Abstract

Lawhon, Sara Dyann. Genetic and environmental regulation of virulence genes in *Salmonella enterica* serovar Typhimurium. (Under direction of Craig Altier and Paul Orndorff)

The purpose of this research has been to assess the effect of the genetic regulator, CsrA, and environmental conditions on the expression of virulence genes in *Salmonella enterica* serovar Typhimurium. CsrA is an RNA binding protein that alters messenger RNA stability in *E. coli* and regulates virulence genes located on the *Salmonella* pathogenicity island 1 (SPI-1). This work demonstrates that CsrA in *S. typhimurium* like its counterpart in *E. coli* regulates expression of genes required for flagellar synthesis and suggests a role for CsrA in the positive regulation of utilization of propanediol and ethanolamine, vitamin B$_{12}$ synthesis, and expression of maltoporin, which transports maltose and maltodextrins across the bacterial cell membrane. Propanediol, ethanolamine, and maltodextrins are byproducts of digestion likely present in the intestinal tract. Vitamin B$_{12}$ is required for utilization of propanediol and ethanolamine. The sensor kinase BarA and its response regulator SirA control levels of CsrA indirectly through the expression of an untranslated RNA CsrB, which binds CsrA. SirA regulates expression of *Salmonella* virulence genes and is required for *Salmonella* enteropathogenesis. BarA and SirA have the opposite effects on propanediol and ethanolamine utilization, and vitamin B$_{12}$ synthesis as CsrA, thus supporting the model of indirect regulation of CsrA by BarA/SirA. In addition, the
short chain fatty acids (SCFA) acetate, propionate, and butyrate are present in the ileum and colon at differing total concentrations and relative percentages. SCFAs representing the ileum and acetate alone are able to restore SPI-1 invasion gene expression to a *barA* mutant but not to a *sirA* mutant. Additionally, ileal SCFAs increase the expression of SPI-1 virulence genes required for invasion of epithelial cells and increase the expression of genes required for survival within epithelial cells and macrophages. SCFAs representing the colon decrease expression of genes required for epithelial cell invasion and decrease the expression of genes required for flagellar synthesis and maltose transport while increasing the expression of genes required for glycerol and propanediol metabolism.
Genetic and environmental regulation of virulence genes in
Salmonella enterica serovar Typhimurium.

by
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Dedication

This dissertation is dedicated to my husband, Ian W. Muise, and to my parents, John T. and Mary Lawhon, without whose support it would not have been initiated or finished.
Biography

Sara Dyann Lawhon was born on September 26, 1969 in Austin, Texas to John T. and Mary E. H. Lawhon. She attended Texas A&M University in College Station, Texas where she received two Bachelor of Science degrees, one in Biomedical Science in 1991 and another in Veterinary Science in 1994. She graduated from the College of Veterinary Medicine at Texas A&M University in 1997 with a degree in Veterinary Medicine. She married Ian W. Muise in 1998. She completed a residency in Comparative Infectious Diseases at North Carolina State University in 2001.
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Literature Review

Salmonella Infection

*Salmonella enterica* serovar Typhimurium causes enteritis and systemic disease in susceptible host species and can be carried asymptptomatically by food animals, including poultry and swine. Human infection is typically marked by self-limiting enteritis but may progress to systemic infection characterized by fever, nausea, vomiting, diarrhea, neutropenia, and in severe cases death. In addition to its effects on human hosts, *S. typhimurium* causes systemic disease in mice reminiscent of human typhoid or enteric fever caused by *S. typhi*. Because of this relationship, infection of mice with *S. typhimurium* has been widely employed as a model of human infection with *S. typhi*. Both diseases result from fecal-oral transmission of the bacteria. Once ingested by a new host, *Salmonella* traverses the gastrointestinal tract where it encounters a series of complex environments and reaches the ileum, the primary site of *Salmonella* infection (Carter and Collins, 1974). In the ileum, *Salmonella* can infect epithelial cells and M-cells. Preferential infection of the ileum is not due solely to the presence of specialized cells such as M-cells because *Salmonella* can colonize other regions of the small intestine and can infect colonic epithelium in mice treated with streptomycin (Meynell and Subbaiah, 1963). As the colon lacks M-cells, it is likely that the antibiotic alters the environment of the colon, thereby making infection possible. One way that streptomycin changes the intestinal environment is by reducing the population of anaerobic bacteria in the colon. This in turn decreases the level of short chain
fatty acids (SCFAs) which inhibit the growth of *Salmonella* (Meynell and Subbaiah, 1963; Meynell, 1963; Bohnhoff et al., 1964a; Bohnhoff et al., 1964b). In the ileum, *Salmonella* induces its uptake by the intestinal epithelial cells through the action of a type III secretion system encoded at centisome 63 on the *Salmonella* chromosome (Galan and Curtiss, 1989; Galan et al., 1992; Behlau and Miller, 1993; Mills et al., 1995). This 40 kb region of the chromosome is designated the *Salmonella* Pathogenicity Island 1 (SPI-1). The SPI-1 type III secretion system consists of the secretion apparatus, transcriptional regulators, and secreted effector proteins that are translocated to the host enterocyte cytoplasm. SPI-1 is required for invasion of the intestinal epithelium but is not required for systemic infection (Murray and Lee, 2000).

Epithelial cells infected by *Salmonella* produce interleukin 1 (IL-1), IL-8, pathogen-elicited epithelial chemoattractant (PEEC), prostaglandin E2 (PGE2), and chemokines. IL-1, IL-8, PEEC, and PGE2 attract polymorphonuclear cells (PMN) to the intestinal epithelium (Eckmann et al., 1993; Jung et al., 1995; McCormick et al., 1995a; McCormick et al., 1995b; McCormick et al., 1998). The recruited PMN migrate between the epithelial cells (enterocytes) and into the intestinal lumen by expressing tight junction proteins (Parkos et al., 1992; McCormick et al., 1993; McCormick et al., 1995a; McCormick et al., 1995b). Once in the intestinal lumen, the PMN phagocytose luminal *Salmonella*. The PMN also secrete additional IL-1, IL-6, and IL-8, which helps recruit macrophages to the site of infection. The PMN and recruited macrophages also phagocytose bacteria as
the bacteria cross the basolateral membrane of the enterocytes and enter the lamina propria of the intestine. Once internalized by the macrophages, *Salmonella* is protected from destruction by the immune system. Within the macrophage, *Salmonella* withstands the acidic environment of the phagolysosome and replicates (Carrol et al., 1979; Alpuche-Aranda et al., 1994; Rathman et al., 1996). Phagocytosed bacteria are disseminated throughout the host by macrophages and go on to cause systemic disease, including infection of the liver and spleen. In addition to the well-studied infection of and dissemination by macrophages, another population of CD18$^+$ cells, dendritic cells, has also recently been identified as a mechanism for *Salmonella* dissemination throughout the host (Vazquez-Torres et al., 1999). Dendritic cells are recruited to the intestinal epithelium in response to the presence of bacteria (Rescigno et al., 2001). Once present, the dendritic cells express tight junction proteins (just as the PMNs do), enabling them to form tight junctions with the enterocytes. This allows the dendritic cells to extend into and sample the intestinal lumen without disrupting the epithelial border (Rescigno et al., 2001). The dendritic cells are specialized cells whose function is not completely understood, although they are known to sample antigens at mucosal surfaces and function as antigen presenting cells by trafficking to regional lymph nodes where they interact with T-cells and B-cells (Reviewed Iwasaki and Kelsall, 1999; Neutra et al., 2001). *Salmonella* induces greater levels of dendritic cell recruitment than does *Escherichia coli* and is able to survive within membrane bound vacuoles inside dendritic cells (Niedergang et al., 2000; Rescigno et al.,...
2001). For these reasons, dendritic cells have been proposed to offer an additional site where Salmonella may be protected from the adaptive immune system.

**Salmonella Pathogenicity Island I**

Engulfment of Salmonella by the normally non-phagocytic enterocytes requires the expression of invasion genes that comprise the type III secretion system encoded at SPI-1. The expression of the SPI-1 type III secretion system is regulated by genetic regulators both within SPI-1 such as HilC/HilD, HilA, and InvF, and outside SPI-1 such as PhoP/PhoQ, BarA/SirA, and CsrA/CsrB. Additionally, environmental conditions similar to those encountered in the intestinal tract, such as near-neutral pH, high osmolarity, and reduced oxygen tension, are known to regulate SPI-1 invasion genes (Galan and Curtiss, 1990; Francis et al., 1992; Lee et al., 1992). Genetic and environmental control of invasion gene expression is coordinated within the pathogenicity island at the level of HilA (Lee et al., 1992; Bajaj et al., 1996). HilA is a transcriptional regulator of the OmpR/ToxR family (Bajaj et al., 1995). Both HilC and HilD are positive regulators of *hilA* expression (Schechter et al., 1999; Lucas and Lee, 2001). InvF regulates expression of secreted effector proteins both dependently and independently of *hilA* (Eichelberg and Galan, 1999; Lostroh et al., 2000). Invasion genes are repressed by PhoP/PhoQ, which responds to low magnesium concentration, and are positively regulated by BarA/SirA (Behlau and Miller, 1993; Pegues et al., 1995; Ahmer et al., 1999; Altier et al., 2000a). The signal for BarA has not yet
been identified. Genetic evidence suggests that SirA interacts with BarA (Altier et al., 2000a). Regulation of invasion genes by CsrA/CsrB is complex. Csr stands for carbon storage regulator. Originally identified in *E. coli*, the csr system is a global regulatory system that functions at the level of mRNA stability and regulates glycogen synthesis and gluconeogenesis in *E. coli* (Romeo et al., 1993; Liu et al., 1995; Romeo, 1996; Liu and Romeo, 1997; Romeo, 1998; Wei et al., 2001). The csr system consists of CsrA, a 61 amino acid protein, and CsrB, an untranslated RNA. CsrB regulates the level of CsrA by binding the protein (Liu et al., 1997). CsrA is a post-transcriptional regulator of mRNA stability that can act either to enhance degradation of mRNA, as in the case of *glgC*, or to stabilize it, as in the case of *flhDC* (Wei et al., 2001; Baker et al., 2002). Loss of CsrA results in increased levels of enzymes involved in glycogen storage and decreased ability of *E. coli* to grow on acetate as a sole carbon source (Liu et al., 1995; Baker et al., 2002). In *S. typhimurium*, CsrA regulates SPI-1 invasion gene expression and invasion of epithelial cells (Altier et al., 2000b). Additionally, both loss and over expression of csrA decrease invasion gene expression, suggesting that the level of csrA must be tightly controlled for optimal invasion (Altier et al., 2000b). Loss of CsrB also decreases invasion gene expression and epithelial cell invasion (Altier et al., 2000a).

The type III secretion system translocates proteins encoded within SPI-1 including AvrA, SipABCD, and proteins encoded elsewhere on the *Salmonella* chromosome including SopE, SopE2, SopB, and SopD. Once inside the
enterocyte, SipA, a secreted effector protein, binds to the host cell’s actin cytoskeleton preventing its depolymerization and inducing membrane-ruffling (Zhou et al., 1999). The host cell membrane then surrounds and engulfs the bacteria (Takeuchi, 1967). SipA also functions as an activator of the signaling cascade, leading to production of PEEC by enterocytes, which in turn induces PMN migration to the intestinal lumen (Lee et al., 2000). SipB activates caspase-1, which also functions as interleukin-1 converting enzyme (ICE) thereby inducing apoptosis within the epithelial cells (Hersh et al., 1999). The activation of this enzyme results in the production and secretion of interleukin-1 (II-1) by the infected epithelial cells. SopE activates Rho GTPases including Cdc42 and Rac, which influence cytoskeletal rearrangements (Hardt et al., 1998). SopE2 is a homolog of SopE and has similar functions (Bakshi et al., 2000; Stender et al., 2000). SopB is an inositol phosphate phosphatase that induces an increase in inositol phosphate, thereby blocking chloride channel closure thus inducing secretory diarrhea (Norris et al., 1998). Loss of SopB decreases fluid secretion and the influx of PMNs, but sopB mutants still cause diarrhea in calves when the dose is high (Galyov et al, 1997, Watson et al., 1998, Tsolis et al., 1999).

**Salmonella Pathogenicity Island 2**

SPI-2 encodes a second type III secretion system required for *Salmonella*’s survival in macrophages and intracellular vesicles (Miller et al., 1989; Ochman et al., 1996; Cirillo et al., 1998; Hensel et al., 1998; Uchiya et al., 1999). Expression of SPI-2 is induced intracellularly in macrophages and epithelial cells (Valdivia and
Falkow, 1997; Pfeifer et al., 1999). *In vitro*, SPI-2 is induced in *Salmonella* grown in minimal media with low levels of magnesium or calcium or by phosphate starvation, conditions thought to replicate the environment of the phagolysosome or intracellular vesicles (Valdivia and Falkow, 1997; Cirillo et al, 1998; Deiwick et al. 1999). Genetic regulators of SPI-2 include PhoP/PhoQ, which responds to low levels of magnesium, EnvZ/OmpR, which may respond to low osmolarity in the phagolysosome, and SsrA/SsrB, which responds to an as yet undetermined signal (Garcia-Vescovi et al., 1996; Deiwick et al. 1999; Lee et al., 2000). SPI-2 gene expression is dependent on SsrA/SsrB, which in turn is regulated by EnvZ/OmpR (Cirillo et al., 1998; Deiwick et al., 1999; Lee et al., 2000). SsrB has significant homology to UvrY of *E. coli* and SirA of *Salmonella*, while the predicted structure of SsrA is that of a phospho-relay type sensor kinase similar to BarA or ArcA (Deiwick et al., 1999). Recent work has shown that SsrA/SsrB regulate genes outside SPI-2, specifically *pipB*, which is encoded at SPI-5 (Knodler et al., 2002). Also encoded at SPI-2 is the anaerobic tetrathionate reductase, *ttrABC* and an associated two component regulatory system, *ttrRS* (Hensel et al., 1997; Hensel et al., 1999). This will be discussed in detail below.

**Flagellar regulation and chemotaxis**

Flagellar synthesis in *E. coli* and *S. typhimurium* requires the coordinated expression of seventeen operons. These operons are expressed in stages early, middle and late. The early genes consist primarily of the central regulatory genes *flhD* and *flhC*, which encode transcriptional activators that are transcribed in
response to a variety of environmental and global regulatory signals (Reviewed Chilcott and Hughes, 2000). Regulators of flagellar synthesis include cyclic AMP-cAMP receptor protein (cAMP-CRP) (Yokota and Gots, 1970; Silverman and Simon, 1974), heat shock proteins (Shi et al., 1992), DNA supercoiling (Shi et al., 1993; Li et al., 1993; Kutsukake, 1997), phosphatidylethanolamine and phosphatidylglycerol synthesis (Shi et al., 1993; Mizushima et al., 1994), acetyl phosphate through OmpR (Shin and Park, 1995), and the carbon storage regulator, CsrA (Wei et al., 2001).

FlhDC regulate the transcription of the middle genes that encode the hook-basal body of the flagella. The middle genes include fliA, which encodes the alternative transcription factor, $\sigma^{28}$ (Ikebe et al., 1999). The expression of the late flagellar genes, which includes genes that encode the external filament and the chemotaxis proteins, requires $\sigma^{28}$. During synthesis of the hook-basal body, $\sigma^{28}$ is prevented from binding the promoters of the late genes by FlgM (Reviewed Chilcott and Hughes, 2000). Once the basal body is complete, FlgM is secreted by the cell, thereby freeing $\sigma^{28}$ and initiating transcription of the late genes. Middle genes include those that encode structure and assembly of the hook-basal body of the flagella including flgAMN, flgBCDEFGHIJKL, flhBAE, fliAZY, fliDST, fliE, fliFGHIJK, fliLMNOPQR and the transcriptional regulators FlgM and $\sigma^{28}$, encoded by flgM and fliA respectively. FlgM binds to $\sigma^{28}$ forming an inactive complex.

When the hook-basal body is formed, FlgM is secreted from the cell. This frees
allowing it to initiate transcription of the late genes. The late genes are those required for the late assembly stage and for motility, chemotaxis, and aerotaxis and include \( \text{flgMN, flgKL, fliC, fliDST, fljBA, motAB, cheAW, cheRBYZ, tar, tsr, and aer.} \)

In addition to its role in coordinated movement of the bacteria, flagella also play a significant role in invasion and the host immune response. Flagellar mutants have decreased ability to invade epithelial cell monolayers and induce less fluid secretion in bovine ileal loops than their wild type counterparts (Schmitt et al., 1996; Schmitt et al., 2001). FliA also regulates SPI-1 invasion gene expression (Eichelberg and Galan, 2000). \textit{Salmonella} produces two forms of the structural protein flagellin FliC and FljB, which are subject to phase variation. Flagellin induces proinflammatory mediators in host epithelial cells by traversing the epithelial cells and binding to Toll-like receptor 5 (TLR5) on the basolateral surface of the enterocytes (Eaves-Pyles et al., 2001 Gewirtz et al., 2001; Reed et al., 2002). FliE mutants are able to interact with the apical surface of intestinal epithelial cells but are unable to induce the cells to produce IL-8 and have reduced signaling through NF-\(\kappa\)B (Reed et al., 2002). Flagellin also induces recruitment of dendritic cells by stimulating epithelial cell secretion of CCL20, also called MIP-3 alpha and the ligand for dendritic cell receptor CCR6 (Sierro et al., 2001).

The intestinal environment

The gastrointestinal tract is a complex system that functions to break down food into smaller molecules that can be readily absorbed by the specialized
epithelium of the small intestine. A number of pathogens and parasites have exploited this unique environment, and the ability of *S. typhimurium* to sense and respond to the intestinal environment enhances its ability to survive and replicate and to find new hosts. The intestinal tract is awash in breakdown products of protein, fat, and carbohydrate metabolism. Among these are amino acids, triglycerides, glucose, maltose and maltodextrins, and SCFAs. Additional SCFAs are produced by bacterial fermentation in the colon. Retrograde leakage of the ileocecal valve probably accounts for the increased presence of SCFAs in the ileum as compared to the rest of the small intestine. The ileum is also unique in its ability to absorb vitamin B$_{12}$. Vitamin B$_{12}$ is released from food during digestion. It is first bound to haptocorrin. Then, in the small intestine, it is transferred to intrinsic factor. In the ileum, the B$_{12}$-intrinsic factor complex attaches to a receptor on the enterocytes. The receptor-ligand complex is endocytosed and degraded in lysosomes. B$_{12}$ is then transferred to transcobalamin and carried to the rest of the body. The ileum is also unique in its elevated ability to exchange bicarbonate for chloride. The gradient for this exchange is 10 times greater in the ileum than elsewhere in the small intestine. To effectively colonize the intestine and invade the intestinal epithelium, *Salmonella* must not only survive this complex intestinal environment, it must exploit it. To this end, the complicated makeup of the intestinal milieu may provide signals that regulate bacterial gene expression. Some of these environmental conditions and the resultant bacterial response will be described in greater detail below.
Short chain fatty acids

Short chain fatty acids (SCFAs) are produced primarily by anaerobic bacteria found in the cecum and colon of mammalian species as a result of carbohydrate fermentation (Macfarlane et al., 1992). The predominant SCFAs found in the gastrointestinal tract are acetate, propionate, and butyrate. These vary in both total concentration and relative proportion to each other depending upon the location within the gastrointestinal tract. In the small intestine, particularly the distal ileum, acetate is the predominant SCFA, comprising 85% of the total SCFA concentration, while propionate and butyrate in equal proportions make up the remainder (Argenzio and Southworth, 1974a; Argenzio and Southworth, 1974b). In the cecum and colon the total level of SCFA increases from the 30 to 40 mM present in the ileum to approximately 200 mM depending on diet (Argenzio and Southworth, 1974a; Argenzio and Southworth, 1974b; Cummings et al., 1987). In general, SCFAs are considered to inhibit the growth of bacteria. However, Salmonella utilizes the products of ackA-pta and the prpBCDE operon to phosphorylate acetate and propionate and to convert them to acetyl-CoA and propionyl-CoA respectively. Ultimately acetyl-CoA and propionyl-CoA are converted to pyruvate for use in either the TCA cycle or gluconeogenesis.

Maltose

Maltose and maltodextrins are found in high levels in the intestinal tracts of most mammals as byproducts of oligosaccharide metabolism. Starch comprises half of the carbohydrate present in the diet and consists of chains of glucose that
are linked together with either $\alpha$ 1-6 bonds or $\alpha$ 1-4 bonds. Salivary and pancreatic amylase cleaves starch at $\alpha$ 1-4 linkages yielding maltose and maltotriose. Maltose is converted to glucose, which has its own transport system, by maltase. The uptake of maltodextrins by \textit{S. typhimurium} and \textit{E. coli} utilizes a maltose transport system that consists of an outer membrane pore, a translocation complex, and a maltose binding protein (MBP). The translocation complex is encoded by \textit{malFGK}$_2$, the maltose binding protein (MBP) by \textit{malE}, and \textit{lamB} encodes a specific pore for maltodextrins that also serves as the receptor for phage $\lambda$ (Reviewed in Boos and Shuman, 1998). The system also includes several enzymes required for the catabolism of maltodextrins. Among these are \textit{malP} and \textit{malQ}, which encode essential enzymes for maltodextrin metabolism, maltodextrin phosphorylase and amylomaltase respectively and \textit{malS}, which encodes a nonessential maltodextrin metabolizing enzyme, periplasmic $\alpha$-amylase. The maltose system is regulated by MalT, a transcriptional regulator required for transcription at \textit{mal} promoters (Reviewed Boos and Shuman, 1998). MalT is activated by binding ATP and maltotriose (Richet and Raibaud, 1989; Dardonville and Raibaud, 1990). Expression of \textit{malT} is subject to catabolite repression and requires the presence of cAMP/CAP (Debarbouille and Schwartz, 1979; Chapon 1982; Chapon and Kolb, 1983). Mutants lacking adenylate cyclase or CAP are unable to grow on maltose (Reviewed in Boos and Shuman, 1998). MBP has a high affinity for maltose and maltodextrins, but not all substrates that
MBP binds are transported into the cell. *mal* gene expression is low in cells actively growing on rich media such as Luria Bertani broth and probably reflects concentration of internal cAMP and catabolite repression (Reviewed in Boos and Shuman, 1998). High levels of *mal* expression in *E. coli* grown in the absence of maltose or maltodextrins suggest endogenous production of a MalT inducer. To date the only known inducer of *malT* in vitro is maltotriose (Raibaud and Richet, 1987). Endogenous maltotriose is synthesized either from glycogen or from trehalose. Glycogen is thought to be converted to maltotriose through the action of *glgX*, whose amino acid sequence shares homology with amylases (Romeo et al., 1988). *malQ* mutants are constitutive for expression of the maltose system unless they carry secondary mutations in *glgA*, which encodes glycogen synthase or *glgC*, which encodes ADP-glucose pyrophosphorylase. The presence of these secondary mutations results in loss of constitutive expression of the maltose system and the mutant regains its ability to be induced by maltose (Decker et al., 1993). In the absence of glycogen, trehalose is able to induce the *mal* genes (Klein and Boos, 1993). Trehalose is metabolized into glucose and glucose-1-phosphate (Rimmele and Boos, 1994). The presence of free glucose induces the *mal* genes. Further, growth of *E. coli* on micromolar concentrations of glucose or on glycerol also results in increased expression of the *mal* genes (Death and Ferenci, 1994). This contradicts the previously noted findings for growth on rich media where glucose transport inhibits *mal* gene expression. This inhibition is the result of interaction of unphosphorylated enzyme IIA\textsuperscript{Glc} of the
phosphoenolpyruvate dependent sugar phosphotransferase system (PTS) and malK and is not present in glucose limiting conditions or in trehalose metabolism (Reviewed in Boos and Shuman, 1998). Thus control of the mal operon is achieved by integrating recognition of internal free glucose levels into control of expression of malT.

Loss of the ability to utilize maltose has been associated with decreased virulence of enteropathogenic E. coli (EPEC) and Vibrio cholerae. EPEC infections are characterized by close association of the bacteria with the host cell, but invasion does not occur. EPECs carrying mutations in espB, an EPEC secretory protein, are defective in expression of maltoporin (Kumar et al., 2001). Expression of maltoporin is restored by complementing the espB mutation. Complementation also restores the ability of the mutant to induce cytopathic changes in cultured cells to wild type levels. Purified maltoporin itself is not cytopathic and its role in virulence is not fully understood, although it is possible that maltoporin offers an alternate route of protein secretion. EspB is translocated to the host cell cytoplasm and to the cell membrane, and maltoporin may provide an alternate means of secreting this protein. Maltoporin has also been associated with secretion of additional proteins required for virulence. One example of this is the decreased secretion of cholera toxin and decreased production of hemagglutinin and hemagglutinin protease in malQ and malF mutants of V. cholerae (Lang et al., 1994). However, it remains clear how the production of these virulence factors is regulated by the maltose system.
Vitamin $B_{12}$

Regulation of genes associated with vitamin $B_{12}$ synthesis.

Unlike *E. coli*, *Salmonella* is able to synthesize vitamin $B_{12}$ *de novo*. $B_{12}$ synthesis occurs under anaerobic conditions and requires the expression of the $cob$ operon, which is located at 44 min on the *Salmonella* chromosome and includes $cob$ and $cbi$ genes (Roth et al., 1993; Reviewed Roth et al., 1996). There are two $B_{12}$ co-enzymes, adenosylcobalamin and methylcobalamin. Adenosylcobalamin is involved in conversion of methylmalonyl-CoA to succinyl CoA and as such is important in catabolism of odd chain fatty acids. Synthesis of vitamin $B_{12}$ requires expression of genes associated with threonine synthesis, glycine synthesis, and synthesis of the adenosyl cobalamide precursor. Threonine is utilized as part of the aminopropanol side chain (Lowe and Turner, 1970; Kurumaya and Kajiwara, 1990). Threonine is also converted to glycine. Glycine is used in the synthesis of dimethylbenzimidazole, which is attached to the corrin ring as part of the nucleotide loop of $B_{12}$ (Reviewed Roth et al., 1996). The glycine cleavage system converts tetrahydrofolate to 5,10 methylene tetrahydrofolate. The methyl group from methylene tetrahydrofolate is donated to cobalamin, generating methylcobalamin, which in turn donates the methyl group to homocysteine in the synthesis of methionine. In addition to synthesis of $B_{12}$, *Salmonella* also possesses the genes that encode a $B_{12}$ transport system. This system requires several proteins including an outer membrane protein BtuB that, in conjunction with TonB, binds $B_{12}$ and translocates it to the periplasmic space.
Once in the periplasmic space, B$_{12}$ binds to BtuF, which in turn interacts with an inner membrane translocation system formed by BtuC and BtuD (Reviewed Roth et al., 1996). As stated earlier, the distal ileum is the primary site of vitamin B$_{12}$ absorption in most mammalian species and is well studied in humans. As such it is most likely present in highest concentration at this site, suggesting a possible role for it as a signal for *Salmonella*. Additionally, vitamin B$_{12}$ is required by *Salmonella* for use of two additional carbon sources that are likely present in abundance in the intestinal tract, ethanolamine and propanediol, to be discussed in greater detail below.

Regulation of genes associated with vitamin B$_{12}$ utilization.

