

# Virulence Gene Regulation by L-Arabinose in *Salmonella enterica*

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**ABSTRACT** Invasion of the intestinal epithelium is a critical step in *Salmonella enterica* infection and requires functions encoded in the gene cluster known as *Salmonella* Pathogenicity Island 1 (SPI-1). Expression of SPI-1 genes is repressed by L-arabinose, and not by other pentoses. Transport of L-arabinose is necessary to repress SPI-1; however, repression is independent of L-arabinose metabolism and of the L-arabinose-responsive regulator AraC. SPI-1 repression by L-arabinose is exerted at a single target, HilD, and the mechanism appears to be post-translational. As a consequence of SPI-1 repression, L-arabinose reduces translocation of SPI-1 effectors to epithelial cells and decreases *Salmonella* invasion *in vitro*. These observations reveal a hitherto unknown role of L-arabinose in gene expression control and raise the possibility that *Salmonella* may use L-arabinose as an environmental signal.

**KEYWORDS** *Salmonella* pathogenicity island 1; *Salmonella* invasion; L-arabinose; HilD

INVASION of epithelial cells by *Salmonella enterica* requires the expression of genes located in a 40-kb region known as *Salmonella* pathogenicity island 1 (SPI-1) (Lostroh and Lee 2001; Altier 2005; Jones 2005). SPI-1 is a cluster of ~40 genes organized in several transcriptional units that encodes a Type 3 Secretion System (TTSS) and effector proteins that are translocated into epithelial cells (Lostroh and Lee 2001). Inside the epithelial cell, the effector proteins trigger cytoskeleton rearrangements necessary for *Salmonella* invasion (Darwin and Miller 1999a). SPI-1 also encodes transcriptional activators responsible for its own expression: HilA, HilC, HilD, and InvF (Lostroh and Lee 2001; Ellermeier and Slauch 2007). HilA is a transcriptional activator of the OmpR/ToxR family (Lee *et al.* 1992; Bajaj *et al.* 1995) and activates transcription of genes encoding components of the TTSS and the transcriptional activator InvF (Bajaj *et al.* 1996). In association with the SicA chaperone, InvF boosts transcription of the *sicA/sip* operon, which encodes effector

proteins (Darwin and Miller 1999b; Eichelberg and Galan 1999). Transcription of *hilA* is directly activated by HilC and HilD, both members of the AraC/XylS family of transcriptional regulators (Schechter and Lee 2001). HilC and HilD relieve repression of the *hilA* promoter by the nucleoid proteins H-NS and Hha (Olekhovich and Kadner 2006) and can activate expression of the *invF* and *sicA/sip* transcriptional units independently of HilA (Rakeman *et al.* 1999; Akbar *et al.* 2003). Furthermore, HilD activates transcription of *hilC* and of *hilD* itself (Ellermeier *et al.* 2005) by direct binding to both promoters (Olekhovich and Kadner 2002). Together with a transcriptional activator encoded outside SPI-1, RtsA (Ellermeier and Slauch 2003), SPI-1 transcription factors form a regulatory network that governs SPI-1 expression in response to environmental and physiological factors (Supporting Information, Figure S1) (Jones 2005; Ellermeier and Slauch 2007).

During passage through the digestive tract, *Salmonella* encounters environmental conditions that affect SPI-1 expression. In the stomach, SPI-1 is repressed at acidic pH (Behlau and Miller 1993; Bajaj *et al.* 1996), thus preventing invasion. The proximal part of the small intestine is under the influence of digestive fluids coming from the stomach, and the pH remains slightly acidic. In the duodenum, SPI-1 is repressed by bile, thereby inhibiting invasion of the proximal section of the small intestine (Prouty and Gunn 2000). In the large intestine, SPI-1 is repressed by the short chain fatty acids propionate and butyrate, synthesized by the

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intestinal microbiota (Lawhon *et al.* 2002). The gradients that repress SPI-1 along the digestive tract, however, leave a region, the ileum, that permits SPI-1 expression. As a consequence, the ileum is the portion of the small intestine that *Salmonella* preferentially invades (Jones *et al.* 1994).

In this study, we describe that the pentose L-arabinose represses SPI-1 expression. L-Arabinose is the second most abundant pentose in nature (Schadel *et al.* 2010; Seiboth and Metz 2011) and is found in hemicellulose and pectin in plant cell walls (Abedon *et al.* 2006; Schadel *et al.* 2010). *Salmonella* can use L-arabinose as the sole carbon source (Gutnick *et al.* 1969). L-Arabinose is transported into *Salmonella* cells by a permease encoded by the *araE* gene (Lee *et al.* 1981, 1982). Inside the cell, L-arabinose is sequentially transformed into L-ribulose, L-ribulose-5-phosphate, and D-xylulose-5-phosphate by the action of L-arabinose isomerase, ribulokinase, and L-ribulose-5-phosphate-4-epimerase, respectively (Englesberg 1961; Englesberg *et al.* 1962). L-Ribulose-5-phosphate and D-xylulose-5-phosphate are substrates for the pentose phosphate pathway, which produces glycolytic intermediates (Figure S2). L-Arabinose isomerase, ribulokinase, and L-ribulose-5-phosphate-4-epimerase are encoded by the *araA*, *araB*, and *araD* genes, respectively (Englesberg 1961; Englesberg *et al.* 1962), arranged in a single transcriptional unit, the *araBAD* operon (Gross and Englesberg 1959; Englesberg 1961; Englesberg *et al.* 1962; Lee *et al.* 1984). Expression of the *araBAD* operon and the *araE* gene is induced by L-arabinose (Lee *et al.* 1980, 1982) and requires the transcriptional regulator AraC (Englesberg *et al.* 1965; Lee *et al.* 1981). The *araC* gene is located upstream of the *araBAD* operon, but it is transcribed in divergent orientation (Lee *et al.* 1984). AraC bound to L-arabinose activates transcription from the *araBAD* and *araE* promoters. However, in the absence of L-arabinose, AraC acts as a repressor (Schleif 2010).

The phenomenon described in this study, SPI-1 repression by L-arabinose, is independent of L-arabinose catabolism and of the regulatory protein AraC. We describe epistasis analysis indicating that the target of L-arabinose within SPI-1 is the transcription factor HilD and present evidence that the inhibitory mechanism may be post-translational. HilD inhibition causes SPI-1 repression, which results in impaired secretion of SPI-1 effectors and reduced invasion of epithelial cells.

## Materials and Methods

### Bacterial strains, plasmids, bacteriophages, and strain construction

All the *S. enterica* strains listed in Table 1 belong to serovar Typhimurium and derive from the mouse-virulent strain American Type Culture Collection (ATCC) 14028. For simplicity, *S. enterica* serovar Typhimurium is often abbreviated as *S. enterica*. Targeted gene disruption was achieved using pKD13 or pKD3 (Datsenko and Wanner 2000). Antibiotic resistance cassettes introduced during strain construction

were excised by recombination with plasmid pCP20 (Datsenko and Wanner 2000). The oligonucleotides used for disruption (labeled “UP” and “DO”) are listed in Table S1, together with the oligonucleotides (labeled “E”) used for allele verification by the polymerase chain reaction. For the construction of transcriptional and translational *lac* fusions in the *Salmonella* chromosome, FRT sites generated by excision of Km<sup>r</sup> cassettes (Datsenko and Wanner 2000) were used to integrate either pCE37 or pCE40 (Ellermeier *et al.* 2002). An exception was the *hilD::lac477* translational fusion, constructed using the method described by M. Hensel and co-workers (Gerlach *et al.* 2007). Unless specified otherwise, all *lac* fusions used in this study are translational. Addition of a 3xFLAG epitope tag to protein-coding DNA sequences was carried out using plasmid pSUB11 (Km<sup>r</sup>, 3xFLAG) as template (Uzzau *et al.* 2001). Plasmid pIZ1902 (pXG10-*hilD*) was constructed by cloning a DNA fragment encompassing the *hilD* transcription start point and the *hilD* transcriptional terminator (Lopez-Garrido *et al.* 2014) on BrfBI-*NheI* restriction sites in pXG10 (Urban and Vogel 2007). Transductional crosses using phage P22 HT105/1 *int201* (Schmieger 1972 and G. Roberts, unpublished results) were used for strain construction operations involving chromosomal markers. The transduction protocol was described elsewhere (Garzon *et al.* 1995). To obtain phage-free isolates, transductants were purified by streaking on green plates. Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5.

Expression of *araE* from a heterologous promoter was achieved by replacing its native promoter with the P<sub>LtetO</sub> promoter (Lutz and Bujard 1997). A fragment containing the *cat* gene and the P<sub>LtetO</sub> promoter was amplified by PCR using pXG1 as template (Urban and Vogel 2007) and primers P<sub>LtetO</sub>UP and P<sub>LtetO</sub>DO (Table S1). The PCR product was treated with DpnI to remove template traces. The construction was inserted in the chromosome by  $\lambda$  Red recombinase-mediated recombination (Datsenko and Wanner 2000), and Cm<sup>r</sup> colonies were selected. Insertion of the construction was verified by PCR using a pair of primers specific for the *cat* gene and the target gene (Table S1).

### Growth conditions

Luria–Bertani (LB) broth was used as standard liquid medium. Solid media were prepared by the addition of 1.5% agar. L-Arabinose, D-arabinose, D-xylose, or sucrose was added from 20% stocks prepared in distilled water. Batch cultures were performed in LB or LB supplemented with the appropriate sugar and incubated at 37° with shaking at 220 × g. Samples were taken when the cultures had reached stationary phase (OD<sub>600</sub> 2–2.5). For translocation and invasion assays, an overnight bacterial culture of the appropriate strain was diluted 1:50 in LB and LB + 0.2% L-arabinose and was incubated at 37° until stationary phase (OD<sub>600</sub> ~2). Carbon-free medium (NCE) (Maloy and Roth 1983) supplemented with the appropriate carbon source was used as minimal medium. Green plates were prepared according to Chan *et al.* (1972), except that methyl blue

