Abstract

HUANG, YANYAN. Genetic and Environmental Mediators of *Salmonella* Infection. (Under the direction of Craig Altier and Jay F. Levine).

The purpose of the research is to identify genetic determinants and environmental signals of infection in *Salmonella enterica* serovar Typhimurium. We established pigs as an animal model to identify *Salmonella* genes specifically expressed *in vivo* by using a recombinase-based *in vivo* expression system. A total of 55 clones from a genomic library of ~ 10,000 random *Salmonella* DNA fragments were isolated from the tonsils and small intestinal tracts of pigs. Characterization of *in vivo* induced genes by sequencing showed that genes involved in adhesion, colonization, virulence, stress response, and a two-component regulator were specifically induced after infection of pigs. High temperature and osmolarity induced a number of these *in vivo* expressed genes. We identified formate as an environmental signal that induces invasion gene expression in *Salmonella.* The effect of formate required a pH below neutrality, and we found that the distal ileum of mice had the appropriate formate concentration and pH to elicit invasion of *Salmonella*. We further found that formate plays a role in inducing invasion gene expression by changing carbon flux. To identify the formate regulon, *Salmonella* DNA microarrays were employed to compare gene expression of wild type *Salmonella* grown with or without formate. A part of formate hydrogenlyase complex, encoded by the *hyc* operon, was induced by the additional formate. A large class of genes involved in the respiratory electron transfer system was also affected by formate. Nitrate reductase and succinate dehydrogenase were repressed, while

fumarate reductase was induced by formate. Thus, in the presence of formate, fumarate may be a more preferred electron acceptor than nitrate. A number of genes involved in vitamin B_{12} synthesis, aromatic acid synthesis, flagella synthesis, lipopolysaccharide synthesis, and two-component regulators were also affected by formate. Surprisingly, genes affected by formate overlapped with some *in vivo* induced genes, suggesting that formate present in the distal ileum could be a signal for *Salmonella* infection of animal hosts.

Genetic and Environmental Mediators of *Salmonella* Infection

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Dedication

This dissertation is dedicated to my daughter, Angelie Xu, to my husband, Daode Xu, and to my parents, Zheng Huang and Meiqin Jiang, without whose support it would not have been initiated or finished.

Biography

Yanyan Huang was born on September 21, 1977 in Guixi, Jiangxi province of China. She got her Bachelor of Science degree in Freshwater Fishery and Master of Science degree in Zoology at Nanchang University in 1999 and 2002 respectively. She worked in Chinese Academy of Sciences for one year and joined the College of Veterinary Medicine at North Carolina State University in 2003.

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In recent years, diseases caused by bacterial pathogens have become a major public health concern in this country. Once thought to be of declining importance due to the advent of effective antibiotics, these diseases have shown a surprising resurgence as of late. One reason for this is the threat of wide-spread foodborne disease outbreaks, owing to the large scale production, processing, and distribution of foodstuffs in this country and throughout the world (Kaferstein and Abdussalam, 1999). Diseases caused by foodborne bacteria are estimated to affect nearly 4.2 million people in the United States each year, and to kill approximately 1,300 of those affected (Mead *et al.*, 1999). One of the most important foodborne pathogens is *Salmonella.*

Salmonella is a genus of rod-shaped gram-negative enterobacteria that causes intestinal infection. The infection caused by *Salmonella* is called salmonellosis and can range from asymptomatic to systemic disease. All pathogenic *Salmonella* are considered to be of the single species *enterica,* but they belong to one of 2449 serotypes (Brenner *et al.*, 2000). *Salmonella enterica* serovar Typhimurium causes overt disease in susceptible animal hosts and can be carried asymptomatically by important food-producing species, such as chickens, turkeys, and pigs. Human infection with *S.* Typhimurium typically occurs through ingestion of contaminated food and food products. Most people infected with *S.* Typhimurium develop diarrhea, fever, and abdominal cramps within 12 to 72 hours

of infection. The illness usually lasts 4 to 7 days and most people recover without treatment. However, in some people, such as the elderly, young children and people with compromised immunity, the diarrhea may be so severe that the patient must be hospitalized. In these patients, the *Salmonella* infection may spread from the intestines to the bloodstream, and then to other body sites, potentially resulting in death unless the person is treated promptly with antibiotics. Non-typhoidal salmonellosis is second to only campylobacterosis in number of cases, some 1.4 million each year (Mead *et al.*, 1999). Salmonellosis remains, however, the leading foodborne cause of death in this country (Mead *et al.*, 1999). Besides human infection, *S.* Typhimurium causes tyhoid fever in mice similar to human typhoid fever caused by S. Typhi. Therefore, infection of mice with *S.* Typhimurium has been widely employed as a model of human infection with *S.* Typhi; as such the pathogenesis of *S.* Typhimurium as a cause of overt diseases has been actively studied.

Transmission of *S.* Typhimurium is traditionally believed to occur by the fecal-oral route with invasion through the intestinal wall and Peyer's patches. However, *Salmonella* have also been transmitted following exposure to aerosols or dust (Clemmer *et al.*, 1960; Darlow *et al.*, 1961), suggesting that involvement of the upper respiratory tract may be equally important in the dissemination of *Salmonella*. A variety of potentially pathogenic bacteria can be isolated from the tonsils of pigs following inoculation, and it is thought that many *Salmonella* use the palatine and pharyngeal tonsil as a reservoir or point of entry to the systemic circulation (Gram *et*

al., 2000; Thibodeau *et al.*, 1999). In a study of distribution of persistent *Salmonella, Salmonella* was recovered most frequently from tonsils (93.5% positive), followed by intestinal tract (71%), and mandibular lymph nodes (54.8%) (Wood and Rose, 1992). Therefore, mechanisms within the tonsils may be responsible for the uptake and dissemination of *Salmonella*.

The pathogenesis of *Salmonella* infection is complex and an early step is bacterial penetration of intestinal epithelium, which is a remarkable two-way interaction between the bacteria and the host cells. Many of the genes required for epithelial penetration are found within *Salmonella* pathogenicity island 1 (SPI 1) (Behlau and Miller, 1993; Galan and Curtiss, 1989b; Groisman and Ochman, 1993; Jones and Falkow, 1994; Lee *et al.*, 1992; Mills *et al.*, 1995). These genes encode the components and substrates of a type III secretion system that exports signaling effectors to the bacterial surface and into adjacent eukaryotic cells. The signaling effectors then induce cytoskeletal changes in these host cells that lead to bacterial engulfment (Collazo and Galan, 1997; Fu and Galan, 1998b; Hardt *et al.*, 1998b; Kubori *et al.*, 1998; Zhou *et al.*, 1999). *Salmonella* pathogenicity island 1 genes have been shown to be important to *Salmonella* virulence using both the mouse model for septicemia (Galan and Curtiss, 1989a; Jones and Falkow, 1994; Miller and Mekalanos, 1990) and the calf model for enterocolitis (Tsolis *et al.*, 1999). The regulatory circuit of the SPI 1 invasion process is complex. A number of genetic determinants and environmental stimuli involved in regulation of SPI 1 invasion genes will be discussed in detail later.

Salmonella in pigs

Swine represent an important source of *Salmonella* serotypes causing disease in humans. Between 1983 and 1987, 2.9% of all *Salmonella* outbreaks were due to pork products (Tauxe, 1991). Ham and pork were implicated in 18% of the outbreaks caused by the consumption of contaminated meat reported to the CDC during the years 1988 to 1992, (Bean *et al.*, 1997). Antimicrobial-resistant *Salmonella* that pose a threat to public health are also shed by pigs. With increasing frequency, *Salmonella* isolates obtained from pigs are resistant to one or multiple antimicrobials. Recent studies have shown that half of *Salmonella* isolates obtained from commercially raised swine in the United States are multiresistant (Farrington *et al.*, 2001; Gebreyes *et al.*, 2000).

There are two distinct clinical syndromes in swine caused by *Salmonella*. Bacteremia is caused by serotype Choleraesuis, often the *Kunzendorf* variant (Schwartz, 1999; Wilcock *et al.*, 1976). Clinical signs are severe, including fever, anorexia, and lethargy, and may be potentiated by concomitant infection with porcine reproductive and respiratory syndrome virus (Wills *et al.*, 2000). The disease can lead to ulcerative colitis, hepatic necrosis, or pneumonia, and produces a high rate of mortality (Schwartz, 1999; Turk *et al.*, 1992). The serotype is host-adapted to swine, but can also cause bacteremia in humans. Infection of humans by serotype Choleraesuis is rare, but important, since it produces a severe disease syndrome (Saphra and Wassermann, 1954). Swine can also suffer clinical disease in the form

of enterocolitis caused by one of a number of serotypes, Typhimurium being the most notable. Enterocolitis is most commonly seen in naïve pigs, or in those suffering from other illnesses (Schwartz, 1999).

Although *Salmonella* can cause clinical disease in swine, the vast majority of infections are subclinical. These infections can be caused by a large number of serotypes; more than 30 have been isolated from pigs on farms, and more than 50 have been identified from swine carcasses at slaughter (Gebreyes *et al.*, 2000). Surveys have shown that up to six serotypes can be isolated from clinically normal pigs on a single farm (Davies *et al.*, 1998). A national survey for fecal *Salmonella* shedding by pigs most frequently produced the *Salmonella enterica* serotypes Derby, Agona, Typhimurium (Copenhagen), Branderburg, Mbendaka, Typhimurium, and Heidelberg (National Animal Health Monitoring System, USDA). Among this group, Typhimurium, Heidelberg, and Agona are also known to be common isolates from humans. It is also possible that Typhimurium (Copenhagen) is a common human isolate, but the typing schemes used to identify isolates from humans in the past have not included this variant. *Salmonella* is also found among pork products both in processing plants and retail stores. Pork and pork sausage samples taken from plants were positive for *Salmonella* at a rate of 5.8% (Duffy *et al.*, 2001). Between 3.3% and 8.3% of whole-muscle pork from stores was positive for *Salmonella* (Duffy *et al.*, 2001; Zhao *et al.*, 2001), while pork sausage was contaminated to a greater degree, from 7.3% to 12.5% (Dahl *et al.*, 1997; Duffy *et al.*, 2001).

Since subclinical infection in swine leads to rapid spread of *Salmonella* and threatens public health, a number of studies have attempted to identify the environmental and managerial factors that affect the shedding of *Salmonella* by pigs in commercial swine herds. On the farm, general cleanliness has been cited as the most important factor in reducing *Salmonella* spreading (Berends *et al.*, 1997; Dahl *et al.*, 1997; Funk and Gebreyes). Such factors as farm hygiene, including cleaning and disinfection, housing, and feed and water sources affect *Salmonella* shedding by pigs (Berends *et al.*, 1997). Biosecurity, including the number of people and other domestic species at the site, also appears to play an important role (Berends *et al.*, 1997; Funk and Gebreyes). The use of barns with slotted floors has been shown to decrease *Salmonella* prevalence in swine finishing barns (Davies *et al.*, 1997). *Salmonella* has been isolated from feed on swine farms, but often in association with other risk factors such as lack of pest control or mixing of feeds on the farm, suggesting that contamination of feed occurs at the farm and that prudent measures to improve farm sanitation might be an effective deterrent to infection (Harris *et al.*, 1997). However, improvements in hygienic measures are costly. The use of all in/all out management, a method used to successfully decrease the incidence of a number of other infectious diseases of swine, does not seem to offer a significant advantage over its alternative, continuous flow, in decreasing *Salmonella* prevalence (Davies *et al.*, 1997).

Once animals leave the farm, transport to the slaughterhouse presents an additional risk to the contamination of pork products with *Salmonella.* Serotypes not

found in fecal samples from pigs while on farms have been isolated from their intestinal contents at slaughter (Hurd *et al.*, 2001a), suggesting infection during transport or at the slaughterhouse. Swine can be rapidly infected experimentally with *Salmonella*, harboring viable organisms less than one hour after infection (Hurd *et al.*, 2001b). The organism can be cultured from the intestinal contents of a larger proportion of animals after transport than on the farm (Isaacson *et al.*, 1999). The effect of transport may however be offset by withholding feed from animals for 24 hours prior to slaughter, suggesting that feed withdrawal is a means of decreasing the number of *Salmonella* in the intestinal tracts of transported animals (Isaacson *et al.*, 1999). Contamination of animals in the slaughterhouse itself can be important. Samples obtained from pens used for lairage, the holding of animals prior to slaughter, show them often to be contaminated with *Salmonella,* thus better cleaning and reduced lairage time might reduce bacterial contamination of meat (Lo Fo Wong *et al.*, 2002).

Genetic regulators required for *Salmonella* infection

The ability to invade host epithelial cells is an essential step in the pathogenesis of *Salmonella* Typhimurium. The invasion process requires a type III secretion system (TTSS) which delivers effector proteins from the cytoplasm of *Salmonella* to the cytosol of host cells. Type III secretion system is a complex apparatus composed of a group of proteins. *Salmonella* Typhimurium has two distinct TTSSs, one encoded within *Salmonella* pathogenicity island 1, located at centisome 63, and another in *Salmonella* pathogenicity island 2 (SPI 2), located at

centisome 31 (Galan, 1999; Shea *et al.*, 1996). SPI1 is believed to be required for the initial interaction of *Salmonella* with the intestinal epithelium (Galan, 1999). SPI 2 is implicated to be essential for the establishment of systemic infection (Ochman *et al.*, 1996; Shea *et al.*, 1996).

Salmonella pathogenicity island 1, a 40Kb region, encodes all of the proteins necessary to assemble a supramolecular structure termed the needle complex and some of secreted effector proteins of the TTSS (Galan and Curtiss, 1989a; Groisman and Ochman, 1993; Kubori *et al.*, 1998; Kubori *et al.*, 2000). The genetic regulation of SPI 1 is mediated by regulators both within SPI 1, such as HilA, HilC/HilD, and InvF, and outside SPI 1, such as PhoP/PhoQ, BarA/SirA, CsrA, and the small RNAs CsrB and CsrC (Altier *et al.*, 2000a; Bajaj *et al.*, 1995; Darwin and Miller, 1999; Fortune *et al.*, 2006; Lawhon *et al.*, 2002; Miller *et al.*, 1989; Pegues *et al.*, 1995; Schechter and Lee, 2001). HilA is a OmpR/ToxR family member that activates the expression of *S.* Typhimurium invasion genes (Bajaj *et al.*, 1995). HilC and HilD, at the top of the cascade of invasion gene regulation, induce *hilA* expression (Eichelberg and Galan, 1999). *invF* is one of the targets of HilA and also itself encodes a transcriptional activator that is required for expression of genes encoding proteins secreted by the SPI1 type III secretion apparatus (Darwin and Miller, 1999; Kaniga *et al.*, 1994). The targets of InvF overlap some sets of targets of HilA both within and outside SPI 1 (Ahmer *et al.*, 1999; Darwin and Miller, 1999; Eichelberg and Galan, 1999). A two-component regulatory, system PhoP/PhoQ, represses invasion gene expression and is involved in *Salmonella* survival in

macrophages (Groisman and Saier, 1990; Miller *et al.*, 1989; Miller and Mekalanos, 1990; Miller, 1991). Invasion genes are positively regulated by another regulator outside SPI 1, the two-component regulator BarA/SirA (Ahmer *et al.*, 1999; Altier *et al.*, 2000b; Johnston *et al.*, 1996; Teplitski *et al.*, 2003). SirA regulates invasion gene expression both dependently and independently of BarA (Altier *et al.*, 2000b; Lawhon *et al.*, 2002; Teplitski *et al.*, 2003). The expression of invasion genes controlled by SirA is mediated through induction of *hilA* and *hilC* and by controlling *csrB* and *csrC* (Fortune *et al.*, 2006; Teplitski *et al.*, 2003). The *csr* (Carbon Storage Regulator) system originally identified in *E. coli* is a global regulatory system that modulates mRNA stability and affects glycogen synthesis and gluconeogenesis (Liu *et al.*, 1995; Liu and Romeo, 1997; Romeo *et al.*, 1993; Romeo, 1998). CsrA in *S.* Typhimurium has been identified as a global regulator that mediates the expression of SPI 1 invasion genes, maltose transport, ethanolamine utilization, propanediol metabolism, B_{12} synthesis, and production of hydrogen sulfide (Altier *et al.*, 2000a; Lawhon *et al.*, 2003). CsrA is a specific RNA-binding protein and binds to a second component of the *csr* control system, the untranslated CsrB and CsrC (Altier *et al.*, 2000a; Fortune *et al.*, 2006; Gudapaty *et al.*, 2001; Liu and Romeo, 1997; Romeo, 1998). CsrA, CsrB, and CsrC all affect invasion gene expression, but CsrB and CsrC counteract the post-transcriptional regulator CsrA (Altier *et al.*, 2000a; Fortune *et al.*, 2006; Gudapaty *et al.*, 2001; Romeo, 1996). Mutation or over-expression of *csrA* results in repression of invasion genes expression (Altier *et al.*, 2000a). It is proposed that CsrB and CsrC compete with invasion gene mRNAs for binding of CsrA (Altier, 2005). In this way, CsrB and CsrC activate invasion gene expression.

CsrB and CsrC are activated by BarA, that is, BarA controls invasion gene expression by activating CsrB and CsrC, which reduce the concentration of CsrA (Altier, 2005).

The TTSS secreted proteins encoded within SPI 1 are comprised of AvrA and SipABCD (Hardt and Galan, 1997; Schesser *et al.*, 2000). Other TTSS secreted proteins encoded elsewhere on the *Salmonella* chromosome include SopE, SopE2, SopB, SopD, and SptP (Bakshi *et al.*, 2000; Hardt and Galan, 1997; Kaniga *et al.*, 1996; Wood *et al.*, 2000; Zhou and Galan, 2001). In *S.* Typhimurium, the *sip* genes are clustered and required for bacterial invasiveness (Hueck *et al.*, 1995; Kaniga *et al.*, 1995a; Kaniga *et al.*, 1995b; Zhou *et al.*, 1999). SipA, localizes within host cells, induces membrane-ruffling and promotes bacterial internalization (Zhou *et al.*, 1999). SipB activates apoptosis within the epithelial cells (Hersh *et al.*, 1999) while SipC possesses functions of both translocation of effectors and actin modulation (Chang *et al.*, 2005; Hayward and Koronakis, 1999; Kaniga *et al.*, 1995b). The actin nucleation activity of SipC plays an essential role in *Salmonella*-induced membrane ruffles and subsequent bacterial invasion (Chang *et al.*, 2005). SipB, SipC, and SipD have been proposed to form a channel in the host cell membrane to translocate *Salmonella* SPI 1 secreted proteins into epithelial cells (Scherer *et al.*, 2000). Bacterial engulfment is the consequence of cellular responses, characterized by Cdc42- and Rac-dependent actin cytoskeleton rearrangment (Cain *et al.*, 2004; Chen *et al.*, 1996; Fu and Galan, 1999). SopE and its homolog SopE2 have an impact on cytoskeletal rearrangements (Bakshi *et al.*, 2000; Hardt *et al.*, 1998a).

Salmonella inositol polyphosphatase SopB exhibits overlapping functions with SopE and SopE2 (Zhou and Galan, 2001). Tyrosine phosphatase SptP of *Salmonella* is a target of TTSS and its translocation requires the function of the secreted proteins, SipB, SipC, and SipD (Fu and Galan, 1998a, b). SptP is an effector protein and disrupts the actin cytoskeleton (Fu and Galan, 1998a, b).

Another *Salmonella* pathogenicity island is a 40kb region termed SPI 2, which plays a vital role in systemic infection by *Salmonella* (Ochman and Groisman, 1996; Ochman *et al.*, 1996; Shea *et al.*, 1996). SPI 2 genes are induced inside host cells, not only inside phagocytes but also infected epithelial cells (Pfeifer *et al.*, 1999). The expression of SPI 2 genes is regulated by a two-component regulator within SPI 2, SsrA/SsrB, and regulators outside the SPI 2, PhoP/PhoQ and OmpR/EnvZ (Lee *et al.*, 2000; Miller, 1991; Shea *et al.*, 1996). The expression of *ssrAB* itself is dependent on the global regulator EnvZ/OmpR (Lee *et al.*, 2000). PhoP/PhoQ is required for the activity of SPI 2, but represses SPI 1 (Bajaj *et al.*, 1996; Behlau and Miller, 1993; Fahlen *et al.*, 2000; Groisman *et al.*, 1989; Miller *et al.*, 1989; Miller, 1991). Therefore, PhoP/PhoQ may function as a switch that turns off SPI 1, required for invasion, and turns on SPI 2, important for survival in macrophage after *Salmonella* enters host cells. It also suggests that *Salmonella* may use different strategies to respond to host cell environments by switching gene expression.

