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Low-pH Rescue of Acid-Sensitive *Salmonella enterica* Serovar Typhi Strains by a Rhamnose-Regulated Arginine Decarboxylase System

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For *Salmonella*, transient exposure to gastric pH prepares invading bacteria for the stresses of host-cell interactions. To resist the effects of low pH, wild-type *Salmonella enterica* uses the acid tolerance response and the arginine decarboxylase acid resistance system. However, arginine decarboxylase is typically repressed under routine culture conditions, and for many live attenuated *Salmonella* vaccine strains, the acid tolerance response is unable to provide the necessary protection. The objective of this study was to enhance survival of *Salmonella enterica* serovar Typhi vaccine strains at pHs 3.0 and 2.5 to compensate for the defects in the acid tolerance response imposed by mutations in *rpoS*, *phoPQ*, and *fur*. We placed the arginine decarboxylase system (*adiA* and *adiC*) under the control of the P_{araBAD} or P_{rhaBAD} promoter to provide inducible acid resistance when cells are grown under routine culture conditions. The rhamnose-regulated promoter P_{rhaBAD} was less sensitive to the presence of its cognate sugar than the arabinose-regulated promoter P_{araBAD} and provided tighter control over *adiA* expression. Increased survival at low pH was only observed when *adiA* and *adiC* were coregulated by rhamnose and depended on the presence of rhamnose in the culture medium and arginine in the challenge medium. Rhamnose-regulated acid resistance significantly improved the survival of Δ*aroD* and Δ*phoPQ* mutants at pHs 3 and 2.5 but only modestly improved the survival of a *fur* mutant. The construction of the rhamnose-regulated arginine decarboxylase system allowed us to render *S.* Typhi acid resistant (to pH 2.5) on demand, with survival levels approximately equivalent to that of the native arginine decarboxylase system.

Before orally ingested enteric pathogens such as *Salmonella* can reach their target host cells, they must first survive their encounter with the low pH of the human stomach—~2.0 following a fast (1). This is an extremely hostile environment, and thus *Salmonella* contains multiple inducible systems to aid in survival at low pH (2, 3). The best studied of these systems is the acid tolerance response (ATR). Cells exposed to moderately low pH synthesize numerous acid shock proteins. Although the specific functions of these proteins are largely unknown, jointly they mitigate the proton damage experienced by the cell during low-pH challenge (pH 3.0) (4, 5). The acid tolerance response is a complex multicomponent system coordinated by a number of global regulatory proteins. In the stationary phase, RpoS is a key regulator of the acid tolerance response. Not only does the acid tolerance response of an *rpoS* mutant fail to provide the same level of protection as in a wild-type strain, but *rpoS* mutants are unable to sustain the acid tolerance response, resulting in rapid cell death upon pH 3.0 challenge (4, 6). In log-phase cells, the *Salmonella* virulence proteins PhoP, PhoQ, and Fur regulate the acid tolerance response. Fur controls a subset of acid shock proteins essential for protecting the cell against organic acid challenge, while PhoP and PhoQ coordinate protection against inorganic acid challenge (7, 8).

An inability to resist low pH reduces the ability of *Salmonella* to reach the small intestine and increases the number of cells required to initiate a successful infection (9). To create a live attenuated *Salmonella* vaccine, it is necessary to introduce mutations that attenuate the virulence of the vaccine strain, and, unfortunately, these mutations often simultaneously decrease the ability to survive at low pH. The vast majority of live attenuated *Salmonella* vaccines for humans are constructed from *Salmonella enterica* serovar Typhi strain Ty2, an *rpoS* mutant (10). In addition to the *rpoS* mutation derived from its parent strain Ty2, the licensed typhoid vaccine strain Ty21a carries *galE* and *tvi* mutations as well as a number of other, less well-characterized mutations (11–13). The strain is sensitive to low pH, due at least in part to its inability to mount a functional acid tolerance response (14). Another vaccine strain, Ty800, contains a deletion of the *phoPQ* locus. This strain is safe and reasonably immunogenic in humans (15), but one would expect that the combination of the Δ*phoPQ* deletion and *rpoS* mutation would render this strain exquisitely sensitive to acidic pH (6, 8). A similar situation occurs for the vaccine strains χ9639(pYA4088) and χ9640(pYA4088) (16). These strains are also safe and immunogenic in humans (S. E. Frey, K. R. Lottenbach, H. Hill, T. P. Blevins, Y. Yu, Y. Zhang, K. E. Brenneman, S. M. Kelly-Aehle, C. McDonald, A. Jansen, and R. Curtiss III, submitted for publication), but the mutation in their *fur* locus leaves them vulnerable to low pH. For each of these vaccine strains, humans immunized with a dose of less than 10^9 CFU fail to develop an immune response.

Most vaccine researchers avoid the problem low gastric pH poses by coating their vaccine in a protective enteric capsule (e.g., Ty21a) or by coadministration of an antacid (usually sodium bicarbonate) at the time of immunization (17–22). Preventing vaccine exposure to low pH increases the number of viable cells that reach the intestine and improves vaccine immunogenicity (22, 23). The disadvantage of bypassing the acidic environment of the stomach is that the low-pH encounter serves as an important signal to *Salmonella*, allowing it to recognize entry into a host envi-

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ronment. Exposure to acid (along with other host signals) stimulates upregulation of the genes that confer resistance to the short-chain fatty acids (24), antimicrobial peptides (25), and osmotic stress (6) found in the intestine. Also, induction of the acid tolerance response has been linked to upregulation of SPI-1 and SPI-2 and an increase in epithelial cell invasion in the intestine (26–28). Thus, transient exposure to low pH prepares the invading bacteria for the stresses of the intestine and for host-cell interactions. Therefore, it is possible that if the survival rate of live attenuated *Salmonella* vaccine strains at low pH can be improved, we not only can eliminate the need for low-pH bypass strategies but also can improve the ability of the vaccine strain to interact with host tissues to enhance immunogenicity.

As a first step toward this goal, we explored methods to increase the low-pH survival of *S.* Typhi strains containing *rpoS*, Δ*phoPQ*, or *fur* mutations. Each of these mutations renders strains acid sensitive, and each has been incorporated into live attenuated vaccine strains. One robust means used by *Salmonella* to resist low-pH challenge is the arginine decarboxylase acid resistance system (29). This system consists of arginine decarboxylase (AdiA) and an arginine-agmatine antiporter (AdiC) (30). Acid resistance is conferred by the activity of AdiA, which consumes one proton from the intracellular environment with each reaction cycle and causes a rapid rise in intracellular pH (31, 32). AdiC then transports agmatine, the product of arginine decarboxylation, to the periplasm in exchange for another arginine substrate molecule (30, 33). The combined activities of AdiA and AdiC allow *Salmonella enterica* serovar Typhimurium to resist pH 2.5 for more than 2 h (2).

Because the arginine decarboxylase system functions independently of the acid tolerance response, we hypothesized that synthesis of AdiA and AdiC would confer high levels of acid resistance on strains containing mutations that affect acid tolerance, such as *rpoS*, *phoPQ*, and *fur*. However, the arginine decarboxylase system is tightly regulated and is not normally available to cells grown under standard vaccine culture conditions (34). Therefore, we replaced the native promoter of arginine decarboxylase with *araBAD* (P_{araBAD}) or *rhaBAD* (P_{rhaBAD}) promoters. The placement of the system under the control of these promoters allows expression *in vitro* and during the initial stages of infection, but results in rapid downregulation once the vaccine invades host tissue and the regulatory sugar is no longer available (35). After selecting the promoter with optimal sugar-dependent expression and activity of the arginine decarboxylase system (P_{rhaBAD}), our objectives were twofold. First, we determined whether the rhamnose-regulated arginine decarboxylase system could rescue *rpoS*, Δ*phoPQ*, and *fur* mutants during low-pH challenge if cells were cultured in the presence of rhamnose but without any other environmental signals that would induce either decarboxylase activity or the acid tolerance response. Second, we determined whether the rhamnose-regulated system functioned equivalently to the native arginine decarboxylase system, by comparing the level of acid resistance in strains carrying our engineered rhamnose-regulated system induced by growth with rhamnose to that in strains carrying the native arginine decarboxylase system induced by anaerobic growth in unbuffered rich medium.

MATERIALS AND METHODS

DNA manipulation and plasmid construction. Chromosomal DNA from *S.* Typhi Ty2 was isolated using the Wizard genomic DNA purification kit (Promega, Madison, WI). Plasmid DNA was isolated using the QIAprep spin miniprep kit (Qiagen, Valencia, CA) or the Wizard Plus midiprep DNA purification system (Promega). DNA inserts were amplified by PCR using the Phusion DNA polymerase (New England BioLabs, Ispwich, MA) or the Easy-A high-fidelity PCR cloning enzyme (Agilent, Santa Clara, CA). Restriction and modification enzymes for cloning (New England BioLabs) were used in accordance with the manufacturer's instructions.

Construction of *S.* Typhi mutants. The bacterial strains and plasmids used in this study are listed in Table 1. The primers used during the construction of plasmids are listed in Table S1 in the supplemental material. To construct the Δ*aroD1299* mutant, two DNA fragments adjacent to the *aroD* gene were amplified from the chromosome of Ty2. Primers Aro-1 and -2 were used for the upstream fragment, while primers Aro-3 and -4 were used for the downstream fragment. These fragments were digested with BamHI, ligated using T4 DNA ligase, reamplified by PCR with primers Aro-1 and -4, and cloned into the AhdI sites of pYA4278 via TA overhangs to generate the suicide vector pYA4895. The Δ*aroD1299* deletion was introduced into Ty2 by conjugation using the antibiotic resistance selection-*sacB* counterselection method described by Kaniga (36). The resulting strain (χ11548) exhibits aromatic amino acid auxotrophy and carries a deletion of the complete coding sequence of *aroD* that spans 759 bp.