**Table 1** Strains used in this study

Strain designation	Genotype or description	Source
ATCC 14028	Wild type	ATCC
SV5999	$\Delta araA$	This study
SV5297	$\Phi(invF'-lacZ^+)$ (Hyb)	Lopez-Garrido and Casadesus (2010)
SV6000	$\Delta araA \Phi(invF'-lacZ^+)$ (Hyb)	This study
SV6205	$\Delta araA \Phi(hilA'-lacZ^+)$ (Hyb)	This study
SV6206	$\Delta araA \Phi(sipB'-lacZ^+)$ (Hyb)	This study
SV6207	$\Delta araA \Phi(prgH'-lacZ^+)$ (Hyb)	This study
SV6209	$\Delta araA invF::3xFLAG$	This study
SV6208	$\Delta araA hilA::3xFLAG$	This study
SV6210	$\Delta araA sipB::3xFLAG$	This study
SV6211	$\Delta araA prgH::3xFLAG$	This study
SV5284	$\Phi(hilA'-lacZ^+)$ (Hyb)	Lopez-Garrido and Casadesus (2010)
SV6218	$\Delta xylA \Phi(hilA'-lacZ^+)$ (Hyb)	This study
SV6213	$\Delta araA \Delta araE \Phi(hilA'-lacZ^+)$ (Hyb)	This study
SV6212	$\Delta araBAD \Delta araC \Phi(hilA'-lacZ^+)$ (Hyb)	This study
SV7138	$\Delta araE \Delta araC \Phi(hilA'-lacZ^+)$ (Hyb)	This study
SV6244	$\Delta araA P_{LtetO-araE} \Phi(hilA'-lacZ^+)$ (Hyb)	This study
SV6245	$\Delta araBAD \Delta araC P_{LtetO-araE} \Phi(hilA'-lacZ^+)$ (Hyb)	This study
SV6219	$\Delta araA \Phi(hilC'-lacZ^+)$ (Hyb)	This study
SV6222	$\Delta araA \Delta hilD \Phi(hilC'-lacZ^+)$ (Hyb)	This study
SV6220	$\Delta araA \Phi(rtsA'-lacZ^+)$ (Hyb)	This study
SV6223	$\Delta araA \Delta hilD \Phi(rtsA'-lacZ^+)$ (Hyb)	This study
SV6221	$\Delta araA \Phi(invH'-lacZ^+)$ (Hyb)	This study
SV6224	$\Delta araA \Delta hilD \Phi(invH'-lacZ^+)$ (Hyb)	This study
SV6417	$\Delta araA \Phi(hilD-lacZ1)$	This study
SV6419	$\Delta araA \Phi(hilD'-lacZ477)$ (Hyb)	This study
SV6421	$\Delta araA \Phi(hilD-lacZ930)$	This study
SV7139	$\Delta araA P_{LtetO-hilD} \Phi(hilD-lacZ930)$	This study
SV7140	$\Delta araA P_{LtetO-hilD}$	This study
SV6657	$\Delta araA P_{LtetO-hilD} \Phi(hilA'-lacZ^+)$ (Hyb)	This study
SV6658	$\Delta araA P_{LtetO-hilD} \Phi(invF'-lacZ^+)$ (Hyb)	This study
SV6659	$\Delta araA \Phi(rtsA'-lacZ^+)$ (Hyb)/pXG10	This study
SV6660	$\Delta araA \Delta spi-1 \Phi(rtsA'-lacZ^+)$ (Hyb)/pXG10	This study
SV6661	$\Delta araA \Delta spi-1 \Phi(rtsA'-lacZ^+)$ (Hyb)/pXG10- <i>hilD</i>	This study
SV6199	$\Delta araE$	This study
SV6197	$\Delta araC$	This study
SV6243	$P_{LtetO-araE}$	This study
SV6201	$\Delta xylA$	This study
SV6423	$\Delta araA \Phi(sipA'-cya^+)$ (Hyb)	This study
SV6424	$\Delta araA \Delta araE \Phi(sipA'-cya^+)$ (Hyb)	This study
SV6425	$\Delta araA \Delta prgH \Phi(sipA'-cya^+)$ (Hyb)	This study

(Sigma Chemical, St. Louis) was substituted for aniline blue.

### pH curves

An overnight culture of *Salmonella* was diluted 1:50 in LB and in LB containing 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1% L-arabinose. The cultures were incubated at 37° until OD<sub>600</sub> ~2.5. The cultures were then centrifuged for 20 min at 2600 × g, and the pH of the supernatant was determined using a pH meter Basic 20 (Crison Instruments, Barcelona, Spain).

### Protein extracts and Western blot analysis

Total protein extracts were prepared from bacterial cultures grown in LB or LB + L-arabinose at 37° until stationary phase (final OD<sub>600</sub> ~2.5). Bacterial cells were collected by centrifugation (16,000 × g, 2 min) and suspended in 100 μl

of Laemmli sample buffer [1.3% SDS, 10% (v/v) glycerol, 50 mM Tris-HCl, 1.8% β-mercaptoethanol, 0.02% bromophenol blue, pH 6.8]. Proteins were resolved by Tris-Tricine-PAGE using 12% gels. Conditions for protein transfer have been described elsewhere (Jakomin *et al.* 2008). Optimal dilutions of primary antibodies were as follows: anti-Flag M2 monoclonal antibody (1:5,000, Sigma Chemical) and anti-GroEL polyclonal antibody (1:20,000, Sigma). Goat anti-mouse horseradish peroxidase-conjugated antibody (1:5000, Bio-Rad, Hercules, CA) or goat anti-rabbit horseradish-peroxidase-conjugated antibody (1:20,000, Santa Cruz Biotechnology, Heidelberg, Germany) were used as secondary antibodies. Proteins recognized by the antibodies were visualized by chemoluminescence using the luciferin-luminol reagents in a LAS3000 mini imaging system (Fujifilm, Tokyo). For quantification, the intensity of the bands was determined

using the MultiGauge software (Fujifilm). GroEL was used as loading control.

### **RNA extraction and Northern blot analysis**

A 2-ml aliquot from a stationary culture ( $OD_{600} \sim 2$ ) was centrifuged at  $16,000 \times g$ ,  $4^\circ$ , for 5 min. The pellet was resuspended in  $100 \mu\text{l}$  of a solution of lysozyme (3 mg/ml, Sigma Chemical). Cell lysis was facilitated by three consecutive freeze–thaw cycles. After lysis, RNA was extracted using 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA), according to manufacturer instructions. Finally, total RNA was resuspended in  $30 \mu\text{l}$  of RNase-free water for subsequent uses. The quality and quantity of the RNA was determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). For Northern blot analysis,  $10 \mu\text{g}$  of total RNA was loaded per well and electrophoresed in denaturing 1% agarose formaldehyde gels. Transfer and fixation to Hybond-N<sup>+</sup> membranes (GE Healthcare, Little Chalfont, UK) were performed by vacuum using 0.05 M NaOH. Filters were then hybridized using an internally labeled [ $\alpha$ -<sup>32</sup>P]UTP riboprobe specific for the upstream (5') 300 nucleotides of the *hilD* coding sequence, generated by *in vitro* transcription using the MEGAscript T7 Transcription Kit (Invitrogen). Hybridization was carried out at  $65^\circ$ . As a control of RNA loading and transfer efficiency, the filters were hybridized with a riboprobe for the RNase P messenger RNA (mRNA) gene (*rnpB*). Images of radioactive filters were obtained with a FLA-5100 imaging system (Fujifilm), and quantification was performed using MultiGauge software (Fujifilm).

### **$\beta$ -Galactosidase assays**

Levels of  $\beta$ -galactosidase activity were assayed using the  $\text{CHCl}_3$ –sodium dodecyl sulfate permeabilization procedure (Miller 1972). Data are averages and standard deviations of three or more independent experiments.

### **Preparation of HeLa cells for infection assays**

HeLa cells (ATCC CCL2) were cultured in tissue culture medium (Dulbecco's modified essential medium supplemented with 10% fetal calf serum and 2 mM L-glutamine). For routine cultivation,  $60 \mu\text{g/ml}$  penicillin and  $100 \mu\text{g/ml}$  streptomycin were added to the culture medium. The day before infection,  $\sim 1.5 \times 10^5$  HeLa cells were seeded, using 24-well plates (Costar, Corning, NY). Each well contained 1 ml of tissue culture medium without antibiotics. Cells were grown at  $37^\circ$ , 5%  $\text{CO}_2$ , to obtain 80% confluency. One hour before infection, the culture medium was removed and replaced by 0.5 ml fresh tissue culture medium without antibiotics.

### **Translocation assays**

Aliquots of bacterial suspensions were added to reach a multiplicity of infection of 15:1 bacteria/HeLa cell. Plates were centrifuged at  $200 \times g$  for 5 min and incubated at  $37^\circ$ , 5%  $\text{CO}_2$ , for 10 min. Infected cells were then washed three

times with PBS and lysed with a 0.1 M HCl solution containing 0.1% Triton X-100. Quantification of cAMP was performed using the Direct Cyclic AMP Enzyme Immunoassay Kit (Array Designs, Ann Arbor, MI) following the manufacturer's instructions.

### **Invasion assays**

Bacterial cultures were grown overnight at  $37^\circ$  in LB, diluted into fresh medium (1:50), and incubated at  $37^\circ$  until stationary phase ( $OD_{600} \sim 2$ ). Bacterial suspensions were added to reach a multiplicity of infection of 50:1 bacteria: HeLa cell. HeLa cells were infected for 30 min, washed three times with PBS, incubated in fresh tissue culture medium containing  $100 \mu\text{g/ml}$  gentamicin for 1.5 hr, and washed three times with PBS. Numbers of viable intracellular bacteria were obtained by lysing infected cells with 1% Triton X-100 (prepared in PBS) and subsequent plating. Invasion rates were calculated as the ratio between viable intracellular bacteria and viable bacteria added to infect the HeLa cells.

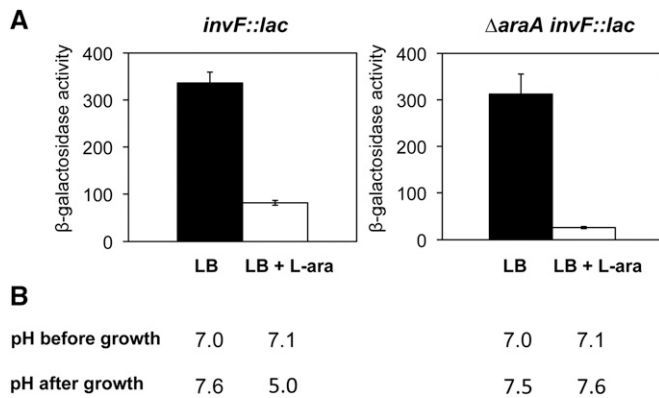
## **Results**

### ***SPI-1* expression is downregulated in the presence of L-arabinose**

pBAD vectors are a set of plasmids that contain the  $P_{\text{BAD}}$  promoter of the L-arabinose operon and the regulatory gene *araC* (Guzman *et al.* 1995). In the presence of L-arabinose, transcription from the  $P_{\text{BAD}}$  promoter is turned on, while in its absence transcription is extremely low. Use of pBAD vectors thus allows conditional, L-arabinose-dependent expression of cloned genes (Guzman *et al.* 1995). We considered the possibility of using pBAD vectors to study SPI-1 expression. As a preliminary control, we examined whether SPI-1 expression was affected by the presence of L-arabinose (in a strain without pBAD). For this purpose, we measured the  $\beta$ -galactosidase activity of an *invF::lac* fusion in LB and in LB + 0.2% L-arabinose. As shown in Figure 1A, *invF::lac* expression was reduced around fourfold in LB + L-arabinose. This observation suggested that the presence of L-arabinose in the culture medium might repress expression of SPI-1 genes.

