

Enterobacterial Common Antigen Mutants of *Salmonella enterica* Serovar Typhimurium Establish a Persistent Infection and Provide Protection against Subsequent Lethal Challenge

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Infection with *Salmonella* spp. is a significant source of disease globally. A substantial proportion of these infections are caused by *Salmonella enterica* serovar Typhimurium. Here, we characterize the role of the enterobacterial common antigen (ECA), a surface glycolipid ubiquitous among enteric bacteria, in *S. Typhimurium* pathogenesis. Construction of a defined mutation in the UDP-*N*-acetylglucosamine-1-phosphate transferase gene, *wecA*, in two clinically relevant strains of *S. Typhimurium*, TML and SL1344, resulted in strains that were unable to produce ECA. Loss of ECA did not affect the gross cell surface ultrastructure, production of lipopolysaccharide (LPS), flagella, or motility. However, the *wecA* mutant strains were attenuated in both oral and intraperitoneal mouse models of infection ($P < 0.001$ for both routes of infection; log rank test), and virulence could be restored by complementation of the *wecA* gene in *trans*. Despite the avirulence of the ECA-deficient strains, the *wecA* mutant strains were able to persistently colonize systemic sites (spleen and liver) at moderate levels for up to 70 days postinfection. Moreover, immunization with the *wecA* mutant strains provided protection against a subsequent lethal oral or intraperitoneal challenge with wild-type *S. Typhimurium*. Thus, *wecA* mutant (ECA-negative) strains of *Salmonella* may be useful as live attenuated vaccine strains or as vehicles for heterologous antigen expression.

Salmonella enterica serovars are enteropathogens that display a broad range of host specificities and are common causes of gastroenteritis worldwide (47, 48). In the United States alone, nontyphoidal *Salmonella* isolates cause an estimated 1.4 million cases of salmonellosis annually (12, 48), which results in up to \$50 million per year in medical expenses and work absences (9). More than 2,500 different serotypes of *Salmonella* have been implicated in diarrheal disease (4, 12, 58); however, the majority of enteric salmonellosis cases are caused by a small subset of these serotypes (12, 16, 58), including *Salmonella enterica* serovar Typhimurium. Infection with *S. Typhimurium* can result in a debilitating inflammatory diarrhea that is often accompanied by fever, malaise, vomiting, muscle aches, and abdominal cramps (47).

Salmonella serotypes that cause diarrheal disease are ingested in contaminated food and water and colonize the small intestine during passage through the digestive tract. Entry into the host intestinal tissue is thought to occur preferentially via antigen-sampling microfold (M) cells, although these pathogens can also invade enterocytes (28). Prior to M-cell entry, however, *Salmonella* must first survive/evade host defenses, such as the low pH of the stomach, bile salts, and various other innate immune mechanisms (16).

A subset of the molecules that permit survival of bacteria in these initial steps in pathogenesis are located on the bacterial cell surface. The expression of these bacterial surface components has also been associated with virulence. Multiple reports have demonstrated that *Salmonella* is less virulent in the absence of cell surface proteins, such as OmpS, lipoproteins, and flagella (10, 13, 17, 34, 36, 54). Similarly, attenuation of virulence also occurs in the absence of bacterial surface polysaccharides, such as lipopolysaccharide (LPS) (20). Recently, these studies have been corroborated utilizing a screen for genes that contribute to the ability of *S. Ty-*

phimurium to establish an infection; several of the genes identified are involved in cell surface polysaccharide biosynthesis (14). Moreover, the role of LPS in resistance to antimicrobial effector molecules, systemic disease, and induction of proinflammatory cytokines has been well characterized (8, 19, 22, 25, 35, 53, 56). In contrast, the contributions of other major cell surface polysaccharides to *Salmonella* virulence have not been as clearly delineated.

The cell surface of Gram-negative enteric bacteria contains an additional glycolipid that is ubiquitous among members of the family *Enterobacteriaceae*, the phosphoglyceride-linked enterobacterial common antigen (ECA_{PG}) (52). The carbohydrate component of ECA_{PG} is a heterotrimeric repeat, $\rightarrow 3\text{-}\alpha\text{-D-Fuc4NAc-(1}\rightarrow 4\text{)-}\beta\text{-D-ManNAcA-(1}\rightarrow 4\text{)-}\alpha\text{-D-GlcNAc}$, where Fuc4NAc is 4-acetamido-4,6-dideoxy-D-galactose, ManNAcA is *N*-acetyl-D-mannosaminuronic acid, and GlcNAc is *N*-acetyl-D-glucosamine (18, 51). ECA polysaccharide chains are linked to diacylglycerol through phosphodiester linkage through the potentially reducing terminal GalNAc residue, and the phosphoglyceride anchors the

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molecules in the outer leaflet of the outer membrane (52). ECA_{PG} accounts for approximately 0.2% of the cellular dry weight of the bacterial cell (52). Two related forms (ECA_{LPS} and ECA_{CYC}) have also been described (18), but ECA_{LPS}, which is linked to the lipid A core region, is present on only a subset of enteric bacteria, and ECA_{CYC} is not surface expressed (18, 52). The genes involved in ECA_{PG} biosynthesis are chromosomally encoded in the *wec* gene cluster (also known as *rfe*) (32, 39, 52) and include *wecA* through *wecG*, as well as the *wzx*, *wzy*, and *wzz* genes, which are involved in the addition of the ECA polysaccharide chains to the lipid carrier and transfer of ECA_{PG} to the bacterial cell surface (6, 49). Although the structure and biochemical composition of ECA_{PG} has been well characterized, relatively few studies have investigated the biological function of this major cell surface glycolipid, and a conclusive role for ECA_{PG} *in vivo* has yet to be elucidated.

Previous studies suggest that ECA_{PG} (referred to below as ECA) may contribute to organic acid resistance in Shiga toxin-producing *Escherichia coli* O157:H7 (7). In addition, ECA may play a role in the virulence of *Yersinia enterocolitica* (64) and was shown to be linked to pustule formation in *Haemophilus ducreyi* infections (5). In the context of *Salmonella*, several intriguing observations have been made: (i) Mayer and Schmidt (37) showed that *S. Typhimurium* ECA does not elicit endotoxin-like activity, (ii) passive immunization with anti-ECA antibodies does not protect against salmonellosis (1), and (iii) ECA plays a role in the resistance of bacteria to host bile salts (41). Moreover, studies in the 1970s, using poorly defined mutations, reported that the virulence of ECA mutant strains of *S. Typhimurium* is attenuated in mice (61). Ramos-Morales et al. (49) described a role for two ECA-specific loci (*wecA* and *wecD*) in bile resistance, as well as virulence. Most recently, a comprehensive study by Chaudhuri et al. (14), which identified *S. Typhimurium* genes required for infection of BALB/c mice, showed that among transposon mutations that were ranked by the severity of their attenuation, several genes within the ECA biosynthesis (*wec*) gene cluster, including *wecA*, *wecB*, *wecE*, *wecD*, and *wecC*, were found to have high attenuation scores (14). Collectively, these data suggest that ECA may play a broad role in bacterial virulence, and taken together with the studies presented here, they suggest that ECA is important for *Salmonella* pathogenesis.