The ΔP_{fur81}::TT (transcription terminator) *araC* P_{BAD} *fur* mutation was introduced into *S.* Typhi Ty2 via P22 HT *int* transduction (37) using a lysate grown on χ9269 containing a chromosomally integrated copy of pYA4181 (35) to create the *S.* Typhi strain χ11118. The presence of the ΔP_{fur81}::TT *araC* P_{BAD} *fur* mutation in *S.* Typhi was confirmed by PCR using the primers Fur-1 and -2. Arabinose-dependent synthesis of Fur was verified by Western blotting.

To remove the entire *adi* locus [Δ(*adiA-adiC*)*-4806*; hereafter Δ(*adiA-adiC*)], the upstream and downstream flanking regions in Ty2 were amplified using PCR primers Adi-1 and -2 and primers Adi-3 and -4, respectively. The flanking regions were digested with BamHI and ligated together with T4 DNA ligase. The resulting product was reamplified by PCR using primers Adi-1 and -4 and cloned into the AhdI sites of pYA4278 to generate the suicide vector pYA5066. The Δ(*adiA-adiC*) mutation carried by pYA5066 was moved into Ty2 to create χ11500. This strain carries a 4,806-bp deletion of the *adi* locus (complete coding sequences of *adiA*, *adiY*, and *adiC* and the *adiY* and *adiC* promoters) (Fig. 1). The absence of the *adi* locus was confirmed by PCR and by arginine decarboxylase assay.

To produce strains in which *adiA* expression could be regulated exclusively by the presence or absence of a specific sugar, we engineered the following promoter substitution mutations: $\Delta P_{adiA203}$::TT *araC* P_{araBAD} *adiA* (regulated by arabinose) and $\Delta P_{adiA276}$::TT *rhaSR* P_{rhaBAD} *adiA* (regulated by rhamnose). For simplicity, these mutations will be referred to as P_{araBAD} *adiA* and P_{rhaBAD} *adiA*, respectively. For the arabinose-regulated construct, the DNA regions flanking the *adiA* promoter were amplified by PCR from Ty2 using primers Adi-5 and -6 for the upstream region and primers Adi-7 and -8 for the downstream region. Both flanking regions were cloned into pYA3700 (using SphI and BglII for the upstream region and KpnI and SacI for the downstream region) to generate pYA5075. The DNA segment containing the flanking regions and arabinose promoter was amplified by PCR using Adi-5 and -8, and the PCR product was cloned into the AhdI sites of pYA4278 to create the suicide vector pYA5089. To generate the rhamnose-regulated construct, the *araC* P_{araBAD} promoter of pYA5089 was removed by XhoI and XbaI double digestion. The *rhaSR* P_{rhaBAD} promoter from pYA5081 was amplified by PCR with the Rha-1 and -2 primers and cloned into pYA5089 using XhoI and XbaI to produce the suicide vector pYA5093. pYA5089 and pYA5093 were introduced into χ11548 by conjugation to produce χ11552 and χ11564, respectively. The juxtaposition of *adiA* with the appropriate promoter was verified by PCR with the Ara-1 and Adi-9 primers (χ11552) or Rha-3 and Adi-9 primers (χ11564) and by the arginine decarboxylase

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype[a]	Source, derivation, or reference
Strains		
E. coli		
BL21(DE3)	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
χ7213	*thr-1 leuB6 fhuA21 lacY1 glnV44 recA1* Δ*asdA4* Δ(*zhf-2*::Tn*10*) *thi-1* RP4-2-Tc::Mu [λ*pir*]	54
S. Typhi		
χ3769 (Ty2)	Wild-type; *cys trp rpoS*	55
χ8444	Δ*phoPQ23*	56
χ11118	ΔP_{fur81}::TT *araC* P_{araBAD} *fur*	Ty2
χ11500	Δ(*adiA-adiC*)*-4806*	Ty2
χ11548	Δ*aroD1299*	Ty2
χ11552	Δ*aroD1299* Δ$P_{adiA203}$::TT *araC* P_{araBAD} *adiA*	χ11548
χ11564	Δ*aroD1299* Δ$P_{adiA276}$::TT *rhaSR* P_{rhaBAD} *adiA*	χ11548
χ11568	Δ*aroD1299* Δ$P_{adiA276}$::TT *rhaSR* P_{rhaBAD} *adiA* Δ(P_{adiY}-*adiY*-P_{adiC})*-119 adiC*	χ11564
χ11622	Δ*phoPQ23* Δ$P_{adiA276}$::TT *rhaSR* P_{rhaBAD} *adiA* Δ(P_{adiY}-*adiY*-P_{adiC})*-119 adiC*	χ8444
χ11623	ΔP_{fur81}::TT *araC* P_{araBAD} *fur* Δ$P_{adiA276}$::TT *rhaSR* P_{rhaBAD} *adiA* Δ(P_{adiY}-*adiY*-P_{adiC})*-119 adiC*	χ11118
χ11636	Δ*aroD1299* Δ(P_{adiY}-*adiY*-P_{adiC})*-119 adiC*	χ11548
Shigella flexneri 2457T	*S. flexneri* 2a, wild type; Pcr$^+$ Mal$^-$ λ^r	57
Plasmids		
pET28a	Protein synthesis vector, T7 promoter; Kan^r	Novagen
pYA3700	Vector containing tightly regulated TT *araC* P_{araBAD} cassette	58, 59
pYA4181	Suicide vector to generate ΔP_{fur81}::TT *araC* P_{araBAD} *fur* mutation	35
pYA4278	Suicide vector; *sacB mobRP4 oriR6K* Cm^r	60
pYA4895	Suicide vector to generate Δ*aroD1299* mutation	pYA4278
pYA5066	Suicide vector to generate Δ(*adiA-adiC*)*-4806* mutation	pYA4278
pYA5072	Suicide vector to generate Δ(P_{adiY}-*adiY*-P_{adiC})*-119 adiC* mutation	pYA4278
pYA5075	Intermediate vector for creation of Δ$P_{adiA203}$::TT *araC* P_{araBAD} *adiA*	pYA3700
pYA5081	Suicide vector specifying tightly regulated *rhaSR* P_{rhaBAD} cassette	61
pYA5085	Protein synthesis vector with N-terminal His tag on AdiA	pET28a
pYA5089	Suicide vector to generate Δ$P_{adiA203}$::TT *araC* P_{araBAD} *adiA* mutation	pYA4278, pYA5075
pYA5093	Suicide vector to generate Δ$P_{adiA276}$::TT *rhaSR* P_{rhaBAD} *adiA* mutation	pYA5089, pYA5081

[a] In genotype descriptions, the subscripted designation number refers to a composite deletion and insertion of the indicated gene. P, promoter; TT, T4 ip III transcription terminator; Cm^r, chloramphenicol resistance; Kan^r, kanamycin resistance.

assay. In both strains, 203 bp of the intergenic region between *melR* and *adiA* (including the −10 and −35 sites of the *adiA* promoter) were deleted and replaced with either TT *araC* P_{araBAD} (χ11552) or TT *rhaRS* P_{rhaBAD} (χ11564). The strong transcription terminator T4 ip III was placed between the upstream *melR* gene and *araC* or *rhaSR* to prevent expression of antisense RNA. A strong Shine-Dalgarno site (AGGA) was inserted 10 bp upstream of the ATG start codon of *adiA* (Fig. 1).

The *adiC* gene was fused into an operon with *adiA* resulting in the Δ(P_{adiY}-*adiY*-P_{adiC})*-119 adiC* mutation (hereafter, *adiAC*). The DNA regions flanking *adiY* were amplified by PCR from Ty2 using primers Adi-10 and -11 for the upstream region and primers Adi-12 and -13 for the downstream region. The two DNA segments were joined by overlap PCR and reamplification with Adi-10 and -13. The final PCR product was ligated into pYA4278 at the AhdI sites to produce the suicide vector pYA5072. The suicide vector was introduced into χ11564 and χ11548 by conjugation to produce χ11568 (Δ*aroD* P_{rhaBAD} *adiAC*) and χ11636 (Δ*aroD adiAC*), respectively. The presence of the *adiAC* operon was confirmed by PCR using Adi-14 and -15. Both strains harbor a 1,078-bp deletion that spans the transcription terminator following *adiA*, *adiY*, and the promoter of *adiC*. The *adiA* and *adiC* genes are separated by a 119-bp intergenic sequence expected to decrease expression of *adiC* from the promoter upstream of *adiA* (Fig. 1).

Growth conditions and culture media. Experiments testing the regulation of arabinose- and rhamnose-controlled genes were conducted in the carbohydrate-free medium purple broth (BD Biosciences, Franklin Lakes, NJ). The use of this carbohydrate-free medium allowed us to accurately control the concentrations of rhamnose or arabinose to evaluate their effects on promoter activity.