Although *Salmonella* remain asymptomatic in most cases of infection of pigs, the genetic regulators of this process have not been characterized thus far. Because colonization is the common step in the animals following exposure to

Salmonella, genetic factors required for colonization may be also important for *Salmonella* carriage. Genes for synthesis of lipopolysaccharide have been shown to be essential for *Salmonella* colonization of chickens (Turner *et al.*, 1998). Fimbriae of *S.* Typhimurium play a vital role in intestinal colonization (Humphries *et al.*, 2001; Tanaka *et al.*, 1981). The attachment of *S.* Typhimurium to intestinal mucosa has been implicated as an important factor for bacterial colonization (Soerjadi *et al.*, 1982). However, no specific genes have been identified as genetic factors required for *Salmonella* carriage in pigs without clinical disease. Further experimental investigation is needed to reveal the genetic mechanism by which *Salmonella* survive and replicate in clinically healthy animals.

Environmental signals for gene expression in *Salmonella*

Genetic regulation must be coordinated with varied environmental factors to mediate gene expression. SPI 1 genes have been shown to be regulated by pH, oxygen tension, osmolar stress, growth phase, and bile (Bajaj *et al.*, 1996; Galan and Curtiss, 1990; Lee and Falkow, 1990; Lee *et al.*, 1992; Lundberg *et al.*, 1999; Prouty and Gunn, 2000; Prouty *et al.*, 2004). A pH below 7.0 induces SPI 1 invasion gene expression while high levels of oxygen represses such expression (Bajaj *et al.*, 1996). Invasion genes are also induced by high osmolarity and repressed by bile (Galan and Curtiss, 1990; Prouty *et al.*, 2004). Because all of these conditions are present in the gastrointestinal tract, the site of *Salmonella* colonization, it is reasonable that other conditions in the intestinal tract may also act as signals to induce gene expression. For example, short chain fatty acids, the major byproducts

of digestion in the intestine, have been shown to affect *Salmonella* invasion gene expression (Gantois *et al.*, 2006; Lawhon *et al.*, 2002). Acetate induces SPI 1 gene expression through SirA (Lawhon *et al.*, 2002) while butyrate represses SPI 1 gene expression (Gantois *et al.*, 2006). SPI 2 gene regulation also depends on environmental factors such as low concentration of Mg^{2+} or Ca^{2+} (Deiwick *et al.*, 1999). PhoP/PhoQ regulates gene expression of SPI 2 in response to low levels of magnesium (Garcia Vescovi *et al.*, 1996). Low osmolarity and low pH have been implicated to regulate SPI 2 genes through the sensor of OmpR/EnvZ (Lee *et al.*, 2000).

The gene expression of *Salmonella* is co-regulated by genetic regulators and environmental signals. Since SCFAs, such as acetate and butyrate, have been identified as important signals for *Salmonella* infection (Gantois *et al.*, 2006; Lawhon *et al.*, 2002), other SCFAs present in the intestinal tracts may also play roles in altering gene expression. One such SCFA, formate, will be described in greater detail below along with the bacterial response.

Formate

Formate is a key metabolite in the energy metabolism of many bacteria and is present in both aerobically and anaerobically grown cells. Depending upon the physiological condition, formate may be secreted or converted into CO_2 and H_2 via the formate hydrogenlyase (FHL) reaction (Ordal and Halvorson, 1939; Stephenson and Stickland, 1932). Its synthesis and degradation are integrated into a network of metabolic routes. Anaerobically and in the absence of external electron acceptors,

pyruvate is cleaved predominantly by pyruvate-formate-lyase (PFL) thus yielding formate and acetyl-CoA. The regulation of both the activity and the synthesis of PFL is complex. PFL is oxygen sensitive (Leonhartsberger *et al.*, 2002), so formate is made primarily under anaerobic conditions. Recently, 2-ketobutyrate was discovered to produce formate and propionyl-CoA (Hesslinger *et al.*, 1998). The formate levels in *E. coli* are also controlled by the posttranslational deformylation of N-formayl-methionine residues and during purine biosynthesis (reviewed by(Leonhartsberger *et al.*, 2002). Therefore, formate is produced in both aerobic and anaerobic conditions. Formate consumption occurs by both aerobic and anaerobic degradation routes also. A formate dehydrogenase-O (FDH-O) appears to couple formate oxidation with ubiquinone reduction in aerobic cells and under conditions of nitrate respiration (Pinsent, 1954; Sawers, 1994). However, formate dehydrogenase-N (FDH-N) is the predominant enzyme in nitrate reducing cells and is synthesized during anaerobiosis and under nitrate respiratory conditions (Enoch and Lester, 1975, 1982; Sawers, 1994). Finally, formate dehydrogenase-H (FDH-H) is a component of the FHL complex, which catalyzes the decomposition of formate to H_2 and CO_2 during fermentative growth (Neidhardt *et al.*, 1996). Therefore, under aerobic conditions, formate concentrations in *Salmonella* should be limited, since PFL is inactive and FDH-O efficiently consumes the low amounts formed via the non-fermentative routes (Rossmann *et al.*, 1991).

Four transcriptional units on the *E. coli* chromosome have been shown to be regulated by the intracellular concentration of formate (Birkmann *et al.*, 1987b;

Birkmann and Bock, 1989; Bohm *et al.*, 1990; Leonhartsberger *et al.*, 2002; Sauter *et al.*, 1992). First, the expression of the monocistronic *fdhF* gene, encoding formate dehydrogenase H, is determined by the concentration of formate (Birkmann *et al.*, 1987b). Second, the *hyc* operon, containing *hycABCDEFGH*, is included in the formate regulon (Bohm *et al.*, 1990). HycA acts as anti-activator counteracting the activity of FhlA (reviewed by (Leonhartsberger *et al.*, 2002). HycBCDEFGH are components of formate hydrogenlyase systems that accept electrons from FDH-H and reduce protons to molecular hydrogen (Bohm *et al.*, 1990; Sauter *et al.*, 1992). HycB is proposed to serve as the membrane docking target of FDH-H; HycC and HycD are predicted to be membrane integral proteins; HycE and HycG are components of hydrogenase 3; HycF seems to be an electron carrier; and the function of HydH is unknown. Third, the *hyp* operon inversely oriented to the *hyc* transcriptional unit consists of six genes: *hypABCDE* and *fhlA* (Sankar *et al.*, 1988; Schlensog and Bock, 1990). HypA and HypC are involved in the maturation of hydrogenase 3 (Maier and Bock, 1996). HypB, HypD, and HypE participate in the maturation of all three hydrogenases (Maier and Bock, 1996). FhlA, encoded by the last gene of this operon, serves as the central regulator of the formate regulon (Sankar *et al.*, 1988; Schlensog and Bock, 1990). Fourth, a transcriptional unit located downstream of the *hyc* operon is responsive to formate (Leonhartsberger *et al.*, 2002). It consists of two genes: *hydN* and *hypF* (Maier *et al.*, 1996). HydN possibly plays a role in electron transfer (Maier *et al.*, 1996) while HypF is involved in the maturation of all three hydrogenases (Maier *et al.*, 1996). All four transcriptional units from *E. coli* have homologs in *Salmonella* and their arrangement is the same in

both bacteria. Thus, genes responsive to formate in *Salmonella* may be the same as those in *E. coli.*

Some physiological conditions that induce the expression of the formate regulon genes have been identified (Pecher *et al.*, 1983; Peck and Gest, 1957; Wimpenny and Cole, 1967). The most important factor is the pH of the medium, which has to be less than 7.0 to induce the expression of the genes of the formate regulon (Leonhartsberger *et al.*, 2002). It is also known that the pH in the small intestine is around 6.7, which induces invasion gene expression in *Salmonella* (Bajaj *et al.*, 1996). Formate is also abundant in the ileum as a fermentation product of microflora in the gastrointestinal tract, but much less so in the large intestine (Laerke *et al.*, 2000). We have previously shown that acetate can induce SirA activation independent of BarA by its conversion to acetyl phosphate through the action of acetate kinase and phosphotransacetylase, encoded by *ackA* and *pta,* respectively (Lawhon *et al.*, 2002). This suggests that acetate present in the intestinal tract may serve as a signal and *Salmonella* may sense this signal. Therefore, formate may also act as an environmental signal, produced by the normal microflora of the intestinal tract, to which *Salmonella* responds and gene expression is induced. Genes with expression altered by formate may allow for survival and long-term *Salmonella* carriage. For example, recent evidence has shown that formate can protect *Salmonella* from killing by a cationic peptide (Barker *et al.*, 2000). Thus, formate provides an excellent candidate for an environmental signaling molecule of *Salmonella* infection.

Conclusion

Salmonella have become one of the most important foodborne pathogens. Non-typhoidal salmonellosis ranks first in numbers of deaths and second in illness numbers among bacterial foodborne diseases. Asymptomatic carriage in food-producing animals is the important source of spread for *Salmonella* by food contamination. Pork and pork products are important sources of salmonellosis. This study identified genes specifically expressed in pigs and environmental signals inducing those *in vivo* expressed genes. Some of genes may be important for *Salmonella* subclinical carriage in pigs, which in the future may help to deduce the mechanism by which *Salmonella* persists in animals without causing clinical disease. Some of genes identified to be required for asymptomatic carriage could be potential candidates for vaccine development in the future. Pigs might also serve as an animal model to study salmonellosis in humans.

Formate is a key molecule in the intestinal tract, since one-third of sugar carbon is converted to formate by fermentation. The small intestine, the *Salmonella* colonization site, is a location in which formate is one of the major products produced by food digestion and of fermentation by enterobacteria. We discovered that formate induced SPI 1 gene expression and invasion of cultured epithelial cells. This effect of formate required below neutral pH, present in the small intestinal tract, and is proposed to be achieved by changing carbon flux. We also defined the *Salmonella* formate regulon and identified possible genetic regulators on which formate acts. A number of genes induced in pigs and by formate overlapped, which

suggests that formate may play a role in *Salmonella* asymptomatic persistence. Therefore, formate may be the important environmental signal in the intestinal tract for both *Salmonella* invasion and subclinical carriage. Further studies are needed to test whether changing animal diets can change the intestinal formate concentration to affect *Salmonella* shedding and invasion. In this way, we may in the future discover new therapies and prevention strategies for *Salmonella* infection.

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Genome-wide screen of *Salmonella* genes expressed during pig infection using *in vivo* expression technology

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Summary

Pigs are a food-producing species that readily carry *Salmonella* but, in the great majority of cases, do not show clinical signs of disease. Little is known about the functions required by *Salmonella* to be maintained in pigs. We have devised a recombinase-based promoter trapping strategy to identify genes with elevated expression during pig infection with *Salmonella enterica* serovar Typhimurium. A total of 55 clones with *in vivo*-induced promoters were selected from a genomic library of ~10,000 random *Salmonella* DNA fragments fused to the recombinase *cre*, and the cloned DNA fragments were analyzed by sequencing. Thirty-one genes encoding proteins involved in bacterial adhesion and colonization (including *bcfA*, *hscA, rffG,* and *yciR*), virulence (*metL*), heat shock (*hscA*), and a sensor of a two-component regulator (*hydH*) were identified. Among the 55 clones, 19 were isolated from both the tonsils and the intestine, while 23 were identified only in the intestine, and 13 only in tonsils. High temperature and increased osmolarity were identified as environmental signals that induced *in vivo* expressed genes, suggesting possible signals for expression

Introduction

Serovars of *Salmonella enterica* infect a variety of hosts, from domestic livestock to humans. The outcomes of *Salmonella* infection can range from asymptomatic carriage to severe disease. The two common disease syndromes caused by *Salmonella*, septicemia and enteritis, have been actively studied, the former through the use of a mouse model, and the latter primarily with calves (Galan and Curtiss, 1989a; Jones and Falkow, 1994; Miller and Mekalanos, 1990; Tsolis *et al.*, 1999). After decades of effort, the genetic factors utilized by *Salmonella* to cause both enteric and systemic infection are becoming clearer. *Salmonella* Pathogenicity Islands (SPI) 1 through 5 have been shown to be required for functions essential to *Salmonella* virulence, including the penetration of epithelial cells and survival in macrophages (Behlau and Miller, 1993; Chan *et al.*, 2005; Galan and Curtiss, 1989a; Groisman and Ochman, 1993; Lee *et al.*, 1992; Mills *et al.*, 1995). Thus, these SPIs are essential to producing overt disease in a wide range of animal species.

Another important aspect of *Salmonella* infection is its persistence and asymptomatic carriage in animals that serve as reservoirs for contamination of human food. Salmonellosis remains the leading cause of death and is second only to campylobacterosis in illness numbers in United States among bacterial foodborne diseases (Mead *et al.*, 1999). Pigs are a food-producing species that readily carry *Salmonella* but, in the great majority of cases, do not show clinical signs of diseases.

Surveys have shown that up to six serotypes can be isolated from clinically normal pigs on a single farm (Davies *et al.*, 1998). Pork products were implicated in 2.9% of all *Salmonella* outbreaks during the years 1983 to 1987 (Tauxe, 1991). Between 1988 and 1992, 18% of the outbreaks caused by consumption of contaminated meat reported to the CDC were due to ham and pork (Bean *et al.*, 1997). Swine also shed antimicrobial-resistant *Salmonella* that pose a threat to food safety. With increasing frequency, *Salmonella* isolates obtained from pigs are resistant to one or multiple antimicrobials. Recent studies have shown that at least half, and in some cases over 90%, of *Salmonella* isolates obtained from commercially raised swine in the United States are multiresistant (Farrington *et al.*, 2001; Gebreyes *et al.*, 2000). Therefore, the high rate of unapparent infections makes pigs potential incubators of *Salmonella,* allowing the expansion of bacterial population and threatening human health. It also makes pigs an important species for the study mechanisms by which *Salmonella* is maintained in animal species that fail to show overt disease.

To effectively survive and persist in animals, *Salmonella* must coordinate gene expression in response to varied environments during the process of infection. Little is known about genetic factors required for *Salmonella* carriage in clinically healthy animal hosts with persistent shedding of bacteria in feces. A characteristic that all *Salmonella* infections share is colonization of the gastrointestinal tract, and so genetic factors important for colonization may be required. In chickens, another important species carrying *Salmonella* without causing overt diseases, mutants of lipopolysaccharide biosynthesis have shown reduced intestinal colonization (Turner

et al., 1998). Fimbrial adhesions are also thought to be potential factors for mediating attachment to intestinal surfaces by *S.* Typhimurium (Humphries *et al.*, 2001), and there is evidence that non-motile mutants of *Salmonella* are deficient in colonization (Barrow *et al.*, 1988). In addition, the colonization factors of *Salmonella* are thought to be host specific (Morgan *et al.*, 2004). It is unknown whether the genetic factors required for *Salmonella* persistence in pigs differ from those used in other animal species.

Besides the genetic factors, a variety of environmental conditions present within animal hosts have been shown to provide signals that control *Salmonella* gene expression. An early step in the pathogenesis of *Salmonella* infection is bacterial penetration of intestinal epithelium. Many of the genes required for epithelial penetration are found within SPI1. It has been demonstrated that the regulation of invasion genes requires a coordinated response to varied environmental signals. Low oxygen, pH below neutrality, high osmolarity, all conditions of the ileum, have been implicated in the induction of SPI1 invasion genes (Bajaj *et al.*, 1996). Transcription of invasion genes has also been shown to be repressed by bile (Prouty and Gunn, 2000), and we have previously shown that acetate can induce invasion gene expression in *Salmonella* (Lawhon *et al.*, 2002). The environmental conditions present in animals thus might also provide plausible signals for other *Salmonella* functions required for life in animal hosts.

The goal of the current study was to identify *Salmonella* genes induced during infection of the pig and the environmental signals plausibly inducing the expression

of those *in vivo* induced genes. In the past decade, many techniques have been developed to study bacterial genes that are expressed during infection of animal hosts, such as signature-tagged mutagenesis (STM) (Turner *et al.*, 1998), differential fluorescence induction (DFI) (Valdivia and Falkow, 1997), *in vivo* expression technology (IVET) (Mahan *et al.*, 1993), and microarray analysis (Lawley *et al.*, 2006). Here, we have used a recombinase-based *in vivo* expression technology in combination with a signature-tagging approach to identify genes expressed during infection of pigs with *Salmonella enterica* serovar Typhimurium as a means to identify genes that may be important for *Salmonella* carriage in pigs. The results indicated that *Salmonella* Typhimurium induces a variety of genes in this animal host, including those involved in adhesion, two-component regulation, survival in macrophages, and anaerobic metabolism, as well as unknown functions. Furthermore, we demonstrate that environmental conditions present in pigs, elevated temperature and increased osmolarity, induce the expression of some of these *in vivo*-induced genes.

Materials and methods

Library screening for *in vivo* induced genes. For this approach, we used a recombinase-based system previously developed to identify differentially expressed *Salmonella* genes (Altier and Suyemoto, 1999). We created a library of *Salmonella* genomic DNA fragments by partial digestion of total genomic DNA from *S.* Typhimurium strain 798, originally isolated from a pig (Wood *et al.*, 1989), with *Hae*III, *Alu*I, or *Rsa*I, then size fractionated the DNA to isolate fragments of 1-2 kb. These

three libraries were pooled and fragments were cloned into the *Pml*I site of the ampicillin-marked plasmid pCA19, placing them upstream of a promoterless derivative of the phage P1 recombinase *cre.* A derivative of strain ATCC14028s carrying a chloramphenicol resistance marker and a pair of chromosomal *loxP* sites flanking *npt* (kanamycin resistance) and *sacB* (sucrose susceptibility) was transformed with this library, with an initial selection on MOPS minimal agar with ampicillin (100 μg/ml), kanamycin (50 μg/ml) and chloramphenicol (25 μg/ml). (Strain ATCC14028s was used in these experiments because strain 798 carrying the *sacB* cassette proved not to be sucrose susceptible; ATCC14028s also infects pigs in high numbers, similar to 798). Selection on kanamycin removed constitutively active promoters from the library, thus leaving DNA fragments with no promoter activity and regulated promoters not expressed on laboratory media.

Approximately 10^4 independent library transformants were pooled for administration to pigs. Approximately $1X10^{10}$ bacteria, representing this pool of 10^4 clones, were administered orally to two 7-week-old pigs. After 48 hours, pigs were sacrificed and the contents of the entire intestinal tract were harvested. Cecal and colonic contents were diluted in PBS, passed through a gauze filter, and then centrifuged to concentrate bacteria. Ileal contents were in small volume and of liquid consistency, and so were used directly. All samples were plated onto *Salmonella-Shigella* agar with added chloramphenicol, ampicillin, and 5% sucrose. On this medium, only bacteria that had lost the *loxP* cassette, along with the intervening

sacB, should have been present due to the differential expression of *cre*. The loss of the *loxP* cassette was verified by susceptibility to kanamycin.

Verification of *in vivo*-induction. Plasmids carrying *in vivo* induced genes were individually re-introduced into the strain with the intact chromosomal *loxP* gene cassette by transduction with phage P22. Only the transductants remaining kanamycin resistant when grown on MOPS minimal medium were retained for further study. We next tested the gene expression of those transductants in pigs again using a modified signature tagging method. A sample of overnight culture of each strain was fixed to a nylon membrane using a dot-blot apparatus according to the manufacture's directions (Millipore, Billerica, MA). The membranes were incubated with the colony side up in denaturing solution (0.5 M NaOH and 1.5 M NaCl) for five minutes twice, and in neutralizing solution (0.5 M Tris pH 7.5 and 1.5 M NaCl) for ten minutes twice. 2XSSC was used to wash the membranes, and the membranes were fixed by UV crosslinking at 1200 W. Each strain was applied to five membranes; each membrane contained 20-35 strains. Equal volumes of these 20-35 strains were pooled to create an inoculum for two 7-week-old pigs, with each pig receiving approximately $1X10^{10}$ bacteria. After 48 hours, pigs were sacrificed. The contents of 10-15 cm of the distal ileum were harvested, diluted in MOPS minimal base, passed through a filter stomacher bag, and then centrifuged to concentrate bacteria. For tonsil samples, both sides of the tonsils of the soft palate were swabbed using two cotton swabs, which were washed with MOPS minimal base and centrifuged to concentrate bacteria. All samples were plated onto

Salmonella-Shigella agar with added chloramphenicol (for the chromosomal marker), ampicillin (for the plasmid marker), and 5% sucrose. Sucrose resistant bacteria were recovered as four output pools, one each for the ileum and the tonsils for each pig. Plasmid DNA was prepared from the inoculated and output pools using a Qiagen plasmid midi kit (Qiagen, Valencia, CA). The DNA inserts present in the pools were PCR amplified using the single primer 5'-GCGGCCGCACGTGCGGCCGC, homologous to sequence that flanks the inserted DNA fragment on both its ends in pCA19. PCR products were purified using a Qiagen PCR purification kit (Qiagen, Valencia, CA). The purified PCR products were labeled using a second PCR amplification with a DIG Probe Synthesis Kit (Roche Applied Science, Indianapolis, IN), and these PCR products were used as probes to hybridize to the membrane containing the corresponding inoculated bacteria for each pig. We hybridized membranes with probes from five sources: one made from the input pool, two made from the output pools for the tonsils (one from each pig), and two for the intestinal contents (one from each pig). Hybridizations were done using DIG Easy Hyb (Roche Applied Science, Indianapolis, IN) overnight at 42^{o}C, and a DIG Wash and Block Buffer set and Biotin Luminescent Detection Kit were used according manufacturer's directions for washing and detection. Blots were detected using a luminescent imager (Boehringer Mannheim Lumi-Imager).