Utilization of ethanolamine and propanediol as carbon and energy sources requires B$_{12}$ (Rondon and Escalante-Semerena, 1992; Lawrence and Roth, 1995; Walter et al., 1997). Both ethanolamine and propanediol are carbon sources present in the gastrointestinal tract. Ethanolamine is a component of both procaryotic and eucaryotic cell membranes, while propanediol is a product of the breakdown of rhamnose, a component of plant cell walls, and fucose, a glycoconjugate found on the surface of intestinal epithelial cells (Badia et al., 1985; Obradors et al., 1988; Bry et al., 1996). Ethanolamine is utilized as a carbon source by both *Salmonella* and *E. coli* and is converted to acetyl-CoA, which can enter the TCA cycle and the glyoxalate shunt (Reviewed Roth et al. 1996). *Salmonella* uses propanediol as a carbon source while *E. coli* lacks the genes required for propanediol metabolism. Propanediol is converted to propionyl-CoA,
which joins oxaloacetate via the 2-methyl-citrate pathway and is converted to succinate and pyruvate (Horswill and Escalante-Semerena, 1999; Tsang et al., 1998). B$_{12}$ is synthesized anaerobically, while early work demonstrated only aerobic metabolism of ethanolamine and propanediol. Recent work has demonstrated that the utilization of tetrathionate as an electron acceptor allows metabolism of ethanolamine and propanediol in the absence of oxygen (Price-Carter et al. 2001). A proposed mechanism of reduction of tetrathionate suggests reduction of tetrathionate to thiosulfate by enzymes encoded by the *ttr* operon (Barrett and Clark, 1987; Hensel et al., 1999; Price-Carter et al. 2001). Thiosulfate is then reduced to sulfite and hydrogen sulfide by enzymes encoded by the *phs* operon (Heinzinger et al., 1995).

**Conclusion**

The intestinal tract provides an interface between the host and the virtually unlimited variety of molecules that result from the breakdown of protein, fats, and carbohydrates. In the ileum, these include SCFAs, maltodextrins, vitamin B$_{12}$, ethanolamine, and propanediol. The ability of *Salmonella* and other pathogens to exploit these and other byproducts of metabolism either as signals or as energy sources enhances their ability to colonize the intestinal tract and potentially to spread to additional hosts. Ongoing work studying the effect of exposure to acid, bile, and bicarbonate on gene expression in Salmonella and other enteric pathogens provide ready examples of fertile new areas of investigation of bacterial response to complex environments. Further analysis of the physiology of the
gastrointestinal tract may offer insight into additional signals used by *Salmonella* to fine tune gene expression.
References


Intestinal short chain fatty acids alter *Salmonella typhimurium* invasion gene expression and virulence through BarA/SirA

**Summary**

*Salmonella typhimurium* causes enteric and systemic disease by invading the intestinal epithelium of the distal ileum, a process requiring the invasion genes of *Salmonella* pathogenicity island 1 (SPI-1). BarA, a sensor kinase postulated to interact with the response regulator SirA, is required for the expression of SPI-1 invasion genes. We found, however, that a *barA* null mutation had little effect on virulence using the mouse model for septicemia. This confounding result led us to seek environmental signals present in the distal ileum that might supplant the need for BarA. We found that acetate restored the expression of invasion genes in the *barA* mutant, but had no effect on a *sirA* mutant. Acetate had its effect only at a pH that allowed its accumulation within the bacterial cytoplasm and not with the deletion of *ackA* and *pta*, the two genes required to produce acetyl-phosphate. These results suggest that the rising concentration of acetate in the distal ileum provides a signal for invasion gene expression by the production of acetyl-phosphate in the bacterial cytoplasm, a pathway that bypasses *barA*. We further found that a Δ(*ackA-pta*) mutation alone had no effect on virulence, but in combination with Δ(*barA*) it increased the oral LD$_{50}$ by 24-fold. Thus, the combined loss of the BarA- and acetate-dependent pathways is required to reduce virulence. Two other short chain fatty acids (SCFA), propionate and butyrate, present in high concentrations in the cecum and colon had effects opposite to
those of acetate: neither restored invasion gene expression in the barA mutant, and both, in fact, reduced expression in the wild type strain. Further, a combination of SCFA found in the distal ileum restored invasion gene expression in the barA mutant, while colonic conditions failed to do so and also reduced expression in the wild type strain. These results suggest that the concentration and composition of SCFA in distal ileum provide a signal for productive infection by Salmonella, while those of the large intestine inhibit invasion.

Introduction

Salmonella enterica serovar typhimurium is the leading cause of human death due to foodborne illness in the United States. In humans, S. typhimurium causes gastroenteritis that can develop into systemic disease. S. typhimurium also colonizes the gastrointestinal tracts of a variety of food-producing animals including cattle, poultry and swine and causes gastroenteritis in cattle, presenting reservoirs for human infection. An early step in Salmonella pathogenesis is the penetration of intestinal epithelial cells (Takeuchi, 1967; Jones et al., 1994). This requires the expression of invasion genes, encoded by Salmonella pathogenicity island I (SPI-1), one of several pathogenicity islands required for full virulence (Mills et al., 1995; Sanderson et al., 1995). SPI-1 is located at centisome 63 and encodes a type III secretion system (TTSS) and associated effector proteins (Mills et al., 1995; Sanderson et al., 1995). The effector proteins are translocated by the TTSS apparatus into the intestinal epithelial cells of the eucaryotic host, inducing actin cytoskeletal rearrangements (Francis et al., 1992, 1993; Pace et al., 1993;
Kaniga et al., 1996; Collazo and Galán, 1997; Fu and Galán, 1998; Hardt et al., 1998a, Hardt et al., 1998b; Kubori et al., 1998; Norris et al., 1998; Zhou et al., 1999). These rearrangements result in ruffling of the epithelial cell membrane and cause engulfment of the bacteria by the host cell. Two additional pathogenicity islands are required for the enteric phase of pathogenesis, SPI-4 and SPI-5. SPI-4 is a 27 kb fragment present at centisome 92 that may encode a type 1 secretion system (Wong et al., 1998). SPI-4 is also the site of a Tn10 insertion that renders Salmonella unable to replicate within macrophages (Baumler et al., 1994; Wong et al., 1998). SPI-5 encodes SopB, a Salmonella secreted protein. SopB is an inositol phosphatase that is translocated into the eucaryotic host cell by the SPI-1 TTSS (Galyov et al., 1997; Hong and Miller, 1998; Norris et al., 1998; Wood et al., 1998). Once present in the eucaryotic cell, SopB causes an increase in inositol phosphate that results in closure of the chloride channels, affecting electrolyte transport and causing fluid secretion (Norris et al., 1998, Eckmann et al., 1997). Loss of SopB reduces but does not completely abrogate fluid accumulation and polymorphonuclear leukocyte (PMN) migration in the bovine ligated loop model (Galyov et al., 1997). Genes in SPI-4 and SPI-5 are controlled by HilA, a transcriptional regulator encoded within SPI-1, and by SirA, a regulator of hilA (Ahmer et al., 1999).

The genes of SPI-1 are under the complex control of a number of transcriptional regulators, encoded both within and outside of SPI-1. A central regulator encoded within SPI-1 is HilA, a member of the OmpR/ToxR family (Bajaj
et al., 1995; Bajaj et al., 1996). Within SPI-1, HilA controls *invF*, which encodes a transcriptional regulator of the AraC family (Kaniga et al., 1994). HilA and InvF jointly control the expression of SPI-1 secreted effector proteins, but independently control components required for the secretion of those proteins (Eichelberg and Galán, 1999; Darwin and Miller, 1999). InvF is also subject to control independent of HilA (Rakeman et al., 1999; Altier et al., 2000a; Altier et al., 2000b). The expression of *hilA* is itself controlled by two additional SPI-1 regulators: HilC and HilD (Eichelberg et al., 1999; Schechter et al., 1999). Both function by binding to a region upstream of the *hilA* promoter that is required for both environmental and genetic control of *hilA*, thus derepressing *hilA* expression (Lucas and Lee, 2001).

Externally encoded regulators of SPI-1 genes include PhoP/PhoQ, BarA/SirA, and the *csr* system. PhoP/PhoQ is a negative regulator of HilA, activated by conditions of low magnesium concentration such as those encountered in the macrophage phagolysosome and the cytoplasm of intestinal epithelial cells (Bajaj et al., 1996; Soncini et al., 1996). The physiologic role of PhoP/Q may be to reduce the expression of invasion genes once the bacteria are internalized by intestinal epithelial cells (Fields et al., 1989; Galán and Curtiss, 1989; Miller et al., 1989). BarA/SirA are positive regulators of *hilA* transcription. BarA is a sensor kinase of the phosphorelay type required for invasion gene expression and for bacterial penetration of epithelial cells (Altier et al., 2000a). The signal to which BarA responds is as yet unknown. On the basis of genetic evidence, SirA has been proposed as the cognate response regulator for BarA. SirA regulates *hilA*, and is
required for invasion gene expression and enteropathogenesis (Ahmer et al., 1999, Altier et al., 2000a). Loss of either barA or sirA reduces SPI-1 invasion gene expression (Ahmer et al., 1999; Altier et al., 2000a). Overproduction of BarA does not suppress a sirA mutation, but overproduction of SirA does suppress a barA mutation (Altier et al., 2000a), indicating that BarA requires SirA for its function, but that SirA, at least when overexpressed, can act independently of BarA. The csr system is comprised of csrB, an untranslated RNA, and CsrA, a small protein that has been shown in E. coli to regulate gene expression post-transcriptionally by modulating the half-life of target messages (Liu et al., 1995; Liu and Romeo, 1997; Wei et al., 2001). csrB binds CsrA, titrating it, and thereby reducing the level of free CsrA protein (Liu et al., 1997). In S. typhimurium, loss of CsrA causes a severe reduction in the expression of SPI-1 genes, at least in part through its effects on hilC and hilD (Altier et al., 2000b). Overexpression of CsrA also reduces expression of invasion genes, suggesting that levels of CsrA must be tightly controlled for maximal invasion gene expression. Other organisms such as Erwinia carotovora and Pseudomonas fluorescens also utilize homologs of the csr system to regulate genes involved in pathogenesis. In E. carotovora, rsmA and rsmB regulate expression of pectate lyase, polygalacturonase, cellulase and protease while in P fluorescens, rsmA controls hydrogen cyanide synthesis and extracellular protease (Murata et al., 1994; Cui et al., 1995; Liu, Y. et al., 1998; Aarons et al., 2000).
Induction of invasion genes requires coordinated response to the varied environmental signals present in the gastrointestinal tract. Environmental conditions control expression of SPI-1 invasion genes through induction and repression of *hilA* (Bajaj et al., 1996). Conditions that induce *hilA* expression and therefore SPI-1 invasion gene expression include near-neutral pH, low oxygen tension, and increased osmolarity (Galán and Curtiss, 1990; Lee and Falkow, 1990; Lee et al., 1992; Bajaj et al., 1996). These conditions are likely present in the distal ileum, the primary site of *Salmonella* infection (Carter and Collins, 1974).

The pH of the intestinal tract varies with diet, but the small intestine and colon typically range between 6 and 7 (Bohnhoff et al., 1964a; Argenzio and Southworth 1974a; Cummings et al, 1987). The lumen of the small intestine and colon is typically considered anaerobic while the brush border of the small intestine is considered microaerophilic. Osmolarity in the small intestine is high at greater than 300 mOsm (Fordtran and Ingelfinger, 1968). In addition to its effects on *hilA*, conditions of high osmolarity induce changes in DNA supercoiling that affect invasion gene transcription (Galán and Curtiss, 1990). In addition to these conditions, *hilA* is repressed by PhoP/PhoQ (Bajaj et al., 1996). PhoP/PhoQ is activated by low-cation concentrations, implying that *hilA* is repressed by these conditions (Garcia-Vescovi et al., 1996; Groisman, 1998).

The mammalian gastrointestinal tract also contains high levels of short chain fatty acids (SCFA), including acetate, propionate, and butyrate, as the result of the breakdown of food by digestive processes and the action of resident colonic
bacteria, particularly members of the species *Lactobacillus* and *Bacteroides*. SCFA can provide a significant source of energy for the host, and high levels of SCFA inhibit growth of some pathogenic bacteria including *Salmonella* (Bohnhoff et al., 1964a; Meynell, 1963). Mice treated with streptomycin have reduced colonic flora and resultant decreases in levels of SCFA, making them more susceptible to *S. typhimurium* infection (Bohnhoff et al., 1964b; Meynell and Subbaiah, 1963; Meynell, 1963). Altering the dietary levels of cellulose and carbohydrates to alter SCFA levels has long been proposed as a method for changing the intestinal flora of food-producing animals to prevent colonization by pathogens (Bailey et al., 1991; Durant et al., 1999; Fukata et al., 1999; Naughton et al., 2001). SCFA vary in concentration through the gastrointestinal tract. Levels in the small intestine, the site of *Salmonella* invasion, are low at between 20 to 40mM total SCFA, while levels in the colon are high, ranging from 130 to 300 mM, depending on animal species and diet (Bohnhoff et al., 1964a; Argenzio and Southworth, 1974a; Argenzio and Southworth, 1974b; Macfarlane et al., 1992; Cummings et al., 1987). In contrast to the inhibitory effects of SCFA on colonization of the gastrointestinal tract by *Salmonella*, recent studies in poultry have shown that alteration in pH and SCFA levels can increase *Salmonella* invasion gene expression, specifically expression of *hilA* (Durant et al., 2000). Specifically, loss of lactobacilli in the crop results in increases in pH and decreases in SCFA. These changes increase expression of *hilA* two-fold. This suggests that, although high levels of SCFA inhibit growth and colonization of the
gastrointestinal tract by *S. typhimurium*, low levels of SCFA may induce invasion
gene expression and increase host susceptibility.

Here we investigate the role of SCFA and BarA/SirA on *Salmonella*
invasion and virulence. We report that acetate, the predominant SCFA in the
distal ileum, is able to compensate for the loss of *barA*, but not *sirA*, in invasion
gene expression in the form of acetyl-phosphate. We also find that SCFA in
concentrations and proportions that mimic the ileum and colon have differing
effects. Ileal conditions restore invasion gene expression in the *barA* mutant while
colonic conditions do not supplant the *barA* defect and in fact decrease invasion
gene expression in wild type bacteria. These results are consistent with a
mechanism in which SCFA serve as a signal for bacterial invasion of the ileum but
reduce invasion in the colon, and suggest an explanation for the long-held
observation that *Salmonella* preferentially invades the distal ileum.

**Results**

*BarA is not required for virulence in mice*

*S. typhimurium* requires the expression of invasion genes, found in
*Salmonella* pathogenicity island 1 (SPI-1) and encoding a type III secretion
apparatus, to produce enteric salmonellosis. We have previously shown that
BarA, a sensor kinase postulated to interact with the response regulator SirA, is
required for the expression of SPI-1 genes and for the invasion of cultured
epithelial cells (Altier et al., 2000a). To investigate the role of BarA in virulence,
we inoculated BALB/c mice orally with wild type S. typhimurium (ATCC 14028s), and with an isogenic Δ(barA) mutant. While mice do not develop gastroenteritis, they are susceptible to Salmonella and develop septicemia that first requires bacterial penetration of the intestinal epithelium. We found that loss of barA had little effect on lethality in mice, increasing the LD$_{50}$ only 3.9-fold (Table 1). Mice that died in the group inoculated with the barA mutant had only a slightly longer time until death than did mice inoculated with the wild type (Figure 1). We similarly tested the virulence of a sirA::Tn10 mutant. When administered to mice in the same concentration as the wild type strain, none of the mice died (three groups of five mice each, using 10-fold differences in bacterial numbers), producing an LD$_{50}$ at least 9.3-fold higher than the wild type (Figure 1 and Table 1). These results indicate that the loss of barA has little if any effect on virulence in mice and suggest that the requirements for BarA and SirA are different in an animal host.

Acetate suppresses the effects of a barA mutation on invasion gene expression and invasion

To explain our findings that BarA is required for invasion in vitro, but apparently not in vivo, we speculated that environmental conditions in the mouse gastrointestinal tract might induce the expression of SPI-1 genes independent of barA, supplanting the need for BarA. The ileum, cecum, and colon of mammals are rich in short chain fatty acids (SCFA), specifically acetate, propionate, and butyrate, with acetate present in concentrations of approximately 10 mM to 30 mM in the distal ileum, the site of Salmonella invasion (Argenzio and Southworth,
1974a; Argenzio and Southworth, 1974b; Macfarlane et al., 1992; Cummings et al., 1987). We therefore tested the hypothesis that SCFA in physiologically relevant concentrations could restore invasion gene expression in the barA mutant, using lacZY operon fusions to the SPI-1 genes hilA, invF, and sipC. We found that the loss of barA reduced the expression of these three genes: hilA by 7-fold, invF by 14-fold, and sipC by 18-fold (Figure 2A-C). However, the addition of 30 mM sodium acetate in medium buffered to pH 6.7 restored the expression of all three genes to a level greater than that of the wild type. This effect was not due simply to an increase in medium osmolarity, since an equivalent concentration of sodium chloride produced no increase in expression. The loss of sirA reduced expression of the SPI-1 genes from 5- to 21-fold, but in contrast to the response of the barA mutant, the addition of acetate failed to restore expression in the sirA mutant (Figure 2A-C). We also found that 15 mM acetate had effects similar to those of 30 mM acetate for all three fusions in both the barA and sirA mutant backgrounds (data not shown). These results show that acetate in concentrations and at a pH found in the distal ileum of mammals can induce the expression of invasion genes independent of BarA, but not without SirA. To confirm the effect of acetate on the invasion, we next examined the penetration of cultured HEp-2 epithelial cells by the barA mutant. When grown in 30 mM NaCl, the barA mutant was 3-fold less efficient in epithelial cell invasion than was the wild type (Figure 3A). Growth in 30 mM sodium acetate, however, restored invasion to the level of wild type grown under the same conditions.
We next investigated the possibility that acetate is an extracellular signal for invasion gene regulation. The acetate anion, being charged, does not freely enter bacteria; however, the undissociated form of acetate, being uncharged, can penetrate readily to the bacterial cytoplasm. Inside the bacterium, the chemical equilibrium favors dissociation to the acid anion and a hydrogen ion. This process continues until the concentration of undissociated acetate is the same inside and outside the bacterium. Thus, at equilibrium, the concentration of acid anion in the cytoplasm reflects the final pH difference between inside and out, with acetate concentrating in the cytoplasm when the external pH is lower than that within the bacterium. The pH of the distal ileum is approximately 6.7 (Argenzio and Southworth, 1974a, Argenzio and Southworth, 1974b, and Cummings et al., 1987). To test whether acetate acts as an extracellular signal or whether instead internalized acetate might restore invasion gene expression to the \textit{barA} mutant, we grew wild type, the \textit{barA} mutant, and the \textit{sirA} mutant strains in the presence of 30 mM acetate in media buffered to either pH 6.7 (external pH lower than internal pH) or pH 8.0 (external pH higher than internal pH). We found that acetate restored invasion gene expression in the \textit{barA} mutant to greater than wild type levels at pH 6.7, but had no significant effect on the expression of \textit{hilA}, \textit{invF}, or \textit{sipC} in the \textit{barA} mutant at pH 8.0 (Figure 2A-C). We also found that, as for pH 6.7, acetate at pH 8.0 had no effect on expression of these three genes in the wild type or in the \textit{sirA} mutant (Figure 2A-C). Therefore, acetate restores invasion
gene expression only under conditions that promote its accumulation in the bacterial cytoplasm.

*Phosphorylation of acetate is required for suppression of the effect of the barA mutation.*

Once inside bacteria, acetate can be phosphorylated to produce acetyl-phosphate. Since acetyl-phosphate is known to transfer phosphate groups to both sensor kinases and response regulators of two-component regulators and thus activate them (Georgellis et al., 1999), we considered the possibility that acetate induces invasion gene expression only after its conversion to acetyl-phosphate. In *Salmonella*, there are two reversible reactions through which acetyl-phosphate is produced. Acetate kinase, encoded by *ackA*, catalyzes acetyl-phosphate production from exogenous acetate. A second reaction is catalyzed by phosphotransacetylase, encoded by *pta*, and accounts for endogenous production of acetyl-phosphate from acetyl-CoA. The two genes are adjacent to each other, and deletions encompassing both genes are viable in *S. typhimurium* but unable to produce acetyl-phosphate (LeVine et al., 1980). To eliminate the production of acetyl-phosphate, we made a Δ(*ackA*-pta) mutant, then tested the effect of the deletion on invasion gene expression using lacZY fusions to *hilA*, *invF*, and *sipC*. We found that the expression of these genes was decreased 3–fold due to loss of *ackA* and *pta* alone (Figure 2A-C). A Δ(*barA*), Δ(*ackA*-pta) double mutant showed a reduction in expression ranging from 3.6-fold, for *hilA*, to 6.4-fold for *invF*, when compared to the wild type. These decreases were similar to those observed for
the barA and sirA mutants. Unlike the Δ(barA) mutant, however, invasion gene expression was not restored in the Δ(barA), Δ(ackA-pta) mutant by acetate at pH 6.7, nor was there any effect of acetate at pH 8.0. These results indicate that, when bacteria are grown in LB broth, acetyl-phosphate is required for the induction of invasion gene expression both in the presence and absence of barA. Consistent with these results, we also found that HEp-2 cell invasion by the Δ(ackA-pta) mutant was decreased as compared to wild type, but that supplementation of the media with acetate did not reverse the invasion defect of this mutant. Invasion of epithelial cells was also reduced in the Δ(barA), ackA-pta mutant and was similarly not restored by the presence of acetate (Figure 3A).

To assess the importance of acetyl-phosphate production to virulence, we tested the effect of the Δ(ackA-pta) mutant and the Δ(barA), Δ(ackA-pta) double mutant on lethality in mice. We found that the LD₅₀ of the Δ(ackA-pta) mutant was nearly identical to that of the wild type strain (Table 1). The LD₅₀ of the Δ(barA), Δ(ackA-pta) mutant, however, was 24-fold greater than that of the wild type, while, as shown above, the Δ(barA) mutation alone produced only a mild virulence defect (a 3.9-fold increase). This indicates that loss of ackA-pta has little effect on Salmonella virulence, but that the loss of both barA and ackA-pta substantially decreases the virulence of Salmonella in mice. We conclude that either intact barA or ackA-pta is sufficient for full virulence in the mouse model, but that loss of all of these loci attenuates the effect of Salmonella infection.
Short-chain fatty acids propionate and butyrate inhibit invasion gene expression.

Although acetate is the predominant SCFA in the distal ileum of mammals, two other SCFA, propionate and butyrate, are present in high concentrations in the colon and cecum. We next tested whether these two SCFA could, like acetate, induce invasion gene expression in a $\text{barA}$ mutant at the pH of the ileum (pH 6.7). We found that propionate and butyrate did not restore invasion gene expression in the $\text{barA}$ mutant. As shown in Figure 4, neither restored the expression of $\text{hilA}$, $\text{invF}$, or $\text{sipC}$ in the $\text{barA}$ mutant, when supplied at a concentration of 30 mM. Surprisingly, propionate and butyrate significantly decreased expression of invasion genes in the wild type strain, 3- to 7-fold. We also examined the effects of these SCFA in a $\text{sirA}$ mutant, finding that propionate and butyrate elicited decreases in $\text{invF}$ and $\text{sipC}$ expression (8-fold for $\text{invF}$ and 4-fold for $\text{sipC}$) when compared to acetate or sodium chloride. This effect also proved to be specific for invasion genes; expression of several random fusions was not altered by propionate or butyrate (not shown). These results show that butyrate and propionate have effects on invasion gene expression opposite to those of acetate.

Because the gastrointestinal tract contains a mixture of acetate, propionate, and butyrate, we tested whether a mixture of SCFA would have effects different from those of any single SCFA. The ileum is the typical site of *Salmonella* invasion, although the organism can commonly be cultured from the large intestine of animals as well. To examine the effects of intestinal SCFA on the control of *Salmonella* invasion, we used two mixtures of SCFA, one to represent the ileum
and the other to represent the colon. The concentration of total SCFA in the distal ileum is approximately 30 mM, and is comprised of 85% acetate, 7.5% propionate, and 7.5% butyrate (Argenzio and Southworth, 1974a; Argenzio and Southworth, 1974b; Cummings et al., 1987). This equates to 25 mM acetate in combination with 2.5 mM propionate and 2.5 mM butyrate. The colon contains approximately 200 mM total SCFA and is comprised of 55% acetate, 35% propionate, and 10% butyrate, equating to 110 mM acetate, 70 mM propionate, and 20 mM butyrate.

We grew the wild type, the *barA* mutant, the *sirA* mutant, and the *ackA-pta* mutant, alone and in combination with Δ(*barA*), each carrying *lacZY* fusions to *hilA*, *invF*, or *sipC*, in LB broth supplemented with each combination of SCFA, or with sodium chloride at the appropriate concentration as controls. The pH of the ileum and colon are similar at around 6.7 (Argenzio and Southworth, 1974a; Argenzio and Southworth, 1974b), therefore, we buffered the media to pH 6.7. We found that in the wild type background, the concentration and ratio of SCFA that mimics conditions of the distal ileum had effects similar to those of acetate alone (Figure 5). Using concentrations and ratios of SCFA typical of the colon, we found a 3- to 26-fold decrease in invasion gene expression, similar to the effects of propionate or butyrate alone, despite the presence of 110 mM acetate. In the *barA* mutant, the ileal SCFA mixture increased invasion gene expression 5- to 14-fold, restoring expression to greater than wild type levels, similar to the effects of acetate alone. Using colonic concentrations and ratios of SCFA, however, there was no restoration of invasion gene expression in the *barA* mutant. In the *sirA* mutant,
ileal SCFA conditions had no effect on any of the invasion gene fusions. Colonic conditions, however, further reduced the already depressed expression of \textit{invF} and \textit{sipC} in the \textit{sirA} mutant (36- and 8-fold, respectively), suggesting a SirA-independent means of control for these genes. As had been observed using acetate alone, invasion gene expression could not be restored using ileal conditions in either the \(\Delta(\text{ackA-pta})\) or \(\Delta(\text{ackA-pta})\), \(\Delta(\text{barA})\) strains. We also tested the effect of ileal and colonic mixtures of SCFA on the invasion of HEp-2 epithelial cells. We found that ileal SCFA conditions restored invasion of the \textit{barA} mutant to wild type levels, but that colonic SCFA conditions significantly reduced invasion of the wild type (Figure 3B).

The two SCFA mixtures tested differed in both their total concentrations and their ratios of SCFA. To determine which of these factors is important in the control of invasion genes, we also tested a mixture containing the ratio of SCFA found in the colon, but at the concentration that would be found in the ileum. This mixture contained 30 mM total SCFA with 55% acetate, 35% propionate, and 10% butyrate (16.5 mM acetate, 10.5 mM propionate, and 3 mM butyrate). Under these conditions, expression of the three invasion genes in the wild type strain was not significantly different from that observed using the ileal SCFA mixture, but was 3- to 40-fold greater than that using the colonic mixture (Figure 5), suggesting that high propionate or butyrate concentrations are required to suppress invasion gene expression. These results, taken together, show that the conditions of the ileum
induce invasion gene expression independent of BarA, but colonic conditions suppress expression by virtue of high propionate or butyrate concentrations.