### ***SPI-1* repression in the presence of L-arabinose is independent of L-arabinose catabolism**

The carbon sources for enteric bacteria in LB are catabolizable amino acids, not sugars (Sezonov *et al.* 2007). Under such conditions, enteric bacteria undergo gluconeogenesis, and the culture medium is slightly alkalinized during growth. However, if a catabolizable sugar is added to LB, *Salmonella* uses it as a carbon source and undergoes glycolysis. As a consequence, pyruvic acid is produced and the culture medium is acidified (Figure S2). Because *S. enterica* is able to regulate SPI-1 expression in response to pH (Bajaj *et al.* 1996), we considered the possibility that repression of SPI-1 by L-arabinose could be an indirect effect of acidification. We measured the pH of LB and LB + 0.2% L-arabinose before inoculation (before growth) and when the bacterial



**Figure 1** Regulation of *invF* by L-arabinose. (A) β-Galactosidase activity of an *invF::lac* fusion in LB (black bars) and in LB + 0.2% L-arabinose (white bars). (Left) Activities measured in an AraA<sup>+</sup> background. (Right) Activities measured in an AraA<sup>-</sup> background. (B) pH of the culture medium measured before bacterial inoculation (before growth) and after the bacterial culture had reached an OD<sub>600</sub> of ~2.5 (after growth).

population had reached an OD<sub>600</sub> of ~2.5 (after growth) (Figure 1B, left). Before growth, both LB and LB + L-arabinose showed a pH near neutrality (7.0 and 7.1, respectively). However, after bacterial growth the pH of LB was slightly alkaline (7.6) while the pH of LB + L-arabinose was 5.0. This difference indicated that catabolism of L-arabinose by *Salmonella* acidifies indeed the culture medium and supported the possibility that SPI-1 repression by L-arabinose might be a consequence of acidification. To test this hypothesis, we investigated the effect of L-arabinose in a mutant lacking L-arabinose isomerase (AraA), the enzyme that catalyzes the first step of L-arabinose catabolism (Englesberg 1961). As expected, an AraA<sup>-</sup> mutant was unable to grow in minimal medium with L-arabinose as the sole carbon source (Figure S3). More interestingly, growth of the AraA<sup>-</sup> mutant did not cause acidification of the culture medium upon growth in LB + 0.2% L-arabinose (pH 7.6 compared with 7.5 in LB without L-arabinose) (Figure 1B). Absence of acidification was likewise observed at higher L-arabinose concentrations (Figure S3). Thus, if SPI-1 repression in the presence of L-arabinose was due to culture medium acidification, it should not be observed in an AraA<sup>-</sup> mutant. However, to our surprise, an *invF::lac* fusion was found to be repressed >10-fold by 0.2% L-arabinose in an AraA<sup>-</sup> background (Figure 1A, right), suggesting that SPI-1 repression is exerted by L-arabinose itself, rather than being a consequence of culture medium acidification. To avoid indirect effects caused by medium acidification, further experiments were performed in an AraA<sup>-</sup> background.

### The entire SPI-1 is repressed by L-arabinose even at low concentrations

To investigate whether L-arabinose repressed only a subset of SPI-1 genes or the entire island, we analyzed the expression of additional SPI-1 genes (Figure 2). We chose genes encoded in different transcriptional units and representing

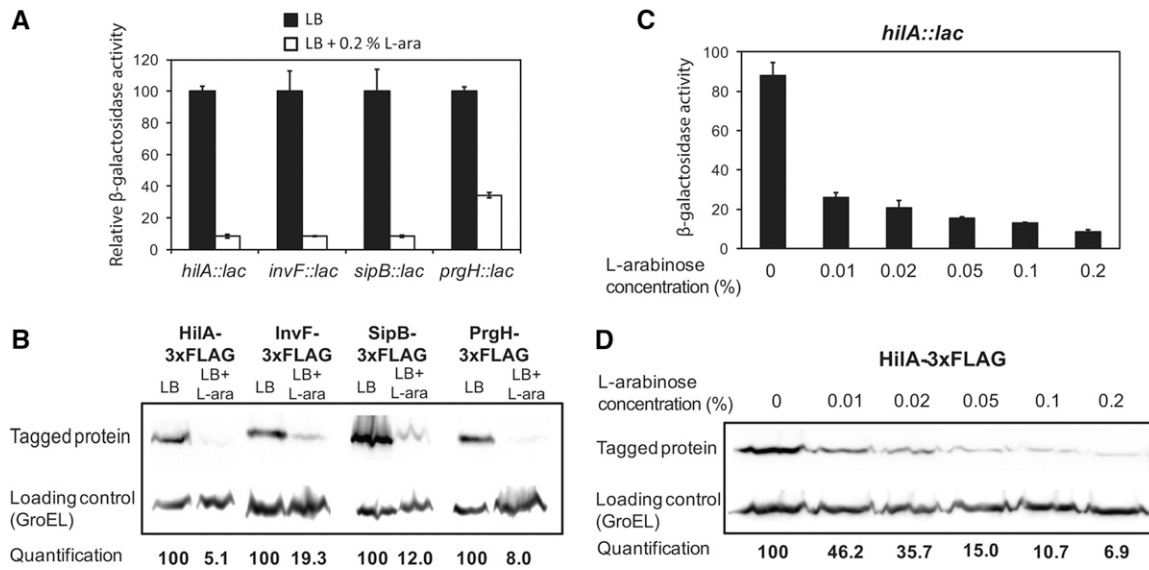
different functional categories: *hilA*, which encodes a major transcriptional activator of SPI-1; *invF*, which encodes a transcriptional activator of a subset of SPI-1 genes; *sipB*, which encodes an effector protein; and *prgH*, which encodes a component of the SPI-1 TTSS. SPI-1 gene expression was compared in LB and LB + 0.2% L-arabinose by two independent methods: (1) measuring the β-galactosidase activity of *lac* fusions (Figure 2A); and (2) determining the protein levels by Western blot (Figure 2B). Both the β-galactosidase activities and the protein levels decreased in the presence of L-arabinose (Figure 2).

Next we analyzed the effect of different concentrations of arabinose on SPI expression, using the central SPI-1 regulator HilA as a reporter. The effect of varying the concentration of L-arabinose concentration was tested again by β-galactosidase assays and Western blotting. A gradual decrease in *hilA* expression as the L-arabinose concentration increased was observed in both the β-galactosidase assays (Figure 2C) and the Western blot analyses (Figure 2D). Note that, at the lower concentration of L-arabinose assayed (0.01%, equivalent to 66.7 μM), *hilA* expression was still reduced (more than threefold in β-galactosidase assays and more than twofold in Western blots). Dose-dependent repression suggests the occurrence of an analog response in which L-arabinose is used by *Salmonella* as a signal.

### Effect of pentoses other than L-arabinose on SPI-1 expression

In addition to L-arabinose, *Salmonella* can metabolize other pentoses (Gutnick *et al.* 1969). An example is D-xylose, the most abundant pentose in nature (Seiboth and Metz 2011). The first reaction in D-xylose catabolism is catalyzed by D-xylose isomerase, encoded by the *xylA* gene (Shamanna and Sanderson 1979). To examine the effect of D-xylose on SPI-1 expression, we constructed a strain carrying a *xylA* deletion. The *xylA* mutant is unable to use D-xylose as sole carbon source (Figure S4), thus avoiding a change in the pH of the culture medium due to D-xylose catabolism. Expression of a *hilA::lac* fusion was monitored in a XylA<sup>-</sup> background, growing the strain in LB, LB + 0.2% D-xylose, and LB + 1% D-xylose. As shown in Figure 3 (center), the presence of D-xylose in the culture medium did not alter *hilA::lac* expression. However, the same fusion was strongly repressed in the presence of either 0.2 or 1% L-arabinose (Figure 3, left).

D-Arabinose, the D-stereoisomer of arabinose, cannot be used by *Salmonella* as sole carbon source (Gutnick *et al.* 1969). However, certain *Salmonella* mutants can metabolize D-arabinose (Old and Mortlock 1977). In such mutants, D-arabinose induces the synthesis of two enzymes usually involved in L-fucose metabolism: L-fucose isomerase and L-fuculokinase. These enzymes have a bifunctional activity and can convert D-arabinose into D-ribulose-1-phosphate. Figure 3 (right) shows that *hilA::lac* expression is similar in LB, LB + 0.2% D-arabinose, and LB + 1% D-arabinose, thus ruling out any effect of D-arabinose on SPI-1 expression.



**Figure 2** Dose-dependent regulation of SPI-1 by L-arabinose. (A)  $\beta$ -Galactosidase activities of *hilA::lac*, *invF::lac*, *sipB::lac*, and *prgH::lac* fusions in the absence (black bars) and in the presence (white bars) of 0.2% L-arabinose, measured in AraA<sup>-</sup> background. Due to disparity between the  $\beta$ -galactosidase activities of the fusions, the  $\beta$ -galactosidase activities of individual *lac* fusions in LB without L-arabinose have been normalized to 100. The actual  $\beta$ -galactosidase activities in LB were 410, 308, 5384, and 1184 Miller units for *hilA::lac*, *invF::lac*, *sipB::lac*, and *prgH::lac*, respectively. (B) Levels of HiIA, InvF, SipB, and PrgH in protein extracts from AraA<sup>-</sup> strains grown in LB with and without 0.2% L-arabinose. Proteins tagged with the 3xFLAG epitope were detected by Western blotting using anti-FLAG commercial antibodies. The loading control was GroEL in all cases. (C)  $\beta$ -Galactosidase activity of a *hilA::lac* fusion in an AraA<sup>-</sup> strain grown in the presence of increasing concentrations of L-arabinose. (D) Level of HiIA-3xFLAG in protein extracts from an AraA<sup>-</sup> strain grown in increasing concentrations of L-arabinose. The HiIA level was normalized to GroEL for quantification.

The conclusion from these experiments was that the effect of L-arabinose on SPI-1 expression is specific, rather than a property shared with other pentoses such as D-xylose and D-arabinose.

### SPI-1 repression requires L-arabinose transport

Trying to understand better SPI-1 repression by L-arabinose, we investigated whether L-arabinose exerted its repressive action from outside the cell or needed to be transported inside. In *Salmonella*, L-arabinose is transported by a specific permease encoded by the *araE* gene (Lee *et al.* 1981, 1982). Note that *Salmonella* does not harbor the AraFG transport system found in *Escherichia coli* (Kolodrubetz and Schleif 1981). A *Salmonella* AraE<sup>-</sup> mutant constructed *ad hoc* was unable to grow in minimal medium containing 0.1% L-arabinose as the sole carbon source grew slowly in the presence of 0.2% L-arabinose and grew like the wild-type strain in 1% L-arabinose (Figure S3). These observations suggest that the AraE permease is essential for transport of L-arabinose when the extracellular concentration is low; higher L-arabinose concentrations, however, may permit transport by AraE-independent routes. This view is supported by the ability of an AraE<sup>-</sup> mutant to acidify the culture medium when grown on high concentrations of L-arabinose (Figure S3). Note that the wild-type strain acidified the culture medium below pH 5 with 0.2% L-arabinose, while the AraE<sup>-</sup> mutant needed 1% L-arabinose to produce a similar acidification.