Sequencing. Sequences were obtained using an Applied Biosystems Inc. 3130xl Genetic Analyzer (ABI, Foster City, CA) or by MClab DNA sequencing service (San Francisco, CA). The sequencing primers were: 5'-CATTTTCCAGGTATGCTCAG,

which is located in *cre*, and 5'-AGTAGGTTGAGGCCGTTG, located upstream from the inserted DNA fragment in pCA19. The location of each of the cloned fragments was determined by comparison to the sequenced genome of *S. enterica* serovar Typhimurium strain LT2 (Genbank AE006468).

β-galactosidase assays. β-galactosidase assays were performed using an enhanced β-galactosidase assay kit (CRPG) following the manufacturer's directions (Genlantis, San Diego, CA) for a 96-well microtiter plate assay, with minor alterations. We used one drop of chloroform and one drop of 0.1% SDS to lyse cells. Levels of β-galactosidase expression were measured by the catalytic hydrolysis of the chlorophenol red-β-D-galactopyranoside (CRPG) substrate to a dark red product. The β-galactosidase levels were calculated using the equation: β-galactosidase = (1000 * absorbance at 595 nm)/ (sample volume in ml * time of reaction * absorbance at 600 nm). Absorbance was read using a Power Wave Xs 96 well plate reader (Bio-tech instruments Inc. Winooski, VT). To assess the effect of temperature on gene expression, strains were grown in 96 well sealed plates as standing overnight cultures in LB broth at 30^{o} C, 37^{o} C, and 42^{o} C. To test the effects of osmolarity, strains were grown aerobically in 96 well plates as standing overnight cultures in MOPS minimal medium at 37^{o} C with or without the addition of 0.4 M NaCl. Triplicate cultures of each strain were assayed for *lacZ* expression by CRPG enhanced β-galactosidase assays.

Statistical analysis. For β-galactosidase assays, two-sample t-tests were performed to determine which means differed at $p \leq 0.05$ (The SAS System for Windows 8 and MINITAB Release 14).

Results and discussion

Recombinase-based screening for *in vivo*-induced genes in the pig. The high rate of unapparent infections makes pigs potential incubators of *Salmonella*, allowing the expansion of bacterial populations in the animal host. Therefore, we sought to establish an experimental model using pigs to study mechanisms of *Salmonella* infection in clinically healthy animals. We reasoned that there might be bacterial genes required for survival that are expressed specifically in pigs. We selected such *in vivo*-induced genes using a recombinase-based system that we had developed to identify *Salmonella* genes that are differently expressed when bacteria are exposed to specific environmental conditions (Altier and Suyemoto, 1999). This system consists of a cassette integrated into the *Salmonella* chromosome that harbors *npt*, encoding kanamycin resistance, and *sacB*, for sucrose susceptibility, flanked by a pair of *loxP* sites. On a plasmid is a promoterless derivative of *cre* encoding the Cre recombinase of phage P1 that recognizes the *loxP* sites as its targets. The fusion of an active promoter to *cre* induces recombination of the two *loxP* sites and deletion of the intervening DNA, allowing selection on sucrose. Fusion of promoters active only when exposed to a specific environment, in this case after infection of pigs, induces bacterial conversion to sucrose resistance only after bacteria have been exposed to that environment, thus selecting for differentially expressed bacterial genes.

For these experiments, we constructed a library of approximately 10,000 random *Salmonella* DNA fragments fused to *cre*. The library was first pre-selected on medium containing kanamycin to eliminate constitutive promoters from the population. It was then administered to two pigs, and the intestinal contents were cultured on selective medium containing 5% sucrose. On this medium, only bacteria that have lost the *loxP* cassette, along with the intervening *sacB*, should be present, due to the differential expression of *cre*. From the genomic library, 173 fragments were identified as being specifically expressed after infection of pigs. To verify the specificity of *in vivo* induction of these putative promoters, each plasmid was reintroduced into the strain carrying the intact *loxP* cassette by P22 transduction. Of 173 transductants, 154 remained kanamycin resistant when grown on MOPS minimal medium. The remaining 19 had undergone Cre-mediated recombination of the *loxP* sites, and thus converted to kanamycin susceptibility, therefore representing constitutively expressed promoters that had escaped our original pre-selection. To further verify *in vivo*-induced gene expression, we used a modified signature tagging method. The 154 strains were divided into five groups of 20-35 each, and each group was used to infect two pigs. Sucrose-resistant *Salmonella* isolates were again isolated from the animals, this time from both the ileum and the tonsils, and these populations of bacteria were used to make pooled probes by PCR amplifying the cloned fragments of each. These probes ware used in colony blots to determine which members of the input pool had reproducibly converted to sucrose resistance after animal infection. From the original 154, we isolated 55 promoter fragments that were expressed again in both of the pigs that had received the

bacterial pool. Of these, 19 were isolated from both the tonsils and the intestine, while 23 were identified only in the intestine, and 13 only in tonsils. To characterize these *in vivo*-expressed genes, we sequenced the cloned fragments and compared them to the *Salmonella* genome database (McClelland *et al.*, 2001). Thirty-one cloned fragments of the 55 clones corresponded to 32 unique genes with known or putative functions (Table 1), as some clones carried more than one gene and some clones were found more than once. Six of the cloned fragments, corresponding to *hydH, hpaB/hpaR, wecC/rffG, yciR,* STM1731, and STM0611/0612/0613, were found twice, and one cloned fragment containing three genes (STM2755/2756/2757) was found three times. In addition, 21 clones found in this screen, representing 18 chromosomal loci, were in an orientation relative to *cre* opposite to that of annotated gene predicted to be carried on the fragment (see details below). Among those reverse-orientated fusions, *ygiK* was recovered four times independently, as it occurred on fragments of different sizes, while *rpoN* and STM1368 was similarly independently identified twice. Besides the 31 clones with known functions and 21 clones with reverse-orientated fusions, we failed to obtain readable sequences for three cloned fragments. These results therefore show that by using a promoter trap strategy in combination with a signature tagging approach, large *Salmonella* genomic libraries can be produced and screened to identify *in vivo*-induced genes.

Genes for synthesis of fimbriae and LPS. Three genes identified by this screen have previously been implicated in the colonization of animals (Humphries *et al.*, 2005; Toth *et al.*, 1999; Turner *et al.*, 1998). One cloned fragment included *bcfA,*

encoding a fimbrial subunit. Fimbriae have been shown to function as intestinal colonization factors in *Salmonella* serovars (Humphries *et al.*, 2001; Tanaka and Katsube, 1978; Tanaka *et al.*, 1981). The expression of serotype Typhimurium fimbrial antigens is induced during the infection of mice (Humphries *et al.*, 2005). *bcfA* is specifically expressed during the infection of bovine ligated ileal loops, but not *in vitro* (Humphries *et al.*, 2003). One other cloned locus found in this screen included two adjacent genes, *wecC* and *rffG,* involved in the production of enterobacterial common antigen (ECA) (Meier-Dieter *et al.*, 1990), a cell surface glycolipid present in all gram-negative enteric bacteria (Kuhn *et al.*, 1988). We found this fragment twice, on different sized fragments, from the tonsils of one pig and from both the tonsils and the intestine of another pig. This gene and its homologues have been shown to be important for colonization in a number of bacterial pathogens. Mutation of *rffG* produces reduced virulence in the plant pathogen *Erwinia carotovora* subsp. *atroseptica* when inoculated into potato plant stems (Toth *et al.*, 1999). It has been shown that *rffG* in *Escherichia coli* is a functional homologue of *rfbB*, with both of these genes encoding dTDP-glucose hydratases (Marolda and Valvano, 1995). *S.* Typhimurium has both *rfbB* and *rffG*, which also encode dTDP-glucose hydratases (Toth *et al.*, 1999), and a mutant of *rfbB* in *S.* Typhimurium has been shown to exhibit reduced intestinal colonization of chicks (Turner *et al.*, 1998). Thus, the importance of these genes identified by our screen is supported by studies in other animal and plant species and suggests that the method is sufficient to identify genes important to the existence of *Salmonella* in pigs.

Sensor of a zinc tolerance two-component system. Two clones encoding a sensor of the HydH/HydG two-component regulator were found in this screen, with both carrying *hydH*, but on fragments of different sizes. HydH/HydG has also been designated ZraS/ZraR (zinc-resistance associated sensor/regulator), as it is involved in zinc tolerance (Leonhartsberger *et al.*, 2001). It has been proposed that ZraS/ZraR senses high zinc concentrations and activates the expression of *zraP* to contribute to zinc tolerance (Leonhartsberger *et al.*, 2001). The level of *hydG* mRNA has also been shown to be increased by three-fold in *E. coli* after the addition of $ZnSO_4$ (Lee *et al.*, 2005). The dietary zinc requirement for swine is 50-100 ppm, which is more than that for other tested livestock (Berger, 1987). Therefore, it is possible that *hydH* was expressed in both the tonsils and the intestine of pigs during *Salmonella* infection in response to the high zinc concentration present in pigs, due to zinc supplementation of feed. Alternatively, it has also been shown that *hydH* of *E. coli* is expressed during infection of the human gut (John *et al.*, 2005). Thus, the two component zinc tolerance system HydH/HydG may be important for bacteria during life within animal hosts.

yciR, encoding GGDEF and EAL domains. Another gene identified in this screen was *yciR*, also designated *gcpE* (GGDEF domain containing protein E, *gcpE*) (Garcia *et al.*, 2004). We cloned *yciR* twice independently, on different sized fragments, once from tonsils and once from the intestine. *yciR* encodes a protein containing GGDEF and EAL domains, representing a class of proteins found in many bacterial species. *S.* Typhimurium has twelve proteins containing GGDEF

domains and fourteen proteins with EAL domains (Garcia *et al.*, 2004; Tatusov *et al.*, 2000). Such proteins control the intracellular concentration of the global second messenger c-di-GMP, with the GGDEF domain stimulating c-di-GMP production and the EAL domain c-di-GMP degradation (Simm *et al.*, 2004). c-di-GMP has been identified as a global regulator responsible for motility, adhesion, biofilm formation, and virulence (Mendez-Ortiz and Membrillo-Hernandez, 2005). Deletion of *yciR* of *S.* Typhimurium affects cellulose production and biofilm formation (Garcia *et al.*, 2004), while a different EAL domain protein has been indicated to control bacterial survival in mice (Hisert *et al.*, 2005). Together, these findings suggest that *yciR* and the modulation of c-di-GMP levels may be involved in *Salmonella* colonization or survival in pigs.

Genes for assembly of Fe-S clusters and the heat shock response. Another cloned fragment plausibly affecting *Salmonella* persistence in pigs contained the *hscBA-fdx* operon, which is involved in the assembly of Fe-S clusters (Tokumoto and Takahashi, 2001) and probably co-transcribed with the Fe-S cluster *iscSUA* genes (Zheng *et al.*, 1998). HscA, a chaperone, has been shown to be regulated by the Fe-S cluster assembly protein IscU and the co-chaperone HscB (Silberg *et al.*, 2004). The ferredoxin (Fdx) is proposed to be involved in electron transfer (Giel *et al.*, 2006). Therefore, this *hscBA-fdx* operon plays a central role in the assembly machinery of Fe-S clusters, which function in a number of cellular processes, including gene regulation (Johnson *et al.*, 2005). It has been shown that the bacterial species *Xenorhabdus nematophila* requires an intact *iscRSUA-hscBA-fdx*

operon to colonize *Steinernema carpocapsae* nematodes (Martens *et al.*, 2003). Thus, this operon might also be important for *S.* Typhimurium colonization or carriage in animals. In particular, within this operon *hscA* encodes a 66-kilodalton heat shock protein, which is a homologue of the heat shock protein DnaK (Hoff *et al.*, 2002). It has been shown that *Salmonella* heat shock proteins are induced upon infection of macrophages (Buchmeier and Heffron, 1990), and a heat shock protein of the size of HscA has been shown to be responsible for binding of *S.* Typhimurium to intestinal mucus (Ensgraber and Loos, 1992). These findings could thus implicate *hscA* as a gene affecting *Salmonella* colonization and persistence in pigs.

Genes involved in the degradation of aromatic compounds. A clone carrying two adjacent genes, *hpaB* and *hpaR,* was found twice on different cloned fragments. *hpaB* and *hpaR* are both components of the 4-hydroxyphenylacetate (4-HPA) degradative pathway in *E. coli* (Galan *et al.*, 2003; Martin *et al.*, 1991). *hpaB* and the gene adjacent to it, *hpaC,* form a single transcription unit and encode the large and small components of a two component 4-HPA 3-monooxygenase. *hpaB* encodes the flavoprotein, whereas *hpaC* codes for a coupling oxidoreductase (Galan *et al.*, 2000; Prieto *et al.*, 1993), which increases the hydroxylase activity of HpaB (Prieto and Garcia, 1994). In *E. coli*, the *hpa* catabolic genes are organized in two transcribed operons in the same orientation: the *upper* operon (*hpaBC*) and the *meta* operon (*hpaGEDFHI*) (Prieto *et al.*, 1996; Roper *et al.*, 1993). The *hpa* pathway of *E. coli* is regulated by two proteins, HpaA as an activator and HpaR as a repressor, reverse-oriented to the two operons (Galan *et al.*, 2003; Prieto *et al.*,

1996). In *S.* Typhimurium*,* the gene arrangement of the *hpa* operons is different from that in *E. coli*; the *upper* operon (*hpaBC*) and the *meta* operon (*hpaGEDFHI*) are divergently transcribed. *hpaR* of *Salmonella* is transcribed in the same orientation as *hpaBC*, but opposite to that of *hpaGEDFHI*. Therefore, it is not yet clear whether the promoter activity identified from this clone originates from the promoter of *hpaB* or that of *hpaR*. The regulatory circuits of these aromatic catabolic pathways have also not been well established in *Salmonella*. Although aromatic compounds are highly abundant in soil and water, it has also been suggested that there are sources of aromatic compounds in the gastrointestinal tract, a majority of them being derived from the fermentation of aromatic amino acids and with some provided by plant materials (Goldman, 1983). Thus, the fact that *hpaB* or *hpaR* of *Salmonella* was induced in pigs suggests that *Salmonella Typhimurium* is able to degrade certain aromatic compounds when living in an animal host.

Virulence functions. Only one gene identified in our screen, *metL*, has been previously shown to be involved in *Salmonella* virulence. This gene encodes the bifunctional enzyme aspartokinase II-homoserine dehydrogenase II (AKII-HDII), and was carried on a clone isolated from the intestine. AKII-HDII catalyzes two independent proximal steps in the prokaryotic biosynthetic pathways that convert aspartate to lysine, threonine, and methionine (Zakin *et al.*, 1983), and an *S.* Typhimurium mutant of *metL* exhibits reduced virulence in mice (De Groote *et al.*, 1996). *metL* might therefore be important for *Salmonella* maintenance in pigs as well. No genes in any of the *Salmonella* pathogenicity islands were found in this

study. This was not unexpected, as mutants of SPI1 or SPI2 genes have been shown to maintain their ability to colonize the chick intestine (Morgan *et al.*, 2004), suggesting that *S.* Typhimurium is much less reliant upon SPI1 and SPI2 to establish and maintain infection in animals that fail to show overt disease, such as pigs and chickens.

Genes required for vitamin B_{12} synthesis. One cloned fragment carried the two adjacent genes *cbiF* and *cbiG*, which regulate vitamin B_{12} synthesis (Roth *et al.*, 1996). *S.* Typhimurium synthesizes B_{12} only during anaerobic growth and can use B_{12} as a co-factor in at least three reactions (reviewed by (Roth *et al.*, 1996)). B_{12} synthesis requires the expression of a single 20-gene operon, named *cob*, which maps near minute 44 of the *S.* Typhimurium chromosome and includes three *cob* and 17 *cbi* genes (Roth *et al.*, 1993). Propanediol, a byproduct of food digestion, induces the transcription of the *cob* operon dependent upon PocR, a regulatory protein of the AraC-family (Chen *et al.*, 1994). Vitamin B_{12} is required for degradation of ethanolamine and propanediol, both of which are carbon sources present in the gastrointestinal tract (Lawrence and Roth, 1995; Rondon *et al.*, 1995). It is therefore possible that *Salmonella* induces the *cob* operon to utilize these nutrient sources while living in the intestinal tract. The *cbiF/cibG* clone identified in this screen, however, was found only in tonsils. It remains possible that *cbiF/cbiG* was also induced in intestinal tract, but that we failed to find it there. For this fusion, and all other fusions described in this work, the specific location of induction can not be made with complete certainty, as the design of the screen does not ensure the

complete recovery of all isolates from both body sites tested. Alternatively, it is possible that environmental signals exist specifically in the tonsil that induce the *cob* operon, either as a requirement for life in the tonsil itself, or as a prelude to passage into the intestinal tract. Our previous work has shown that the invasion of epithelial cells by *Salmonella* is co-regulated with propanediol and ethanolamine catabolism, including *cob* expression (Lawhon *et al.*, 2002; Lawhon *et al.*, 2003). It is therefore possible that environmental cues encountered by *Salmonella* coordinately regulate functions important to life in an animal host, including both metabolic and virulence functions.

Genes of other functions. Other genes found in this screen included a protease (*sppA*), an integrase (*rlgA*), a gene used for cysteine synthesis (*cysQ*) (Neuwald *et al.*, 1992), and those with the putative functions shown in Table 1. Therefore, our screen has identified diverse classes of *Salmonella* genes that appear to be induced *in vivo*.

Reverse-oriented fusions. We also found that 21 of the fragments identified in this screen were cloned in the direction that placed the annotated gene contained on the fragment in an orientation opposite to that of *cre*, thus producing no obvious promoter fusion. Examination of the sequence of these clones showed that they did not carry portions of adjacent genes in the opposite orientation, nor were they composed of concatenated DNA fragments from disparate regions of the genome. Misannotaion of the genome is unlikely as an explanation, because most of cloned fragments carried genes with recognized functions, such as *shdA, rpoN,* and *wcaL*

(Table 1), while three cloned fragments with putative functions in *Salmonella* (STM1368, *ygiK,* and *yciA/yciB*) had homologues with recognized functions in another organism. Similar identification of reverse-oriented fusions has been repeatedly observed by others, but without complete explanation (Camilli and Mekalanos, 1995; Mahan *et al.*, 1993; Rainey, 1999; Rollenhagen and Bumann, 2006; Wang *et al.*, 1996). Although some of these reverse-oriented fusions may contain no genuine promoter element, it is possible that others of them do. Three of the 21 were found independently twice or more in our screen, while four of these were induced by elevation of temperature and five were induced by osmolarity, as described below. One proposed explanation is that these fusions represent promoters that act to control gene expression by an antisense regulation mechanism (Osorio, 2003; Silby *et al.*, 2004). Further investigation of these clones will be required to elucidate their functions.

Identification of environmental factors that induce gene expression. A number of environmental conditions likely to be present within animal hosts have been studied as possible stimuli for *Salmonella* gene expression. Specifically, for *Salmonella* invasion genes, oxygen tension, osmolarity, growth phase, pH, and the presence of bile have all been implicated in the control of gene expression (Bajaj *et al.*, 1996; Ernst *et al.*, 1990; Galan and Curtiss, 1990; Lee and Falkow, 1990; Lundberg *et al.*, 1999; Prouty and Gunn, 2000). We therefore next determined whether our *in vivo*-induced genes responded to similar conditions. The reporter plasmid used in this study carries a promoterless *lacZ* immediately downstream from

cre (Altier and Suyemoto, 1999), and so we used β-galactosidase assays to assess changes in gene expression in response to environmental signals. One plausible means to induce expression in pigs is via a change in temperature. Pigs have a normal body temperature of ~39-40°C, higher than that of humans. To test the effects of temperature, we tested the expression of clones when strains were grown at 30° C, 37° C, and 42° C. We found that nine of the cloned fragments induced *lacZ* expression with statistical significance at 42° C and/or 37° C when compared to growth at 30° C (Figure 1). Two of reverse-orientated fusions, *shdA* and *stbD*, were also significantly increased at 42° C versus 37° C. *fdx-hscA* was induced by high temperature, as expected, as *hscA* is a heat-shock protein. *hydH*, the sensor kinase of the zinc tolerance two-component system, was also induced by high temperature. Other induced genes included those encoding a putative hydrogenase (STM0611/0612), an inner membrane protein (*ybbP*), and a putative catalase (STM1731). Four of nine clones induced by high temperature were reverse-oriented fusions, those that contained *ygiK, wcaL, shdA,* and *stbD.* As described above, these fragments carried no identified promoter elements that would induce the conditional expression of the *lacZ* fusion. The fact that they were significantly induced by changes in temperature, however, suggests that transcription at these loci might indeed occur in response to altered environmental conditions.