*BarA and acetate induce csrB expression*

Invasion gene expression is controlled in *Salmonella* by the *csr* regulatory system, as well as by BarA/SirA (Altier et al., 2000a; Altier et al., 2000b). *csr* regulation consists of CsrA, a protein that affects RNA stability (Liu et al., 1995), and *csrB*, an untranslated RNA that opposes the action of CsrA (Liu et al., 1997). We sought next to determine whether BarA or acetate function in the control of invasion genes through *csr*. Using densitometric analysis of Northern hybridizations, we found that the level of *csrB* in the Δ(barA) mutant was reduced 3-fold when grown in medium buffered to pH 8.0 without acetate (Figure 6). The addition of 30 mM acetate at this pH had no effect on the level of *csrB*. When grown at pH 6.7 without acetate, *csrB* was similarly decreased, 2-fold less than the wild type. However, the addition of 30 mM acetate at pH 6.7 fully restored *csrB* to its wild type level. These results show that BarA is required for the expression of *csrB* and, like SPI-1 genes themselves, that acetate can suppress the loss of BarA in the expression of *csrB*. Similar analyses for *csrA* detected no control of *csrA* message level by either BarA or acetate (not shown).

**Discussion**

In our previous work, we have proposed that BarA, a sensor kinase, and SirA, a response regulator, constitute a two-component regulator that induces the
expression of SPI-1 genes in response to an unidentified extracellular signal. In this work we have shown that acetate in a concentration found in the distal ileum of animals can induce the expression of SPI-1 genes through a pathway that involves SirA but not BarA. In order to produce this effect, acetate must be converted to acetyl-phosphate in the bacterial cytoplasm. We therefore propose a model in which acetate and BarA provide two distinct routes to activate SirA and ultimately SPI-1 expression (Figure 7). At the pH of the distal ileum (approximately 6.7), acetate ion accumulates within bacteria due to the pH difference that exists between the bacterial cytoplasm and the ileal contents. Once within bacteria, acetate may be converted to acetyl-phosphate by acetate kinase \((ackA)\). In addition, endogenous acetyl-phosphate can be produced from acetyl-CoA through the action of phosphotransacetylase \((pta)\) when the level of acetyl-CoA exceeds metabolic needs. The resultant acetyl-phosphate might then phosphorylate BarA, SirA, or both, thereby inducing invasion gene expression. Phosphorylation by acetyl-phosphate is a well-known means of activating two-component regulators. Acetyl-phosphate has been shown to induce the expression of OmpR-dependent genes (McCleary and Stock, 1994), and to act as a phospho-donor for OmpR in the presence or absence of EnvZ, its cognate sensor kinase (Matsubara and Mizuno, 1999). CheY in \(E. coli\) is also activated by acetate (Wolfe et al., 1988). In this case, acetyl-phosphate is produced by both \(ackA\) and \(pta\) (Barak et al., 1998).
For bacteria grown in LB broth, we have found that both \textit{barA} and \textit{ackA-pta} are important for invasion gene regulation. Expression of SPI-1 genes was reduced in the $\Delta(\textit{ackA-pta})$ mutant, even though \textit{barA} was intact in this strain, as well as in the $\Delta(\textit{barA})$ mutant. Also, invasion gene expression in the $\Delta(\textit{barA})$, $\Delta(\textit{ackA-pta})$ mutant was not decreased to a greater extent than in mutants of either \textit{barA} or \textit{ackA-pta} alone. These findings, taken together, suggest that in LB broth acetate and BarA function together to provide the signal for invasion gene induction through SirA. In LB without the addition of exogenous acetate, the acetate signal would presumably be due to endogenous production of acetyl-phosphate from acetyl-CoA by means of \textit{pta}. Since the \textit{barA} mutant also had reduced expression of SPI-1 genes, it must also play a role in the pathway of gene induction. One possibility is that the primary effect of endogenously produced acetyl-phosphate is to activate BarA, rather than SirA, by phosphorylation. Recently, acetate was shown to increase autophosphorylation of ArcB, a sensor kinase of the phosphorelay type similar to BarA (Georgellis et al., 1999). We propose that supplying acetate exogenously increases intracellular concentrations of acetyl-phosphate sufficiently to phosphorylate SirA as well, thus activating invasion genes even in the absence of BarA. In contrast to invasion gene expression in laboratory media, either BarA or the capacity to produce acetyl-phosphate was sufficient for \textit{Salmonella} virulence in mice. The loss of \textit{barA} or \textit{ackA-pta} had little effect on LD$_{50}$, while the loss of both attenuated virulence. This
suggests that there exist in the mouse intestinal tract two independent signals for
the expression of SPI-1 genes, acetate being one and an unknown substance,
acting through BarA, being the other. In contrast to previously published reports
(Ahmer et al., 1999), in this study the sirA mutant appeared to have reduced
virulence. This disparity in LD$_{50}$ may result from growth conditions and bacterial
preparation methods that differed between the two studies.

While acetate induced the expression of *Salmonella* invasion genes, two
other SCFA found primarily in the colon and cecum, propionate and butyrate, had
the opposite effect. The mechanism of inhibition by propionate and butyrate
remains unclear, although the effect appears to be independent of acetate kinase
and phosphotransacetylase, as both propionate and butyrate repress invasion
gene expression in the Δ(ackA-pta) mutant. High concentrations of propionate
and butyrate also inhibited the already low expression of *invF* and *sipC* in a sirA
mutant, suggesting that their effects may be independent of SirA, although the low
expression that exists in the sirA mutant makes such a finding difficult to interpret.
Further experiments are needed to elucidate the mechanism of propionate and
butyrate repression of invasion gene expression.

The loss of BarA also resulted in reduced *csrB* expression, but acetate was
able to restore *csrB* expression in the Δ(barA) strain. *csrB* is an untranslated
regulatory RNA that binds to the regulator CsrA, opposing its action (Liu et al.,
1997). CsrA is a post-transcriptional regulator that can have either a positive or a
negative effect on gene expression, depending upon its target, by altering
message stability (Liu et al., 1997; Wei et al., 2001). The indirect effect of BarA thus would be to reduce the level of active CsrA. We have shown previously that both the overexpression of csrA and its loss reduce the expression of SPI-1 genes (Altier et al., 2000b). It is possible therefore that induction of csrB by BarA or acetate serves to produce a concentration of free CsrA required for maximal invasion gene expression. Unlike other regulators of invasion gene expression that act at the level of transcription, post-transcriptional regulation by CsrA may act as a mechanism for fine-tuning levels of invasion gene products. Despite its control of csrB, BarA must play some other role in the control of SPI-1 genes as well. A null mutant of barA has a more severe invasion defect than does a csrB mutant (Altier et al., 2000a), indicating a BarA-dependent mechanism of invasion gene control that does not include csrB.

*S. typhimurium* is able to sense the environmental conditions required for productive invasion, such as pH, osmolarity, and oxygen tension (Galán and Curtiss, 1990; Lee and Falkow, 1990; Lee et al., 1992; Bajaj et al., 1996), typically invading the distal ileum of the mammalian gastrointestinal tract (Carter and Collins 1974). *S. typhimurium* invades colonic epithelium and causes colitis only under aberrant intestinal conditions, such as those found in streptomycin-treated mice. In these animals, antibiotic treatment reduces the number of lactobacilli and *Bacteroides sp.* present in the colon, causing subsequent reductions in SCFA and increasing colonic pH (Bohnhoff et al., 1964b; Meynell and Subbaiah, 1963; Meynell, 1963). We have shown here that the types and concentrations of SCFA
present can greatly alter the expression of invasion genes. A mixture of SCFA that replicates conditions of the distal ileum induced expression of SPI-1 genes, while the SCFA conditions found in the colon repressed these same genes. It is likely then that Salmonella can use the SCFA conditions of the mammalian intestinal tract as a signal for invasion. Low total SCFA (~30 mM) with a predominance of acetate induces invasion, while high total SCFA (~200 mM) with greater concentrations of propionate and butyrate suppress it.

These opposing effects of SCFA on Salmonella invasion may have important implications for the areas of probiotic and prebiotic therapy. SCFA in the gastrointestinal tract are produced by constituents of the normal gastrointestinal flora including Lactobacillus sp., Bifidobacterium sp., and Bacteroides sp., while enteric pathogens such as E. coli, Shigella and Salmonella produce only low levels of acetate and are virtually unable to produce propionate and butyrate (Krishnan et al., 1998). Probiotics are bacteria, such as Lactobacillus, consumed by humans and animals with the intention of achieving beneficially intestinal effects. Similarly, prebiotics are nutritional supplements designed to encourage the growth of these beneficial bacterial species. Both have been proposed to reduce intestinal colonization by bacterial pathogens (reviewed in Gibson, 1999; Isolauri et al., 2002). One proposed mechanism for the effects of these approaches is the alteration of SCFA levels in the intestinal tract. It is clear that greatly increasing SCFA can reduce Salmonella growth in broth and in animals (Bohnhoff et al., 1964a; Meynell, 1963; Wolin, 1969; Luli and Strohl, 1990; Kouassi and Shelef, 1998).
1996; Durant et al., 1999), however, alterations in SCFA concentrations and ratios that favor acetate and decrease total amount of propionate and butyrate might make the organisms more invasive as well. It remains a concern, therefore, that such approaches might have the unintended consequence of increasing Salmonella virulence.

Experimental Procedures

Bacterial strains

Table 2 lists the chromosomal mutations used in this study. Salmonella strains (isogenic derivatives of ATCC 14028s) containing multiple genetic elements were constructed by sequential P22 transductions (Sternberg and Maurer 1991). Selection for each mutation utilized the corresponding antibiotic resistance shown in the table with the exception of the unmarked $\Delta(barA)$ mutation. Other mutations were moved into the unmarked $\Delta(barA)$ strain. To construct the $\Delta(ackA-pta)$ deletion, a 2.7 kb fragment carrying ackA and its upstream flanking region and approximately the 5' half of pta was PCR amplified from the S. typhimurium 14028s chromosome. The fragment was cut with BsrBRI and ligated, removing the distal portion of ackA and the proximal portion of pta (position 2448326 to 2449821 of Genbank sequence NC_003197). A kanamycin resistance gene was inserted into a PstI site near the resulting single BsrBRI site, and the deletion-insertion was exchanged into the Salmonella chromosome by temperature shifts using pMAK705 (Hamilton et al., 1989). The presence of the
deletion was confirmed by negative PCR reactions (the inability to produce either 
the wild type \textit{ackA} or \textit{pta} fragments). The deletion-insertion was transferred to 
other strains by P22 transduction with selection for kanamycin resistance.

Random \textit{lacZY} fusions were created by transduction of the \textit{hilA::lacZY} fusion, 
marked by tetracycline resistance, into a \textit{hilA339::kan} strain, maintaining selection 
for both tetracycline and kanamycin to select for new transposon insertions. Four 
such fusions producing varying shades of blue on X-gal were used to test the 
specificity of propionate and butyrate on the expression of invasion genes.

\textit{Virulence studies}

Six-week old, female BALB/c mice were inoculated orally with \textit{Salmonella} 
\textit{typhimurium} 14028s wild type, \textit{\Delta(barA)}, \textit{sirA::Tn10d}, \textit{\Delta(ackA-pta)}, and \textit{\Delta(barA)}, 
\textit{\Delta(ackA-pta)} strains. Strains were grown overnight with aeration in E-minimal 
medium with 0.4\% glycerol as a carbon and energy source. The bacteria were 
washed and resuspended in PBS prior to inoculation. Bacteria were titered to 
determine the inoculum. Mice were divided into three groups of five mice. For 
each strain mice were given either \(2 \times 10^6\), \(2 \times 10^5\), or \(2 \times 10^4\) bacteria except for 
the \textit{\Delta(barA)}, \textit{\Delta(ackA-pta)} strain. Mice given the \textit{\Delta(barA)}, \textit{\Delta(ackA-pta)} strain were 
given \(2 \times 10^7\), \(2 \times 10^6\), or \(2 \times 10^5\) bacteria. Mice were allowed to drink 30 \(\mu\)l of a 
10\% sodium bicarbonate solution. Five minutes later, mice were allowed to drink 
the bacterial solution from a pipet tip. Mice were monitored for 30 days. The \textit{LD}_{50} 
was determined as previously described (Reed and Muench, 1938).
\( \beta \)-galactosidase assays

Triplicate (or more) cultures of each bacterial strain to be assayed were grown standing at 37\(^\circ\)C and assayed for \( \beta \)-galactosidase activity as described previously (Miller, 1992). Cultures were grown in LB media supplemented with either 100 mM HEPES, pH 8, or 100mM MOPS, pH 6.7. Cultures were further supplemented with sodium chloride, sodium acetate, sodium propionate, or sodium butyrate either singly at 15 mM or 30 mM or in combination to replicate gastrointestinal SCFA levels (Ileal mixture: 25.5 mM sodium acetate, 2.25 mM sodium propionate, and 2.25 mM sodium butyrate. Colonic mixture: 110 mM sodium acetate, 70 mM sodium propionate, and 20 mM sodium butyrate. Mixture containing ileal total concentration and colonic percentages: 16.5 mM sodium acetate, 10.5 mM sodium propionate, and 3 mM sodium butyrate).

Northern analysis

Wild-type, \( \Delta csrB::kan \), and \( \Delta(barA) \) strains were grown overnight with aeration in LB media. Bacteria were then subcultured 1:50 and grown to late-log phase (OD\(600\) of 0.8) in LB media supplemented with either 100 mM HEPES, pH 8, or 100 mM MOPS, pH 6.7, and either 30 mM sodium chloride or 30 mM sodium acetate. Total RNA was isolated using a SV Total RNA Isolation System kit (Promega, Madison) according to manufacturer’s protocol. RNA concentration was determined spectrophotometrically by measuring OD\(260\), and 5 \(\mu\)g was loaded in each lane of a formaldehyde gel and run for 1.5 hr at 100V. RNA was
transferred to a nylon membrane (Roche, Indianapolis), fixed by ultraviolet crosslinking. Pre-hybridization and hybridization were performed at 37°C with gentle agitation (Sambrook et al., 1989). The membrane was hybridized with the previously described 593 bp csrB region as probe (Altier et al. 2000a). Probe was detected by chemiluminescence using a Boehringer Mannheim Lumi-Imager. Band intensity was determined by densitometry using LumiAnalyst 3.0 software (Boehringer Mannheim).

**Invasion assays**

HEp-2 cells were grown in 24 well plates to confluence in RPMI 1640 with 5% fetal calf serum. Bacteria were grown overnight as static cultures in LB broth with the appropriate concentration of SCFA or with sodium chloride as controls. Approximately 10^6 bacteria were added to cells, for a multiplicity of infection of 10 bacteria/cell. Plates were then centrifuged for 10 min at 800 X g and incubated for 1 h at 37°C in 95% air with 5% CO₂. 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 bacterial culture was tested in triplicate.
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 (least square differences t-test at a $p \leq 0.05$) were used to determine which means differed (The SAS System for Windows 8).
References


Table 1 Virulence of *S. typhimurium* strains in BALB/c mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>LD50</th>
<th>Attenuation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>$4.1 \times 10^5$</td>
<td>-</td>
</tr>
<tr>
<td>$\Delta (barA)$</td>
<td>$1.6 \times 10^6$</td>
<td>3.9-fold</td>
</tr>
<tr>
<td>$sirA::Tn10$</td>
<td>$3.8 \times 10^6$</td>
<td>&gt;9.3-fold</td>
</tr>
<tr>
<td>$\Delta (ackA-pta)$</td>
<td>$4.9 \times 10^5$</td>
<td>1.2-fold</td>
</tr>
<tr>
<td>$\Delta (barA),\Delta (ackA-pta)$</td>
<td>$9.9 \times 10^6$</td>
<td>24-fold</td>
</tr>
</tbody>
</table>
Table 2 Mutations used.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Description</th>
<th>Resistance</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔcsrB::kan</td>
<td>319 bp internal deletion of csrB, substituted by kan cassette</td>
<td>Kan</td>
<td>Altier et al. (2000a)</td>
</tr>
<tr>
<td>Δ(barA)</td>
<td>Deletion of barA ORF</td>
<td>none</td>
<td>Altier et al. (2000a)</td>
</tr>
<tr>
<td>sirA::Tn10d</td>
<td>Insertion in sirA</td>
<td>Tet</td>
<td>Johnston et al. (1996)</td>
</tr>
<tr>
<td>sirA::cam</td>
<td>Cam cassette inserted into the tetA gene of sirA::Tn10d</td>
<td>Cam</td>
<td>Altier et al. (2000a)</td>
</tr>
<tr>
<td>Δ(ackA-pta)</td>
<td>Deletion of ackA-pta, substituted by kan cassette</td>
<td>Kan</td>
<td>This work</td>
</tr>
<tr>
<td>Φ(invF'-lacZY')</td>
<td>Operon fusion; same as invF::lacZY11-5</td>
<td>Tet</td>
<td>Hueck et al. (1995)</td>
</tr>
<tr>
<td>Φ(sipC'-lacZY')</td>
<td>Operon fusion; same as sspC</td>
<td>Tet</td>
<td>Bajaj et al. (1996)</td>
</tr>
<tr>
<td>Φ(hilA'-lacZY')</td>
<td>Operon fusion; same as hilA::lacZY-080</td>
<td>Tet</td>
<td>Bajaj et al. (1996)</td>
</tr>
</tbody>
</table>

Cam, Kan, and Tet resistance to ampicillin, chloramphenicol, kanamycin and tetracycline respectively.
Figure 1. Time to death of mice challenged with wild type (diamonds), $\Delta (\text{barA})$ (squares) and sirA::Tn10d (circles). BALB/c mice were challenged orally with $10^6$ bacteria and followed for 30 days.
Figure 2. Acetate restores invasion gene expression in a Δ(barA) mutant. Wild type, Δ(barA), sirA::cam, Δ(ackA-pta), and Δ(barA),Δ(ackA-pta) strains carrying transcriptional lacZY fusions to hilA (A), invF (B), and sipC (C) were grown in LB broth supplemented with 30 mM sodium chloride or 30 mM sodium acetate and buffered to pH 8.0 or pH 6.7. Error bars represent standard error of the mean.
Figure 3. Invasion of HEp-2 cells. Invasion is shown with the wild type strain set to 100. Values represent the mean ± standard error of the mean with each strain tested in triplicate. (A) Strains were grown in either LB with 30 mM sodium acetate or 30 mM sodium chloride at pH 6.7 prior to invasion. Significant differences for each strain are represented by an asterisk. (B) Strains were grown in LB with short chain fatty acid concentrations representing the distal ileum or the colon. The distal ileum was represented by a mixture of SCFA containing 25.5 mM sodium acetate, 2.25 mM sodium propionate, and 2.25 mM sodium butyrate. The colon was represented by a mixture containing 110 mM sodium acetate, 70 mM sodium propionate, and 20 mM sodium butyrate. Sodium chloride at 30 mM and 200 mM were used as controls. Asterisks indicate a significant difference in invasion as compared to the appropriate sodium chloride control.
Figure 4. Propionate and butyrate inhibit invasion gene expression. Wild type, \( \Delta(barA) \), \( sirA::cam \), and \( \Delta(ackA-pta) \) strains carrying transcriptional lacZY fusions to \( hilA \) (A), \( invF \) (B), and \( sipC \) (C) were grown in LB broth supplemented with 30 mM sodium chloride, 30 mM sodium propionate, or 30 mM sodium butyrate and buffered to pH 6.7. Error bars represent standard error of the mean.
Figure 4

A

B

C

- Sodium chloride, pH 6.7
- Sodium propionate, pH 6.7
- Sodium butyrate, pH 6.7
Figure 5. Gastrointestinal levels of short-chain fatty acids (SCFA) regulate invasion gene expression. Wild type, Δ(barA), sirA::cam, Δ(ackA-pta), and Δ(barA),Δ(ackA-pta) strains carrying transcriptional lacZY fusions to hilA (A), invF (B), and sipC (C) were grown in LB broth supplemented with mixtures of short-chain fatty acids representing the distal ileum and the colon and buffered to pH 6.7. A mixture containing a total concentration of 30 mM SCFA using the proportions found in the colon was also tested. (16.5 mM sodium acetate, 10.5 mM sodium propionate, and 3 mM sodium butyrate). Error bars represent standard error of the mean.
Figure 5

A

β-galactosidase (Miller Units)

B

β-galactosidase (Miller Units)

C

β-galactosidase (Miller Units)

30 mM Sodium chloride
200 mM Sodium chloride
Ileal conditions
Colonic conditions
30 mM Colonic percentages
Figure 6. Intracellular acetate restores expression of CsrB in a Δ(barA) mutant. Northern analysis was performed using total bacterial RNA prepared from ΔcsrB::kan (lane 1), wild type (lanes 2-5), and Δ(barA) strains (lanes 6-9) with a 593 bp csrB region as probe. Strains were grown in LB broth buffered to pH 8.0 (lanes 1, 2, 3, 6, and 7) or to pH 6.7 (lanes 4, 5, 8, and 9) and supplemented with 30 mM sodium chloride (lanes 1, 2, 4, 6, and 8) or 30 mM sodium acetate (lanes 3, 5, 7, and 9).
Figure 7. A model for the regulation of invasion gene expression by acetate and BarA. Acetyl-phosphate is produced in the bacterial cytoplasm from acetate by acetate kinase, *ackA*, or from acetyl-CoA by phosphotransacetylase, *pta*. Acetyl-phosphate phosphorylates either the response regulator SirA directly, or the sensor kinase BarA, which in turn transfers its phosphate groups to SirA. Alternatively, in the mammalian intestinal tract, BarA can be activated by an undefined signal independent of acetyl-phosphate.
Global regulation by CsrA in *Salmonella typhimurium*

**Summary**

CsrA is a regulator of invasion genes in *Salmonella enterica* serovar Typhimurium. To investigate the wider role of CsrA in gene regulation, we compared the expression of *Salmonella* genes in a csrA mutant to those in the wild type using a DNA microarray. As expected, we found that expression of *Salmonella* pathogenicity island 1 (SPI-1) invasion genes was greatly reduced in the csrA mutant, as were genes outside the island that encode proteins translocated into eucaryotic cells by the SPI-1 type III secretion apparatus. The flagellar synthesis operons *flg* and *fli* were also poorly expressed, and the csrA mutant was aflagellate and non-motile. The genes of two metabolic pathways likely to be used by *Salmonella* in the intestinal milieu also showed reduced expression: the *pdu* operon for utilization of 1,2-propanediol, and the *eut* operon for ethanolamine catabolism. Reduced expression of reporter fusions in these two operons confirmed the microarray data. Moreover, csrA was found to coordinately regulate the *cob* operon for synthesis of vitamin B₁₂, required for the metabolism of either 1,2-propanediol or ethanolamine. Additionally, the csrA mutant poorly expressed the genes of the *mal* operon, required for transport and use of maltose and maltodextrins, and did not produce maltoporin, normally a dominant protein of the outer membrane. These results show that csrA controls a number of gene classes in addition to those required for invasion, some of them unique to
Salmonella, and suggests a coordinated bacterial response to conditions that exist at the site of bacterial invasion, the intestinal tract of a host animal.

**Introduction**

Originally identified in *Escherichia coli*, CsrA is a post-transcriptional regulator that alters stability of its target messages. In *E. coli*, CsrA activates glycolysis, acetate metabolism, and motility, while repressing gluconeogenesis and glycogen biosynthesis (Romeo et al., 1993; Liu et al., 1995; Liu and Romeo, 1997). CsrA has been shown to function as either a positive or a negative regulator, depending upon its target. The post-transcriptional regulation of two messages, those of *glgCAP*, which encodes glycogen synthetase, and *flhDC*, which encodes the central regulator of flagellar synthesis, has been studied in detail (Romeo et al., 1993; Liu et al., 1995; Liu and Romeo, 1997; Baker et al., 2002; Wei et al., 2001). In the case of *glgCAP*, CsrA binds to the Shine-Dalgarno sequence and to a site in the *glgCAP* leader sequence, thereby blocking ribosomal binding and message translation (Baker et al., 2002). In contrast, CsrA binding to the *flhDC* transcript is thought to stabilize the message and allow its translation (Wei et al., 2001). The precise CsrA binding site within the *flhDC* message has not been fully determined, but it lies within the upstream untranslated region of the message. One hypothesis is that CsrA acts as a positive regulator by binding to its target message and protecting it from endonucleolytic attack (Wei et al., 2001). A second component of the csr control system is the untranslated RNA CsrB. In *E. coli*, approximately 18 CsrA molecules can bind to a single molecule of CsrB,
presumably sequestering the protein (Liu et al., 1997). CsrA is thought to bind CsrB at repeated sequence elements similar to the Shine-Dalgarno sequence (Liu et al., 1997). CsrA also activates transcription of csrB, possibly through indirect regulation of a transcription factor (Gudpaty et al. 2001). In E. coli, CsrB has a short half-life (approximately 2 min). The induction of CsrB by CsrA may serve to modulate CsrA activity by titrating the protein. This would result in tight control of the level of active CsrA and would provide an indirect mechanism by which CsrA could control its own activity, since studies have shown that CsrA does not directly control its own expression (Gudpaty et al. 2001).

Both csrA and csrB in Salmonella enterica serovar Typhimurium (hereafter S. typhimurium) share strong sequence homology with their counterparts in E. coli (Altier et al., 2000a; Altier et al., 2000b). Although CsrA is a global regulator in E. coli that controls central functions such as carbon metabolism (Romeo et al., 1993; Liu et al., 1995; Sabnis et al., 1995; reviewed in Romeo, 1996; Wei et al., 2000; Baker et al., 2002), in S. typhimurium, it has been shown to regulate specialized virulence determinants not found in E. coli (Altier et al., 2000b). The ability of S. typhimurium to invade intestinal epithelial cells, an early step in pathogenesis, is encoded by a type III secretion system located at centisome 63 of the chromosome in Salmonella Pathogenicity Island 1 (SPI-1). Genetic and environmental control of SPI-1 invasion gene expression is multi-factorial and is coordinated within the pathogenicity island by HilA (Bajaj et al., 1995; Bajaj et al., 1996). Regulators of HilA include those encoded within SPI-1, HilC (also known
as SprA and SirC) and HilD, and regulators encoded outside SPI-1, PhoP/PhoQ and BarA/SirA (Behlau and Miller, 1993; Bajaj et al., 1995; Johnston et al., 1996; Ahmer et al., 1999; Altier et al., 2000a; Lucas and Lee, 2001). Both HilC and HilD are positive regulators of hilA expression (Schechter et al., 1999; Lostroh et al., 2000; Lucas and Lee, 2001). Altering the levels of CsrA results in decreased expression of SPI-1 invasion genes and a decreased ability of mutant bacteria to invade cultured epithelial cells (Altier et al., 2000b). Regulation of SPI-1 invasion genes by CsrAB is, however, complex. Loss of CsrA or a presumed reduction in free CsrA by the over-expression of CsrB decreases epithelial cell invasion and the expression of SPI-1 genes, but over-expression of CsrA or loss of CsrB have these same effects (Altier et al., 2000a; Altier et al., 2000b). These findings indicate that the csr system of regulation can have both positive and negative effects on invasion and suggest that tight control of the level of active CsrA is required for optimal invasion. The csr system is also controlled by BarA/SirA, since barA and sirA are required for full expression of CsrB (B. Ahmer, personal communication; Lawhon et al. in press).