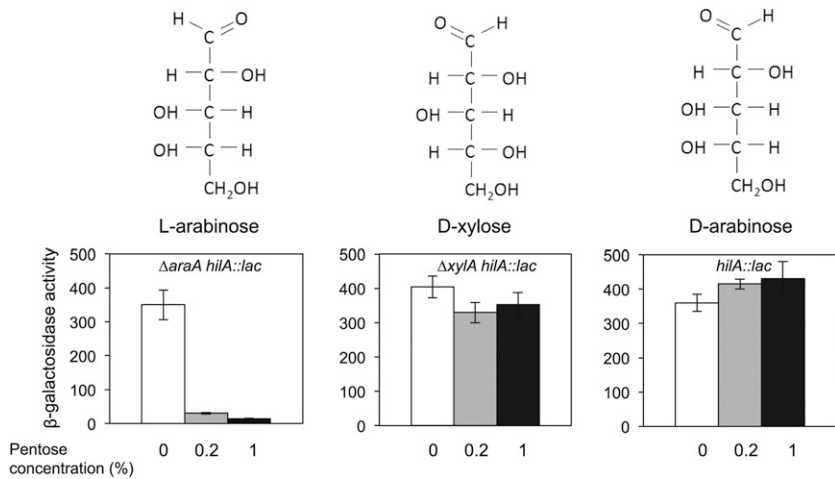
If L-arabinose needs to be inside the cell to repress SPI-1, we reasoned, repression should not be observed in an AraE<sup>-</sup>

mutant grown at low L-arabinose concentrations. At higher concentrations, however, L-arabinose would enter the cell through AraE-independent pathways, repressing SPI-1. If, on the contrary, extracellular L-arabinose was able to repress SPI-1 expression, repression should be observed in AraE<sup>+</sup> and AraE<sup>-</sup> backgrounds at both low and high L-arabinose concentrations. To ascertain between these alternative possibilities, we examined the expression of a *hilA::lac* fusion in isogenic AraA<sup>-</sup> and AraA<sup>-</sup> AraE<sup>-</sup> strains grown on different concentrations of L-arabinose (Figure 4A). The *araE* mutation nearly suppressed *hilA* repression in the presence of 0.2% L-arabinose. In the presence of higher L-arabinose concentrations, however, *hilA* remained significantly repressed. On the other hand, in all the L-arabinose concentrations assayed, repression was higher in the AraE<sup>+</sup> background than in the AraE<sup>-</sup> background. Altogether, these observations indicate that L-arabinose needs to be transported inside the cell to repress SPI-1.

To rule out the possibility that repression at high L-arabinose concentrations might be caused by high osmolarity, *hilA::lac* expression was examined also in the presence of sucrose (up to 3%). Expression of *hilA::lac* remained unaffected under all the sucrose concentrations assayed (Figure S5).

### SPI-1 repression by L-arabinose is independent of AraC

In the bacterial cytoplasm, L-arabinose binds AraC and causes an allosteric change that enables AraC to activate the expression of genes involved in L-arabinose catabolism, including *araE* (Schleif 2010). The evidence that L-arabinose



**Figure 3** Chemical structure of the pentoses L-arabinose, D-xylose, and D-arabinose (Top) and their effect on SPI-1 expression (Bottom). β-Galactosidase activity of a *hilA::lac* fusion in LB and LB + 0.2% and 1% of the appropriate pentose. Experiments with L-arabinose and D-xylose were performed in AraA<sup>-</sup> and XylA<sup>-</sup> backgrounds, respectively.

needs to be inside the cell to repress SPI-1 raised the possibility that activation of AraC by L-arabinose might be directly responsible for SPI-1 repression. To test this hypothesis, we compared the effect of increasing concentrations of L-arabinose on the expression of a *hilA::lac* fusion in AraA<sup>-</sup> and AraA<sup>-</sup> AraC<sup>-</sup> backgrounds. Lack of AraC suppressed *hilA* repression by 0.2% L-arabinose (Figure 4A, third column). However, this observation did not necessarily indicate that AraC is involved in L-arabinose repression. As AraC activates *araE* transcription, suppression in an AraC<sup>-</sup> background might be caused by a decrease of intracellular L-arabinose due to lack of AraE permease. In fact, *hilA* expression was increasingly repressed in an AraC<sup>-</sup> background as the L-arabinose concentration increased, and the repression pattern was very similar to that observed in an AraE<sup>-</sup> mutant (Figure 4A, second column). To ascertain whether AraC was directly involved in SPI-1 regulation or merely necessary for AraE permease synthesis, two different approaches were undertaken:

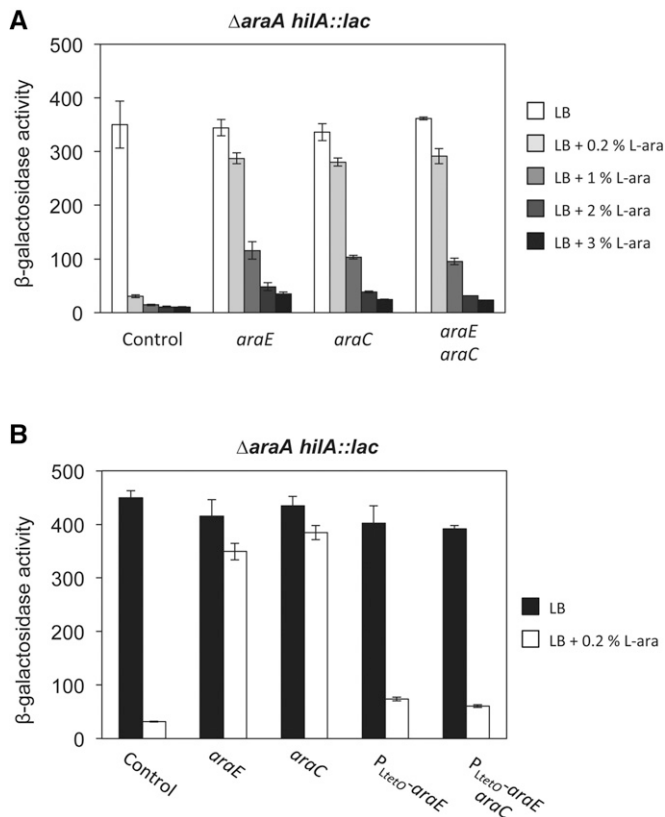
1. If suppression of SPI-1 regulation by L-arabinose in AraC<sup>-</sup> background was due to lack of AraE permease, an *araE* mutation should be epistatic over an *araC* mutation. Thus, a double-mutant AraE<sup>-</sup> AraC<sup>-</sup> and an AraE<sup>-</sup> mutant should display the same phenotype. However, if the effect of the *araC* mutation was independent of AraE permease, *araE* and *araC* mutations should have additive effects. As shown in Figure 4, an AraE<sup>-</sup> AraC<sup>-</sup> double mutant and an AraE<sup>-</sup> mutant displayed the same phenotype.
2. If AraC is needed for AraE permease synthesis only, constitutive *araE* expression should prevent AraC-mediated suppression of SPI-1 repression. AraC-independent expression of *araE* was achieved by replacing the native *araE* promoter with the P<sub>L<sub>letO</sub></sub> promoter (Lutz and Bujard 1997). The resulting strain grew as well as the wild type in minimal medium containing 0.1% L-arabinose (Figure S6). In addition, it acidified the culture medium like the wild type when grown in the presence of different concentrations of L-arabinose, suggesting that AraE permease was produced indeed (Figure S6).

When *araE* was expressed constitutively, lack of AraC no longer suppressed *hilA* repression by L-arabinose (Figure 4B).

Altogether, the above observations suggest that, once inside the cell, L-arabinose represses SPI-1 expression by an AraC-independent mechanism.

#### L-Arabinose-dependent repression of SPI-1 is transmitted via HilD

SPI-1 expression is controlled by a regulatory network of SPI-1-encoded transcriptional activators. On top of the network are the transcriptional activators HilA, HilC, and HilD (Figure S1). The fact that *hilA* expression is regulated by L-arabinose might indicate either that L-arabinose directly regulates *hilA* or that the regulatory input is transmitted to *hilA* via HilC and/or HilD. Lack of HilC reduces *hilA* expression 2- to 3-fold (Lopez-Garrido and Casades 2010), while addition of 0.2% L-arabinose to the culture medium represses *hilA* >10-fold. This discrepancy makes unlikely that HilC is responsible for transmission of L-arabinose regulation. In contrast, lack of HilD reduces *hilA* expression >100-fold (Lopez-Garrido and Casades 2010). Thus, we examined the possibility that L-arabinose might regulate SPI-1 expression via HilD. For this purpose, we analyzed the effect of L-arabinose on the expression of three HilD-activated genes (*hilC*, *rtsA*, and *invH*). Strains carrying *hilC::lac*, *rtsA::lac*, or *invH::lac* fusions in AraA<sup>-</sup> HilD<sup>+</sup> and AraA<sup>-</sup> HilD<sup>-</sup> backgrounds were used, and β-galactosidase activities were measured in the presence and in the absence of 0.2% L-arabinose. Expression of all three genes was found to decrease in the presence of L-arabinose in a HilD<sup>+</sup> background (Figure 5). In a HilD<sup>-</sup> background, expression of the *hilC::lac*, *rtsA::lac*, and *invH::lac* fusions is reduced but not completely abolished, and similar levels of β-galactosidase activity are detected in LB and LB + L-arabinose (Figure 5). Loss of L-arabinose repression in a *hilD* background thus provides evidence that L-arabinose-dependent regulation of SPI-1 requires a functional *hilD* gene.