For induction of the native arginine decarboxylase system, strains were propagated in tryptic soy broth (TSB) (BD Biosciences) with 0.4% glucose under anaerobic conditions. This causes the medium pH to fall below 5.0 during culture. In all other acid resistance experiments, strains were grown aerobically in minimal E medium (pH 7.0) with 0.4% glucose (EG medium) (38). For our experiments, 22 μg/ml L-cysteine, 20 μg/ml L-tryptophan, and 0.1% Casamino Acids were added to EG medium to supplement the growth of all strains (EGA medium). For strains with the Δ*aroD1299* mutation, 20 μg/ml L-tryptophan, 2 μg/ml p-aminobenzoic acid, and 2.5 μg/ml 2,3-dihydroxybenzoate were added to all media. EGA medium was additionally supplemented with 50 μg/ml L-phenylalanine and 20 μg/ml L-tyrosine. Rhamnose was added to 0.1% or to 0.4% in the case of strain χ11623, as indicated. Strains containing the ΔP_{fur81}::TT *araC* P_{araBAD} *fur* mutation were supplied with 0.2% arabinose unless otherwise indicated. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Pittsburgh, PA) unless otherwise indicated.

Measurement of *adiA* expression by semiquantitative PCR. Strains were grown in purple broth with various concentrations of rhamnose or arabinose to an optical density at 600 nm (OD_{600}) of 0.6. Total cellular

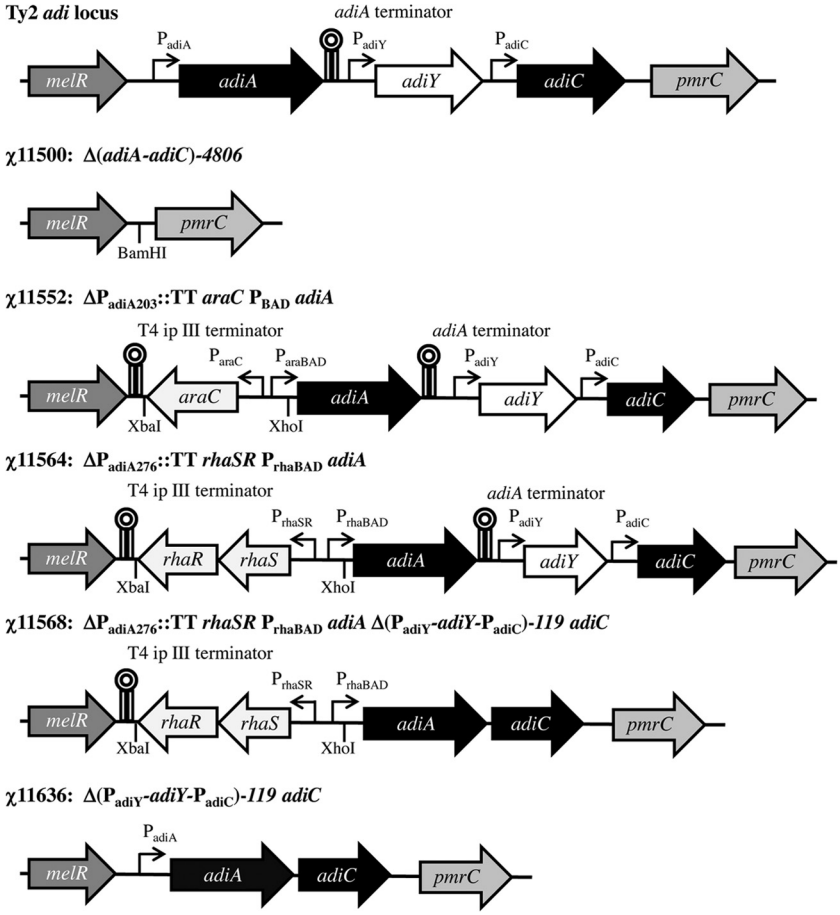

FIG 1 Schematic diagram of arginine decarboxylase mutations. The genes and associated regulatory sequences for the Δ(*adiA-adiC*)*-4806*, $\Delta P_{adiA203}$::TT *araC* P_{araBAD} *adiA*, $\Delta P_{adiA276}$::TT *rhaSR* P_{rhaBAD} *adiA*, and $\Delta(P_{adiY}$-*adiY*-$P_{adiC})$*-119 adiC* mutations are shown above, along with the archetypal strain number. The wild-type arginine decarboxylase locus (*adi*) of *S.* Typhi Ty2 is depicted for comparative purposes. The diagram is approximately to scale. The promoters and transcription terminator are labeled on the figure.

RNA was isolated using the RNeasy minikit (Qiagen) and was treated with RNase-free DNase (Qiagen). cDNA was generated via reverse transcription (RT)-PCR using 1 μg of cellular RNA with the TaqMan reverse transcriptase kit (Life Technologies, Grand Island, NY) under the following conditions: 10 min at 25°C for optimal random hexamer primer binding and then 45 min at 48°C for extension followed by 5 min at 95°C to heat inactivate the transcriptase. Semiquantitative PCR of the *gapA* (control) and *adiA* transcripts was performed using the GoTaq DNA polymerase system (Promega) using primers SQ-1 and SQ-2 for *gapA* and SQ-3 and SQ-4 for *adiA* under the following conditions: 2.5 min at 95°C for template denaturation, followed by 28 cycles of 40 s at 95°C, 30 s at 48°C for primer annealing, and 1 min at 72°C for primer extension. The semiquantitative PCR primer sequences are listed in Table S1 in the supplemental material (SQ-1 to SQ-4). PCR products were electrophoresed on a 2% agarose gel in the presence of ethidium bromide and visualized with the ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA). Images were analyzed in Adobe Photoshop CS4 (Adobe Systems, Inc., San Jose, CA) in order to establish histogram values for the fluorescence signal intensity of the PCR products. Signal intensity values for *adiA* were normalized to the value obtained with the single-gene-expression control *gapA* for each culture.

Preparation of antiserum against arginine decarboxylase protein. *Escherichia coli* BL21(DE3) harboring pYA5085 was used for the synthesis of His-tagged AdiA protein. Cells were grown in LB at 37°C to the mid-log phase (OD_{600} of 0.6). The growth medium was supplemented with 0.2 g/liter pyridoxine to augment protein folding and enzyme activity (39). Protein synthesis was induced with 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) (Amresco, Solon, OH) for 4 h at 37°C. Cells were collected by centrifugation and disrupted using lysozyme (3 mg/g cells) and deoxycholic acid (120 mg/g cells) (40). His-tagged AdiA protein from the soluble fraction was purified over Talon metal affinity resin (BD Biosciences) in accordance with the manufacturer's instructions, except that 10% ethanol was added to the elution buffer. Purified protein was stored in 20 mM HEPES–50 mM NaCl (pH 8.0) (31).

One juvenile New Zealand White rabbit (Charles River Laboratories, Wilmington, MA) was immunized with 200 μg of AdiA emulsified in Freund's complete adjuvant and boosted with an additional 200 μg of AdiA emulsified in Freund's incomplete adjuvant 4 and 8 weeks after the initial injection. Serum was collected 3 weeks following the final immunization.

Western blot procedure. Strains were grown overnight at 37°C in purple broth containing various concentrations of rhamnose or arabinose. The amount of total cellular protein in each sample was normalized by absorbance at 280 nm using the NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE). Equal amounts of total cellular protein (100 μg) were mixed with 2× SDS-PAGE buffer, boiled, and electrophoresed on a 10% acrylamide gel (41). Separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using Towbin's wet transfer method (42), blocked in 5% skim milk, and then probed with rabbit antiserum (final dilution, 1:10,000) for the presence of AdiA (35).

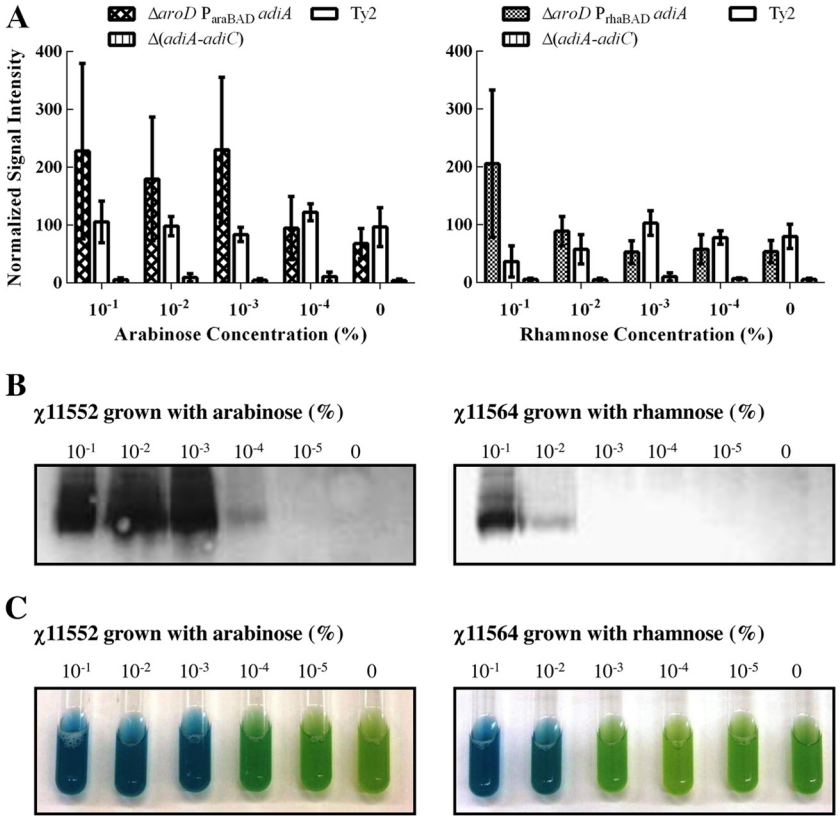

FIG 2 Regulation of *adiA* by the *araC* P_{araBAD} and *rhaSR* P_{rhaBAD} promoters. χ11552 (Δ*aroD* P_{araBAD} *adiA*) and χ11564 (Δ*aroD* P_{rhaBAD} *adiA*) were cultured in the presence of various concentrations of arabinose or rhamnose (ranging from 10^{-1} to 10^{-5}%), normalized and assayed by semiquantitative PCR for the level of *adiA* transcript (A), probed for the presence of AdiA by Western blotting (B), or tested for arginine decarboxylase activity via colorimetric assay (C). mRNA data are plotted as the means and standard errors of the means (SEM) from three independent experiments. Western blot and enzyme assay data are representative of three independent assays. In the colorimetric arginine decarboxylase assay, active enzyme raises the assay medium pH above 5.0, resulting in a color change from yellow-green (negative) to blue (positive).