Homologues of CsrA and CsrB are widespread among the eubacteria and regulate virulence in Pseudomonas and Erwinia species (White et al., 1996). The CsrA/CsrB homologues, RsmA (PrrB) and RsmB, respectively, in Pseudomonas fluorescens regulate production of hydrogen cyanide, encoded by hcnABC, and the major exoprotease, encoded by aprA, (Blumer et al., 1999; Heeb et al., 2002). In Pseudomonas aeruginosa, RsmA is a negative regulator of hydrogen cyanide production.
production, stapholytic activity, and N-acylhomoserine lactone synthesis required for quorum sensing (Pessi et al., 2001). The genes that encode these products are also under the control of the two-component regulator, GacS/GacA, homologous to BarA/SirA, which is required for expression of PrrB, the CsrB homolog (Aarons et al., 2000). Consistent with this, over-expression of RsmA has effects similar to a deletion of GacA, including increased expression of hcnABC in both P. fluorescens and P. aeruginosa (Blumer and Haas, 2000; Blumer et al., 1999; Pessi et al., 2001). Like its counterpart in E. coli, RsmA appears to act at the ribosomal binding site (RBS) of target sequences (Blumer et al., 1999, Pessi et al., 2001). In Erwinia carotovora, RsmA represses production of virulence factors pectate lyase, cellulase, and protease and regulates production of N-acylhomoserine lactone (Chatterjee et al., 1995; Cui et al., 1995). Over-expression of RsmA in E. carotovora also inhibits motility and flagellar synthesis in Erwinia carotovora, although the effect of loss of rsmA on these functions has not been determined (Mukerjee et al., 1996). Like other bacterial species, regulation of RsmA in Erwinia appears to be under the control of a two-component regulator, ExpS/ExpA (Cui et al., 2001; Hyytiainen et al., 2001). Unlike E. coli, however, in Erwinia RsmA is proposed to degrade the message of RsmB rather than to stabilize it (Chatterjee et al., 2002).

Here we investigate the global effects of CsrA in Salmonella. We use genomic analysis, employing a S. typhimurium microarray, supported by phenotypic and genotypic characterization of a csrA mutant. As previously shown,
we find that loss of csrA reduces the level of expression of SPI-1 invasion genes. We also find regulation of pathways not previously identified in other bacterial species, including those for maltose transport and ethanolamine utilization, and those not present in *E. coli*, such as propanediol metabolism, B$_{12}$ synthesis, and production of hydrogen sulfide.

**Results**

*Regulation of invasion genes by CsrA*

To test the global effects on *S. typhimurium* gene expression induced by the loss of *csrA*, we used a DNA microarray that includes 4465 of the 4609 predicted orfs (97%) of *S. typhimurium* LT2. We hybridized cDNA from a virulent wild type *S. typhimurium* strain (ATCC 14028s) and the isogenic *csrA* mutant grown under the same conditions to the microarray and compared gene expression. We found genes of several regulons to have reduced expression in the mutant. One such class was the invasion genes of SPI-1. Of the 37 genes known to be a part of this island, 24 were reduced in their expression by 3- to 10-fold (Table 1). Among these were *hilA* (5-fold reduced), a central regulator of SPI-1 and *invF* (10-fold reduced), a SPI-1 regulator that is regulated both by *hilA* and independent of *hilA*. Also reduced 10-fold were SPI-1 genes encoding secreted effectors and secretion apparatus structural proteins, including *sipABCD*, *invCBAEG*, *spaOP*, and *prgHIJK*. In addition to control of SPI-1 expression, genes outside the island but encoding proteins known to be translocated by the type III secretion system of SPI-1 were also reduced in their expression (Table 1).
These included sopE2, reduced 5-fold, and sopB, located in SPI-5 and reduced 10-fold. sopA, encoding another secreted effector protein required for enteropathogenesis was also reduced in its expression, by 3-fold. An additional SPI-5 gene showed reduced expression, pipC, which exists in an operon with sopB. Conversely, pipA, required for enteropathogenesis and also encoded in SPI-5, had a 3-fold increase in its expression. Of the remaining 13 genes that are part of SPI-1, six were reduced in expression, but less than 3-fold, four were increased in their expression, and three were not detected above the level of nonspecific controls on the array. The four genes with increased expression (sitABCD) encode a fur-regulated iron transporter known not to be under the control of SPI-1 regulatory elements. These results demonstrate that genome analysis using the Salmonella DNA microarray is supportive of our previous results showing that the loss of csrA greatly reduces the expression of SPI-1 invasion genes and epithelial cell invasion and also show that csrA controls the expression of other virulence genes outside SPI-1.

Regulation of flagellar synthesis and chemotaxis by CsrA

In Salmonella and E. coli, flagellar biosynthesis and chemotaxis/aerotaxis are coordinately regulated by flhDC (Reviewed Chilcott and Hughes, 2000). Flagellar genes are expressed in three stages, early, middle, and late. The two early genes, flhD and flhC, form an operon through which environmental control of flagellar synthesis is coordinated. In E. coli, CsrA binds to and stabilizes flhDC mRNA (Wei et al., 2001), thus predicting a reduction of flhDC message in a csrA
mutant. Microarray analysis showed no significant effect of the loss of *csrA* on *flhD* expression and only a slightly decreased level of *flhC* expression (a ratio of mutant to wild type of 0.7). However, genes regulated by *flhDC*, including *flgM, flgK, flgL, fliAZ, fliDT, fliC, fljBA, motAB* were significantly decreased (at least 3-fold) in the *csrA* mutant (Table 2). Additional genes with 2-fold or less reduced expression included *flgDEG, flgN, fliY, fliS*, and *fliJL*. These comprise both middle and late genes required for the synthesis and assembly of flagella, as well as transcriptional regulators of flagellar gene expression (*flgM* and *fliA*). Also regulated by *flhDC* are genes associated with chemotaxis/aerotaxis, *cheA, tsr*, and *aer*. These were also decreased between 3- and 5-fold in the *csrA* mutant (Table 2). Mutants of the flagellar genes would be predicted to have profound defects in the synthesis of flagella and in motility. To confirm the findings of the microarray, we examined the wild type, the *csrA* mutant, and the *csrA* mutant complemented with *csrA* on a low-copy-number vector for the presence of flagella by transmission electron microscopy (Figure 1). The *csrA* mutant had no detectable flagella (Figure 1B), while the complemented mutant had flagella indistinguishable from those of the wild type (Figure 1C and 1A, respectively). The *csrA* mutant was non-motile when grown on semi-solid LB agar (0.35% agar), a phenotype also restored to that of wild type in the complemented mutant (Figure 1D). We also extracted outer membrane proteins from the wild type, the *csrA* mutant, and the complemented mutant to assess the presence of flagellin. As shown in Figure 2, two bands of apparent molecular weights 56 kDa and 53 kDa were missing in the
mutant, but present in the wild type and complemented mutant. *S. typhimurium* phase 2 flagellin, encoded by *fljB*, and phase 1 flagellin, encoded by *fliC*, have these apparent molecular weights (Schmitt et al., 1996). Protein sequencing showed the proteins to have the amino terminal sequence of AQXINTNSLS and XQVINTNS, respectively. The predicted amino-terminal sequence of both phase 1 and phase 2 flagellin is MAQVINTNSLS, with no other *Salmonella* protein having a similar amino-terminal sequence. Thus, these outer membrane proteins missing in the *csrA* mutant represent the two phase-variants of flagellin. Therefore, as in *E. coli*, *S. typhimurium csrA* is essential for the production of flagella and for motility.

*Regulation of genes associated with vitamin B12 synthesis and utilization*

Unlike *E. coli*, *S. typhimurium* is able to synthesize vitamin B$_{12}$ *de novo*. B$_{12}$ synthesis occurs under anaerobic conditions and requires the *cob* operon, including *cob* and *cbi* genes that encode enzymes required for the production of the precursors to vitamin B$_{12}$, adenosyl cobalamin and the corrin ring (reviewed in Roth et al., 1996). In the *csrA* mutant, we found 3- to 10-fold reductions in the expression of genes required for synthesis of vitamin B$_{12}$, specifically genes within the *cob* operon, *cbiAFGHKMPo* and *cobSTU* (Table 3). We tested regulation of *cbi* by *csrA* using a *lacZ* fusion in the *cbi* operon to measure β–galactosidase expression. We found a 4-fold decrease in the expression of the *cbi* operon in the *csrA* mutant (Figure 3A). This decrease in expression was restored to the wild type level in the complemented mutant. Additionally, genes used for the
synthesis of threonine are required for the production of vitamin B\textsubscript{12}, since threonine forms the 1-amino-2-propanol side chain of vitamin B\textsubscript{12} in other bacterial species and is thought to do so in \textit{Salmonella} (Lowe and Turner, 1970; Kurumaya and Kajiwara, 1990), and can also be converted to glycine. Glycine is utilized in the formation of 5,6-dimethylbenzimidazole, which is joined to the corrin ring in the synthesis of B\textsubscript{12}. Genomic analysis showed a number of genes required for threonine synthesis to be controlled by \textit{csrA}. Specifically, \textit{tdcBCDEG} of the threonine synthesis operon were reduced 3- to 10-fold, while \textit{tdcA}, the transcriptional regulator of the operon, was reduced 2.5-fold (Table 3).

In addition to genes required for the synthesis of B\textsubscript{12}, microarray analysis established \textit{csrA} regulation of genes in two operons, \textit{eut} and \textit{pdu}, that function in carbon metabolism and require B\textsubscript{12}. The 17 genes in the \textit{eut} operon are required for ethanolamine usage as a carbon and nitrogen source. Ethanolamine is a phospholipid component of both procaryotic and eucaryotic cell membranes. We found a 3- to 10-fold reduction in the expression of \textit{eutPQDMEJHBCKL}, members of the operon required for ethanolamine degradation, and of \textit{eutR}, a regulator that induces the \textit{eut} operon in the presence of ethanolamine and vitamin B\textsubscript{12} (Table 3). The remaining genes of the \textit{eut} operon, \textit{eutS}, \textit{eutG}, and \textit{eutA} were not detected above the level of nonspecific controls on the array. We further tested the regulation of the \textit{eut} operon by \textit{csrA} using a \textit{lacZ} fusion to the \textit{eut} operon and found a 4-fold reduction in the expression of the fusion in the \textit{csrA} mutant as compared to wild type. The decreased expression seen in the mutant
was restored to wild type levels by plasmid complementation of the csrA mutant (Figure 3B). We also found that genes required for the degradation of propanediol were more poorly expressed in the csrA mutant. Propanediol is a byproduct of catabolism of rhamnose and fucose, both of which are found in the mammalian gastrointestinal tract (Badia et al., 1985; Obradors et al., 1988), with rhamnose being a byproduct of the breakdown of cellulose, and fucose a glycoconjugate present on the cell surface of intestinal epithelial cells (Bry et al., 1996).

Propanediol usage requires B₁₂ and the expression of the genes of the pdu operon, which mediate the conversion of propanediol to propionyl-CoA and are located adjacent to the cob operon. Using microarray analysis, we found a 2.5-fold decrease in the expression of pduA and pduC, proposed to encode a shell protein of the polyhedral organelles that are involved in degradation of propanediol and the large subunit of dehydratase, respectively (Table 3). We tested a lacZ fusion to the pdu operon and found a 10-fold decrease in β-galactosidase production in the csrA mutant as compared to wild type. Complementation fully restored pdu expression to the csrA mutant (Figure 3C). An additional B₁₂-dependent reaction is the production of methionine. The glycine cleavage system converts glycine to 5,10-methelene tetrahydrofolate, an intermediate of this pathway. Genomic analysis also showed that expression of the components of the glycine cleavage complex, gcvHPT, was reduced 3- to 5-fold (Table 3).

The anaerobic utilization of ethanolamine and propanediol as carbon and energy sources by S. typhimurium requires tetrathionate as a terminal electron
Tetrathionate is reduced to thiosulfate by tetrathionate reductase, encoded by the ttr operon, located in SPI-2, and thiosulfate is further reduced to sulfite and hydrogen sulfide by thiosulfate reductase activity encoded by the phs operon. By microarray analysis, we found a slight increase in expression (less than 2-fold) of the ttr operon in the csrA mutant (Table 3). To further investigate the effect of csrA on ttr, we measured β–galactosidase production from a lacZ fusion to ttr and found a 3-fold increase in the expression of ttr in the csrA mutant (Figure 3D). This increase was reduced to wild type levels in the csrA mutant carrying the complementing plasmid (Figure 3D). We also found a three-fold decrease in expression of phsA and phsC in the csrA mutant by microarray analysis (Table 3). To examine the effects of the reduced expression of the phs operon, we grew strains on triple sugar iron agar, which turns black with bacterial production of hydrogen sulfide. The csrA mutant failed to produce sufficient hydrogen sulfide to change the color of the medium, but the complemented mutant had restored production of hydrogen sulfide (Figure 4). These results, taken together, show that csrA regulates the production of vitamin B₁₂, as well as three metabolic pathways that require B₁₂: ethanolamine and propanediol utilization, and methionine synthesis. Further, csrA regulates genes required for the reduction of tetrathionate, necessary for the anaerobic use of ethanolamine and propanediol.
Regulation of maltose operon by CsrA

Maltose and maltodextrins are present in high concentrations in the intestinal tracts of animals as byproducts of starch metabolism. Maltose and maltodextrin are transported through a pore, consisting of maltoporin encoded by *lamB*, which serves as a channel for sugar migration across the outer membrane. Both transport and utilization of these compounds are regulated by MalT, a regulator required for transcription at *mal* promoters (reviewed in Boos and Shuman, 1998). Genomic analysis using the microarray demonstrated decreased expression of the maltose system in the *csrA* mutant (Table 4). The ratio of expression of *malT* in the *csrA* mutant as compared to wild type was reduced only modestly, to 0.7. Expression of genes under the control of MalT was, however, significantly reduced in the *csrA* mutant, approximately three- to ten-fold below the levels of the wild type (Table 4). Loss of *csrA* reduced expression of *malE*, encoding the maltose binding protein, and *malFGK₂*, encoding the translocation complex. Genes used for maltose and maltodextrin metabolism were also reduced in expression, *malP*, encoding maltodextrin phosphorylase, and *malS*, which encodes a nonessential maltodextrin metabolizing enzyme, periplasmic α-amylase, as well as *malM*, a periplasmic protein of unknown function. Expression of *lamB*, encoding maltoporin, the specific pore for maltodextrins and the receptor for phage λ, was reduced 3-fold in the *csrA* mutant. Additionally, loss of *csrA* reduced expression of *treA*, a periplasmic trehalase 3-fold. Trehalose can act as an inducer of the *mal* genes. To confirm the effect of *csrA* on maltose acquisition,
we purified outer membrane proteins from the csrA mutant to be compared with those of the wild type and the complemented mutant (Figure 2). The csrA mutant showed a greatly reduced amount of a protein with an apparent molecular weight of 48 kDa. Complementation of the mutant with a low-copy-number plasmid carrying csrA restored the protein to the wild type level. Production of maltoporin in E. coli is known to be induced by maltose and repressed by glucose. We found that a protein of this apparent molecular weight was present in outer membrane preparations of E. coli DH5a when grown in maltose, but not in glucose, but that S. typhimurium produced the protein during growth with either sugar (data not shown). To identify this outer membrane protein, we determined its amino terminal sequence to be MDFHGYAR. The amino terminal sequence of processed S. typhimurium maltoporin, lacking its signal sequence, is VDFHGYAR, differing only at the initial methionine. No other Salmonella protein is predicted to have a similar sequence. Therefore, the combination of sequence analysis, molecular weight, and maltose induction in E. coli suggests that the protein is indeed maltoporin. Although reduced in its expression of maltoporin, the csrA mutant was still able to grow on minimal medium with 0.2% maltose provided as the sole carbon source (data not shown). Thus, although it produced less maltoporin, the csrA mutant remained able to import sufficient maltose for growth.
Regulation of carbon metabolism by CsrA

CsrA was originally described as a regulator of carbon metabolism in *E. coli*. The *Salmonella* microarray showed no genes of central metabolic pathways to be altered in their expression by 3-fold or more in the csrA mutant. Expression of genes in the glycogen biosynthetic pathway, specifically *glgA*, *glgP*, and *glgB*, all known to be negatively regulated by CsrA in *E. coli*, was in fact slightly decreased in the *S. typhimurium* csrA mutant, while *glgC* was not detected above the level of nonspecific controls present on the array. Conversely, two glyoxylate shunt genes under the positive control of CsrA in *E. coli*, *aceA* and *aceB*, were induced 2-fold in the *S. typhimurium* csrA mutant. Thus, the effect of *Salmonella* CsrA on carbon metabolism appears to be less pronounced and not always consistent with that of *E. coli*.

Discussion

The *csr* system for post-transcriptional regulation is found in a variety of bacterial species. In *E. coli*, in which the system has been best studied, it functions to regulate central carbon metabolism (hence the name *csr*, for carbon storage regulator), and motility (reviewed by Romeo, 1998). In pathogens such as *Erwinia* and *Pseudomonas* species, homologues of *csr* have been shown to regulate a number of virulence functions. We have previously shown that CsrA and CsrB control an important virulence trait in *S. typhimurium*, the ability to penetrate intestinal epithelial cells by the action of the SPI-1 type III secretion apparatus (Altier et al., 2000b).
In this work, we have studied the global effects of CsrA by examining genome-wide changes in expression caused by the loss of csrA. We found that in S. typhimurium, CsrA has a number of novel regulatory roles. As expected, CsrA regulated the expression of SPI-1 genes, including those that encode transcriptional regulators, secreted effector proteins, and components of the type III secretion apparatus. It also, however, controlled expression of genes outside SPI-1 that produce secreted proteins translocated into eucaryotic cells by the SPI-1 secretion system. Among these was sopB, which is known to be controlled by the SPI-1 regulator InvF (Eichelberg and Galan, 1999), and so likely comes under CsrA control indirectly. Expression of genes for two other secreted effectors, sopA and sopE2, was also reduced in the csrA mutant. Control of these genes by SPI-1 regulatory elements has not been demonstrated, but our work suggests the coordinated control of these secreted effectors with the apparatus by which they are secreted. We found only one gene required for enteropathogenesis, pipA, to have significantly increased expression in the csrA mutant. The function of PipA has not been elucidated, and the significance of this finding is currently unknown. As in E. coli, we also found that CsrA was required for motility. The csrA mutant was deficient in the expression of flagella and chemotaxis genes, lacked flagella, and was non-motile. Among Salmonellae, control of flagellar synthesis and chemotaxis is coordinated with the expression of SPI-1 genes. In S. typhi, invasion gene expression and penetration of epithelial cells is greatly reduced with the loss of FliA, the flagellar sigma factor required for the expression of late
flagellar operons. In *S. typhimurium*, however, the effect of a *fliA* mutation is much less pronounced (Eichelberg and Galan, 2000). It is possible, therefore, that some portion of the effect on invasion by CsrA is due to its control of flagellar genes, including *fliA*. Due to the profound defect in invasion of a *S. typhimurium csrA* mutant, however, it is unlikely that control of flagellar genes provides the only mechanism by which CsrA regulates invasion.

Although the classes of genes induced by CsrA in *S. typhimurium* at first appear to be unrelated, they may be linked by their use within the intestinal tract of an animal host. The invasion genes of SPI-1 are obviously important in this environment, being required for bacterial penetration of the intestinal epithelium. Flagella are also important in the intestinal milieu, since flagellar synthesis and the flagellar export apparatus contribute to enteritis caused by *S. typhimurium* (Schmitt et al., 2001). Our analysis has shown CsrA to be required for both production and secretion of flagella. In addition to these virulence functions, CsrA controls genes required for the metabolism of three nutrients found in the intestinal tract: maltodextrins, propanediol and ethanoloamine. Maltodextrins are present in high concentration in the intestinal tracts as byproducts of starch metabolism. They are processed in *Salmonella* by the same means as is maltose, and CsrA appears to be required for both their transport and metabolism through the control of maltoporin, maltose binding protein, the maltose translocation complex, and an enzyme needed for their metabolism, maltodextrin phosphorylase. The utilization of propanediol is also regulated by CsrA. The degradation of propanediol to
propionaldehyde and then to propionyl-CoA to yield carbon and energy is catalyzed by genes of the pdu operon, which require CsrA for full expression. Propanediol is the fermentation product of rhamnose or fucose (Badia et al., 1985). Rhamnose is derived from the breakdown of dietary starches, and so is created by the intestinal microflora. Fucose is a product of the intestinal epithelial cells themselves. Fucosylated glycans on the surface of intestinal epithelial cells can be cleaved by bacterial fucosidase to liberate fucose. It is clear that members of the normal microflora are capable of producing fucosidases and can also induce the production of fucosylated glycoconjugates by intestinal epithelial cells (Bry et al., 1996). The similar catabolism of ethanolamine to acetyaldehyde and then acetyl-CoA uses the eut genes, also induced by CsrA. Like propanediol, ethanolamine is a carbon and energy source common in the intestinal tract, since it is a component of the membranes of both bacteria and eukaryotic cells. The utilization of both propanediol and ethanolamine also requires vitamin B\textsubscript{12}, its synthesis encoded by the cob operon, which is controlled by CsrA as well. Thus, CsrA induces the enzymes required for the degradation of common intestinal nutrients, as well as for the cofactor, B\textsubscript{12}, required for their action. In addition to B\textsubscript{12}, the anaerobic degradation of propanediol and ethanolamine requires tetrathionate as the terminal electron acceptor (Price-Carter et al., 2001). We have shown here that CsrA controls genes required for tetrathionate metabolism and for the production of hydrogen sulfide, the final product of tetrathionate reduction. The importance of tetrathionate as a terminal electron acceptor in the
intestinal tract is, however, unknown. Our results, taken together, therefore suggest that CsrA in *Salmonella* coordinates the regulation of functions valuable for bacterial life in the intestinal tract of an animal host.

Genome-wide analysis using DNA microarrays leads to the identification of genes with the most pronounced reproducible changes in their expression. Transcriptional regulators might go unnoticed, since only small changes in their expression could cause detectable alterations in their regulons. Similarly, regulator activity might be controlled post-translationally via modification (e.g. phosphorylation or methylation). It is therefore likely that our work has failed to identify important targets of CsrA control. One potential example of an undetected regulator is *flhDC*, known in *E. coli* to be controlled by CsrA, but showing little change in expression in the *S. typhimurium csrA* mutant. It remains possible that CsrA controls flagella through *flhDC* in *Salmonella*, but investigation of this question will require other methods. Similarly, DNA microarrays leave open the question of the direct targets of regulation. CsrA controls post-transcriptionally by altering message stability or by blocking the Shine-Dalgarno sequence from recognition by ribosomes. It is likely that most of the genes that we have identified are components of regulons under the control of CsrA and are not themselves the direct targets of its action. Identification of the targets, and elucidation of the pathways by which CsrA controls these genes, will also require other methods. Another caveat of the microarray used in this study is that its construction was based upon the sequence of the *S. typhimurium* type strain LT2, which is a
laboratory strain. Because the experiments were performed in a fully virulent wild type strain, 14028s, any genes unique to 14028s or highly divergent from LT2 were not assayed. Therefore, some genes regulated by CsrA in 14028s may not have been detected by our LT2 microarray.

Although CsrAB and their homologues exist in a number of bacterial species, the functions that they regulate are quite diverse. In fact, the only role of S. typhimurium CsrA that we have shown to be in common with that of E. coli is in the regulation of flagellar synthesis. Beyond this, several of the regulated functions in S. typhimurium do not exist in the closely related E. coli. Invasion genes are part of an acquired pathogenicity island, SPI-1. The utilization of propanediol and the synthesis of B\(_{12}\) are encoded by the linked cob and pdu operons in a region that was acquired through horizontal transfer and is absent in E. coli (Price-Carter et al., 2001). Similarly, ttr for tetrathionate reduction is a part of a second pathogenicity island, SPI-2 (Hensel et al., 1999). We can also find no evidence of regulation by S. typhimurium CsrA of carbon metabolism genes similar to that in E. coli. Thus, this ubiquitous regulator appears to have been adapted in Salmonella to the control of specific functions necessary for bacterial life in the intestinal tract, including those required for virulence.

**Experimental Procedures**

**Strains and growth conditions**
Strains and plasmids used in this study are listed in Table 5. Strains were grown standing at 37°C in Luria Bertani (LB) broth buffered to pH 8.0 with 100 mM HEPES throughout, except were otherwise noted.

**RNA Isolation**

Total bacterial RNA was isolated from mid-log cultures by killing the bacteria with the addition of 0.15 volumes of 95% ethanol, 5% phenol pH 4.3, pelleting the bacteria, resuspending the pellet in 10 mM Tris, 1 mM EDTA (TE) containing 0.5 mg/ml lysozyme, adding 1 ml of 10% SDS, and incubating this suspension at 64°C for 2 minutes. After incubation, 11 ml 1 M sodium acetate at pH 5.2 was added. An equal volume of phenol was then added and the suspension was incubated at 64°C for 6 minutes with frequent mixing. Cultures were centrifuged at 7,000 g for 10 min at 4°C. The aqueous layer was removed and mixed with an equal volume of chloroform and centrifuged at 7,000 x g for 5 min at 4°C. The aqueous layer was removed and a 1/10 volume of 3 M sodium acetate at pH 5.2 was added. Nucleic acid was precipitated with cold isopropanol and pelleted by centrifugation at 10,000 x g for 25 min at 4°C. The pellet was washed with 80% ethanol and resuspended in 1 ml of nuclease-free water. To this suspension, 20 µl of 1M Tris (pH 8.3) and 10 µl of 1 M magnesium chloride and a total of 500 U of RNase inhibitor and 250 U of Rnase-free DNase were added, and the mixture was incubated at 37°C for 30 minutes. The RNA sample was then extracted once each with phenol and phenol/chloroform, and twice with
chloroform. A one-tenth volume of 3M sodium acetate at pH 5.2 was added and RNA was precipitated with isopropanol, washed with 80% ethanol, and resuspended in nuclease-free water. The RNA concentration was measured with a spectrophotometer.

**DNA Microarrays**

A total of 50 µg of RNA was transcribed to DNA and labeled with Cy3 or Cy5 conjugated dUTP using reverse transcriptase (Superscript II®, Invitrogen) and random hexamers as primers. The RNA was then hydrolyzed by incubating labeled probes with 0.1 M sodium hydroxide final concentration at 65°C for 10 min. The sodium hydroxide was neutralized with the addition of hydrochloric acid to a final concentration of 0.1 M. Unincorporated nucleotides were removed using PCR purification kit (Qiagen) according to manufacturer’s instructions. Equal volumes of labeled probes from wild type and the csrA mutant strain were mixed with an equal volume of hybridization solution consisting of 50% formamide, 10X SSC, and 0.2% SDS. Slides were prehybridized in 25% formamide, 5X SSC, and 0.1% SDS at 42°C. Probes were hybridized simultaneously to a chip containing three replicate arrays spotted onto CMT-UltraGAPS® (Corning) slides. A second chip was hybridized with the dyes reversed to normalize for any differences in incorporation or fluorescence of each dye. Chips were scanned using a ScanArray 5000 laser scanner (GSI Lumonics), signals were recorded with ScanArray 2.1 software and then quantified using Quantarray 3.0 software (Packard BioScience).
Ratios were calculated between the two conditions (i.e. mutant strain/wild type) (Eisen and Brown, 1999). Genes with signals less than two standard deviations (SD) above background controls in both conditions (experiment and control) were considered not detected.