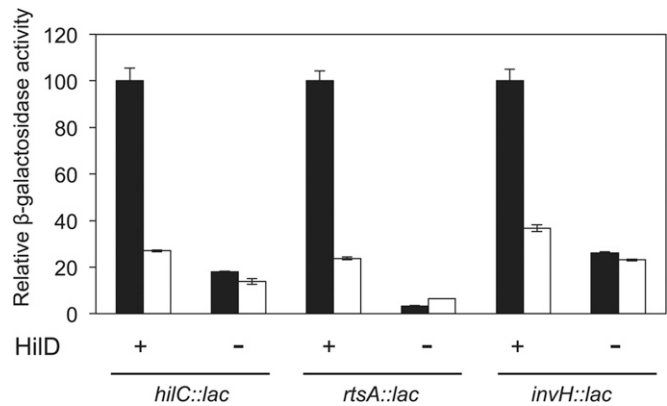


**Figure 4** Roles of AraE and AraC in SPI-1 regulation by L-arabinose. (A)  $\beta$ -Galactosidase activity of a *hilA::lac* fusion in AraA<sup>-</sup> (control), AraA<sup>-</sup> AraE<sup>-</sup>, AraA<sup>-</sup> AraC<sup>-</sup>, and AraA<sup>-</sup> AraE<sup>-</sup> AraC<sup>-</sup> backgrounds.  $\beta$ -Galactosidase activities were measured in LB and LB + 0.2, 1, 2, and 3% L-arabinose. (B)  $\beta$ -Galactosidase activity of an *hilA::lac* fusion in AraA<sup>-</sup> (control), AraA<sup>-</sup> AraE<sup>-</sup>, AraA<sup>-</sup> AraC<sup>-</sup>, AraA<sup>-</sup>  $P_{LtetO-araE}$ , and AraA<sup>-</sup> AraC<sup>-</sup>  $P_{LtetO-araE}$  backgrounds, grown in LB and LB + 0.2% L-arabinose.

### Regulation of HilD by L-arabinose is post-transcriptional

The evidence that L-arabinose regulates SPI-1 expression through HilD suggested that *hilD* expression itself might be controlled by L-arabinose. To examine this possibility, we monitored the expression of two different *hilD::lac* fusions in LB and LB + 0.2% L-arabinose: (1) *hilD::lac1*, a transcriptional fusion in which the *lacZ* gene is inserted exactly at the transcription start point (Lopez-Garrido and Casadesus 2012); and (2) *hilD::lac477*, a translational fusion in which the *lacZ* gene is inserted at position 477 of the *hilD*-coding sequence (Figure 6A). To our surprise, similar  $\beta$ -galactosidase levels were observed in both fusions in the absence and in the presence of L-arabinose (Figure 6B). This observation suggested that L-arabinose does not regulate *hilD* transcription or *hilD* translation initiation. However, when the level of *hilD* mRNA was analyzed by Northern blotting (Figure 6, panel C), a twofold reduction was detected in the presence of L-arabinose.

The discrepancy between *hilD::lac* fusion expression (not responsive to L-arabinose) and *hilD* mRNA level (responsive to arabinose) led us consider the following possibility: the *hilD::lac* fusions used above generate *hilD* null mutations,

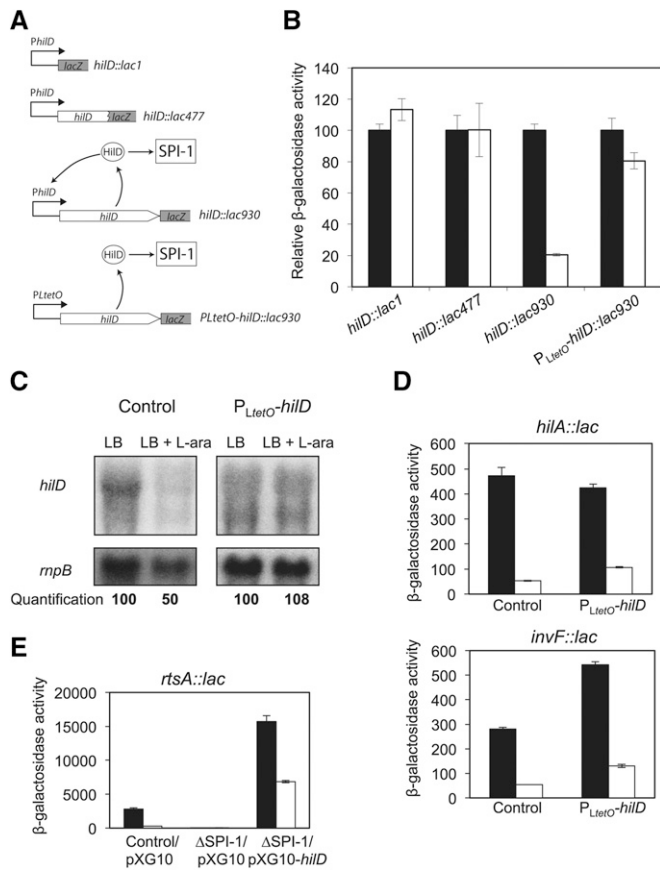


**Figure 5**  $\beta$ -Galactosidase activities of *hilC::lac*, *rtsA::lac*, and *invH::lac* fusions in isogenic AraA<sup>-</sup> HilD<sup>+</sup> and AraA<sup>-</sup> HilD<sup>-</sup> backgrounds. Black bars represent  $\beta$ -galactosidase activities in LB. White bars represent  $\beta$ -galactosidase activities in LB + 0.2% L-arabinose. Due to disparity between the  $\beta$ -galactosidase activities of the different fusions, the  $\beta$ -galactosidase activities of individual *lac* fusions in the HilD<sup>+</sup> background grown in LB without L-arabinose have been normalized to 100. The actual  $\beta$ -galactosidase activities were 320, 2513, and 1134 Miller units for *hilC::lac*, *rtsA::lac*, and *invH::lac*, respectively.

while Northern blotting was carried out in a HilD<sup>+</sup> background. Transcription of *hilD* undergoes autogenous activation by the HilD protein (Ellermeier *et al.* 2005). If L-arabinose impaired the ability of HilD to activate gene transcription, the reduction in the *hilD* mRNA level observed in the presence of L-arabinose might be due to loss of the HilD positive feedback loop. If such were the case, *hilD* transcription should be L-arabinose-dependent in a strain carrying a functional HilD protein. To examine this possibility, we used a *hilD::lac* transcriptional fusion constructed immediately downstream of the *hilD* stop codon (*hilD::lac930*). This fusion leaves the *hilD*-coding sequence intact, thus allowing autogenous activation of *hilD* transcription (Figure 6A) (Lopez-Garrido and Casadesus 2012). Figure 6B shows that the  $\beta$ -galactosidase activity of the *hilD::lac930* fusion is reduced around fivefold in the presence of 0.2% L-arabinose. To determine whether this regulatory pattern reflected the occurrence of HilD autoregulation, we expressed *hilD* from the heterologous promoter  $P_{LtetO}$  (Figure 6A) (Lopez-Garrido *et al.* 2014). The  $\beta$ -galactosidase activity of the *hilD::lac930* fusion transcribed from  $P_{LtetO}$  was similar in LB and LB + 0.2% L-arabinose (Figure 6B). Furthermore, the level of *hilD* mRNA did not decrease in the presence of L-arabinose when *hilD* was transcribed from  $P_{LtetO}$  (Figure 6C). Altogether, these results suggest that the target of L-arabinose is the HilD protein. A conceivable model is that L-arabinose might interfere with HilD activity, thus affecting transcription of *hilD* itself and of the remaining SPI-1 genes. This hypothesis is supported by the following observations:

1. The effect of L-arabinose on the activity of *hilA::lac* and *invF::lac* fusions was monitored upon *hilD* expression from  $P_{LtetO}$ . Expression of both fusions was reduced around fourfold in the presence of 0.2% L-arabinose (Figure 6D).





**Figure 6** Post-translational regulation of HilD by L-arabinose. (A) Diagram of the *hilD::lac* fusions used in this study. (B) Relative  $\beta$ -galactosidase activities of *hilD::lac1*, *hilD::lac 477*, *hilD::lac930*, and *P<sub>LtetO</sub>-hilD::lac930* fusions in LB (black bars) and LB + 0.2% L-arabinose (white bars). Measurements were performed in an AraA<sup>-</sup> background. To facilitate visual perception of differences, the  $\beta$ -galactosidase activities of individual *lac* fusions in LB without L-arabinose have been normalized to 100. The actual  $\beta$ -galactosidase activities in LB were 177, 61, 494, and 220 Miller units for *hilD::lac1*, *hilD::lac 477*, *hilD::lac930*, and *P<sub>LtetO</sub>-hilD::lac930*, respectively. (C) Level of *hilD* mRNA in extracts from AraA<sup>-</sup> (control) and AraA<sup>-</sup> *P<sub>LtetO</sub>-hilD* strains, grown in LB and LB + 0.2% L-arabinose. *hilD* mRNA was detected by Northern blotting using a <sup>32</sup>P-labeled riboprobe complementary to the 5' region of *hilD* mRNA. *mnpB* mRNA was used as loading control. (D)  $\beta$ -Galactosidase activities of *hila::lac* and *invF::lac* fusions in LB (black bars) and LB + 0.2% L-arabinose (white bars). Measurements were performed in AraA<sup>-</sup> (control) and AraA<sup>-</sup> *P<sub>LtetO</sub>-hilD* strains. (E)  $\beta$ -Galactosidase activity of an *rtsA::lac* fusion in LB (black bars) and LB + 0.2% L-arabinose (white bars) in the following backgrounds: AraA<sup>-</sup> with pXG10 empty plasmid, AraA<sup>-</sup>  $\Delta$ SPI-1 with pXG10 empty plasmid, and AraA<sup>-</sup>  $\Delta$ SPI-1 with pXG10-*hilD* plasmid.

2. The effect of L-arabinose on the activity of a *rtsA::lac* fusion was also monitored. The *rtsA* gene is located outside SPI-1 but is directly activated by HilD. This trait permits study of *rtsA* regulation in the absence of SPI-1, expressing *hilD* ectopically. For this purpose, we cloned *hilD* on pXG-10 under the control of the *P<sub>LtetO</sub>* promoter. Regulation of an *rtsA::lac* fusion by L-arabinose was examined in three different backgrounds: AraA<sup>-</sup> with the pXG-10 vector (empty),

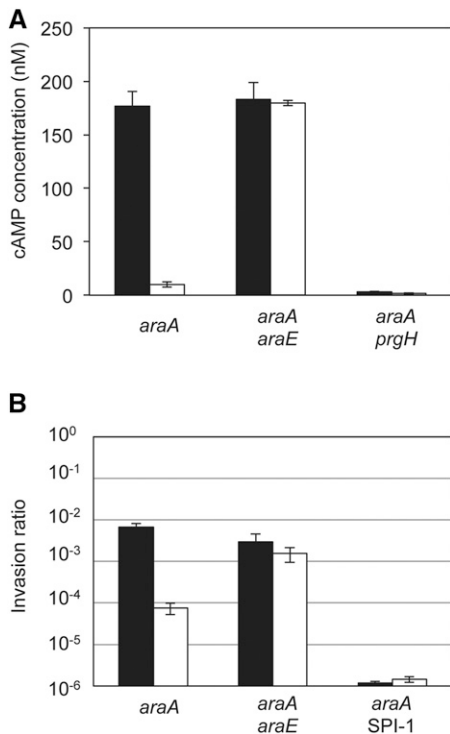
AraA<sup>-</sup>  $\Delta$ SPI-1 with the pXG-10 vector (empty), and AraA<sup>-</sup>  $\Delta$ SPI-1 with pIZ1902 (pXG10-*hilD*). As shown in Figure 6E, the *rtsA::lac* fusion was regulated by L-arabinose in a strain with SPI-1. Deletion of SPI-1 decreased  $\beta$ -galactosidase activity and suppressed regulation by L-arabinose. However, ectopic expression of *hilD* from a plasmid restored both the level of *rtsA::lac* expression and regulation by L-arabinose in the absence of SPI-1.