The results presented in this paper describe a thorough *in vitro* and *in vivo* characterization of ECA mutant strains (*wecA*::Gm) of *S. Typhimurium*. Our studies demonstrate that disruption of the *wecA* locus results in abrogation of ECA production and that the resulting strains are attenuated *in vivo*. Importantly, these *wecA*-null strains are not cleared by the host but establish a persistent infection. Moreover, the *wecA* mutants can protect against a subsequent lethal challenge in a well-established murine model of salmonellosis. Our results highlight the possibility of using ECA-negative strains of *Salmonella* as live attenuated vaccine strains. Furthermore, the capacity of the ECA mutants to establish a persistent infection suggests the possibility of using ECA-negative strains of *S. Typhimurium* as vehicles for heterologous antigen delivery.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. Bacterial stocks were maintained in LB broth plus 40% glycerol at -80°C . Bacteria were routinely grown as indicated at 37°C in LB or Trypticase soy (Difco) broth (TSB) or

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Plasmids		
pTOPO-TA	Cloning vector	Invitrogen
pTA-BRABR1	pTOPO-TA:: <i>wecA</i>	This study
pBR322	Cloning vector	Promega
pRL140	pBR322:: <i>wecA</i>	This study
pUC-Gm	pUC::Gm	This study
pRL144	pBR322:: <i>wecA</i> ::Gm	This study
pMAK705 ^{ts}	Temperature-sensitive vector; Cm ^r	This study
pRL145	pMAK705 ^{ts} :: <i>wecA</i> ::Gm	This study
pACYC184	Complementation vector; Tet ^r	55
pGEM-T Easy	Commercial cloning vector; Amp ^r	Promega
pDSM643	pGEM-T Easy:: <i>wecA</i> ; Amp ^r	This study
pDSM647	pACYC184:: <i>wecA</i> ; Tet ^r	This study
<i>E. coli</i> strains		
Top10	Cloning strain	Laboratory strain
DH5 α	Cloning strain	Laboratory strain
Top10F'	Cloning strain; F+	Laboratory strain
PR4131	<i>E. coli</i> DH5 α (pRL140)	This study
PR4135	<i>E. coli</i> DH5 α (pRL144)	This study
PR4136	<i>E. coli</i> Top10F'(pRL145)	This study
<i>S. enterica</i> strains		
DSM644	Wild-type <i>S. Typhimurium</i> TML	23
DSM645	TML Δ <i>wecA</i> ; Gent ^r	This study
DSM649	TML Δ <i>wecA</i> (pDSM647) Gent ^r Tet ^r	This study
DSM753	Wild-type <i>S. Typhimurium</i> SL1344	62
DSM754	SL1344 Δ <i>wecA</i> ; Gent ^r	This study
DSM732	SL1344 Δ <i>wecA</i> (pDSM647) Gent ^r Tet ^r	This study

on LB or Trypticase soy agar containing 12.5 $\mu\text{g/ml}$ tetracycline and/or 20 $\mu\text{g/ml}$ gentamicin (Gm) where applicable. Plasmids and PCR products were purified using Qiagen reagents. Growth kinetics were measured in either TSB or minimal N medium (42) supplemented with 0.1% Casamino Acids.

Construction of *wecA*::Gm mutant strains. *S. Typhimurium* ECA mutant strains (*wecA*::Gm) were constructed in both TML and SL1344 wild-type backgrounds. A 1.5-kb fragment containing the *wecA* gene was PCR amplified from genomic DNA from the wild-type TML strain using primers BRA (5'-TACCCGTAAGAAGAGCTGCTCACC-3') and BR (5'-CAGCCCATAAAGTACGAAACAACCC-3'). This product was cloned into pTOPO-TA to yield plasmid pTA-BRABR1. Insertion of the *wecA* gene was confirmed by restriction digestion and sequencing. The 1.5-kb fragment containing the *wecA* gene was liberated from pTA-BRABR1 by EcoRI digestion and subsequently cloned into the EcoRI site of pBR322 to yield plasmid pRL140. An 800-bp Gm resistance cassette was liberated from plasmid pUC-GM using XbaI and then cloned into a SpeI site within the coding region of the *wecA* gene contained within pRL140 to create pRL144 (*wecA*::Gm). A 3.07-kb fragment containing the *wecA*::Gm cassette was then digested from pRL144 using PstI and ClaI and subsequently cloned into the PstI and ClaI sites in pMAK705^{ts} to create plasmid pRL145. pRL145 was electroporated into TML and SL1344, and transformants were selected on LB containing Gm at 30°C . Single-colony Gm^r transformants were subcultured in Gm and incubated overnight at 44°C to promote plasmid loss; growth would occur only if the *wecA*::Gm cassette had recombined into the bacterial chromosome. Gentamicin-resistant strains were passaged multiple times at 44°C to ensure complete loss of the vector pMAK705^{ts}::*wecA*::Gm. Gm^r/Cm^s colonies were then selected and screened by PCR to confirm the mutation of *wecA*. To ensure that insertion of the gentamicin resistance cassette into *wecA* did not ab-

rogate expression of the downstream gene (*wzzE*), we performed semi-quantitative reverse transcription PCR for both TML and SL1344 wild-type and *wecA* mutant strains. Expression of *wzzE* in the *wecA* mutant strains was similar to that in the respective wild-type strains (data not shown). Mutant strains were further characterized as described below.

Complementation of the *wecA* mutation. The *wecA* gene and promoter from the wild-type strain TML was PCR amplified using high-fidelity Phusion enzyme (Finnzymes, Espoo, Finland) and primers *WecAF1* (5'-gcggccgcCCTGACTATCATCGCGACGCGC-3') and *WecAR1* (5'-ccggcgCATTTCAGCGCTCACCGCGCG-3'), which incorporate a *NotI* and *SacII* site, respectively (the restriction enzyme recognition sites are indicated by lowercase letters). The 1,715-bp product was gel purified, 3' A overhangs were added, and the full-length fragment was subcloned into pGemT-Easy (Promega, Madison, WI) to create plasmid pDSM643. The correct insert size was confirmed by *EcoRI* digestion, and the digested product was subsequently cloned into the *EcoRI* site of pACYC184 (55) to create plasmid pDSM647. The complementation plasmid pDSM647 was transformed into *wecA*-null strains DSM645 and DSM754 in order to create strains DSM649 and DSM732, respectively. Transformants were selected on 12.5 µg/ml tetracycline, and complementation of ECA production was verified as described below.