**Electron microscopy**

A droplet of LB broth containing approximately $10^8$ bacteria/ml was placed onto a formvar- and carbon-coated 2000 mesh copper grid, and liquid was removed after three minutes by wicking with filter paper. A droplet of 2% aqueous phosphotungstic acid (PTA) at pH 7.2 was placed on the grid before the surface dried completely. After 30 seconds, the PTA was completely removed by wicking with filter paper. The grid was examined with a transmission electron microscope.

**Protein isolation**

Outer membrane proteins were isolated as described previously (Kumar et al., 2001). Overnight cultures were centrifuged for 5 minutes at 7000 x g. Pellets were washed once with 20 mM Tris, 10 mM EDTA pH 8 (TE), then resuspended in the same buffer. Bacteria were disrupted by sonication for 1 minute, followed by a 2 minute rest, then an additional 1 minute sonication. Samples were centrifuged for 5 minutes at 7000 x g to remove debris, and the resulting supernatant was centrifuged for 1 hour at 100,000 x g. The pellet was resuspended in TE and the protein concentration was estimated using Bradford reagent. A one-tenth volume of 10% Sarcosyl was added, and samples were incubated at 4°C for 1 hour. Samples were again centrifuged for 1 hour at 100,000 x g. Pellets were
resuspended in 1% SDS and boiled for 10 minutes. Proteins were separated using 10% SDS-PAGE and stained with Coomassie brilliant blue R-250. For sequencing, proteins were transferred to a PVDF membrane, and amino-terminal sequence was determined by the Protein Sequencing Core Facility at the University of North Carolina.

**β-galactosidase assays**

Triplicate or more overnight cultures of each bacterial strain were grown overnight without aeration at 37°C and assayed as described previously (Miller, 1992). Strains carrying eut-38::MuD were grown in LB broth buffered to pH 8.0 with 100 mM HEPES as above but were also supplemented with 10 mM ethanolamine and 15 nM cobinamide dicyanide to induce expression of the eut operon.

**Motility assays**

Bacteria were grown as aerated overnight cultures, and 10 µl of each was spotted onto semi-solid (0.35%) LB agar. Plates were incubated at 37°C for 3-5 hours in a humidified incubator and strains were assessed for motility. The csrA mutant strain was further assessed until 48 hours after inoculation.

**Statistical analysis**

For β-galactosidase 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 (least square differences t-test at a p
≤0.05) were used to determine which means differed (The SAS System for Windows 8e). For the microarrays, analysis of variance (ANOVA) based on mixed model analysis was utilized (The SAS System for Windows 8e). Changes in gene expression were considered significant if the t-statistic of the log(2) of the fold change for a given gene had a p-value of less than 0.05 (Wolfinger et al., 2001).
References


Romeo, T. (1996) Post-transcriptional regulation of bacterial carbohydrate metabolism: evidence that the gene product CsrA is a global mRNA decay factor. 14$^{th}$ forum in Microbiology.


Table 1. Regulation of invasion genes by csrA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Ratio of Expression (csrA mutant/wild type)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SPI-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hilA</td>
<td>invasion genes transcription activator</td>
<td>0.2</td>
</tr>
<tr>
<td>hilC</td>
<td>SPI-1 transcriptional regulator</td>
<td>0.1</td>
</tr>
<tr>
<td>hilD</td>
<td>SPI-1 transcriptional regulator</td>
<td>0.2</td>
</tr>
<tr>
<td>invA</td>
<td>invasion protein</td>
<td>0.1</td>
</tr>
<tr>
<td>invB</td>
<td>surface presentation of antigens; secretory proteins</td>
<td>0.1</td>
</tr>
<tr>
<td>invC</td>
<td>surface presentation of antigens; secretory proteins</td>
<td>0.1</td>
</tr>
<tr>
<td>invE</td>
<td>invasion protein</td>
<td>0.2</td>
</tr>
<tr>
<td>invF</td>
<td>invasion protein, transcriptional regulator</td>
<td>0.1</td>
</tr>
<tr>
<td>invG</td>
<td>invasion protein; outer membrane</td>
<td>0.1</td>
</tr>
<tr>
<td>invI</td>
<td>surface presentation of antigens; secretory proteins</td>
<td>0.3</td>
</tr>
<tr>
<td>invJ</td>
<td>surface presentation of antigens; secretory proteins</td>
<td>0.2</td>
</tr>
<tr>
<td>prgH</td>
<td>cell invasion protein</td>
<td>0.1</td>
</tr>
<tr>
<td>prgI</td>
<td>cell invasion protein; cytoplasmic</td>
<td>0.2</td>
</tr>
<tr>
<td>prgK</td>
<td>cell invasion protein; lipoprotein, may link inner and outer membranes</td>
<td>0.1</td>
</tr>
<tr>
<td>sicA</td>
<td>surface presentation of antigens; secretory proteins</td>
<td>0.4</td>
</tr>
<tr>
<td>sicP</td>
<td>chaperone, related to virulence</td>
<td>0.1</td>
</tr>
<tr>
<td>sipA</td>
<td>cell invasion protein</td>
<td>0.1</td>
</tr>
<tr>
<td>sipB</td>
<td>cell invasion protein</td>
<td>0.1</td>
</tr>
<tr>
<td>sipC</td>
<td>cell invasion protein</td>
<td>0.1</td>
</tr>
<tr>
<td>sipD</td>
<td>cell invasion protein</td>
<td>0.1</td>
</tr>
<tr>
<td>spaO</td>
<td>surface presentation of antigens; secretory proteins</td>
<td>0.2</td>
</tr>
<tr>
<td>spaP</td>
<td>surface presentation of antigens; secretory proteins</td>
<td>0.5</td>
</tr>
<tr>
<td>sprB</td>
<td>transcriptional regulator</td>
<td>0.2</td>
</tr>
<tr>
<td>sptP</td>
<td>protein tyrosine phosphate</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>SPI-5</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sopB</td>
<td><em>Salmonella</em> outer protein: homologous to <em>ipgD</em> of <em>Shigella</em></td>
<td>0.1</td>
</tr>
<tr>
<td>pipC</td>
<td>Pathogenicity island encoded protein; homologous to <em>ipgE</em> of <em>Shigella</em></td>
<td>0.2</td>
</tr>
<tr>
<td>pipA</td>
<td>Pathogenicity island encoded protein</td>
<td>3.4</td>
</tr>
<tr>
<td>Located outside <em>Salmonella</em> pathogenicity islands</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sopE2</td>
<td>Type III-secreted protein effector: invasion-associated protein</td>
<td>0.2</td>
</tr>
<tr>
<td>sopA</td>
<td>Secreted effector protein of <em>Salmonella dublin</em></td>
<td>0.3</td>
</tr>
</tbody>
</table>
Table 2. Regulation of flagellar synthesis and chemotaxis by *csrA*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Ratio of Expression (csrA mutant/wild type)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flagellar Biosynthesis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>flgD</em></td>
<td>initiation of hook assembly</td>
<td>0.4</td>
</tr>
<tr>
<td><em>flgE</em></td>
<td>hook protein (1st module)</td>
<td>0.4</td>
</tr>
<tr>
<td><em>flgG</em></td>
<td>cell-distal portion of basal-body rod</td>
<td>0.4</td>
</tr>
<tr>
<td><em>flgK</em></td>
<td>hook-filament junction protein 1 (2nd module)</td>
<td>0.1</td>
</tr>
<tr>
<td><em>flgL</em></td>
<td>hook-filament junction protein</td>
<td>0.2</td>
</tr>
<tr>
<td><em>flgM</em></td>
<td>anti-FliA (anti-sigma) factor; also known as RflB protein</td>
<td>0.3</td>
</tr>
<tr>
<td><em>flgN</em></td>
<td>believed to be export chaperone for FlgK and FlgL</td>
<td>0.4</td>
</tr>
<tr>
<td><em>fliA</em></td>
<td>sigma F (sigma 28) factor of RNA polymerase; transcription of late genes</td>
<td>0.2</td>
</tr>
<tr>
<td><em>fliC</em></td>
<td>flagellin, filament structural protein (2nd module)</td>
<td>0.1</td>
</tr>
<tr>
<td><em>fliD</em></td>
<td>filament capping protein; enables filament assembly</td>
<td>0.02</td>
</tr>
<tr>
<td><em>fliT</em></td>
<td>possible export chaperone for FliD</td>
<td>0.1</td>
</tr>
<tr>
<td><em>fljA</em></td>
<td>repressor of <em>fliC</em></td>
<td>0.2</td>
</tr>
<tr>
<td><em>fljB</em></td>
<td>phase 2 flagellin (filament structural protein)</td>
<td>0.02</td>
</tr>
<tr>
<td><em>motA</em></td>
<td>proton conductor component of motor</td>
<td>0.1</td>
</tr>
<tr>
<td><em>motB</em></td>
<td>enables flagellar motor rotation</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Aerotaxis/Chemotaxis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>aer</em></td>
<td>aerotaxis sensor receptor, senses cellular redox state or proton motive force</td>
<td>0.1</td>
</tr>
<tr>
<td><em>cheA</em></td>
<td>sensory kinase, transduces signal between chemo-signal receptors and</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>CheB and CheY</td>
<td></td>
</tr>
<tr>
<td><em>cheW</em></td>
<td>purine-binding chemotaxis protein; regulation</td>
<td>0.3</td>
</tr>
<tr>
<td><em>cheY</em></td>
<td>chemotaxis regulator, transmits chemoreceptor signals to flagellar motor</td>
<td>0.4</td>
</tr>
<tr>
<td><em>cheZ</em></td>
<td>chemotactic response; CheY protein phosphatase</td>
<td>0.4</td>
</tr>
<tr>
<td><em>tsr</em></td>
<td>methyl-accepting chemotaxis protein I, serine sensor receptor</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Table 3. Regulation of vitamin B<sub>12</sub> synthesis and of metabolic pathways requiring B<sub>12</sub> by csrA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Ratio of Expression (csrA mutant/wild type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthesis of Vitamin B&lt;sub&gt;12&lt;/sub&gt; precursor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cbiA</td>
<td>adenosyl cobalamide precursor</td>
<td>0.4</td>
</tr>
<tr>
<td>cbiF</td>
<td>adenosyl cobalamide precursor</td>
<td>0.3</td>
</tr>
<tr>
<td>cbiG</td>
<td>adenosyl cobalamide precursor</td>
<td>0.2</td>
</tr>
<tr>
<td>cbiH</td>
<td>adenosyl cobalamide precursor</td>
<td>0.4</td>
</tr>
<tr>
<td>cbiK</td>
<td>adenosyl cobalamide precursor</td>
<td>0.3</td>
</tr>
<tr>
<td>cbiM</td>
<td>adenosyl cobalamide precursor</td>
<td>0.4</td>
</tr>
<tr>
<td>cbiP</td>
<td>adenosyl cobalamide precursor</td>
<td>0.4</td>
</tr>
<tr>
<td>cbiO</td>
<td>adenosyl cobalamide precursor</td>
<td>0.4</td>
</tr>
<tr>
<td>Threonine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tdcA</td>
<td>transcriptional activator of tdc operon</td>
<td>0.4</td>
</tr>
<tr>
<td>tdcB</td>
<td>threonine dehydratase, catabolic</td>
<td>0.1</td>
</tr>
<tr>
<td>tdcC</td>
<td>HAAAP family, L-threonine/ L-serine permease, anaerobically inducible</td>
<td>0.3</td>
</tr>
<tr>
<td>tdcD</td>
<td>propionate kinase/acetate kinase II</td>
<td>0.1</td>
</tr>
<tr>
<td>tdcE</td>
<td>pyruvate formate-lyase 4/ 2-ketobutyrate formate-lyase</td>
<td>0.1</td>
</tr>
<tr>
<td>tdcG</td>
<td>L-serine deaminase</td>
<td>0.2</td>
</tr>
<tr>
<td>thrA</td>
<td>aspartokinase I,</td>
<td>0.3</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eutB</td>
<td>ethanolamine ammonia-lyase, heavy chain</td>
<td>0.1</td>
</tr>
<tr>
<td>eutC</td>
<td>ethanolamine ammonia-lyase, light chain</td>
<td>0.1</td>
</tr>
<tr>
<td>eutD</td>
<td>putative phosphotransacetylase</td>
<td>0.3</td>
</tr>
<tr>
<td>eutE</td>
<td>putative aldehyde oxidoreductase</td>
<td>0.1</td>
</tr>
<tr>
<td>eutH</td>
<td>putative transport protein,</td>
<td>0.3</td>
</tr>
<tr>
<td>eutJ</td>
<td>paral putative heatshock protein (Hsp70)</td>
<td>0.1</td>
</tr>
<tr>
<td>eutK</td>
<td>putative carboxysome structural protein</td>
<td>0.2</td>
</tr>
<tr>
<td>eutL</td>
<td>putative carboxysome structural protein</td>
<td>0.1</td>
</tr>
<tr>
<td>eutP</td>
<td>putative cobalamin adenosyltransferase</td>
<td>0.1</td>
</tr>
<tr>
<td>eutM</td>
<td>putative detoxification protein</td>
<td>0.3</td>
</tr>
<tr>
<td>eutN</td>
<td>putative detox protein</td>
<td>0.2</td>
</tr>
<tr>
<td>eutQ</td>
<td>putative ethanolamine utilization protein</td>
<td>0.1</td>
</tr>
<tr>
<td>eutP</td>
<td>putative ethanolamine utilization protein</td>
<td>0.3</td>
</tr>
<tr>
<td>eutR</td>
<td>putative regulator (AraC/XylS family)</td>
<td>0.3</td>
</tr>
<tr>
<td>Propanediol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pduA</td>
<td>polyhedral bodies</td>
<td>0.4</td>
</tr>
<tr>
<td>pduC</td>
<td>dehydratase, large subunit</td>
<td>0.4</td>
</tr>
<tr>
<td>Glycine cleavage system</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gcvH</td>
<td>glycine cleavage complex protein H, carrier of aminomethyl moiety</td>
<td>0.3</td>
</tr>
<tr>
<td>gcvP</td>
<td>glycine cleavage complex protein P, glycine decarboxylase</td>
<td>0.2</td>
</tr>
<tr>
<td>gcvT</td>
<td>glycine cleavage complex protein T, aminomethyltransferase</td>
<td>0.3</td>
</tr>
<tr>
<td>Tetrathionate metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ttrA</td>
<td>Tetrathionate reductase complex</td>
<td>1.2</td>
</tr>
<tr>
<td>ttrB</td>
<td>Tetrathionate reductase complex, subunit B</td>
<td>1.9</td>
</tr>
<tr>
<td>ttrC</td>
<td>Tetrathionate reductase complex, subunit C</td>
<td>1.8</td>
</tr>
<tr>
<td>Hydrogen sulfide production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>phsA</td>
<td>membrane anchoring protein</td>
<td>0.3</td>
</tr>
<tr>
<td>phsB</td>
<td>iron- sulfur subunit; electron transfer</td>
<td>0.4</td>
</tr>
<tr>
<td>phsC</td>
<td>membrane anchoring protein</td>
<td>0.3</td>
</tr>
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</table>
Table 4. Regulation of the maltose operon by csrA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Ratio of Expression (csrA mutant/wild type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>malE</td>
<td>maltose transport protein, substrate recognition for transport and chemotaxis</td>
<td>0.2</td>
</tr>
<tr>
<td>malF</td>
<td>maltose transport protein</td>
<td>0.2</td>
</tr>
<tr>
<td>malG</td>
<td>maltose transport protein</td>
<td>0.1</td>
</tr>
<tr>
<td>malS</td>
<td>alpha-amylase</td>
<td>0.1</td>
</tr>
<tr>
<td>malK</td>
<td>maltose transport protein; phenotypic repressor of the mal regulon</td>
<td>0.2</td>
</tr>
<tr>
<td>malP</td>
<td>maltodextrin phosphorylase</td>
<td>0.1</td>
</tr>
<tr>
<td>malM</td>
<td>periplasmic protein of mal regulon</td>
<td>0.3</td>
</tr>
<tr>
<td>lamB</td>
<td>phage lambda receptor protein; maltose high affinity receptor facilitates diffusion of maltose and maltose oligosaccharides</td>
<td>0.3</td>
</tr>
<tr>
<td>treA</td>
<td>periplasmic trehalase</td>
<td>0.3</td>
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</table>
Table 5 Strains and plasmids used.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Resistance</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14028s <em>Salmonella typhimurium</em></td>
<td></td>
<td>ATCC</td>
</tr>
<tr>
<td>Δ(csrA)::Cam</td>
<td>Cam</td>
<td>Altier et al., 2000b</td>
</tr>
<tr>
<td>cbID24::MudJ</td>
<td>Kan</td>
<td>Andersson and Roth, 1989</td>
</tr>
<tr>
<td>trrB123::MudJ</td>
<td>Kan</td>
<td>Price-Carter et al., 2001</td>
</tr>
<tr>
<td>metE205 ara-9 pdu12::MudJ</td>
<td>Kan</td>
<td>Walter et al., 1997</td>
</tr>
<tr>
<td>eut-38::MudJ</td>
<td>Kan</td>
<td>J. Roth</td>
</tr>
<tr>
<td>Δ(csrA)::Cam, cbID24::MudJ</td>
<td>Cam, Kan</td>
<td>this work</td>
</tr>
<tr>
<td>Δ(csrA)::Cam, trrB123::MudJ</td>
<td>Cam, Kan</td>
<td>this work</td>
</tr>
<tr>
<td>Δ(csrA)::Cam, metE205, ara-9, pdu12::MudJ</td>
<td>Cam, Kan</td>
<td>this work</td>
</tr>
<tr>
<td>Δ(csrA)::Cam, eut-38::MudJ</td>
<td>Cam, Kan</td>
<td>this work</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pFF584</td>
<td>Str, Sp</td>
<td>Altier et al., 2000a</td>
</tr>
<tr>
<td>pCA132 0.7-kb csrA fragment on pFF584</td>
<td>Str, Sp</td>
<td>Altier et al., 2000a</td>
</tr>
</tbody>
</table>

Cam, Kan, Str, and Sp resistance to ampicillin, chloramphenicol, kanamycin, streptomycin and spectinomycin respectively.
Figure 1. CsrA is required for production of flagella and for motility. Transmission electron microscopy was used to detect flagella on the wild type (A), and on the csrA mutant complemented with a plasmid carrying csrA (C), but the csrA mutant (B), had no detectable flagella. In panel D, the wild type (1), and the complemented csrA mutant were motile when grown on semi-solid (0.35%) LB agar, but the csrA mutant (2) was non-motile.
Figure 2. Loss of csrA alters the expression of *Salmonella* outer membrane proteins. Three outer membrane proteins of apparent molecular weight 56, 53, and 48 kDa (arrow heads) were present in the wild type (lane 1) and the complemented csrA mutant (lane 3), but absent or in greatly reduced concentration in the csrA mutant (lane 2). Amino-terminal protein sequencing indicated these to be type 2 and type 1 flagellin and maltoporin, respectively.
Figure 3. CsrA regulates the utilization of ethanolamine and propanediol and vitamin B$_{12}$ synthesis. β-galactosidase production from lacZ fusions to pdu (A), eut (B), cbi (C), and ttr (D) were used to measure expression in the wild type (black bars), the csrA mutant (gray bars), the complemented csrA mutant (hatched bars), and the csrA mutant with the appropriate cloning vector without csrA (white bars). Error bars represent standard error of the mean. Asterisks indicate significant difference in β-galactosidase production as compared to wild type.
Figure 4. Regulation of hydrogen sulfide production by *csrA*. Wild type (tube 1), and the complemented *csrA* mutant (tube 3) produced hydrogen sulfide on triple sugar iron agar, but the *csrA* mutant (tube 2) failed to do so.
Regulation of virulence gene expression by intestinal short chain fatty acids and BarA/SirA

Summary

Short chain fatty acids (SCFAs) are present in the intestinal tract and are known to regulate invasion genes in Salmonella enterica serovar Typhimurium. To investigate the broader role of SCFA, we compared gene expression of a virulent wild type strain grown in differing SCFA mixtures. We found that SCFAs in concentrations present in the ileum increase the expression of SPI-1 invasion genes while SCFA concentrations typical of the colon reduce expression of SPI-1. We also found that ileal SCFAs induce the expression of the SPI-2 type III secretion system apparatus and secreted effector proteins while colonic SCFAs have no effect on the expression of SPI-2 genes. Ileal SCFAs also increase the expression of katE and sifA, the latter of which is important in macrophage survival. Colonic SCFAs reduce the expression of the flagellar synthesis operons flg and fli, the genes of the mal operon, which encodes genes required for maltose transport, and the genes of the tdc operon required for threonine synthesis. Colonic SCFAs increase the expression of genes required for glycerol metabolism. We also found that ileal SCFAs elevated expression of genes encoding enzymes of the tricarboxylic acid cycle (TCA cycle) while colonic SCFAs had little effect on these enzymes. We also investigated the effects of loss of BarA and SirA on gene expression. We found, as previously demonstrated, reduced expression of SPI-1 invasion genes in both barA and sirA mutants. The genes of two metabolic
pathways likely to be used by Salmonella in the intestinal environment showed increased expression in both the barA and sirA mutants: the pdu operon for utilization of 1, 2-propanediol, and the eut operon for ethanolamine catabolism. Consistent with these findings was the coordinated regulation by barA and sirA of the cob operon for synthesis of vitamin B\textsubscript{12}, required for the metabolism of either 1, 2-propanediol or ethanolamine. We also found that the sirA mutant poorly expressed the genes of the mal operon while the opposite was true for the barA mutant, possibly indicating a role for acetyl-phosphate in the regulation of maltoporin expression. Additionally, we found elevated expression of genes encoding enzymes of the TCA cycle in the sirA mutant. We also found decreased catalase production in both the barA mutant and the sirA mutant. These results show that SCFA and BarA/SirA control a number of gene classes in addition to those required for invasion and suggest a coordinated bacterial response to conditions that exist at the site of bacterial invasion, the intestinal tract of a host animal.

**Introduction**

Salmonella enterica serovar Typhimurium causes enteritis in humans and cattle and systemic disease in mice. Following oral ingestion, bacteria navigate the gastrointestinal tract, attach to, and invade M-cells and enterocytes of the intestinal epithelium (Takeuchi, 1967). Invasion of intestinal epithelial cells requires the expression of Salmonella pathogenicity island 1 (SPI-1) located at centisome 63 on the Salmonella chromosome and encodes a type III secretion
system. Expression of the type III secretion system is coordinately regulated by genetic and environmental factors, which control expression of \textit{hilA}, encoding a transcriptional regulator of the OmpR/ToxR family (Bajaj et al., 1995; Bajaj et al., 1996). Genetic regulators of \textit{hilA} include HilC and HilD, both located within SPI-1, and PhoP/PhoQ, CsrA/CsrB, and BarA/SirA, located outside SPI-1 (Behlau and Miller, 1993; Johnston et al., 1996; Ahmer et al., 1999; Altier et al., 2000b; Lucas and Lee, 2001). The two-component regulator PhoP/PhoQ is known to respond \textit{in vitro} to low magnesium concentration and has been shown to modulate \textit{hilA} expression (Bajaj et al., 1996). CsrA is a post-transcriptional regulator of mRNA stability known to regulate \textit{hilC} and \textit{hilD} (Altier et al., 2000a). The level of active CsrA is regulated by a regulatory RNA, CsrB. CsrB in \textit{E. coli} is known to bind CsrA (Liu et al., 1997). In \textit{S. typhimurium} CsrB levels are regulated in part by BarA/SirA (Ahmer personal communication; Chapter 2). BarA is a sensor kinase of the his-asp-his phosphorelay type thought to activate the response regulator SirA (UvrY in \textit{E. coli}) (Altier et al., 2000b; Suzuki et al., 2001). BarA/SirA regulates \textit{hilA} expression and epithelial cell invasion (Ahmer et al., 1999; Altier et al., 2000b).

\textit{S. typhimurium} preferentially invades enterocytes in the ileum (Carter and Collins, 1974). The environment of the ileum is complex and consists of secreted enzymes, bile, and extruded cells from the intestinal epithelium as well as byproducts of digestion of protein, fats, and carbohydrates. Each of these is hydrolyzed into lower molecular weight fragments. Proteins are cleaved to
oligopeptides and amino acids. Fats are cleaved into triglycerides, which are processed into short and medium chain fatty acids. Dietary carbohydrates, consist predominantly of starch, are hydrolyzed to maltose and maltodextrins and subsequently to glucose. Additionally, propanediol and ethanolamine are, likely to be present in the intestine. Propanediol is a fermentation byproduct of rhamnose derived from cellulose and fucose, a glycoconjugate present on intestinal epithelial cells. Ethanolamine is a component of prokaryotic and eukaryotic cell membranes (Badia et al., 1985; Obradors et al., 1988; Bry et al., 1996). Salmonella uses vitamin B$_{12}$ in the metabolism of ethanolamine and propanediol. Vitamin B12 is released from food sources as a result of digestion. In the intestinal lumen, B12 is bound to intrinsic factor and preferentially absorbed in the ileum where the B12-intrinsic factor complex binds to a specialized receptor, found primarily in the ileum. Unlike E. coli, S. typhimurium is able to synthesize vitamin B$_{12}$ in the form of adenosylcobalamin. Therefore, the ileum contains an abundance of amino acids, short chain fatty acids (SCFAs), maltodextrins, ethanolamine, propanediol, and vitamin B$_{12}$.

Previously we found that acetate and SCFA concentrations present in the gastrointestinal tract influence the expression of SPI-1 genes and epithelial cell invasion. Specifically, SCFAs in concentrations present in the ileum increase expression of SPI-1 invasion genes while SCFAs in concentrations likely present in the colon have the opposite effect. Initial studies of a barA mutant indicated decreased SPI-1 invasion gene expression and decreased invasion of epithelial
cells (Altier et al., 2000b). Oral inoculation of balb/C mice demonstrated little difference in the LD$_{50}$ of the $barA$ mutant as compared to wild type, suggesting that an environmental condition or signal present in the gastrointestinal tract could override the $barA$ defect. SCFAs in concentrations found in the ileum restored invasion gene expression to a $barA$ mutant. Genetic evidence demonstrates that the genes $ackA$ and $pta$, which encode acetate kinase and phosphotranacetylase respectively and phosphorylate acetate, are required for this effect. Loss of $ackA$ and $pta$ had little effect on LD$_{50}$ in balb/C mice while loss of $barA$ and $ackA$-$pta$ increased the LD$_{50}$ 24-fold suggesting a role for both $barA$ and $ackA$-$pta$ in vivo. Loss of $sirA$ also increased the LD$_{50}$ greater than 9-fold. These findings suggest a model in which either $barA$ or acetyl-phosphate phosphorylates SirA and suggest a role for SCFA regulation of gene expression through two-component regulators in *Salmonella*.