These observations support the view that L-arabinose may interact with the HilD protein and confirms that HilD alone is sufficient for transmission of L-arabinose-dependent regulation to other *Salmonella* genes.

### Translocation of SPI-1 effectors and epithelial cell invasion are reduced in the presence of L-arabinose

To determine whether SPI-1 repression by L-arabinose affected the interaction between *Salmonella* and host cells, we analyzed translocation of SPI-1 effectors into eukaryotic HeLa cells (Figure 7A). A fusion of the SPI-1 gene *sipA* with the *cyaA* gene of *Bordetella pertussis* was used for this purpose. *Bordetella* adenylate cyclase requires calmodulin to synthesize cAMP (Wolff *et al.* 1980; Greenlee *et al.* 1982; Glaser *et al.* 1989). Thus, cAMP will be produced only if the SipA-CyaA fusion protein is translocated into eukaryotic cells. The *sipA::cyaA* fusion was introduced in isogenic AraA<sup>-</sup>, AraA<sup>-</sup> AraE<sup>-</sup>, and AraA<sup>-</sup> PrgH<sup>-</sup> backgrounds, and the strains were grown in LB and LB + 0.2% L-arabinose before mixing with HeLa cells. Translocation was estimated by measuring the amount of cAMP and was strongly reduced in the presence of L-arabinose in the AraA<sup>-</sup> background (Figure 7A). However, in the AraA<sup>-</sup> AraE<sup>-</sup> background, addition of L-arabinose did not reduce translocation, reflecting the need of L-arabinose transport to repress SPI-1 expression (Figure 7A). The AraA<sup>-</sup> PrgH<sup>-</sup> strain was used as negative control since *prgH* encodes an essential component of the SPI-1 T3SS. These results confirmed that SPI-1 repression by L-arabinose impairs the interaction between *Salmonella* and epithelial host cells.

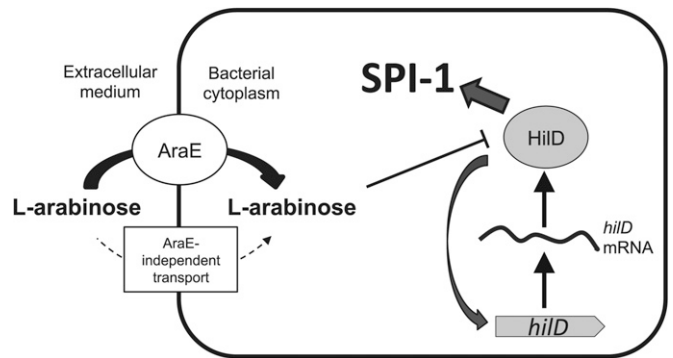
We then analyzed if the impaired translocation of SPI-1 effectors caused a reduction in *Salmonella* invasion. The ability to invade epithelial HeLa cells was determined for isogenic AraA<sup>-</sup>, AraA<sup>-</sup> AraE<sup>-</sup>, and AraA<sup>-</sup>  $\Delta$ SPI-1 strains, grown on either LB or LB + 0.2% L-arabinose. Invasion by an AraA<sup>-</sup> strain decreased almost 100-fold in the presence of arabinose. As expected, inactivation of AraE permease nearly suppressed invasion inhibition in the presence of L-arabinose. In the absence of SPI-1, invasion was reduced >5000-fold, and no further reduction was observed when L-arabinose was added to the culture medium (Figure 7B). These observations suggest that SPI-1 repression in the presence of L-arabinose impairs *Salmonella* interaction with eukaryotic epithelial cells, causing inhibition of invasion.



**Figure 7** (A) Translocation of a SipA-CyaA fusion protein into HeLa cells. Translocation was determined as the amount of cAMP produced upon infection. Black bars represent translocation levels of a bacterial culture in LB, and white bars represent translocation levels of a bacterial culture in LB + 0.2% L-arabinose. (B) Invasion of eukaryotic HeLa cells by *Salmonella* strains grown in LB (black bars) or LB + 0.2% L-arabinose (white bars). Invasion rates were determined as the ratio of viable intracellular bacteria vs. viable bacteria added to infect the HeLa cells. Note that a logarithmic scale has been used for the y-axis.

## Discussion

Regulation of virulence genes by sugars has been previously reported. In the Gram-positive pathogen *Listeria monocytogenes*, expression of virulence genes regulated by the master regulator PrfA is repressed in the presence of sugars transported by the phosphoenolpyruvate-sugar phosphotransferase system (PTS) (Park and Kroll 1993; Milenbachs *et al.* 1997; De Las Heras *et al.* 2011). However, repression is not observed in the presence of non-PTS sugars (Ripio *et al.* 1997; Joseph *et al.* 2008; Stoll *et al.* 2008; De Las Heras *et al.* 2011). In *Streptococcus pyogenes*, production of surface M protein, a major virulence determinant, is affected by the sugar source (Pine and Reeves 1978). Transcription of the gene encoding the M protein is indirectly activated by carbon catabolic repression (CCR) through the virulence gene regulator Mga. CCR also controls virulence gene expression in *Clostridium perfringens* (Varga *et al.* 2004) and *Staphylococcus aureus* (Morse *et al.* 1969). In *S. enterica*, evidence exists that PTS-dependent sugars may repress expression of invasion genes: Crp<sup>-</sup> Cya<sup>-</sup> mutants of *S. enterica* serovar Choleraesuis are attenuated in pigs (Kennedy *et al.* 1999), which correlates with the inability of Crp<sup>-</sup> mutants to secrete SPI-1 TTSS effectors (Chen *et al.* 2010). In addition, it has



**Figure 8** Model of SPI-1 regulation by L-arabinose. L-Arabinose is transported inside the cell by AraE permease. AraE-independent transport may also occur when L-arabinose is provided at high concentrations. Inside the cell, L-arabinose inhibits the HilD protein. Repression of *hilD* by L-arabinose is further transmitted to the rest of SPI-1. The regulatory effect is amplified by the feedback loop that activates *hilD* transcription.

been reported that Mlc, a global regulator of carbon metabolism that can be induced by the PTS-sugars glucose and mannose (Plumbridge 2002), activates SPI-1 expression by repressing transcription of the *hilE* gene, which encodes a negative regulator of SPI-1 (Lim *et al.* 2007).

The ability of L-arabinose, a non-PTS sugar, to regulate the expression of genes involved in L-arabinose catabolism has been known for half a century (reviewed in Schleif 2010). Like L-arabinose catabolism, SPI-1 repression requires transport of L-arabinose by the AraE permease, a requirement that admits two alternative interpretations: (1) L-arabinose has to be inside the cell to repress SPI-1; or (2) transport of L-arabinose by AraE is necessary for SPI-1 repression. The latter possibility seems unlikely because a signal transduction system associated with AraE has not been described. Furthermore, the view that SPI-1 repression is exerted by L-arabinose itself is supported by two observations: (1) *Salmonella* AraE<sup>-</sup> mutants can grow with 1% L-arabinose as sole carbon source, indicating the existence of alternative transport mechanisms; and (2) L-arabinose represses SPI-1 expression in *Salmonella* AraE<sup>-</sup> mutants if provided at concentrations of 1% or higher.

Epistasis analysis indicates that the target of L-arabinose is the transcription factor HilD, and experiments with gene fusions suggest that regulation is post-transcriptional. Because HilD is an AraC-like transcription factor, it is tempting to propose that L-arabinose might regulate HilD activity by directly binding to the protein. A tentative model of SPI-1 regulation by L-arabinose is depicted in Figure 8.

L-Arabinose is a plant-derived sugar, and the possession of a specific system for L-arabinose catabolism may enable *Salmonella* to use L-arabinose as carbon source in L-arabinose-rich environments. In addition, L-arabinose may serve as a signal for SPI-1 repression under certain circumstances. The observation that *Salmonella* grown in the presence of L-arabinose fails to invade epithelial cells (Figure 7) suggests that L-arabinose might inhibit invasion in the animal intestine. L-Arabinose is poorly absorbed during digestion by

mammals (Cori 1925) and chickens (Wagh and Waibel 1967), and its presence in the intestine after ingestion is supported by experimental evidence:  $P_{BAD}$  promoter expression is induced in the intestine of mice fed with plant components (Loessner *et al.* 2009). Furthermore, L-arabinose catabolism is required for efficient colonization of the large intestine by commensal and pathogenic strains of *E. coli* (Fabich *et al.* 2008). L-Arabinose supports efficient growth of *Salmonella in vitro* and might be a good carbon source in the intestine. Thus, the presence of L-arabinose in the intestine might be detected by *Salmonella* as a signal for repression of invasion. If such were the case, L-arabinose-rich compounds in the diet could prevent infections by *Salmonella*. Consistent with this possibility, dietary addition of arabinoxylooligosaccharides, made of L-arabinose and D-xylose, has been shown to protect poultry against oral infection by *S. enterica* serovar Enteritidis (Eckhaut *et al.* 2008).

SPI-1 repression by L-arabinose could also play a role outside the animal host. As a plant-derived sugar, L-arabinose accumulates in the soil. It has been shown that *Salmonella* is able to persist in the soil for long periods (Islam *et al.* 2004). Because synthesis of the SPI-1 TTSS causes growth retardation (Sturm *et al.* 2011; Ali *et al.* 2014), SPI-1 repression by L-arabinose may improve the fitness of *Salmonella* in the soil. On the other hand, *Salmonella* can colonize plant surfaces (Barak *et al.* 2002; Brandl and Mandrell 2002) as well as intercellular spaces inside the plants (Franz *et al.* 2007). Plant colonization by *Salmonella* might be used as a strategy for recolonizing animal hosts (Tyler and Triplett 2008). *Salmonella* mutants lacking components of the SPI-1 TTSS colonize plants more efficiently than the wild type (Iniguez *et al.* 2005), perhaps because the presence of a functional TTSS in the *Salmonella* surface triggers a defense response by the plant (Iniguez *et al.* 2005). In such a scenario, detection of L-arabinose during plant colonization might contribute to turning down SPI-1 expression.

An additional implication of this study concerns the use of L-arabinose and other sugars for ectopic induction of gene expression, a tool routinely used in biological research. Metabolizable sugars can produce changes in cell physiology. In addition, as shown above, sugars can have metabolism-independent effects, potentially interfering with the subject of study.

