figure 1) (73, 82, 92, 120-123). Fructose-1,6-bisphosphatase is inhibited by adenosine monophosphate (AMP), ADP, glucose-6-phosphate, and phospho(enol)pyruvate (92, 122).

Three other reactions, involving TCA intermediates, are considered part of gluconeogenesis (73, 120). The enzyme phospho(enol)pyruvate carboxykinase (*pckA*) irreversibly converts oxaloacetate to phospho(enol)pyruvate, requires ATP, and is inhibited by NADH, phospho(enol)pyruvate, and high concentrations of ATP (Figure 1) (73, 93, 120, 124). Two highly-regulated malate oxidoreductases, an NADP-dependent malic enzyme (*maeB*) and an NAD-dependent malic enzyme (*sfcA*), irreversibly convert malate to pyruvate (73, 120, 123, 125, 126). The NADP-dependent malic enzyme is activated by NH_4^+ and K^+ (125). This enzyme is both competitively inhibited by malonate and glutarate and also allosterically inhibited by oxaloacetate, acetyl-CoA, NAD^+ , NADP^+ , and cAMP (125). The NAD-dependent malic enzyme is allosterically activated by L-aspartate and allosterically inhibited by ATP, coenzyme A, and acetyl-CoA (126). Malate also activates this enzyme by an unknown mechanism (126).

For the purposes of this study, a gluconeogenic substrate is considered any substrate which enters the gluconeogenic pathway such as acetate, alanine, glycerol, citrate, fumarate, malate, oleate, phospho(enol)pyruvate, pyruvate, and succinate.

The fate of pyruvate in *S. typhimurium* metabolism. The oxidation of hexoses, such as glucose, to pyruvate by the EMP pathway generates two molecules of pyruvate and two molecules of NADH (127). To maintain glycolytic flux, the NADH must be oxidized to NAD^+ (127). One option for regenerating these reducing equivalents in the absence of oxygen is by a process called fermentation (127, 128). Fermentation occurs by depositing the reducing equivalents on partially oxidized metabolic intermediates, which are then excreted from the cell (127). The fermentation products comprise a mixture of ethanol, and acetic, formic, lactic, and succinic acids (127). Since the carbon atoms in the metabolic intermediates are only partially oxidized and the difference in reduction potentials between the primary electron donor and terminal electron acceptor is small, the fermentation processes yield little energy (129).

Another option for regenerating these reducing equivalents which occurs in the presence of oxygen is a process called aerobic respiration; this process involves an electron transport system and oxidative phosphorylation (129-134). In this process which utilizes O_2 as the final electron acceptor, all the substrate molecules can be oxidized completely to CO_2 , and a far higher yield of ATP is theoretically possible (129-131). Two hydrogen atoms, each consisting of one proton and one electron, are transferred from NADH to a series of other carrier compounds (NADH dehydrogenases, flavoproteins, nonheme iron-sulfur proteins, quinones, and cytochromes) embedded within the cell membrane (130-134). This electron transport chain separates the protons from the electrons during the transport process (131, 133, 134). The protons are extruded into the periplasm; the net result is the generation of a pH gradient and an electrical potential across the membrane, with the inside of the cytoplasm electrically negative and alkaline, and the outside of the membrane electrically positive and acidic (131, 133, 134). Membrane-bound catalytic ATPases act as proton channels which drive the formation of ATP from ADP plus inorganic phosphate (131, 133, 134). This ATP-producing process is known as oxidative phosphorylation (131, 133, 134).

Aerobic respiration via the TCA cycle and oxidative phosphorylation require that pyruvate first be converted to acetyl-CoA (131, 133, 134). Acetyl-CoA is synthesized by a linker reaction between the EMP pathway and the TCA cycle (72). The pyruvate dehydrogenase multienzyme complex (*aceEF*, *lpdA*), one of the most complicated enzyme systems known, converts pyruvate to acetyl-CoA (92, 135). NAD^+ is reduced to NADH and CO_2 is generated in the reaction (135). Inhibitors of the reaction include glyoxylate, 2-oxobutanoate, pyruvate, and high concentrations of NAD^+ (135).

The TCA cycle and glyoxylate bypass. The TCA cycle is an inducible pathway with the levels of the enzymes responding primarily to the presence of oxygen and to the carbon source(s) available (74). The levels of the TCA cycle enzymes are also regulated at the transcriptional level by the interaction of two global regulatory systems: catabolite (glucose) repression and the ArcA-ArcB two-component regulatory system (136). The full TCA cycle is functional only during aerobic growth on acetate or fatty acids (Figure 1) (74). However, growth on acetate or fatty acids requires the induction and function of an anapleurotic pathway, the glyoxylate shunt, to replenish the dicarboxylic acid intermediates consumed in amino acid biosynthesis. (74, 137, 138).

Citrate synthase (*gltA*), the rate-limiting step in the TCA cycle, converts oxaloacetate plus acetyl-CoA to citrate (Figure 1) (74, 92, 139). The synthesis of the enzyme is subject to catabolite repression and induced by O_2 when acetate is the carbon source (74, 139). The enzyme is activated by acetyl-CoA and K^+ ions and inhibited by 2-oxoglutarate, ATP, NADH, oxaloacetate, and NAD^+ (74, 139).

There are two aconitases in *S. typhimurium*, aconitate hydrotase 1 (*acnA*) and aconitate hydrotase 2 (*acnB*) (Figure 1) (74, 92, 93, 140, 141). Both catalyze the reversible isomerization of citrate and isocitrate via *cis*-aconitate and contain a labile iron-sulfur cluster (74, 140, 141). The main role of the aconitate hydrotase 1 enzyme is one of a maintenance or survival enzyme during nutritional or oxidative stress and it is inhibited by NO_3^- by an unknown mechanism (140). The aconitate hydrotase 2 enzyme functions as the main catabolic enzyme (141).

Next, isocitrate dehydrogenase (*icdA*) converts isocitrate to α -ketoglutarate (Figure 1) (74, 92, 142). Nicotinamide adenine dinucleotide phosphate (NADP^+) is reduced to NADPH and CO_2 is generated in the reaction (74, 142). Isocitrate dehydrogenase was the first bacterial enzyme shown to be regulated by phosphorylation/dephosphorylation (74, 142). The enzyme is inactivated by phosphorylation which also affects the binding of NADP^+ (74, 142).

The enzymes encoded by the three genes, *sucA*, *sucB*, and *lpdA*, are required for the 2-oxoglutarate dehydrogenase complex (Figure 1) (74, 92, 143). The substrate, α -ketoglutarate, is channeled through the catalytic reactions via a classical swinging arm carrying the substrate molecules to each successive active site (74, 143). The final products are succinyl-CoA and CO_2 (74, 143). NAD^+ is also reduced to NADH (74, 143).

Succinyl-CoA synthetase next catalyzes the reaction which yields succinate, coenzyme A and ATP from succinyl-CoA (Figure 1) (74, 144). This tetrameric enzyme contains two α -subunits (*sucD*) and two β -subunits (*sucC*) (74, 92, 144). This is the only reaction in the TCA cycle that employs substrate-level phosphorylation (74, 144).

Succinate is subsequently converted to fumarate by succinate dehydrogenase (Figure 1) (74, 145). Succinate dehydrogenase is encoded by four genes: the *sdhA* gene encodes a flavoprotein subunit containing a covalently bound flavin adenine dinucleotide (FAD) moiety; the *sdhB* gene encodes an iron-sulfur protein; the *sdhC* and *sdhD* genes encode two very hydrophobic membrane proteins which serve to anchor the hydrophilic flavoprotein and iron-sulfur protein subunits to the cytoplasmic membrane and also participate in electron transport (74, 92, 145). Succinate dehydrogenase is made under aerobic conditions and FAD is also temporarily reduced to FADH_2 during the reaction (74, 145). Enzyme synthesis is regulated by catabolite repression (74). Activation of the enzyme by covalent attachment of FAD to the SdhA enzyme subunit is promoted by intermediates of the TCA cycle (74).

S. typhimurium contains three distinct fumarases, encoded by the *fumA*, *fumB*, and *fumC* genes, which all convert fumarate to malate (Figure 1) (74, 92, 146-148). The cell adapts to changing environmental oxygen concentrations by utilizing different isozymes (74, 146-148). Fumarase A is the

major active enzyme under microaerophilic conditions (1 to 2% oxygen) and is constitutively synthesized under fermentation and microaerophilic conditions (146). Fumarase B also has some activity under microaerophilic conditions (147). Both fumarase A and fumarase B decrease activity when oxygen levels are greater than 15% (146, 147). Fumarase C is highly active under aerobic conditions (21% oxygen) (148). This provides the cell with an active fumarase under highly oxidative conditions (>4% oxygen), a situation where fumarase A is inactivated (146, 148).

The last reaction in the TCA cycle is the conversion of malate to oxaloacetate by the dimeric enzyme malate dehydrogenase (*mdh*) (Figure 1) (74, 92, 149). Enzyme synthesis is subject to catabolite repression and is co-regulated with *fumA*; *mdh* and *fumA* have a common catabolite repressor protein (CRP) binding site upstream (149). NADH is an allosteric inhibitor of malate dehydrogenase (149). NAD⁺ is also reduced to NADH in this final reaction in the TCA cycle (149).

The glyoxylate bypass in *S. typhimurium* is an inducible anapleurotic pathway within the TCA cycle required for growth on carbon sources such as acetate or fatty acids (74, 137, 138). This pathway allows the net conversion of acetyl-CoA to metabolic intermediates (74). Strains lacking this pathway fail to grow on these carbon sources since acetate carbon entering the TCA cycle is quantitatively lost as CO₂ with no means to replenish the TCA precursor metabolites consumed for amino acid and heme biosynthesis (Figure 1) (68, 69, 74).

The first enzyme in the glyoxylate bypass is isocitrate lyase (*aceA*), which catalyzes the conversion of isocitrate to glyoxylate and succinate (Figure 1) (74, 92, 150). Isocitrate lyase is activated by phosphorylation and inhibited by Ca²⁺, Cl⁻, oxalate, *cis*-aconitate, 3-phosphoglycerate, 2-oxoglutarate, phospho(enol)pyruvate, succinate, glycolate, methylmalonate, and hydroxymalonnate (150). Isocitrate lyase competes with the TCA cycle enzyme, isocitrate dehydrogenase, for isocitrate (74). Another enzyme, isocitrate dehydrogenase kinase/phosphatase (*aceK*), is needed to decrease the activity of isocitrate dehydrogenase to allow isocitrate lyase to effectively compete for isocitrate (74, 92, 151). The second enzyme in the glyoxylate bypass is malate synthase A (*aceB*), which catalyzes the conversion of glyoxylate plus acetyl-CoA to malate and coenzyme A (74, 92, 152). Malate synthase A is induced by growth on acetate and repressed by most other carbon sources (152).

In *S. typhimurium*, isocitrate dehydrogenase (*icdA*) is regulated by phosphorylation; the function of this phosphorylation is to control the flow of isocitrate through the glyoxylate bypass (Figure 1) (74, 92, 142). During growth on acetate, approximately 75% of the isocitrate dehydrogenase is converted to the inactive phosphorylated form by isocitrate dehydrogenase kinase/phosphatase (74, 151). Inhibition of the isocitrate dehydrogenase slows the TCA cycle and this forces isocitrate through the glyoxylate bypass (74, 151). Although the glyoxylate bypass can provide metabolic intermediates, the TCA cycle is more efficient at generating energy (74). The cell must, therefore, precisely balance the flux of isocitrate between these two competing pathways during growth on acetate; the energy requirements of the cell appear to be monitored through AMP levels (74). AMP activates the phosphatase moiety and inhibits the kinase moiety of isocitrate dehydrogenase kinase/phosphatase (74, 151). Isocitrate dehydrogenase kinase/phosphatase also controls the glyoxylate bypass during transitions between carbon sources (74, 151).

The genes which encode the metabolic and regulatory enzymes of the glyoxylate bypass reside in the same operon, *aceBAK*, containing a single promoter (74, 92, 150-152). The operon is regulated by a repressor encoded by the *icIR* gene (74, 138). Derepression of the *aceBAK* operon occurs upon adaptation to growth on acetate or fatty acids, presumably involving some metabolic intermediate; FadR, a repressor of fatty acid degradation, mediates a part of the derepression process when the substrate is a fatty acid (74, 138). Expression of the operon is also upregulated by the histone-like protein, IHF (74).

The pentose phosphate pathway. In addition to the formation of the precursor metabolites, ribose-5-phosphate and erythrose-4-phosphate, the pentose phosphate pathway may be required to provide NADPH for biosynthesis and survival within phagocytic cells (Figure 1) (57, 68, 69, 73). Another function of this pathway is the long route between glucose-6-phosphate to fructose-6-phosphate; this would effectively be a cycle for the complete oxidation of hexose monophosphate to CO₂ (73). The pentose phosphate pathway is also needed for growth on pentoses and for the portion of gluconate metabolism not utilizing the Entner-Doudoroff pathway (73).

The pentose phosphate pathway in *S. typhimurium* utilizes the following enzymes (genes): glucose-6-phosphate dehydrogenase (*zwf*), 6-phosphogluconolactonase (*pgl*), gluconate-6-phosphate dehydrogenase (*gnd*), ribosephosphate isomerase (*rpiA*), ribose-5-phosphate isomerase (*rpiB*), D-ribulose-5-phosphate 3-epimerase (*rpe*), transketolase 2 (*tktB*), transketolase 1 (*tktA*), transaldolase A (*talA*), and transaldolase B (*talB*) (Figure 1) (92, 93, 153-163).

The Entner-Doudoroff pathway. The inducible Entner-Doudoroff pathway in *S. typhimurium* is employed for the metabolism of gluconate (73). The Entner-Doudoroff pathway can be properly considered as one of three pathways found in nature, in addition to the Embden-Meyerhof-Parnas and pentose phosphate pathways, that feed into the "bottom half" of glycolysis, which is central to all intermediary metabolism (Figure 1) (164).

Two gluconokinases, thermosensitive D-gluconate kinase (*idnK*) and gluconate kinase 2 (*gntK*), plus ATP phosphorylates gluconate and yield gluconate-6-phosphate (Figure 1) (73, 93, 165, 166). Gluconate-6-phosphate is converted to 2-keto-3-deoxy-gluconate-6-phosphate (KDPG) by 6-phosphogluconate dehydratase (*edd*) (73, 164, 167, 168). The final step in the Entner-Doudoroff pathway is the conversion of KDPG into glyceraldehyde-3-phosphate and pyruvate by 2-keto-3-deoxy-gluconate-6-phosphate aldolase (*eda*) (73, 169, 170). The *eda* gene encodes for a multifunctional enzyme: 2-keyto-4-hydroxyglutarate aldolase participates in the regulation of intracellular levels of glyoxylate and oxaloacetate decarboxylase activity participates in the dissimilation of glyoxylate to pyruvate (169, 170). Competitive inhibitors include glyceraldehyde-3-phosphate and gluconate-6-phosphate (169). The *edd-eda* operon is repressed by GntR and is induced (derepression of GntR) by gluconate, although a high basal expression of *eda* occurs regardless of the carbon source (92, 164, 171).

The fatty acid β -oxidation pathway. Although enzymes in the aerobic fatty acid β -oxidation pathway degrade both long and short chain fatty acids, it is the long chain compounds that induce the enzymes of this pathway (172). The first step in fatty acid degradation is the activation of the free fatty acid to an acyl-CoA thioester by acyl-CoA synthetase (*fadD*) (Figure 1) (93, 172, 173). This initial activation step requires coenzyme A (CoA-SH) and two high-energy phosphate equivalents

from ATP per molecule of fatty acid (173). Each turn of the cycle yields an acetyl-CoA, an NADH, and an FADH₂ (172, 173). The cycle utilizes the enzymes acyl-CoA dehydrogenase (*yafH*), *cis*- Δ^3 -*trans*- Δ^2 -enoyl-CoA isomerase (*fadB*), enoyl-CoA hydratase (*fadB*), 3-hydroxyacyl-CoA epimerase (*fadB*), 3-hydroxyacyl-CoA dehydrogenase (*fadB*), and 3-ketoacyl-CoA thiolase (*fadA*) (92, 173, 174-176). One of the products of β -oxidation, acetyl-CoA, is fed into the TCA cycle and glyoxylate bypass, which facilitates its conversion to metabolic intermediates (74, 137, 173). The reducing equivalents produced, NADH and FADH₂, can be regenerated in the electron transport chain producing ATP via oxidative phosphorylation (131, 133, 134, 173).

When even numbered fatty acids are broken down, a two carbon compound remains, acetyl-CoA (172). When odd numbered fatty acids are broken down, a three carbon compound remains, propionyl-CoA; this is further catabolized by the reactions of propionate catabolism (172). Unsaturated fatty acids require additional metabolic reactions by an isomerase, an epimerase, and a reductase reactions to enter the acid β -oxidation pathway (172). Expression of the genes of the β -oxidation pathway are usually subjected to strong catabolite repression (173). The *fadBA* operon also is negatively regulated by the FadR repressor; acyl-CoA relieves this repression (173).

Fructose catabolism. The same general PTS system as glucose is utilized by fructose for entry into the cell; fructose is simultaneously transported and phosphorylated by group translocation (73, 90). The general PTS proteins, EI (*ptsH*) and Hpr (*ptsI*) are the soluble cytoplasmic proteins that participate in the phosphorylation of all PTS carbohydrates (Figure 1) (90-92). The EII^{fru} enzyme complex (*fruA*, *fruB*) is fructose-specific, possesses three domains in the FruA protein (IIB'-IIB-IIC) and three domains in the FruB protein (IIA-IIM-IIH) (91, 93, 177, 178). Domains IIA-IIB-IIB' are localized to the cytoplasmic side of the membrane (177, 178). The domain IIB' is required for high affinity binding of FruB to FruA, but does not participate in phosphoryl transfer (177, 178). Domain IIA is the first phosphorylation site, IIM is a central domain of unknown function, and IIH is an HPr-like domain called FPr (fructose-inducible HPr) (177, 178). Fructose-1-phosphate is the product of this series of reactions (91, 177, 178).

Fructose-1-phosphate kinase (*fruK*) converts fructose-1-phosphate to fructose-1,6-bisphosphate in a reaction which requires ATP or GTP; the product enters the glycolytic (EMP) pathway (91, 92, 179). Fructose-1-phosphate kinase is reversibly inhibited by fructose-1,6-bisphosphate and ADP (179).

The fructose operon (*fruBKA*) is subject to positive control by the cAMP/cAMP receptor protein (CRP) complex (91, 177, 178). The *cra* gene, formerly the *fruR* gene (fructose repressor), encodes a Cra (catabolite repressor/activator) repressor protein of the fructose operon; *cra* does not map near the *fruBKA* operon (91, 177, 178).

Growth of *S. typhimurium* utilizing a non-limiting glucose source. The branched, biosynthetic form of the TCA cycle utilizing a non-limiting glucose source was separated from the above pathways because of its importance to this study. The TCA cycle is split into a oxidative branch terminating at α -ketoglutarate and a reductive branch terminating at succinyl-CoA (Figure 2) (74, 78). The oxidative branch functions as described in the section above titled: The TCA cycle and glyoxylate bypass. However, the reductive branch of the TCA cycle functions backwards utilizing the same reversible enzymes except succinate dehydrogenase (*sdhCDAB*), which is replaced by fumarate reductase (*frdABCD*) (74, 78, 93, 180).

Fumarate reductase is composed of four subunits and two domains (180). The catalytic domain consists of two subunits: one with a covalently-bound flavin cofactor and the fumarate binding site; the other contains three iron-sulfur clusters (180). This catalytic domain is attached to the cytoplasmic side of the cytoplasmic membrane by the anchor domain, which consists of two subunits that interact with quinone and contain heme (180). The enzyme also has two quinol-binding sites, Qp and Qd (180). The covalent attachment of FAD to the enzyme (A subunit apoprotein) is stimulated by citrate, isocitrate, succinate, and fumarate, acting as possible allosteric effectors (180). Fumarate reductase is inhibited by oxaloacetate and malonate (180).