Here we investigate the effects of intestinal SCFA and loss of $barA$ and $sirA$ on gene expression in *Salmonella*. We use genomic analysis, employing a *S. typhimurium* microarray, supported by phenotypic and genotypic characterization of altered gene expression. As previously described, we find regulation of SPI-1 invasion genes by both ileal and colonic SCFAs and loss of $barA$ and $sirA$. We further find increased expression of the genes of SPI-2, which encode a type III secretion system required for systemic *Salmonella* infection, in cultures grown with ileal SCFAs. Additionally, we find that ileal SCFAs increased expression of $katE$, which encodes catalase, and $sifA$, which is required for macrophage survival. We
also find regulation by colonic SCFAs of pathways required for flagellar synthesis and chemotaxis, maltose transport, threonine synthesis, and glycerol metabolism or synthesis. In the barA and sirA mutants, we identify regulation of pathways including those for maltose transport, B₁₂ synthesis, ethanolamine utilization, and propanediol metabolism. We also found decreased catalase production in the barA mutant and the sirA mutant. Additionally, we identify increased expression of the genes that encode enzymes associated with the TCA cycle in the barA and sirA mutants, and wild type cultures grown with ileal SCFAs.

**Results**

*Regulation of Salmonella pathogenicity island 1 by intestinal SCFA and BarA/SirA*

To test the global effects induced by the SCFAs typical of the ileum and colon on *S. typhimurium* gene expression, we used a DNA microarray that includes 4465 of the 4609 predicted orfs (97%) of *S. typhimurium* LT2. We hybridized to the microarray cDNA from wild type *S. typhimurium* strain (ATCC 14028s) grown in Luria-Bertani (LB) broth buffered to pH 6.7 and supplemented with SCFA mixtures that represent either the ileum or the colon or with equivalent levels of sodium chloride in order to compared gene expression. The ileum was represented by a mixture of SCFAs that consisted of 85% sodium acetate, 7.5% sodium propionate, and 7.5% sodium butyrate with a total concentration of 30 mM SCFA. The colon was represented by a mixture of SCFAs that consisted of 55% sodium acetate, 35% sodium propionate, and 10% sodium butyrate with a total concentration of 200 mM SCFA. Addition of the SCFA mixtures did not alter the
pH of the media, which remained 6.7. We also hybridized cDNA from wild type S. typhimurium strain (ATCC 14028) and the isogenic barA mutant or sirA mutant grown in LB broth buffered to pH 8.0 to the microarray and compared gene expression. We found genes of several regulons to have altered expression in both mutants and in the SCFA mixtures (Table 1). One such class was the invasion genes of SPI-1. We found reduced expression of SPI-1 invasion genes in the barA and sirA mutants and in wild type grown with colonic SCFAs, and found increased expression of SPI-1 genes in wild type grown with the ileal SCFA. Of 37 genes known to be a part SPI-1, 23 were reduced in their expression in the barA mutant by 3- to 10-fold while an additional 5 were reduced 2-fold (Table 2). Among these were hilA, a central regulator of SPI-1 (5-fold reduced) and invF, a SPI-1 regulator that is regulated by both hilA and independent of hilA (2-fold reduced). Regulators hilA, hilC and hilD, were decreased in expression 3- and 10-fold respectively. Also reduced 10-fold were SPI-1 genes encoding secreted effectors and secretion apparatus structural proteins, including sipABCD. Of the remaining 9 genes, none was detected above the level of nonspecific controls on the array. In addition to control of SPI-1 expression, genes outside the island but encoding proteins known to be translocated by the type III secretion system of SPI-1 were also reduced in their expression (Table 2). These included sopE2, reduced 10-fold, and sopA, encoding another secreted effector protein required for enteropathogenesis was reduced in its expression, by 2-fold. Additionally two genes in SPI-5 gene showed reduced expression, pipB and pipC were reduced 3-
and 10-fold respectively. Like the barA mutant, we found decreased expression of SPI-1 genes in the sirA mutant (Table 2). Of the 37 SPI-1 genes, 25 were reduced in expression 2- to 5-fold. Among these hilA, hilC, and invF were reduced 2-fold and hilD was reduced 3-fold. Three genes, iacP, iagB, and invE were reduced, but less than 2-fold and the remaining five genes were not detected above the level of nonspecific controls on the array. Genes outside SPI-1 that encode proteins translocated by the type III secretion system of SPI-1 including sopE2, sopB, and pipC, which exists in an operon with sopB, were reduced 2-fold. Just as loss of barA and sirA reduced SPI-1 invasion gene expression, growing wild type in LB supplemented with colonic SCFA reduced expression of SPI-1 genes. Of the 37 SPI-1 genes, 11 were decreased in expression including hilD and invF, 13 were unaffected, 12 were not detected including hilA, and 1 was increased in expression (Table 2). Also reduced in expression were sopE2 and sopA (2-fold) and sopB (10-fold). One gene iacP, thought to encode an acyl carrier protein was increased in expression. The reason for this elevation in expression remains unclear.

Unlike loss of barA or sirA, or growth of wild type in colonic SCFAs, ileal SCFAs increased expression of invasion genes approximately 2- to 3-fold (Table 2). Of the 37 genes of SPI-1, 5 were upregulated 3-fold including regulators hilA and hilD, 19 were increased in expression between 1.5 and 2-fold including hilC, and the remaining 9 genes were relatively unaffected by colonic SCFAs with relative expression ratios of 0.7 to 1.3 (cDNA from cultures supplemented with ileal
SCFAs compared to cDNA from cultures grown with supplemental 30 mM sodium chloride. We further assessed the effect of ileal and colonic SCFA on the expression of *hilD* using densitometric analysis of Northern hybridizations. We found that the level of *hilD* in cultures grown with ileal SCFA was 1.5-fold greater than in cultures grown with 30 mM sodium chloride. Additionally, we found no expression of *hilD* in cultures grown with colonic SCFA (Figure 1). In addition to invasion genes, SPI-1 contains four genes, *sitABCD*, that encode a *fur*-regulated iron transporter known not to be under the control of SPI-1 regulatory elements. These genes were unaffected by growth in either SCFA mixture and were undetected on the *barA* and *sirA* microarrays. Thus, genome analysis using the *Salmonella* DNA microarray is supportive of our previous results showing that the loss of *barA*, the loss of *sirA*, or growth in colonic SCFAs greatly reduces the expression of SPI-1 invasion genes and epithelial cell invasion, and that *barA* and *sirA* control the expression of other virulence genes outside SPI-1. These results also indicate that colonic SCFAs regulate expression of these secreted effectors outside SPI-1. Ileal SCFAs increased expression of SPI-1 genes 2- to 3-fold. Previously, we found no increase in expression of *lacZ* reporter fusions to SPI-1 genes and no increase in invasion of cultured epithelial cells as a result of growing wild type in ileal SCFAs.

*Regulation of Salmonella pathogenicity island 2 by ileal SCFA*

*Salmonella* pathogenicity island 2 (SPI-2) encodes a type III secretion system required to cause systemic disease in mice. SPI-2 is regulated by genetic
regulators PhoP/PhoQ, OmpR/EnvZ, and SsrA/SsrB and by environmental conditions such as low concentrations of magnesium or calcium, and by phosphate starvation (Deiwick et al., 1999). Low levels of magnesium may regulate SPI-2 through PhoP/PhoQ (Garcia-Vescovi et al., 1996). SsrA/SsrB is required for SPI-2 gene expression and is regulated by OmpR/EnvZ (Lee et al., 2000). Microarray analysis revealed increased SPI-2 gene expression in wild type cultures grown with ileal SCFAs as compared to wild type grown with supplemental sodium chloride. We found 3-fold increased expression of response regulator, ssrB and genes that encode secreted effector proteins and structural proteins, sseABC, ssaC and ssaL (Table 3). Northern blot analysis of sseB expression confirmed that ileal conditions increased levels of sseB and revealed a smaller band, possibly a second message, when cultures were grown in the presence of SCFA (Figure 2). We also found 1.5- to 2-fold increased expression of other SPI-2 genes including ssrA, ssaD, sseDG, and ssaGJKMVNOQR. We found 2-fold downregulation of ssal and no effect on ssaBE, sseE, sscB, sseF, and ssaPST. These results indicate that ileal SCFAs regulate the expression of SPI-2 genes. Also located at SPI-2 are the genes that encode tetrathionate reductase, ttrABC, and the two component regulatory system required for regulation of the structural genes, ttrR/ttrS. The ttr genes are not required for systemic pathogenesis of S. typhimurium (Hensel, 1999). Of these genes, ttrA, ttrR, and ttrS were not increased in expression by ileal conditions and ttrBC were not present above the level of nonspecific controls (Table 3). In addition to
regulation of SPI-2, ileal SCFAs induced increased expression of \textit{katE}, which is regulated by \textit{rpoS} and encodes catalase, and \textit{sifA}, which is required for macrophage survival (2.3- and 2.5-fold respectively). Expression of \textit{katE} was decreased 2-fold in both the \textit{barA} and \textit{sirA} mutants.

\textit{Regulation of flagellar synthesis and chemotaxis by colonic SCFA}

In \textit{S. typhimurium}, flagellar biosynthesis and chemotaxis/aerotaxis are coordinately regulated by \textit{flhDC} \cite{Reviewed Chilcott and Hughes, 2000}. Flagellar genes are expressed in three stages, early, middle, and late. The two early genes, \textit{flhD} and \textit{flhC}, form an operon through which environmental control of flagellar synthesis is coordinated. Microarray analysis showed no significant effect of colonic SCFA on \textit{flhDC} expression (ratios of growth condition to control of 1.4 to 1.2 respectively). However, genes regulated by \textit{flhDC}, including \textit{flgM}, \textit{flgK}, \textit{flgL}, \textit{flgN}, \textit{fliAZ}, \textit{fliDS}, \textit{fliC}, \textit{fliB}, \textit{motAB} were significantly decreased (at least 3-fold) in the cultures grown in colonic conditions (Table 4). Additionally, \textit{flgE} was reduced in expression 2-fold. These comprise both middle and late genes required for the synthesis and assembly of flagella, as well as transcriptional regulators of flagellar gene expression (\textit{flgM} and \textit{fliA}). Also regulated by \textit{flhDC} are genes associated with chemotaxis/aerotaxis, \textit{cheWA}, \textit{cheZYBR}, \textit{tsr}, and \textit{aer}. These were also decreased between 3- and 10-fold in cultures grown in colonic conditions (Table 4). Mutants of the flagellar genes would be predicted to have profound defects on the synthesis of flagella and in motility. To confirm the findings of the microarray, we examined motility of wild type grown on semi-solid LB agar (0.35% agar).
buffered to pH 6.7 and supplemented either with colonic SCFA or with 200 mM sodium chloride and found that wild type was non-motile when grown with colonic SCFA (data not shown). In contrast to the effects of colonic SCFA on the regulation of flagellar genes, loss of barA only slightly increased expression of flagellar genes and loss of sirA had little effect on flagellar gene expression (Table 4). Seven genes were increased in expression in the barA mutant 1.5 to 1.8 fold, including flhC. In the sirA microarray results only flgE and flgK were increased in expression 1.5 fold. To evaluate the findings of the microarray, we examined the wild type, the barA mutant, and the sirA mutant for the presence of flagella by transmission electron microscopy, but found no difference between the strains (data not shown). We also tested motility of the barA and sirA mutants as well as an ackA-pta mutant and a barA, ackA-pta mutant for motility on semi-solid LB agar supplemented with colonic SCFA. Two of these mutants, sirA and barA, developed small rings around the sites of inoculation on the motility plates. It was unclear whether this represented motility or not.

**Regulation of vitamin B\textsubscript{12} synthesis and utilization by BarA/SirA**

Vitamin B\textsubscript{12} synthesis in *S. typhimurium* occurs under anaerobic conditions and requires the cob operon including cob and cbi genes, which encode enzymes required for the production of the precursors to vitamin B\textsubscript{12}, adenosylcobalamin and the corrin ring (reviewed in Roth et al., 1996). In the barA and sirA mutants, we found increased expression of genes required for synthesis of vitamin B\textsubscript{12}, specifically genes within the cob operon (Table 5). In the sirA mutant, expression
of cbiM and cbiF was increased 3-fold. Expression of cbiAGHKLNOP, cobD, and cobSU was increased approximately 2-fold. Expression of several additional genes was increased but less than 2-fold. In the barA mutant, cbiF and cobQ were increased 2-fold (Table 5). Additional genes were increased slightly in expression, but less than 2-fold. Threonine forms the 1-amino-2-propanol side chain of vitamin B₁₂ in other bacterial species and is thought to do so in Salmonella (Lowe and Turner, 1970; Kurumaya and Kajiwara, 1990), and can be converted to glycine. Glycine is utilized in the formation of 5, 6-dimethylbenzimidazole, which is joined to the corrin ring in the synthesis of B₁₂. Genomic analysis showed a number of genes required for threonine synthesis to be controlled by sirA. Specifically, tdcABCD of the threonine synthesis operon were increased 2- to 3-fold and tdh, which encodes threonine 3-dehydrogenase, and thrA, which encodes aspartokinase, were increased 3-fold (Table 5). Additionally, expression of thrS, which encodes threonine tRNA synthetase, was increased 1.7-fold in the sirA mutant. In the barA mutant, three genes, tdh, and thrAB were increased in expression 2-fold. Expression of three additional genes, tdcAB, and thrS was increased 1.5-fold. Unlike regulation of the cob operon and threonine synthesis by sirA and barA, intestinal SCFAs had no effect on the cob operon, ileal SCFAs had no effect on threonine synthesis and colonic SCFAs reduced expression of genes required for threonine synthesis genes 2 to 5-fold. Among the genes with decreased expression were tdcABDEG, thrABC and thrS (Table 5). One gene was increased in expression 2-fold, thrL. These results indicate that barA and sirA
regulate expression of genes required for production of vitamin B12 and that colonic SCFA play a role in negative regulation of threonine synthesis.

In addition to genes required for the synthesis of B$_{12}$, microarray analysis found $barA$ and $sirA$ regulation of genes in two operons, $eut$ and $pdu$, that function in carbon metabolism and require B$_{12}$. The 17 genes in the $eut$ operon are required for ethanolamine usage as a carbon and nitrogen source. Ethanolamine is a phospholipid component of both procaryotic and eucaryotic cell membranes. In both the $sirA$ mutant and the $barA$ mutant, we found a 2- to 5-fold increase in the expression of 11 of the members of the operon required for ethanolamine degradation, including $eutR$, a regulator that induces the $eut$ operon in the presence of ethanolamine and vitamin B$_{12}$ (Table 5). Two genes, $eutJ$ and $eutS$, were increased in expression 1.6-fold in the $barA$ mutant and 4-fold in the $sirA$ mutant. Unlike the $barA$ and $sirA$ mutants, ileal SCFA had no effect on the expression of genes in the $eut$ operon while two genes, $eutP$ and $eutR$, were increased in expression on the colonic SCFA microarray. We further tested the regulation of the $eut$ operon by intestinal SCFA using a $lacZ$ fusion to the $eut$ operon and found no effect on the expression of the fusion by either of the SCFA mixtures as compared to cultures supplemented with equivalent levels of sodium chloride (Figure 3B). We also found increased expression of the genes required for the degradation of propanediol in the $barA$ and $sirA$ mutants than in wild type (Table 5). Propanediol is a byproduct of catabolism of rhamnose and fucose, both of which are found in the mammalian gastrointestinal tract (Badia et al., 1985;
Obradors et al., 1988), with rhamnose being a byproduct of the breakdown of cellulose, and fucose a glycoconjugate present on the cell surface of intestinal epithelial cells (Bry et al., 1996). Propanediol usage requires B_{12} and the expression of the genes of the pdu operon, which mediate the conversion of propanediol to propionyl-CoA and are located adjacent to the cob operon. Using microarray analysis, we found in the sirA mutant a 2-fold increase in the expression of pduDGJOP, which encode proteins associated with propanediol dehydratase and polyhedral bodies (Table 5). Six genes, pduACHMQW, were increased in expression slightly (1.5-fold or less). Like the sirA mutant, we found increased expression of eleven genes in the barA mutant (Table 5). Unlike the barA and sirA mutants, we found little effect on genes required for propanediol utilization by intestinal SCFAs. Ileal SCFA had no effect on expression of pdu genes. On the colonic SCFA microarray, we detected only three genes of the pdu operon above the level of the nonspecific controls. All three had ratios of expression greater than one, suggesting increased expression of these genes in cultures grown in colonic SCFA. We tested a lacZ fusion to the pdu operon and found the reverse to be true (Figure 3A). Instead of increased expression of the pdu operon in cultures grown in colonic SCFA, we found no β-galactosidase production in these cultures and little β-galactosidase production in cultures grown with 30 mM sodium propionate or 30 mM sodium butyrate (Figure 3A). An additional B_{12}-dependent reaction is the production of methionine. The glycine cleavage system converts glycine to 5, 10-methelene tetrahydrofolate, an
intermediate of this pathway. We also found increased expression (2-fold) of genes encoding the glycine cleavage system in both the sirA and barA mutants (Table 5). Intestinal SCFAs only a slightly increased expression of gcvA, a regulator of the gcv operon (1.5-fold) and had no other effect on genes of this system. The anaerobic utilization of ethanolamine and propanediol as carbon and energy sources by S. typhimurium requires tetrathionate as a terminal electron acceptor (Price-Carter et al. 2001). Tetrathionate is reduced to thiosulfate by tetrathionate reductase, encoded by the ttr operon (located in SPI-2), and thiosulfate is further reduced to sulfite and hydrogen sulfide by thiosulfate reductase activity encoded by the phs operon. We found no effect of ileal or colonic SCFA on the ttr operon, which was confirmed by measuring β-galactosidase production from a lacZ fusion to ttr (Figure 3D). Only one gene of the ttr operon, ttrS, which encodes a sensor kinase that regulates this operon, was detected on both the barA and sirA microarrays. This gene was unaffected on the barA microarray and increased two-fold on the sirA microarray (Table 5). We also found a 2-fold increase in expression of phsA in the sirA mutant by microarray analysis (Table 5). These results, taken together, show that barA and sirA regulate the production of vitamin B₁₂, as well as two metabolic pathways that require B₁₂, specifically ethanolamine and propanediol utilization, and methionine synthesis and that ileal SCFAs have no role in regulation of these genes while colonic SCFAs increase genes required for propanediol utilization.
Regulation of maltoporin and maltose transport by colonic SCFA and BarA/SirA

Maltose and maltodextrins are present in high concentrations in the intestinal tracts of animals as byproducts of oligosaccharide metabolism. Maltose and maltodextrin are transported through a pore, consisting of maltoporin encoded by \textit{lamB}, which serves as a channel for sugar migration across the outer membrane. Both transport and utilization of these compounds are regulated by MalT, a regulator required for transcription at \textit{mal} promoters (Reviewed Boos and Shuman, 1998). Genomic analysis using microarrays demonstrated decreased expression of the maltose system in the \textit{sirA} mutant and wild type grown under colonic conditions and increased expression in the \textit{barA} mutant (Table 6). Loss of \textit{sirA} reduced expression of five of the ten genes of this system, including \textit{lamB}, which encodes maltoporin, the specific pore for maltodextrins and the receptor for phage \textit{\lambda}. Additionally, loss of \textit{sirA} reduced expression of \textit{treA}, a periplasmic trehalase 2-fold. Trehalose can act as an inducer of the \textit{mal} genes. Unlike loss of \textit{sirA}, loss of \textit{barA} increased expression of eight of the ten genes of the maltose system (Table 6). Like the \textit{sirA} mutant, colonic SCFA reduced expression of the maltose system genes (Table 6). Unlike the \textit{sirA} mutant, colonic SCFA increased expression of \textit{treA} and \textit{treF}, which encodes a cytoplasmic trehalase, 2-fold. Ileal SCFA had little effect on the expression of maltose system genes but did increase expression of \textit{lamB} and \textit{malE}, which encodes the maltose binding protein, approximately 2-fold. In addition to control of maltoporin expression by \textit{sirA}, microarray analysis also revealed that loss of \textit{sirA} elevated expression of outer
membrane proteins encoded by *ompCFW* (3- to 4-fold). Expression of these proteins was unaffected by loss of *barA* (data not shown). Loss of *barA* had the opposite effect on expression of the maltose transport system from loss of *sirA*. These results indicate that loss of *sirA* and colonic SCFA negatively regulate the expression of the maltose transport system while loss of *barA* increases expression of the genes encoded by these systems.

*Regulation of metabolism by intestinal SCFA and BarA/SirA*

Microarray analysis also revealed altered expression of genes central to carbon metabolism. Genes that encode enzymes required in the TCA cycle and glycogen synthesis were increased in expression by loss of *barA*, loss of *sirA*, and exposure to ileal SCFAs (Table 7). Loss of *sirA* resulted in increased expression of genes encoding enzymes in each step of the TCA cycle and genes involved in central carbon metabolic pathways. Among the genes with increased expression in the *sirA* mutant were genes associated with the conversion of phosphoenolpyruvate to pyruvate specifically *pykA*, which encodes pyruvate kinase II, and conversion of pyruvate to acetyl-CoA specifically *aceE*, which encodes pyruvate dehydrogenase. Additionally, genes required for the conversion of acetate to acetyl-phosphate and acetyl-CoA, specifically *ackA-pta*, which encode acetate kinase and phosphotransacetylase were increased in expression in the *sirA* mutant. Also increased in expression by loss of *sirA* were *pps*, which encodes phosphoenolpyruvate synthase, *fba*, which encodes fructose-bisphosphate aldolase, *fbp*, which encodes fructose-bisphosphatase, and *gnd*,
which encodes gluconate-6-phophatase. Loss of *sirA* also increased expression of enzymes associated with glycogen breakdown including *glgP*, which encodes glycogen phosphorylase, *agp*, which encodes glucose-1-phosphatase, and *pgm*, which encodes phosphoglucomutase. Loss of *barA* had a less effect on gene expression in these pathways but did increase the expression of several genes that encode enzymes required in the TCA cycle. Among these were genes that encode TCA cycle enzymes including *gltA*, which encodes citrate synthase, *sdhB*, which encodes succinate dehydrogenase, *sucAB*, *sucCD*, and *fumBC*, which encode fumurase. Like loss of *barA* and loss of *sirA*, ileal SCFAs also increased expression of some of the genes in the central carbon pathways 1.5- to 3-fold. Ileal SCFAs increased expression of *fbaB*, which encodes 3-oxoacyl-synthase I, *gapA*, which encodes glyceraldehyde-3-phosphate dehydrogenase, *glgX*, which encodes glycosyl hydrolase, *gnd*, and *gpmA*, which encodes phosphoglucomutase 1. Additionally, ileal conditions increased expression of *eda*, which encodes 2-keto-3-deoxygluconate 6-phosphate aldolase, and *edd*, which encodes 6-phosphogluconate dehydratase. Colonic conditions had little effect on the central carbon pathways except for a 4-fold increase in the expression of *fbaB* required for the conversion of fructose bisphosphate to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate and 2-fold increased expression of *sucAB*, and *sucCD*, which encode 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase respectively. Colonic SCFAs did increase expression (2- to 4-fold) of
genes required for glycerol metabolism (Table 7). It is unclear what the signal for increased expression of the central carbon pathways is in the SCFA mixtures.

Also increased in expression 1.5- to 3-fold in the \textit{sir}A and \textit{bar}A mutants were genes required for production of formate dehydrogenase (Table 7). Among the genes increased in expression by loss of \textit{sir}A are \textit{fdol}, which encodes formate dehydrogenase, \textit{hybABCDE}, which are associated with hydrogenase-2, \textit{hypA}, which encode a guanine-nucleotide binding protein, and \textit{hypBCDE}, which encode putative hydrogenase formation proteins, and \textit{hypO}, thought to encode a putative hydrogenase. Loss of \textit{sir}A also increased expression of \textit{fdnG}, which is thought to encode an oxidoreductase. Similarly, in the \textit{bar}A mutant, \textit{fdol}, which encodes a subunit of formate dehydrogenase, \textit{hybB}, \textit{hydH}, which encode a sensor kinase, \textit{hypBC}, and \textit{hypO} were increased in expression 1.5- to 2.0-fold. Intestinal SCFAs had little effect on genes associated with formate hydrogenase except for \textit{hydG}, which was increased in expression under both ileal and colonic conditions, and \textit{hypA}, which was increased under colonic conditions.

In addition to effects on central carbon metabolism, we found 2-fold increased expression of the mannose specific transport system in the \textit{sir}A mutant. Specifically, expression of \textit{man}XZ, which encodes mannose specific enzyme II and \textit{man}A, which encodes mannose-6-phosphate isomerase, was increased in the \textit{sir}A mutant. It is unclear why these genes have increased expression, but it is possible that mannose is another carbohydrate abundantly available in the
gastrointestinal tract that serves as an energy or carbon source for *S. typhimurium*.

Additionally, we found increased expression of *asnA*, which encodes asparagine synthetase (2-fold) and *ansB*, which encodes a periplasmic L-asparaginase (3-fold) in the *sirA* mutant and decreased expression of *asnA* and *ansB*, which encodes a periplasmic L-asparaginase in wild type cultures grown with intestinal SCFAs. The importance of asparagine in the gastrointestinal tract and the relationship of expression of *asnA* and *ansB* to the intestinal tract is unclear.

Finally, we found increased expression (2-fold) of *cadA*, which encodes lysine decarboxylase, in cultures grown with intestinal SCFAs. Increased expression of this gene and of other amino acid decarboxylases has been associated with induction of the acid tolerance response. By decarboxylating amino acids the bacteria are able to decrease at least temporarily the level of cytoplasmic hydrogen ions.