***In vitro* characterization of *wecA::Gm* and *wecA* complementation strains.** Serotyping of wild-type and *wecA* mutant strains was performed with *Salmonella* strain-specific O:4 and O:9 antisera (Difco Laboratories, BD Systems, Sparks, MD) according to the manufacturer's instructions. ECA phenotypes were characterized by passive hemagglutination and immunoblotting. Passive hemagglutination was conducted using a 1:320 dilution of anti-ECA antibody as previously described (50). ECA immunoblotting and LPS/O-antigen silver staining were conducted as follows. Whole-cell lysates were prepared as previously described (27). Briefly, 2 optical density (OD) units/ml of overnight culture were pelleted, resuspended in 200 µl of lysis buffer (0.065 M Tris-HCl [pH 6.8], 2% [wt/vol] SDS 5% [vol/vol] β-mercaptoethanol, 10% [vol/vol] glycerol, 0.05% [wt/vol] bromophenol blue), and incubated at 95°C for 15 min. The lysates were cooled to room temperature and treated with 20 µg proteinase K (Ambion) at 60°C for 1 h. Equal volumes of each lysate were subjected to 12% SDS-PAGE using a 4% stacking layer and subsequently stained using the method of Tsai and Frasch (60). For preparation of immunoblots, non-proteinase K-treated lysates were prepared as described above and transferred at a constant current of 200 mA for 3 h using a semidry transfer apparatus (Thermo Scientific, Rochester, NY). Membranes were probed with a 1:10,000 dilution of murine anti-ECA monoclonal antibody 898 (1, 27). ECA glycoconjugates were detected using a 1:20,000 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were developed using the SuperSignal West Pico chemiluminescent substrate kit (Thermo Scientific/Pierce) and imaged using a LAS-3000 Intelligent Dark Box with LAS-3000 Lite capture software (Fujifilm, Stamford, CT).

Electron microscopy. Transmission electron microscopy was performed at the Biomedical Instrumentation Center (BIC) of the Uniformed Services University of the Health Sciences (USUHS). Overnight stationary-phase cultures were harvested by centrifugation, fixed with 2% electron microscopy (EM) grade glutaraldehyde fixation solution, and examined using a Phillips CM100 electron microscope without staining.

Motility assays. Swimming and swarming motility assays were performed as previously described (11, 59). Briefly, LB plates containing 0.25% (swimming motility) or 0.5% (swarming motility) agar were inoculated with each strain, as indicated. For swimming motility assays, a single colony of each strain was inoculated in the center of each plate and incubated overnight at room temperature. To analyze swarming motility, the LB-based plates contained 0.5% glucose, and each strain was inoculated in the center of each plate from liquid cultures and incubated overnight at 37°C.

***In vivo* characterization experiments.** Animal experiments were approved by the USUHS Animal Care and Use Committee and were per-

formed in accordance with guidelines set forth by the National Institutes of Health (NIH). *S. Typhimurium* strains were cultured overnight at 37°C in TSB with shaking at 225 rpm. Overnight cultures were diluted ~1:100 and grown to exponential phase (optical density at 600 nm, ~0.6). Bacterial cultures were harvested by centrifugation, standardized to a density of 0.6 OD unit/ml in isotonic saline, and subsequently diluted to the appropriate dosage as indicated in the text. Doses of bacterial suspensions were administered in isotonic saline at a volume of 0.2 ml for oral gavage or at a volume of 0.5 ml for intraperitoneal (i.p.) injection. Serial dilutions of each inoculum were plated to confirm the dose. Female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used for all experiments. For oral infections only, animals were fasted for 4 h prior to inoculation and for 1 h postinoculation. The animals were monitored daily for mortality and onset of end-stage disease symptoms.

Bacterial loads at systemic sites. To assess the bacterial loads at systemic sites, mice were inoculated with 1×10^4 CFU of wild-type or *wecA*-null strains as indicated. Spleen and liver tissues were harvested at days 3, 5, 7, 10, 14, 21, 28, 35, 45, 48, 56, 63, and 70 postinfection. Each tissue was homogenized, and an aliquot was serially diluted and plated to determine bacterial counts.

***In vivo* attenuation and complementation of the *wecA* mutation.** Mice were inoculated with the TML wild-type, *wecA* mutant, or *wecA* complemented strain (DSM644, DSM645, and DSM649, respectively) at a dose of 1×10^5 , 1×10^6 , or 1×10^7 CFU by oral gavage. The i.p. infections were performed using 1×10^2 CFU of each bacterial strain, as indicated. Animals were monitored daily for mortality and onset of end-stage disease symptoms. Where indicated, the mean time to death (MTD) was calculated as follows: MTD is equal to the sum of all *x* values divided by the total number of mice dead, where *x* is the number of days postinfection multiplied by the number of mice that died that day.

Immunization and lethal challenge studies. Immunizations with *wecA* mutant strains were administered by oral gavage or i.p. injection and were given either 30 or 60 days prior to lethal challenge, as detailed in the text for each experiment. Vaccination doses were as indicated in the text for each individual experiment. The postvaccination oral and i.p. lethal challenges were performed as described above.

Statistical analyses. Statistical analyses for colonization experiments were performed using a two-tailed Student's *t* test. The statistical significance of differences in percent survival for oral and i.p. infection experiments was determined using the Kaplan-Meier method and a log rank test as indicated in the text.

RESULTS

***In vitro* characterization of *S. Typhimurium wecA* mutant strains.** Previous studies implicated ECA as a virulence factor of *S. Typhimurium* and indicated that *wecA* mutants are attenuated *in vivo* (49, 61). However, the mutant strains used in those studies were poorly defined (11) or the *in vivo* experiments with those strains were conducted only in competition with the wild-type strain (49). Furthermore, complementation of the mutant strains was not attempted in any of these studies. Thus, the observed mutant phenotypes could not be conclusively linked solely to the absence of ECA expression. Therefore, to clearly delineate the role of ECA as a virulence factor, we analyzed well-defined ECA mutants in a series of *in vitro* and *in vivo* studies. We created isogenic *wecA* mutant strains in two clinically relevant *S. Typhimurium* strain backgrounds, TML (DSM644) (23) and SL1344 (DSM753) (62), as described above. The *wecA* gene encodes a UDP-N-acetylglucosamine-1-phosphate transferase and is the first gene in a gene cluster that also encodes the downstream enzymatic components of the ECA biosynthetic pathway. Since *WecA* catalyzes the first step in ECA biosynthesis, we created a nonpolar mutation in the gene to eliminate the possibility of changes in the bacterial cell due to the build-up of ECA intermediates. Deletion of the

TABLE 2 Serotyping and ECA passive hemagglutination

Strain	Genotype	ECA ^a	PHA ^b	Serotyping ^c	
				O:4	O:9
Saline control		NA	–	–	–
TML					
DSM644	WT	+	+	+	–
DSM645	<i>wecA</i> ::Gm	–	–	+	–
DSM649	<i>wecA</i> ::Gm Complemented	+	+	NT	NT
SL1344					
DSM753	WT	+	+	+	–
DSM754	<i>wecA</i> ::Gm	–	–	+	–
DSM732	<i>wecA</i> ::Gm Complemented	+	+	NT	NT

^a NA, not applicable; +, positive; –, negative.

^b PHA, passive hemagglutination; +, hemagglutination; –, no hemagglutination.