Oxaloacetate is converted to aspartate by aspartate aminotransferase (*aspC*) in this branched, biosynthetic form of the TCA cycle (Figure 2) (74, 93, 181). Glutamate is also a reactant and 2-oxoglutarate a product of this reaction (181). The dimeric aspartate aminotransferase enzyme is

inhibited by 2-methyl aspartate (181). Aspartate ammonia lyase (*aspA*) next converts aspartate to fumarate and NH_3 (74, 92, 182). Aspartate ammonia lyase is activated by aspartate and inhibited by citrate and n-propanol (182).

The full TCA cycle is not required for growth of *S. typhimurium* utilizing a non-limiting glucose source because the bulk of energy is derived from glycolysis (74, 78). In this glucose-rich environment, cells produce excess acetyl-CoA which drains through acetyl phosphate, to further produce ATP with the associated secretion of acetate (Figure 2) (78, 79). As cell density increases, the build-up of acetate also serves to increase the size of the acetyl phosphate pool (78).

Regulation by catabolite repression. Catabolite repression is often called the glucose effect because glucose, which yields the highest return of ATP per unit of expended energy, usually strongly represses operons for the utilization of other carbon sources (183). Catabolite repression in *S. typhimurium* involves the cytoplasmic sensor of carbon and energy concentrations, cAMP, and the dimeric CRP protein; cAMP binds to CRP at specific DNA sequences in cAMP-CRP-sensitive promoters, induces bends in the DNA, and interacts with RNA polymerase to promote transcriptional initiation (184, 185). However, the mechanism of cAMP-CRP regulation varies; CRP can function not only as an activator, but also as a repressor depending where it binds relative to the promoter (184, 185).

The synthesis of cAMP is controlled through the regulation of activity of adenylate cyclase (186). This enzyme, which catalyzes the formation of cAMP from ATP, is more active when cellular concentrations of catabolites are low and less active when catabolite concentrations are high (186). The synthesis of cAMP is regulated by a protein phosphorylation mechanism that is catalyzed by the PTS system (184). PTS permeases function as transmembrane signal transduction devices: when one of the PTS sugars (i.e. glucose, fructose, mannitol) such as glucose is present extracellularly, the *crr*-encoded enzyme IIA^{Glc} protein becomes dephosphorylated as the phosphoryl groups are transferred to incoming sugar molecules via the sugar-specific permease proteins (91, 94, 184). This process results in both allosteric deactivation of adenylate cyclase, allosteric inhibition of the non-PTS permeases (i.e. lactose, maltose, and glycerol permeases), and catabolic enzymes that generate cytoplasmic inducers

(91, 184). Exogenous glucose both inhibits the synthesis of cAMP and stimulates the efflux of cAMP from the cell cytoplasm (184).

Almost all genes that encode enzymes and transport proteins which initiate the metabolism of an exogenous carbon source are under cAMP-CRP control; the expression of the genes is consequently subject to catabolite repression (184). The levels of the TCA cycle enzymes are regulated at the transcriptional level by catabolite repression (136). Phospho(enol)pyruvate carboxylase (*ppc*) is under cAMP control as well (184). One of the primary responses to a limitation of a specific nutrient is the activation of the cAMP-CRP regulon; this allows higher-affinity uptake of the nutrient present in low concentration or the utilization of an alternative carbon source (187).

Many proteins in addition to carbon catabolic enzymes have been shown to be subject to catabolite repression (183). Flagellar synthesis, as well as chemotaxis are subject to cAMP control (184). Finally, genes involved in pH regulation, extracellular macromolecule degradation, ubiquinone synthesis, intracellular glycogen metabolism, nitrogen utilization, organic phosphate ester utilization, thiol-sulfate reduction, iron uptake, drug (antibiotic) resistance, colicin induction and recognition, and toxin production are all regulated directly or indirectly by cAMP-CRP (184). Many of these processes are related to carbon metabolism (184).

Regulation of central carbohydrate metabolism via the *csrA* gene. The carbon storage regulator gene, *csrA*, exhibits pleiotropic regulation of central carbohydrate metabolism in *E. coli* (82). The regulatory effects of CsrA peak during the transition from exponential phase into early stationary phase (82). The CsrA regulatory protein dramatically affects the biosynthesis of glycogen through its negative control of two glycogen genes, *glgC* (ADP-glucose pyrophosphorylase) and *glgB* (glycogen branching enzyme) (82). Intracellular carbon flux is also directed by CsrA in both the glycolytic (EMP pathway) and gluconeogenic pathways (82). CsrA exerts negative regulation of gluconeogenesis with a decrease in the specific activity of phospho(enol)pyruvate carboxykinase (*pckA*), phospho(enol)pyruvate synthetase (*pps*), and fructose-1,6-bisphosphatase (*fbp*) (Figure 1) (82). Positive regulation of glycolysis by CsrA is reported in the increased specific activity of glucosephosphate isomerase (*pgi*), 6-phosphofructokinase-I (*pfkA*), triose phosphate isomerase (*tpi*),

enolase (*eno*), and pyruvate kinase I (*pykF*) (82). In contrast, the expression of genes in the pentose phosphate pathway is weakly or negligibly affected by CsrA (82). *S. typhimurium* possesses a nearly identical homologue of this *csrA* gene (92, 188).

Regulation of central carbohydrate metabolism by the ArcA-ArcB two-component signal transduction system. The two component regulatory ArcA-ArcB system functions as a major control system for the regulation of expression of genes encoding enzymes in both aerobic and anaerobic catabolic pathways (189, 190). Over forty operons are controlled by the ArcA-ArcB system (189). Almost all of the genes of the TCA cycle and glyoxylate shunt are subjected to repression by the ArcA-ArcB two component regulatory system (Figure 1) (191). The exception is the *sdhCDAB-sucABCD* operon, containing five ArcA binding sites, which is primarily initiated and regulated at the upstream *sdh* promoter (192). ArcA positively regulates this operon at the two upstream ArcA binding sites and represses the operon at the remaining ArcA binding sites flanking or within the *sdh* promoter (192). This operon also contains one internal promoter, P_{suc} (192).

The expression of Arc-regulated genes varies in response to environmental O_2 , although this compound is not thought to be the signal detected by the ArcB sensory kinase (190, 193). ArcB probably senses the redox state of the cell through the detection of an electron transport component in reduced form (189, 193). The phosphorylated form of the response regulator ArcA (ArcA-P) is predicted to reach peak levels in anoxic cells as the ArcB kinase activity progressively increases during the transition from aerobic to anaerobic growth (193). However, significant levels of ArcA-P are apparent in aerobic cells, and differential patterns of expression of the members of the ArcA regulon can be attributed, at least in part, to the affinity of ArcA-P for DNA binding sites located in the transcriptional regulatory regions of its targeted operons (193).

ArcA plays a vital role in adjusting catabolism to oxygen-restricted growth conditions through regulation of carbon flux via the TCA cycle and electron flux via terminal cytochrome oxidases, rather than to adjust catabolism to fully aerobic or anaerobic conditions (189). Studies of the intracellular redox state ($NAD^+/NADH$) under varying O_2 concentrations, of an ArcA⁻ mutant, strongly suggest that ArcA is actually a microaerobic redox regulator (189).

Acetyl phosphate and the activation of two-component signal transduction systems. It has been estimated that there may be as many as fifty different two-component signal transduction systems in *E. coli* (24). Acetyl phosphate has been shown to phosphorylate numerous response regulator proteins *in vitro* and has been shown to influence several two-component signal transduction pathways *in vivo* (25, 190). Studies have shown that a major secondary source of phosphoryl groups is from a small molecule phospho-donor, acetyl phosphate (79). When *E. coli* is incubated with acetyl phosphate, the following response regulator protein components of two-component signal transduction systems, become phosphorylated: CheB, NtrC, PhoB, OmpR, and ArcA (24, 79). These phosphorylated response regulator proteins control the expression of genes or operons involved in chemotaxis, nitrogen regulation, phosphate-specific transport systems, outer membrane proteins involved in osmo-regulation, and enzymes in both aerobic and anaerobic catabolic pathways respectively (24, 79, 189, 190).

The demonstration that acetyl phosphate can serve as a substrate for response regulator autophosphorylation revealed some subtle aspects of the two-component signal transduction pathways (24). These pathways can be regulated by controlling the levels of phospho-donors, phospho-histidine kinases, or by controlling the rates of dephosphorylation of the active response regulators (24).

The function of acetyl phosphate in two-component signal transduction systems may be to adjust the sensitivity and/or the magnitude of an adaptive response such as shifts in metabolism and transitions in the expression of virulence factors (79). If acetyl phosphate contributes to the basal levels of phospho-response regulators within a cell, then higher levels of acetyl phosphate would produce a more sensitive system; a smaller stimulus would be required to produce enough phosphorylated response regulator to trigger a response (79).

***Salmonella* vaccines and vaccine candidates.** Two *Salmonella typhi* vaccines, the live oral *S. typhi* Ty21a and a vaccine based on the Vi polysaccharide capsule of *S. typhi*, currently are licensed in several countries (194). The *S. typhi* Ty21a is a galactose epimerase (*galE*) mutant: the galactose required for the synthesis of smooth LPS O antigen is derived from UDP-galactose, which is synthesized from UDP-glucose by the enzyme galactose epimerase (195). The result is an avirulent

strain with rough LPS (195). This property, coupled with the fact that *S. typhi* Ty21a is also Vi antigen negative, is a factor in the safety of this live vaccine strain *in vivo* (195). The Vi antigen, a capsular polysaccharide composed of N-acetylglucosamine, is produced by the most virulent *S. typhi* strains (196). Each of the above vaccines, while effective, still suffers from drawbacks such as fevers after administration, incomplete protection, and a loss of protection with time (194, 195). Therefore, new attenuated *S. typhi* live oral vaccines are being developed (194, 195).

Live oral typhoid fever vaccine candidates, based on deletions in genes necessary for *Salmonella* pathogenesis, have shown progress (197, 198). For example, the live oral *S. typhimurium* Δ *cya* (adenylate cyclase) Δ *crp* (cAMP receptor protein) vaccine is based on deletions in a global regulatory system and is effective in animals and humans (197, 198). The live oral *S. typhi* Δ *cya* Δ *crp* vaccine becomes even more effective when a third deletion in the *cdt* gene (deep tissue colonization) is introduced (199, 200).

Another typhoid fever live oral vaccine, avirulent and immunogenic in mice and cattle, is based on *aroC* and *aroD* deletions in *S. typhimurium*; this strain is auxotrophic for aromatic compounds which are unavailable in mammalian cells (198, 201, 202). This Δ *aroC* and Δ *aroD* double mutant is unable to synthesize chorismic acid, a precursor of the aromatic compounds *p*-aminobenzoic acid and 2,3-dihydroxybenzoate, and no other pathway for their synthesis exists in *S. typhimurium* (198, 201). As a consequence of this auxotrophy, the bacteria cannot proliferate within mammalian cells, yet they reside and replicate long enough to stimulate protective immune responses (198, 201, 202). Again, the introduction of a third deletion in the *htrA* gene (a protease DO precursor heat shock protein) in a *S. typhi* strain increase the effectiveness of this vaccine candidate in clinical trials (54, 199, 200).

Another live oral typhoid fever vaccine candidate, based on deletions in genes of the *phoP-phoQ* two-component signal transduction system (regulation of virulence genes), is avirulent and immunogenic in mice and humans (200, 203, 204). A recent *Salmonella enterica* serovar Typhimurium live oral vaccine contains a deletion in the DNA adenine methylase (*dam*) gene (205). DNA adenine methylase plays an important role in the timing and targeting of a number of cellular

functions including DNA replication, segregation of chromosomal DNA, and mismatch DNA repair (61). The Dam⁻ vaccine strain was avirulent in day-of-hatch chicks (broiler chickens) and elicited cross protective immune responses against challenge by homologous serovars of Typhimurium and heterologous serovars of Enteritidis (205, 206). The vaccine strain also proved to be highly attenuated for virulence in mice and conferred protection against murine typhoid fever (206).

The use of attenuated *Salmonella* strains as live vaccines is a safe and effective means of inducing significant humoral and secretory antibody responses in animal species humans, cattle, sheep, rabbits, fowl, and mice (195). Live *Salmonella* invade the Peyer's patches, where they present their numerous antigens directly to the T and B lymphocytes of the mucosal-associated lymphoid tissues (195). This elicits the mucosal immune system to produce antigen-specific immune responses (195). Due to the significant humoral and secretory antibody responses, live attenuated *Salmonella* strains show great promise for carriers of antigenic determinants from other pathogenic microorganisms (195, 200).

Other *S. typhimurium* strains attenuated for virulence. A strain of *S. typhimurium*, with a mutation in *ptsH*-encoded enzyme I (EI) of the phospho(enol)pyruvate: sugar phosphotransferase system (PTS), has been reported as attenuated for virulence in an intraperitoneal mouse model; the log (attenuation) values were 2.3 below the wild-type *Salmonella* strain (90-92, 207). The general PTS proteins, EI and HPr, transfer phosphoryl groups from phospho(enol)pyruvate the sugar-specific transporters (EII^{sugar}) in the PTS system (91, 207). The PTS system is also an integral part of cAMP-CRP regulation (catabolite repression) and the genes of its regulon (183-185).

The outer membrane of *S. typhimurium* contains LPS; lipid A is the major component of this endotoxin that stimulates inflammatory cytokine release and inducible nitric oxide synthase (iNOS) responses in the murine host (7-9, 22, 208). A *S. typhimurium* strain with a deletion in the *waaN* gene, which encodes the enzyme that catalyzes one of the two secondary acylation reactions that complete lipid A biosynthesis, was constructed (9). The *waaN* mutant strain, which synthesize a full-length LPS molecule containing the O antigen and lack only the secondary acyl chain, were intravenously injected into BALB/c mice at a dose of with 10² bacteria per mouse (9). Extremely high bacterial

counts of 10^9 in the liver and spleen were observed (the lethal load of the wild-type parental strain is 10^8 bacteria per organ), and only approximately 10% of the mice expired (9). Most of the remaining mice carrying these extremely high *waaN* mutant bacterial loads recovered and eventually cleared the bacteria from the organs (9). This study implied that death in a mouse typhoid infection is directly dependent on the toxicity of lipid A and suggested that this is mediated via pro-inflammatory cytokine and/or the iNOS responses (9).

The Vaccine Candidate *Salmonella typhimurium* SR-11 Fad⁻. The live oral vaccine candidate, *Salmonella typhimurium* SR-11 Fad⁻, was constructed by Tn10d::*cam* transposon mutagenesis of the SR-11 parent strain (209, 210). The mutant strain was selected for its inability to catabolize oleate and citrate as sole carbon sources (210). It was phenotypically designated Fad⁻ (Fatty acid) for its inability to catabolize fatty acids as a sole carbon source (210). The SR-11 Fad⁻ strain also was unable to utilize acetate and isocitrate as carbon sources, but could utilize glucose, and glycerol (210).

The disease caused by *Salmonella typhimurium* in mice mimics typhoid fever in humans and is well accepted as a model for human typhoid (20, 21). *Salmonella typhimurium* SR-11 Fad⁻, administered perorally, was completely avirulent in BALB/c mice at a dose as high as 10^9 colony forming units (cfu) (210). In contrast, the SR-11 parent strain proved lethal at doses of greater than 10^4 cfu (210). The vaccine candidate also was found to be protective in BALB/c mice (33, 210) as well as avirulent and protective in chickens (Dr. Paul S. Cohen: personal communication). A single oral dose of 10^7 SR-11 Fad⁻ cells protected BALB/c mice against a lethal dose of 10^9 cells of the virulent SR-11 *S. typhimurium* parent strain (33, 210).

The *S. typhimurium* SR-11 Fad⁻ strain was also found to be slightly attenuated relative to the SR-11 wild-type parental strain when administered intraperitoneally: 100% of the BALB/c mice survived an intraperitoneal injection of 5×10^1 SR-11 Fad⁻ bacteria whereas 100% of the BALB/c mice were killed by the same dosage of SR-11 wild-type cells (210). However, only 50% of the BALB/c mice survived an intraperitoneal injection of 1.5×10^2 SR-11 Fad⁻ bacteria and 100% mortality was observed in doses greater than 1.5×10^3 cells (210).

Attenuated strains of *Salmonella* generally exhibit higher virulence with intraperitoneal injection versus peroral inoculation; this unnatural route for *Salmonella* infection bypasses the M cells (filled with a cluster of B cells, T cells, and macrophages) and the mucosal-associated lymphoid tissue carrying intraepithelial lymphocytes (T-cells which express unusual T-cell receptors and exhibit limited diversity for antigens), the lamina propria, (large numbers of B cells, plasma cells, activated helper-T cells, and macrophages in loose clusters), and the Peyer's patches (nodules consisting of 30-40 organized lymphoid follicles) (11, 21, 29, 30, 33, 34).

SR-11 Fad⁻ is a *cra* (*fruR*) mutant. Southern hybridization with a probe specific for the chloramphenicol resistant cassette in SR-11 Fad⁻, followed by cloning of a 4.5 kb *Pst* I fragment containing the mini-Tn10d::*cam* into pBluescript II SK (+) yielded the plasmid pJHA7 (33). Sequencing of pJHA7 revealed the gene interrupted by the mini-transposon was the *cra* gene (33). Furthermore, complementation with a wild-type *cra* gene in the plasmid pJHA8 (Table 1) restored both virulence in BALB/c mice as well as the ability of SR-11 Fad⁻ to utilize fatty acids and gluconeogenic substrates as sole carbon sources (33, 211).

The Cra protein: a global regulator of carbon and energy metabolism in glycolysis and gluconeogenesis. In *E. coli* and *S. typhimurium* the *cra* gene (catabolite repressor/activator), formerly the *fruR* gene, encodes the 150 kDa homotetrameric DNA-binding Cra protein (212). The Cra protein, a global regulator of carbon and energy metabolism in glycolysis and gluconeogenesis, represses transcription of genes encoding glycolytic enzymes (specific enzymes in the EMP and Entner-Doudoroff pathways) but activates transcription of genes encoding enzymes involved in biosynthesis (specific enzymes in the TCA cycle, the glyoxylate shunt, and the gluconeogenic pathway) (Figure 3) (213-217).

The Cra protein recognizes an imperfect palindromic DNA sequence (TGAA, A or T, C • C or G, any nucleotide, T, A or C, A or C, A or T) which it binds to asymmetrically (217). If the Cra binding site is upstream of the promoter, it activates transcription of the target operon or gene; if it overlaps or is downstream of the promoter, Cra represses transcription of the target operon or gene (217). Transcription of at least 20 genes is upregulated up to ten-fold and downregulated by a

maximum of eleven-fold by the Cra protein (214, 217, 218). The Cra protein acts independently of cAMP-CRP transcriptional regulation (catabolite repression) (217, 219, 220). Two effector molecules of the Cra protein, μM concentrations of fructose-1-phosphate and mM concentrations of fructose-1,6-bisphosphate, inactivate the DNA-binding protein (Figure 4) (212, 217, 219, 221). Therefore, growth on glucose or fructose may inactivate the Cra DNA-binding protein via these two effectors (222, 223). Derepression of the glycolytic pathway and deactivation of the gluconeogenic pathway by the effectors of Cra also help to ensure the preferential utilization of carbon sources such as glucose and fructose (223).

The Cra global regulatory protein also represses the *pts* operon: *ptsH* (PTS general enzyme EI), *ptsI* (PTS general enzyme Hpr), and *crr* (glucose specific enzyme IIA^{Glc}) constitute the *pts* operon (Figure 3) (90, 91, 94, 212, 215, 222). Cra (FruR) mutants were first isolated as suppressor mutations which allowed *ptsH* mutants to grow on PTS carbohydrates; the suppression resulting from the constitutive synthesis of the HPr-like domain of the *fruB*-encoded diphosphoryl transfer protein because it can substitute for HPr (212). Furthermore, data has shown that under certain physiological conditions, Cra modulates the activity of adenylate cyclase, the cAMP biosynthetic enzyme (217). Since the PTS system is also an integral part of cAMP-CRP regulation (catabolite repression), the global regulator Cra is intertwined with other global regulators that exert pleiotropic effects on the transcription of operons and genes involved in central carbon metabolism (72, 73, 183-185, 217).