Bound primary antibody was detected by the addition of goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma-Aldrich). Blots were developed with NBT/BCIP (nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl phosphate) (Amresco) and photographed using the ChemiDoc XRS system.

Arginine decarboxylase assays. Arginine decarboxylase enzyme activity was measured using a modified version of the rapid glutamate decarboxylase assay previously described (43). Strains were grown overnight (18 h) to the stationary phase in purple broth, washed once in phosphate-buffered saline (PBS) (40), and normalized to an OD_{600} value of 0.7. Five milliliters of normalized cells was pelleted, resuspended in 2.5 ml arginine decarboxylase assay medium (1 g L-arginine, 0.05 g bromocresol green, 90 g NaCl, and 3 ml Triton X-100 per liter of distilled water [adjusted to pH 3.4]), and vortexed for 30 s. Assay tubes were incubated at 37°C for 5 to 30 min, scored, and photographed.

Acid resistance assays. Acid resistance was determined essentially as described previously (44, 45), with the following modifications. Strains were grown overnight to stationary phase in minimal EGA medium at pH 7.0 (38) or in TSB with 0.4% glucose. Cultures were normalized to the same OD_{600} and then pelleted and washed once in EGA medium (pH 7.0) containing no growth supplements. Cells were pelleted a second time and resuspended at a density of 1×10^9 CFU/ml in EGA medium containing 1 mM L-arginine at pH 3.0, 2.5, or 2.0. Low-pH challenge was conducted at 37°C, and samples were collected immediately after resuspension (time [*t*] 0) and hourly for 4 h. Samples were serially diluted and plated onto LB agar to assess bacterial viability.

Statistical analyses. All statistical analyses were performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA). Survival curves for 4-h acid resistance assays were compared using two-way repeated measures (mixed model) analysis of variance (ANOVA) with Bonferroni's posttest. Data from 1-h acid resistance challenges were compared using the paired *t* test.

RESULTS AND DISCUSSION

Comparison of *adiA* regulation from arabinose- and rhamnose-regulated promoters. To allow expression of the arginine decarboxylase system during aerobic growth, we constructed *S.* Typhi strains in which *adiA* expression was regulated by either the P_{araBAD} or P_{rhaBAD} promoter. For safety, the sugar-regulated *adiA* constructs were introduced into *S.* Typhi strain χ11548, which carries an attenuating Δ*aroD* mutation (18, 46). Thus, in strains χ11552 (Δ*aroD* P_{araBAD} *adiA*) and χ11564 (Δ*aroD* P_{rhaBAD} *adiA*), *adiA* expression responds to the level of exogenous arabinose or rhamnose, respectively. In the absence of the regulating sugar, both strains expressed low levels of *adiA* transcript consistent with background levels observed in Ty2 cultured under noninducing conditions for *adiA* (Fig. 2A). Both strains increased production of the *adiA* mRNA transcript when 0.1% (10^{-1}%) of the appropriate sugar was added. The two promoters drove production of essentially equivalent amounts of *adiA* transcript at this concentration, consistent with previous results (47). As the amount of regulatory sugar present in the culture was decreased, the activities of the two promoters decreased differentially. Strain χ11552 (P_{araBAD} *adiA*) continued to express *adiA* mRNA at arabinose con-

centrations as low as 0.001% (10^{-3}%). Only when the arabinose concentration fell below 0.001% (10^{-3}%) did the amount of *adiA* transcript return to background levels. In contrast, strain χ11564 (P_{rhaBAD} *adiA*) expressed *adiA* transcript only in the presence of 0.1% (10^{-1}%) rhamnose and produced background levels of *adiA* mRNA at lower rhamnose concentrations.

AdiA protein synthesis and enzyme activity levels presented a pattern similar to that of the mRNA. χ11552 (P_{araBAD} *adiA*) synthesized AdiA over a wide range of arabinose concentrations (10^{-1} to 10^{-4}% arabinose), while in χ11564 (P_{rhaBAD} *adiA*), AdiA was detected over a narrower range of rhamnose concentrations (10^{-1} to 10^{-2}% rhamnose) (Fig. 2B). Interestingly, χ11552 (P_{araBAD} *adiA*) produced greater amounts of AdiA protein in the presence of 0.1% arabinose than did χ11564 (P_{rhaBAD} *adiA*) in the presence of 0.1% rhamnose. Arginine decarboxylase activity was detected in χ11552 (P_{araBAD} *adiA*) cultures grown in the presence of arabinose concentrations as low as 10^{-3}% (Fig. 2C). An intermediate reaction suggestive of low levels of enzyme activity was observed at 10^{-4}% arabinose. In contrast, arginine decarboxylase activity was observed in χ11564 (P_{rhaBAD} *adiA*) only at rhamnose concentrations greater than 10^{-2}%. While the largest amounts of AdiA in both strains were observed at the arabinose and rhamnose concentrations that increased levels of *adiA* transcript, small amounts of AdiA were also detected at sugar concentrations that did not produce a measurable increase in the amount of *adiA* transcript present, which could reflect differences in the sensitivities of the assays or differences in the stabilities of the *adiA* mRNA transcript and AdiA protein.

Comparison of the arabinose-regulated P_{araBAD} and rhamnose-regulated P_{rhaBAD} promoters indicated that P_{rhaBAD} was less sensitive to its regulatory sugar than P_{araBAD}. The reduced sensitivity of the P_{rhaBAD} promoter made it an ideal choice to regulate the arginine decarboxylase system since it allowed tighter control of gene expression in media containing trace amounts of rhamnose, such as LB and TSB. In addition, the promoter will not be active if the cell encounters trace amounts of the inducing sugar *in vivo*, which should enhance its safety as a vaccine. An additional advantage of the P_{rhaBAD} promoter was its decreased level of AdiA protein synthesis. Protein overproduction has been shown to impair the efficacy of *Salmonella* vaccines (48, 49). For these reasons, we selected the $\Delta P_{adiA276}$::TT *rhaSR* P_{rhaBAD} *adiA* mutation for use in further studies.

Coregulation of *adiA* and *adiC* is necessary for survival during pH 3.0 challenge. Our goal in introducing the P_{rhaBAD} *adiA* construct into *S.* Typhi was to provide arginine-dependent acid resistance when cells were grown under conditions when this system is not normally induced (noninducing conditions). To test this, we performed low-pH challenges on cells grown aerobically in minimal EGA medium. However, while χ11564 (Δ*aroD* P_{rhaBAD} *adiA*) exhibited rhamnose-inducible arginine decarboxylase activity under these conditions (data not shown), the survival profile of χ11564 (Δ*aroD* P_{rhaBAD} *adiA*) at pH 3.0 did not differ from that of Ty2 or its parent strain, χ11548 (Δ*aroD*) (Fig. 3). We reasoned that the P_{rhaBAD} promoter in strain χ11564 (Δ*aroD* P_{rhaBAD} *adiA*) does not drive *adiC* expression due to the presence of a transcriptional terminator downstream of *adiA* and the intervening *adiY* gene. To coregulate expression of both *adiA* and *adiC*, the intergenic region between the two genes, including the regulatory gene *adiY* and the *adiC* promoter, was deleted, resulting in the fusion of *adiA* and *adiC* into a single operon under the control of the native

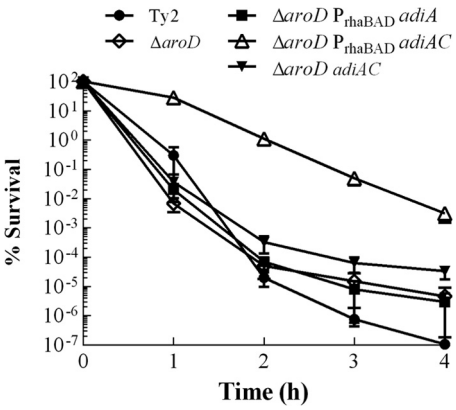

FIG 3 Coregulation of *adiA* and *adiC* by rhamnose is required for survival during pH 3 challenge. χ11564 (Δ*aroD* P_{rhaBAD} *adiA*), χ11568 (Δ*aroD* P_{rhaBAD} *adiAC*), and χ11636 (Δ*aroD adiAC*) were grown to the stationary phase in EGA medium in the presence of 0.1% rhamnose and then challenged with pH 3.0 EG medium containing 1 mM arginine. Survival was monitored by plating on LB agar containing all necessary supplements. The data shown are the means and SEM from three independent assays.

adiA promoter, resulting in strain χ11636 (*adiAC*) (Fig. 1). The sensitivity of χ11636 to pH 3.0 challenge was not significantly different from those of Ty2 and χ11548 (Δ*aroD*) ($P = 0.327$) (Fig. 3). However, when the *adiAC* operon fusion was placed under transcriptional control of the P_{rhaBAD} promoter and the resulting strain (χ11568 Δ*aroD* P_{rhaBAD} *adiAC*) was grown in the presence of 0.1% rhamnose, it displayed a 1,000- to 10,000-fold increase over Ty2 in the number of viable cells present at all time points during pH 3.0 challenge ($P < 0.0001$) (Fig. 3). Thus, our ability to rescue χ11568 at low pH via rhamnose induction of the *adiA* system indicates that the activity of the arginine decarboxylase system alone is sufficient for low-pH survival in *S.* Typhi, despite the presence of the *rpoS* mutation in Ty2. This is not surprising, since the induction and activity of the native *adiA* system in *Salmonella* are independent of *rpoS* (2).