^c NT, not tested; +, expressed; –, not expressed.

wecA gene resulted in an ECA-negative phenotype, as determined by passive hemagglutination (Table 2) and immunoblotting (data not shown). Because components of the ECA biosynthetic pathway are also known to be involved in LPS synthesis in some bacteria (52, 64) and because LPS is important for *Salmonella* colonization (33, 43), we wanted to ensure that non-ECA cell surface polysaccharides were unaltered in our *wecA* mutant strains. To this end, DSM645 (TML *wecA*::Gm) and DSM754 (SL1344 *wecA*::Gm) were serotyped using *S. Typhimurium* O:4 antisera to ensure that our *S. Typhimurium* strains retained expression of the wild-type O:4 antigen (Table 2). In addition, the LPS/O-antigen profile of the *wecA* mutant and wild-type strains was evaluated by silver staining. As depicted in Fig. 1A, the *wecA* mutant strains (DSM645 and DSM754) retained LPS profiles indistinguishable from those of the respective parental wild-type strains. Furthermore, analysis of cell surface structure by transmission electron microscopy revealed no gross abnormalities in the *wecA* mutant strains (see Figure S1 in the supplemental material).

To ensure that the ECA-negative phenotype of DSM645 and DSM754 was due to mutation of the *wecA* gene, *wecA* complementation strains were constructed as described in Materials and Methods. Functional complementation of the ECA-negative phenotype was observed in both DSM649 (TML *wecA*::Gm with or without pACYC184::*wecA*) and DSM732 (SL1344 *wecA*::Gm with or without pACYC184::*wecA*) by both passive hemagglutination (Table 2) and immunoblotting (data not shown). The *in vitro* growth of the *wecA* mutant and complementation strains was comparable to that of the parental wild-type strains (Fig. 1B and C), with the exception of a slight but reproducible difference in the growth of the TML wild-type and mutant strains in rich media; however, the growth kinetics were more similar in minimal media, which may more accurately represent *in vivo* growth conditions.

Recently, ECA biosynthesis was shown to be a checkpoint for flagellar biosynthesis in another enteric bacterium, *Serratia marcescens* (11). Since previous studies have shown a link between the presence of flagella and virulence in *S. Typhimurium* (10), we sought to determine whether ECA expression was linked to *S. Typhimurium* flagellar biosynthesis. To this end, we determined

whether the *S. Typhimurium* *wecA* mutant strains displayed any defects in production of flagella or motility. As shown in Fig. S1 in the supplemental material, the *wecA* mutant strains (DSM645 and DSM754) appear to retain normal numbers and localization of flagella. Furthermore, the *wecA* mutant strains appear to retain wild-type levels of swimming motility (see Fig. S2 in the supplemental material). Similarly, the mutant and complementation strains retained the ability to swarm (see Fig. S2 in the supplemental material). In some replicates of the assay, the swarming speeds of the mutant strains appeared slightly slower than those of the parental wild-type strains when assessed at early time points (data not shown). However, after 24 h of growth, the mutant strains were able to swarm to the edge of the plate as well as the wild-type strains. Collectively, our *in vitro* data suggest that the *wecA*-null strains are both genetically and functionally isogenic with their respective parental wild-type strains, except for the absence of ECA.

***wecA* mutant strains are attenuated *in vivo*.** While previous

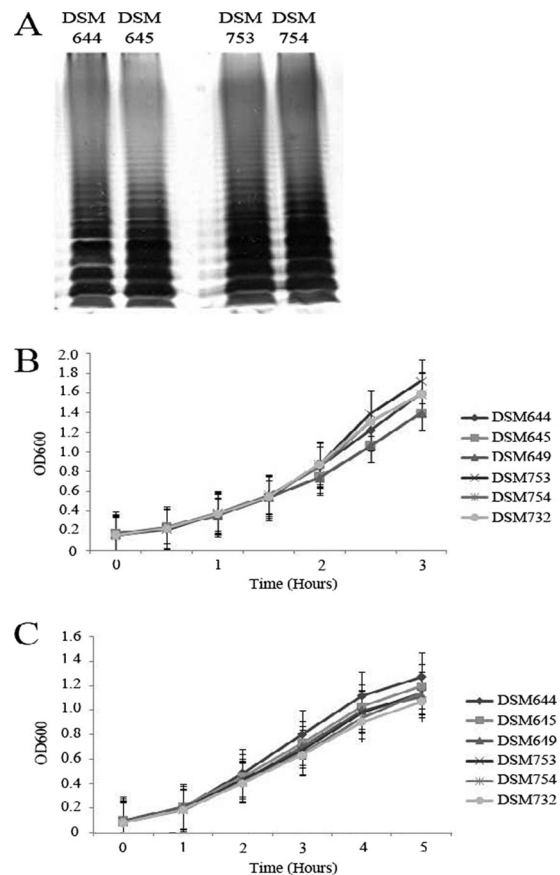


FIG 1 *In vitro* characterization of *wecA* mutant and complementation strains of *S. enterica* serovar Typhimurium. (A) LPS/O-antigen profiles of TML wild-type (DSM644), TML *wecA* mutant (DSM645), SL1344 wild-type (DSM753), and SL1344 *wecA* mutant (DSM754) strains. Silver staining was performed as described in Materials and Methods. (B and C) Growth kinetics of TML wild-type (DSM644), TML *wecA* mutant (DSM645), and TML *wecA* complementation (DSM649) strains and growth kinetics of SL1344 wild-type (DSM753), SL1344 *wecA* mutant (DSM754), and SL1344 *wecA* complementation (DSM732) strains in TSB (B) and minimal N medium (C). Overnight cultures were diluted to an optical density of ~0.1 and incubated at 37°C with shaking. Growth was monitored by the OD at 600 nm (OD600). The data points shown are the averages of 2 or 3 independent experiments, and the error bars represent the standard errors of the mean.

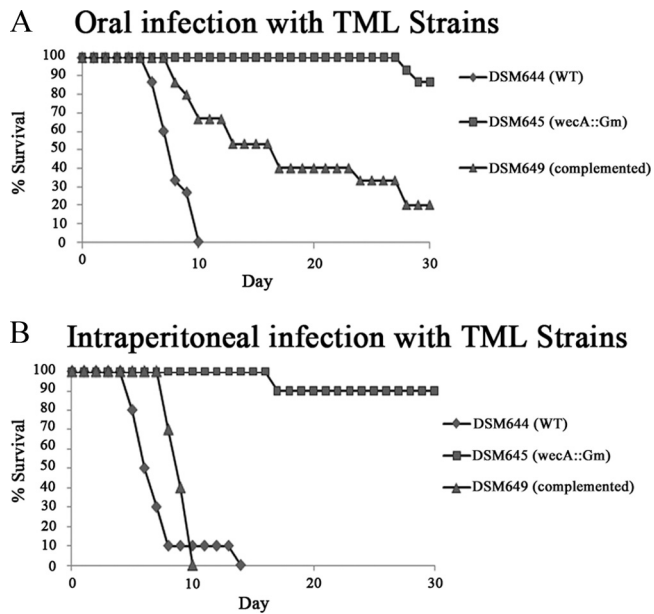


FIG 2 Attenuation and complementation of the TML *wecA* mutant strain. (A) Groups of 5 C57BL/6 mice were orally inoculated with 10^5 , 10^6 , or 10^7 CFU of the TML wild-type (DSM644), TML *wecA* mutant (DSM645), or TML *wecA* complementation (DSM649) strain. The percent survival data for each strain represent combined data from all inoculation doses tested. (B) Groups of 8 to 10 C57BL/6 mice were intraperitoneally inoculated with 10^2 CFU of the TML wild-type (DSM644), TML *wecA* mutant (DSM645), or TML *wecA* complementation (DSM649) strain. The percent survival data for each strain represent combined data from two biologically independent experiments. The differences in percent survival between the wild-type and *wecA* mutant strains, and *wecA* mutant and complementation strains, are statistically significant ($P < 0.0001$; log rank test) for both panels A and B. The percent survival for each of the individual doses is shown in Fig. S3 in the supplemental material.