The catabolite repressor/activator Cra protein was initially characterized as the fructose repressor (FruR: fructose repressor) (217). Cra is a transcriptional repressor of the *fruB(F)KA* operon: *fruA* and *fruB* encoding the EII^{fru} enzyme complex of the PTS system and *fruK* encoding fructose-1-phosphate kinase (Figure 3) (177-179, 212, 224, 225). *S. typhimurium* and *E. coli* mutants defective in the *cra* (*fruR*) gene are unable to grow on gluconeogenic substrates such as acetate, lactate, pyruvate, alanine, and all the TCA cycle intermediates, as sole carbon sources (212, 217, 218, 225).

Cra has also been reported to repress the transcription the following operons or genes: *adhE* (the fermentative bi-functional enzyme ethanol dehydrogenase), *epd* (*gapB*) (a component of the erythrose-4-phosphate dehydrogenase enzyme which plays a role in the *de novo* biosynthesis of the

pyridoxal-5'-phosphate coenzyme), *nirBD* (nitrate reductase enzyme) and *mtlADR* (mannitol transport proteins and catabolism enzymes) (92, 219, 221, 223, 227-230).

The goal of this study. The initial goal of this study was to determine the extent to which gluconeogenesis is linked to virulence and pathogenesis in *S. typhimurium* SR-11, since *S. typhimurium* SR-11 Fad^+ , is unable to undergo gluconeogenesis and is avirulent in BALB/c mice (33, 210). To that end, the construction of several mutations in the gluconeogenic pathway was planned to determine the virulence of these strains. A second goal of this study was to determine the regulation of the *cra* gene by assaying for *cra* promoter activity during growth on various gluconeogenic and glycolytic substrates and how deletions in the gluconeogenic pathway would alter *cra* promoter activity.

Materials and Methods

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Agarose gel electrophoresis. Agarose gels were made with a concentration of 0.85% (wt/vol) of molecular biology grade agarose (Fisher Scientific, Fair Lawn, NJ) or DNA grade low melt agarose (Fisher Scientific, Fair Lawn, NJ) in TAE buffer (pH 8.5; 40 mM Tris-acetate; 1.0 mM EDTA; 5 Prime→3 Prime, Inc., Boulder, CO) with ethidium bromide (Sigma Chemical Co., St. Louis, MO) at a final concentration of 0.2-0.4 µg/ml (234). Three different DNA standard markers were used: a 100 bp DNA Step Ladder (Promega Corporation, Madison, WI), a 1 kb DNA Step Ladder (Promega Corporation, Madison, WI) and a 1 kb DNA Ladder (Promega Corporation, Madison, WI).

Agarose gel photography. Agarose gels were viewed on a Fotodyne ultra-violet transilluminator (Fotodyne, Inc., New Berlin, WI) and photographed with a Kodak DC12 Zoom Digital Camera (Eastman Kodak Company, Rochester, NY) (235). Kodak Digital Science 1D software (version 3.0.2; Eastman Kodak Company, Rochester, NY) was used to analyze the digital photographs. Text and graphics were added with Adobe® Photoshop® (version 4.0 LE; Adobe Systems, Inc.).

Antibiotics. Antibiotics were purchased from Sigma Chemical Co. (St. Louis, MO) and Fisher Scientific (Fair Lawn, NJ) and added to media at a final concentration, except where noted, as follows: 100 µg/ml or 150 µg/ml ampicillin; 30 µg/ml chloramphenicol; 40 µg/ml kanamycin monosulfate, 50 µg/ml nalidixic acid; 100 µg/ml streptomycin sulfate; and 10 µg/ml tetracycline hydrochloride.

Bacterial growth media. Luria broth (LB; Lennox formulation; Difco Laboratories, Detroit, MI and Fisher Scientific, Fair Lawn, NJ) and Luria agar plates (Lennox formulation; Difco Laboratories, Detroit, MI and Fisher Scientific, Fair Lawn, NJ) were used routinely unless noted.

M9 minimal medium was adjusted to pH 7.0 ± 0.1 and separately supplemented with various glycolytic and gluconeogenic substrates (Sigma Chemical Co., St. Louis, MO and Fisher Scientific, Fair Lawn, NJ) at the following anhydrous concentrations: D-fructose (0.2% [wt/vol]), D-glucose

(0.2% [wt/vol]), D-gluconic acid potassium salt (0.2% [wt/vol]), glycerol (0.2% [vol/vol]), potassium acetate (0.4% [wt/vol]), D-alanine (0.2% [wt/vol]), sodium citrate (0.2% [wt/vol]), fumaric acid disodium salt (0.4% [wt/vol]), L(-)malic acid (0.6% [wt/vol]), phospho(enol)pyruvate monosodium salt (0.4% [wt/vol]), pyruvic acid sodium salt (0.4% [wt/vol]), oleic acid sodium salt (5mM), and succinic acid disodium salt (0.6% [wt/vol]) (236).

M9 minimal agar plates, containing 15 g/L of noble agar (Difco Laboratories, Detroit, MI) and separately supplemented with the various glycolytic and gluconeogenic substrates, were utilized for determination of growth (utilization of substrate by the bacteria) (236). The M9 minimal agar plates were incubated at 37°C and the results were recorded at 24 hours and 48 hours. M9 minimal medium (broth) were used for determination of growth, rate of growth, and β -galactosidase assays (for a detailed protocol see " β -galactosidase assays for *cra* promoter activity" in this section) (232, 233, 236, 237).

SOB medium contained 2% (wt/vol) Bacto™ tryptone (Difco Laboratories, Detroit, MI), 0.5% (wt/vol) yeast extract (Difco Laboratories, Detroit, MI), 10 mM NaCl (Sigma Chemical Co., St. Louis, MO), 2.5 mM KCl (Sigma Chemical Co., St. Louis, MO), 10 mM $MgCl_2 \cdot 6 H_2O$ (Sigma Chemical Co., St. Louis, MO), and 10 mM $MgSO_4 \cdot 7 H_2O$ (Sigma Chemical Co., St. Louis, MO) (238). SOC medium included SOB medium plus 20 mM glucose (238). Both SOB and SOC media were used in allelic replacement protocols where noted.

Fusaric acid selection medium contained 1% (wt/vol) tryptone, 0.5 % (wt/vol) yeast extract, 1% (wt/vol) NaCl, 0.2% (wt/vol) D-glucose, 0.005% (wt/vol) chlorotetracycline HCl (Sigma Chemical Co., St. Louis, MO), 1% (wt/vol) $Na_2HPO_4 \cdot H_2O$ (Sigma Chemical Co., St. Louis, MO), 2% (wt/vol), 100 μM $ZnCl_2$ (Sigma Chemical Co., St. Louis, MO), and fusaric acid @ 20 μg /ml (Sigma Chemical Co., St. Louis, MO) (239).

All liquid bacterial cultures were grown overnight at 37°C, with aeration, in the appropriate antibiotic(s) except where noted. Agar plates were incubated at 37°C for 12-18 hours except where noted. MacConkey (Difco Laboratories, Detroit, MI) and XLD (Difco Laboratories, Detroit, MI)

selective and differential agar plates were used to characterize all transformants, transconjugants, and segregants.

Isolation of genomic DNA. (Note: volume of reagents used and/or centrifugation speed and time varied according to the initial volume of the bacterial cultures and/or bacterial strain (240)). Genomic DNA was isolated by a modified protocol from *Current Protocols in Molecular Biology* (241). Bacterial cells from an overnight culture were pelleted by centrifugation, resuspended in TE Buffer (10 mM Tris-HCl [Tris[hydroxymethyl]aminomethane hydrochloride]; 1 mM EDTA [ethylenediaminetetraacetic acid]; pH 8.0, (5 Prime→ 3 Prime, Inc., Boulder, CO), lysed with sodium dodecyl sulfate (SDS) (BioRad Laboratories, Richmond, CA), and incubated at 37°C for one hour in proteinase K (Sigma Chemical Co., St. Louis, MO) to degrade protein. Hexadecyltrimethylammonium bromide (10% CTAB in 0.7 M NaCl) was added to the lysate and incubated at 65°C for 15 minutes. An equal volume of chloroform/isoamyl alcohol (24:1) (Sigma Chemical Co., St. Louis, MO) was added to the lysate. The aqueous phase, containing the DNA, was extracted after centrifugation. A second extraction with phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma Chemical Co., St. Louis, MO), removed residual CTAB precipitate. The DNA was precipitated in 100% isopropanol (Sigma Chemical Co., St. Louis, MO) and pelleted by centrifugation. The DNA pellet was rinsed in 70% ethanol to remove excess salt, lyophilized, and resuspended in TE buffer. Incubation (37°C for greater than 1 hour) of the genomic DNA in RNase A (Sigma Chemical Co., St. Louis, MO) degraded most of the contaminating RNA. The DNA underwent a final phenol/chloroform/isoamyl extraction and precipitation in 100% ethanol to remove the RNase A and any residual proteins (242).

Genomic DNA also was isolated by using the G NOME[®] genomic DNA isolation kit (BIO 101, Vista, CA). The protocol from the manufacturer was modified by increasing the initial volume of overnight liquid bacterial cell culture to 10 ml per miniprep. The kit used a "Cell Suspension Solution," "RNase Mixx," "Cell Lysis/Denaturing Solution," "Protease Mixx," and "Salt-Out Mixture" of unspecified composition and replaced the chloroform steps of the standard protocol with incubation at 4°C followed by centrifugation (241). A final precipitation of the genomic DNA in 100% ethanol completed the manufacturer's protocol (242).

Genomic DNA also was isolated by using the Wizard® genomic DNA Prep Kit (Promega Corporation, Madison, WI). The manufacturer's protocol was followed using up to 3 ml of an overnight culture of bacteria. All centrifugation steps were performed at 14,000 x g in an Eppendorf microcentrifuge (#5415C; Brinkmann Instruments, Inc., Westbury, NY). The isolated genomic DNA was incubated in an excess of DNA rehydration solution (10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8.0) at 65°C for greater than one hour or a combination of incubation at 65°C followed by overnight incubation at room temperature. All genomic DNA was frozen at -20°C for short-term storage and -78°C for long-term storage.

Isolation of plasmid DNA. Quantities of plasmid DNA greater than 10 µg were isolated by modified alkaline lysis (243). A minimum of 100 ml of bacterial cells from an overnight liquid culture were pelleted by centrifugation and resuspended in GTE Buffer (50 mM glucose; 10 mM EDTA [ethylenediaminetetraacetic acid]; 25 mM Tris-HCl [Tris[hydroxymethyl]aminomethane hydrochloride]; pH 8.0). The resuspended cells were added to a lysis solution, containing 0.2 N NaOH (Sigma Chemical Co., St. Louis, MO) and 1.0% SDS (wt/vol), and incubated on ice for 10 minutes. An appropriate volume of potassium acetate solution (5 M KOAc, pH 4.8) was mixed into the cell lysate and centrifuged. The supernatant, containing the plasmid DNA, was extracted with phenol/chloroform/isoamyl alcohol and precipitated in 100% isopropanol at -20°C for 30 minutes. The plasmid DNA was pelleted by centrifugation, rinsed in 1 ml of 70% ethanol to remove excess salt, lyophilized, and resuspended in TE buffer. Incubation (37°C for greater than 1 hour) of the plasmid DNA in RNase A degraded the RNA contaminant. The plasmid DNA underwent a phenol/chloroform/isoamyl extraction to remove the RNase A and any residual proteins. A final concentration by precipitation in 100% ethanol was performed (242).

Quantities of plasmid DNA less than 10 µg were isolated by Wizard™ Plus Miniprep DNA Purification Systems (Promega Corporation, Madison, WI). The protocol from the manufacturer was modified by increasing the initial volume of overnight liquid bacterial cell culture to 25 ml per miniprep. The bacterial cells were pelleted by centrifugation and resuspended in Cell Resuspension Solution (50 mM Tris, pH 7.5; 10 mM EDTA; 100 µg/ml RNase A). Cell Lysis Solution (0.2 N

NaOH; 1.0% SDS) was added to the resuspended cells, mixed by inversion of the microcentrifuge tubes, and incubated at room temperature for 5 minutes. Neutralization Solution (1.32 M KOAc) was mixed into the cell lysate and centrifuged. The supernatant, containing the plasmid DNA, was pipetted into a 3 ml syringe (Becton Dickinson and Co., Franklin Lakes, NJ) attached to a Wizard™ Minicolumn. Wizard™ DNA Purification Resin was added to the lysate and plunged through the minicolumn. The minicolumn was rinsed with Column Wash Solution (80 mM KOAc; 8.3 mM Tris-HCl, pH 7.5; 40 μ M EDTA; 55% ethanol), removed from the syringe and dried by centrifugation. Plasmid DNA was eluted off the minicolumn by the addition of TE buffer and centrifugation.

Plasmid DNA was also isolated by utilizing the PERFECTprep® Plasmid Mini (5 Prime→ 3 Prime, Inc., Boulder, CO) and Eppendorf Perfectprep Plasmid Mini kits (Brinkmann Instruments, Inc., Westbury NY). Two to three ml of bacterial culture was used for each prep. The protocol from the manufacturer was modified as follows: all microcentrifugation steps at 16,000 x g were increased to 60 seconds (vs. 30 seconds) for *Salmonella* strains; the DNA Binding Matrix Suspension (composition unknown) was preheated to 37°C before use; the Elution Buffer (70 μ l of TE buffer, pH 8.0) was preheated to 65°C to facilitate the elution of plasmid DNA from the DNA Binding Matrix; and all isolated plasmid DNA was incubated at for 65°C for greater than 15 minutes to heat-inactivate any residual nucleases. All plasmid DNA was frozen at -20°C for short-term storage and -78°C for long-term storage.

Purification and concentration of DNA. DNA was purified, when necessary, by extraction with phenol/chloroform/isoamyl alcohol (242). Phase Lock Gel™ (5 Prime→ 3 Prime, Inc., Boulder, CO) microcentrifuge tubes were used during the extraction process according to the manufacturer's protocol: an equal volume of DNA sample (genomic or plasmid) and phenol/chloroform/isoamyl alcohol were added to a Phase Lock Gel™ microcentrifuge tube, mixed, and centrifuged (14,000 x g) for two minutes. A thick density barrier was formed between the upper aqueous phase, containing the DNA, and the protein-laden interface and the lower organic phase. The upper aqueous phase was then simply poured off into sterile microcentrifuge tubes. A second extraction of the trapped organic phase with TE buffer and phenol/chloroform/isoamyl alcohol removed any remaining DNA. The DNA

samples were pooled, concentrated by ethanol precipitation, lyophilized, and resuspended in an appropriate volume of TE buffer .

DNA was also purified and concentrated directly by ethanol precipitation (242). GenElute™ LPA, a linear polyacrylamide DNA carrier, (Supelco, Bellefonte, PA) was used in most ethanol precipitations of plasmid DNA and amplicons. The protocol from the manufacturer was modified by increasing the sodium acetate (Sigma Chemical Co., St. Louis, MO) concentration to 0.3 M to facilitate a more complete precipitation of the DNA in 100% ethanol (Quantum Chemical CO, Tuscola, IL). Precipitated DNA was pelleted by centrifugation (12,000 x g), lyophilized or air-dried at 50°C, and resuspended in TE buffer or Molecular Biology Grade water (5 Prime→ 3 Prime, Inc., Boulder, CO). All purified and concentrated DNA was frozen at -20°C for short-term storage and plasmid DNA was frozen at -78°C for long-term storage.

Purification and concentration of DNA from agarose gels. DNA, including amplicons, was also purified and recovered from agarose gels. Bands of electrophoresed genomic or plasmid DNA were cut from agarose gels and added to GenElute™ Minus EtBr Spin Columns (Supelco, Bellefonte, PA) pre-washed in TE Buffer. The columns were inserted onto microcentrifuge tubes and centrifuged (12,000 x g) for 10 minutes. The columns retained the agarose and ethidium bromide while the DNA was eluted by the TE buffer. The DNA was then concentrated, when necessary, by ethanol precipitation.

A second method was also employed for the recovery of DNA from low melt agarose gels. After electrophoresis, the band containing the DNA was cut from of the gel and placed in a 0.5 ml sterile microcentrifuge tube (Fisher Scientific, Fair Lawn, NJ). The microcentrifuge tube was heated to 55°C in a heating block (FisherBrand Dry Bath Incubator; Fisher Scientific, Fair Lawn, NJ). After the agarose melted, an equal volume of 10 % (vol/vol) glycerol (Sigma Chemical Co., St. Louis, MO) was added. The aqueous solution containing the DNA now remained liquid at room temperature and was utilized directly in restriction endonuclease digests and ligase reactions (244, 245). The purified and/or concentrated DNA isolated from agarose gels was frozen at -20°C for storage.

Polymerase chain reactions. The polymerase chain reaction (PCR) was utilized for the amplification of specific DNA (246). A GeneAmp PCR System 9600 Thermocycler (Applied Biosystems, Foster City, CA) was employed for the 25 μ l, 50 μ l, or 100 μ l reactions. Primers were designed (Table 2) and ordered from GibcoBRL[®] (Life Technologies, Inc., Rockville, MD), Life Technologies[™] (Invitrogen Corporation, Rockville, MD), and Integrated DNA Technologies (Coralville, IA) (247). Three different DNA polymerases were utilized: DyNAzyme[™] II, DyNAzyme[™] EXT (Finnzymes, MJ Research, Inc., Incline Village, NV), and Expand[®] High Fidelity PCR System (Roche Diagnostics Corp., Indianapolis, IN) polymerases (248). Each PCR reaction contained 0.016-0.08 U/ μ l polymerase, 1.5 mM MgCl₂ in supplied PCR buffer, 0.4-1.6 mM of each deoxynucleoside triphosphate, 0.4 μ M of each primer, nanogram quantities of template DNA, and nuclease-free molecular biology grade water (248). PCR reactions were optimized with up to 3.0 mM MgCl₂ and/or 2.5 % (vol/vol) dimethyl sulfoxide (DMSO) supplied in the PCR kits (249). Each PCR reaction began with an initial template DNA denaturation for 5-7 minutes at 94°C and ended with a final primer extension for 7 minutes at 72°C to ensure that all amplicons were fully extended (250). The temperatures and times of the DNA denaturation, primer annealing, and primer extension steps (30-35 cycles) varied for each specific PCR reaction (251).

Quantification of DNA. Concentrations of DNA were estimated by ethidium bromide dot quantitation (252). Sample DNA, in a series of dilutions in TE Buffer and ethidium bromide (1.0 μ g/ml final concentration), was spotted onto Parafilm[®] M (American Can Company, Greenwich, CT) and viewed on a ultra-violet transilluminator. A series of standards, lambda DNA cut with *Hind* III (Gibco BRL, Gaithersburg, MD), was spotted above the sample DNA. Comparison of the fluorescence of the sample to these standards provided an estimate of the DNA concentration in the sample (252).

The concentration of DNA was also estimated by comparing the intensity of sample DNA, after agarose gel electrophoresis, to a standardized 1kb DNA step ladder (Promega Corporation, Madison, WI).

Endonuclease digests. Restriction endonucleases were purchased from GibcoBRL® (Gaithersburg, MD), New England Bio Labs (Beverly, MA), Promega Corporation (Madison, WI), and TaKaRa Biomedicals (Fisher Scientific, Fair Lawn, NJ). The manufacturer's protocols for restriction endonuclease digestions were modified for volumes of 20-100µl using nuclease-free, deionized, distilled water (Sigma Chemical Co., St. Louis, MO) (244). Bovine serum albumin (0.01 % [wt/vol]; supplied with enzyme) was added to each endonuclease digestion. DNA was digested from one hour to overnight with an excess of enzyme (>5U/µg DNA).

Calf intestinal alkaline phosphatase. Calf intestinal alkaline phosphatase (CIAP) (Promega Corporation, Madison, WI) was employed for the hydrolysis of 5'-phosphate groups vector DNA, following incubation with a restriction enzyme (253). Three successive CIAP reactions, following the manufacturer's protocol, were performed on all vectors digested with a single restriction endonuclease prior to ligation to an insert.

Ligase reactions. T4 DNA ligase (Promega Corporation, Madison, WI) reactions were performed according to the manufacturer's protocol for greater than 12 hours at 16°C. The molar ratios of vector:insert DNA routinely used were 1:1, 1:3, 1:5, 3:1, and 5:1 (245).