Survival of strain χ11568 during pH 3.0 challenge is rhamnose and arginine dependent. A number of acid resistance and acid tolerance mechanisms have been described in stationary-phase *Salmonella*. To confirm that the acid resistance phenotype of χ11568 (Δ*aroD* P_{rhaBAD} *adiAC*) was attributable to the rhamnose-regulated arginine decarboxylase system, χ11568 was tested for survival at pH 3.0 in the absence of rhamnose and arginine. When cultured in minimal EGA medium without rhamnose, χ11568 (Δ*aroD* P_{rhaBAD} *adiAC*) displayed a survival profile during pH 3.0 challenge indistinguishable from that of the wild-type Ty2 and χ11548 (Δ*aroD*) strains (Fig. 4A). Addition of rhamnose to the EGA culture medium restored the acid resistance of χ11568 (Δ*aroD* P_{rhaBAD} *adiAC*), resulting in a 1,000- to 10,000-fold higher survival rate when rhamnose was provided ($P = 0.001$).

The acid resistance of χ11568 (Δ*aroD* P_{rhaBAD} *adiAC*) also depended on the presence of arginine in the challenge medium (Fig. 4B). The percentage of viable χ11568 (Δ*aroD* P_{rhaBAD} *adiAC*) cells during challenge rapidly declined over 4 h in the absence of arginine, with few survivors detected after the first 2 h. However, cells that were challenged in the presence of 1 mM arginine showed a marked increase in survival ($P = 0.003$). The arginine requirement for survival at low pH confirms that the acid resistance we observed was due to the *Salmonella* arginine decarboxylase system

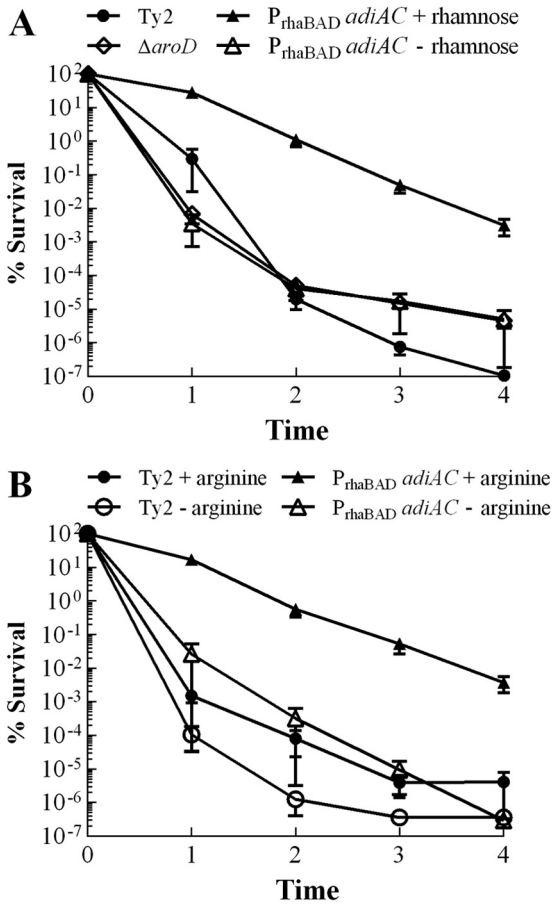

FIG 4 Acid resistance depends on the presence of rhamnose and arginine. Ty2, χ11548 (Δ*aroD*), and χ11568 (Δ*aroD* P_{rhaBAD} *adiAC*) were grown to the stationary phase in EGA medium and then challenged with EG medium (pH 3.0). (A) Strains cultured in the presence or absence of 0.1% rhamnose. (B) Strains challenged in the presence or absence of 1 mM arginine. Survival in all assays was monitored by plating on LB agar containing all necessary supplements. The data shown are the means and SEM from three independent assays.

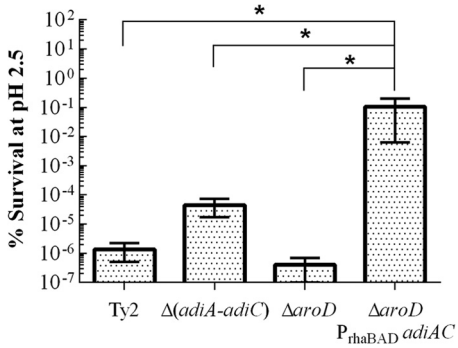

FIG 5 Acid resistance of a Δ*aroD1299* mutant containing rhamnose-regulated arginine decarboxylase at pH 2.5. Ty2, χ11500 (Δ*adiA-adiC*), χ11548 (Δ*aroD*), and χ11568 (Δ*aroD* P_{rhaBAD} *adiAC*) were grown to the stationary phase in EGA medium containing 0.1% rhamnose and then challenged with EG medium containing 1 mM arginine at pH 2.5 for 1 h. Survival in all assays was monitored by plating on LB agar containing all necessary supplements. The data shown are the means and SEM from three independent assays. Pairs of data marked with an asterisk are significantly different ($P < 0.05$).

and not to the stationary-phase acid tolerance response or the oxidative acid resistance response (AR1), as neither of these systems requires arginine (3, 44). Interestingly, even though cells were cultured in aerobic minimal medium to prevent induction of the native arginine decarboxylase system in wild-type *S.* Typhi strain Ty2 (2), we observed an arginine-dependent increase ($P = 0.022$) in resistance to pH 3 challenge (Fig. 4B). This suggests that arginine decarboxylase is expressed at low levels in *S.* Typhi during stationary-phase culture—a conclusion consistent with the low, but detectable, levels of *adiA* transcript observed in Ty2 (Fig. 2).

The rhamnose-regulated arginine decarboxylase system also provided a substantial benefit to *S.* Typhi survival during pH 2.5 challenge (Fig. 5). After 1 h at pH 2.5, χ11568 (Δ*aroD* P_{rhaBAD} *adiAC*) survived significantly better than its Δ*aroD* parent (χ11548) ($P = 0.010$), wild-type strain Ty2 ($P = 0.010$), and the arginine decarboxylase deletion mutant χ11500 (Δ*adiA-adiC*) ($P = 0.035$). Of the 10^9 CFU that were challenged, over 10^5 CFU of χ11568 (Δ*aroD* P_{rhaBAD} *adiAC*) remained viable after 1 h. However, the arginine decarboxylase system did not appear to be able to protect *S.* Typhi for longer than 1 h at pH 2.5, as we did not detect any viable cells after the first hour of challenge (data not shown). This is quite different from what occurs in *S.* Typhimurium, where the arginine decarboxylase system protects cells for more than 2 h at pH 2.5 (2).

Rhamnose-dependent acid resistance is equivalent to acid resistance in cells grown under decarboxylase-inducing conditions. We next compared the level of acid resistance afforded by the rhamnose-regulated system to the acid resistance provided by the native system. Strains were grown anaerobically with 0.1% rhamnose in unbuffered rich medium where the pH was allowed to fall below pH 5.0 during growth (native inducing conditions). The arginine decarboxylase deletion mutant χ11500 (Δ*adiA-adiC*) rapidly succumbed to challenge at both pHs 3.0 and 2.5 (Fig. 6A and B). Ty2 and χ11548 (Δ*aroD*) displayed a high degree of acid resistance at pH 3.0 (greater than 10^4 CFU/ml were viable after 4 h), but succumbed to pH 2.5 after 2 h. In contrast, the highly acid-resistant *Shigella flexneri* strain 2457T exhibited >70% viability for 4 h at pH 3.0, and viability only decreased by 1 log after 4 h at pH 2.5. Strain χ11568 (Δ*aroD* P_{rhaBAD} *adiAC*) was not able to match the acid resistance profile of *Shigella*, although it displayed a survival profile equivalent to those of *S.* Typhi Ty2 and χ11548 (Δ*aroD*) grown under these conditions ($P = 0.210$). No protection was afforded against pH 2.0 challenge (data not shown), consistent with previous reports for *Salmonella* (3, 50).

Rescue of Δ*phoPQ23* and ΔP_{fur81}::TT *araC* P_{araBAD} *fur* mutants at pHs 3.0 and 2.5. Based on our successful rescue of a Δ*aroD* mutant with the rhamnose-regulated arginine decarboxylase system, we wanted to evaluate the system in *S.* Typhi strains carrying attenuating mutations known to affect low-pH survival. Therefore, we introduced the rhamnose-regulated *adiA* system into two *S.* Typhi strains containing mutations that result in well-characterized acid sensitivities (7, 8): Δ*phoPQ* mutant χ8444 and *fur* mutant χ11118 (Table 1). The resulting strains, χ11622 (Δ*phoPQ* P_{rhaBAD} *adiAC*) and χ11623 (P_{araBAD} *fur* P_{rhaBAD} *adiAC*), exhibited rhamnose-dependent arginine decarboxylase activity (data not shown).