reports implied that ECA may play a role in *S. Typhimurium* virulence (49, 61), those studies utilized poorly defined mutants, competitive indices, or short-term infection studies. Although the reports were suggestive, we wished to definitively establish an *in vivo* role for ECA using the more robust single-strain model of infection. To this end, we inoculated five C57BL/6 mice per group with 1×10^5 , 1×10^6 , or 1×10^7 CFU of the TML wild-type (DSM644) or *wecA* mutant (DSM645) strain by oral gavage. As depicted in Fig. 2A, all 15 of the mice infected with the wild-type TML strain (DSM644) succumbed to infection by day 10, with an MTD of 7.1 days. The MTDs for animals infected with 1×10^5 , 1×10^6 , and 1×10^7 CFU were 7.4, 7.4, and 6.4 days, respectively (see Fig. S3 in the supplemental material). These data are similar to those shown previously with the same strain of *S. Typhimurium* and the same strain of mice (44). In contrast, only 2 of the 15 mice infected with the *wecA* mutant strain DSM645 succumbed to infection (~87% survival), with an MTD of 27.5 days (1 animal infected with 1×10^6 CFU died on day 28, and 1 animal infected with 1×10^7 CFU died on day 27 [see Fig. S3 in the supplemental material]). This difference in percent survival was highly significant ($P < 0.001$; log rank test). In addition, complementation of the *wecA* mutation in *trans* partially restored virulence; 20% of the mice infected with the *wecA* complementation strain (DSM649) survived for the duration of the experiment, with an MTD of 13.6 days (Fig. 2A). The MTDs for animals infected with 1×10^5 , 1×10^6 , and 1×10^7 CFU of DSM649 were 11.5, 18.6, and 12.2 days,

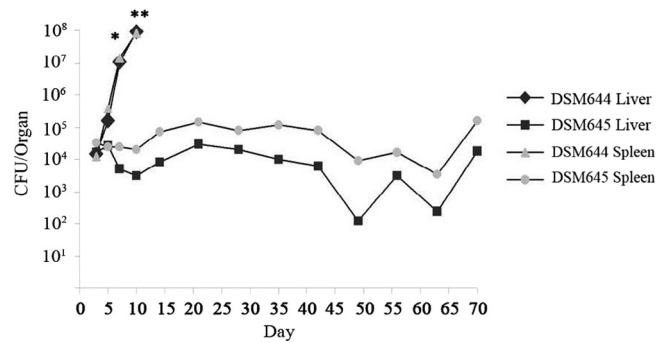


FIG 3 Bacterial loads at systemic sites of infection. C57BL/6 mice were orally inoculated with 10^4 CFU of the TML wild type (DSM644) or TML *wecA* mutant (DSM645). Groups of 7 or 8 mice were sacrificed on days 3, 5, 7, 10, 14, 21, 28, 35, 45, 48, 56, 63, and 70 postinfection. Bacterial counts from liver and spleen were determined by plating serially diluted tissue homogenates and are shown as the geometric mean \log_{10} CFU per organ. Student's *t* test was used to compare the colonization levels of TML and TML *wecA* mutant strains for each tissue site. *, $P < 0.05$; **, not enough animals remained to do statistical tests for the time point.

respectively. The difference in percent survival between the *wecA* mutant and *wecA* complementation strains was also significant ($P < 0.001$; log rank test). In addition, the difference in percent survival between the wild-type and complemented strains was also significant ($P < 0.001$; log rank test), which highlights the fact that only partial complementation was achieved.

We next tested the virulence of the *wecA* mutant strain (DSM645) in the murine single-strain i.p. infection model. C57BL/6 mice were inoculated with 1×10^2 CFU of either the wild-type strain DSM644 or the *wecA* mutant strain DSM645 (Fig. 2B). Whereas all of the mice infected with the wild-type strain succumbed to infection (MTD, 5.4 days), only 1 mouse infected with the *wecA* mutant strain died at day 16 postinfection (90% survival). These results were similar to those in the oral infection experiments, where the differences in percent survival were highly significant ($P < 0.001$; log rank test). Once again, complementation of *wecA* in *trans* restored virulence; all mice infected with DSM649 succumbed to infection (MTD, 7.3 days). The difference in survival between the *wecA* mutant and complemented strains was significant ($P < 0.001$; log rank test). Similar to the oral infection experiments, the difference in percent survival between the wild-type and complemented strains was also significant ($P = 0.008$; log rank test), indicating partial complementation. Collectively, these data indicate that the *wecA* mutant strain is significantly attenuated in both the oral and i.p. single-strain infection models and that the attenuation of virulence is due to the loss of ECA expression, since virulence could be at least partially restored upon complementation of the *wecA* mutation.

***wecA* mutant strains establish a persistent infection.** A key component of *Salmonella* virulence is the ability to establish colonization and persist within the host (16, 24, 46). Given the dramatic changes in lethality we observed with the ECA mutant strain, we next sought to determine whether the *wecA* mutant strain retained the ability to efficiently disseminate and colonize within the host. Groups of mice received 1×10^4 CFU of the TML wild-type (DSM644) or *wecA* mutant (DSM645) strain by oral gavage and were sacrificed at 3- to 5-day intervals postinfection (Fig. 3). The spleen and liver were harvested from each mouse, and

TABLE 3 Oral immunization with ECA mutants and subsequent oral challenge at day 30 with wild-type *S. Typhimurium* strains^a

Background	Immunization			Challenge			
	Strain	Dose	No. survived/total	Strain	Dose	No. survived/total	% Survival
Control	Saline	NA	7/7 ^b	DSM644	1.0 × 10 ³	0/7 ^b	0
	Saline	NA	4/4	DSM644	1.7 × 10 ⁵	0/4	0
	Saline	NA	4/4	DSM644	1.7 × 10 ⁶	0/4	0
TML	DSM645	1.0 × 10 ²	3/3	DSM644	1.0 × 10 ³	3/3	100
	DSM645	1.0 × 10 ³	14/15 ^b	DSM644	1.0 × 10 ³	7/14 ^b	50
	DSM645	1.0 × 10 ⁴	13/15 ^b	DSM644	1.0 × 10 ³	9/13 ^b	69.2
	DSM645	1.0 × 10 ⁵	14/15 ^b	DSM644	1.0 × 10 ³	8/14 ^b	57.1
	DSM645	1.0 × 10 ⁶	11/15 ^b	DSM644	1.0 × 10 ³	7/11 ^b	63.6
	DSM645	1.0 × 10 ⁷	11/15 ^b	DSM644	1.0 × 10 ³	7/11 ^b	63.6
	Total		66/78			41/66	62.1
SL1344	DSM754	1.3 × 10 ⁴	8/8	DSM753	1.7 × 10 ⁵	4/8	50
	DSM754	1.3 × 10 ⁴	8/8	DSM753	1.7 × 10 ⁶	5/8	62.5
	DSM754	1.3 × 10 ⁵	7/7	DSM753	1.7 × 10 ⁵	4/7	57.1
	DSM754	1.3 × 10 ⁵	8/8	DSM753	1.7 × 10 ⁶	6/8	75
	DSM754	1.3 × 10 ⁶	10/10	DSM753	1.7 × 10 ⁵	6/10	60
	DSM754	1.3 × 10 ⁶	10/10	DSM753	1.7 × 10 ⁶	6/10	60
	Total		51/51			31/51	60.7
Total			117/129			72/117	61.5

^a DSM644, TML wild type; DSM645, TML *wecA* mutant; DSM753, SL1344 wild type; DSM754, SL1344 *wecA* mutant.