Transformations. *E. coli* and *S. typhimurium* competent recipient cells were prepared for all transformations according to a modified protocol from *Current Protocols in Molecular Biology* (254). An overnight culture of bacterial cells was grown in Luria broth containing the appropriate antibiotics; the fresh overnight culture was diluted (1:100) into Luria broth lacking the antibiotic and grown to an optical density (absorbance @ 600 nm) of 0.5 to 1.0. The cells were chilled on ice and pelleted by centrifugation (4000 x g) in a Sorvall® Super Speed RC2-B centrifuge (Ivan Sorvall, Inc., Norwalk, CT) for 15 minutes in a cold Sorvall® SS-34 rotor (Dupont Instruments, Norwalk, CT). The supernatant was poured off, and the cells were resuspended in the same volume of cold, sterile, distilled, deionized water (ddiH₂O). The bacterial cells were pelleted by centrifugation, washed again in sterile ddiH₂O, and washed once in 20 ml of 10% (vol/vol) glycerol (Sigma Chemical Co., St. Louis, MO). The washed cells were resuspended in 10% glycerol to a final concentration of approximately

10^{10} cells/ml. These competent recipient cells were used immediately and/or frozen at -78°C for up to 6 months (254).

Electroporation with high voltage was utilized to introduce DNA into the competent recipient cells (254). Each transformation contained 40 μl of chilled competent recipient cells and 1-2 μl of ligase reaction, intact plasmid DNA, or concentrated PCR-amplified DNA fragments. These were electroporated in 0.2 cm electrode gap cuvettes (Bio-Rad Laboratories, Hercules, CA) using a Bio-Rad Pulse Controller and Bio-Rad Gene Pulser (Bio-Rad Laboratories, Hercules, CA) at 2.5 kV, 25 μF , and 200 Ω . Time constants ranged between 4.0 ms to 4.8 ms. The electroporated cells were then incubated, shaking, for 1 hour at 37°C in Luria broth.. After incubation, 100-300 μl aliquots of the transformation mixture were spread plated onto Luria agar plates containing the appropriate antibiotic (254).

Determination of growth rate. SR-11 strains were adapted to M9 minimal media separately supplemented with various carbon sources with the appropriate antibiotic(s). The cultures were back-diluted into fresh homologous media to an optical density (absorbance @ 500 nm (OD_{500})) of approximately 0.1 utilizing a Ultraspec 2000[®] UV/Visible Spectrophotometer (Pharmacia Biotech, Cambridge, England) using FisherBrand[®] disposable semi-PS cuvettes (Fisher Scientific, Fair Lawn, NJ) (255). The cultures were grown to an $\text{OD}_{500} > 1.0$ at 37°C with aeration. Data points were recorded every 45-60 minutes throughout the experiment, then graphed with Slide Write Plus for Windows[®] (version 4.0; Advanced Graphics Software, Inc., Carlesbad, CA). The rate of growth (generation time) was calculated from data points in the log (exponential) phase using the formula:

$$\text{Generation Time (minutes)} = \frac{(t_2 - t_1) \log 2}{\log [(OD_2)/(OD_1)]}$$

Where t_1 and t_2 are the first and second time points in minutes and OD_1 and OD_2 are their respective OD_{500} readings (237).

β -galactosidase assays for *cra* promoter activity. SR-11 strains, containing the *cra* promoter-*lacZ* transcriptional fusion, were adapted to M9 minimal media separately supplemented with various carbon sources with the appropriate antibiotic(s). The cultures were back-diluted to an OD₅₀₀ of ≈ 0.1 into fresh homologous media containing 150 $\mu\text{g/ml}$ of ampicillin. The cultures were grown at 37°C with aeration to an OD₅₀₀ of ≈ 1.0 , then 1 ml samples were removed in triplicate for each culture.

β -galactosidase assays were performed by modified protocols by Miller (232) and Ausubel (233). The OD₆₀₀ of the 1 ml samples was measured then centrifuged @14,000 x g in microcentrifuge for 5-10 minutes. The supernatant was removed and the pellet was resuspended in 1 ml of Z buffer (pH 7.0; 1.61% [wt/vol] Na₂HPO₄ • 7H₂O (Sigma Chemical Co., St. Louis, MO), 0.55% [wt/vol] NaH₂PO₄ • H₂O (Sigma Chemical Co., St. Louis, MO), 0.075% [wt/vol] KCl (Sigma Chemical Co., St. Louis, MO), 0.0245% [wt/vol] MgSO₄ • 7H₂O (Sigma Chemical Co., St. Louis, MO), and 0.27% [vol/vol] β -mercaptoethanol (Sigma Chemical Co., St. Louis, MO)). One drop of toluene (Sigma Chemical Co., St. Louis, MO) was added to each sample, vortexed, and incubated at 37°C for 30 minutes in a heating block with the caps of the microcentrifuge tubes open to allow the toluene to evaporate. One hundred microliters of 5 mM ONPG (orhonitrophenyl- β -D-galactopyranoside; 5 mM in Z buffer; Sigma Chemical Co., St. Louis, MO) was added to each sample and incubated at 37°C for an additional for 45-64 minutes. Five hundred microliters of 1 M Na₂CO₃ (Sigma Chemical Co., St. Louis, MO) was added to each sample to stop the reaction and the elapsed time was recorded. The samples were centrifuge for 5 minutes @ 14,000 x g and the OD₄₂₀ and OD₅₅₀ of the supernatant was measured. The β -galactosidase activity was calculated using the formula:

$$\text{Units of } \beta\text{-galactosidase activity} = \frac{1000 [(OD_{420}) - 1.75(OD_{550})]}{t \cdot v (OD_{600})}$$

Where t = the time, in minutes, incubated at 37°C and v = the volume, in milliliters, of culture used in the assay.

Virulence assays (33, 210). Female BALB/c mice (Charles River Laboratories, Wilmington, MA) weighing 13-15 g were housed, at no more than 4 per sterile cage, with pine shavings as bedding. The mice were fed Prolab RMH2000 pellets (PMI Nutrition International, Inc., Brentwood, MO) and sterile deionized, distilled water. The cages were placed in an HEPA-filtered isolation unit (Britz-Heidbrink, Inc., Wheatlands, WY) to prevent contamination. Cages, food, water, and bedding were changed every other day throughout the entire experiment. All experiments were performed in accordance with institutional guidelines for animal care (IACUC Approval Number: A98-03-030).

All *S. typhimurium* SR-11 strains utilized in the virulence assays were grown in Luria broth, with the appropriate antibiotic(s), at 37°C, with aeration, for 18-19 hours. One ml of each strain was centrifuged (10,500 x g) for 5 minutes (certain strains required a second centrifugation to completely pellet the cells) in a microcentrifuge and the supernatant was carefully removed. The pelleted cells were washed twice in 1 ml of phosphate buffered saline with gelatin (pH 7.2; 0.85% [wt/vol] NaCl, 0.03% [wt/vol] KH₂PO₄, 0.06% [wt/vol] Na₂PO₄, and 0.1% [wt/vol] gelatin (Sigma Chemical Co., St. Louis, MO)). The washed cells were finally resuspended in 0.5 ml of phosphate buffered saline with gelatin and kept on ice.

The 30 day-old female BALB/c mice were moved to beddingless cages and food and water was withheld for 4 hours. At that time, 50 µl of 10% (wt/vol) sodium bicarbonate was administered to each mouse after placing the tip of a micropipet just behind the incisors. Approximately 30 minutes later, 20 µl of the above strains (ranging between 7.2×10^7 and 4.3×10^8 bacteria) were separately administered perorally to mice in quadruplicate (sham infected mice were fed only the sodium bicarbonate). Food, water, and bedding was returned after peroral inoculation. The inocula were calculated by serial dilutions of the bacterial cultures into 5 ml saline (0.85% [wt/vol] NaCl) dilution tubes, followed by 100 µl spread plates onto MacConkey agar plates containing the appropriate antibiotic(s).

The SR-11 infected mice were observed at least three times daily for signs of illness such as ruffled fur, eye infections, lethargy, labored breathing, and death. Within 12 hours from the time of death, the livers and spleens were removed, weighed, and homogenized (Pyrex #7725 homogenizer;

Fisher Scientific, Fair Lawn, NJ) in 1 ml of Luria broth. The homogenates were serially diluted into 5 ml saline dilution tubes and spread plated onto MacConkey agar containing the appropriate antibiotic(s). The cfu/organ were recorded. Surviving SR-11 infected BALB/c mice were sacrificed by carbon dioxide asphyxiation 9-21 days post peroral inoculation. The cfu/liver and cfu/spleen were also counted and recorded as above.

The *S. typhimurium* SR-11 *cradl craz* integrant control strain. A control strain, *S. typhimurium* SR-11 *cradl craz* integrant, containing a *cra* promoter-*lacZ* transcriptional fusion, was obtained from Mary P. Leatham (256). The strain was constructed by conjugative-dependent allele replacement utilizing the suicide vector pML55*cradl-craz* (Table 1, Figure 5) (256, 257). The control strain carries a partial upstream *ilvI* gene, an upstream *IlvH* gene, the upstream intergenic sequence, and the *cra* promoter ligated to the promoterless *lacZ* gene from the plasmid pCB267 (Figure 5) (256, 258). Approximately 947 bp of DNA upstream of the *cra* promoter were engineered into this construct to retain any possible regulatory sequences (256, 259). The 3' end of the *lacZ* reporter gene is ligated to approximately 200 bp of the remaining truncated *cra* gene, followed by approximately 534 bp of downstream DNA (a partial *yabB* gene) (Figure 5) (256). Since SR-11 *cradl craz* integrant is a partial diploid, containing both a wild-type *cra* gene and a *cra* promoter ligated to a reporter gene (Figure 5), it is able to utilize fatty acids and gluconeogenic substrates as sole carbon sources (231, 256, 257). The SR-11 *cradl craz* integrant strain was used as a control to measure the *cra* promoter activity (β -galactosidase assays) during growth on glycolytic and gluconeogenic substrates.

Construction of *S. typhimurium* SR-11 mutants by allelic exchange. An *S. typhimurium* SR-11 *fbp*⁻ mutant was constructed by conjugative-dependent allele replacement (239, 257). Since the genome was not fully sequenced and the homology between *S. typhimurium* and *E. coli* was approximately 85% identical at the nucleotide level, the *E. coli* fructose-1,6-bisphosphatase (*fbp*) DNA sequence was obtained using PubMed and Entrez Protein (66, 260-262). The *S. typhimurium* DNA sequence of the *fbp* gene was then pieced together from contig518 and contig1460 of the *S. typhimurium* LT2 unfinished fragments of the complete genome using BLASTn (263, 264). ORF Finder was utilized to determine the translational start and stop codons of the *fbp* gene (265). The

DNA sequence of the *fbp*, as well as the gene directly upstream (*yifG*) and directly downstream (*yifF*), were processed in Webcutter to find the restriction endonuclease sites (266). Primers for PCR were designed to make a permanent deletion in the *fbp* gene in which a chloramphenicol resistance gene could be inserted for selection purposes. The primers were also designed for the amplicon to be ligated into both the phagemid cloning vector pBluescript II SK (+) and the suicide vector pLD55 for conjugation and allelic exchange (Table 2) (257, 267, 268)

Using *S. typhimurium* SR-11 wild-type genomic DNA as a template and the *yifF*/forward and *yifG*/reverse primers (Table 2), a 3089 base pair (bp) fragment was amplified by PCR. This amplicon contained 1023 bp upstream (a partial *yifG* gene) and 1072 bp downstream (a partial *yifF* gene) of the *fbp* gene. The amplicon was digested with *Bam* HI and *Not* I and ligated into the *Bam* HI/*Not* I-double digested cloning vector pBluescript II SK (+). The ligase reaction was electroporated into *E coli* HB101 competent recipient cells. The resulting plasmid, pJHA-*fbp*⁺, was isolated and verified by PCR and restriction mapping (Figure 6) (269).

A deletion of 878 bp from the *fbp* gene was made by amplifying around pJHA-*fbp*⁺ (Figure 7) by PCR using the *fbp*-DEL/forward and *fbp*-DEL/reverse deletion primers (Table 2). The amplicon was digested with *Bam* HI, ligated to itself, and electroporated into *E coli* XL1-Blue MRF' competent cells. The result of the transformation yielded the plasmid pΔ*fbp* (Figure 8). The chloramphenicol resistance cassette from pJHA7 (Table 1) was digested with *Bam* HI, ligated into *Bam* HI-digested pΔ*fbp*, and electroporated into *E coli* XL1-Blue MRF' to produce the plasmid pΔ*fbp*Cam (Figure 9). The plasmids pJHA-*fbp*⁺, pΔ*fbp*, and pΔ*fbp*Cam were verified by PCR and restriction mapping. These plasmids were also sequenced (270-272) by Intervet International B.V. (Boxmeer, Netherlands) to confirm the *fbp* gene was cloned and deleted.

The plasmid pΔ*fbp*Cam (Figure 9) was digested with *Bam* HI and *Not* I and ligated into the *Bam* HI/*Not* I-double digested suicide vector pLD55 (Table 1). This ligase reaction was electroporated into the host strain *E. coli* S17-1 λ *pir* and the suicide vector p55Δ*fbp*Cam was subsequently isolated and verified by PCR and restriction mapping (Figure 10) (257, 273).

The temperature-sensitive donor strain *E. coli* S17-1 λ pir (p55 Δ fbpCam) was grown overnight at 30°C in LB containing ampicillin and chloramphenicol to retain the plasmid (273). An overnight culture of the recipient strain, *S. typhimurium* SR-11 wild-type, was grown in LB containing nalidixic acid. A conjugation was performed by mixing 200 μ l each of the donor and recipient strains in 5 ml of 10 mM MgSO₄ (273, 274). The mixture was suctioned through a Millipore® Vacuum Filtration System (Millipore Corporation, Bedford, MA) containing a sterile 0.45 μ m cellulose nitrate filter (Micro Filtration Systems, Dublin, CA). The membrane was placed, bacteria side up, on a pre-warmed LB agar plate and incubated for 5 hours at 37°C. After incubation, the membrane filter removed and vortexed in 5 ml of sterile 0.85% (wt/vol) saline. Aliquots of 50, 100, 200, and 300 μ l of the cells were spread plated onto LB agar plates containing chloramphenicol and nalidixic acid to select for partial diploid integrants (275). PCR, using colonies as template DNA, was utilized to screen for the correct exconjugant which successfully achieved homologous recombination with integration of the suicide vector p55 Δ fbpCam into its genome (257). The exconjugant was designated *S. typhimurium* SR-11 Δ fbpCam I-3 integrant (Figure 10).

Since *S. typhimurium* SR-11 Δ fbpCam I-3 integrant is a partial diploid, containing both a wild-type and deleted *fbp* gene (Figure 11), a second recombinational event was required to select for a *fbp*⁻ segregant (Figure 12). This was accomplished by positive selection for the loss of tetracycline resistance (239). An overnight culture of SR-11 Δ fbpCam I-3 integrant was grown in LB supplemented with chloramphenicol and subcultured, at an approximate concentration of 10⁶ colony forming units (cfu)/ml, into fusaric acid selection medium supplemented with chloramphenicol. The culture was incubated at 37°C, with aeration, for 48 hours then serially diluted to approximately 10³ cfu/ml (276). One hundred μ l of the 10³, 10⁴, and 10⁵ dilutions were spread plated onto fusaric acid agar plates containing chloramphenicol. Chloramphenicol-resistant, nalidixic acid-resistant, tetracycline-sensitive segregant colonies were screened for the absence of growth on M9 minimal agar media supplemented separately with various gluconeogenic substrates. One such colony was chosen and designated *S. typhimurium* SR-11 Δ fbpCam AX-3 segregant (Figure 12). The deletion of the *fbp* gene in the genome of this strain was confirmed by PCR.

Mutations in the *maeB* gene (encoding the gluconeogenic NADP-dependent malic enzyme), *sfcA* gene (encoding the gluconeogenic NAD-dependent malic enzyme), and the *pta* gene (encoding the enzyme phosphotransacetylase) were constructed in *S. typhimurium* SR-11 wild-type (277-280) (Figure 1). These separate mutations were constructed by allelic exchange with a slightly modified one-step inactivation of chromosomal genes using PCR products method as described by Datsenko and Wanner (281). The sequences, open reading frames, translational start and stop codons, the upstream and downstream genes, and the restriction endonuclease sites were determined for each gene as described above for the *fbp* gene (282-297, 298-301).

Deletion primers for PCR (Table 2) were designed with 45 nucleotides of homology within the wild-type *maeB*, *sfcA* and *pta* genes. The primers were also engineered with 20 nucleotides of homology to the kanamycin resistance gene in pKD4 (Table 1). Using pKD4 as template DNA and the deletion primers, five 100 μ l PCR reactions for each gene to be deleted were performed. The amplicons now contained a kanamycin resistant gene flanked by sequences homologous to the wild-type *maeB*, *sfcA* or *pta* genes (Figure 13). The PCR reactions were pooled (500 μ l/gene to be deleted), purified, and concentrated to approximately 10 μ l.

S. typhimurium SR-11 (pKD46) was grown in SOB media, containing 150 μ g/ml of ampicillin and 20 mM L-arabinose (the gene encoding λ Red recombinase, which greatly enhances the rate of homologous recombination, is located the plasmid pKD46 and is controlled by an arabinose-inducible promoter), at 30°C to an OD₆₀₀ of $\approx$ 0.6 and then made electrocompetent (254, 255). Two microliters of the concentrated linear DNA amplicons from above were electroporated into 40 μ l of fresh electrocompetent cells and incubated at 37°C in 1 ml of SOC media for 1 hour. Five 100 μ l aliquots were spread plated onto LB plates, containing either 50, 60, or 70 μ g/ml of kanamycin, and incubated at 37°C to cure any transformants of the temperature-sensitive plasmid. The remainder of the electroporated culture was incubated overnight at room temperature and then spread plated as above. Transformants which successfully underwent homologous recombination (Figure 13) were kanamycin-resistant and ampicillin-sensitive. Deletions in the *maeB*, *sfcA* or *pta* genes were

confirmed by PCR utilizing flanking confirmation primers (Table 2). These strains were designated *S. typhimurium* SR-11 *maeB*⁻, SR-11 *sfcA*⁻, and SR-11 *pta*⁻ respectively (Table 1).

The strains *S. typhimurium* SR-11 *cradl craz* integrant (256), SR-11 *maeB*⁻ *cradl craz* integrant, SR-11 *sfcA*⁻ *cradl craz* integrant, and SR-11 *pta*⁻ *cradl craz* integrant (Table 1) were constructed to assay for *cra* promoter activity (231). The strains were constructed by conjugative-dependent allele replacement which utilized the donor strain *E. coli* S17-1 λ *pir* (pML55*cradl-craz*) (Table 1). The suicide vector pML55*cradl-craz* contained a *cra* promoter-*lacZ* transcriptional fusion, plus DNA sequences flanking the *cra* gene, ligated into the suicide vector pLD55 (Table 1) (257). Exconjugants which successfully achieved homologous recombination with integration of the suicide vector pML55*cradl-craz* into its genome were selected on LB plates containing ampicillin and kanamycin (257). The integrants were confirmed on LB plates containing tetracycline, XLD plates, and LB plates containing approximately 70 μ g/ml of X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; Sigma Chemical Co., St. Louis, MO) (302). The integrants are partial diploids, containing both a wild-type *cra* gene and a *cra* promoter-*lacZ* transcriptional fusion; these strains were therefore also confirmed for growth on M9 minimal agar plates separately supplemented with various carbon sources. The kanamycin resistance cassette, as well as the *cra* promoter-*lacZ* transcriptional fusion (using *cra*-COMP/forward and *lacZ*/reverse primers), were confirmed by PCR.