Previous studies have demonstrated that an increase in osmolarity has a global effect on gene expression in *E. coli* (Weber and Jung, 2002; Weber *et al.*, 2006; Weber *et al.*, 2005). In the gastrointestinal tracts of mammals, bacteria are

faced with hyperosmolarity (Russell and Jarvis, 2001). High osmolarity has been implicated in the induction of *Salmonella* invasion genes (Galan and Curtiss, 1990), and so might induce other genes required for infection and survival. Therefore, we tested osmolar stress by growing our strains with the addition of 0.4 M sodium chloride to the media (Weber *et al.*, 2006). Nine of the clones were induced by increased osmolarity (Figure 2). These included genes encoding the sensor kinase *hydH*, an integrase (*rlgA*), and a predicted amino acid ABC transporter (STM1634). Increased osmolarity also induced the reverse-orientated fusions carrying *shdA* and *pslt 068.* Thus, osmolarity is likely a signal for the induction of *Salmonella* genes in pigs and induces some transporters and membrane proteins. We also tested anaerobiosis, increased zinc by addition of $ZnSO_4$, iron limitation by addition of the iron chelating agent 2, 2'-dipyridyl, rich vs. minimal medium, and cold shock as possible inducers of gene expression. However, none of these conditions induced the expression of any *in vivo*-induced genes identified in this study.

Conclusions. In this work, we performed a comprehensive *Salmonella* genomic library screen to identify genes differentially expressed upon infection of pigs using a recombinase-based *in vivo* expression technology. As the first such screen in this animal species to be reported, we identified some common colonization factors that likely verify the utility of the approach. These genes included *bcfA, wecC*, *rffG,* and *yciR*, involved in surface adherence and isolated from both tonsils and the intestinal tract. An early step for *Salmonella* colonization is adherence to cell surfaces. Thus, regardless of the location, *Salmonella* likely uses these colonization factors to

establish itself in the body. We also found novel factors not previously known to be induced upon bacterial interaction with an animal host. These included *hydH*, which may indicate that zinc acts as a signal during *Salmonella* infection, and *hpaB,* the product of which catalyzes the degradation of 4-HPA, suggesting the use of aromatic acids as energy sources. Interestingly, we also identified a gene, *yciR*, with putative diguanylate cyclase and phosphodiesterase functions used for the production and degradation of c-di-GMP. As *yciR* has been shown to be used for biofilm formation in *Salmonella* (Garcia *et al.*, 2004), and as c-di-GTP as a second messenger has recently been identified as important in other pathogenic bacteria (D'Argenio *et al.*, 2002; Galperin, 2004; Romling *et al.*, 2005; Tamayo *et al.*, 2007), the induction of this gene may play an important role for *Salmonella* after animal infection. Although no screen of this type can provide an exhaustive account all genes induced *in vivo*, this study provides information for further research on *Salmonella* survival and colonization in animals that carry the pathogen without clinical signs, which may lead to new therapies or prevention strategies to reduce the contamination of the human food supply.

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Table 1. *Salmonella* genes induced during infection of pigs.

Category	Gene	Function	Tissue
Adherence	*wecC/rffG*	UDP-N-acetyl-D-mannosaminuronic acid dehydrogenase/dTDP-glucose	intestine and tonsil
	bcfA	fimbrial subunit	intestine and tonsil
	fdx-hscA	involved in assembly of Fe-S clusters	intestine
	yciR	putative diguanylate cyclase/phosphodiesterase	intestine and tonsil
Two-component regulator	*hydH*	sensory kinase in two component regulatory system with HydG	intestine and tonsil
Aromatic hydroxylase	*hpaB/hpaR*	4-hydroxyphenylacetate catabolism	intestine and tonsil
Virulence	*metL*	aspartokinase II-homoserine dehydrogenase II	intestine
Metabolism	*cysQ*	sulfite biosynthetic protein	intestine and tonsil
	sppA	protease IV	intestine and tonsil

Table 1 continued

	cbiF/cbiG	synthesis of vitamin B_{12} adenosyl cobalamide presursor	tonsil
Putative function	STM0611/0612/0613	putative hydrogenase protein	intestine
	oadB	putative sodium ion pump	tonsil
	rlgA	putative integrase	tonsil
	STM2689	pseudogene	intestine
	STM1731	putative catalase	intestine and tonsil
	STM2755/2756/2757	putative sugar phosphate aminotransferase/ putative hexulose 6 phosphate synthase	tonsil
	yiiG	putative cytoplasmic protein	intestine
	STM0325	putative IS3 transposase	intestine
	STM4489	putative superfamily I DNA helicases	intestine
	STM4320/ *pheR*	putative *merR* family bacterial regulatory protein	intestine
	ybbP	putative inner membrane protein	intestine and tonsil
	STM1634/1635	putative amino acid ABC transporter permease	intestine and tonsil

Table 1 continued

	ybgH	putative POT family transport protein	intestine
Reverse-orientated fusions	*ygiK*	putative inner membrane protein	intestine and tonsil
	STM1368	putative Na^+-dicaboxylate symptorter	intestine and tonsil
	yciA/yciB	intracellular septation protein A/B	intestine
	sitC	fur regulated *Salmonella* iron transporter	intestine
	kduD	2-deoxy-D-gluconate 3-dehydrogenase	intestine
	parA	plasmid partition protein A	intestine
	galP	galactose/proton symporter	intestine
	shdA	similar to the C-terminal region of AIDA	intestine
	pslt068	putative ParB-like nuclease	intestine
	napA	periplasmic nitrate reductase	intestine and tonsil
	hflK	FtsH modulator	intestine and tonsil
	stbD	putative fimbrial usher	intestine and tonsil

Table 1 continued

	rpoN	DNA-directed RNA polymerase subunit N	intestine and tonsil
	slt-trpR	soluble lytic murein transglycosylase	intestine and tonsil
	pslt026	putative periplasmic protein	intestine and tonsil
	wcaL	putative glycosyl transferase	tonsil

Figure legends

Figure 1. Effects of temperature on *in vivo* induced gene fusions. Strains carrying a fusion of the gene shown to *lacZ* were grown as standing overnight cultures in LB at 30° C (white bars), 37° C (grey bars), and 42° C (black bars). Triplicate cultures of each strain were assayed for *lacZ* expression using CRPG enhanced β-galactosidase assays. Single asterisks (*) show a significant increase ($p \leq 0.05$) when the strain was grown at 42° C or 37° C as compared to 30° C. Double asterisks (**) show a significant increase ($p \leq 0.05$) when grown at 42° C as compared to 37° C. The β-galactosidase concentration was calculated in defined in the Materials and Methods. Error bars show standard deviation.

Figure 2. Effects of osmolarity on *in vivo* induced gene fusions. Strains carrying a fusion of the gene shown to *lacZ* were grown as standing overnight cultures in MOPS minimal medium (grey bars), and with the addition of 0.4 M NaCl (black bars). Triplicate cultures of each strain were assayed for *lacZ* expression using CRPG enhanced β-galactosidase assays. All fusions shown produced a significant increase ($p \leq 0.05$) in expression due to the addition of the NaCl. The β-galactosidase concentration was calculated in defined in the Materials and Methods. Error bars show standard deviation.

Figure 1.

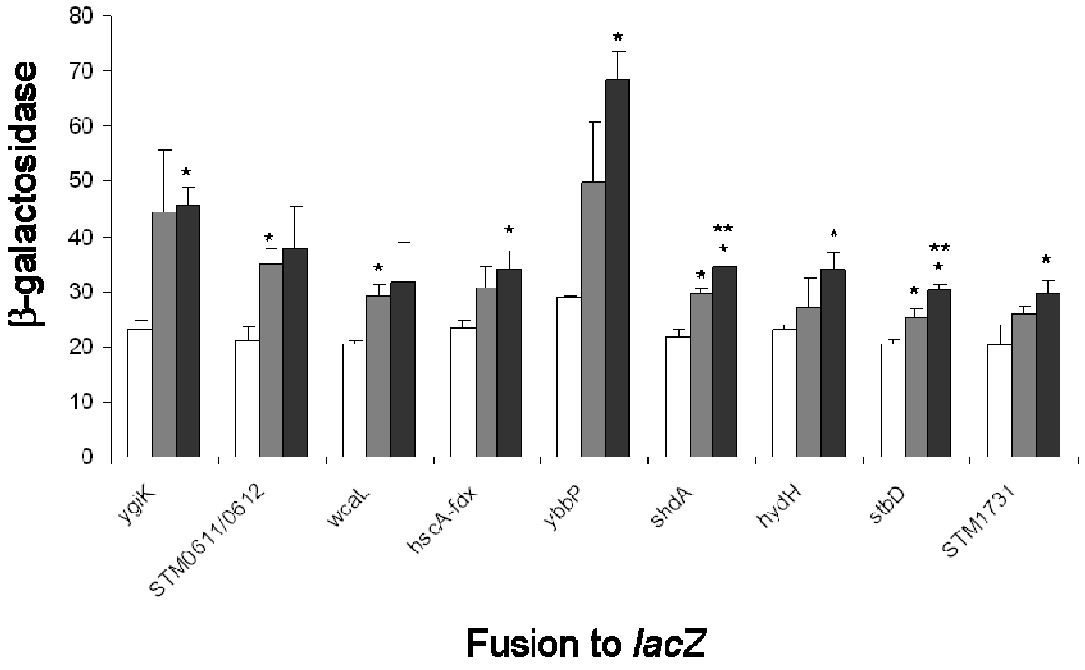

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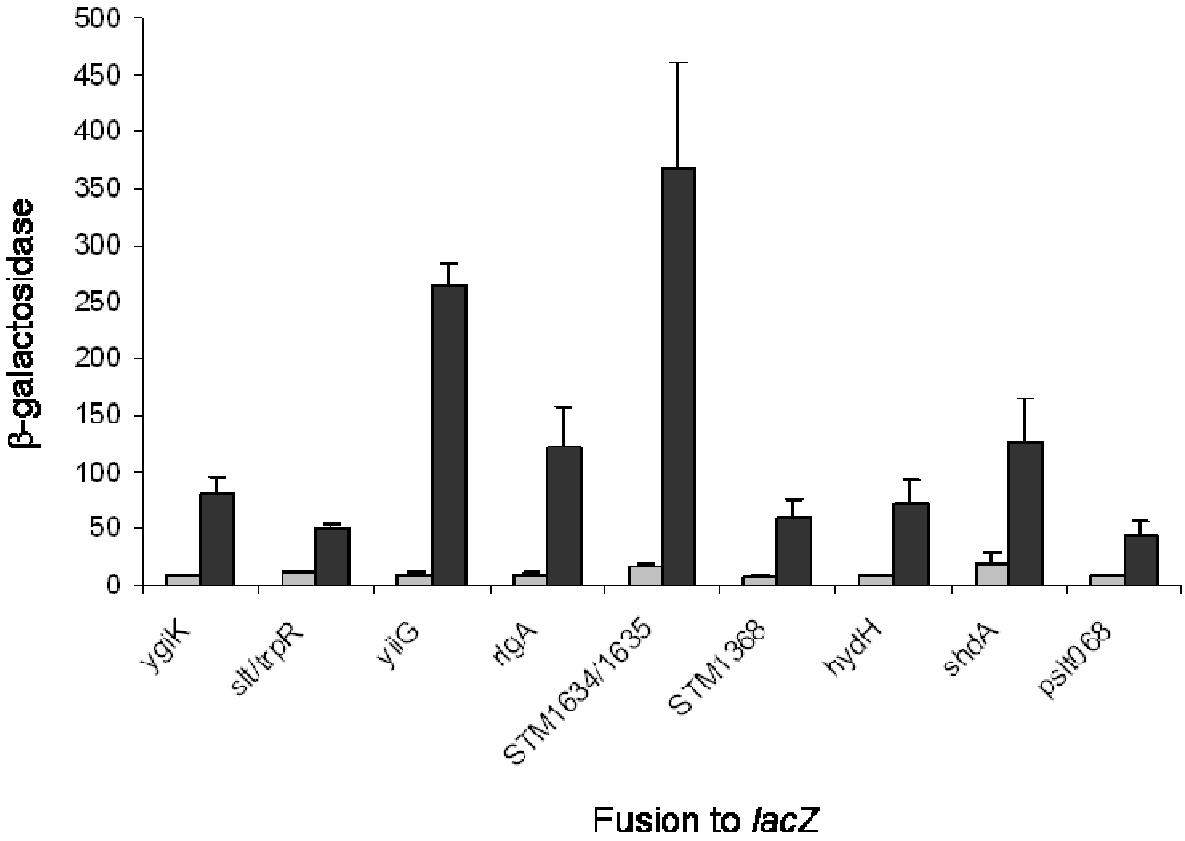
Figure 2.



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Formate acts as a diffusible signal to induce *Salmonella* invasion

Summary

To infect an animal host, *Salmonella* must penetrate the intestinal epithelial barrier. This process of invasion requires a type III secretion system encoded within *Salmonella* pathogenicity island I (SPI1). We found that a mutant of acetate kinase and phosphotransacetylase (*ackA-pta*) was deficient in invasion and SPI1 expression, but that invasion gene expression was completely restored by supplying media conditioned by growth of the wild type strain, suggesting that a signal produced by the wild type but not by the *ackA-pta* mutant was required for invasion. This mutant also excreted 68-fold less formate into culture medium, and the addition of sodium formate to cultures restored both the expression of SPI1 and the invasion of cultured epithelial cells by the mutant. The effect of formate was pH-dependent, requiring a pH below neutrality, and studies in mice showed that the distal ileum, the preferred site of *Salmonella* invasion in this species, had the appropriate formate concentration and pH to elicit invasion, while the cecum contained no detectable formate. Furthermore, we found that the primary routes of formate metabolism played no role in its activity as a signal, but that disrupting the ability of formate to enter central carbon metabolism did prevent its effect, suggesting a role for formate in inducing invasion by changing carbon flux.

Introduction

Salmonella has evolved an elaborate mechanism to promote its penetration of the intestinal epithelium, an early step in its pathogenesis. It harbors in its genome *Salmonella* pathogenicity island 1 (SPI1), a 40 kb region encoding some 40 genes (Behlau and Miller, 1993; Galan and Curtiss, 1989; Groisman and Ochman, 1993; Jones and Falkow, 1994; Lee *et al.*, 1992; Mills *et al.*, 1995). This region produces the components of a type III secretion apparatus and secreted proteins that are exported to the bacterial surface and into adjacent eukaryotic cells. These proteins then signal the eukaryotic cell to induce cytoskeletal changes that lead to bacterial engulfment (Collazo and Galan, 1997; Fu and Galan, 1998; Hardt *et al.*, 1998b; Kubori *et al.*, 1998; Zhou *et al.*, 1999). SPI1 invasion genes are known to be important for both septicemia and enterocolitis caused by *Salmonella* (Galan and Curtiss, 1989; Jones and Falkow, 1994; Miller and Mekalanos, 1990; Tsolis *et al.*, 1999).

The genetic regulation of SPI1 is complex. Much of the response to environmental conditions requires HilA, a SPI1 transcriptional regulator of the OmpR/ToxR family (Bajaj *et al.*, 1995; Bajaj *et al.*, 1996). Among the targets of HilA is the SPI1 gene *invF*, which itself encodes a transcriptional regulator (Kaniga *et al.*, 1994). HilA and InvF have overlapping, but not identical, sets of targets, both within and outside SPI1 (Ahmer *et al.*, 1999; Darwin and Miller, 1999; Eichelberg and Galan, 1999). HilA, in turn, is subject to multiple controls, with two additional SPI1 regulators, HilC and HilD, inducing *hilA* expression (Eichelberg *et al.*, 1999;

Johnston *et al.*, 1996; Schechter *et al.*, 1999). In addition to control by transcriptional regulators within SPI1, invasion genes are also under the control of several regulators outside the island (Ellermeier and Slauch, 2003; Iyoda *et al.*, 2001; Lucas *et al.*, 2000; Nakayama *et al.*, 2003; Schechter *et al.*, 2003; Wilson *et al.*, 2001). Among these is the two-component regulator BarA/SirA. BarA is a sensor kinase of the phosphorelay type, and SirA is its cognate response regulator, with both being required for invasion gene expression and bacterial penetration of epithelial cells (Ahmer *et al.*, 1999; Altier *et al.*, 2000b; Johnston *et al.*, 1996; Teplitski *et al.*, 2003). SirA induces invasion through induction of *hilA* and *hilC* and by its control of *csrB* and *csrC* (Fortune *et al.*, 2006; Teplitski *et al.*, 2003). The latter two encode untranslated RNAs that oppose the action of the post-transcriptional regulator CsrA, also known to affect invasion (Altier *et al.*, 2000a). Thus, SirA regulates SPI1 genes both directly, through induction of *hilA* and *hilC*, and indirectly, by its control of the csr regulatory system.

The regulation of invasion genes also requires a coordinated response to the varied environmental signals present in the gastrointestinal tract. Environmental conditions control the induction of SPI1 genes through HilA (Bajaj *et al.*, 1996), and include pH, oxygen tension, osmolarity, growth phase, and bile (Bajaj *et al.*, 1996; Galan and Curtiss, 1990; Lee and Falkow, 1990; Lee *et al.*, 1992; Lundberg *et al.*, 1999; Prouty and Gunn, 2000; Prouty *et al.*, 2004). In addition, short chain fatty acids (SCFAs) appear to play an important role in regulating invasion genes. We have previously shown that acetate can induce invasion in a *barA* mutant, but

requires *sirA* to do so. This effect also requires *ackA-pta*, which encodes acetate kinase and phosphate acetyltransferase, and mediates the interconversion of acetate to acetyl-phosphate and acetyl-CoA (Figure 8), suggesting that one of these products is required for the BarA-independent induction of SirA (Lawhon *et al.*, 2002). The mammalian gastrointestinal tract contains high levels of SCFAs, including formate, acetate, propionate, and butyrate, as the result of the breakdown of food by digestive processes and the action of resident intestinal bacteria. SCFA types and concentrations vary through the gastrointestinal tract. Levels in the small intestine, the site of *Salmonella* invasion, are between 20 to 40 mM total SCFA, while levels in the colon range above 100 mM, depending on animal species and diet (Argenzio *et al.*, 1974; Argenzio and Southworth, 1975; Bohnhoff *et al.*, 1964; Cummings *et al.*, 1987; Macfarlane *et al.*, 1992). Also varying in the gastrointestinal tract is the proportion and the distribution of these SCFA. Studies in animals have shown that acetate and formate predominate in the distal small intestine, but that propionate and butyrate are in higher concentrations in the cecum and colon (Argenzio and Southworth, 1975; Laerke *et al.*, 2000). It has been demonstrated that while acetate induces invasion genes, propionate and butyrate can repress them (Durant *et al.*, 2000; Gantois *et al.*, 2006; Lawhon *et al.*, 2002), suggesting that these SCFAs provide environmental cues that allow *Salmonella* to recognize specific regions of the intestinal tract.

Although both environmental signals and genetic regulators of *Salmonella* invasion are known, in most cases the means by which the environment signals

Salmonella to invade have not been elucidated. SirA is required for both repression by bile and activation by acetate (Lawhon *et al.*, 2002; Prouty and Gunn, 2000), but the pathways for responses to others signals have not been defined. Here we describe a novel means by which invasion genes are induced by formate, a common constituent of the mammalian small intestine. We show that formate produced by *Salmonella* grown in laboratory media acts as a diffusible signal that induces invasion. This effect is independent of the known induction pathway utilizing BarA/SirA and the csr regulatory system, and requires that formate enter the bacterial cytoplasm to have its effect. We also provide genetic evidence that formate itself is not the direct signal for invasion but instead must be metabolized to produce constituents of central carbon metabolism to have its effect.