^b Combined results from 2 independent experiments.

aliquots of homogenized tissue were serially diluted and plated to determine bacterial counts. Mice infected with the wild-type TML (DSM644) bacteria succumbed to infection within 10 days postinfection. In contrast, mice infected with the *wecA* mutant strain (DSM645) survived until the experiment was terminated at day 70 postinfection. Moreover, bacterial counts from mice infected with the wild-type bacteria (DSM644) rapidly increased until host death, whereas mice infected with the *wecA* mutant strain (DSM645) displayed a moderate level of persistent infection throughout the experiment (Fig. 3). This persistent infection was accompanied by splenomegaly and hepatomegaly (data not shown), which often typify persistent *S. Typhimurium* colonization (41). These data demonstrate that the *wecA* mutant strain of *S. Typhimurium* is able to efficiently colonize mice and establish a persistent infection.

Oral immunization protects against a subsequent oral challenge. Since the virulence of the *wecA* mutant strain was significantly attenuated but the strain was still able to establish a persistent infection, we next sought to determine whether immunization/vaccination with the mutant strains could induce a protective immune response. To this end, mice were orally immunized with 1 × 10², 1 × 10³, 1 × 10⁴, 1 × 10⁵, 1 × 10⁶, or 1 × 10⁷ CFU of the TML *wecA* mutant strain DSM645. On day 30 postimmunization, the mice were challenged orally with 1 × 10³ CFU of the wild-type TML strain DSM644 (Table 3). Of the 78 total mice vaccinated, 66 survived until the day of challenge; of the 66 mice that were challenged with wild-type *S. Typhimurium*, 41 mice (62.1%) were protected for the duration of the experiment (30 days).

To ensure that the capacity of the *wecA* mutant strain to protect mice against a subsequent lethal challenge with wild-type bacteria was not *S. Typhimurium* strain dependent, we conducted a similar experiment with the *S. Typhimurium* wild-type strain SL1344.

Mice were orally immunized with 1 × 10⁴, 1 × 10⁵, or 1 × 10⁶ CFU of the SL1344 *wecA* mutant strain DSM754; 30 days postimmunization, groups of vaccinated mice were challenged orally with 1 × 10⁵ or 1 × 10⁶ CFU of the SL1344 wild-type strain DSM753. As shown in Table 3, all 51 mice immunized with the SL1344 *wecA* mutant strain survived until the day of challenge. Moreover, 31 of the 51 challenged mice were fully protected against subsequent lethal challenge; the percent survival was similar across all dosage regimens. Although a higher percentage of mice vaccinated with the SL1344 mutant strain survived until the day of challenge, the degree of protection was similar to that of groups immunized with the TML *wecA* mutant: 62.1% versus 60.7%.

Since the potential to use the *wecA* mutants as live vaccines or vehicles for heterologous antigen delivery would be enhanced if the bacteria persisted in the host for longer than 30 days, we next considered whether the immunity induced by the *wecA* mutant strain would provide protection for longer periods. To address this question, mice were orally immunized with 1 × 10², 1 × 10³, 1 × 10⁴, 1 × 10⁵, 1 × 10⁶, or 1 × 10⁷ CFU of the TML *wecA* mutant strain DSM645 on day 0. On day 45 postimmunization, the mice were orally challenged with 1 × 10⁴ CFU of the wild-type TML strain DSM644 (Table 4). Twenty-four of the 30 immunized mice (80%) survived until the day of challenge. Of the 24 mice challenged on day 45, 12 survived (50%); the highest degree of protection was seen in the mice immunized with 1 × 10⁶ or 1 × 10⁷ CFU (100%). Taken together, these data indicate that oral immunization with a *wecA* mutant strain of *S. Typhimurium* can induce a protective immune response and that protection may last for at least 45 days postimmunization.

Intraperitoneal immunization protects against subsequent intraperitoneal or oral challenge. To determine whether i.p. immunization with the *wecA* mutant strain could also induce pro-

TABLE 4 Oral immunization with the TML ECA mutant and subsequent oral challenge at day 45 with wild-type TML strain mutant

Immunization		Challenge		
Dose	No. survived/total	Dose	No. survived/total	% Survival
Saline	5/5	1.0×10^4	0/5	0
1.0×10^2	4/5	1.0×10^4	0/4	0
1.0×10^3	5/5	1.0×10^4	1/5	20
1.0×10^4	5/5	1.0×10^4	3/5	60
1.0×10^5	3/5	1.0×10^4	1/3	33
1.0×10^6	4/5	1.0×10^4	4/4	100
1.0×10^7	3/5	1.0×10^4	3/3	100
Total ^a	24/30	Total:	12/24	50

^a The totals exclude animals immunized with saline.

tective immunity, mice were injected with 1×10^1 , 1×10^2 , 1×10^3 , or 1×10^4 CFU of DSM645. On day 30 postimmunization, the surviving mice were challenged with either an i.p. lethal dose (1×10^2 CFU) or an oral lethal dose (1×10^4 CFU) of the wild-type TML strain (DSM644) (Table 5). Thirty-nine of the 40 mice immunized i.p. with either 1×10^1 or 1×10^2 CFU survived until the day of challenge (97.6%); 32 of the 39 mice challenged were then fully protected (82.1%) against an intraperitoneal or oral challenge of 1×10^2 or 1×10^4 CFU, respectively. An immunizing dose of 1×10^3 or 1×10^4 CFU of the *wecA* mutant strain was more virulent than the lower immunization doses; only 6 of the 14 mice immunized at these doses survived (42.9%). Clearly, the *wecA* mutant can still be lethal if the immunizing dose is too high.