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# GENETICS

Supporting Information

[www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.178103/-/DC1](http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.178103/-/DC1)

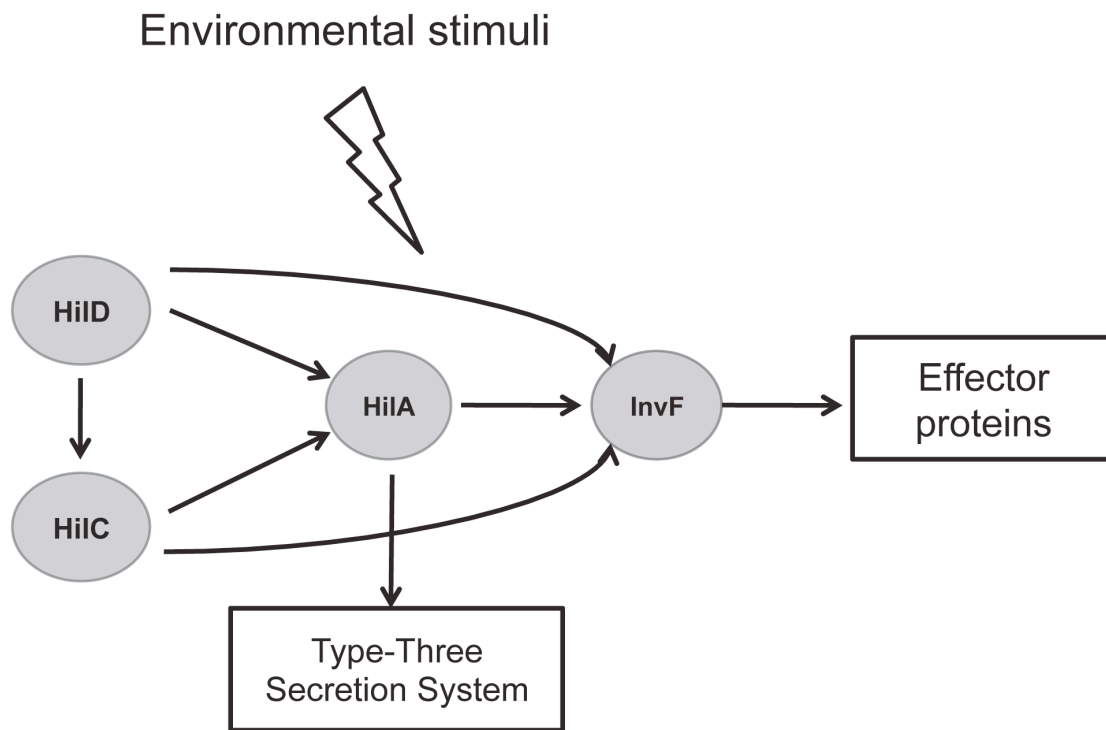
## Virulence Gene Regulation by L-Arabinose in *Salmonella enterica*

Javier López-Garrido, Elena Puerta-Fernández, Ignacio Cota, and Josep Casadesús

Virulence gene regulation by L-arabinose  
in *Salmonella enterica*

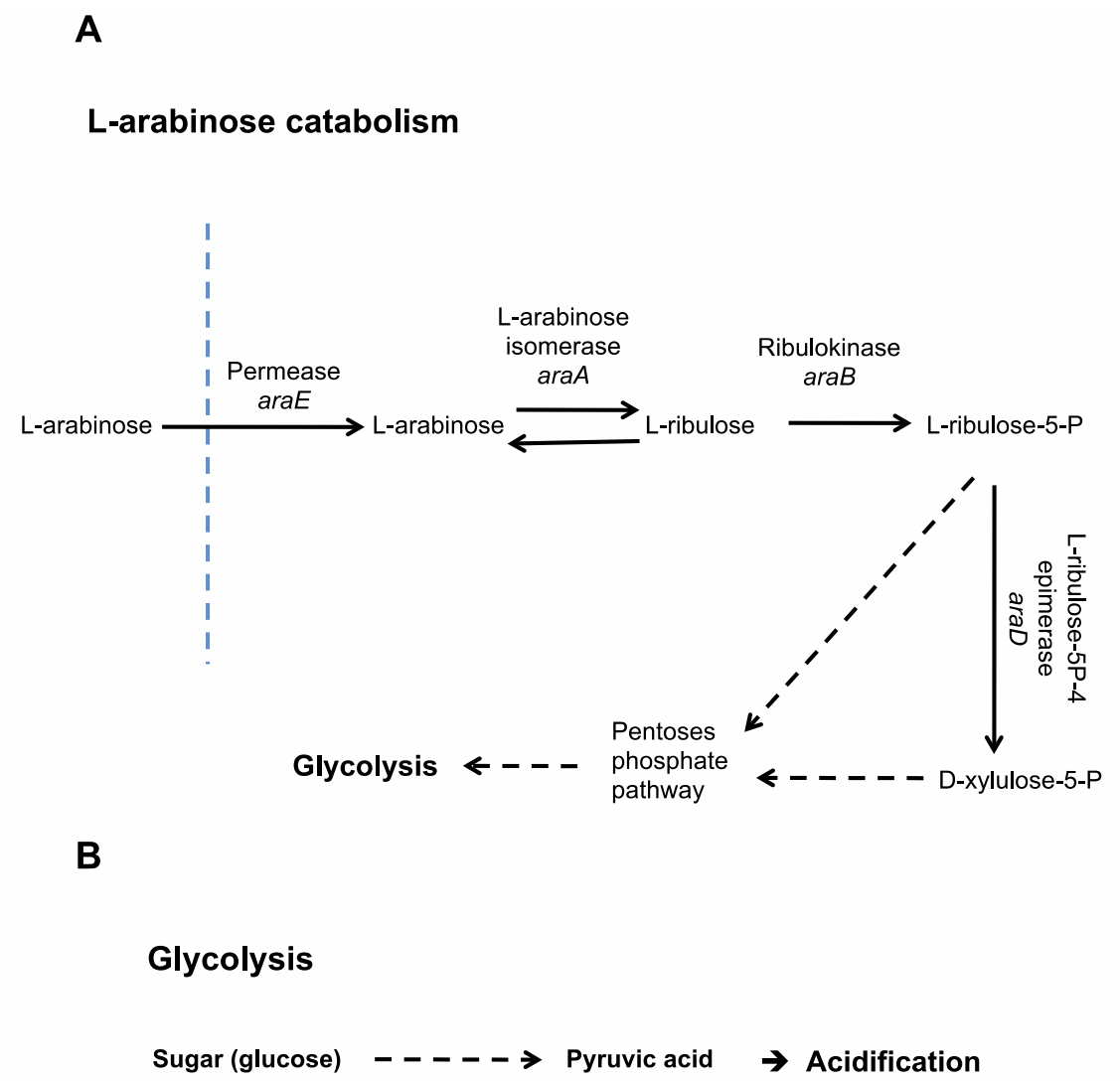
Javier López-Garrido, Elena Puerta-Fernández, Ignacio Cota,  
and Josep Casadesús

**SUPPORTING INFORMATION**

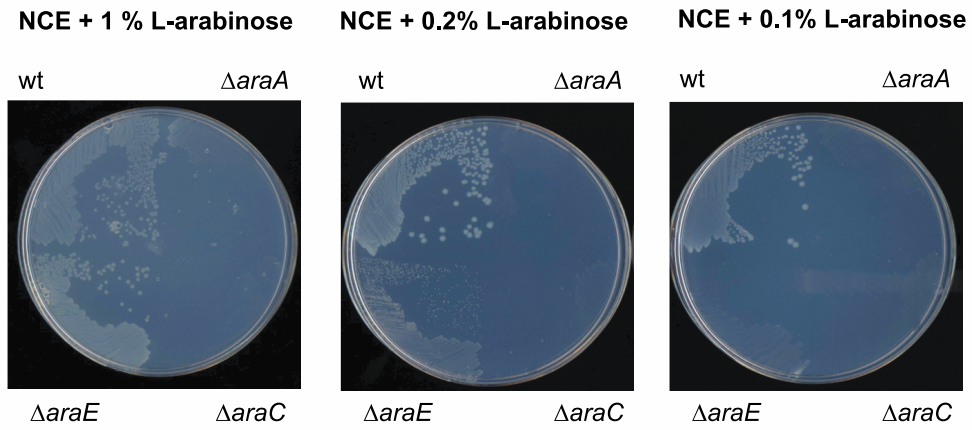
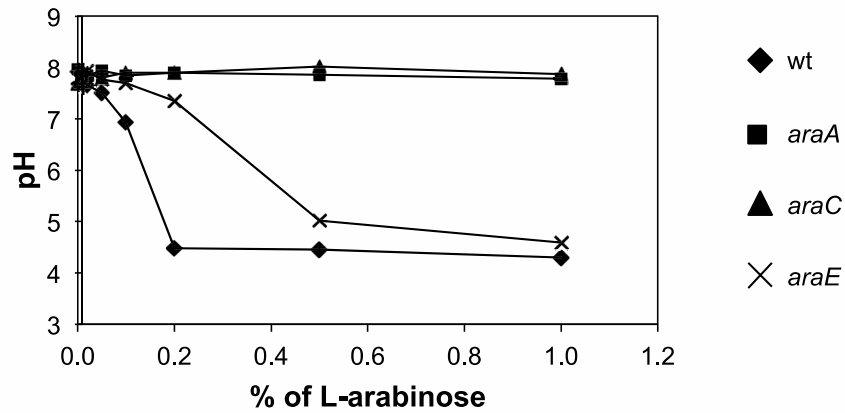