To evaluate the ability of the rhamnose-regulated arginine decarboxylase system to rescue χ11622 (Δ*phoPQ* P_{rhaBAD} *adiAC*), the strain was grown in aerobically in minimal EGA medium to the stationary phase at pH 7.0 in the presence of 0.1% rhamnose

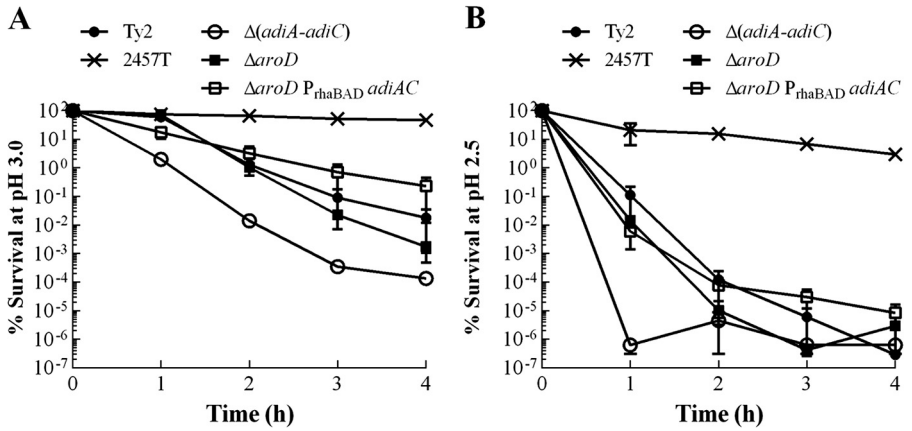

FIG 6 Comparison of acid resistance provided by native and rhamnose-regulated arginine decarboxylase. *S. flexneri* 2457T, Ty2, χ11500 (Δ*adiA-adiC*), χ11548 (Δ*aroD*), and χ11568 (Δ*aroD* P_{rhaBAD} *adiAC*) were grown overnight in TSB medium with 0.4% glucose and 0.1% rhamnose under anaerobic conditions. Cells were challenged for 4 h with EG medium containing 1 mM arginine at pH 3.0 (A) or pH 2.5 (B). Survival in all assays was monitored by plating on LB agar containing all necessary supplements. The data shown are the means and SEM from three independent assays.

and then was challenged at either pH 3.0 or 2.5. Under these growth conditions, which do not induce the native *adiA* system, the Δ*phoPQ23* mutant χ8444 displayed a survival profile similar to that of wild-type Ty2 ($P = 0.996$) (Fig. 7A). In contrast, the rhamnose-regulated arginine decarboxylase system in strain χ11622 (Δ*phoPQ* P_{rhaBAD} *adiAC*) provided approximately a 1,000-fold increase in viability at pH 3.0 over both the parent Δ*phoPQ* mutant (χ8444) and the wild-type strain Ty2 ($P = 0.034$). At pH 2.5, the viability of χ11622 (Δ*phoPQ* P_{rhaBAD} *adiAC*) after 1 h significantly exceeded that of the other *S.* Typhi strains: $P = 0.009$ for χ8444, $P = 0.010$ for Ty2, and $P = 0.0232$ for χ11500 [Δ(*adiA-adiC*)] (Fig. 7B). When strains were grown under native *adiA*-inducing conditions and challenged at pH 3.0 or 2.5, the rhamnose-inducible arginine decarboxylase system in χ11622 (grown with 0.1% rhamnose) provided protection equivalent to that of the native system (see Fig. S1 in the supplemental material).

The success of our system at rescuing the Δ*phoPQ* mutant may be due to two factors. First, the strains were challenged during stationary phase, when PhoP and PhoQ are less important for acid tolerance (51). Second, mutations that inactivate *phoP* or *phoQ* cause a well-characterized sensitivity to inorganic acid (8). At low pH, inorganic acids exist almost exclusively in their dissociated state (free proton with conjugate base), which makes them ideal candidates for neutralization by arginine decarboxylase (which will consume the free protons in the decarboxylase reaction, which immediately raises the intracellular pH and stops further cytoplasmic damage by the free protons). Thus, the arginine decarboxylase system is well poised to compensate for the acid sensitivity imposed by a Δ*phoPQ* mutation.

We next examined the impact of the arginine decarboxylase system on a *fur* mutant [χ11623 (P_{araBAD} *fur* P_{rhaBAD} *adiAC*)]. Although *fur* expression in χ11118 (P_{araBAD} *fur*) is conditional, depending on the presence of arabinose in the culture medium (35), the strain displayed the phenotype of a *fur* knockout mutant in the acid resistance assay irrespective of the arabinose concentration (see Fig. S2 in the supplemental material). Therefore, we decided to work with it and the rhamnose-regulated arginine decarboxylase daughter strain [χ11623 (P_{araBAD} *fur* P_{rhaBAD} *adiAC*)] only in the absence of arabinose. We observed no difference in

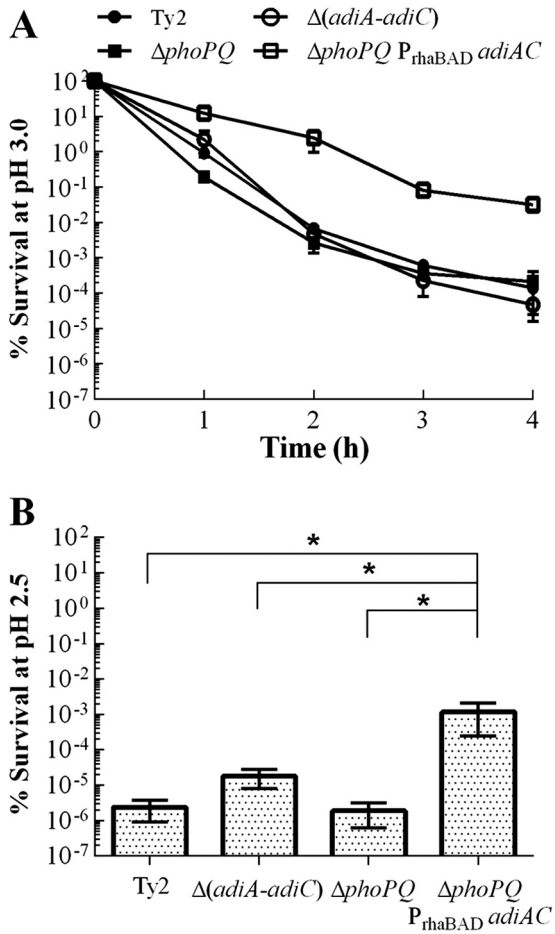

FIG 7 Acid resistance of a Δ*phoPQ23* mutant containing rhamnose-regulated arginine decarboxylase. Ty2, χ11500 (Δ*adiA-adiC*), χ8444 (Δ*phoPQ*), and χ11622 (Δ*phoPQ* P_{rhaBAD} *adiAC*) were grown to the stationary phase in EGA medium containing 0.1% rhamnose and then challenged with EG medium containing 1 mM arginine at pH 3.0 for 4 h (A) or pH 2.5 for 1 h (B). Survival in all assays was monitored by plating on LB agar containing all necessary supplements. The data shown are the means and SEM from three independent assays. Pairs of data marked with an asterisk are significantly different ($P < 0.05$).

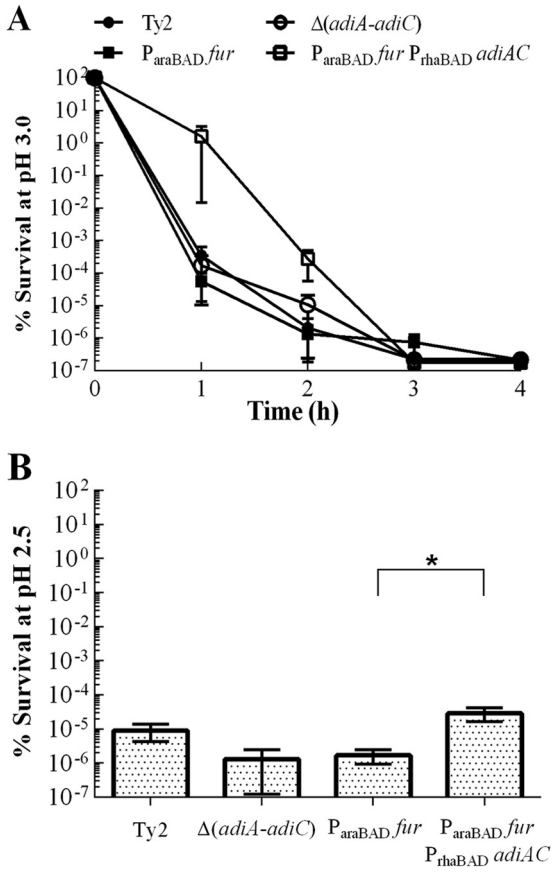

FIG 8 Acid resistance of a ΔP_{fur81}::TT *araC* P_{araBAD} *fur* mutant containing rhamnose-regulated arginine decarboxylase. Arginine decarboxylase rescue was performed by growing strains in EGA medium to stationary phase in the absence of arabinose and presence of 0.1% rhamnose and challenging them with EG medium containing 1 mM arginine at pH 3.0 for 4 h (A) or pH 2.5 for 1 h (B). Survival in all assays was monitored by plating on LB agar containing all necessary supplements. The data shown are the means and SEM from three independent assays. Pairs of data marked with an asterisk are significantly different ($P < 0.05$). χ11500, Δ(*adiA-adiC*); χ11118, P_{araBAD} *fur*; χ11623, P_{araBAD} *fur* P_{rhaBAD} *adiAC*.