**Discussion**

In this work, we have studied the global effects of BarA and SirA by examining genome-wide changes in expression caused by the loss of these genes. We have also studied the roles of intestinal SCFAs in the regulation of *S. typhimurium* gene expression. We have found coordinate regulation of some operons by BarA/SirA and intestinal SCFAs. This is not surprising since BarA/SirA is known to have a role in the enteric phase of *Salmonella* infection. We have
found that a barA mutant is as virulent as wild type in vivo and in vitro, acetyl-phosphate is able to override the barA defect. We found, as previously determined, that in S. typhimurium, BarA and SirA regulate the expression of SPI-1 genes and genes regulated by SPI-1 that exist outside SPI-1 (Table 1) (Ahmer et al., 1999; Altier et al., 2000b). Among these were sopB and pipC, which coexist in SPI-5 as an operon, and are controlled by the SPI-1 regulator InvF (Eichelberg and Galan, 1999). As with loss of barA and sirA, we also found decreased expression of SPI-1 genes, sopB, sopE2, and sopA in response to colonic SCFAs. Further, the opposite effect was found in cultures grown with ileal SCFAs. We previously found that ileal SCFAs did not increase expression of SPI-1 genes using lacZY reporter fusions and found no increase in epithelial cell invasion resulting from growing cultures with ileal SCFAs, while here the microarrays indicate increased expression of SPI-1 genes in response to ileal SCFAs. The simplest explanation for these disparate findings is that the β-galactosidase assay is less sensitive than the microarray due in part to the stable nature of β-galactosidase and therefore does not detect the increased expression of SPI-1. Also, smaller changes in expression level can be detected using the microarray because the technique is more direct and more sensitive, measuring fluorochrome labeled cDNA copies of mRNA instead of measuring enzyme activity and relating it to message level. It is clear that the increase in SPI-1 gene expression detected by the array does not increase invasion of cultured epithelial cells. In Figure 1, we confirm by northern analysis that hilD mRNA is increased slightly when wild type is
grown with supplemental ileal SCFA (lane 4) as compared to sodium chloride (lane 2). This suggests that something, possibly post-transcriptional regulation, occurs to prevent the increased expression of SPI-1 genes from increasing invasion of epithelial cells. It is likely that the overlapping regulation of SPI-1 invasion genes that we have noted between BarA, SirA, and intestinal SCFAs reflects the physiologic role of acetate and possibly propionate and butyrate in signaling through SirA. BarA and SirA are known to regulate CsrB, which in turn controls the level of CsrA. CsrA has both positive and negative effects on invasion gene expression, as both loss and overexpression of CsrA reduce invasion gene expression (Altier et al., 2000a). This suggests that the level of CsrA must be tightly controlled for optimal invasion gene expression. CsrB is thought to modulate the level of active CsrA by binding the protein at one of its predicted binding sites. BarA and SirA regulate invasion gene in part through CsrB, but are not thought to control SPI-1 invasion gene expression solely through CsrB, as loss of either BarA or SirA has a greater effect on invasion gene expression than loss of CsrB alone (Altier et al., 2000b). Ileal SCFAs are able to induce SPI-1 invasion gene expression in the absence of BarA but not in the absence of SirA. This effect requires the presence of acetate kinase and phosphotransacetylase as strains lacking ackA and pta, the genes that encode these enzymes, and barA exhibit poor expression of SPI-1 (Chapter 2). These findings suggest that acetate restores SPI-1 gene expression to the barA mutant, but that it must be phosphorylated to do so. The model for this pathway is that acetate, either alone
or as a component of the ileal SCFA mixture, crosses the bacterial cell membrane and is phosphorylated (Figure 5). The resultant acetyl-phosphate then phosphorylates SirA, which in turn induces invasion gene expression. The colonic SCFA mixture also contains acetate but in a lower proportion of the total mixture. Colonic SCFAs reduced the expression of SPI-1 invasion genes in the wild type (Chapter 2). This effect was due to the increased level of propionate and butyrate, which individually reduced SPI-1 expression. It is unclear whether these SCFAs act directly on SPI-1 genes or through regulators of SPI-1 located outside the pathogenicity island. Colonic SCFAs could reduce SPI-1 expression by acting to increase CsrB expression either through SirA or independent of SirA, or by lowering the cytoplasmic pH and inducing acid responsive genes such as PhoP/PhoQ. PhoP/PhoQ is known both to be induced by acidic pH and to repress SPI-1 (Adams et al., 2001). Regulation of CsrB by intestinal SCFAs appears complex. Acetate alone restored CsrB to wild type levels in the barA mutant but had only slight effects on wild type levels of CsrB (Chapter 2). Ileal SCFAs actually decreased CsrB levels in wild type while the colonic SCFA mixture increased CsrB to greater than wild type levels (Figure 4). This indicates that expression of CsrB may be increased by the colonic SCFAs in response to the higher level of acetate present in the colonic mixture or in response to decreased cytoplasmic pH due to the high level of organic acid present in this mixture. SCFAs including acetate, propionate, and butyrate are known to induce expression of acid tolerance or acid resistance genes (Kwon and Ricke, 1998).
Acid tolerance refers to the improved ability of *Salmonella* and other enterobacteria to withstand low pH if previously exposed to mildly acidic conditions (Reviewed Bearson et al., 1997). This complex pathway is regulated by the alternative sigma factor $\sigma^S$, encoded by RpoS, and by PhoP/PhoQ in exponential phase and in part by OmpR in stationary phase (Bearson et al., 1998; Adams et al., 2001). We found an additional indication that colonic SCFAs may induce acid stress. Colonic SCFAs increased expression of lysine decarboxylase, which protonates lysine to cadaverine in a temporary measure to reduce intracellular acid.

Microarray analysis also indicated that colonic SCFAs reduce the expression of genes required for flagellar synthesis (Table 3). We confirmed this finding by motility assays using plates made with LB agar (0.35%) supplemented either with 200 mM sodium chloride or with colonic SCFAs. We found that wild type was nonmotile when grown on plates containing colonic SCFAs. This fits our model for invasion gene regulation by colonic SCFAs. Both motility and flagellin production are repressed by acid-mediated signaling through PhoP/PhoQ (Adams et al., 2001). Additionally, in *E. coli*, CsrA stabilizes the message of *flhDC*, the flagellar gene master regulator (Adams et al., 2001; Wei et al., 2001). The repression of flagellar gene expression by colonic SCFA could occur through either of these mechanisms or through both. In our analysis of the microarrays, we did not find that SirA, as previously reported (Goodier and Ahmer, 2001), regulated flagellar gene expression. Instead, we found little change in expression
of flagellar genes in the sirA mutant. This is likely due to differences in growth
conditions between the two studies, specifically growth in broth cultures as
opposed to growth on motility agar. The microarray showed slightly increased
expression of flagellar gene expression in the barA mutant. We previously
demonstrated that CsrA regulates flagellar gene expression, that loss of CsrA
results in loss of flagella, and that loss of BarA results in decreased expression of
CsrB. Thus, loss of BarA would presumably increase flagellar gene expression by
indirectly increasing the level of CsrA. These observations further support the
proposed model in which BarA and SirA regulate proposed targets by opposing
the effects of CsrA. While we saw no effect of loss of SirA on flagellar genes on
the microarray, we did observe slight motility in the sirA mutant and the barA
mutant grown on semi-solid LB agar supplemented with the colonic SCFA mixture.
None of the other strains tested including wild type, a csrB mutant, an ackA-pta
mutant, and a barA, ackA-pta mutant showed any sign of motility. This would
support regulation of motility by colonic SCFAs in part through BarA/SirA. This
probably does not occur through regulation of CsrB, as the CsrB mutant showed
no sign of motility in the presence of colonic SCFAs.

In addition to regulation of invasion genes and flagella, we found several
other groups of genes regulated by both BarA/SirA and intestinal SCFAs.
Although the classes of genes induced by BarA/SirA and intestinal SCFAs in S.
typhimurium at first appear to be unrelated, they may be linked by their use within
the intestinal tract of an animal host. The invasion genes of SPI-1 are clearly
important in this environment, being required for bacterial penetration of the intestinal epithelium. We also found that BarA, SirA, and intestinal SCFAs control genes required for the metabolism of three nutrients found in the intestinal tract: maltodextrins, propanediol and ethanolamine. Maltodextrins are present in high concentration in the intestinal tract as byproducts of starch metabolism and are processed in *Salmonella* by the same means as maltose through uptake by the maltose transporter and maltoporin (Reviewed Boos and Shuman, 1998). Loss of *sirA* and colonic SCFAs negatively regulate both transport and metabolism of maltodextrins through the control of maltoporin, maltose binding protein, the maltose translocation complex, and an enzyme needed for their metabolism, maltodextrin phosphorylase (Table 6). This contradicts the model in which SirA is a negative regulator of CsrA through CsrB, because CsrA is a positive regulator of maltoporin expression (Chapter 3). This suggests that regulation of maltoporin by SirA, like SPI-1 invasion gene regulation, can occur independently of CsrA. Loss of *barA* resulted in increased expression of the maltose transport system. One explanation for the differential regulation by BarA and SirA is that endogenously produced acetyl-phosphate may act as a phosphate donor for SirA in the *barA* mutant, leading to increased expression of maltoporin. Endogenously produced phosphate transfer has been demonstrated for ArcA/ArcB in *E. coli* (Georgellis et al., 1999). Colonic SCFAs may regulate expression of the maltose transport system by acid induced signaling through PhoP/PhoQ. In *E. coli*, loss of PhoQ with concurrent growth on pyruvate to increase internal acetyl-phosphate (to
provide a phosphate donor for PhoP) reduced expression of *malE*, which encodes the maltose binding protein, and *malK*, which encodes a protein that forms part of the maltose transporter (Reviewed Boos and Shuman, 1998). This effect may have been induced by the high level of acetyl-phosphate rather than PhoP, but introducing *phoP* on a multicopy plasmid had the same effect of reducing expression of *malEK*.

BarA and SirA also negatively regulate the utilization of propanediol. Propanediol offers an additional carbon source that is likely present in the intestinal tract. Its utilization requires expression of the *pdu* operon. The pathway for propanediol degradation is conversion to propionaldehyde by propanediol dehydratase, which is encoded by *pduCDE*, and then to propionyl-CoA by propionaldehyde dehydrogenase. Expression of the *pdu* operon was increased in the *barA* and *sirA* mutants whereas its expression was decreased in the *csrA* mutant. Loss of *barA* or *sirA* and the subsequent decrease in the level of CsrB ostensibly results in increased levels of CsrA, thereby increasing expression of the *pdu* operon. It is unclear whether BarA/SirA have any effect on propanediol utilization independent of CsrA. In addition to regulation of propanediol usage by BarA/SirA, we found increased expression of two members of the *pdu* operon, *pduL* and *pduM*, by colonic SCFAs. We found differing results using a *lacZ* fusion to *pdu* (Figure 3). The *lacZ* reporter fusion indicated decreased expression of the *pdu* operon in colonic SCFAs. Only three genes in the *pdu* operon were detected on the colonic SCFA microarray and of these *pduL* and *pduM* were increased.
more than 1.5-fold while pduE was increased slightly. These genes are all encoded downstream of the lacZ fusion used to assess expression. The product of pduE is known to form part of the propanediol dehydratase enzyme complex, but the function of the products of pduL and pduM is unknown. It is possible that pduL and pduM are differentially regulated or that they are required in metabolism of propionate, although there is no strong evidence for this. It is also possible that the increased level of mRNA does not result in increased protein synthesis. We noted earlier that SPI-1 genes were increased in expression by ileal SCFAs, but that this increase had no functional effect. Propanediol usage as a carbon and energy source requires the conversion of propanediol to propionyl-CoA, which is subsequently converted either to propionyl-phosphate or to pyruvate and succinate, which enter the TCA cycle or are used for gluconeogenesis. This pathway requires the expression of the prp operon, the same pathway used for metabolism of propionate. The colonic SCFA mixture contains high levels of propionate. This propionate is likely converted to propionyl-CoA and enters the TCA cycle or is used for gluconeogenesis. It is possible that the high levels of propionate result in feedback inhibition of the pdu operon resulting in the decreased expression of this operon indicated by the lacZ fusion. Propionate metabolism requires expression of the cob operon, although the defect caused by loss of the cob operon can be overridden by supplementing strains with propanediol. This fact in combination with the physical location of the cob operon (adjacent to the pdu operon), the mutual requirement for the transcription factor
PocR, and the ability of propanediol to override the effect of loss of CobB, has led to the hypothesis that an as yet unknown protein of the pdu operon (PduL or PduM?) may function as a transcriptional activator of the prp operon in conjunction with or instead of CobB (Tsang et al., 1999). Additional studies of the uncharacterized genes of the pdu operon might clarify the role of SCFAs on their induction and on their importance to propionate metabolism. In addition to regulating utilization of propanediol, BarA/SirA also regulated expression of the eut operon, required for the utilization of ethanolamine as a carbon source. Loss of either of these genes resulted in increased expression of this operon. This differed from loss of csrA, which resulted in decreased expression of the eut operon. These combined findings support the model (Figure 5) in which BarA and SirA regulate gene expression by altering the level of CsrA through control of CsrB levels. It is unclear whether BarA/SirA regulate eut independent of CsrA.

The utilization of both propanediol and ethanolamine also requires vitamin B\textsubscript{12}, which is encoded by the cob operon and is negatively controlled by BarA and SirA. In addition to B\textsubscript{12}, the anaerobic degradation of propanediol and ethanolamine requires tetrathionate as the terminal electron acceptor (Price-Carter et al., 2001). The importance of tetrathionate as a terminal electron acceptor in the intestinal tract remains unknown. We found that SirA negatively controls genes required for tetrathionate metabolism and for the production of hydrogen sulfide, the final product of tetrathionate reduction. We have previously shown that CsrA is a positive regulator of expression of vitamin B\textsubscript{12} synthesis, tetrathionate
reduction, production of hydrogen sulfide, and threonine synthesis (Chapter 3). Loss of barA and loss of sirA have the opposite effect on these genes. The same is true for regulation of the gcv operon, which was increased in expression in the barA and sirA mutants and decreased in the csrA mutant. Regulation of B12 and gene products required for its production and role in propanediol and ethanolamine utilization, tetrathionate reductase, threonine synthesis, and hydrogen sulfide, follows the same pathway as regulation of propanediol and ethanolamine utilization. That is, each of these pathways is positively regulated by CsrA and negatively regulated by BarA/SirA. It follows that these genes would be coordinately regulated because of the requirement for B12 for utilization of these carbon sources and because the cob and pdu operons are adjacent on the Salmonella chromosome and known to be regulated by the same transcriptional regulator PocR.

We also demonstrated that BarA and SirA regulated genes are required for central carbon metabolism and for the TCA cycle. In our previous work with the CsrA microarray we did not find any changes in central carbon metabolism of at least 3-fold. We also found that expression of other genes required for carbon metabolism and known to be regulated by CsrA in E. coli were either not regulated by CsrA in Salmonella or were regulated oppositely to those in E. coli. Here we find that BarA/SirA are negative regulators of the TCA cycle, while in the CsrA microarray, we found decreased expression of citrate synthase, encoded by gltA, and decreased expression of 2-oxoglutarate dehydrogenase, encoded by sucA
and required for the conversion of 2-oxoglutarate to succinyl-CoA. This supports our model outlined in Figure 5 in which BarA/SirA oppose the effects of CsrA by increasing levels of CsrB. We found that ileal SCFAs increased expression of genes encoding several enzymes required for the TCA cycle. Ileal SCFAs may increase expression of these genes in one of two ways. First, acetate in the ileal SCFA mixture may cross the cell membrane, become phosphorylated, and in turn may phosphorylate BarA/SirA leading to increased expression of CsrB and decreased availability of CsrA. Second, acetate may cross the cell membrane, become phosphorylated by acetate kinase, be converted to acetyl-CoA by phosphotransacetylase, and enter the TCA cycle resulting in increased need for TCA cycle enzymes. It is unclear which of these mechanisms occurs in cultures supplemented with the ileal SCFA mixture. We also found that both intestinal SCFA mixtures increased expression of fbaB, which encodes 3-oxyacyl-[acyl-carrier protein]-synthase, which catalyzes the conversion of fructose bisphosphate to dihydroxyacetone and glyceraldehyde-3-phosphate. It seems likely that increased expression of this enzyme results from increased gluconeogenesis. Both acetate and propionate in the intestinal SCFA mixtures could cross the bacterial cell membrane, become converted to acetyl-Co and propionyl-CoA (catalyzed by pta and prpE respectively) and enter the TCA cycle or serve as substrates for gluconeogenesis. We also noted that colonic SCFAs increased expression of sucABCD which are required for conversion of 2-oxoglutarate to succinyl-CoA and succinate. We mentioned previously that propionate is
converted propionyl-CoA which is metabolized to pyruvate and succinate by the prp operon which would require expression of sucABCD. From these findings we conclude that the elevated propionate in the colonic mixture is converted to pyruvate and succinate which in turn function as substrates for the TCA cycle and gluconeogenesis.

Microarray analysis also revealed that ileal SCFAs induced the expression of SPI-2 genes. This finding was unexpected but was supported by northern analysis of sseB expression (Figure 2). Three two-component regulators, PhoP/PhoQ, EnvZ/OmpR, and SsrA/SsrB regulate SPI-2. Of these, SsrB is required for expression of SPI-2 and is regulated by EnvZ/OmpR. There are two likely mechanisms for increased expression of SPI-2 by ileal SCFAs. One of these is phosphorylation of OmpR by acetyl-phosphate. The other is decreased cytoplasmic pH due to the buildup of SCFAs resulting in induction of the acid tolerance response. In the bacterial cytoplasm, ackA-pta phosphorylates acetate to acetyl-phosphate, and OmpR can be phosphorylated by either its cognate sensor kinase EnvZ or by acetyl-phosphate. Phosphorylation of OmpR results in its activation and subsequent regulation of SsrA/SsrB. Additionally, exposure to acetate or propionate induces the acid tolerance response, which in turn induces transcription of ompR possibly through CRP although the mechanism has not been determined (Kwon and Ricke, 1998; Bang et al., 2000; Bang et al., 2002). Further investigation is needed to determine if induction of SPI-2 genes by ileal SCFAs occurs through OmpR, SsrB, or PhoP and whether the increased
expression of SPI-2 genes by ileal SCFAs results in improvement in macrophage survival.

These combined results suggest a model in which BarA/SirA and intestinal SCFAs regulate *Salmonella* gene expression in the environment of the intestine, in part through modulation of the level of CsrA. We have identified BarA/SirA regulation of several pathways required for utilization of carbon sources likely to exist in the gastrointestinal tract including propanediol, ethanolamine, and maltodextrin. We have also found that regulation of vitamin B\textsubscript{12} synthesis, which is required for two of these pathways, is regulated by BarA/SirA. We have found overlapping areas of regulation between intestinal SCFAs and BarA/SirA including SPI-1 invasion gene expression and maltoporin expression, which further support a pathway through which acetyl-phosphate phosphorylates SirA. We also identified a function of ileal SCFAs that is independent of BarA/SirA, regulation of SPI-2 gene expression possibly in response to decreased internal pH of the bacteria or more likely through phosphorylation of an alternate two component regulator such as EnvZ/OmpR or SsrA/SsrB. Clearly, these pathways are complex and require more detailed study to determine definitively the mechanisms through which they are regulated.

**Experimental Procedures**

*Strains and growth conditions*

Strains and plasmids used in this study are listed in Table 5. Strains were grown standing at 37°C in Luria Bertani (LB) broth buffered to pH 8.0 with 100 mM
HEPES throughout, except where otherwise noted. Motility assays were performed on semi-solid (0.35%) LB agar.

**RNA Isolation**

For microarrays, total bacterial RNA was isolated from mid-log cultures by killing the cells with the addition of 0.15 volumes of 95% ethanol, 5% phenol pH 4.3, pelleting the bacteria, resuspending the pellet in 10 mM Tris, 1 mM EDTA (TE) containing 0.5 mg/ml lysozyme, adding 1 ml of 10% SDS, and incubating this suspension at 64°C for 2 minutes. After incubation, 11 ml 1 M sodium acetate at pH 5.2 was added. An equal volume of phenol was then added and the suspension was incubated at 64°C for 6 minutes with frequent mixing. Cultures were centrifuged at 7,000 g for 10 min at 4°C. The aqueous layer was removed and mixed with an equal volume of chloroform and centrifuged at 7,000 x g for 5 min at 4°C. The aqueous layer was removed and a 1/10 volume of 3 M sodium acetate at pH 5.2 was added. Nucleic acid was precipitated with cold isopropanol and pelleted by centrifugation at 10,000 x g for 25 min at 4°C. The pellet was washed with 80% ethanol and resuspended in 1 ml of nuclease-free water. To this suspension, 20 µl of 1M Tris (pH 8.3) and 10 µl of 1 M magnesium chloride and a total of 500 U of RNase inhibitor and 250 U of Rnase-free DNase were added and the mixture was incubated at 37°C for 30 minutes. The RNA sample was then extracted once each with phenol and phenol/chloroform, and twice with chloroform. A one-tenth volume of 3M sodium acetate at pH 5.2 was added and
RNA was precipitated with isopropanol, washed with 80% ethanol, and resuspended in nuclease-free water. The RNA concentration was measured with a spectrophotometer.

**Northern Analysis**

For northern blots, RNA was isolated using a SV Total RNA Isolation System kit (Promega, Madison) according to manufacturer's protocol. RNA concentration was determined spectrophotometrically by measuring OD$_{260}$, and 5 µg was loaded in each lane of a formaldehyde gel and run for 1.5 hr at 100V.

RNA was transferred to a nylon membrane (Roche, Indianapolis), fixed by ultraviolet crosslinking. Pre-hybridization and hybridization were performed at 37°C with gentle agitation (Sambrook et al., 1989). The membranes were hybridized with probes to sseB, csrA, a previously described 509 bp hilD region as probe or with the previously described 593 bp csrB region as probe (Altier et al. 2000b). The probe for sseB was PCR amplified using with oligonucleotides 5’ ATGTCTTCAGGAAACATCTTATGGG 3’ and 5’ TCATGAGTACGTTCGCGCTAT 3’ as primers. The probe for csrA was PCR amplified with oligonucleotides 5’ GGAATTCAAGGAGCAAAGAATGCTG 3’ and 5’ GCTCTAGACCTTAGAAGACTGGCTGGG 3’ as primers. Probes were detected by chemiluminescence using a Boehringer Mannheim Lumi-Imager.

Band intensity was determined by densitometry using LumiAnalyst 3.0 software (Boehringer Mannheim).

**DNA Microarrays**
A total of 50 µg of RNA was transcribed to DNA and labeled with Cy3 or Cy5 conjugated dUTP using reverse transcriptase (Superscript II®, Invitrogen) and a random hexamer as a primer. The RNA was then hydrolyzed by incubating labeled probes with 0.1M sodium hydroxide final concentration at 65°C for 10 min. The sodium hydroxide was neutralized with the addition of hydrochloric acid to a final concentration of 0.1 M. Unincorporated nucleotides were removed using PCR purification kit (Qiagen) according to manufacturer’s instructions. Equal volumes of labeled probes from wild type and the csrA mutant strain were mixed with an equal volume of hybridization solution consisting of 50% formamide, 10X SSC, and 0.2% SDS. Slides were prehybridized in 25% formamide, 5X SSC, and 0.1% SDS at 42°C. Probes were hybridized simultaneously to a chip containing three replicate arrays spotted onto CMT-ultraGAPS® slides. A second chip was hybridized with the dyes reversed to normalize for any differences in incorporation or fluorescence of each dye. Chips were scanned using a ScanArray 5000 laser scanner, signals were recorded with ScanArray 2.1 software and then quantified using Quantarray 2.1 or 3.0 software. Ratios were calculated between the two conditions (e.g. experiment / control or mutant strain / wild type). Genes with signals less than two standard deviations (SD) above background controls in both conditions (experiment and control) are considered as not detected (ND)

Electron microscopy
A droplet of LB broth containing approximately $10^8$ bacteria/ml was placed onto a formvar- and carbon-coated 2000 mesh copper grid, and liquid was removed after three minutes by wicking with filter paper. A droplet of 2% aqueous phosphotungstic acid (PTA) at pH 7.2 was place on the grid before the surface dried completely. After 30 seconds, the PTA was completely removed by wicking with filter paper. The grid was examined with a transmission electron microscope.

**β-galactosidase assays**

Triplicate or more overnight cultures of each bacterial strain were grown overnight without aeration at 37°C and assayed as described previously (Miller, 1992). Strains carrying the eut-38::Md fusion were grown in LB broth with 100 mM HEPES pH 8.0, 10 mM ethanolamine and 15 nm cobinamide dicyanide.

**Statistical analysis**

For β-galactosidase 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 (least square differences t-test at a p ≤0.05) were used to determine which means differed (The SAS System for Windows 8e). For the microarrays, analysis of variance (ANOVA) based on mixed model analysis was utilized (The SAS System for Windows 8e). Changes in gene expression were considered significant if the t-statistic of the log(2) of the fold change for a given gene had a p-value of less than 0.05 (Wolfinger et al., 2001).
References


Table 1 Summary of gene regulation by intestinal short chain fatty acids (SCFA) and barA/sirA (Based on microarray analysis).

<table>
<thead>
<tr>
<th></th>
<th>Ileal SCFA</th>
<th>Colonic SCFA</th>
<th>Δ(barA)</th>
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<tr>
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<tr>
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<td>Vitamin B$_{12}$ synthesis</td>
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<td>Threonine</td>
<td>-</td>
<td>↓</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td>Maltose transport</td>
<td>-</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>*katE</td>
<td>↑</td>
<td>ND</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>*sifA</td>
<td>↑</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Glycerol metabolism</td>
<td>-</td>
<td>↑</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose-1-phosphate, agp</td>
<td>↑</td>
<td>ND</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>Mannose</td>
<td>-</td>
<td>↓</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>↑</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fumarate reductase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>Formate dehydrogenase</td>
<td>-</td>
<td>-</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Asparagine synthetase</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>Lysine decarboxylase</td>
<td>↑</td>
<td>↑</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = Gene expression not present above the level of microarray controls
- = No change as compared to wild type grown with 30mM sodium chloride for ileal or colonic SCFA or as compared to wild type grown under the same condition for barA and sirA mutants
↑ = Gene expression is increased in expression as compared to control cDNA
↓ = Gene expression is decreased in expression as compared to control cDNA
* = The pdu operon is increased in expression on the microarray but is decreased using transcriptional lacZ fusions (Figure 3).
Table 2 Regulation of *Salmonella typhimurium* SPI-1 invasion genes by intestinal short chain fatty acids (SCFA) and barA/sirA

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Ileal SCFA</th>
<th>Colonic SCFA</th>
<th>barA</th>
<th>sirA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPI-1 Invasion genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>avrA</em></td>
<td>putative inner membrane protein</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>hilA</em></td>
<td>invasion genes transcription activator</td>
<td>2.7</td>
<td>-</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td><em>hilC</em></td>
<td>SPI-1 transcriptional regulator</td>
<td>2.3</td>
<td>1.4</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td><em>hilD</em></td>
<td>SPI-1 transcriptional regulator</td>
<td>3.0</td>
<td>0.3</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td><em>iapC</em></td>
<td>putative acyl carrier protein</td>
<td>1.3</td>
<td>2.2</td>
<td>-</td>
<td>0.7</td>
</tr>
<tr>
<td><em>iagB</em></td>
<td>cell invasion protein</td>
<td>2.2</td>
<td>1.1</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td><em>invA</em></td>
<td>invasion protein</td>
<td>1.8</td>
<td>0.2</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td><em>invB</em></td>
<td>surface presentation of antigens</td>
<td>1.6</td>
<td>0.7</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td><em>invC</em></td>
<td>surface presentation of antigens</td>
<td>1.9</td>
<td>0.5</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td><em>invE</em></td>
<td>invasion protein</td>
<td>1.1</td>
<td>0.8</td>
<td>0.2</td>
<td>0.7</td>
</tr>
<tr>
<td><em>invF</em></td>
<td>invasion protein, transcriptional regulator</td>
<td>1.2</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td><em>invG</em></td>
<td>invasion protein; outer membrane</td>
<td>2.7</td>
<td>-</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td><em>invH</em></td>
<td>invasion protein</td>
<td>1.9</td>
<td>-</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td><em>invI</em></td>
<td>surface presentation of antigens</td>
<td>1.5</td>
<td>0.8</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td><em>invJ</em></td>
<td>surface presentation of antigens</td>
<td>2.9</td>
<td>0.4</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td><em>orgA</em></td>
<td>pseudogene; frameshift</td>
<td>1.7</td>
<td>0.3</td>
<td>0.1</td>
<td>0.3</td>
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<tr>
<td><em>prgH</em></td>
<td>cell invasion protein</td>
<td>2.7</td>
<td>0.1</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td><em>prgI</em></td>
<td>cell invasion protein; cytoplasmic</td>
<td>1.8</td>
<td>0.8</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td><em>prgJ</em></td>
<td>cell invasion protein; cytoplasmic</td>
<td>1.8</td>
<td>0.7</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td><em>prgK</em></td>
<td>cell invasion protein; lipoprotein</td>
<td>1.9</td>
<td>-</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td><em>sicA</em></td>
<td>surface presentation of antigens</td>
<td>2.0</td>
<td>0.2</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td><em>sicP</em></td>
<td>chaperone, related to virulence</td>
<td>0.7</td>
<td>-</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td><em>sipA</em></td>
<td>cell invasion protein</td>
<td>1.9</td>
<td>0.1</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td><em>sipB</em></td>
<td>cell invasion protein</td>
<td>1.8</td>
<td>-</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td><em>sipC</em></td>
<td>cell invasion protein</td>
<td>2.5</td>
<td>0.1</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td><em>sipD</em></td>
<td>cell invasion protein</td>
<td>1.8</td>
<td>-</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td><em>spaO</em></td>
<td>surface presentation of antigens</td>
<td>1.9</td>
<td>-</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td><em>spaP</em></td>
<td>surface presentation of antigens</td>
<td>1.3</td>
<td>1.1</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td><em>spaQ</em></td>
<td>surface presentation of antigens</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>spaR</em></td>
<td>surface presentation of antigens</td>
<td>1.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>spaS</em></td>
<td>surface presentation of antigens</td>
<td>1.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>sprB</em></td>
<td>transcriptional regulator</td>
<td>1.8</td>
<td>0.8</td>
<td>0.4</td>
<td>0.5</td>
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<tr>
<td><em>spI</em></td>
<td>protein tyrosine phosphate</td>
<td>1.2</td>
<td>0.3</td>
<td>0.1</td>
<td>0.2</td>
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<td>SPI-5</td>
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<tr>
<td><em>sopB</em></td>
<td>Salmonella outer protein</td>
<td>1.8</td>
<td>0.1</td>
<td>-</td>
<td>0.6</td>
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<tr>
<td><em>pipC</em></td>
<td>Pathogenicity island encoded protein</td>
<td>2.1</td>
<td>-</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td><em>pipA</em></td>
<td>Pathogenicity island encoded protein</td>
<td>0.8</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>pipB</em></td>
<td>Pathogenicity island encoded protein</td>
<td>1.5</td>
<td>-</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td><em>pipD</em></td>
<td>Pathogenicity island encoded protein</td>
<td>1.4</td>
<td>0.8</td>
<td>0.8</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Located outside Salmonella pathogenicity islands
<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Ileal SCFA</th>
<th>Colonic SCFA</th>
<th>barA</th>
<th>sirA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>sopE2</em></td>
<td>Type III-secreted protein effector</td>
<td>2.3</td>
<td>0.5</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td><em>sopA</em></td>
<td>Secreted effector protein of <em>S. dublin</em></td>
<td>1.7</td>
<td>0.5</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td><em>sopD</em></td>
<td>secreted protein</td>
<td>1.8</td>
<td>1.4</td>
<td>0.3</td>
<td>0.4</td>
</tr>
</tbody>
</table>

- represents gene expression level below the level of controls.
All values represent the median relative fold change in gene expression between either the growth condition and appropriate control or between the mutant and wild type grown under the same condition.