**Figure S1** Diagram representing the regulatory network of SPI-1-encoded transcriptional activators.

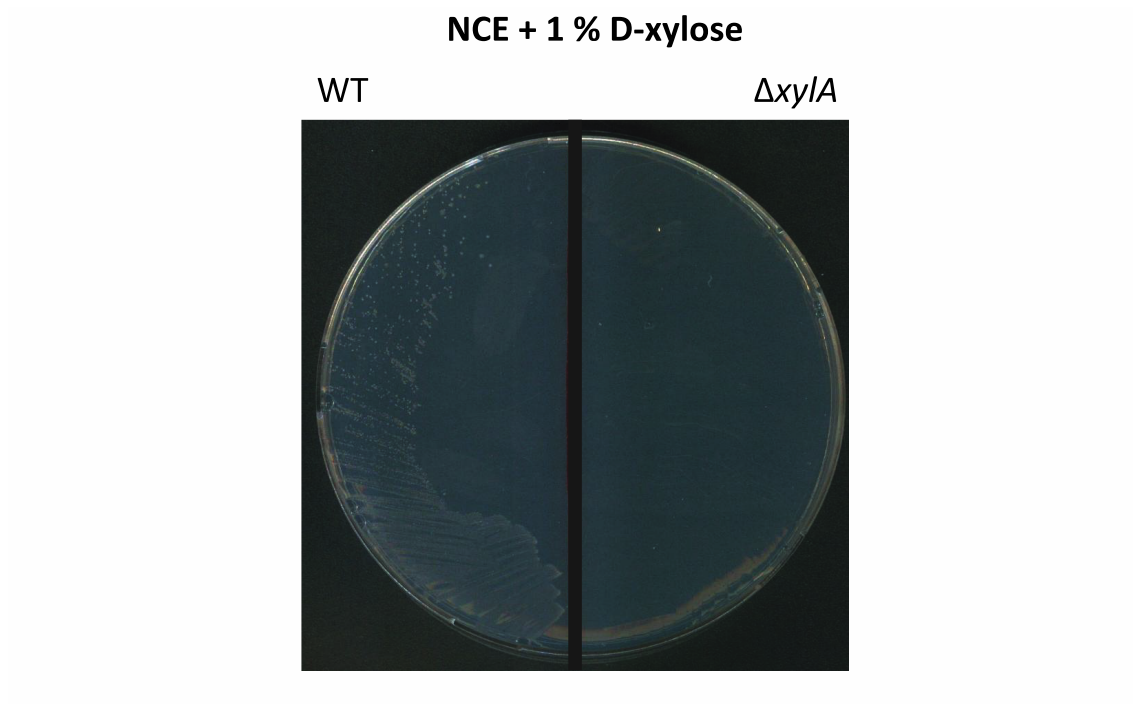




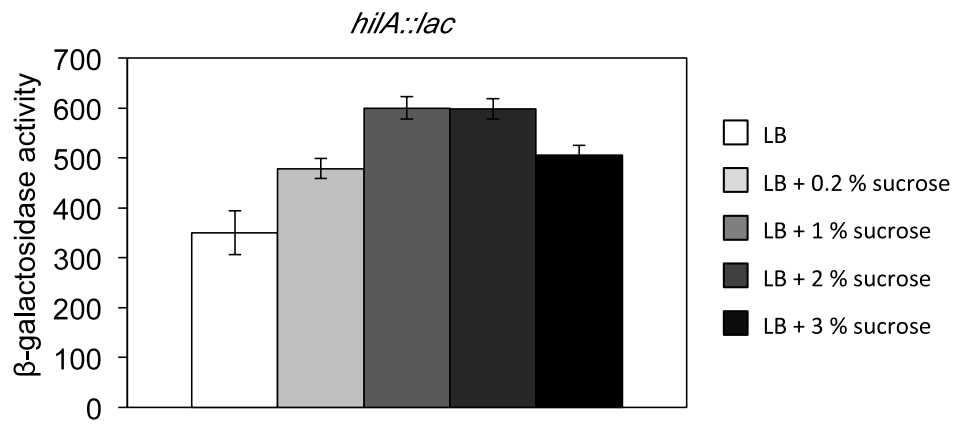
**Figure S2** **A.** L-arabinose catabolism in *Salmonella*. Main steps in L-arabinose catabolism, enzymes involved and genes encoding the enzymes (in italics). **B.** Cause of culture medium acidification upon addition of a metabolizable sugar to LB.

**A****B**

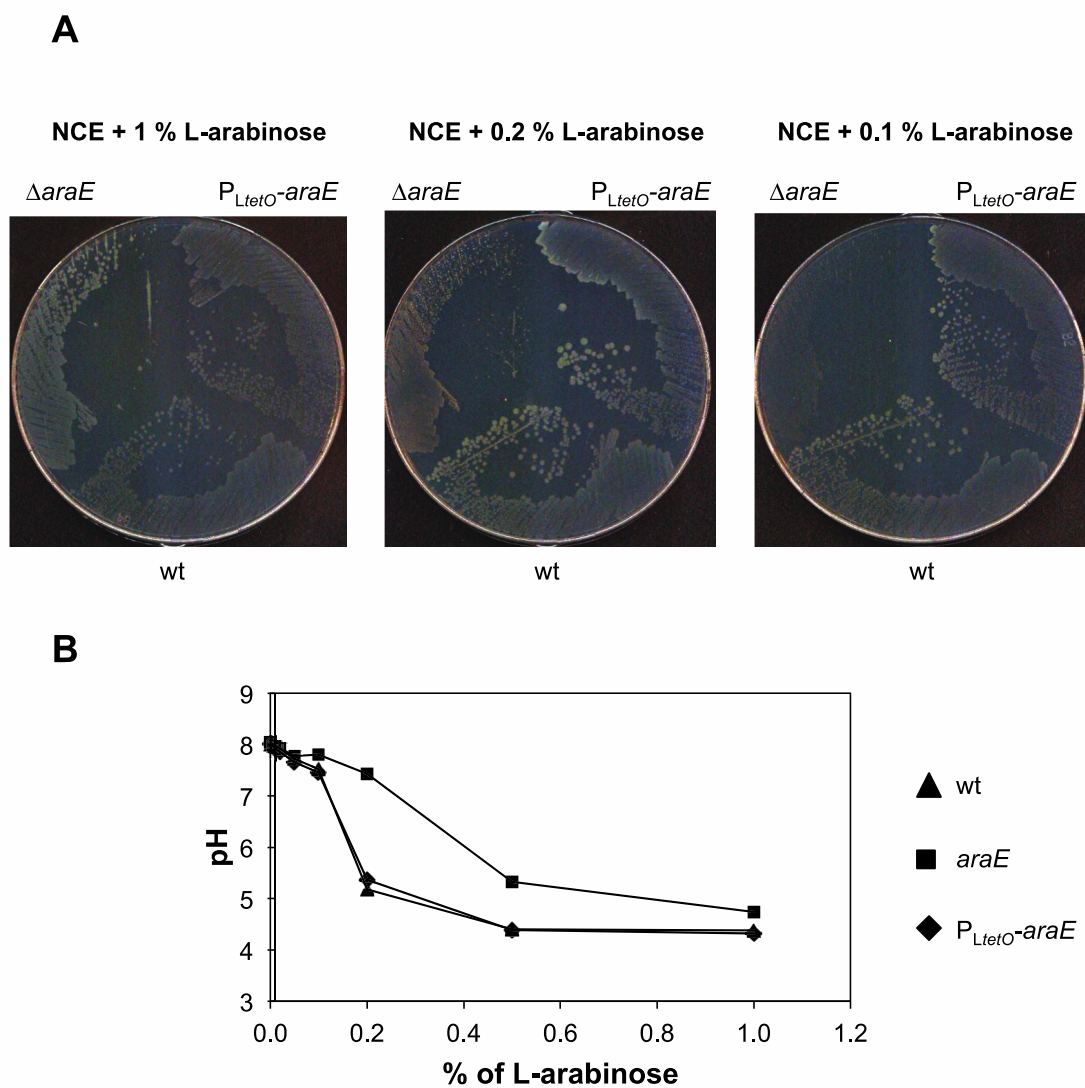
**Figure S3** Phenotypes associated with *araA*, *araC*, and *araE* mutations. **A.** Growth of the wild type,  $AraA^-$ ,  $AraC^-$ , and  $AraE^-$  strains on minimal agar with 1%, 0.2% or 0.1% L-arabinose as sole carbon source. **B.** pH curves of the wild type,  $AraA^-$ ,  $AraC^-$  and  $AraE^-$  strains.



**Figure S4** Growth of the wild type and an isogenic  $XylA^-$  strain on minimal agar with 1% D-xylose as sole carbon source.



**Figure S5**  $\beta$ -galactosidase activity of a *hilA::lac* fusion in LB and LB + 0.2%, 1%, 2%, and 3% sucrose.



**Figure S6** Phenotypes associated with *araE*. **A.** Growth of the wild type,  $AraE^-$ , and  $P_{LtetO-araE}$  strains in minimal medium with 1%, 0.2% or 0.1% L-arabinose as sole carbon source. **B.** pH curves of wt,  $AraE^-$ , and  $P_{LtetO-araE}$  strains.

**Table S1** Oligonucleotides used in this study (5'→3')

Oligonucleotide	Sequence
araAUP	ctgactcattaaggacacgacaatgacgattttgataatattccggggatccgctgacc
araADO	gcaatccgttcaccaattaacgtttgaacccgtaatacagtgtaggctggagctgcttc
araA-E1	tgcgacgtactgaatcgtcc
araA-E2	accaccacatatacgtcagc
xylAUP	cgttacttgccgtcttatctgattatggagctcactatgattccggggatccgctgacc
xylADO	ggccgggctaacgcggagtcgcccggtagatagggtattgtgtaggctggagctgcttc
xylA-E1	aattcatcacagcaaacgg
xylA-E2	caggatagctttacacccg
PLtetO-araE-UP	tgggttaacttaatccatatattgttaaataatagctataggcttaccgcttactgtc
PLtetO-araE-DO	ttaatagagaccatatttctgccacaacagagtaagacgtgctcagtatctctatcactgatag
araEUP	ttcaggctatgtcttactctgttggcaggaaaatagattccggggatccgctgacc
araEDO	cggataacaggcgtcatccggcatgggaggggggattacagtgtaggctggagctgcttc
araE-E1	tataccatagcggtagatggc
araE-E2	agtcgattcccagctcatc
araCUP	tttgtttctctctgaacatcgggggtagagaaatcatgattccggggatccgctgacc
araCDO	gcttatgacatcttggacacatcattcactttttattgtgtaggctggagctgcttc
araC-E1	tcaatgtggacattccagc
araC-E2	gataaagtgtccagcagtg
hilDUP1	agagcattacaactcagatttttcagtaggataaccagtcatatgaatatcctccttag
hilDUP930	aactacgcatcgacattcataaaaatggcgaaccattaacatatgaatatcctccttag
hilD-lacZUP	tgaacatctgaaaacggcgttctcctgtacgaaggatacaccgctgtttacaacgctg
hilD-lacZDO	gcaaatagttctcagaggaacggatgatgtataaatatgcgtgtaggctggagctgcttc
hilDDO2	gcaaatagttctcagaggaacggatgatgtataaatatggtgtaggctggagctgcttc
hilD-E1	agaccattgccaacacacgc

hilD-E2'	atcatcctcaggctggctcc
pXG10-hilD-UP	gtttttatgcataaggaacattaaaataacatcaac
pXG10-hilD-DO	gtttttgctagcggcaaatagttctcagaggg
pXG10-FOR	ttggaacctcttacgtgcc
pXG10-REV	gcatcaccttcaccctctc
hilDriboprobeUP	atggaaaatgtaacctttgtaag
hilDriboprobeDO	gttttttaatacgactcactatagggaggatatacgaaatccatgtggc
rnpBriboprobeUP	gaagaagtgaaactgaccgataagc
rnpBriboprobeDO	taatacgactcactataggccgaagctgaccagacagtcg