Results

Invasion is induced by a diffusible signal that requires *ackA-pta* for its production. In previous work, we showed that either the sensor kinase BarA or acetate was sufficient to induce *Salmonella* virulence in the mouse model of septicemia, and that the *ackA-pta* operon, encoding acetate kinase and phosphate acetyltransferase, was required for acetate to act as an inducing signal for invasion. In laboratory medium, however, invasion genes were poorly expressed in the *ackA-pta* mutant, even in the presence of intact *barA* (Lawhon *et al.*, 2002). These contradictory results, that *ackA-pta* was redundant *in vivo*, but required *in vitro*, led us to reason that a diffusible signal present in the intestinal tract, other than acetate, was necessary for the induction of invasion genes, but that an *ackA-pta* mutant

failed to express invasion genes when grown in laboratory media because it was unable to produce that signal. To test this, we examined the effect of culture supernatants obtained from the wild type, a *sirA* mutant, and an *ackA-pta* mutant on the expression of a β-galactosidase fusion to *sipC*, a SPI1 invasion gene that encodes a secreted effector protein and is under the control of known SPI1 regulators (Figure1). In the *ackA-pta* mutant, expression of *sipC* was approximately 9-fold less than that of the wild type when grown without the addition of conditioned media, but was completely restored to the level of the wild type by media derived from either wild type or *sirA* mutant cultures. Media produced by growth of the *ackA-pta* mutant itself, however, failed to significantly increase *sipC* expression by the *ackA-pta* mutant. In a *barA* mutant, *sipC* expression was reduced approximately 11-fold and was not significantly induced by conditioned media from any of the strains, while expression in the wild type was unchanged by the addition of media conditioned by growth of any of the three strains. Thus, these results indicate that induction of SPI1 invasion genes requires a signaling molecule that is secreted into the culture medium, and that acetate kinase and/or phosphotransacetylase is necessary for the production of the signal. They also show that *barA*, encoding the sensor kinase of the BarA/SirA two-component regulator, is required for invasion gene expression even in the presence of this signal, but that this two-component regulator is not necessary for signal production.

Formate induces invasion. Mutants of *ackA* and *pta* have been extensively studied in *E. coli*, as they are commonly used to optimize culture conditions in the

industrial production of recombinant proteins. It is known that in *E. coli*, *ackA-pta* mutants fail to produce three metabolic products that are normally excreted into the culture media: acetate, ethanol, and formate, the latter two during anaerobic growth (Yang *et al.*, 1999). We reasoned that a normally excreted product that is not produced by an *ackA-pta* mutant might be the signal for invasion. When tested, ethanol had no effect on *sipC* expression (data not shown), and acetate had been shown previously not to restore invasion in an *ackA-pta* mutant (Lawhon *et al.*, 2002). We therefore next considered formate. To ensure that the loss of *ackA* and *pta* in *Salmonella* resulted in the reduced excretion of formate into the culture medium, as it does in *E. coli*, we measured the concentration of formate in culture supernatants using an enzymatic assay. After overnight growth of the wild type strain under conditions identical to those used for the conditioned media experiments shown in Figure 1 (minimal medium supplemented with glucose and amino acids), the formate concentration in the culture supernatant was 8.8 ± 0.23 mM. The *ackA-pta* mutant produced 0.13 ± 0.08 mM formate, a reduction of 68-fold. The addition of 10 mM sodium formate to the medium prior to growth of the *ackA-pta* mutant restored the formate concentration in the supernatant to 7.6 ± 0.43 mM. This showed that some portion of the added formate was metabolized during bacterial growth, but that the addition of sodium formate at this concentration restored the medium concentration of formate to a level near that produced by the wild type.

We next tested the effects of formate on invasion gene expression. For these and all subsequent experiments, we used pH-buffered LB as the growth medium, as

it maintained stable culture pH after overnight growth. We found that the addition of 10 mM sodium formate to an *ackA-pta* mutant restored *sipC* expression to a level greater than that of the wild type strain (Figure 2A). This same concentration of sodium formate, however, had no significant effect in strains that carried null mutations of either *barA* or *sirA*. Similar results were obtained upon the addition of formic acid (data not shown), and the addition of either sodium formate or formic acid did not alter the pH of the culture medium. To determine the concentration of formate required to induce invasion genes, we grew the *ackA-pta* mutant in media containing increasing amounts of sodium formate (Figure 2B). All of the concentrations tested, at 1 mM or greater, significantly induced *sipC* expression to the level of the wild type or higher.

We next directly tested the effect of formate on invasion using cultured epithelial cells (Figure 3). The *ackA-pta* mutant was reduced in its invasion of HEp-2 cells by 2.5-fold compared to the wild type. Similar to the findings for SPI1 gene expression, the addition of 10 mM sodium formate, however, restored invasion 3-fold, to a level comparable to that of the wild type. To assess the importance of BarA/SirA to invasion induced by formate, we tested isogenic *ackA-pta* mutants that also lacked either *sirA* or *barA*. The loss of *sirA* in the *ackA-pta* mutant reduced invasion by 33-fold, and the addition of formate failed to significantly improve invasion. Similarly, the *barA* null mutation in the same strain background reduced invasion by 12-fold without a significant increase in invasion with added formate. These results therefore show that formate, or some product of formate, is the signal

required for induction of invasion, but that the BarA/SirA two-component regulator remains essential for invasion, even in the presence of formate.

pH affects the signaling activity of formate. As the intestinal tract of animals, the site of *Salmonella* invasion, has a pH near or slightly below neutrality, we sought to determine whether the pH of the medium affected the ability of formate to induce invasion. It is also known that weak acids such as formate diffuse into the bacterial cytoplasm more readily at lower pH and so achieve higher concentrations within bacteria. We therefore tested *sipC* expression in *Salmonella* strains grown at either pH 6.7, the approximate pH of the mammalian ileum, or pH 8.0 (Figure 4). At pH 6.7, expression in the *ackA-pta* mutant was 4.5-fold less than that of the wild type, but the addition of 10 mM formate induced *sipC* expression to the level of the wild type without the addition of exogenous formate. Formate also increased expression in the wild type to a lesser degree, approximately 67%. At pH 8, expression in the *ackA-pta* mutant was nearly 9-fold less than in the wild type. At this pH, however, formate did not significantly increase *sipC* expression in either strain. Thus, formate did not affect SPI1 expression under alkaline conditions, but instead required a lower pH, similar to that found within the intestinal tract, suggesting that formate must enter the bacterial cytoplasm to have its effect. To confirm that the pH and formate concentrations tested reflect those found in the intestinal tracts of animals used as models of *Salmonella* infection, we measured these values in mice. We found the pH of the distal ileum of conventionally-raised C57BL/6 mice to be 6.80 ± 0.35. Although the level of formate varied among the mice tested, it was detected in this

same region at a concentration of 8.4 mM ± 6.7 mM, with the lowest concentration detected in any mouse being 3.3 mM. All of these concentrations were therefore well above that required to induce invasion gene expression *in vitro* (Figure 2B). In contrast, we could detect no formate in the cecum of these mice (with a limit of detection of less than 0.08 mM), showing that formate is, indeed, present in the distal ileum in a concentration capable of inducing invasion, but is absent or in very low concentration in the adjacent cecum.

Formate signals independently of BarA/SirA. One possible model for the action of formate is that it induces invasion by activating the sensor kinase BarA. This model is consistent with the genetic requirement for *barA* and *sirA*, but not with the proposed role of formate as a cytoplasmic signal, as suggested by its dependence upon pH. It is known that BarA/SirA induces the small untranslated RNAs *csrB* and *csrC*, which subsequently control invasion (Fortune *et al.*, 2006; Lawhon *et al.*, 2002; Teplitski *et al.*, 2003). We therefore reasoned that the activation of BarA/SirA by formate would lead to the increased expression of these small RNAs. To test this model, we examined the effects of formate on the expression of *csrB* using a *lacZ* fusion (Figure 5). We found, as expected, that the loss of *barA* reduced the expression of *csrB* by 4-fold. However, the *ackA-pta* mutant did not have reduced *csrB* expression, nor did the addition of 10 mM sodium formate alter its expression in this mutant strain. These results therefore show that formate does not function by activating BarA/SirA and that this two-component regulator plays an independent but required role in the induction of invasion.

Oxidation of formate is not required for signaling. *Salmonella* produces formate as a by-product of anaerobic growth. Formate can then be used to maintain redox balance during anaerobic respiration by acting as an electron donor for nitrite, as well as a number of other substrates of anaerobic respiration, and can donate electrons to the quinone pool (Figure 8). These major routes of formate utilization alternatively employ the formate dehydrogenase O (Fdh-O) system, encoded by *fdoGHI*, or the Fdh-N system, encoded by *fdnGHI*. To determine whether products of formate oxidation, rather than the molecule itself, were signals for activation of invasion, we examined mutants of these known formate degradation pathways for their effects on invasion gene expression. We found that loss of either pathway, through mutation of either *fdoG* or *fdnG*, did not change the expression of *sipC* (Figure 6). The loss of both mechanisms for the production of reduced metabolic intermediates, using an *fdoG*, *fdnG* double mutant, also failed to alter expression. Alternatively, *Salmonella* can excrete formate, but under the low pH conditions induced by fermentation, formate can re-enter the bacterium, and there it can be used to stabilize the internal pH by its conversion to CO_2 and H_2. This reaction requires a membrane-bound formate hydrogen lyase complex, an essential component of which is formate dehydrogenase H (Fdh-H), encoded by *fdhF*. We tested a null mutant of *fdhF* and found it to have a small increase (approximately 63%) in the expression of *sipC* (Figure 6.). Thus, as Fdh-H is required for production of molecular hydrogen from formate, H_2 cannot be the signal for invasion. It has also been reported that in *E. coli* the Fdh-H system, which normally produces

H_2 from formate, can play a significant role in the reduction of nitrite by formate (Darwin *et al.*, 1993). We therefore eliminated all of the known routes by which formate can reduce metabolic intermediates by constructing and testing a *fdoG*, *fdnG*, *fdhF* triple mutant, and found that it did not differ from the wild type in its effects on the expression of *sipC* (Figure 6). These results thus show that the metabolic products of the known pathways of formate oxidation are not required to induce invasion.

Formate signals through central carbon metabolism. In *Salmonella*, the major source of formate production is from pyruvate by the action of pyruvate formate-lyase, encoded by *pflB*, the products of which are formate and acetyl-CoA (Figure 8). To determine whether endogenous formate production was required for *Salmonella* invasion gene expression, we tested a null mutant of *pflB*. We first determined the amount of formate produced by this mutant. Unlike growth in minimal medium with glucose as the carbon source, strains grown in pH-buffered LB did not excrete measurable quantities of formate into the culture media, presumably due to the lack of glucose as a primary source of formate production. The addition of 10 mM formate to the wild type strain, however, resulted in 6.8 ± 1.4 mM formate in the medium after overnight growth, while the *pflB* mutant had no detectable formate remaining in the medium, suggesting that exogenous formate was more completely metabolized due to a reduction of endogenous production. Surprisingly, however, when tested for its effects on invasion, we found that in the *pflB* mutant the expression of *sipC* was not reduced, but instead was significantly increased by 3-

fold (Figure 7A). Pyruvate formate-lyase (Pfl) is known to function bidirectionally (Kessler and Knappe, 1996; Knappe *et al.*, 1974), and thus pyruvate can be produced by this enzyme in the presence of a high concentration of formate. Based upon these findings, we hypothesized that exogenously supplied formate might affect invasion by its conversion to pyruvate through *pflB*, and thus that pyruvate or some product of pyruvate constituted the signal for invasion. To test this model directly, we added 50 mM sodium pyruvate to the growth medium and assessed changes in *sipC* expression (Figure 7B). Indeed, we found that the addition of pyruvate increased expression 2.4-fold in the wild type strain. The *pflB* mutant had intrinsically higher expression in the absence of exogenous pyruvate, comparable to that of the wild type grown in the presence of pyruvate, and did not show increased expression with the addition of pyruvate. These findings suggest that an increased level of pyruvate plays an important role in this response and that formate acts only indirectly in inducing invasion.

The second major pathway for pyruvate metabolism in *Salmonella* is through pyruvate dehydrogenase, producing acetyl-CoA and CO_2, an essential component of which is encode by *aceE* (Figure 8). To determine whether pyruvate itself or a metabolic product of pyruvate was required to control invasion, we next tested the effects of an *aceE* mutation. We found that expression of *sipC* in an *aceE* mutant was reduced 3-fold compared to that of the wild type (Figure 7A and 7B), indicating a role for the conversion of pyruvate to acetyl-CoA by this pathway. The eventual fate of the acetyl-CoA produced by pyruvate dehydrogenase is entry into the citric

acid cycle by combining with oxaloacetate to produce citrate through the action of citrate synthase, the product of *gltA* (Figure 8). To determine whether this conversion of acetyl-CoA was required to induce invasion gene expression, we next tested a *gltA* mutant and found that the loss of *gltA* had no effect on *sipC* expression, nor was expression altered in this mutant by the addition of pyruvate (Figure 7B). These results, taken together, therefore show that the conversion of pyruvate to acetyl-CoA by pyruvate dehydrogenase is necessary to affect invasion genes, but that the further metabolism of acetyl-CoA is not required.

These results suggested that the levels of intermediates of carbon metabolism were important to the expression of invasion genes. Although *aceE* and *pflB* are central to these metabolic processes, other routes of metabolism exist. To determine whether these pathways were sufficient, as well as necessary, for the induction of invasion, we further examined the effects of formate and pyruvate on *sipC* expression in mutants of these genes. We found that both formate and pyruvate significantly increased expression in the *aceE* mutant (Figure 7A and 7B), and that formate increased expression in the *pflB* mutant (Figure 7A), showing that pyruvate dehydrogenase and pyruvate formate-lyase do not provide the sole source of metabolites required to induce invasion. We additionally tested one other known route of pyruvate metabolism. In *Salmonella*, there also exists a homologue of the *E. coli tdcE*, which shares strong homology with *pflB* in that organism and has been shown to possess formate pyruvate-lyase activity (Hesslinger *et al.*, 1998). We found that the mutation of *tdcE* alone had no effect on *sipC* expression, both with

and without the addition of formate to the culture medium, and a *pflB*, *tdcE* double mutant had a phenotype identical to that of the *pflB* mutant alone (data not show), indicating that the interconversion of pyruvate and formate through PflB is the major means by which invasion gene expression is affected, and that *tdcE* has no significant role in these effects.

Discussion

To penetrate the intestinal epithelium of an animal host, *Salmonella* secretes effector proteins by means of the type III secretion apparatus of SPI1. The expression of SPI1 invasion genes must be appropriately timed, and *Salmonella* relies upon environmental signals from the intestinal tract to induce these genes. We show here that formate is likely to be such a signal. Our results show that formate can act as a diffusible signal for invasion when *Salmonella* is grown under laboratory conditions. This mechanism of self-induction, however, is almost certainly not required for invasion when *Salmonella* resides in the intestinal tract of an animal. Indeed, our previous results using the mouse model of septicemia showed that an *ackA-pta* mutant deficient in the production of formate maintained its virulence (Lawhon *et al.*, 2002). Instead, *Salmonella* relies upon the formate produced by the resident microbiota of the intestine as a signal for invasion. For this purpose, formate is particularly well suited, as it is produced in high concentration in the mammalian intestinal tract. Importantly, work presented here and previous animal studies have shown formate to be present in measurable concentration in the distal ileum, but absent or in lower concentration both in more proximal portions of the

intestine and more distal, such as the cecum and colon (Laerke and Jensen, 1999; Laerke *et al.*, 2000). The distal ileum is the preferred site for *Salmonella* colonization and penetration in animal models of enteritis and septicemia (Carter and Collins, 1974; Jones *et al.*, 1994; Penheiter *et al.*, 1997; Tsolis *et al.*, 1999). Thus, formate provides a plausible signal for the induction of invasion determinants at the site most appropriate for productive infection, and signaling by formate can explain, at least in part, the tropism of this pathogen for infection of this region of the intestine.

One finding presented in this work, that formate has its effects only at below-neutral pH, indicates that formate functions as a cytoplasmic signal. When the pH of the surrounding medium is lower than that of the bacterial cytoplasm, exogenous formic acid, a weak acid, can concentrate within bacteria. At equilibrium, the concentration of formate in the cytoplasm is dependent upon the difference between cytoplasmic and external pH, and by the external concentration of formate. An external pH of 6.7 with 10 mM formate, as tested here, should allow a cytoplasmic concentration of formate approximately 20-fold greater than that produced at pH 8. Our work has shown that this lower pH is necessary for the activity of formate and thus reflects a requirement for high cytoplasmic formate concentrations.

Formate plays important roles in both anaerobic respiration and fermentation, and thus much is known about its metabolism. Although it can be degraded to produce both hydrogen and reduced metabolic intermediates, neither of these products appears to be required for the capacity of formate to signal the induction of

invasion. Instead, once within the bacterial cytoplasm, genetic evidence presented here suggests that formate enters central carbon metabolism to affect invasion (Figure 8). The regulation of carbon metabolism is complex and interconnected, but one plausible model for the action of formate is that it shifts the metabolism of pyruvate toward oxidative decarboxylation by pyruvate dehydrogenase and away from that by pyruvate formate-lyase. We have shown that invasion gene expression is elevated in a mutant that cannot make formate due to the loss of pyruvate formate-lyase (Pfl). As this enzyme converts pyruvate to acetyl-CoA and formate, it is likely that this induction of invasion is due to an increase in pyruvate concentration in the mutant strain. It is known that Pfl is a bidirectional enzyme (Kessler and Knappe, 1996; Knappe *et al.*, 1974), and so likely can produce pyruvate when supplied with a high concentration of formate. In support of this, the addition of pyruvate itself caused induction in the wild type strain. We have shown further that the loss of the alternative pathway for pyruvate degradation, through pyruvate dehydrogenase (a component of which is encoded by *aceE*), reduced invasion gene expression. The product of pyruvate dehydrogenase is acetyl-CoA, the central molecule of carbon metabolism, and also the product of pyruvate formate-lyase. The by-products of acetyl-CoA production, however, differ in these two enzymatic pathways. Decarboxylation of pyruvate to acetyl-CoA via pyruvate dehydrogenase produces NADH from NAD^+, while with pyruvate formate-lyase, electrons remain with the carboxyl group to create formate, and thus no NADH is formed. It is clear that in *E. coli* the cytoplasmic NADH/NAD+ ratio is used by the bacteria as an

indicator of the redox state and can alter gene expression. A number of enzymes used in carbon metabolism are controlled by the NADH/NAD+ ratio, and this regulation is thought to be achieved through the ArcA/ArcB two-component regulator (Vemuri *et al.*, 2006). ArcA/ArcB itself is known to be activated by changes in redox state (Georgellis *et al.*, 2001; Malpica *et al.*, 2004). Similarly, the PhoP/PhoQ two-component regulator, which in *Salmonella* controls virulence functions, is also affected by changes in acetyl-CoA levels (Lesley and Waldburger, 2003). Thus, the fate of acetyl-CoA and corresponding changes in redox state may provide a comparable means of signaling for the induction of invasion by formate. Such a model of the re-direction of acetyl-CoA metabolism requires the simultaneous operation of pyruvate formate-lyase and pyruvate dehydrogenase, the former of which is thought to function primarily under anaerobic conditions and the latter aerobically. It is known, however, that under microaerobic conditions both of these enzymes are functional, and so both pathways of pyruvate metabolism are available (Alexeeva *et al.*, 2000; Zhu *et al.*, 2007). The small intestine is microaerobic, with an oxygen tension of 30-45 torr (Dawson *et al.*, 1965). This environment therefore likely allows the metabolism of pyruvate by both of these routes, and thus the action of formate in invasion gene induction. It has also long been known that *Salmonella* grown as static cultures produce maximal induction of SPI1; it is thus likely that induction of invasion in laboratory media requires the production of formate and its metabolism by these two pathways.

Although we detected formate in the ileum of mice, its genesis at that site is largely unknown. Presumably, it is produced by the resident microbiota of the small intestine. Recently, there have been great advances in characterizing the microbiota of the large intestine of humans and animals (Gill *et al.*, 2006; Ley *et al.*, 2005; Turnbaugh *et al.*, 2006). The microbiota of the ileum, however, has not been well described. There is evidence as well that the generation of formate depends upon the complex ecology of the intestinal tract. It has been shown that in the large intestine the archaeon *Methanobrevibacter smithii* induces formate production by the bacterium *Bacteroides thetaiotaomicron*, and then uses that formate as a nutrient source (Samuel and Gordon, 2006). Studies have also shown that alteration of the diet of pigs can affect the ileal concentration of formate (Laerke *et al.*, 2000). These findings, along with the fact that very low concentrations of formate are present in the adjacent cecum, suggest that the ileum possesses a microbial ecology that is uniquely suited to the production of this fatty acid.

Induction of *Salmonella* invasion by formate is one of but several means by which this bacterium senses the environment of the intestine and responds by altering its virulence characteristics. As *Salmonella* passes through the intestinal tract during the course of infection, it encounters changing environments. In the proximal small intestine, for example, bile is secreted into the intestinal lumen. Bile has been shown to repress invasion, working at or above the level of BarA/SirA (Prouty and Gunn, 2000), and thus preventing invasion at a site at which it would be unproductive. As the bacteria reach the ileum, the most distal portion of the small

intestine, they encounter increased concentrations of formate and the short chain fatty acid acetate (Argenzio *et al.*, 1974; Argenzio and Southworth, 1975; Cummings *et al.*, 1987; Laerke and Jensen, 1999; Laerke *et al.*, 2000). Both of these constituents induce invasion, formate by entering into central carbon metabolism and acetate through a mechanism that requires SirA but not BarA (Lawhon *et al.*, 2002). Our work suggests that these two environmental cues provide redundant signaling mechanisms, either of which is sufficient to cause disease (Lawhon *et al.*, 2002). Yet to be discovered, however, is the signal for BarA, as none of the environmental conditions known have been shown to activate invasion through this sensor kinase. As *Salmonella* leaves the small intestine and enters the large intestine, it encounters higher concentrations of two other short chain fatty acids, propionate and butyrate, produced by the anaerobic microbiota (Argenzio *et al.*, 1974; Argenzio and Southworth, 1975; Cummings *et al.*, 1987; Macfarlane *et al.*, 1992). Both of these repress invasion, acting by an uncharacterized mechanism, again preventing the unproductive expression of invasion determinants (Gantois *et al.*, 2006; Lawhon *et al.*, 2002). Thus, *Salmonella* uses products of both its animal host and the intestinal microbiota to sense specific regions of the intestine and to promote its virulence.