survival at pH 3.0 between the wild-type strain Ty2, the arginine decarboxylase knockout χ11500 (Δ*adiA-adiC*), and χ11118 (P_{araBAD} *fur*) in our assay ($P = 0.392$) (Fig. 8A). Strain χ11623 (P_{araBAD} *fur* P_{rhaBAD} *adiAC*) displayed greater survival than its parent χ11118 (P_{araBAD} *fur*) for the first hour of challenge at pH 3.0 ($P = 0.010$) indicating that the arginine decarboxylase system could rescue this strain to some degree. However, there was no difference between χ11623 (P_{araBAD} *fur* P_{rhaBAD} *adiAC*) and χ11118 (P_{araBAD} *fur*) for the later time points ($P = 0.337$). A similar trend was observed at pH 2.5 (Fig. 8B). χ11623 (P_{araBAD} *fur* P_{rhaBAD} *adiAC*) survived significantly better after 1 h at pH 2.5 than its acid-sensitive parent χ11118 (P_{araBAD} *fur*) ($P = 0.013$), but it was not significantly different from the wild-type Ty2 ($P = 0.242$) or the arginine decarboxylase mutant χ11500 (Δ*adiA-adiC*) ($P = 0.122$). Even when grown under native *adiA*-inducing conditions, χ11623 displayed very poor survival (see Fig. S1 in the supplemental material).

There are several possible reasons for the difficulty in rescuing strain χ11623 (P_{araBAD} *fur* P_{rhaBAD} *adiAC*). First, unlike *phoPQ* mutants, *fur* mutants are sensitive to organic acids. Inorganic acids such as HCl and organic acids behave quite differently inside the cell, due to differences in their dissociation constants. Our EGA challenge medium contained 10 mM citric acid (38). It is possible that the consumption of free protons by the arginine decarboxylase system is less effective at countering the effects of an organic acid, such as citric acid, than the strong inorganic acid HCl (8, 24, 38, 52). Second, because the ΔP_{fur81}::TT *araC* P_{araBAD} *fur* mutation was introduced into Ty2, the strain also contains a mutation in *rpoS*. RpoS and Fur jointly regulate a number of key effectors responsible for protection against organic acid. Thus, the combination of *fur* and *rpoS* mutations may have rendered χ11623 more sensitive to acid than strains carrying the *rpoS* mutation alone or the combination of *phoPQ* and *rpoS* (4, 6). Finally, the P_{fur81}::TT *araC* P_{araBAD} *fur* mutation may have altered the ability of χ11623 to transport rhamnose, as it required four times the concentration of rhamnose to induce arginine decarboxylase activity as the Δ*aroD* and Δ*phoPQ* mutants (data not shown). Fur is known to regulate expression of a number of outer membrane proteins and other genes that may influence surface structure (53). Thus, it is possible that membrane perturbations due to the lack of Fur in the cell may have resulted in a reduction in rhamnose transport activity by RhaT.

Conclusion. In this work, we constructed an acid resistance system whose expression and activity responded to the presence of a single sugar, either arabinose or rhamnose. Both *adiA* expression and *adiC* expression were required for acid resistance, and the rhamnose-regulated P_{rhaBAD} promoter provided tighter control over *adiA* expression than the arabinose-regulated P_{araBAD} promoter. Rhamnose-dependent acid resistance in *S.* Typhi depended on three things: the presence of rhamnose in the culture medium, the presence of arginine in the challenge medium, and the fusion of *adiA* and *adiC* into an operon under the control of P_{rhaBAD}. The absence of any of these components resulted in rapid cell death at low pH. The level of acid resistance provided by P_{rhaBAD} *adiAC* grown with rhamnose under decarboxylase-inducing conditions was equivalent to the level of acid resistance observed with the native arginine decarboxylase system grown under the same conditions. However, the rhamnose-regulated *adiAC* system was inducible in cells otherwise unprepared for low-pH challenge, thus our rhamnose-regulated system significantly improved the survival of acid-unadapted *aroD*, Δ*phoPQ*, and *fur* mutants at pHs 3 and 2.5. This has far-reaching implications for vaccine development, as high levels of acid resistance can be attained without anaerobic, low-pH culture—a process which is neither efficient nor cost-effective. Instead, cells can be grown in the optimal medium for vaccine formulation as long as rhamnose is included.

The construction of the rhamnose-regulated arginine decarboxylase system allowed us to render *S.* Typhi acid resistant (to pH 2.5) on demand. Importantly, aerobically grown vaccine strains were protected from pHs 3 and 2.5. Since the low pH of the gastric environment poses a significant threat to the success of any live attenuated *Salmonella* vaccine, the rhamnose-regulated arginine decarboxylase system represents a novel means to augment survival in this *in vivo* compartment. Also, because low gastric pH is an important virulence signal, the ability to administer vaccines without stomach pH neutralization may also improve vaccine performance in the host. We plan to address these issues in future studies.