Complementation of the *cra*⁻ and *fbp*⁻ genes. The *S. typhimurium* SR-11 Fad⁻ strain was complemented (211) with the plasmid pJHA8 containing the wild-type *cra* gene (Table 1). The plasmid pJHA8 was constructed with PCR by amplifying the *cra* gene from SR-11 wild-type template genomic DNA and primers (*cra*-COMP/forward and *cra*-COMP/reverse primers) 128 bp upstream of the translational start codon and 293 bp downstream of the translational stop codon (Table 2). The 1.4 kbp amplicon was digested with *Pst* I and ligated into the *Pst* I site of the cloning vector pBR322 yielding the plasmid pJHA8 (303). The plasmid was electroporated into the cloning strain, *E. coli* HB101, and isolated (Table 1). The plasmid was then electroporated into *S. typhimurium* MS1868, a restrictionless strain which modified the plasmid DNA (Table 1) (304). After isolating the modified plasmid DNA, it was electroporated into the SR-11 Fad⁻ strain (Table 1).

The *S. typhimurium* SR-11 Δ *fbp*Cam AX-3 segregant strain was complemented with a plasmid containing the wild-type *fbp* gene (211). Utilizing SR-11 wild-type genomic DNA as a template and the *yifF*/forward and *yifG*/reverse primers (Table 2), a 3089 bp fragment was amplified by PCR. This amplicon contained 1023 bp upstream (a partial *yifG* gene) and 1072 bp downstream (a partial *yifF* gene) of the *fbp* gene. The amplicon was digested with *Bam* HI and *Not* I and ligated into the *Bam* HI/*Not* I-double digested cloning vector pBluescript II SK (+). The resulting plasmid, pJHA-*fbp*⁺ (Figure 6), was electroporated into *S. typhimurium* MS1868, isolated, and then electroporated into the SR-11 Δ *fbp*Cam AX-3 segregant strain.

Statistics using the Student t distribution. To determine if the differences observed in β -galactosidase activities were significant, the student t distribution was calculated using a P value of 0.05. Significance or insignificance was reported with a 95% confidence limit (305).

Construction, confirmation, and characterization of the *S. typhimurium* SR-11 *fbp*⁻ mutant. A mutation in the *fbp* gene, which encodes the gluconeogenic enzyme fructose-1,6-bisphosphatase was constructed in *S. typhimurium* SR-11 (Figure 1). A deletion of 878 bp from the wild-type *fbp* gene was made and replaced with a chloramphenicol resistance cassette. The resulting *S. typhimurium* SR-11 Δ *fbp*Cam AX-3 segregant *fbp*⁻ mutant strain was confirmed by PCR (Figure 14).

The SR-11 *fbp*⁻ mutant strain, like the SR-11 Fad⁻ vaccine strain, was unable to utilize gluconeogenic substrates as sole carbon sources when streaked onto M9 minimal agar plates (Table 3). With the expected exception of the SR-11 *fbp*⁻ mutant's inability to metabolize glycerol as a sole carbon source, the phenotypes observed were identical to the SR-11 Fad⁻ vaccine strain. Furthermore, complementation with a wild-type *fbp* gene on the plasmid pJHA-*fbp*⁺, restored the ability of the SR-11 *fbp*⁻ mutant to utilize gluconeogenic substrates as sole carbon sources on M9 minimal agar plates.

Virulence of the *S. typhimurium* SR-11 Δ *fbp*Cam AX-3 segregant in BALB/c mice. The SR-11 *fbp*⁻ mutant strain was administered perorally (inoculation of approximately 2.4×10^8 cfu) to 30 day-old female BALB/c mice and was found to be virulent (Table 4). The cfu recovered from the livers and spleens of the mice fed the SR-11 *fbp*⁻ mutant strain were similar to the numbers of bacteria recovered from the mice fed the SR-11 wild-type control strain (Table 4). A mortality rate of 100% was observed in the SR-11 *fbp*⁻ mutant at the same inoculum that is 100% avirulent in the SR-11 Fad⁻ vaccine strain.

β -galactosidase assays, for *cra* promoter activity, in the SR-11 *cradl* *craz* integrant. The *cra* promoter activity was highest in SR-11 *cradl* *craz* integrant when grown on gluconeogenic substrates (Table 5). The highest *cra* promoter activity was observed on succinate (3.3 units), followed by malate, pyruvate, citrate, oleate, acetate, and phospho(enol)pyruvate (1.7 units). The β -galactosidase assays in SR-11 *cradl* *craz* integrant were lower when grown on glycolytic substrates versus gluconeogenic substrates (Table 5). For glycolytic substrates, the highest *cra* promoter activity was observed on glucose (1.3 units), followed by gluconate (1.0 units), and lowest on fructose (0.9

units). As expected, the β -galactosidase assays revealed no activity in the SR-11 wild-type control grown on glucose (0.0 units).

The ratio of the β -galactosidase units for each substrate assayed relative to the β -galactosidase units for fructose, the lowest *cra* promoter activity observed (0.9 units), was calculated. The highest ratio, at 3.6, was for the SR-11 *cradl cra* integrant when grown on succinate (Table 6, Figure 15). This translates to 3.6 times more *cra* promoter activity observed when grown on succinate versus fructose. The average increase in *cra* promoter activity, for all the gluconeogenic substrates tested, was 2.5-fold above the *cra* promoter activity for growth on fructose.

The generation times of SR-11 *cradl cra* integrant when grown on gluconeogenic substrates were longer than when grown on glycolytic substrates (Table 5). The generation times on gluconeogenic substrates were greater 100 minutes, whereas on glycolytic substrates, the generation times were less than 91 minutes. The fastest growth rate for the SR-11 *cradl cra* integrant was recorded in M9 minimal broth supplemented with fructose (73 minutes).

Construction, confirmation, and characterization of the *S. typhimurium* SR-11 *maeB* and SR-11 *sfcA* mutants. Mutations in the *maeB* gene (encoding the gluconeogenic NADP-dependent malic enzyme) and the *sfcA* gene (encoding the gluconeogenic NAD-dependent malic enzyme) were constructed in *S. typhimurium* SR-11 (Figure 1). A deletion of 2191bp from the wild-type *maeB* gene and a separate deletion of 1612 bp from the wild-type *sfcA* gene were made; both deletions were replaced with a kanamycin resistance cassette. The resulting *S. typhimurium* SR-11 *maeB* and *S. typhimurium* SR-11 *sfcA* mutant strains were confirmed by PCR (Figure 16, 17, 18).

The *S. typhimurium* SR-11 *maeB* and *sfcA* mutants grew as well as the SR-11 wild-type on M9 minimal agar plates supplemented with glucose or fructose, but grew more slowly, versus the wild-type, on M9 minimal agar plates supplemented with malate or succinate. Noticeably longer generation times for the SR-11 *maeB* and *sfcA* mutants, versus the SR-11 wild-type, were observed in both mutants when grown on malate or succinate in M9 minimal broth (Table 7). The differences in growth rates of all three strains were minor when utilizing glycolytic substrates as sole carbon sources (Table 7).

β -galactosidase assays, for *cra* promoter activity, in the *S. typhimurium* SR-11 *maeB*⁻ *cradl craz* integrant and the SR-11 *sfcA*⁻ *cradl craz* integrant. The *cra* promoter activity in the SR-11 *maeB*⁻ mutant, measured by β -galactosidase assays, was slightly down-regulated (1.1- to 1.4-fold) on the two gluconeogenic substrates and one glycolytic substrate, versus the SR-11 *cradl craz* integrant control strain (Table 8). The greatest down-regulation was observed for growth on succinate (1.4-fold), followed by malate (1.1-fold), and glucose (1.1-fold). The *cra* promoter activity in the SR-11 *maeB*⁻ mutant was essentially the same as the control strain when grown on fructose (Table 8).

The *cra* promoter activity was significantly up-regulated ($P = 0.05$) in the SR-11 *sfcA*⁻ mutant on both gluconeogenic and glycolytic substrates, versus the SR-11 *cradl craz* integrant control strain (Table 8). The greatest up-regulation was observed for growth on malate (2.8-fold), followed by glucose (2.7-fold), fructose (2.5-fold), and succinate (2.3-fold).

Virulence of the *S. typhimurium* SR-11 *maeB*⁻ and SR-11 *sfcA*⁻ mutants in BALB/c mice.

The *S. typhimurium* SR-11 *maeB*⁻ and SR-11 *sfcA*⁻ mutant strains were virulent when administered perorally (inoculation of approximately 1.3×10^8 cfu) to 33 day-old female BALB/c mice. A mortality rate of 100% was observed in BALB/c mice fed either the SR-11 *maeB*⁻ mutant strain or the SR-11 *sfcA*⁻ mutant strain (Table 9). The day of expiration post-peroral inoculation for the BALB/c mice fed the SR-11 *maeB*⁻ and the SR-11 *sfcA*⁻ strains was similar to age-matched BALB/c mice fed the SR-11 wild-type control (Table 9). The cfu recovered from the livers and spleens of the mice fed the SR-11 *maeB*⁻ mutant strain (4.8×10^7 cfu and 3.9×10^7 cfu respectively) were approximately 1/2 of a log below the numbers of bacteria recovered from the mice fed the SR-11 wild-type control strain (Table 9). The cfu from the livers and spleens of the mice fed the SR-11 *sfcA*⁻ mutant strain (1.5×10^7 cfu and 7.9×10^7 cfu respectively) were approximately 1 log below the cfu recovered from the SR-11 wild-type control strain (Table 9).

The *S. typhimurium* SR-11 *sfcA*⁻ mutant strain was also fed (inoculation of approximately 2.1×10^8 cfu) to 50 day-old female BALB/c mice. Again, the SR-11 *sfcA*⁻ mutant strain was found to be virulent with a 100% mortality rate (Table 10). The day of expiration post-peroral inoculation for the BALB/c mice fed the *sfcA*⁻ mutant strain was similar to the age-matched BALB/c mice fed the SR-11

wild-type control (Table 10). The cfu recovered from the livers and spleens of the mice fed the SR-11 *sfcA*⁻ mutant strain were slightly elevated versus the numbers of bacteria recovered from the mice fed the SR-11 wild-type control strain (Table 10).

Construction, confirmation, and characterization of the *S. typhimurium* SR-11 *pta*⁻ mutant. To further examine the regulation of the global regulatory *cra* gene, a mutation in the *pta* gene, which encodes the enzyme phosphotransacetylase was constructed in *S. typhimurium* SR-11 (Figure 1). A deletion of 1911 bp from the wild-type *pta* gene was made and replaced with a kanamycin resistance cassette. The resulting *S. typhimurium* SR-11 *pta*⁻ mutant strain was confirmed by PCR (Figure 19).

The SR-11 *pta*⁻ mutant strain was also unable to utilize acetate as a sole carbon source in both M9 minimal agar plates and M9 minimal broth supplemented with potassium acetate. The generation times of the SR-11 *pta*⁻ *cradl* *craz* integrant were 19 minutes (22%) longer on glucose and 31 minutes (31%) longer on fructose, versus the SR-11 *cradl* *craz* integrant control, in M9 minimal broth (Table 11). The differences in the generation times of the SR-11 *pta*⁻ *cradl* *craz* integrant and the SR-11 *cradl* *craz* integrant control were minor for growth on succinate and malate (Table 11).

β-galactosidase assays, for *cra* promoter activity, in the SR-11 *pta*⁻ *cradl* *craz* integrant.

The *cra* promoter activity was significantly ($P = 0.05$) up-regulated (3.0- to 3.4-fold) in the SR-11 *pta*⁻ *cradl* *craz* integrant using both gluconeogenic and glycolytic substrates, versus the SR-11 *cradl* *craz* integrant control strain (Table 12). The greatest up-regulation was observed for growth on glucose (3.4-fold), followed by succinate (3.1-fold), malate (3.0-fold), and fructose (3.0-fold) (Table 12).

Virulence of the *S. typhimurium* SR-11 *pta*⁻ mutant in BALB/c mice. The *S. typhimurium* SR-11 *pta*⁻ mutant strain was administered perorally (inoculation of approximately 1.6×10^8 cfu) to 33 day-old female BALB/c mice and was found to be avirulent (Table 13). However, the mice showed signs of disease: ruffled fur, eye infections, and lethargy. Two mice recovered by Day₁₄ of the experiment and the remaining two mice were also recovering. The intensity of the disease was not as pronounced as the mice fed the SR-11 wild-type strain. The cfu recovered from the livers and spleens of the mice fed the SR-11 *pta*⁻ mutant strain (6.9×10^6 cfu and 6.7×10^6 cfu respectively), sacrificed on

Day₁₅, were 1.3 logs less than numbers of bacteria recovered from the mice fed the SR-11 wild-type control strain (Table 13).

The link between gluconeogenesis in *S. typhimurium* SR-11 and virulence in BALB/c mice. Like the SR-11 Fad⁻ vaccine strain, gluconeogenesis is interrupted in the SR-11 *fbp*⁻ mutant, yet the SR-11 *fbp*⁻ mutant strain proved virulent in BALB/c mice. This indicates that general interruption of the gluconeogenic pathway is not the reason for the SR-11 Fad⁻ vaccine strain's avirulence in BALB/c mice. The Cra protein may be involved in the regulation of some other cellular function related to virulence or μ M concentrations of glucose or other glycolytic substrate(s) were available *in vivo* for SR-11 *fbp*⁻ mutant strain (growth *in vitro* requires 80-160 μ M glucose). Co-metabolism, utilizing both glycolytic and gluconeogenic substrates, may have occurred in the SR-11 *fbp*⁻ mutant *in vivo*.

The availability of μ M concentrations of glucose or another glycolytic substrate(s) would allow the biosynthesis of essential metabolites such as ribose-5-phosphate for ribonucleotides and deoxynucleotides, erythrose-4-phosphate for aromatic amino acids, and glucose-6-phosphate and pentose-5-phosphate for LPS (69, 76). Without co-metabolism from a separate glycolytic substrate such as glucose, these metabolites would not be biosynthesized in an SR-11 *fbp*⁻ mutant, since gluconeogenesis is blocked at fructose-1,6-bisphosphate and never reaches glucose-6-phosphate and the pentose phosphate pathway (Figure 1). Furthermore, it appears that *in vivo*, mM concentrations of the *cra* effector fructose-1,6-bisphosphate did not accumulate and inactivate the Cra DNA binding protein (Figure 1). The SR-11 *fbp*⁻ mutant, *in vivo*, appears to possess a functioning Cra protein, unlike the SR-11 Fad⁻ vaccine strain which is avirulent in BALB/c mice (Table 4) (33, 210).

A subsequent publication revealed the existence of a second fructose-1,6-bisphosphatase in *E. coli* encoded by the *glpX* gene (306). *S. typhimurium* also contains this redundant fructose-1,6-bisphosphatase enzyme (307). After this research was completed, an SR-11 *fbp*⁻ *glpX*⁻ double mutant, also unable to utilize gluconeogenic substrates as sole carbon sources *in vitro*, was constructed and proved to be virulent in BALB/c mice (308).

The SR-11 Fad⁻, unable to utilize gluconeogenic substrates as sole carbon sources *in vitro* (Table 3), was able to invade and was viable in enclosed vacuoles within M cells of the Peyer's patches

in BALB/c mice (33). Additionally, when SR-11 *Fad⁻ cra⁻* is fed to BALB/c mice, low numbers of the strain (approximately 10^3 cfu) were recovered from both the liver and spleen (Table 4). This suggests that the SR-11 *Fad⁻* and the SR-11 *fbp⁻* mutant both have access *in vivo* to a glycolytic substrate in BALB/c mice.

Clearly, the SR-11 *fbp⁻* mutant's interruption "high" in the gluconeogenic pathway does not render *S. typhimurium* SR-11 avirulent in BALB/c mice (Figure 1, Table 4). Either co-metabolism utilizing both gluconeogenic and glycolytic substrates occurs *in vivo*, or the Cra protein is involved in the regulation of some other cellular function related to virulence. Perhaps the TCA cycle must generate a sufficient concentration of the precursor metabolite phospho(enol)pyruvate via malate or oxaloacetate for virulence (Figure 1). PTS carbohydrate phosphorylation is coupled to carbohydrate translocation across the cytoplasmic membrane; the energy for these processes being provided by the precursor metabolite phospho(enol)pyruvate (91). The general PTS proteins, EI and HPr, transfer phosphoryl groups from phospho(enol)pyruvate to the sugar-specific transporters (EII^{sugar}) in the PTS system (91, 207). A strain of *S. typhimurium*, with a mutation in the *ptsH*-encoded EI enzyme of the PTS system, has been reported as attenuated for virulence in BALB/c mice (207). The PTS system is also an integral part of cAMP-CRP regulation (catabolite repression) and the genes of its regulon (183-185). The live oral *S. typhimurium* Δcya (adenylate cyclase) Δcrp (cAMP receptor protein) vaccine is based on deletions in the cAMP-CRP global regulatory system (197). The above mutant strains, both attenuated for virulence in BALB/c mice, reveal the importance for a fully functional PTS system driven by the precursor metabolite phospho(enol)pyruvate; the SR-11 *fbp⁻* mutant, although blocked in the gluconeogenic pathway at fructose-1,6-bisphosphate, may have generated a sufficient concentration of phospho(enol)pyruvate (Figure 1).

A *S. typhimurium* *sfcA⁻* mutant, deficient in the NAD-dependent malic enzyme, was reported to be avirulent in BALB/c mice (20, 80). Furthermore, four-carbon gluconeogenic substrates such as malate and/or oxaloacetate were noted as clearly important for growth in the murine host (20). Unlike the SR-11 *fbp⁻* mutant, where gluconeogenesis is interrupted up "high" in the gluconeogenic pathway, the reported *sfcA⁻* mutant interrupts gluconeogenesis directly off the TCA cycle (Figure 1). The

difference in the location of the interruption in the gluconeogenic pathway may account for the virulence observed in the SR-11 *fbp*⁻ mutant.

The *cra* promoter activity in the SR-11 *cradl craz* integrant. To further investigate the link between the *cra* gene, gluconeogenesis, and virulence in BALB/c mice, the *cra* promoter activity in the SR-11 *cradl craz* integrant (containing a *cra* promoter-*lacZ* transcriptional fusion) was determined for growth on various glycolytic and gluconeogenic substrates (104, 232, 233). Overall, the *cra* promoter activity was up-regulated (1.6- to 3.1-fold) when grown on gluconeogenic substrates versus glycolytic substrates (Table 5). The average increase in *cra* promoter activity, for all the gluconeogenic substrates tested, was 2.5-fold above the *cra* promoter activity for growth on fructose (Table 6, Figure 15). Except for pyruvate, the *cra* promoter activities were highest for growth on substrates within the TCA cycle. These data are consistent with the function of the Cra protein: up-regulation of transcription of genes encoding enzymes involved in gluconeogenesis (specific enzymes in the TCA cycle, the glyoxylate shunt, and the gluconeogenic pathway) and repression of transcription of genes encoding glycolytic enzymes (specific enzymes in the Embden-Meyerhof-Parnas and Entner-Doudoroff pathways) (Figure 3) (212, 217-219). Furthermore, gluconeogenesis or one of the metabolites in the gluconeogenic pathway appears to up-regulate *cra* promoter activity.

The *cra* promoter activity of SR-11 *cradl craz* integrant during growth on malate was up-regulated by 2.1-fold higher versus growth on glucose (Table 5). *S. typhimurium* contains two redundant malic enzymes: an NAD-dependent malic enzyme (encoded by the *sfcA* gene) and NADP-dependent malic enzyme (encoded by the *maeB* gene) (123, 125, 126). Both enzymes are malate oxidoreductases which convert malate, normally present in the TCA cycle, to pyruvate (Figure 1) (125, 126, 309, 310). Both biochemical reactions, studied more extensively in *E. coli*, are essentially irreversible (81, 309-311). In *E. coli*, it has been suggested that the *sfcA*-encoded NAD-dependent malic enzyme is involved in gluconeogenesis and that the *maeB*-encoded NADP-dependent malic enzyme supplies the cell with NADPH when growing on C₄ carbon sources (312). Another study in *E. coli* suggested that the NADP-dependent malic enzyme is also involved in the supply of acetyl-CoA

from malate, which is utilized for the biosynthesis of lipids as well as for the maintenance of TCA cycle intermediates (310).

During growth on gluconeogenic substrates such as malate (succinate, citrate, pyruvate, acetate, or oleate) the pyruvate pool is divided between conversion to phospho(enol)pyruvate (up the gluconeogenic pathway) and conversion to acetyl-CoA to fuel the TCA cycle; both routes are required for the biosynthesis of precursor metabolites for growth (Figure 1). Because of the high demand for acetyl-CoA to fuel the TCA cycle, it is unlikely that much of the acetyl-CoA is converted to acetyl phosphate (Figure 1).