Experimental procedures

Bacterial strains. Strains used in this study are shown in Table 1. Precise deletions for all strains described in this study were made using a one-step inactivation method (Datsenko and Wanner, 2000). PCR primers were designed to

allow the amplification of the kanamycin resistance marker from plasmid pKD4 or the chloramphenicol resistance marker from pKD3. Each primer had at its 5' end 40 bases of homology to the regions immediately flanking the initiation or termination codon of the gene to be deleted, linked to 3' sequence homologous to the plasmid to be amplified. The resulting PCR products were used to transform strain ATCC14028s carrying pKD46, which encodes λ Red recombinase that provides for allelic exchange. Candidate mutants were tested for loss of the appropriate region by PCR amplification. The marked disruptions were then moved into ATCC14028s carrying the *sipC::lacZY* fusion by P22 transduction. To create unmarked mutants when strains with multiple mutations were required, resistance markers were excised using the FLP recombinase (Datsenko and Wanner, 2000). The *csrB::lacZ* fusion was similarly created by first deleting *csrB*, and then integrating *lacZ* at the site of the disruption as described (Ellermeier *et al.*, 2002).

Conditioned Media Assays. Conditioned media was made by growing wild type, *sirA* mutant, or *ackA-pta* mutant strains standing overnight in 1X MOPS minimal medium (Neidhardt *et al.*, 1974) with 0.5% glucose and a combination of 18 amino acids in concentrations equivalent to 0.5% tryptone (Difco), then filter-sterilizing the media. Wild type, *barA*, and *ackA-pta* mutants with the *sipC::lacZY* fusion were grown standing overnight in 50% 2X MOPS minimal medium with 1% glucose and 50% of either fresh or conditioned media. Fresh media consisted of 1X MOPS minimal with 0.5% glucose and amino acids.

β-galactosidase Assays. For β-galactosidase assays used to assess the effects of formate, bacteria were grown as standing overnight cultures in LB with the addition of 100 mM MOPS pH 6.7. Sodium formate was added at a concentration of 10 mM, while control cultures received 10 mM NaCl. To assess the effects of pyruvate, sodium pyruvate or NaCl were added at 50 mM. Triplicate cultures of each bacterial strain were used unless otherwise noted. For assays to assess the role of pH, strains were grown standing overnight in LB with either 100 mM HEPES pH 8.0 or 100 mM MOPS pH 6.7. All cultures were assayed for β-galactosidase activity as described previously (Miller, 1992).

Invasion assays. For invasion assays, bacteria were grown overnight as standing cultures in LB broth buffered with 100 mM MOPS pH 6.7 and with either 10 mM sodium chloride or 10 mM sodium formate. Bacteria were added to HEp-2 cells grown to confluence in 24 well plates for a multiplicity of infection of approximately 10 bacteria/cell. Plates were incubated for 1 h at 37°C, medium was removed, the cells were washed three times with phosphate-buffered saline, and the medium was replaced by medium supplemented with gentamicin (20 μg/ml). Cells were incubated for an additional hour, the medium was removed, and monolayers were washed three times with phosphate-buffered saline. The cells were lysed with 1% Triton X-100 for 5 min, and the bacterial titers of the lysates were determined by colony counts. Each strain was tested in quadruplicate for each condition.

Determination of Formate Concentrations. Formate concentrations in media were measured using a formic acid enzymatic bioanalysis detection kit, following the

manufacturer's directions (r-biopharm). This method measured formate concentration by the enzymatic conversion of formate and NAD^+ to bicarbonate and NADH through the action of formate dehydrogenase. NADH concentration was then measure by UV light absorbance at a wavelength of 340 nm.

Animal experiments. Three seven-week-old female C57BL/6 mice were used to determine the pH and the formate concentrations of the intestinal tract. To measure pH, the ileum of the mice was transected at the junction of the cecum immediately after euthanasia, and an Orion Micro Combination pH Electrode (Thermo Electron Corp., Beverly, MA) was inserted retrograde ~2 mm into the distal ileum. To determine formate concentrations, cecal and ileal samples were acidified using a 1% solution of H_2SO_4, flash frozen in liquid nitrogen, and stored at -80° C. Before analysis, samples were thawed and the mass of each sample was recorded. Samples were analyzed by HPLC as previously described (Siegfried *et al.*, 1984), using crotonic acid as the internal standard. Volatile fatty acids were separated using a Supelcogel H (carbohydrate) column (250 x 4.6 mm with 9 µm particles; Supelco; Bellefonte, PA) with a guard column (Supelcogel H; Supelco). Twenty µl of sample was injected and a flow rate of 0.17 ml/min was used to adjust for the narrower bore of the Supelcogel H column. All samples were run for 70 minutes at 39°C using a mobile phase of 0.015 N sulfuric acid and 0.25 mM EDTA. A Beckman System Gold (Beckman Coulter; Fullerton, CA) was used with the UV detector set at 210 nm, and results were analyzed using a VFA standard and the 32 Karat Software (Beckman Coulter).

Statistical Analysis. For β-galactosidase and invasion assays, a one-way analysis of variance was used to determine whether the mean of at least one strain differed from that of any of the others. Then, multiple comparison tests were used to determine which means differed at $p \leq 0.05$ (The SAS System for Windows 8 and MINITAB Release 14).

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Table 1. Strains used in this study.

Genotype	Resistance	Source or reference
Wild type; ATCC 14028s	None	American Type Culture Collection
sipC::lacZY	Tet	(Bajaj *et al.*, 1996)
Δ*barA*	None	(Altier *et al.*, 2000b)
sirA::cam	Cam	(Altier *et al.*, 2000b)
Δ*(ackA-pta)*	Kan	(Lawhon *et al.*, 2002)
Δ*fdoG*	Kan	This study
Δ*fdnG*	Kan	This study
Δ*fdhF*	Kan	This study
csrB::lacZ	Cam	This study
Δ*pflB*	Kan	This study
Δ*aceE*	Cam	This study
Δ*gltA*	Cam	This study
Δ*tdcE*	Cam	This study

Figure Legends

Figure 1. Effects of conditioned media on *sipC* expression. Wild type, *barA*, and *ackA-pta* mutants with a *sipC::lacZY* fusion were grown standing overnight in 50% 2X MOPS minimal medium with 1% glucose and 50% of either fresh or conditioned media. Fresh media (grey bars) consisted of 1X MOPS minimal medium with 0.5% glucose and amino acids. Conditioned media was made by growing the wild type (striped bars), *sirA* mutant (white bars), or *ackA-pta* mutant (black bars) standing overnight in this same media, then filter-sterilizing the culture supernatant. Expression of *sipC* was assessed using β-galactosidase assays. Values represent the mean for each condition tested in triplicate. Asterisks (*) show a significant difference ($p \leq 0.05$) for the strain with the genotype shown when grown with the addition of conditioned media as compared to the fresh media control. Error bars show standard deviation.

Figure 2. Effects of formate on *sipC* expression. (A.) Strains were grown as standing overnight cultures in LB with 100 mM MOPS pH 6.7 and with 10 mM sodium chloride (grey bars) or 10 mM sodium formate (black bars). Triplicate cultures of each strain were assayed for *sipC::lacZ* expression by β-galactosidase assays. (B.) Overnight standing cultures of the wild type (grey bars) and *ackA-pta* mutant (black bars) with the *sipC::lacZ* fusion were grown in LB with 100 mM MOPS pH 6.7 and with 0, 1, 5, 10, 20 or 30 mM sodium formate. Triplicate cultures for

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Figure 3. Invasion of epithelial cells. Bacteria were grown overnight as standing cultures in LB broth buffered with 100 mM MOPS pH 6.7 and with either 10 mM sodium chloride (grey bars) or 10 mM sodium formate (black bars). Bacteria were added to HEp-2 cells and invasion was assessed using a gentamicin protection assay. Invasion is shown as compared to the wild type strain set to 100. Each strain and condition was tested in quadruplicate. Single asterisks (*) show a significant difference ($p \leq 0.05$) for the strain with the genotype shown when grown with the addition of formate as compared to the NaCl control. Double asterisks (**) show a significant difference for the mutant strain compared to the wild type when both were grown without formate. Error bars show standard deviation.

Figure 4. Effects of media pH on *sipC* expression in response to formate. Wild type and *ackA-pta* mutants with a *sipC::lacZY* fusion were grown standing overnight in LB with 100 mM HEPES pH 8.0 or 100 mM MOPS pH 6.7, as indicated, and with either 10 mM sodium chloride (grey bars) or 10 mM sodium formate (black bars). Expression of *sipC* was assessed using β-galactosidase assays. Values represent

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Figure 5. Expression of *csrB* in response to formate. Strains carrying a *csrB::lacZ* fusion and the designated mutation were grown in LB with 100 mM MOPS pH 6.7 and with either 10 mM NaCl (grey bars) or 10 mM sodium formate (black bars). Values represent the mean for each condition tested in triplicate. Error bars show standard deviation.

Figure 6. Effects of formate metabolism on *sipC* expression. Strains with the genotype shown and with the *sipC::lacZ* fusion were grown as standing overnight cultures in LB with 100 mM MOPS pH 6.7. Triplicate cultures of each strain were assayed for *sipC::lacZ* expression by β-galactosidase assays. Double asterisks (**) show a significant difference for the mutant strain when compared to the wild type. Error bars show standard deviation.

Figure 7. Effects of central carbon metabolism pathways on invasion gene expression in response to formate and pyruvate. Strains were grown as standing overnight cultures in LB with 100 mM MOPS pH 6.7 and with (A.) 10 mM

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Figure 8. Pathways for the integration of formate into central carbon metabolism.

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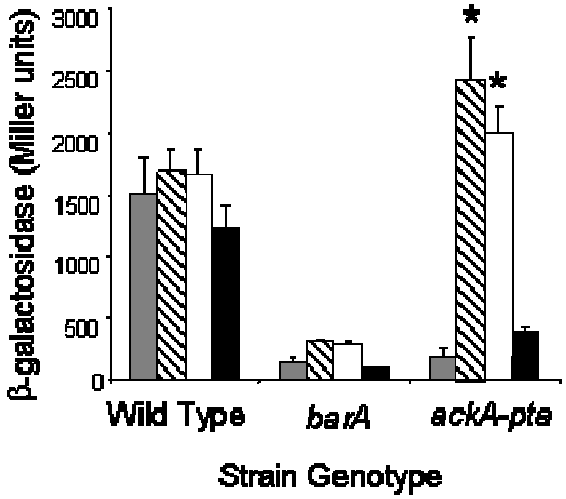

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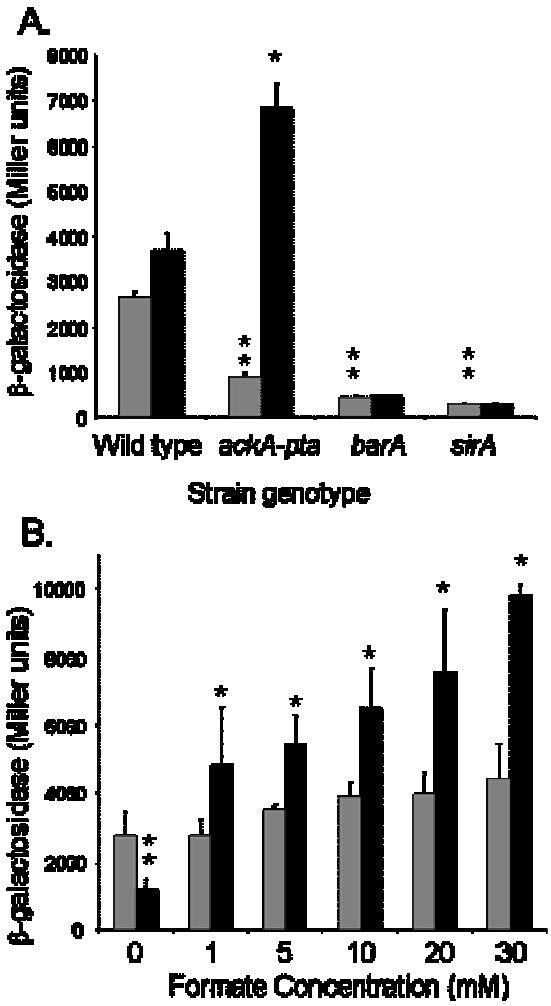

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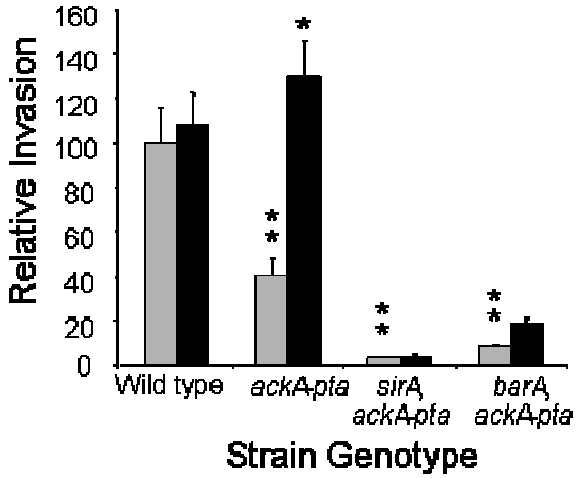

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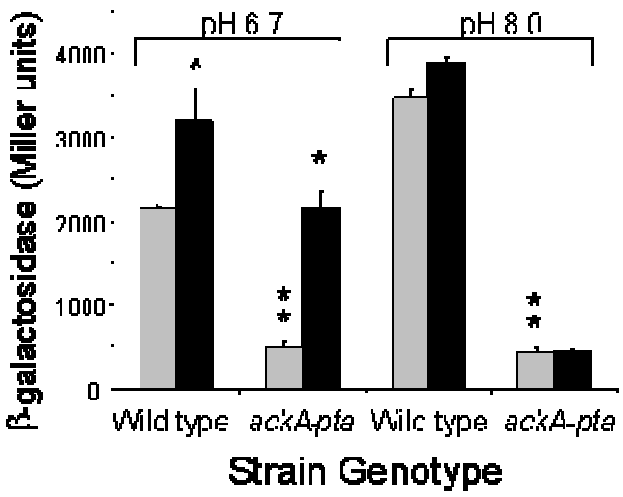

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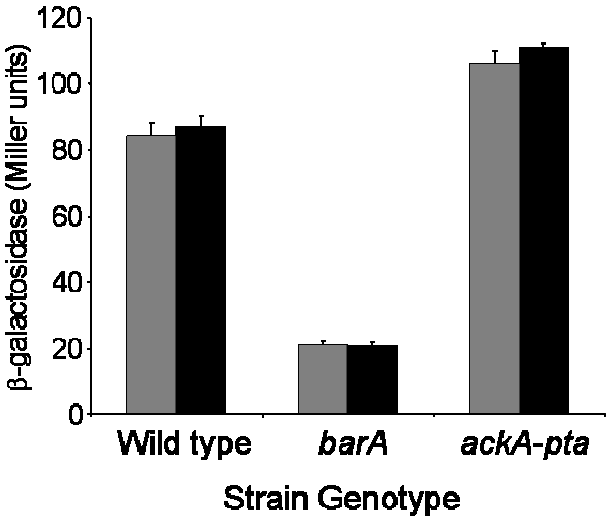

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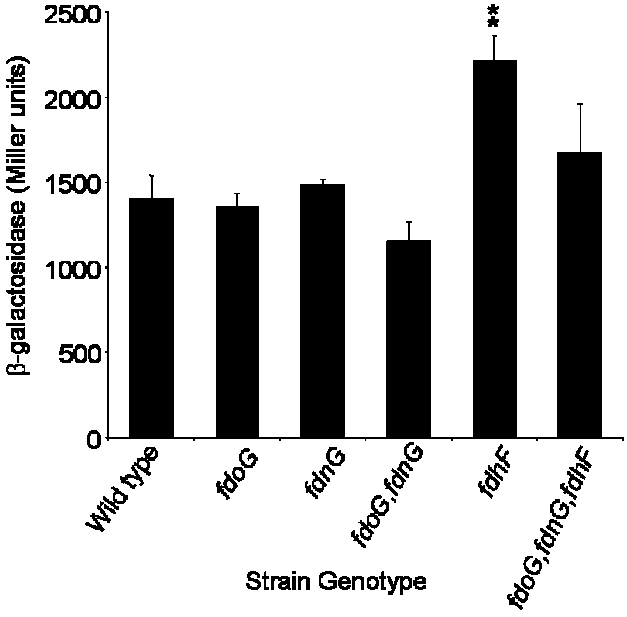

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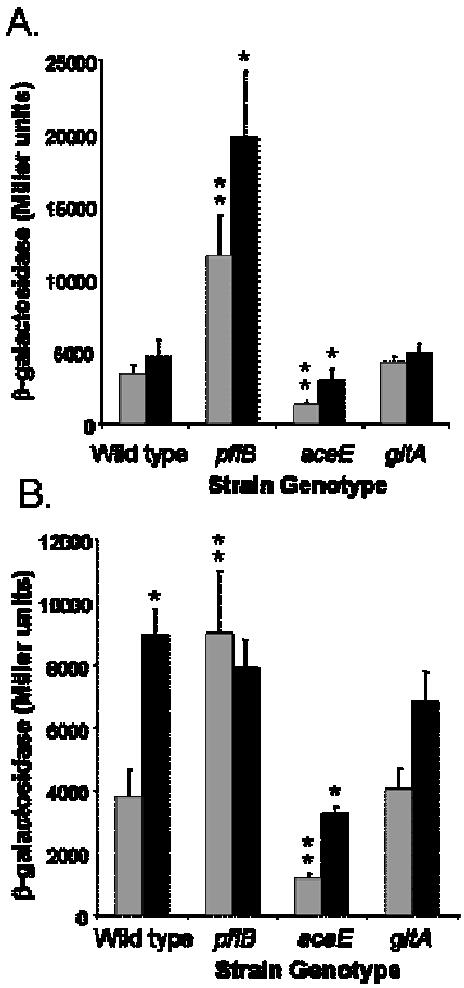

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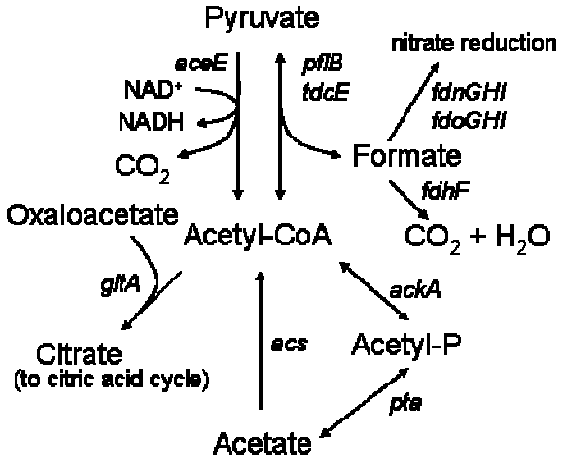

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Global gene regulation by formate in *Salmonella enterica* serovar Typhimurium

Summary

Formate is an important molecule since it is the major product of enterobacterial fermentation and can work as an electron donor or acceptor in the electron transfer system. The present study identified the formate regulon of *Salmonella* using a *Salmonella* DNA microarray. This global genomic analysis compared the transcriptional profile of wild type *Salmonella* grown with sodium formate or with sodium chloride. The *hyc* operon genes, encoding the hydrogenase 3 and electron carriers for formate hydrogenlyase, were induced by the addition of formate. Thus, formate may primarily be degraded into CO_2 and H_2 by formate hydrogenlyase. Genes required for synthesis of vitamin B_{12} were induced while genes for aromatic acid degradation were repressed by formate. In addition, genes encoding a number of enzymes of the electron transfer system were affected by formate, showing that formate participates in electron transfer. The expression of genes of nitrate reductase and succinate dehydrogenase was decreased while genes encoding fumarate reductase were induced by formate. The repression of nitrate reductase and succinate dehydrogenase suggests that formate present in the medium could inhibit the activity of these two enzymes. Formate may function as an electron donor while fumarate functions as an electron acceptor. In the presence of formate, *Salmonella* may prefer fumarate as an electron acceptor rather than nitrate.