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REFERENCES

1. **Verdu E, Viani F, Armstrong D, Fraser R, Siegrist HH, Pignatelli B, Idstrom JP, Cederberg C, Blum AL, Fried M.** 1994. Effect of omeprazole on intragastric bacterial counts, nitrates, nitrites, and N-nitroso compounds. Gut **35**:455–460.
2. **Kieboom J, Abee T.** 2006. Arginine-dependent acid resistance in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **188**:5650–5653.
3. **Lin J, Lee IS, Frey J, Slonczewski JL, Foster JW.** 1995. Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri*, and *Escherichia coli*. J. Bacteriol. **177**:4097–4104.
4. **Foster JW.** 1993. The acid tolerance response of *Salmonella typhimurium* involves transient synthesis of key acid shock proteins. J. Bacteriol. **175**: 1981–1987.
5. **Foster JW, Spector MP.** 1995. How *Salmonella* survive against the odds. Annu. Rev. Microbiol. **49**:145–174.
6. **Lee IS, Lin J, Hall HK, Bearson B, Foster JW.** 1995. The stationary-phase sigma factor sigma S (RpoS) is required for a sustained acid tolerance response in virulent *Salmonella typhimurium*. Mol. Microbiol. **17**:155–167.
7. **Hall HK, Foster JW.** 1996. The role of *fur* in the acid tolerance response of *Salmonella typhimurium* is physiologically and genetically separable from its role in iron acquisition. J. Bacteriol. **178**:5683–5691.
8. **Bearson BL, Wilson L, Foster JW.** 1998. A low pH-inducible, PhoPQ-dependent acid tolerance response protects *Salmonella* Typhimurium against inorganic acid stress. J. Bacteriol. **180**:2409–2417.
9. **Wilmes-Riesenberg MR, Bearson B, Foster JW, Curtiss R, III.** 1996. Role of the acid tolerance response in virulence of *Salmonella typhimurium*. Infect. Immun. **64**:1085–1092.
10. **Robbe-Saule V, Norel F.** 1999. The *rpoS* mutant allele of *Salmonella typhi* Ty2 is identical to that of the live typhoid vaccine Ty21a. FEMS Microbiol. Lett. **170**:141–143.
11. **Germanier R, Furer E.** 1975. Isolation and characterization of Gal E mutant Ty 21a of *Salmonella typhi*: a candidate strain for a live, oral typhoid vaccine. J. Infect. Dis. **131**:553–558.
12. **Germanier R, Furer E.** 1983. Characteristics of the attenuated oral vaccine strain "*S. typhi*" Ty 21a. Dev. Biol. Stand. **53**:3–7.
13. **Hone D, Morona R, Attridge S, Hackett J.** 1987. Construction of defined *galE* mutants of *Salmonella* for use as vaccines. J. Infect. Dis. **156**:167–174.
14. **Hone DM, Harris AM, Levine MM.** 1994. Adaptive acid tolerance response by *Salmonella typhi* and candidate live oral typhoid vaccine strains. Vaccine **12**:895–898.
15. **Hohmann EL, Oletta CA, Killeen KP, Miller SI.** 1996. *phoP*/*phoQ*-deleted *Salmonella typhi* (Ty800) is a safe and immunogenic single-dose typhoid fever vaccine in volunteers. J. Infect. Dis. **173**:1408–1414.
16. **Shi H, Wang S, Roland KL, Gunn BM, Curtiss R, III.** 2010. Immunogenicity of a live recombinant *Salmonella* vaccine expressing *pspA* in neonates and infant mice born from naive and immunized mothers. Clin. Vaccine Immunol. **17**:363–371.
17. **Tacket CO, Sztein MB, Losonsky GA, Wasserman SS, Nataro JP, Edelman R, Pickard D, Dougan G, Chatfield SN, Levine MM.** 1997. Safety of live oral *Salmonella typhi* vaccine strains with deletions in *htrA* and *aroC aroD* and immune response in humans. Infect. Immun. **65**:452–456.
18. **Tacket CO, Hone DM, Curtiss R, III, Kelly SM, Losonsky G, Guers L, Harris AM, Edelman R, Levine MM.** 1992. Comparison of the safety and immunogenicity of Δ*aroC* Δ*aroD* and Δ*cya* Δ*crp Salmonella typhi* strains in adult volunteers. Infect. Immun. **60**:536–541.
19. **Kirkpatrick BD, Tenney KM, Larsson CJ, O'Neill JP, Ventrone C, Bentley M, Upton A, Hindle Z, Fidler C, Kutzko D, Holdridge R, Lapointe C, Hamlet S, Chatfield SN.** 2005. The novel oral typhoid vaccine M01ZH09 is well tolerated and highly immunogenic in 2 vaccine presentations. J. Infect. Dis. **192**:360–366.
20. **DiPetrillo MD, Tibbetts T, Kleanthous H, Killeen KP, Hohmann EL.** 1999. Safety and immunogenicity of *phoP*/*phoQ*-deleted *Salmonella typhi* expressing *Helicobacter pylori* urease in adult volunteers. Vaccine **18**:449–459.
21. **Gilman RH, Hornick RB, Woodard WE, DuPont HL, Snyder MJ, Levine MM, Libonati JP.** 1977. Evaluation of a UDP-glucose-4-epimeraseless mutant of *Salmonella typhi* as a live oral vaccine. J. Infect. Dis. **136**:717–723.
22. **Black R, Levine MM, Young C, Rooney J, Levine S, Clements ML, O'Donnell S, Hugues T, Germanier R.** 1983. Immunogenicity of Ty21a attenuated *Salmonella typhi* given with sodium bicarbonate or in enteric-coated capsules. Dev. Biol. Stand. **53**:9–14.
23. **Levine MM, Ferreccio C, Black RE, Germanier R.** 1987. Large-scale field trial of Ty21a live oral typhoid vaccine in enteric-coated capsule formulation. Lancet **i**:1049–1052.
24. **Baik HS, Bearson S, Dunbar S, Foster JW.** 1996. The acid tolerance response of *Salmonella typhimurium* provides protection against organic acids. Microbiology **142**:3195–3200.
25. **Groisman EA, Kayser J, Soncini FC.** 1997. Regulation of polymyxin resistance and adaptation to low-Mg^{2+} environments. J. Bacteriol. **179**: 7040–7045.
26. **Rychlik I, Barrow PA.** 2005. *Salmonella* stress management and its relevance to behaviour during intestinal colonisation and infection. FEMS Microbiol. Rev. **29**:1021–1040.
27. **Durant JA, Corrier DE, Ricke SC.** 2000. Short-chain volatile fatty acids modulate the expression of the *hilA* and *invF* genes of *Salmonella typhimurium*. J. Food Prot. **63**:573–578.
28. **Lee AK, Detweiler CS, Falkow S.** 2000. OmpR regulates the two-component system SsrA-SsrB in *Salmonella* pathogenicity island 2. J. Bacteriol. **182**:771–781.
29. **Richard H, Foster JW.** 2004. *Escherichia coli* glutamate- and arginine-dependent acid resistance systems increase internal pH and reverse transmembrane potential. J. Bacteriol. **186**:6032–6041.
30. **Gong S, Richard H, Foster JW.** 2003. YjdE (AdiC) is the arginine: agmatine antiporter essential for arginine-dependent acid resistance in *Escherichia coli*. J. Bacteriol. **185**:4402–4409.
31. **Andrell J, Hicks MG, Palmer T, Carpenter EP, Iwata S, Maher MJ.** 2009. Crystal structure of the acid-induced arginine decarboxylase from *Escherichia coli*: reversible decamer assembly controls enzyme activity. Biochemistry **48**:3915–3927.
32. **Blethen SL, Boeker EA, Snell EE.** 1968. Arginine decarboxylase from *Escherichia coli*. I. Purification and specificity for substrates and coenzyme. J. Biol. Chem. **243**:1671–1677.
33. **Iyer R, Williams C, Miller C.** 2003. Arginine-agmatine antiporter in extreme acid resistance in *Escherichia coli*. J. Bacteriol. **185**:6556–6561.
34. **Auger EA, Redding KE, Plumb T, Childs LC, Meng SY, Bennett GN.** 1989. Construction of *lac* fusions to the inducible arginine- and lysine decarboxylase genes of *Escherichia coli* K12. Mol. Microbiol. **3**:609–620.
35. **Curtiss R, III, Wanda SY, Gunn BM, Zhang X, Tinge SA, Ananthnarayan V, Mo H, Wang S, Kong W.** 2009. *Salmonella enterica* serovar Typhimurium strains with regulated delayed attenuation in vivo. Infect. Immun. **77**:1071–1082.
36. **Kaniga K, Delor I, Cornelis GR.** 1991. A wide-host-range suicide vector for improving reverse genetics in gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. Gene **109**:137–141.
37. **Kang HY, Dozois CM, Tinge SA, Lee TH, Curtiss R, III.** 2002. Transduction-mediated transfer of unmarked deletion and point mutations through use of counterselectable suicide vectors. J. Bacteriol. **184**:307–312.
38. **Vogel HJ, Bonner DM.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97–106.
39. **De Biase D, Tramonti A, John RA, Bossa F.** 1996. Isolation, overexpression, and biochemical characterization of the two isoforms of glutamic acid decarboxylase from *Escherichia coli*. Protein Expr. Purif. **8**:430–438.
40. **Sambrook J, Russell DW.** 2001. Molecular cloning: a laboratory manual, vol 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
41. **Laemmli UK.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680–685.
42. **Towbin H, Staehelin T, Gordon J.** 1989. Immunoblotting in the clinical laboratory. J. Clin. Chem. Clin. Biochem. **27**:495–501.
43. **Rice EW, Johnson CH, Dunnigan ME, Reasoner DJ.** 1993. Rapid glutamate decarboxylase assay for detection of *Escherichia coli*. Appl. Environ. Microbiol. **59**:4347–4349.

44. **Castanie-Cornet MP, Penfound TA, Smith D, Elliott JF, Foster JW.** 1999. Control of acid resistance in *Escherichia coli*. J. Bacteriol. **181**:3525–3535.
45. **Berk PA, Jonge R, Zwietering MH, Abee T, Kieboom J.** 2005. Acid resistance variability among isolates of *Salmonella enterica* serovar Typhimurium DT104. J. Appl. Microbiol. **99**:859–866.
46. **Hoiseth SK, Stocker BA.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature **291**:238–239.
47. **Haldimann A, Daniels LL, Wanner BL.** 1998. Use of new methods for construction of tightly regulated arabinose and rhamnose promoter fusions in studies of the *Escherichia coli* phosphate regulon. J. Bacteriol. **180**:1277–1286.
48. **Galen JE, Wang JY, Chinchilla M, Vindurampulle C, Vogel JE, Levy H, Blackwelder WC, Pasetti MF, Levine MM.** 2010. A new generation of stable, nonantibiotic, low-copy-number plasmids improves immune responses to foreign antigens in *Salmonella enterica* serovar Typhi live vectors. Infect. Immun. **78**:337–347.
49. **Wang S, Li Y, Shi H, Sun W, Roland KL, Curtiss R, III.** 2011. Comparison of a regulated delayed antigen synthesis system with in vivo-inducible promoters for antigen delivery by live attenuated *Salmonella* vaccines. Infect. Immun. **79**:937–949.
50. **Foster JW.** 1999. When protons attack: microbial strategies of acid adaptation. Curr. Opin. Microbiol. **2**:170–174.
51. **Foster JW, Hall HK.** 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. J. Bacteriol. **172**:771–778.
52. **McHan F, Shotts EB.** 1993. Effect of short-chain fatty acids on the growth of *Salmonella typhimurium* in an in vitro system. Avian Dis. **37**:396–398.
53. **Troxell B, Fink RC, Porwollik S, McClelland M, Hassan HM.** 2011. The Fur regulon in anaerobically grown *Salmonella enterica* sv. Typhimurium: identification of new Fur targets. BMC Microbiol. **11**:236. doi:10.1186/1471-2180-11-236.
54. **Roland K, Curtiss R, III, Sizemore D.** 1999. Construction and evaluation of a Δ*cya* Δ*crp Salmonella typhimurium* strain expressing avian pathogenic *Escherichia coli* O78 LPS as a vaccine to prevent airsacculitis in chickens. Avian Dis. **43**:429–441.
55. **Felix A, Pitt RM.** 1951. The pathogenic and immunogenic activities of *Salmonella typhi* in relation to its antigenic constituents. J. Hyg. (Lond.) **49**:92–110.
56. **Brenneman KE, McDonald C, Kelly-Aehle SM, Roland KL, Curtiss R, III.** 2012. Use of RapidChek SELECT *Salmonella* to detect shedding of live attenuated *Salmonella enterica* serovar Typhi vaccine strains. J. Microbiol. Methods **89**:137–147.
57. **Formal SB, Dammin GJ, Labrec EH, Schneider H.** 1958. Experimental *Shigella* infections: characteristics of a fatal infection produced in guinea pigs. J. Bacteriol. **75**:604–610.
58. **Wang S, Li Y, Shi H, Scarpellini G, Torres-Escobar A, Roland KL, Curtiss R, III.** 2010. Immune responses to recombinant pneumococcal PsaA antigen delivered by a live attenuated *Salmonella* vaccine. Infect. Immun. **78**:3258–3271.
59. **Santander J, Wanda SY, Nickerson CA, Curtiss R, III.** 2007. Role of RpoS in fine-tuning the synthesis of Vi capsular polysaccharide in *Salmonella enterica* serotype Typhi. Infect. Immun. **75**:1382–1392.
60. **Kong Q, Yang J, Liu Q, Alamuri P, Roland KL, Curtiss R, III.** 2011. Effect of deletion of genes involved in lipopolysaccharide core and O-antigen synthesis on virulence and immunogenicity of *Salmonella enterica* serovar Typhimurium. Infect. Immun. **79**:4227–4239.
61. **Kong W, Brovold M, Tully J, Benson L, Curtiss R, III.** 2012. Abstr. 112th Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, DC. http://gm.asm.org.