DISCUSSION

These studies were undertaken to more clearly understand the biological role of one of the major glycolipids on the surface of Gram-negative bacteria, ECA_{PG}. Compared with the long history of structure and function studies of other major bacterial surface glycolipids, such as LPS, definitive studies of ECA function are rare. Although a clear physiological role for the enterobacterial common antigen has not been determined, ECA has been linked to virulence in several species of bacteria (5, 7, 49, 61). Despite this link to pathogenesis, the function of ECA seems to differ in each species. For example, whereas ECA production in *S. marcescens* is linked to flagellar assembly and motility (11), ECA imparts resis-

tance to organic acids in pathogenic *E. coli* (7). Additionally, while ECA is involved in the swarming motility of *S. marcescens* (11), this phenomenon has not been reported in other Gram-negative bacterial species. Since each of these functions is essential for the survival of these organisms in their respective niches, these data perhaps suggest that although ECA is present in many bacteria, each species has evolved unique ways to utilize ECA or ECA biosynthesis in a manner that is most conducive to survival of the species. Thus, it is possible that Gram-negative bacteria that target different host sites may utilize ECA in different ways. This speculation is supported by studies that indicate ECA is important in the pathogenesis of bacterial species that colonize very different sites within the host (3, 5, 7, 11, 31, 49). As such, we were interested in determining the role of ECA in *S. Typhimurium* biology.

An early report indicated that an ECA mutant strain of *S. Typhimurium* was attenuated in an intraperitoneal infection model (61); however, due to the limitations of genetic techniques at that time, the mutations responsible for the ECA-negative phenotype could not be confirmed as completely isogenic, and restoration of virulence by complementation was not achieved. Nevertheless, these data imply a role for ECA in the virulence of *S. Typhimurium*. The role of this molecule in virulence is further supported by the work of Chaudhuri et al. and Ramos-Morales et al., who independently reported that ECA-negative strains were attenuated in competitive tail vein and both oral and intraperitoneal short-term infection models (14, 49). Intrigued by these findings, we sought to further evaluate the role of ECA in *S. Typhimurium* pathogenesis.

To this end, we created genetically defined ECA mutant (*wecA::Gm*) and complementation (Δ *wecA* plus pACYC184::*wecA*) strains in both the TML and SL1344 strain backgrounds of *S. Typhimurium* (Table 1). *In vitro* characterization of these strains showed that disruption of the *wecA* gene resulted in the complete loss of ECA production and that ECA production was fully restored in the complementation strains (Table 2 and data not shown). Further *in vitro* characterization showed no major changes in the growth kinetics (Fig. 1B and C), O-antigen production (Table 2), LPS/O-antigen profile (Fig. 1A), or motility (see Fig. S2 in the supplemental material) of the ECA-negative strains. Conversely, we found that the ECA mutant strain was severely attenuated *in vivo*; significantly fewer animals were killed by the ECA mutant strain than by the wild type in both the oral and intraperitoneal routes of infection (Fig. 2A and B). Finally, we

TABLE 5 Intraperitoneal immunization with the TML ECA mutant and subsequent challenge at day 30 with wild-type TML strain

Immunization			Challenge			
Route	Dose	No. survived/total	Route	Dose	No. survived/total	% Survival
i.p.	Saline	10/10	i.p.	1.5×10^2	0/10	0
i.p.	Saline	9/10	Oral	1.0×10^4	0/9	0
i.p.	1.4×10^1	10/10	i.p.	1.5×10^2	7/10	70
i.p.	1.4×10^2	9/10	i.p.	1.5×10^2	8/9	88.9
i.p.	1.4×10^3	6/9	i.p.	1.5×10^2	2/6	30
i.p.	1.4×10^4	0/5	i.p.	N/A ^a	NA ^a	NA
i.p.	1.0×10^1	10/10	Oral	1.0×10^4	8/10	80
i.p.	1.0×10^2	10/10	Oral	1.0×10^4	9/10	90
Total ^b		45/54			34/45	75.5

^a NA, not applicable.

^b Totals exclude animals immunized with saline.

determined that either oral or intraperitoneal vaccination with the ECA mutant strain provided protection against subsequent lethal challenge with wild-type *S. Typhimurium*.

Interestingly, Ramos-Morales et al. previously reported that the intraperitoneal competitive defect seen with an ECA mutant strain of *S. Typhimurium* was modest compared to the defect seen during oral infections (49). These results suggested that ECA performs an essential function during oral infection that is less crucial during intraperitoneal infection or that the presence of ECA on the wild-type bacteria is able to partially complement the ECA mutant phenotype during intraperitoneal, but not oral, infection. The authors went on to show a role for *S. Typhimurium* ECA in bile resistance and suggested that bile sensitivity may be responsible for the oral defect (49). Surprisingly, we found that in single-strain infections, the ECA mutant strain displayed comparable virulence defects regardless of the route of infection (Fig. 2A and B). Moreover, we were unable to show increased bile sensitivity with either of our defined *wecA* mutant strains (data not shown). While the reason(s) for the discrepancies between the studies remains unclear, they may be due to the models employed (competitive versus single-strain infections) or subtle differences among the *S. Typhimurium* strains used (strain 14028 versus strains TML and SL1344). Regardless, the data clearly indicate that ECA is important for *Salmonella* colonization and virulence, though the actual biological function of ECA remains open to debate.

A key component of *S. Typhimurium* pathogenesis in the murine model of salmonellosis is the ability of the pathogen to disseminate and colonize systemic sites. During oral infection, this process requires that bacteria efficiently cross the intestinal barrier, establish and survive in an intracellular niche, and then disseminate to peripheral sites. The results of our *in vitro* analyses showed no evidence that ECA expression plays a role in bacterial uptake; wild-type and ECA mutant strains showed similar levels of internalization into INT 407 cells *in vitro* (data not shown). As a consequence of these observations, we postulated that the attenuation of ECA mutant strains was due to the inability of the bacteria to disseminate throughout the host. However, as illustrated in Fig. 3, the ECA mutant strain was not only able to colonize systemic sites, such as the liver and spleen, but was also able to persist for up to 70 days postinfection. Thus, attenuation of the ECA mutants is not due to an inability to properly disseminate within the host but may be a reflection of the ECA mutants' inability to attain high numbers within the host (Fig. 3).

Previous studies have shown that mutations in other genes also lead to the ability of *S. Typhimurium* to establish persistent infections *in vivo*. These genes include (but are not limited to) those associated with metabolic pathways (e.g., *aroD*, *aroA*, *purA*, *purE*, and *surA*) and regulatory factors (*ompR*, *rpoS*, *rpoE*, and *phoP*). Many strains with mutations in such genes have been considered potential live-vaccine candidates (13, 15, 17, 21, 26, 40, 54, 57). However, for many of these strains, the ability to achieve persistence requires the use of an intravenous, intraperitoneal, or highly concentrated oral (1×10^8 -CFU) dose; thus, utilizing these strains as vaccine candidates may be challenging. In contrast, the ECA mutant strain was able to establish a persistent infection with a moderate oral dose of 10^4 CFU; colonization levels for the ECA mutant were approximately 10^3 to 10^4 CFU/spleen and approximately 10^4 to 10^5 CFU/liver (Fig. 3). These colonization values are slightly higher than those reported in the studies using *aroA*, *purA*, and *clpP* mutant strains (36, 45, 63). Given these findings, we

hypothesized that higher numbers of persistent bacteria within these organs could result in increased or prolonged immune stimulation within the host and that the ECA mutant might serve as a viable vaccine to protect against subsequent *S. Typhimurium* challenge. Indeed, we found that ECA mutants could provide protection against subsequent lethal challenge by both the oral and i.p. routes of infection. Moreover, this protection lasted as long as 45 days postvaccination and could be achieved with both the TML- and SL1344-derived ECA mutant strains.