Introduction

The enteric bacterium *Salmonella* enteric serovar Typhimurium is a facultative anaerobe. Besides using oxygen as an electron acceptor, it can respire using alternative terminal electron acceptors, such as nitrate and fumarate. Accordingly, cells synthesize many enzymes to undergo oxidation and reduction and obtain energy through this process. Under aerobic conditions, *Salmonella* primarily produces dehydrogenases including formate dehydrogenase, *sn*-glycerol-3-phosphate dehydrogenase, NADH dehydrogenase, and succinate dehydrogenase. The reductases produced include fumarate reductase, nitrate reductase, and cytochrome o and d. For the purpose of electron transport, the two enzymes are linked by a quinone. In response to different growth conditions, *Salmonella* regulates the production of the corresponding enzymes. For example, nitrate reductase is induced in the presence of nitrate during anaerobic growth (Stewart, 1988; Wang *et al.*, 1999). Fumarate reductase is produced optimally only when cells are supplied with fumarate and grown anaerobically (Cecchini *et al.*, 2002; Hirsch *et al.*, 1963). Expression of many of these genes encoding respiratory enzymes have been shown to be controlled at the transcriptional level (Cecchini *et al.*, 1995; Iuchi and Lin, 1987a, b; Stewart and Berg, 1988).

Formate is a particularly important molecule in the energy metabolism of many bacteria since as much as one-third of the sugar carbon is converted to formate by fermentation (Rossmann *et al.*, 1991). Without external electron acceptors, formate is oxidized by the formate hydrogenlyase (FHL) system, which

catalyses the cleavage of intracellular formate to H_2 and CO_2 (Ordal and Halvorson, 1939; Sawers *et al.*, 1986; Stephenson and Stickland, 1932). FHL is a membrane-bound complex and consists of formate dehydrogenase-H (FDH-H), hydrogenase 3 and intermediate electron carriers (Sawers *et al.*, 1985; Sawers *et al.*, 1986). FDH-H is encoded by *fdhF*, while hydrogenase 3 and the intermediate electron carriers are encoded by the *hyc* operon, which includes nine genes (McClelland *et al.*, 2001; Schlensog and Bock, 1990). FhlA is a transcriptional activator necessary for the expression of the *hyc* operon and *fdhF* (Bohm *et al.*, 1990). HycA, coded by the proximal promoter gene of the *hyc* operon, is thought to be an anti-activator counteracting transcriptional activation by FhlA (Sauter *et al.*, 1992). Although it is believed that FHL is only produced under anaerobic conditions and in the absence of alternative terminal electron acceptors such as nitrate, the repression of *hyc* transcription by oxygen and nitrate can be relieved by the addition of exogenous formate (Rossmann *et al.*, 1991). Formate as an effector molecule thus is essential for the activity of FhlA as an activator (Maupin and Shanmugam, 1990; Schlensog *et al.*, 1994). The regulatory network in which formate functions as an effector has been defined in *Escherichia coli,* designated as the formate regulon (Leonhartsberger *et al.*, 2002; Rossmann *et al.*, 1991). *Salmonella* Typhimurium has the homologs to all genes of the formate regulon of *E. coli*, and the same order of all genes, and so those genes might be controlled by the same mechanisms (McClelland *et al.*, 2001; Rossmann *et al.*, 1991).

Short chain fatty acids (SCFAs) are the favored products of bacterial metabolism from polysaccharides, oligosaccharides, proteins, peptides and glycoproteins (Cummings *et al.*, 1987). Formate is one of the end products of fermentation by the microflora in the intestine (Cummings *et al.*, 1987). Although short chain fatty acids are thought to inhibit bacteria growth, our previous data showed that acetate and formate induce *Salmonella* invasion genes (Lawhon *et al.*, 2002). It is also known the pH in the small intestine, the *Salmonella* colonization site, is around 6.7 (Bajaj *et al.*, 1996), a level required for acetate and formate to induce invasion gene expression (Lawhon *et al.*, 2002). However, the genetic mediators on which formate acts remain unclear. Recent studies regarding formate metabolism or signal transduction have been done in anaerobically grown cells for known formate regulon genes or at the protein level (Kirkpatrick *et al.*, 2001; Rossmann *et al.*, 1991). Little is known about how *Salmonella* global gene transcription would respond to the presence of exogenous formate, a condition that it would experience in the intestinal tract.

In this work, we used microarray analysis to study the steady state responses of *S.* Typhimurium global gene expression to exogenous formate at pH 6.7. As expected, some known formate regulon genes identified in *E. coli* were induced by the addition of formate. The major class of genes affected by formate was involved in the respiratory electron transfer system. We also report additional genes induced by the addition of formate, including genes involved in vitamin B_{12} synthesis, flagellar

synthesis, lipopolysaccharide synthesis, and a two-component regulator. Aromatic acid synthesis, however, was repressed by additional formate.

Results

To test the global effects induced by formate on *S.* Typhimurium gene expression, we used a DNA microarray that consisted of gene-specific PCR products covering the 4463 (99.4%) predicted Open Reading Frames (ORFs) of the *S.* Typhimurium LT2 genome. We hybridized to the microarray cDNA from wild type *S.* Typhimurium strain (ATCC14028s) grown in Luria-Bertani (LB) broth buffered to pH 6.7 and supplemented with 10 mM sodium formate or with the equivalent level of sodium chloride. We measured the formate concentration in the culture just before we isolated RNA. The formate concentration in the wild type with sodium chloride was below the detection limit (0.4 mM). The formate concentration with added formate was 4.74±0.71 mM, which is sufficient for formate to have impact on gene expression of *Salmonella* (unpublished results). We found genes of several regulons to be induced, while some genes were repressed by the addition of exogenous sodium formate.

Genes within the known formate regulon regulated by formate. One class of genes identified in this screen was the previously characterized genes of formate regulon (Table 1), which suggest that formate under these experimental conditions does act to affect gene expression. Except for *hycE* (1.7-fold induced), the *hycBCDFGH* genes coding for components of the formate hydrogen-lyase systems

were induced over three-fold. Among these, *hycH,* involved in processing of the large subunits of the hydrogenase (HycE), was the most induced (6.4-fold). *hycI,* the product of which was proposed to function in the maturation of the formate hydrogen-lyase complex, was also induced 3.2-fold. In addition, *hydN*, whose expression is responsive to formate, was induced 3.9-fold. *hydN* has a putative role in electron transfer from formate to hydrogen and is located downstream of the *hyc* operon. However, *hycA,* encoding the transcriptional repressor of *hyc* operon, was also induced 3.4-fold. As *hycA* is part of the *hyc* operon, it is reasonable that the expression of *hycA* is, in turn, regulated by FhlA and formate (see details in discussion). These results demonstrate that genome analysis using a *Salmonella* DNA microarray and our experimental conditions are supportive of previous results showing that the expression of the *hyc* operon and *hydN* is controlled by the addition of formate at pH 6.7.

Hydrogenase 2 regulation by formate. *Salmonella* Typhimurium possesses genes for three putative homologous membrane-associated hydrogenases, designated hydrogenase 1, 2, and 3 (Maier *et al.*, 2004; Sawers *et al.*, 1986). *Salmonella* hydrogenase 1 and 2 are immunologically equivalent to hydrogenase 1 and 2 of *E. coli* (Sawers *et al.*, 1986). In *E. coli,* hydrogenase 2 has been shown to be involved in hydrogen uptake and can be differentially induced to high levels when bacteria are grown on non-fermentable carbon sources such as hydrogen and fumarate or glycerol and fumarate (Ballantine and Boxer, 1985, 1986; Lee *et al.*, 1985). Thus, the function of hydrogenase 2 was proposed to be to allow the cells to

gain energy from the oxidation of molecular hydrogen in a respiratory capacity (Menon *et al.*, 1994). The hydrogenase 2 enzymes are encoded by the polycistronic *hybOABCDEFG* operon (Menon *et al.*, 1990; Menon *et al.*, 1994). The transcription of the *hyb* operon was enhanced under anaerobic fermentative conditions and repressed by the presence of nitrate, but was not affected by addition of formate to anaerobic growth medium (Richard *et al.*, 1999). ArcA acts to suppress *hyb* expression under anaerobic conditions, and the Nar regulatory system with two two-component regulators, NarL/NarX and NarP/NarQ, works to repress *hyb* expression in the presence of nitrate (Richard *et al.*, 1999). The possible activators for the induction of *hyb* under anaerobic conditions remain unknown. Three genes at the end of the *hyb* operon showed a varied level of gene expression in our experiments. *hybE* and *hybF* were both induced 4.1-fold, while the expression of *hybG* was decreased 2.1-fold. Genetic studies shows that HybG works as a putative chaperone and is responsible for the maturation of hydrogenase 1 and 2 (Blokesch *et al.*, 2001; Butland *et al.*, 2006; Hube *et al.*, 2002). In *E. coli,* further protein interaction studies have demonstrated that HybG binds to HyaB and HybC, the large subunit of hydrogenase 1 and 2 respectively (Butland *et al.*, 2006). There is no *hyaB* in *S.* Typhimurium but *hybC* was also repressed by formate (data not shown). HybF of *E. coli* is involved in the maturation of not only hydrogenase 1 and 2, but also hydrogenase 3 (Hube *et al.*, 2002). HybE is classified as a hydrogenase 2-specific chaperone which interacts specifically with the precursor forms of both β-subunit and α-subunit of hydrogenase 2 to prevent premature interaction between

the partner subunits (Dubini and Sargent, 2003). Thus, HybF and HybG have redundant functions in maturation of hydrogenase 1 and 2. Our results showed that HybF was active while HybG was inactive in the presence of formate, suggesting that HybF and HybG may function in response to different conditions. The activation of HybF involved in maturation of hydrogenase 3 was consistent with the induction of the *hyc* operon encoding hydrogenase 3. The fact that the repressor of *hyb* operon NarL was repressed by formate was consistent with the induction of *hybE* and *hybF*.

Nitrate reductase negative regulation by formate. Although nitrate reductases of *E. coli* have been actively studied (Darwin *et al.*, 1998; Stewart, 1988, 1994; Wang *et al.*, 1999), little has been published regarding nitrate reductase in *Salmonella.* According to the annotated complete genome sequences of *Salmonella* Typhimurium, *Salmonella* has three distinct nitrate reductases (McClelland *et al.*, 2001). One is encoded by the operon *napABCDFH*, which is homologous to the *nap* operon in *E. coli.* In *E. coli,* Nap is believed to be a periplasmic nitrate reducatase that is expressed in response to nitrate-limiting conditions (Potter *et al.*, 1999; Stewart *et al.*, 2002; Wang *et al.*, 1999). NapA and NapB are periplasmic proteins that interact with each other and interact with NapC and NapD in the cytoplasm (Nilavongse *et al.*, 2006). NapC, located in cytoplasmic membrane, is thought to be the quinol dehydrogenase of the system (Berks *et al.*, 1995a; Berks *et al.*, 1995b; Brondijk *et al.*, 2002; Simon *et al.*, 2003). NapD is a cytoplasmic protein, which is proposed to play a role in NapA maturation (Potter and Cole, 1999). NapF locates to the cytoplasm of *E. coli* and plays an unidentified role in the post-translational

modification of NapA (Nilavongse *et al.*, 2006). NapG and NapH function to transfer electrons from ubiquinol to NapAB (Brondijk *et al.*, 2002). A second major respiratory nitrate reductase, encoded by the *narGHIJ* operon, is located in the cytoplasmic membrane in *E. coli* (Zumft, 1997). The *narZYWV* operon encodes a third nitrate reductase, which is believed to be constitutively expressed (Blaso *et al.*, 1990). The activity of nitrate reductases in *E. coli* is inhibited by oxygen (Stickland, 1931). *E. coli* has two pairs of two-component regulators of nitrate reductase: NarQ/NarP and NarX/NarL, both of which are present in *Salmonella* as well (Stewart, 1994; Wang and Gunsalus, 2003). NarL and NarP are the transcriptional regulators while NarQ and NarX are nitrate sensing proteins (Stewart, 1994; Wang and Gunsalus, 2003). We found that eight nitrate reductase genes were aerobically repressed by exogenous formate. Among these, six genes of the seven-gene *napABCDFGH* operon were repressed from 2.2-fold to 5.2-fold, without changing the expression of *napG.* The expression of *narJ* from the *narGHIJ* operon was also decreased 2.6-fold. The gene coding the response regulator NarL of a two-component regulator system NarL/NarX was repressed 3.2-fold. No change for the expression of the *narZYWV* operon was observed in this study. This is reasonable, as *narZYWV* is considered to be the constitutively expressed nitrate reductase (Blaso *et al.*, 1990). Thus, the addition of formate to culture medium aerobically represses the expression of structural genes (*napABCDFH* and *narJ*) and a regulator (*narL*) of two-component regulator system in nitrate reductase.

Fumarate reductase and succinate dehydrogenase regulation by formate.

Salmonella Typhimurium has two distinct cytoplasmic membrane-bound enzymes that have been reported to reversibly interconvert fumarate and succinate, but with different substrate affinities and at different rates (Hirsch *et al.*, 1963). One of them is fumarate reductase, which functions more effectively in reduction (Cole, 1982; Hirsch *et al.*, 1963). The other is succinate dehydrogenase and is more suited to oxidation (Hirsch *et al.*, 1963). However, either fumarate reductase or succinate dehydrogenase can catalyze both reduction and oxidation. The enzymes are encoded at different genetic loci. Fumarate reductase is encoded by the *frdABCD* operon while succinate dehydrogenase is made by the *sdhCDAB* operon (Jones and Gunsalus, 1985, 1987; McClelland *et al.*, 2001). The expression of *frdC* and *frdD* was increased 2.0- and 2.7-fold respectively by formate. *frdCD* are thought to encode membrane anchored polypeptides that specifically bind the catalytic FrdAB subunits (Lemire *et al.*, 1982). *frdAB*, encoding the catalytic part of fumarate reductase, were also induced around 1.5-fold (Table 2). Our results consistently showed that the repressor of the *frd* operon, *narL,* was repressed 3.2-fold by formate (Iuchi and Lin, 1987a). All genes encoding succinate dehydrogenase were also repressed by formate. Among these, the transcription of *sdhB* was decreased 2.7-fold and that of *sdhC* 3.5-fold by formate. The transcriptional change of *sdhAD* was less than two-fold. Thus, the fumarate reductase was induced while succinate dehydrogenase was repressed when exogenous formate was added in aerobic culture. This result is supported by the fact that fumarate reductase genes are

coordinately regulated in response to the cellular availability of potential terminal electron acceptors (Cecchini *et al.*, 1995; Cecchini *et al.*, 2002; Cole and Wimpenny, 1968; Lemire *et al.*, 1982). It also suggests that fumarate reductase can be synthesized aerobically when formate is present. NarL could be the mediator for formate to induce fumarate reductase.

Vitamin B_{12} biosynthesis induction by formate. Vitamin B_{12} is a large molecule with a molecular weight of 1580, and at least 25 enzymes are uniquely involved in its synthesis (Roth *et al.*, 1996). Most of the genes involved in vitamin B_{12} synthesis are clustered in the a single operon (*cob*) mapping to 41 minutes on the chromosome (Bobik *et al.*, 1992; Jeter *et al.*, 1984). The *cob* operon includes 17 *cbi* genes needed for the synthesis of cobinamide, a precursor of vitamin B_{12} (Chen *et al.*, 1995). Propanediol has been shown to induce the expression of the *cob* operon (Bobik *et al.*, 1992; Rondon and Escalante-Semerena, 1992) and vitamin B_{12} is required for the activity of propanediol dehydratase, glycerol dehydratase, and ethanolamine ammonia lyase (Roth *et al.*, 1996). Four *cbi* genes (*cbiAKNO*) were induced more than two-fold with the addition of formate (Table 2). Thus, exogenous formate could induce vitamin B_{12} synthesis. Although it is believed that *Salmonella* Typhimurium makes cobalamin (vitamin B_{12}) only under anaerobic conditions (Jeter *et al.*, 1984), our results suggest that the addition of formate induce the synthesis of vitamin B_{12} under aerobic condition.

Other genes regulated by formate. Other genes regulated by formate were involved in many cellular functions, including cell surface proteins, ATP-binding

cassette (ABC) transporters, aromatic acid catabolism, heat shock, RNAase and ribosomal RNA subunits, a two-component regulatory system, and cytochrome d (Table 2). Among these, we found that formate specifically induced the expression *fliO* and *fliE* for biosynthesis of flagella, *rfaH* and *rfaP* for lipopolysaccharide synthesis, and *ompW* encoding an outer membrane protein known to be required for *Salmonella* virulence (Baumler *et al.*, 1997; Chan *et al.*, 2005; Sharma and Chaturvedi, 2006). Formate is present in the ileum, the *Salmonella* colonization site; this result thus suggests that formate may be a signal for *Salmonella* colonization. The ABC transporter genes were all repressed by formate, but it is unclear why this would be. The acetyl-CoA synthetase (*acs*) and putative pyruvate formate lyase (*pflF*) genes were aerobically repressed by formate. Since formate is mainly made by pyruvate formate lyase during anaerobic growth, it is reasonable that pyruvate formate lyase was inactive aerobically and the added formate did not induce it. *Salmonella* has two gene clusters encoding 4-hydroxyphenylacetate catabolism, with the two clusters being divergently transcribed (McClelland *et al.*, 2001). The first is comprised of three genes, *hpaBCR*. HpaC and HpaB form a two component 4-HPA 3-monooxygenase, with HpaB as the large subunit and HpaC as the small subunit (Galan *et al.*, 2000; Prieto and Garcia, 1994; Prieto *et al.*, 1996). HpaR is a repressor for genes coding 4-hydroxyphenylacetate catabolism (Galan *et al.*, 2003). A second gene cluster includes *hpaGEDFHIXA* (McClelland *et al.*, 2001). HpaA is thought to be an activator of this gene cluster (Prieto and Garcia, 1997). Our microarray data showed that the second gene cluster, *hpaGEDFHIXA*, was

repressed by formate, and in particular the expression of *hpaFGI* was decreased more than two-fold. Consistent with this result, *hpaR* was induced, although the increase was less than two-fold. Thus, genes for degradation of 4-hydroxyphenylacetate were repressed, which suggests that the addition of formate may inhibit 4-hydroxyphenylacetate catabolism. Another noteworthy repressed gene was *sodA* (Superoxide dismutase, SOD*),* the product of which is required for resistance to the early oxygen-dependent microbicidal mechanisms of phagocytes (Tsolis *et al.*, 1995). There were also genes with putative functions identified by microarray (Table 4). Thus, besides affecting the respiratory system of *Salmonella,* formate has an impact on many aspects of cellular functions.

Discussion

Formate plays an important role in metabolism and respiration. It has been demonstrated that the intracellular level of formate affects the transcription of the *hyp* and *hyc* operon genes and of the *fdhF* gene in *E. coli,* identified as the formate regulon (Leonhartsberger *et al.*, 2002; Rossmann *et al.*, 1991). FhlA is the activator of the formate regulon and its level is also controlled by formate (Maupin and Shanmugam, 1990; Sankar *et al.*, 1988; Schlensog and Bock, 1990). Several factors including oxygen, nitrate (anaerobically), pH, molybdate, and formate have also been suggested to control the expression of the formate regulon (Leonhartsberger *et al.*, 2002; Pecher *et al.*, 1983; Peck and Gest, 1957; Wimpenny and Cole, 1967). However, the repression of the FHL complex genes by oxygen

and the requirement for an acidic pH in the medium may be relieved by the single signal of the intracellular concentration of formate (Rossmann *et al.*, 1991). Therefore, formate plays a decisive role in the expression of formate regulon genes. This present study was designed to identify the formate regulon of *Salmonella* by the comparison of global gene expression between wild type *Salmonella* grown with and without exogenous formate.

The largest class of induced genes was the *hyc* operon genes, whose products are the components of FHL complex. It is known that formate can be dissipated through FHL or three formate dehydrogenase isoenzymes (FDH-H, FDH-N, FDH-O). Our results showed that none of the isoenzymes were induced by formate, which is in partial agreement with previous reports. FDH-N is reported to be induced under anaerobic growth conditions when nitrate is present (Chaudhry and MacGregor, 1983; Enoch and Lester, 1975) . FDH-H synthesis requires not only formate but also anaerobic conditions (Birkmann *et al.*, 1987a). However, FDH-O is synthesized under aerobic and nitrate respiratory conditions (Abaibou *et al.*, 1995; Sawers *et al.*, 1991). Although FDH-O was not induced by formate, FDH-O has been shown to be constitutively expressed at relative low levels independent of either oxygen or nitrate (Abaibou *et al.*, 1995). Thus, our results suggest that the addition of formate does not induce degradation by formate dehydrogenase isoenzymes, but by FHL, since part of the FHL complex was induced. Under aerobic conditions, FhlA is able to compete for formate with FDH-O (Rossmann *et*

al., 1991). Our results might also indicate that FhlA or part of the FHL complex has more affinity for formate than FDH-O when formate is supplied during aerobic growth.

flhA, coding for the activator of the formate regulon, was not induced by the addition of formate. However, *flhA* is transcribed from three different promoters with a complex control system. Among these, two of them are active only under fermentative conditions (Rossmann *et al.*, 1991). A third promoter is a weak constitutive promoter that is the only expressed promoter under aerobic conditions (Lutz, 1991; Rossmann *et al.*, 1991). Our results showed that *flhA* was not aerobically induced by added formate. The anti-activator of FlhA, HycA, was induced. However, the *hyc* operon, activated by FlhA and assumed to be repressed by HycA, was induced as well. Although HycA counteracts the activity of FhlA, it is not known what role HycA plays in control of the *hyc* operon. One hypothesis is that HycA functions as an exporter to excrete formate (Leonhartsberger *et al.*, 2002). Our results seem to support this hypothesis. The expression of the *hyc* operon was increased, so that HycA and the products of the rest *hyc* operon work together to consume extra formate.