The *cra* promoter activity in SR-11 *cradl craz* integrant grown on the glycolytic substrate glucose was 2.1-fold lower than when grown on malate (Table 5). In these non-limiting glucose growth conditions, an incomplete branched, biosynthetic form of the TCA cycle is utilized (Figure 2) (74, 78). The full TCA cycle is not required under these conditions because the bulk of energy is derived from glycolysis (74, 78). In this glucose-rich environment, cells produce excess acetyl-CoA which drains through acetyl phosphate, to further produce ATP with the associated secretion of acetate (Figure 2) (78, 79). As cell density increases, the build-up of acetate also serves to increase the size of the acetyl phosphate pool (78).

The *cra* promoter activity for SR-11 *cradl craz* integrant cells grown on acetate was 1.4-fold higher than glucose-grown cells (Table 5). *S. typhimurium* contains two pathways for the utilization of acetate: the first pathway is a direct route to acetyl-CoA catalyzed by the *acs* gene product acetyl-CoA synthetase; the second pathway forms the intermediate metabolite, acetyl phosphate, catalyzed by the *ackA*-encoded acetate kinase A enzyme and then acetyl-CoA is formed by the *pta*-encoded phosphotransacetylase enzyme (Figure 1) (279, 280, 313-316). Biochemical studies in *E. coli* on the *acs* pathway and the *pta-ackA* pathway, which both lead to acetyl-CoA, may have clarified their function (Figure 1) (317-319). In these biochemical studies, the *pta* and *ackA* genes were both down-regulated by approximately 2-fold while the *acs* gene was induced by greater than 8-fold in a global expression profiling experiment of acetate-grown *E. coli* using glucose grown cultures as a reference (317). In another experiment, investigating the global regulation of the main metabolic pathways of *E.*

coli based on 2-dimensional electrophoresis, similar results were observed: the *pta*-encoded and *ackA*-encoded enzymes were down-regulated (1.3-fold and 1.7-fold respectively) for aerobic growth on acetate versus the glucose-grown control (318). These combined data for both experiments suggest that the *acs* gene pathway is mainly responsible for acetate uptake, whereas the *pta-ackA* pathway is utilized for acetate excretion (Figure 1) (317-319). Consequently, a limited pool of acetyl phosphate would be expected for aerobic growth in acetate.

Overall, the predicted size of the acetyl phosphate pool, the biochemical pathways utilized, and the data from the β -galactosidase assays, implicate acetyl phosphate in the regulation of the *cra* gene; acetyl phosphate appears to function, indirectly or directly, as a repressor of the *cra* gene. Furthermore, acetyl phosphate is a logical candidate since it is a known global regulator of chemotaxis, the phosphate-specific transport system, and nitrogen regulation (24, 79, 189, 190). Acetyl phosphate also has been proposed to play a role in outer membrane proteins involved in osmo-regulation and the regulation of enzymes in the TCA cycle and glyoxylate shunt (24, 79, 189, 190).

The *cra* promoter activity of the *S. typhimurium* SR-11 *maeB*, SR-11 *sfcA*, and SR-11 *pta* mutants. A *S. typhimurium sfcA* mutant was reported to be avirulent in BALB/c mice and the importance of four carbon gluconeogenic substrates such malate and/or oxaloacetate for growth in the murine host was implied (20, 80). This warranted further study and also suggested that the *sfcA* gene may be involved in the regulation of the *cra* gene or the Cra protein. To test this hypothesis and the above implication that acetyl phosphate appears to function as a repressor of the *cra* gene, deletions in the *maeB*, *sfcA*, and *pta* genes were constructed in *S. typhimurium* SR-11 wild-type.

The generation times of the SR-11 *maeB*, and SR-11 *sfcA* mutants were essentially the same as the SR-11 wild-type for growth in M9 minimal broth supplemented with glucose or fructose (Table 7). However, considerably longer generation times (versus the SR-11 wild-type) for both the SR-11 *maeB* and SR-11 *sfcA* mutants were observed for growth in malate or succinate (Table 7). This suggests that both malic enzymes are required for maximum growth rates when utilizing gluconeogenic substrates. Conversely, both malic enzymes are not required for maximum growth rates when utilizing glycolytic substrates.

Of interest, the SR-11 *maeB*⁻ mutant's growth rate was 12 minutes (11%) slower on succinate and 41 minutes (22%) slower on malate than the SR-11 *sfcA*⁻ mutant grown on these gluconeogenic substrates (Table 7). These differences in growth rates may be due to the reported functions of the malic enzymes: the *sfcA*-encoded NAD-dependent malic enzyme is involved in gluconeogenesis whereas the *maeB*-encoded NADP-dependent malic enzyme supplies the cell with NADPH when growing on C₄ carbon sources and is also involved in the supply of acetyl-CoA from malate, which is utilized for the biosynthesis of lipids as well as for the maintenance of TCA cycle intermediates (310, 312).

Overall, considering the long generation times for growth in gluconeogenic substrates, a deletion in the *pta* gene in the SR-11 *pta*⁻ *cradl* *craz* integrant strain exhibited minor pleiotropic effects for growth utilizing gluconeogenic substrates (Table 11). Conversely, the growth rate of the SR-11 *pta*⁻ *cradl* *craz* integrant was 19 minutes (22%) slower on glucose and 31 minutes (31%) slower on fructose versus the SR-11 *cradl* *craz* integrant control (Table 11). Excess acetyl-CoA cannot drain through acetyl phosphate, to further produce ATP with the associated secretion of acetate in the SR-11 *pta*⁻ *cradl* *craz* integrant (Figure 2). This implies that the *pta* gene, and the ability to produce acetyl phosphate from acetyl-CoA, appear to be required for maximum growth rates when utilizing glycolytic substrates (Table 11). A similar conclusion was reported for the growth of *E. coli* in glucose: the ability to produce acetyl phosphate influences (depresses) the maximal growth rate (79).

The *cra* promoter activity in the SR-11 *maeB*⁻ *cradl* *craz* integrant was slightly down-regulated (1.1- to 1.4-fold) when grown on two gluconeogenic substrates and one glycolytic substrate, versus the SR-11 *cradl* *craz* integrant control strain (Table 8). In the SR-11 *maeB*⁻ *cradl* *craz* integrant, an increase in the concentration of its redundant *sfcA*-encoded NAD-dependent malic enzyme should be expected; mutational analysis has demonstrated that the malic enzymes can compensate for each other in acetate-grown cultures (317). It is possible that the same occurs in glucose-grown, succinate-grown, and malate-grown cultures. This minor down-regulation in the *cra* promoter activity of the SR-11 *maeB*⁻ *cradl* *craz* integrant may be due to an increased concentration of the *sfcA*-encoded malic enzyme.

The *cra* promoter activity was significantly ($P = 0.05$) up-regulated (2.3- to 2.8-fold) in the SR-11 *sfcA*⁻ *cradl craz* integrant grown on both gluconeogenic and glycolytic substrates, versus the SR-11 *cradl craz* integrant control strain (Table 8). This significant up-regulation becomes more striking when focused at the extremely high β -galactosidase units observed: 7.0 units for growth on succinate and 8.2 units for growth on malate (Table 8). The *sfcA*-encoded NAD-dependent malic enzyme appears to function as a transcriptional repressor, or play a role in the transcriptional repression, of the global regulatory *cra* gene.

Similar to the SR-11 *sfcA*⁻ *cradl craz* integrant, the *cra* promoter activity was significantly considerably up-regulated (3.0- to 3.4-fold) in the SR-11 *pta*⁻ *cradl craz* integrant on both gluconeogenic and glycolytic substrates, versus the control strain (Table 12). Although the β -galactosidase activities were slightly lower in the SR-11 *cradl craz* integrant control strain for this set of assays, the ratio of units of β -galactosidase for each substrate, relative to units of β -galactosidase for the SR-11 *cradl craz* integrant control strain, compensates for this and reveals a clearer view of the *cra* promoter activity (Table 12). It appears that a repressor of the *cra* gene ceased to function in the SR-11 *pta*⁻ *cradl craz* integrant: this implicates acetyl phosphate, indirectly or directly, in the regulation of the *cra* gene.

Virulence of the *S. typhimurium* SR-11 *maeB*⁻, SR-11 *sfcA*⁻, and SR-11 *pta*⁻ mutants in BALB/c mice. Both the SR-11 *maeB*⁻ and SR-11 *sfcA*⁻ mutants were virulent in BALB/c mice (Table 9). Since an *sfcA*⁻ mutant strain of *S. typhimurium*, deficient in the NAD-dependent malic enzyme, was reported to be avirulent in mice, a second virulence experiment was performed utilizing more mature mice (20, 80). The SR-11 *sfcA*⁻ mutant strain proved to be virulent in more mature BALB/c mice (Table 10). It was later determined that the *sfcA*⁻ mutant strain reported to be avirulent, was submitted to the American Type Culture Collection (ATCC) as a strain of *S. typhimurium*, but the ATCC characterized this strain as *S. choleraesuis* (a swine pathogen) (320). The pathogenicity of a *S. choleraesuis sfcA*⁻ mutant strain may deviate from the pathogenicity of a *S. typhimurium sfcA*⁻ mutant strain in the mouse model.

The SR-11 *pta*⁻ mutant strain was found to be avirulent in BALB/c mice (Table 13). The mice exhibited symptoms of murine typhoid fever, but the intensity of the disease was not as pronounced as the mice fed the SR-11 wild-type strain. Two mice recovered by Day₁₄ of the experiment and the remaining two mice were also recovering. The cfu recovered from the livers and spleens of the mice fed the SR-11 *pta*⁻ mutant strain (6.9×10^6 cfu and 6.7×10^6 cfu respectively), sacrificed on Day₁₅, were 1.3 logs less than numbers of bacteria recovered from the mice fed the SR-11 wild-type control strain (Table 13). Since the SR-11 *pta*⁻ mutant strain cannot synthesize acetyl phosphate from acetyl-CoA and acetyl phosphate is a known global regulator, it appears that an essential virulence factor was not induced (24, 79).

Mouse typhoid infections are directly dependent on the toxicity of lipid A (9). A *S. typhimurium waaN*⁻, lacking a single acyl chain on its lipid A domain of LPS molecules, was 90% avirulent in BALB/c mice (9). Extremely high counts of bacteria (10^9 cfu) were recovered from the livers and spleens of the mice (9). Most of the mice carrying the high bacterial loads slowly cleared the *Salmonella* from their organs (9). Perhaps acetyl phosphate, regulating over fifty different two-component signal transduction systems in *E. coli*, is involved in the regulation of lipid A biosynthesis (24). The SR-11 *pta*⁻ mutant strain may also have an altered lipid A component, since it cannot synthesize acetyl phosphate from acetyl-CoA.

Another possibility of an essential virulence factor not induced in the SR-11 *pta*⁻ mutant strain is the SPI-2 type III secretion system (22, 56). The two-component signal transduction system, SsrA-SsrB, is required for SPI-2 gene expression of the SPI-2 type III secretion system as well as its translocated effectors (22, 56). SsrA-SsrB is positively regulated by the OmpR-EnvZ two-component system (22, 56). Acetyl phosphate is a major secondary source of phosphoryl groups for response regulators of two-component signal transduction pathways; in *E. coli*, acetyl phosphate has been shown to phosphorylate OmpR *in vivo* (24, 25, 79, 190). Acetyl phosphate is not synthesized in the SR-11 *pta*⁻ mutant strain.

The *sfcA*-encoded NAD-dependent malic enzyme and acetyl phosphate may act in concert as a repressor of the *cra* gene. The SR-11 *sfcA*⁻ and SR-11 *pta*⁻ mutants both exhibited

significant ($P = 0.05$) up-regulation of *cra* promoter activity for growth on both gluconeogenic and glycolytic substrates. This significant up-regulation, combined with their virulence data, suggest that the *sfcA*-encoded NAD-dependent malic enzyme and acetyl phosphate may act in concert as co-repressors of the *cra* gene. Acetyl phosphate may phosphorylate or bind to the tetrameric NAD-dependent malic enzyme causing a conformational change in the enzyme (24, 79, 81, 321). The phosphorylated enzyme or phosphorylated enzyme complex may then bind to the operator downstream of the *cra* promoter which might repress transcription of the *cra* gene. If so, a concomitant depression of β -galactosidase activity would be observed (Figure 20). In the absence of either the *sfcA*-encoded NAD-dependent malic enzyme or acetyl phosphate, negative regulation of the *cra* gene would not occur; transcription of the *cra* gene would be up-regulated and more of the Cra protein would subsequently be produced (Figure 20). A concomitant elevation of β -galactosidase activity should also be observed. It is assumed that the degree of repression of the *cra* gene is also dependent on the concentrations of both the NAD-dependent malic enzyme and acetyl phosphate within the cell.

To date, the NAD-dependent malic enzyme has only been crystallized from the human mitochondrion, which is 57% similar to the *sfcA*-encoded NAD-dependent malic enzyme in *S. typhimurium* (277, 278, 322). X-ray diffraction reveals a helix-turn-helix motif which is a recognition motif common to many proteins that bind DNA (322, 323). However, this helix-turn-helix motif may not exist in the *sfcA*-encoded NAD-dependent malic enzyme. The protein sequence of the NAD-dependent malic enzyme from *S. typhimurium* does not contain the DNA-binding helix-turn-helix consensus sequence of the LacI-GalR family of bacterial transcription regulatory factors (321, 324). The LacI-GalR family of bacterial transcription regulatory factors contains over 25 regulatory DNA-binding proteins including the CRP and Cra proteins (324, 325). The NAD-dependent malic enzyme may bind DNA by an alternative mechanism from the LacI-GalR family of bacterial transcription regulatory factors.

Does the data fully support the hypothesis? One component of the data does not: the units of β -galactosidase in the SR-11 *sfcA*⁻ mutant grown on glucose and fructose (3.8 units and 2.6 units respectively) are not at the same levels observed for growth on succinate and malate (7.0 units and 8.2

units respectively) (Table 8). If the *sfcA*-encoded NAD-dependent malic enzyme is an integral component of a repressor of the *cra* gene, it seems logical to expect similar β -galactosidase activity for growth in both glycolytic and gluconeogenic substrates; the *cra* gene should be derepressed in a SR-11 *sfcA* mutant. The ratio of units of β -galactosidase, for each substrate, relative to units of β -galactosidase for the SR-11 *cradl cra* integrant control strain are similarly increased, but the actual units are not (Table 8). It appears that the transcription of the *cra* gene, like many other genes and operons, is subject to regulation by multiple regulatory proteins or multiple forms of regulation.

The orientation of the *cra* promoter-*lacZ* transcriptional fusion versus the wild-type *cra* gene in the SR-11 *cradl cra* strains. A second possibility, although unlikely, to explain the significant differences in the *cra* promoter activities of the SR-11 *maeB* *cradl cra* integrant, SR-11 *sfcA* *cradl cra* integrant, the SR-11 *pta* *cradl cra* integrant, and the SR-11 *cradl cra* integrant control strain, involves the orientation of the *cra* promoter-*lacZ* transcriptional fusion versus the wild-type *cra* gene. If the orientation of the *cra* promoter-*lacZ* transcriptional fusion and the wild-type *cra* gene, in the SR-11 *maeB* *cradl cra* integrant and the SR-11 *cradl cra* integrant control strain, is inverted versus the other two strains, then there is a slight possibility of an transcriptional regulatory effect occurring in a distant location upstream of the *cra* promoter-*lacZ* transcriptional fusion (Figure 21). This seems highly improbable since approximately 947 bp of DNA upstream of the *cra* promoter was engineered into these constructs to retain any possible regulatory sequences (Figure 5) (256, 259).

Reported regulation of the *cra* gene by catabolite repression. It has been suggested, that FruR (encoded by the *fruR* gene; homologous to the Cra protein in *S. typhimurium*) synthesis in *E. coli* is subject to control by the cyclic AMP receptor protein (CRP) (213). Catabolite repression involves the cytoplasmic sensor of carbon and energy concentrations, cAMP, and the CRP protein; cAMP binds to CRP at specific DNA sequences in promoters, induces bends in the DNA, and interacts with RNA polymerase to promote transcriptional initiation (184, 185). However, the mechanism of cAMP-CRP regulation varies; CRP can function not only as an activator, but also as a repressor depending where it binds relative to the promoter (184, 185). Adenylate cyclase, which catalyzes the formation of cAMP from ATP, is more active when cellular concentrations of catabolites are low and less active when

catabolite concentrations are high (186). The synthesis of cAMP is regulated by a protein phosphorylation mechanism that is catalyzed by the PTS system; exogenous PTS sugars (i.e. glucose, fructose, mannitol) inhibit the synthesis of cAMP (91, 94, 184).

Catabolite repression of the *cra* gene would explain the lower β -galactosidase activity observed in SR-11 *scA⁻* *cradl* *craz* integrant and the SR-11 *cradl* *craz* integrant control strain grown in glycolytic substrates versus gluconeogenic substrates (Table 6, 8). For transcriptional activation, the CRP protein generally binds at two sites upstream of the promoter: -45 to -49 and -70 to -74 bp upstream of the transcriptional start site (326). In order to determine if the *cra* gene is subjected to catabolite repression, the DNA binding consensus sequence of the CRP protein [5'-CGCAATTAATGTGAGTT AGCTCACTCATTAGGCA-3'] was compared to the DNA sequence of the *cra* promoter of *S. typhimurium* (71, 323). Absolutely no significant homology was detected within 200 bp upstream and downstream of the +1 transcription start site; the CRP does not appear to bind to the *cra* promoter and is not subjected to catabolite repression (183).

Putative phosphorylated-ArcA DNA-binding sequence in the promoter of the *cra* gene.

The 10 bp DNA binding site consensus sequence for phosphorylated-ArcA (ArcA-P) is 5'-[A/T] GTTAATTA[A/T]-3' (193). The -35 DNA sequence ("35 bp upstream of the transcriptional start site") of the *cra* promoter is within the DNA sequence 5'-ATGTTAACGATTTTAA-3' and the underlined bases match the first six bases of the ArcA-P DNA binding site consensus sequence (33, 193). A gap of five bp follows and then the remaining four bases (italicized) of the ArcA-P DNA binding site consensus sequence continues. It is possible that the ArcA-P response regulator can bind and repress the transcription of the *cra* gene at this ArcA-P consensus sequence; considerable diversity in the consensus sequence, specifically in the last four bases, occurs in many operons regulated by the ArcA-ArcB two component regulatory system (193). For example, diversity in base substitution of the ArcA-P binding site consensus sequence is encountered in *gluA* (citrate synthase) and the *sdhCDAB* (succinate dehydrogenase) promoters (193). At least eleven other promoters, shown to bind the ArcA-P response regulator, contain base substitutions (including C and G base substitutions) in the last four base sequences of the ArcA-P binding site consensus sequence (193).

Summary. Mutations in the *fbp*, *maeB*, or the *sfcA* genes of *S. typhimurium* SR-11 remained virulent in BALB/c mice when administered perorally. Conversely, a mutation in the *pta* gene attenuated the *S. typhimurium* SR-11 strain, and avirulence was observed in BALB/c mice. The *cra* promoter activity was higher in the *S. typhimurium* SR-11 *crdI* *craZ* integrant control strain when grown on gluconeogenic substrates versus glycolytic substrates. A mutation in the *maeB* gene slightly down-regulated the *cra* promoter activity when grown on either gluconeogenic or glycolytic substrates. In contrast, mutations in either the *sfcA* or the *pta* gene significantly ($P = 0.05$) up-regulated the *cra* promoter activity for growth on both gluconeogenic and glycolytic substrates. This significant up-regulation, combined with their virulence in BALB/c mice, suggest that the *sfcA*-encoded NAD-dependent malic enzyme and acetyl phosphate act in concert as a repressor of the *cra* gene. It also appears that the *cra* gene, like many other genes and operons, may be subject to regulation by multiple regulatory proteins or multiple forms of regulation.