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Table 3 Regulation of *Salmonella enterica* serovar Typhimurium SPI-2 invasion genes by ileal short chain fatty acids (SCFA)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Ileal SCFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ttrA</td>
<td>Tetrathionate reductase complex, Subunit A</td>
<td>1.2</td>
</tr>
<tr>
<td>ttrR</td>
<td>Tetrathionate reductase complex: Response regulator</td>
<td>1.1</td>
</tr>
<tr>
<td>ttrS</td>
<td>Tetrathionate reductase complex: Sensory transduction histidine kinase</td>
<td>0.8</td>
</tr>
<tr>
<td>ssrB</td>
<td>Secretion system regulator: Response regulator</td>
<td>2.8</td>
</tr>
<tr>
<td>ssrA</td>
<td>Secretion system regulator: Sensor component</td>
<td>1.5</td>
</tr>
<tr>
<td>sscB</td>
<td>Secretion system chaperone</td>
<td>1.0</td>
</tr>
<tr>
<td>sseA</td>
<td>Secretion system effector</td>
<td>3.2</td>
</tr>
<tr>
<td>sseB</td>
<td>Secretion system effector</td>
<td>3.0</td>
</tr>
<tr>
<td>sscA</td>
<td>Secretion system chaperone</td>
<td>1.9</td>
</tr>
<tr>
<td>sseC</td>
<td>Secretion system effector</td>
<td>3.2</td>
</tr>
<tr>
<td>sseD</td>
<td>Secretion system effector</td>
<td>1.6</td>
</tr>
<tr>
<td>sseE</td>
<td>Secretion system effector</td>
<td>1.2</td>
</tr>
<tr>
<td>sseF</td>
<td>Secretion system effector</td>
<td>1.3</td>
</tr>
<tr>
<td>sseG</td>
<td>Secretion system effector</td>
<td>1.5</td>
</tr>
<tr>
<td>ssaB</td>
<td>Secretion system apparatus</td>
<td>1.2</td>
</tr>
<tr>
<td>ssaC</td>
<td>Secretion system apparatus</td>
<td>3.2</td>
</tr>
<tr>
<td>ssaD</td>
<td>Secretion system apparatus</td>
<td>1.7</td>
</tr>
<tr>
<td>ssaE</td>
<td>Secretion system apparatus</td>
<td>1.2</td>
</tr>
<tr>
<td>ssaG</td>
<td>Secretion system apparatus</td>
<td>1.7</td>
</tr>
<tr>
<td>ssaI</td>
<td>Secretion system apparatus</td>
<td>0.5</td>
</tr>
<tr>
<td>ssaJ</td>
<td>Secretion system apparatus</td>
<td>2.4</td>
</tr>
<tr>
<td>ssaK</td>
<td>Secretion system apparatus</td>
<td>2.5</td>
</tr>
<tr>
<td>ssaL</td>
<td>Secretion system apparatus</td>
<td>3.3</td>
</tr>
<tr>
<td>ssaM</td>
<td>Secretion system apparatus</td>
<td>1.7</td>
</tr>
<tr>
<td>ssaV</td>
<td>Secretion system apparatus: Homology with the LcrD family of proteins</td>
<td>1.6</td>
</tr>
<tr>
<td>ssaN</td>
<td>Secretion system apparatus: Homology with the YscN family of proteins</td>
<td>1.8</td>
</tr>
<tr>
<td>ssaO</td>
<td>Secretion system apparatus</td>
<td>1.6</td>
</tr>
<tr>
<td>ssaP</td>
<td>Secretion system apparatus</td>
<td>1.2</td>
</tr>
<tr>
<td>ssaQ</td>
<td>Secretion system apparatus</td>
<td>1.6</td>
</tr>
<tr>
<td>ssaR</td>
<td>Secretion system apparatus: Homology with YscR of Yersinia</td>
<td>2.2</td>
</tr>
<tr>
<td>ssaS</td>
<td>Secretion system apparatus: Homology with YscS of Yersinia</td>
<td>0.9</td>
</tr>
<tr>
<td>ssaT</td>
<td>Secretion system apparatus: Homology with YscT of Yersinia</td>
<td>0.9</td>
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</tbody>
</table>

All values represent the median relative fold change in gene expression between cultures grown in LB broth with supplemental ileal SCFA and cultures grown with 30 mM sodium chloride.
Table 4 Regulation of *Salmonella typhimurium* flagellar synthesis and chemotaxis by intestinal short chain fatty acids (SCFA) and barA/sirA

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Ileal SCFA</th>
<th>Colon SCFA</th>
<th>barA</th>
<th>sirA</th>
</tr>
</thead>
<tbody>
<tr>
<td>FlhA</td>
<td>possible export of flagellar proteins</td>
<td>1.1</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FlhB</td>
<td>putative part of export apparatus</td>
<td>1.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FlhC</td>
<td>regulator of flagellar biosynthesis</td>
<td>1.8</td>
<td>1.4</td>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td>FlhD</td>
<td>regulator of flagellar biosynthesis</td>
<td>1.0</td>
<td>1.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FlgA</td>
<td>assembly of basal-body periplasmic P ring</td>
<td>1.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FlgB</td>
<td>cell-proximal portion of basal-body rod</td>
<td>1.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FlgC</td>
<td>cell-proximal portion of basal-body rod</td>
<td>1.2</td>
<td>1.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FlgD</td>
<td>initiation of hook assembly</td>
<td>0.9</td>
<td>-</td>
<td>1.6</td>
<td>1.0</td>
</tr>
<tr>
<td>FlgE</td>
<td>hook protein (1st module)</td>
<td>1.2</td>
<td>0.4</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>FlgF</td>
<td>cell-proximal portion of basal-body rod</td>
<td>1.0</td>
<td>0.8</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>FlgG</td>
<td>cell-distal portion of basal-body rod</td>
<td>1.0</td>
<td>0.7</td>
<td>1.6</td>
<td>1.0</td>
</tr>
<tr>
<td>FlgH</td>
<td>basal-body outer-membrane ring protein</td>
<td>1.1</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FlgI</td>
<td>putative flagella basal body protein</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FlgJ</td>
<td>flagellar biosynthesis</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FlgK</td>
<td>flagellar biosynthesis</td>
<td>0.6</td>
<td>0.1</td>
<td>1.5</td>
<td>1.5</td>
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<tr>
<td>FlgL</td>
<td>hook-filament junction protein</td>
<td>0.6</td>
<td>0.1</td>
<td>1.8</td>
<td>1.1</td>
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<tr>
<td>FlgM</td>
<td>anti-FliA (anti-sigma) factor</td>
<td>0.7</td>
<td>0.3</td>
<td>1.6</td>
<td>0.8</td>
</tr>
<tr>
<td>FlgN</td>
<td>flagellar biosynthesis</td>
<td>1.6</td>
<td>0.2</td>
<td>-</td>
<td>0.9</td>
</tr>
<tr>
<td>FliA</td>
<td>sigma 28 factor of RNA polymerase</td>
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<td>0.3</td>
<td>1.0</td>
<td>1.2</td>
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<tr>
<td>FliB</td>
<td>N-methylation of lysine residues in flagellin</td>
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<td>0.7</td>
<td>1.8</td>
<td>-</td>
</tr>
<tr>
<td>FliC</td>
<td>phase 1 flagellin, filament structural protein</td>
<td>0.8</td>
<td>0.1</td>
<td>1.3</td>
<td>1.4</td>
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<tr>
<td>FliD</td>
<td>filament capping protein</td>
<td>0.7</td>
<td>0.1</td>
<td>1.5</td>
<td>1.4</td>
</tr>
<tr>
<td>FliE</td>
<td>putative flagellar hook-basal body protein</td>
<td>0.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FliF</td>
<td>basal-body membrane-ring and collar protein</td>
<td>1.1</td>
<td>0.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FliG</td>
<td>component of motor switching and energizing</td>
<td>0.9</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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Chemotaxis

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* represents gene expression level below the level of controls.
Table 5 Regulation of genes required for vitamin B-12 synthesis and utilization by intestinal short chain fatty acids (SCFA) and barA/sirA in *Salmonella typhimurium*.

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<td>Tetraphionate metabolism</td>
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<td></td>
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</tr>
<tr>
<td>ttrA</td>
<td>Tetraphionate reductase subunit A</td>
<td>1.2</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ttrB</td>
<td>Tetraphionate reductase subunit B</td>
<td>-</td>
<td>0.9</td>
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<td>ttrR</td>
<td>Tetraphionate reductase response regulator</td>
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<td>0.9</td>
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<td>ttrS</td>
<td>Tetraphionate reductase sensory histidine kinase</td>
<td>0.8</td>
<td>1.0</td>
<td>1.3</td>
<td>2.2</td>
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<td>Hydrogen sulfide production</td>
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<td>phsA</td>
<td>membrane anchoring protein</td>
<td>1.4</td>
<td>0.3</td>
<td>1.1</td>
<td>1.9</td>
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<tr>
<td>phsB</td>
<td>iron-sulfur subunit; electron transfer</td>
<td>0.9</td>
<td>0.5</td>
<td>1.6</td>
<td>1.1</td>
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<tr>
<td>phsC</td>
<td>membrane anchoring protein</td>
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<td>0.4</td>
<td>0.9</td>
<td>1.6</td>
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- represents gene expression level below the level of controls.

All values represent the median relative fold change in gene expression between either the growth condition and appropriate control or between the mutant and wild type grown under the same condition.
Table 6 Regulation of the maltose transport system by intestinal short chain fatty acids (SCFA) and barA/sirA in *Salmonella typhimurium*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Ileal SCFA</th>
<th>Colonic SCFA</th>
<th>barA</th>
<th>sirA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maltose transport system</strong></td>
<td></td>
<td>--------------</td>
<td>------</td>
<td>------</td>
<td></td>
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<tr>
<td><em>lamB</em></td>
<td>phage lambda receptor protein; maltose high-affinity receptor</td>
<td>1.6</td>
<td>0.1</td>
<td>1.9</td>
<td>0.1</td>
</tr>
<tr>
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<td>0.1</td>
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<td>0.04</td>
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<td>maltose transport protein</td>
<td>1.3</td>
<td>0.1</td>
<td>2.1</td>
<td>0.1</td>
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<td>maltose transport protein</td>
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<td>0.1</td>
<td>1.8</td>
<td>0.3</td>
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<td>maltose transport protein</td>
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<td>-</td>
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<tr>
<td><em>malM</em></td>
<td>periplasmic protein of mal regulon</td>
<td>1.0</td>
<td>0.1</td>
<td>3.0</td>
<td>0.2</td>
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<tr>
<td><em>malP</em></td>
<td>maltodextrin phosphorylase</td>
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<td>0.1</td>
<td>1.3</td>
<td>0.2</td>
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<td><em>malS</em></td>
<td>alpha-amylase (2nd module)</td>
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<td>0.3</td>
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<td><em>malT</em></td>
<td>transcriptional activator</td>
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<td>0.6</td>
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<td>1.8</td>
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<td>0.6</td>
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<td><em>treA</em></td>
<td>trehalase, periplasmic</td>
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<td>cytoplasmic trehalase</td>
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<td>0.5</td>
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All values represent the median relative fold change in gene expression between either the growth condition and appropriate control or between the mutant and wild type grown under the same condition.
Table 7 Regulation of the metabolism by intestinal short chain fatty acids (SCFA) and barA/sirA in Salmonella typhimurium

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Ileal SCFA</th>
<th>Colonic SCFA</th>
<th>barA</th>
<th>sirA</th>
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</thead>
<tbody>
<tr>
<td><strong>Carbon metabolism</strong></td>
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<tr>
<td>aceA</td>
<td>isocitrate lyase</td>
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<td>1.1</td>
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</tr>
<tr>
<td>aceB</td>
<td>malate synthase A</td>
<td>0.6</td>
<td>1.2</td>
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<td>-</td>
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<tr>
<td>aceE</td>
<td>pyruvate dehydrogenase</td>
<td>1.1</td>
<td>1.0</td>
<td>0.7</td>
<td>1.7</td>
</tr>
<tr>
<td>aceF</td>
<td>pyruvate dehydrogenase</td>
<td>0.9</td>
<td>1.0</td>
<td>1.2</td>
<td>1.2</td>
</tr>
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<td>aceK</td>
<td>isocitrate dehydrogenase kinase/phosphatase</td>
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<td>ackA</td>
<td>acetate kinase A (propionate kinase 2)</td>
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<td>0.8</td>
<td>1.7</td>
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<td><strong>gg</strong></td>
<td>glucose-1-phosphatase</td>
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<tr>
<td><strong>eda</strong></td>
<td>3-deoxygluconate-6-phosphate aldolase</td>
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<td>1.0</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>edd</strong></td>
<td>6-phosphogluconate dehydratase</td>
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<td></td>
<td>1.1</td>
<td></td>
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<tr>
<td>eno</td>
<td>enolase</td>
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<td>0.7</td>
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<td>fructose-bisphosphate aldolase</td>
<td>1.3</td>
<td>0.9</td>
<td>0.9</td>
<td>1.7</td>
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<td><strong>fbaB</strong></td>
<td>3-oxoacyl-[acyl-carrier-protein] synthase I</td>
<td>4.5</td>
<td>4.1</td>
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<td>fructose-bisphosphatase</td>
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<td>1.3</td>
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<tr>
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<td>fumarase B (fumarate hydratase class I)</td>
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<td>glycogen synthase</td>
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<td>1.4</td>
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<td>1,4-alpha-glucan branching enzyme</td>
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<td>1.5</td>
<td>0.4</td>
<td>0.7</td>
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<td>glucokinase</td>
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<td>1.0</td>
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<td><strong>gltA</strong></td>
<td>citrate synthase</td>
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<td>1.1</td>
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<td><strong>mdh</strong></td>
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<td>-</td>
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<td>1.3</td>
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<td><strong>pfIB</strong></td>
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<td><strong>pykF</strong></td>
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<td>succinate dehydrogenase, flavoprotein subunit</td>
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<td>1.2</td>
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<td>1.4</td>
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<tr>
<td><strong>sdhB</strong></td>
<td>succinate dehydrogenase, Fe-S protein</td>
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<td>1.2</td>
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<td><strong>sdhC</strong></td>
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<td><strong>sdhD</strong></td>
<td>succinate dehydrogenase, hydrophobic subunit</td>
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<td>2-oxoglutarate dehydrogenase</td>
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<td>2.0</td>
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<td><strong>sucB</strong></td>
<td>2-oxoglutarate dehydrogenase</td>
<td>1.7</td>
<td>2.3</td>
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</table>

All values represent the median relative fold change in gene expression between either the growth condition and appropriate control or between the mutant and wild type grown under the same condition.
Table 7 continued

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Ileal SCFA</th>
<th>Colonic SCFA</th>
<th>barA</th>
<th>sirA</th>
</tr>
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<tbody>
<tr>
<td>sucC</td>
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<td>1.7</td>
<td>1.6</td>
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<tr>
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<td>succinyl-CoA synthetase, alpha subunit</td>
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<td>3.1</td>
<td>2.2</td>
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Glycerol metabolism

<table>
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<th>Colonic SCFA</th>
<th>barA</th>
<th>sirA</th>
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<td>glgA</td>
<td>glycerol dehydrogenase</td>
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<td>0.8</td>
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<tr>
<td>glpA</td>
<td>sn-glycerol-3-phosphate dehydrogenase</td>
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<td>2.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>glpC</td>
<td>sn-glycerol-3-phosphate dehydrogenase</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>glpD</td>
<td>sn-glycerol-3-phosphate dehydrogenase</td>
<td>1.0</td>
<td>3.7</td>
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<td>1.0</td>
<td>-</td>
<td>-</td>
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<tr>
<td>gpmB</td>
<td>putative phosphoglyceromutase 2</td>
<td>1.2</td>
<td>-</td>
<td>-</td>
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<tr>
<td>gpsA</td>
<td>glycerol-3-phosphate dehydrogenase</td>
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<td>1.1</td>
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<td>1.3</td>
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</table>

Mannose specific transport system

<table>
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<th>Gene</th>
<th>Function</th>
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<th>Colonic SCFA</th>
<th>barA</th>
<th>sirA</th>
</tr>
</thead>
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<td>mannose-specific enzyme IIAB</td>
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<td>0.2</td>
<td>1.3</td>
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<td>manY</td>
<td>mannose-specific enzyme IIIC</td>
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<td>manZ</td>
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<td>0.7</td>
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Fumarate reductase

<table>
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<th>Gene</th>
<th>Function</th>
<th>Ileal SCFA</th>
<th>Colonic SCFA</th>
<th>barA</th>
<th>sirA</th>
</tr>
</thead>
<tbody>
<tr>
<td>frdA</td>
<td>fumarate reductase, anaerobic</td>
<td>1.2</td>
<td>1.6</td>
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<td>frdB</td>
<td>fumarate reductase, anaerobic</td>
<td>1.3</td>
<td>1.1</td>
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<tr>
<td>frdC</td>
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<td>1.1</td>
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<tr>
<td>frdD</td>
<td>fumarate reductase, anaerobic</td>
<td>1.2</td>
<td>0.6</td>
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<td>1.7</td>
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<td>fumA</td>
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<td>fumB</td>
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<td>fumC</td>
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Formate hydrogenase

<table>
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<th>sirA</th>
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<tr>
<td>fhlA</td>
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<td>fdhD</td>
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<td>fdhE</td>
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<td>fdnG</td>
<td>putative molybdopterin oxidoreductases</td>
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<td>0.7</td>
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<td>1.9</td>
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<td>1.3</td>
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<td>1.4</td>
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<tr>
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<td>formate dehydrogenase-O, Fe-S subunit</td>
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<td>0.9</td>
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<td>1.4</td>
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</tbody>
</table>

All values represent the median relative fold change in gene expression between either the growth condition and appropriate control or between the mutant and wild type grown under the same condition.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>ileal SCFA</th>
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<th>sirA</th>
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<td>fdol</td>
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<td>hybB</td>
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<td>1.9</td>
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<td>0.4</td>
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<tr>
<td>hybD</td>
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<td>1.2</td>
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<td>hybG</td>
<td>hydrogenase-2 operon protein</td>
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<td>hybA</td>
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<td>-</td>
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<td>hybD</td>
<td>hydrogenase 3, membrane subunit</td>
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<td>hybE</td>
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<tr>
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<td>hydG</td>
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<td>hydN</td>
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<td>1.9</td>
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<td>hypB</td>
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Asparagine synthetase

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<th>gene</th>
<th>Description</th>
<th>ileal SCFA</th>
<th>Colonic SCFA</th>
<th>barA</th>
<th>sirA</th>
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</tr>
<tr>
<td>asnC</td>
<td>transcriptional regulator</td>
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<td>-</td>
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<tr>
<td>asNS</td>
<td>asparagine tRNA synthetase</td>
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<td>0.8</td>
<td>1.7</td>
<td>2.5</td>
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<td>aspA</td>
<td>aspartate ammonia-lyase</td>
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<td>aspS</td>
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<td>0.7</td>
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<td>0.2</td>
<td>1.3</td>
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All values represent the median relative fold change in gene expression between either the growth condition and appropriate control or between the mutant and wild type grown under the same condition.
<table>
<thead>
<tr>
<th>Genotype</th>
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<td>ΔcsrB::kan</td>
<td>Kan</td>
<td>Altier et al. (2000b)</td>
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<tr>
<td>Δ(barA)</td>
<td>none</td>
<td>Johnston et al. (1996) and Altier et al. (2000b)</td>
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<tr>
<td>sirA::Tn10d</td>
<td>Tet</td>
<td>Worley et al. (2000)</td>
</tr>
<tr>
<td>ssrB::cam</td>
<td>Cam</td>
<td>Worley et al. (2000)</td>
</tr>
<tr>
<td>Δ(ack-pta)</td>
<td>Kan</td>
<td>This work</td>
</tr>
<tr>
<td>Δ(barA), Δ(ack-pta)</td>
<td>Kan</td>
<td>This work</td>
</tr>
<tr>
<td>cbiD24::MudJ</td>
<td>Kan</td>
<td>Andersson and Roth, 1989</td>
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<tr>
<td>ttrB123::MudJ</td>
<td>Kan</td>
<td>Price-Carter et al., 2001</td>
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<tr>
<td>metE205 ara-9 pdu12::MudJ</td>
<td>Kan</td>
<td>Walter et al., 1997</td>
</tr>
<tr>
<td>eut-38::MudJ</td>
<td>Kan</td>
<td>J. Roth</td>
</tr>
</tbody>
</table>

Cam, Kan, and Tet, resistance to chloramphenicol, kanamycin, and tetracycline respectively.
Figure 1. Regulation of HilD by intestinal SCFA. Northern analysis was performed using total bacterial RNA prepared from ΔcsrA::cam (lane 1), and wild type (lanes 2-6) with a 509 bp hilD region as probe. Strains were grown in LB broth buffered to pH 6.7 (lanes 2-6) and supplemented with 30 mM sodium chloride (lanes 1 and 2) or 200 mM sodium chloride (lane 3) or with mixtures of short-chain fatty acids representing the distal ileum (lane 4) and the colon (lane 5). A mixture containing a total concentration of 30 mM SCFA using the proportions found in the colon was also tested (lane 6). (16.5 mM sodium acetate, 10.5 mM sodium propionate, and 3 mM sodium butyrate).
Figure 2. Regulation of SseB by intestinal SCFA. Northern analysis was performed using total bacterial RNA prepared from ssrB::kan (lane 2), and wild type (lanes 3-7) with a 596 bp sseB region as probe. Strains were grown in LB broth buffered to pH 6.7 (lanes 2-6) and supplemented with 30 mM sodium chloride (lanes 2 and 3) or 200 mM sodium chloride (lane 4) or with mixtures of short-chain fatty acids representing the distal ileum (lane 5) and the colon (lane 6). A mixture containing a total concentration of 30 mM SCFA using the proportions found in the colon was also tested (lane 7). (16.5 mM sodium acetate, 10.5 mM sodium propionate, and 3 mM sodium butyrate). Lane 1 is a molecular weight marker.
Figure 3. Colonic SCFAs regulate the utilization of propanediol. β-galactosidase production from lacZ fusions to pdu (A), eut (B), cbi (C), and ttr (D) were used to measure expression in the wild type grown in LB broth supplemented with 30 mM sodium chloride (black bars), 200 mM sodium chloride (dark gray bars), ileal SCFAs (white bars), colonic SCFAs (light gray bars), SCFAs at total 30 mM concentration, 55% acetate, 35% propionate, 10% butyrate (diagonally hatched bars), 30 mM sodium acetate (horizontally hatched bars), 30 mM sodium propionate (checkerboard), and 30 mM sodium butyrate (stippled). Error bars represent standard error of the mean. Asterisks indicate significant difference in β-galactosidase production as compared to wild type.
Figure 4. Regulation of CsrB by intestinal SCFA. Northern analysis was performed using total bacterial RNA prepared from ΔcsrB::kan (lane 1A), ΔcsrA::cam (lane 1B), and wild type (lanes 2-6) with a 594 bp csrB region as probe (A) or with a 180 bp csrA region as probe (B). Strains were grown in LB broth buffered to pH 6.7 (lanes 2-6) and supplemented with 30 mM sodium chloride (lanes 2 and 3) or 200 mM sodium chloride (lane 4) or with mixtures of short-chain fatty acids representing the distal ileum (lane 5) and the colon (lane 6). A mixture containing a total concentration of 30 mM SCFA using the proportions found in the colon was also tested (lane 7). (16.5 mM sodium acetate, 10.5 mM sodium propionate, and 3 mM sodium butyrate). Lane 1 is a molecular weight marker.
Figure 5. A model for the regulation of invasion gene expression by intestinal SCFA, BarA, and SirA. Acetyl-phosphate is produced in the bacterial cytoplasm from acetate by acetate kinase, \textit{ackA}, or from acetyl-CoA by phosphotransacetylase, \textit{pta}. Acetyl-phosphate phosphorylates either the response regulator SirA directly, or the sensor kinase BarA, which in turn transfers its phosphate groups to SirA. Alternatively, in the mammalian intestinal tract, BarA can be activated by an undefined signal independent of acetyl-phosphate. Acetate is produced by bacterial fermentation in the distal ileum and colon. Ileal SCFAs function predominantly through effects induced by acetate. Colonic SCFAs act in opposition to ileal SCFAs, increasing expression of CsrB thereby decreasing invasion gene expression. Decreased cytoplasmic pH resulting from colonic SCFAs supplementation may also induce regulation through additional two-component regulators such as PhoP/PhoQ.