Another important effect of formate is in the electron transfer system. A number of genes encoding enzymes in the electron transfer system were affected by formate, including nitrate reductase, succinate dehydrogenase, fumarate reductase, cytochrome d complex, dimethyl sulfoxide reductase, and hydrogenase 2. Among these, nitrate reductase and succinate dehydrogenase were repressed, while fumarate reductase was induced. NarL has been proposed to act as a regulator for

nitrate reductase, succinate dehydrogenase, fumarate reductase, hydrogenase 2, and formate hydrogenlyase. (Chiang *et al.*, 1997; Darwin *et al.*, 1998; Iuchi and Lin, 1987a; Richard *et al.*, 1999; Stewart, 1982; Stewart and Berg, 1988; Wang and Gunsalus, 2003). In the presence of nitrate, nitrate reductase is induced, while fumarate reductase, hydrogenase 2, and FHL are repressed through the mediation of NarL (Iuchi and Lin, 1987a; Richard *et al.*, 1999; Wang and Gunsalus, 2003). Our results showed that NarL and nitrate reductase were repressed while part of FHL and hydrogenase 2, and fumarate reductase were induced in the presence of formate under aerobic conditions. Thus, NarL may serve as the global regulator of this network of formate regulation in the presence of exogenous formate. Although fumarate reductase and succinate dehydrogenase catalyze the same reaction, fumarate reductase was induced while succinate dehydrogenase was repressed. Thus, *Salmonella* may prefer fumarate reductase rather than succinate dehydrogenase to catalyze succinate oxidation in the presence of formate under aerobic conditions. Formate could be the electron donor while fumarate may act as the electron acceptor. The mediator for this process may be the two-component regulatory system NarL/NarX. In conclusion, exogenous formate participates in the electron transfer system and is degraded by FHL complex through the mediation of a two-component regulatory system NarL/NarX.

Experimental procedures

Bacterial strains and growth conditions. *Salmonella enterica* serovar Typhimurium ATCC14028 was used in this study. Three independent cultures were grown in Luria-Bertani (LB) broth at 37^{o}C overnight with shaking at 200 rpm. Each overnight culture was diluted 20-fold into fresh LB buffered with 100 mM MOPS pH 6.7 with the addition of either 10 mM sodium formate or 10 mM sodium chloride. These six cultures were then grown for approximately two hours under the same conditions as the overnight culture.

Determination of Formate Concentrations. Formate concentrations in media were measured just before the RNA isolation using a formic acid enzymatic bioanalysis detection kit, following the manufacturer's directions (r-biopharm, Darmstadt, Germany). This method measured formate concentration by the enzymatic conversion of formate and NAD^{+} to bicarbonate and NADH through the action of formate dehydrogenase. NADH concentration was then measure by UV light absorbance at a wavelength of 340 nm. Formate concentration was calculated by the equation provided with the kit.

RNA Isolation. Steady-phase cultures (OD_{600} of 0.6-0.7) were used for RNA Isolation. Cells were harvested by adding 0.15 volumes of 95% ethanol and 5% acidic phenol and then stored on ice for 20 minutes. RNA isolation was performed with an SV total RNA isolation system according the provided manual (Promega, Madison, WI, USA). The quality of RNA was analyzed using a NanoDrop ND-1000

spectrophotometer (Nanodrop, Wilmington, DE) and electrophoresis. The ratio of OD_{260} to OD_{280} for all isolated RNA was between 1.9 and 2.1.

Genomic DNA Preparation. Genomic DNA was prepared as follows. Twenty-five ml of overnight culture was centrifuged to harvest the cells, resuspended in 25 ml of J-buffer (0.1 M Tris pH 8.0, 0.1 M EDTA, 0.15 M NaCl), centrifuged again, and resuspended in 0.8 ml J-buffer. Freshly-made 0.1 ml lysozyme solution (10 mg/ml in 0.25 M Tris, pH 8.0) was used to lyse cells and followed by incubation at 37^{o} C for ten minutes. One hundred μl of boiled RNase solution (1 mg/ml) was added to remove RNA by incubation at 37^{o} C for ten minutes and heating to 70^{o} C for three minutes. To release the DNA, 0.08 ml of 30% sodium lauryl sarcosinate was added and incubated at 70^{o} C for 20 minutes, and then shifed to 37^{o} C for another one hour. Two mg of proteinase K was used to degrade the protein by incubation at 37^{o} C. After two to four hours, another 2 mg of proteinase K was added. The preparation was then transferred to a dialysis bag and dialyzed overnight at 37^{o} C against 0.01 M Tris (pH 8.0), 0.01 M EDTA, and 0.15 M NaCl. Neutral and water-saturated phenol was used to extract the DNA twice. Genomic DNA was subsequently purified by ether extraction twice and dialyzing against TE (10 mM Tris, pH 8.0 and 1 mM EDTA) for several hours.

Genomic DNA labeling. One and half μg of genomic DNA in a total volume of 21 μl was labeled with Cy5 monoreactive dye (Amersham, Piscataway, NJ). First, 20 μl of freshly prepared 2.5X random primer with buffer mixture (12 μl random hexamers

(1μg/μl), 2.5 μl 1 M Tris-HCl (pH 6.8), 0.25 μl 1 M $MgCl_2$, 0.04 μl 2-mercaptoethanol, 5.21 μl sterile water) was annealed to 1.5 μg of genomic DNA by boiling the mixture for five minutes. The mixture was then briefly centrifuged and placed on ice. Five μl of 10X dNTP mixture (6 μl 10mM each dATP, dGTP, and dTTP; 3 μl 10 mM dCTP; 29 μl sterile water) (Sigma, St. Louis, MO) was added to the mixture on ice. Next, 2 μl Cy5-dCTP was added and followed by 2 μl Klenow enzyme (5 U/μl). The mixture was then incubated overnight at 37° C in the dark. The reaction was stopped by adding 5 μl 0.5 M EDTA (pH 8.0). Unincorporated dyes were removed with a QiaQuick PCR purification Kit (Qiagen, Germantown, MD) and the eluted samples were dried in a Speedvac until volume reached 20 μl.

cDNA synthesis and labeling. Twenty-eight to 30 μl of any isolated RNA (50 μg) sample was converted to cDNA in two steps. First, 2 μl of random hexamers N_6 (2 μg/ul) was annealed to 50 μg of total RNA by heating the mixture to 70º C for exactly ten minutes and then cooling on ice. Next, 6 μl of 0.1 M DTT, 12 μl of 1st strand buffer (Superscript II), 1.2 μl of nucleotide mix (25 mM dATP, dTTP, dGTP, 10 mM dCTP), 4 μl of Superscript II (RT) (Invitrogen, Carlsbad, CA), 2 μl of Rnasin (Roche, Indianapolis, IN), 0.8 μl sterile water (Invitrogen, Carlsbad, CA) were added to each reaction mixture. For labeling, 4 μl of Cy3-dCTP (Amersham, Piscataway, NJ) were added in the dark, followed by incubation at 42° C for one hour. All six RNA samples, three of them isolated from the culture with 10 mM sodium chloride and three from the culture with 10 mM sodium formate, were labeled with Cy3 dye. Another 2 μl

more of Superscript II were added and the reaction mixture was incubated for another one hour at 42^{o} C. The RNA template was then degraded by adding 3 μl of 1 M NaOH and incubating at 70^{o} C for ten minutes. Finally, the reaction mixture was neutralized by the addition of 3 μl of 1 M HCl. QiaQuick PCR purification columns (Qiagen, Germantown, MD) were used to remove unincorporated nucleotides. Labeled cDNA was finally eluted in 30 μl of RNase-free water.

Pre-hybridization and hybridization. All *Salmonella* genomic microarrays were provided by Michael McClelland at Sydney Kimmel Cancer Center (San Diego, CA). Microarray slides were washed in 0.1% SDS for two minutes, twice in sterile water for two minutes, and incubated at 42ºC with rotation in 100 ml of Pre-Hyb solution (25 ml of formamide, 25 ml of 20X SSC, 1 ml of 10% SDS, 10 mg BSA, 49 ml of water) for 45 minutes. After pre-hybridization, the microarray slides were washed in water twice for two minutes and dried by centrifugation for 15-20 minutes. The labeled cDNA probe and genomic DNA as a control were then hybridized to a *Salmonella* genomic microarray by the following steps. First, 20 μl of each cDNA and genomic DNA probes were mixed with 40 μl of hybridization solution (50% formamide, 10X SSC, 0.2% SDS). The mixture was heated to 95º C for five minutes, centrifuged for thirty seconds to collect condensation, and cooled to room temperature. The mixtures were then pipetted onto the surface of the printed side between the microarray slide and LifterSlip (Erie scientific company, Portsmouth, NH). The slides were next placed in Corning hybridization chambers (Corning,

Corning, NY) and incubated at 42^{o} C in a hybridization oven overnight. After hybridization, the hybridization chambers were disassembled with the printed array side up. The array slides were washed in the mixture of 2X SSC and 0.1% SDS for five minutes at 42^{o} C, in the mixture of 0.1X SSC and 0.1% SDS for ten minutes at room temperature, in 0.1X SSC for one minute at room temperature for four times, rinsed in water for ten seconds or less, and finally dried by centrifugation.

Microarray quantification and analysis. Hybridized microarrays were scanned using a 4000XL spotted array scanner (Hewlett-Packard Co. Palo Alto, CA) at 633 nm and 543 nm. WebArray software (Xia *et al.*, 2005) was used to quantify the signals. We considered microarray data of the genomic DNA as common references; therefore, we made a two-sample comparison, both of which were subcultured from the same strain but with one sample treated with sodium chloride as a control sample and the other treated with sodium formate as an experimental sample. Background intensities were subtracted for correction. We used Print-tip Loess to normalize the within-array expression log-ratios so that the log-ratio averaged to zero within each array. The scale normalization method was used to scale the log-ratios to have the same median-absolute-deviation (MAD) across arrays (Smyth and Speed, 2003; Yang *et al.*, 2002). Linear Model Statistical Analysis, including false discovery rate (FDR) calculation with spacing LOESS histogram (SPLOSH) (Pounds and Cheng, 2004) was employed to compute moderated t-statistics and log-odds of differential expression by empirical Bayes shrinkage of the standard errors towards a common value. For genomic mapping,

we chose single chromosome. The normalized signal for each gene was then averaged over a total of six arrays. The output file was ranked by the log-differential expression ratio (M), which was the value of $\log_2$ (Experimental sample) minus $\log_2$ (Control Sample).

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Table 1. Formate regulon genes showed by previous study (Leonhartsberger *et al.*, 2002) were regulated by exogenous formate

Gene	M	Function
hycI	1.6	protease involved in processing C-terminal end of HycE
hycD	1.6	hydrogenase 3, membrane subunit (part of FHL complex)
hycA	1.7	transcriptional repressor of hyc and hyp operons
hycC	1.9	hydrogenase 3, membrane subunit (part of FHL complex)
hycG	1.9	hydrogenase activity
hycB	2.1	hydrogenase-3, iron-sulfur subunit (part of FHL complex)
hycF	2.3	hydrogenase 3, putative quinone oxidoreductase
hycH	3.2	processing of HycE (part of the FHL complex)
hydN	2.0	electron transport protein (FeS senter) from formate to hydrogen

M is the log-differential expression ratio

Table 2. Genes involved in respiratory system regulated by formate

Gene	M	Function
nitrate reductase		
napD	-2.6	periplasmic nitrate reductase
napF	-1.5	ferredoxin-type protein: electron transfer
		periplasmic nitrate reductase, large subunit, in complex with
napA	-2.2	NapB
napH	-1.1	ferredoxin-type protein: electron transfer
		periplasmic nitrate reductase, small subunit, cytochrome C550,
napB	-1.2	in complex with NapA
napC	-1.2	periplasmic nitrate reductase, cytochrome c-type protein
		nitrate reductase 1, delta subunit, chaperone required for
narJ	-1.3	molybdenum cofactor assembly in nitrate reductase 1
		response regulator in two-component regulatory system with
		NarX (or NarQ), regulates anaerobic respiration and
narL	-1.6	fermentation (LuxR/UhpA familiy)
succinate dehydrogenase		
sdhB	-1.3	succinate dehydrogenase, Fe-S protein
sdhC	-1.7	succinate dehydrogenase, cytochrome b556
fumarate reductase		
frdC	2.0	fumarate reductase, anaerobic, membrane anchor polypeptide
frdD	2.7	fumarate reductase, anaerobic, membrane anchor polypeptide

Table 2 continued

cytochrome d complex		
cydB	1.2	cytochrome d terminal oxidase polypeptide subunit II
dimethyl sulfoxide reductase		
dmsC	1.4	anaerobic dimethyl sulfoxide reductase, subunit C
hydrogenase 2		
hybE	2.1	putative hydrogenase
hybF	2.1	putative hydrogenase expression/formation protein
	-1.1	
hybG		hydrogenase-2 operon protein

M is the log-differential expression ratio

Table 3. Genes with other functions regulated by formate

Gene	M	Function
4-hydroxyphenylacetate catabolism		
hpaF	-1.3	4-hydroxyphenylacetate catabolism
hpaG	-1.1	4-hydroxyphenylacetate catabolism
hpaI	-1.3	4-hydroxyphenylacetate catabolism
ABC-transporter genes		
yehW	-1.6	putative ABC-type proline/glycine betaine transport systems, permease component
dppC	-1.4	ABC superfamily (membrane), dipeptide transport protein 2
gltI	-1.0	ABC superfamily (bind_prot), glutamate/aspartate transporter
gltL	-2.1	ABC superfamily (atp_bind), glutamate/aspartate transporter
ccmC	-1.1	ABC superfamily (membrane) heme exporter protein, cytochrome c-type biogenesis protein
argT	-1.3	ABC superfamily (bind_prot), lysine/arginine/ornithine transport protein
Heat shock		
ibpA	-1.6	small heat shock protein
ibpB	-1.3	small heat shock protein
Cell surface protein		
fliO	1.1	flagellar biosynthesis
fliE	1.2	putative Flagellar hook-basal body protein

Table 3 continued

		transcriptional activator affecting biosynthesis of
rfaH	1.0	lipopolysaccharide core, F pilin, and haemolysin
		lipopolysaccharide core biosynthesis; phosphorylation of core
rfaP	1.0	heptose
nmpC	1.0	new outer membrane protein; predicted bacterial porin
		outer membrane protein W; colicin S4 receptor; putative
ompW	1.3	transporter
Synthesis of vitamin B_{12}		
cbiK	1.1	synthesis of vitamin B_{12} adenosyl cobalamide precursor
cbiO	1.3	synthesis of vitamin B_{12} adenosyl cobalamide precursor
cbiN	1.3	synthesis of vitamin B_{12} adenosyl cobalamide precursor
cbiA	1.4	synthesis of vitamin B_{12} adenosyl cobalamide precursor
RNA		
rnc	1.5	RNase III, ds RNA
rpmH	1.4	50S ribosomal subunit protein L34
rsmC	1.2	16S rRNA m2G 1207 methylse
ligT	1.0	2'-5' RNA ligase
rpsH	1.0	30S ribosomal subunit protein S8, and regulator
rph	1.1	RNase PH
Other functions		
hsdM	1.1	DNA methylase M, host modification
himD	1.4	integration host factor (IHF), beta subunit; site-specific

Table 3 continued

		recombination
		APC family, gamma-aminobutyrate transport protein, RpoS
gabP	-1.2	dependent
gabD	-1.6	succinate-semialdehyde dehydrogenase I, NADP-dependent
gabT	-1.6	4-aminobutyrate aminotransferase
acs	-1.9	acetyl-CoA synthetase
pflF	-1.3	putative pyruvate formate lyase
sodA	-1.3	superoxide dismutase, manganese
lpfE	-2.0	long polar fimbrial minor protein
citD2	-1.1	putative citrate lyase acyl carrier protein (gamma chain)
csgC	-1.2	putative curli production protein
kdgT	-2.2	2-keto-3-deoxygluconate permease
wza	-1.4	putative polysaccharide export protein, outer membrane
cyoD	-2.3	cytochrome o ubiquinol oxidase subunit IV
ssb	-1.1	ssDNA-binding protein controls activity of RecBCD nuclease
lldP	-1.0	LctP transporter, L-lactate permease
		periplasmic murein tripeptide transport protein, also negative
mppA	1.0	regulator of mulitple antibiotic resistance
apaG	1.0	putative cytoplasmic protein
bcsC	1.0	endo-1,4-D-glucanase
ptxA	1.1	putative PTS enzyme IIsga subunit
malX	1.1	pseudogene; frameshift

Table 3 continued

gph	1.1	phosphoglycolate phosphatase
msyB	1.1	acidic protein suppresses mutants lacking function of protein export
nanE	1.7	putative ManNAc-6P epimerase
gpt	1.1	guanine-hypoxanthine phosphoribosyltransferase
mreC	1.1	rod shape-determining protein
lamB	1.1	phage lambda receptor protein; maltose high-affinity receptor, facilitates diffusion of maltose and maltoseoligosaccharides
lepB	1.2	leader peptidase (signal peptidase I), serine protease
dps	1.2	stress response DNA-binding protein; starvation induced resistance to H_2O_2
syd	1.2	interacts with secY
gudD	1.3	d-glucarate dehydratase
aspA	1.3	aspartate ammonia-lyase (aspartase)
rhoL	1.3	pseudogene; no in-frame start
cspD	1.4	similar to CspA but not cold shock induced
truA	1.4	pseudouridylate synthase I
ispD	1.4	4-phosphocytidyl-2C-methyl-D-erythritol synthase
mutT	1.5	7,8-dihydro-8-oxoguanine-triphosphatase, prefers dGTP
tatA	1.6	component of Sec-independent protein secretion pathway
pgtA	1.6	Phosphoglycerate transport: activator
caiF	1.7	transcriptional regulator of cai and fix operon

Table 3 continued

		response regulator in two-component regulatory system with DpiB, transcriptional regulation of cit operon (citrate fermentation) genes and of plasmid inheritance genes (OmpR
dpiA	1.1	family)
citF	1.2	bifunctional citrate lyase alpha chain/citrate-ACP transferase
dcuB	2.9	Dcu family, anaerobic C4-dicarboxylate transporter

M is the log-differential expression ratio

Table 4. Genes with putative functions regulated by formate

Gene	M	Function
Induced genes		
orf245	1.0	putative cytoplasmic protein
ydiH	1.1	putative cytoplasmic protein
yhcH	1.0	putative cytoplasmic protein
ybhB	1.0	putative Phospholipid-binding protein
ybjN	1.0	putative cytoplasmic protein
yhhL	1.1	putative inner membrane protein
ydgF	1.1	putative membrane transporter of cations and cationic drugs
yfbB	1.1	putative enzyme
ydfZ	1.1	putative cytoplasmic protein
yacG	1.1	putative cytoplasmic protein
yrdD	1.2	putative DNA topoisomerase
yccD	1.2	putative cytoplasmic protein
yqjK	1.2	putative inner membrane protein
yrbA	1.2	putative transcriptional regulator (BolA family)
yqgB	1.2	putative inner membrane protein
ybeB	1.3	putative ACR, homolog of plant lojap protein
yidE	1.3	paral putative transport protein
yadB	1.3	putative glutamyl t-RNA synthetase
ycbL	1.4	putative Metallo-beta-lactamase

Table 4 continued

yfiD	1.9	putative formate acetyltransferase
yneC	1.7	putative inner membrane protein
yccK	1.0	putative sulfite reductase, gamma subunit
yejL	1.0	putative cytoplasmic protein
yjjX	1.5	putative cytoplasmic protein
yqjE	1.4	putative inner membrane protein
Repressed genes		
yfiA	-1.0	ribosome associated factor, stabilizes ribosomes against dissociation
yegN	-1.3	paral putative outer membrane receptor
ybaM	-2.2	putative inner membrane protein
ygaF	-1.4	paral putative sarcosine oxidase-like protein

M is the log-differential expression ratio