Table 1. Bacterial strains and plasmids

Strain or plasmid:	Relevant genotype and/or phenotype:	Source or reference:
<i>Salmonella typhimurium</i> strains (plasmid):		
SR-11	<i>gyr1816</i> /nalidixic acid resistant	(197)
SR-11 <i>Fad</i> ⁻	<i>gyr1816 cra::Tn10d cam</i> /chloramphenicol resistant	(210)
SR-11 <i>Fad</i> ⁻ (pJHA8)	<i>gyr1816 cra::Tn10d cam</i> , (<i>cra</i> ⁺ <i>tet</i>)	(33)
SR-11 Δ <i>fbpCam</i> I-3 integrant	<i>gyr1816 fbp⁻ fbp⁺ cam bla tetAR</i>	This study
SR-11 Δ <i>fbpCam</i> AX-3 segregant	<i>gyr1816 fbp⁻ cam</i>	This study
SR-11 Δ <i>fbpCam</i> AX-3 segregant (pJHA- <i>fbp</i> ⁺)	<i>gyr1816 fbp⁻ cam (bla fbp⁺)</i>	This study
SR-11 (pKD46)	<i>gyr1816 (bla kan P_{araB} γ β <i>exo</i>)/kanamycin resistant arabinose-inducible promoter for λ Red recombinase</i>	This study
SR-11 <i>cradl craz</i> integrant	<i>gyr1816 cra⁺ bla tetAR/cra promoter-lacZ</i> fusion	(256)
SR-11 <i>maeB</i> ⁻	<i>gyr1816 maeB⁻ kan</i>	This study
SR-11 <i>maeB</i> ⁻ <i>cradl craz</i> integrant	<i>gyr1816 maeB⁻ bla tetAR kan/cra promoter-lacZ</i> fusion	This study
SR-11 <i>sfcA</i> ⁻	<i>gyr1816 sfcA⁻ kan</i>	This study
SR-11 <i>sfcA</i> ⁻ <i>cradl craz</i> integrant	<i>gyr1816 sfcA⁻ bla tetAR kan /cra promoter-lacZ</i> fusion	This study
SR-11 <i>pta</i> ⁻	<i>gyr1816 pta⁻ kan</i>	This study
SR-11 <i>pta</i> ⁻ <i>cradl craz</i> integrant	<i>gyr1816 pta⁻ bla tetAR kan /cra promoter-lacZ</i> fusion	This study
MS1868	<i>leu414 hsdL fcls2/restriction⁻ modification⁺ (RM⁺)</i>	K. L. Roland (304)

Table 1. Bacterial strains and plasmids (continued)

Strain or plasmid:	Relevant genotype and/or phenotype:	Source or reference:
<i>E. coli</i> strains (plasmids):		
HB101	F ⁻ <i>thi-1 hsdS20</i> (<i>r_B</i> ⁻ , <i>m_B</i> ⁻) <i>supE44 recA13 ara14 leuB6 proA2 lacY1 galK2 rpsL20</i> (<i>str</i> ^r) <i>xyl-5 mtl-1 λ</i> ⁻ /general cloning strain	Promega (Madison, WI)
XL1-Blue MRF'	Δ(<i>mcrA</i>)183 Δ(<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i> [F' <i>proAB lacI^F</i> ZΔM15 Tn5 (Kan ^r)]/general cloning strain	Stratagene (La Jolla, CA)
S17-1 λ <i>pir</i>	<i>E. coli</i> K-12 Tp ^r Sm ^r <i>recA thi hsdRM</i> ⁺ RP4::2-Tc::Mu::Km tn7, λ <i>pir</i> phage lysogen/Host for pLD55, p55Δ <i>bpCam</i> , and pML55 <i>cradl-craz</i>	V. de Lorenzo (274)

Table 1. Bacterial strains and plasmids (continued)

Strain or plasmid:	Relevant phenotype and/or genotype:	Source or reference:
Plasmids:		
pBR322	cloning vector/ <i>bla</i> <i>tet</i>	(303)
pJHA7	4.5 kb <i>Pst</i> I fragment containing <i>cra::Tn10d cam</i> in pBluescript II SK (+)/ <i>bla</i>	(33)
pJHA8	1.4 kb <i>cra</i> ⁺ gene in <i>Pst</i> I site of pBR322/ <i>tet</i>	This study
pBluescript II SK (+)	phagemid cloning vector/ <i>bla</i> <i>lacZ</i>	(267, 268)
pJHA- <i>fbp</i> ⁺	3.1 kb <i>Not</i> I/ <i>Xho</i> I fragment containing the <i>fbp</i> ⁺ gene in pBluescript II SK (+)/ <i>bla</i>	This study
pΔ <i>fbp</i> Cam	3.5 kb <i>Not</i> I/ <i>Xho</i> I fragment containing a deleted <i>fbp</i> gene ligated to the 1.4 kb chloramphenicol resistance cassette in pBluescript II SK (+)/ <i>cam bla</i>	This study
pLD55	suicide vector for allelic exchange/ <i>bla</i> <i>tetAR</i>	(257)
p55Δ <i>fbp</i> Cam	3.5 kb <i>Not</i> I/ <i>Xho</i> I fragment containing a deleted <i>fbp</i> gene ligated to the 1.4 kb chloramphenicol resistance cassette in pLD55/ <i>cam bla tetAR</i>	This study
pML55cra ⁺ l-craz	5.3 kb <i>Pst</i> I fragment containing a <i>cra</i> promoter- <i>lacZ</i> transcriptional fusion and flanking <i>cra</i> upstream and downstream genes in the suicide vector pLD55/ <i>bla tetAR</i>	(256)
pKD4	template plasmid used in allelic exchange for amplification of the kanamycin resistance cassette by PCR/ <i>bla kan</i>	(281)
pKD46	temperature sensitive plasmid used for allelic exchange with an arabinose-inducible promoter for λ Red recombinase/ <i>bla P_{arab} γ β exo</i>	(281)

Table 2. Primers used in the amplification of DNA by PCR

Primer:	DNA sequence and restriction endonuclease sites:
<i>yifF</i> /forward	5'-ATAAGAAT <u>GCGGCCG</u> TACGGTTATTGGTAGGGAGAGC-3' <i>Not</i> I
<i>yifG</i> /reverse	5'-GCTAGCTCGAGGTTGTGCATGTTATGTTCCGG-3' <i>Xho</i> I
<i>fbp</i> -DEL /forward	5'-ACTAGGGATCCAAGTACAGATTTACCTGAAATGCG-3' <i>Bam</i> HI
<i>fbp</i> -DEL /reverse	5'-ACTAGGGATCCAACGTATTCTGGATATCATTCCGG-3' <i>Bam</i> HI
<i>maeB</i> -DEL /forward	5'-CGTACAGAGGCGATCGGCGTTAATACATGTACCGGTTTAGACACGC ATATGAATATCCTCCTTAG-3'
<i>maeB</i> -DEL /reverse	5'-GCGCCCTTGATTTCCACGAATTTCGGTACCCGGTAAAATTCAGG TGTAGGCTGGAGCTGCTTCG-3'
<i>maeB</i> -Flank /forward	5'-ACTAGCTGCAGCCGGTTAGCGCGAGGATTTGC-3' <i>Pst</i> I
<i>maeB</i> -Flank /reverse	5'-ACTAGCTGCAGCACCGACTATTCTTTGTATTACTACC-3' <i>Pst</i> I
<i>sfcA</i> -DEL /forward	5'-GCACGTTCCCTTTATATCCCTTACGCTGGCCCTGTATTGCTGGAA TGTAGGCTGGAGCTGCTTCG-3'
<i>sfcA</i> -DEL /reverse	5'-CGCCTGTTGCAGCGCTTCCGCAGAGGTTTTCACCGCTACGCCTTG CATATGAATATCCTCCTTAG-3'
<i>sfcA</i> -Flank /forward	5'-ACTAGCTCGAGTCAGTGATGAATATTAAACCAACAGG-3' <i>Xho</i> I
<i>sfcA</i> -Flank /reverse	5'-ACTAGCTCGAGTGCACAATTTAGCCGCATCTTCCG-3' <i>Xho</i> I

Underlined DNA sequences represent restriction endonuclease sites. Italicized sequences are complementary to the flanking sequences in the kanamycin cassette of pKD4 (Table 1). DEL denotes a primer used for deleting, and confirming the the deletion, of a gene. Flank denotes a primer flanking the gene of interest used for confirming the wild-type and deleted genes.

Table 2. Primers used in the amplification of DNA by PCR (continued)

Primer:	DNA sequence and restriction endonuclease sites:
<i>pta</i> -DEL /forward	5'-CTACCGGAACCAGCGTCGGCCTGACCAGCGTCAGCCTCGGCGTCA TGTAGGCTGGAGCTGCTTCG-3'
<i>pta</i> -DEL /reverse	5'-CGATGGAGATCAGGTCGGCAGAACGCTGTACCGCTTTGTAGGTGG CATATGAATATCCTCCTTAG-3'
<i>pta</i> -Flank /forward	5'-ACTAGATTATTGGATGCAGTCGTGTTACCG-3' <i>Ase</i> I
<i>pta</i> -Flank /reverse	5'-ACTAGATTATTAGTTATTCATTGATGCAGCGC-3' <i>Ase</i> I
<i>cra</i> -COMP /forward	5'-ATCGACTGCAGTGCAGAAATCCGTGGTAACCCGG-3' <i>Pst</i> I
<i>cra</i> -COMP /reverse	5'-TAGCTCTGCAGCCTGTTTAACGTGTGCGGTGCC-3' <i>Pst</i> I
<i>lacZ</i> /reverse	5'-TAGATCGAATGCCTTATTTTGTACACCAGACCAACTGG-3' <i>Bsm</i> I

Underlined DNA sequences represent restriction endonuclease sites. Italicized sequences are complementary to the flanking sequences in the kanamycin cassette of pKD4 (Table 1). DEL denotes a primer used for deleting, and confirming the deletion, of a gene. Flank denotes a primer flanking the gene of interest used for confirming the wild-type and deleted genes. COMP denotes a primer utilized to clone, and confirm the cloning, of a gene. A cloned gene, amplified with a COMP primer, was intended to complement a deleted gene. The *lacZ*/reverse primer was utilized to confirm the *cra-lacZ* transcriptional fusion in *S. typhimurium* SR-11 *cradl* *craz* integrant strains.

Table 3. Growth of *Salmonella typhimurium* SR-11 wild-type, SR-11 Fad⁻, and SR-11 Δfbp Cam AX-3 *fbp*⁻ segregant on M9 minimal agar plates supplemented with various carbon sources

Carbon source:	SR-11 wild-type:	SR-11 Fad ⁻ :	SR-11 Δfbp Cam AX-3 <i>fbp</i> ⁻ segregant:
glucose:	(+)	(+)	(+)
gluconate:	(+)	(+)	(+)
alanine:	(+)	(-)	(-)
citrate:	(+)	(-)	(-)
fumarate:	(+)	(-)	(-)
glycerol:	(+)	(+)	(-)
oleate:	(+)	(-)	(-)
pyruvate:	(+)	(-)	(-)
succinate:	(+)	(-)	(-)

The M9 minimal agar plates, supplemented with various carbon sources, were incubated @ 37°C for 45 hours. A (+) denotes growth to at least the third quadrant on the plate. A (-) denotes no growth.

Table 4. Virulence of *Salmonella typhimurium* SR-11 wild-type, SR-11 Fad⁻, and SR-11 Δ fbpCam AX-3 fbp⁻ segregant in 4.0 week-old female BALB/c mice

Strain: [†]	Virulence: (survival)	Day of expiration post peroral inoculation: (mean $\pm$ std. deviation)	Log[cfu/liver]: (mean $\pm$ std. deviation)	Log[cfu/spleen]: (mean $\pm$ std. deviation)
SR-11 wild-type	0/4	7.5 $\pm$ 1.0	8.33 $\pm$ 0.58	8.00 $\pm$ 0.34
SR-11 Fad ⁻	4/4*	N/A [†]	3.41 $\pm$ 0.34*	3.84 $\pm$ 0.31*
SR-11 fbp ⁻	0/4	7.5 $\pm$ 1.0	8.18 $\pm$ 0.15	8.11 $\pm$ 0.20
sham infected	4/4	N/A [†]	N/A [†]	N/A [†]

[†] peroral inoculation of approximately 2.4×10^8 cfu.

* mice sacrificed on Day₁₃ of virulence experiment.

[†] N/A denotes not applicable.

Table 5. Generation time and β -galactosidase activity of *Salmonella typhimurium* SR-11 cradl craz integrant grown in M9 minimal broth supplemented with various carbon sources[♦]

Carbon source:	Mean generation time: (minutes $\pm$ std. deviation)	Units of β -galactosidase*: (mean $\pm$ std. deviation)
succinate:	109 $\pm$ 15 , n =3	3.3 $\pm$ 0.7 , n =9 [♦]
malate:	103 $\pm$ 10 , n =3	2.7 $\pm$ 0.6 , n =9 [♦]
pyruvate:	102 $\pm$ 20 , n =2	2.4 $\pm$ 0.2 , n =3 [♦]
citrate:	93 $\pm$ 5 , n =3	2.2 $\pm$ 0.1 , n =6 [♦]
oleate:	148 $\pm$ 25 , n =2	1.9 $\pm$ 0.1 , n =3 [♦]
acetate:	213 $\pm$ 18 , n =2	1.8 $\pm$ 0.1 , n =3 [♦]
phospho(enol)pyruvate:	78 , n =1	1.7 $\pm$ 0.1 , n =3 [♦]
glucose:	90 $\pm$ 4 , n =7	1.3 $\pm$ 0.2 , n =21 [♦]
gluconate:	67 $\pm$ 4 , n =3	1.0 $\pm$ 0.2 , n =9 [♦]
fructose:	73 $\pm$ 5 , n =4	0.9 $\pm$ 0.1 , n =12 [♦]
SR-11 wild-type:		
glucose:	73 , n =1	0.0 $\pm$ 0.0 , n =3 [♦]

[♦] grown in the presence of 150 μ g/ml of ampicillin to maintain the integrant (257).

* assayed @ OD₅₀₀ of $\approx$ 1.0

[♦] β -galactosidase assays were performed in triplicate for each culture.

Table 6. Ratio of β -galactosidase activity of *S. typhimurium* SR-11 cradl craz integrant grown in M9 minimal broth supplemented with various carbon sources relative to β -galactosidase activity of *Salmonella typhimurium* SR-11 cradl craz integrant grown in M9 minimal broth supplemented with fructose[†]

Carbon source:	Units of β -galactosidase*: (mean $\pm$ std. deviation)	Ratio of β -galactosidase units relative to β -galactosidase units for fructose :
succinate:	3.3 $\pm$ 0.7 , n =9 [†]	3.6
malate:	2.7 $\pm$ 0.6 , n =9 [†]	3.0
pyruvate:	2.4 $\pm$ 0.2 , n =3 [†]	2.6
citrate:	2.2 $\pm$ 0.1 , n =6 [†]	2.4
oleate:	1.9 $\pm$ 0.1 , n =3 [†]	2.1
acetate:	1.8 $\pm$ 0.1 , n =3 [†]	2.0
phospho(enol)pyruvate:	1.7 $\pm$ 0.1 , n =3 [†]	1.8
glucose:	1.3 $\pm$ 0.2 , n =21 [†]	1.4
gluconate:	1.0 $\pm$ 0.2 , n =9 [†]	1.0
fructose:	0.9 $\pm$ 0.1 , n =12 [†]	1.0

[†] grown in the presence of 150 μ g/ml of ampicillin to maintain the integrant (257).

* assayed @ OD₅₀₀ of $\approx$ 1.0

[†] β -galactosidase assays were performed in triplicate for each culture.

Table 7. Generation times of *Salmonella typhimurium* SR-11 *maeB*⁻, SR-11 *sfcA*⁻, and SR-11 wild-type grown in M9 minimal broth supplemented with various carbon sources[†]

Strain:	Carbon source:	Generation time: (minutes)
SR-11 wild-type:	glucose	80
SR-11 <i>maeB</i> ⁻ :	glucose	82
SR-11 <i>sfcA</i> ⁻ :	glucose	89
SR-11 wild-type:	fructose	72
SR-11 <i>maeB</i> ⁻ :	fructose	82
SR-11 <i>sfcA</i> ⁻ :	fructose	77
SR-11 wild-type:	succinate	78
SR-11 <i>maeB</i> ⁻ :	succinate	106
SR-11 <i>sfcA</i> ⁻ :	succinate	94
SR-11 wild-type:	malate	82
SR-11 <i>maeB</i> ⁻ :	malate	186
SR-11 <i>sfcA</i> ⁻ :	malate	145

[†] grown in the presence of 50 µg/ml of nalidixic acid.

Table 8. β -galactosidase activity of *S. typhimurium* SR-11 *maeB* cradl craz integrant, SR-11 *sfcA* cradl craz integrant, and SR-11 cradl craz integrant grown in M9 minimal broth supplemented with various carbon sources[†]

Strain:	Carbon source:	Units of β -galactosidase*: (mean $\pm$ std. deviation)	Ratio of units of β -galactosidase, for each substate, relative to units of β -galactosidase for the SR-11 cradl craz integrant control strain:
SR-11 cradl craz integrant:	succinate	3.1 $\pm$ 0.4 , n =15 [‡]	1.0
SR-11 <i>maeB</i> cradl craz integrant:	succinate	2.0 $\pm$ 0.3 , n =9 [‡]	0.7
SR-11 <i>sfcA</i> cradl craz integrant:	succinate	7.0 $\pm$ 0.7 , n =9 [‡]	2.3
SR-11 cradl craz integrant:	malate	3.0 $\pm$ 0.3 , n =15 [‡]	1.0
SR-11 <i>maeB</i> cradl craz integrant:	malate	2.7 $\pm$ 0.1 , n =6 [‡]	0.9
SR-11 <i>sfcA</i> cradl craz integrant:	malate	8.2 $\pm$ 0.4 , n =6 [‡]	2.8
SR-11 cradl craz integrant:	glucose	1.4 $\pm$ 0.2 , n =27 [‡]	1.0
SR-11 <i>maeB</i> cradl craz integrant:	glucose	1.3 $\pm$ 0.1 , n =9 [‡]	0.9
SR-11 <i>sfcA</i> cradl craz integrant:	glucose	3.8 $\pm$ 0.5 , n =9 [‡]	2.7
SR-11 cradl craz integrant:	fructose	1.1 $\pm$ 0.2 , n =18 [‡]	1.0
SR-11 <i>maeB</i> cradl craz integrant:	fructose	1.1 $\pm$ 0.2 , n =9 [‡]	1.0
SR-11 <i>sfcA</i> cradl craz integrant:	fructose	2.6 $\pm$ 0.5 , n =12 [‡]	2.5

[†] grown in the presence of 150 μ g/ml of ampicillin to maintain the integrants (257).

* assayed @ OD₅₀₀ of $\approx$ 1.0

[‡] β -galactosidase assays were performed in triplicate for each culture.

Table 9. Virulence of *Salmonella typhimurium* SR-11 wild-type, SR-11 *maeB*⁻, and SR-11 *sfcA*⁻ in 4.4 week-old female BALB/c mice

Strain:*	Virulence: (survival)	Day of expiration post peroral inoculation: (mean ± std. deviation)	Log[cfu/liver]: (mean ± std. deviation)	Log[cfu/spleen]: (mean ± std. deviation)
SR-11 wild-type	0/4	7.0 ± 1.2	8.15 ± 0.10	7.98 ± 0.06
SR-11 <i>maeB</i> ⁻	0/4	6.5 ± 1.3	7.68 ± 0.45	7.59 ± 0.25
SR-11 <i>sfcA</i> ⁻	0/4	8.0 ± 1.6	7.19 ± 0.30	6.90 ± 0.43
sham infected	4/4	N/A*	N/A*	N/A*

* peroral inoculation of approximately 1.3×10^8 cfu.

* N/A denotes not applicable.

Table 10. Virulence of *Salmonella typhimurium* SR-11 wild-type and SR-11 *sfcA*⁻ in 7.1 week-old female BALB/c mice

Strain:*	Virulence: (survival)	Day of expiration post peroral inoculation: (mean ± std. deviation)	Log[cfu/liver]: (mean ± std. deviation)	Log[cfu/spleen]: (mean ± std. deviation)
SR-11 wild-type	0/2	8.0 ± 1.4	7.65 ± 1.11	7.49 ± 1.38
SR-11 <i>sfcA</i> ⁻	0/3	7.0 ± 1.7	7.82 ± 0.34	7.69 ± 0.15

* peroral inoculation of approximately 2.1×10^8 cfu.

Table 11. Generation times of *Salmonella typhimurium* SR-11 *pta*⁻ *cradl* *craz* integrant and SR-11 *cradl* *craz* integrant grown in M9 minimal broth supplemented with various carbon sources[†]

Strain:	Carbon source:	Generation time: (minutes)
SR-11 <i>cradl</i> <i>craz</i> integrant:	glucose	75
SR-11 <i>pta</i> ⁻ <i>cradl</i> <i>craz</i> integrant:	glucose	94
SR-11 <i>cradl</i> <i>craz</i> integrant:	fructose	70
SR-11 <i>pta</i> ⁻ <i>cradl</i> <i>craz</i> integrant:	fructose	101
SR-11 <i>cradl</i> <i>craz</i> integrant:	succinate	200
SR-11 <i>pta</i> ⁻ <i>cradl</i> <i>craz</i> integrant:	succinate	213
SR-11 <i>cradl</i> <i>craz</i> integrant:	malate	199
SR-11 <i>pta</i> ⁻ <i>cradl</i> <i>craz</i> integrant:	malate	192

[†] grown in the presence of 150 µg/ml of ampicillin to maintain the integrants (257).

Table 12. β -galactosidase activity of *S. typhimurium* SR-11 *pta*⁻ *cradl* *craz* integrant and SR-11 *cradl* *craz* integrant grown in M9 minimal broth supplemented with various carbon sources[†]

Strain:	Carbon source:	Units of β -galactosidase*: (mean $\pm$ std. deviation)	Ratio of units of β -galactosidase, for each substrate, relative to units of β -galactosidase for the SR-11 <i>cradl</i> <i>craz</i> integrant control strain:
SR-11 <i>cradl</i> <i>craz</i> integrant:	succinate	2.0 $\pm$ 0.1, n=3 [‡]	1.0
SR-11 <i>pta</i> ⁻ <i>cradl</i> <i>craz</i> integrant:	succinate	6.3 $\pm$ 0.2, n=3 [‡]	3.1
SR-11 <i>cradl</i> <i>craz</i> integrant:	malate	1.6 $\pm$ 0.3, n=3 [‡]	1.0
SR-11 <i>pta</i> ⁻ <i>cradl</i> <i>craz</i> integrant:	malate	4.7 $\pm$ 0.7, n=3 [‡]	3.0
SR-11 <i>cradl</i> <i>craz</i> integrant:	glucose	0.7 $\pm$ 0.1, n=3 [‡]	1.0
SR-11 <i>pta</i> ⁻ <i>cradl</i> <i>craz</i> integrant:	glucose	2.5 $\pm$ 0.1, n=3 [‡]	3.4
SR-11 <i>cradl</i> <i>craz</i> integrant:	fructose	1.2 $\pm$ 0.1, n=3 [‡]	1.0
SR-11 <i>pta</i> ⁻ <i>cradl</i> <i>craz</i> integrant:	fructose	3.7 $\pm$ 0.1, n=3 [‡]	3.0

[†] grown in the presence of 150 μ g/ml of ampicillin to maintain the integrants (257).

* assayed @ OD₅₀₀ of $\approx$ 1.0

[‡] β -galactosidase assays were performed in triplicate for each culture.

Table 13. Virulence of *Salmonella typhimurium* SR-11 wild-type and SR-11 *pta*⁻ in 4.4 week-old female BALB/c mice

Strain:†	Virulence: (survival)	Day of expiration post peroral inoculation: (mean ± std. deviation)	Log[cfu/liver]: (mean ± std. deviation)	Log[cfu/spleen]: (mean ± std. deviation)
SR-11 wild-type	0/4	7.0 ± 1.2	8.15 ± 0.10	7.98 ± 0.06
SR-11 <i>pta</i> ⁻	4/4*	N/A†	6.85 ± 1.36*	6.69 ± 1.14*
sham infected	4/4	N/A†	N/A†	N/A†

† peroral inoculation of approximately 1.6×10^8 cfu.

* mice sacrificed on Day₁₅ of virulence experiment.

† N/A denotes not applicable.

Figures

Figure 1. The major metabolic pathways of *Salmonella typhimurium*. The Embden-Meyerhof Pathway, the pentose phosphate cycle, the tricarboxylic acid cycle (TCA), and the Entner-Doudoroff Pathway are shown above and represent the fueling pathways in central metabolism (72, 73). The glyoxylate shunt, when induced, bypasses several reactions in the TCA cycle (74). Two steps in the reversible Embden-Meyerhof pathway are replaced in the gluconeogenic pathway by the *pps*-encoded enzyme phosphoenolpyruvate synthetase and the *fbp*-encoded enzyme fructose-1,6-bisphosphatase (73, 82, 120). The pathways for acetate metabolism, fructose metabolism, and fatty acid oxidation (oleate) are also shown above (172, 173, 177-179). P represents phosphate in the diagram. Diagram highly modified from references (73, 212).

Figure 2. The branched, biosynthetic form of the TCA cycle for aerobic growth of *S. typhimurium* utilizing a non-limiting glucose source. The full TCA cycle is not required under these conditions because the bulk of energy is derived from glycolysis (74, 78). In this rich environment, cells produce excess acetyl-CoA which drains through acetyl phosphate, to further produce ATP with the associated secretion of acetate (78, 79). As cell density increases, the build-up of acetate also serves to increase the size of the acetyl phosphate pool (78). P represents phosphate in the diagram. Diagram highly modified from references (69, 73, 212).

Figure 3. Overview of the transcriptional regulatory effects of the Cra protein on key enzymes in the Embden-Meyerhof pathway, the TCA cycle, the glyoxylate shunt, the gluconeogenic pathway, and the Entner-Doudoroff pathway in *Salmonella typhimurium*; (+) denotes genes positively regulated by Cra; (–) denotes genes negatively regulated by Cra. P represents phosphate in the diagram. Diagram highly modified from reference (69, 73, 212).

Figure 4. Model for transcriptional regulation of target genes by the Cra protein. In the absence of effector molecules (fructose-1-phosphate and/or fructose-1,6-bisphosphate), Cra binds to the operator site (o) in the regulatory region of the target gene or operon. Positively regulated genes (+) are activated and negatively regulated genes (-) are repressed. The binding of effector molecules to the Cra protein causes it to dissociate from the DNA, exerting deactivation or derepression of gene expression. Diagram highly modified from references (212, 217).

Figure 5. A control strain, *S. typhimurium* SR-11 cradl craz integrant, containing a *cra* promoter-*lacZ* transcriptional fusion, was obtained from Mary P. Leatham (256). The strain was constructed by conjugative-dependent allele replacement utilizing the suicide vector pML55cradl-craz (256, 257). The control strain carries a partial upstream *ilvI* gene, an upstream *ilvH* gene, the upstream intergenic sequence, and the *cra* promoter ligated to the promoterless *lacZ* gene from pCB267 (256, 258). Approximately 947 bp of DNA upstream of the *cra* promoter were engineered into this construct to retain any possible regulatory sequences (256, 259). The 3' end of the *lacZ* reporter gene is ligated to approximately 200 bp of the remaining truncated *cra* gene, followed by approximately 534 bp of downstream DNA (a partial *yabB* gene) (256). The SR-11 *maeB* cradl craz integrant, SR-11 *sfcA* cradl craz integrant, the SR-11 *pta* cradl craz integrant were also constructed by this technique employing the suicide vector pML55cradl-craz.

Figure 6. The plasmid pJHA-*fbp*⁺ was mapped with the restriction endonucleases *Not* I, *Pst* I, and *Xho* I. The plasmid contains the entire *fbp* gene with partial upstream and downstream *yifG* and *yifF* genes. The amplicon was ligated into the *Bam* HI/*Not* I sites of the phagemid cloning vector pBluescript II SK (+) (Table 1). The numbers in parentheses, which denote base pairs, are approximate.

Figure 7. The plasmid pJHA-*fbp*⁺ was utilized as template DNA in a PCR reaction, with the primers shown above, to amplify around the plasmid (Table 2). The amplicon effectively deleted 878 bp from the *fbp* gene. The PCR product was then digested with *Bam* HI and ligated to itself. The numbers in parentheses, which denote base pairs, are approximate.

Figure 8. The plasmid pΔfbp was mapped with the restriction endonucleases *Not* I, *Pst* I, and *Xho* I. The plasmid contains a deleted *fbp* gene with partial upstream and downstream *yifG* and *yifF* genes in the cloning vector pBluescript II SK (+) (Table 1). A *Bam* HI site was engineered adjacent to the deleted *fbp* gene for insertion of a chloramphenicol resistance cassette. The numbers in parentheses, which denote base pairs, are approximate.

Figure 9. The plasmid pΔfbpCam was mapped with the restriction endonucleases *Bam* HI, *Not* I, *Pst* I, and *Xho* I. The plasmid contains a chloramphenicol resistance cassette adjacent to the deleted *fbp* gene and is flanked by partial upstream and downstream *yif*G and *yif*F genes. This insert is contained within the *Bam* HI/*Not* I sites of the cloning vector pBluescript II SK (+) (Table 1). The numbers in parentheses, which denote base pairs, are approximate.

Figure 10. The plasmid p55ΔfbpCam was mapped with the restriction endonucleases *Bam* HI, *Not* I, *Pst* I, and *Xho* I. The plasmid contains a chloramphenicol resistance cassette adjacent to the deleted *fbp* gene and is flanked by partial upstream and downstream *yifG* and *yifF* genes. This insert was ligated into the *Xho* I/*Not* I sites of the suicide vector pLD55 (Table 1). The numbers in parentheses, which denote base pairs, are approximate.

Figure 11. The first recombinational event of allelic exchange yielded *S. typhimurium* SR-11 $\Delta fbpCam$ I-3 integrant (257). The integrant is a partial diploid, containing both the wild-type *fbp* and deleted *fbp* genes.

Figure 12. The second recombinational event of allelic exchange yielded *S. typhimurium* SR-11 Δfbp Cam AX-3 segregant (239, 257). The segregant contains the deleted *fbp* gene adjacent to chloramphenicol resistance cassette.

Figure 13. The linear DNA amplicon containing the kanamycin resistance cassette and homologous flanking sequences to the *maeB* gene was electroporated into competent SR-11 wild-type cells containing the plasmid pKD46 (Table 1) (281). The gene encoding λ Red recombinase, which greatly enhances the rate of homologous recombination, is located the plasmid pKD46 and is controlled by an arabinose-inducible promoter. The wild-type gene is replaced by a deleted version and an antibiotic resistance marker in one step (281). This method was also used to make permanent deletions in the *sfcA* and *pta* genes.

Figure 14. PCR amplification of the *fbp* gene in *S. typhimurium* SR-11 wild-type, SR-11 Δ *fbp*Cam I-3 integrant, and SR-11 Δ *fbp*Cam AX-3 segregant. Lane 1: 1 kb DNA ladder. Lane 2: The SR-11 wild-type yields a 3.1 kb band which represents the wild-type *fbp* gene (994 bp) plus approximately 1 kb upstream (a partial *yifG* gene) and approximately 1 kb downstream (a partial *yifF* gene). Lane 3: The SR-11 Δ *fbp*Cam I-3 integrant exhibits two bands: one corresponding to the wild-type band and a 3.5 kb band representing the deleted *fbp* gene plus the chloramphenicol resistance cassette. Lane 4: The SR-11 Δ *fbp*Cam AX-3 segregant yields one 3.5 kb band denoting the deleted *fbp* gene plus the chloramphenicol resistance cassette. The PCR reactions utilized the *yifF*/forward and *yifG*/reverse primers (Table 2), 2.4 mM $MgCl_2$, 2.5 % DMSO, and genomic template DNA from the above strains. The PCR reactions were subjected to an initial denaturation of 94°C for 5 minutes followed by 32 cycles of: 94°C for 45 seconds, 56°C for 45 seconds, and 72°C for 3 minutes. A final extension of 72°C for 7 minutes completed the PCR.

Figure 15. The ratio of β -galactosidase units for each substrate assayed relative to the β -galactosidase units for fructose. β -galactosidase assays, for *cra* promoter activity, were performed in SR-11 *cradl* *craz* integrant grown in M9 minimal broth supplemented separately with D-fructose, D-glucose, D-gluconic acid potassium salt, potassium acetate, sodium citrate, L(-)malic acid, phospho(enol)pyruvate monosodium salt, pyruvic acid sodium salt, oleic acid sodium salt, and succinic acid disodium salt (232, 233). A (+) denotes genes positively regulated by the Cra protein; a (-) denotes genes negatively regulated by the Cra protein. P represents phosphate in the diagram. Diagram highly modified from references (69, 73, 212).

Figure 16. PCR primer diagram for confirmation of the deleted *maeB* gene in the genome of *S. typhimurium* SR-11 *maeB*. The primer pairs *maeB*-Flank/forward & reverse, *maeB*-DEL/forward & reverse, *maeB*-Flank/forward & *maeB*-DEL/reverse, and *maeB*-DEL/forward & *maeB*-Flank/reverse were utilized in separate PCR reactions to confirm, by amplicon size, the deletion in the *maeB* gene. The same scheme was employed to confirm deletions in the *sfcA* and *pta* genes of *S. typhimurium* SR-11 *sfcA* and *S. typhimurium* SR-11 *pta* respectively.

Figure 17. PCR amplification of the *maeB* gene in *S. typhimurium* SR-11 *maeB*⁻ and SR-11 wild-type. Lane 1: 1 kb DNA ladder. Lane 2: The 3.6 kb band (*maeB*-Flank/forward & reverse primers) represents the deleted *maeB* gene, kanamycin cassette, plus partial *talA* upstream and *tnpA*_3 downstream genes. Lane 3: A 2.5 kb band (*maeB*-Flank/forward & *maeB*-DEL/reverse primers) reveals the kanamycin cassette, deleted *maeB* gene, and a partial upstream gene. Lane 4: A 2.6 kb band (*maeB*-Flank/reverse & *maeB*-DEL/forward primers) corresponds to the kanamycin cassette, deleted *maeB* gene, and a partial downstream gene. Lane 5: The 1.5 kb kanamycin resistance cassette (*maeB*-DEL/forward & reverse primers) inserted in the deleted *maeB* gene. Lane 6: A 4.2 kb band (*maeB*-Flank/forward & reverse primers) corresponding to the partial *talA* upstream gene, the wild-type *maeB* gene (2280 bp) plus a partial *tnpA*_3 downstream gene. Lane 7: A (-) DNA control. Lanes 2-5 utilized SR-11 *maeB*⁻ genomic template DNA and Lane 6 utilized SR-11 wild-type genomic template DNA. The PCR reactions contained 1.8 mM MgCl₂ and were subjected to an initial denaturation of 94°C for 5 minutes followed by 32 cycles of: 94°C for 1 minute, 57°C for 1.5 minutes, and 72°C for 3 minutes. A final extension of 72°C for 7 minutes completed the PCR.

Figure 18. PCR amplification of the *sfcA* gene in *S. typhimurium* SR-11 *sfcA*⁻ and SR-11 wild-type. Lane 1: 1 kb DNA ladder. Lane 2: The 3.6 kb band (*sfcA*-Flank /forward & reverse primers) represents the deleted *sfcA* gene, kanamycin cassette, plus entire upstream *rpsV* gene, partial upstream *yddX* gene, and partial downstream *adhP* gene. Lane 3: A 2.7 kb band (*sfcA*-Flank/forward & *sfcA* -DEL/reverse primers) reveals the kanamycin cassette, deleted *sfcA* gene, and upstream genes. Lane 4: A 2.6 kb band (*sfcA*-Flank/reverse & *sfcA*-DEL/forward primers) corresponds to the kanamycin cassette, deleted *sfcA* gene, and a partial *adhP* downstream gene. Lane 5: The 1.5 kb kanamycin resistance cassette (*sfcA*-DEL/forward & reverse primers) inserted in the deleted *sfcA* gene. Lane 6: A 3.7 kb band (*sfcA*-Flank/forward & reverse primers) corresponding to the wild-type *sfcA* gene (1697 bp) plus the entire upstream *rpsV* gene, partial upstream *yddX* gene, and partial downstream *adhP* gene. Lane 7: A (-) DNA control. Lanes 2-5 utilized SR-11 *sfcA*⁻ genomic template DNA and Lane 6 utilized SR-11 wild-type genomic template DNA. The PCR reactions contained 1.8 mM MgCl₂ and subjected to an initial denaturation of 94°C for 5 minutes followed by 32 cycles of: 94°C for 1 minute, 57°C for 1.5 minutes, and 72°C for 3 minutes. A final extension of 72°C for 7 minutes completed the PCR.

Figure 19. PCR amplification of the *pta* gene in *S. typhimurium* SR-11 *pta*⁻ and SR-11 wild-type. Lane 1: 1 kb DNA ladder. Lane 2: A (-) DNA control. Lane 3: A 2.0 kb band (*pta*-Flank/forward & *pta*-DEL/reverse primers) corresponds to the kanamycin cassette, deleted *pta* gene, and the partial upstream *ackA* gene. Lane 4: A 1.9 kb band (*pta*-Flank/ reverse & *pta*-DEL/forward primers) reveals the kanamycin cassette, deleted *pta* gene, and downstream sequences. Lane 5: The 2.3 kb band (*pta*-Flank /forward & reverse primers) represents the partial upstream *ackA* gene, the deleted *pta* gene, the kanamycin cassette, plus downstream sequences. Lane 6: The 1.5 kb kanamycin resistance cassette (*pta*-DEL/forward & reverse primers) inserted in the deleted *pta* gene. Lane 7: A 2.6 kb band (*pta*-Flank/forward & reverse primers) corresponding to the partial upstream *ackA* gene, the wild-type *pta* gene (2155 bp), plus downstream sequences. Lanes 3-6 utilized SR-11 *pta*⁻ genomic template DNA and Lane 7 utilized SR-11 wild-type genomic template DNA. The PCR reactions contained 1.5 mM MgCl₂ and were subjected to an initial denaturation of 94°C for 5 minutes followed by 32 cycles of: 94°C for 1 minute, 54°C for 1.5 minutes, and 72°C for 2.5 minutes. A final extension of 72°C for 7 minutes completed the PCR.

Figure 20. The *sfcA*-encoded, NAD-dependent malic enzyme and acetyl phosphate may act in concert as a repressor of the *cra* gene. Top figure: Acetyl phosphate transfers a phosphoryl group to the tetrameric, *sfcA*-encoded, NAD-dependent malic enzyme then binds to the operator (O) downstream of the *cra* promoter; this represses the transcription of the *cra* gene by RNA polymerase. Middle figure: Acetyl phosphate binds to the *sfcA*-encoded NAD-dependent malic enzyme causing a conformational change in the enzyme; the enzyme complex then binds to the operator downstream of the *cra* promoter and represses the transcription of the *cra* gene by RNA polymerase. Bottom figure: In the absence of either the *sfcA*-encoded, NAD-dependent malic enzyme or acetyl phosphate, negative regulation of the *cra* gene does not occur; RNA polymerase transcribes the *cra* gene and it is subsequently translated into the tetrameric Cra protein.

Figure 21. A second possibility, although unlikely, to explain the significant differences in the *cra* promoter activities of the SR-11 *maeB*⁻ cradl craz integrant, SR-11 *sfcA*⁻ cradl craz integrant, the SR-11 *pta*⁻ cradl craz integrant, and the SR-11 cradl craz integrant control strain, involves the orientation of the *cra* promoter-*lacZ* transcriptional fusion versus the wild-type *cra* gene. If the orientation of the *cra* promoter-*lacZ* transcriptional fusion and the wild-type *cra* gene, in the SR-11 cradl craz integrant control strain and the SR-11 *maeB*⁻ cradl craz integrant strain, is inverted versus the other two strains, then there is a slight possibility of an transcriptional regulatory effect occurring in a distant location upstream of the *cra* promoter-*lacZ* transcriptional fusion. This seems highly improbable since approximately 947 bp of DNA upstream of the *cra* promoter was engineered into these constructs to retain any possible regulatory sequences (256, 259).

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