A major consideration in the future development of any live-attenuated vaccine strain is the ability to maintain the delicate balance between attenuation of virulence and optimal immunogenicity. While definitive *in vivo* studies of the role of ECA have been limited, our work clearly demonstrates that ECA mutant strains of *S. Typhimurium* are significantly attenuated in a murine model of infection. In addition, the data presented here also indicate that ECA-negative strains of *S. Typhimurium* can persist for over 2 months and induce a protective immune response, as indicated by the survival of animals immunized with ECA-negative *S. Typhimurium* strains after a lethal challenge (Tables 3, 4, and 5). However, the precise mechanism of attenuation remains unclear. Molecules expressed on the surfaces of bacterial cells have been shown to serve as pathogen-associated molecular patterns (PAMPs) and are known to act as ligands for immune signaling receptors (2, 29, 38). It is possible that the lack of ECA on the surfaces of the *wecA* mutant strains alters the initial steps in activation of the host immune response and results in a failure to clear the organisms from systemic sites. Since ECA itself has been shown to be minimally immunogenic (30, 52), ECA mutant strains should retain a degree of immunogenicity similar to that of wild-type strains. Therefore, given the results presented here, we propose that ECA mutant strains of *S. enterica* serovar Typhimurium may be good candidates for the development of a live attenuated vaccine against salmonellosis. Furthermore, the ability of the ECA mutant strain to persist at systemic sites suggests the potential to use ECA-negative strains as vehicles for heterologous antigen delivery. Future work will focus on characterization of the host immune response to ECA-negative strains of *S. Typhimurium* and exploration of the efficiencies of these strains as delivery vehicles for heterologous protective antigens.

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REFERENCES

1. Albertson TE, et al. 2003. Multicenter evaluation of a human monoclonal antibody to Enterobacteriaceae common antigen in patients with Gram-negative sepsis. *Crit. Care Med.* 31:419–427.
2. Anderson KV. 2000. Toll signaling pathways in the innate immune response. *Curr. Opin. Immunol.* 12:13–19.
3. Bahrani-Mougeot FK, et al. 2002. Type 1 fimbriae and extracellular

- polysaccharides are preeminent uropathogenic *Escherichia coli* virulence determinants in the murine urinary tract. *Mol. Microbiol.* 45: 1079–1093.
4. Bangtrakulnonth A, et al. 2004. *Salmonella* serovars from humans and other sources in Thailand, 1993–2002. *Emerg. Infect. Dis.* 10:131–136.
 5. Banks KE, et al. 2008. The enterobacterial common antigen-like gene cluster of *Haemophilus ducreyi* contributes to virulence in humans. *J. Infect. Dis.* 197:1531–1536.
 6. Barr K, Klerna J, Rick PD. 1999. The modality of enterobacterial common antigen polysaccharide chain lengths is regulated by o349 of the *wec* gene cluster of *Escherichia coli* K-12. *J. Bacteriol.* 181:6564–6568.
 7. Barua S, et al. 2002. Involvement of surface polysaccharides in the organic acid resistance of Shiga Toxin-producing *Escherichia coli* O157:H7. *Mol. Microbiol.* 43:629–640.
 8. Beaty CD, Franklin TL, Uehara Y, Wilson CB. 1994. Lipopolysaccharide-induced cytokine production in human monocytes: role of tyrosine phosphorylation in transmembrane signal transduction. *Eur. J. Immunol.* 24: 1278–1284.
 9. Buzby JC, Roberts T, Lin CJ, MacDonald JM. 1996. Bacterial foodborne disease: medical costs and productivity losses. U.S. Department of Agriculture, Washington, DC. <http://hdl.handle.net/10113/34242>.
 10. Carsiotis M, Weinstein DL, Karch H, Holder IA, O'Brien AD. 1984. Flagella of *Salmonella typhimurium* are a virulence factor in infected C57BL/6J mice. *Infect. Immun.* 46:814–818.
 11. Castelli ME, et al. 2008. Enterobacterial common antigen integrity is a checkpoint for flagellar biogenesis in *Serratia marcescens*. *J. Bacteriol.* 190: 213–220.
 12. Centers for Disease Control and Prevention. 1994. *Salmonella* surveillance report—annual summary—1990. Centers for Disease Control and Prevention, Atlanta, GA.
 13. Chatfield SN, Dorman CJ, Hayward C, Dougan G. 1991. Role of ompR-dependent genes in *Salmonella typhimurium* virulence: mutants deficient in both ompC and ompF are attenuated in vivo. *Infect. Immun.* 59: 449–452.
 14. Chaudhuri RR, et al. 2009. Comprehensive identification of *Salmonella enterica* serovar Typhimurium genes required for infection of BALB/c mice. *PLoS Pathog.* 5:e1000529.
 15. Curtiss R III, Kelly SM. 1987. *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. *Infect. Immun.* 55:3035–3043.
 16. Darwin KH, Miller VL. 1999. Molecular basis of the interaction of *Salmonella* with the intestinal mucosa. *Clin. Microbiol. Rev.* 12:405–428.
 17. Dorman CJ, Chatfield S, Higgins CF, Hayward C, Dougan G. 1989. Characterization of porin and ompR mutants of a virulent strain of *Salmonella typhimurium*: ompR mutants are attenuated in vivo. *Infect. Immun.* 57:2136–2140.
 18. Erbel PJ, et al. 2003. Identification and biosynthesis of cyclic enterobacterial common antigen in *Escherichia coli*. *J. Bacteriol.* 185:1995–2004.
 19. Erridge C, Bennett-Guerrero E, Poxton IR. 2002. Structure and function of lipopolysaccharides. *Microbes Infect.* 4:837–851.
 20. Fadl AA, et al. 2005. Attenuation of *Salmonella enterica* serovar Typhimurium by altering biological functions of murein lipoprotein and lipopolysaccharide. *Infect. Immun.* 73:8433–8436.
 21. Fang FC, et al. 1992. The alternative sigma factor katF (rpoS) regulates *Salmonella* virulence. *Proc. Natl. Acad. Sci. U. S. A.* 89:11978–11982.
 22. Freudenberg MA, et al. 2008. Lipopolysaccharide sensing an important factor in the innate immune response to Gram-negative bacterial infections: benefits and hazards of LPS hypersensitivity. *Immunobiology* 213:193–203.
 23. Giannella RA, Formal SB, Dammin GJ, Collins H. 1973. Pathogenesis of salmonellosis: studies of fluid secretion, mucosal invasion, and morphologic reaction in the rabbit ileum. *J. Clin. Invest.* 52:441–453.
 24. Haraga A, Ohlson MB, Miller SI. 2008. Salmonellae interplay with host cells. *Nat. Rev. Microbiol.* 6:53–66.
 25. Heumann D, Roger T. 2002. Initial responses to endotoxins and Gram-negative bacteria. *Clin. Chim Acta* 323:59–72.
 26. Hoiseth SK, Stocker BA. 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* 291:238–239.
 27. Ilg K, et al. 2009. O-antigen-negative *Salmonella enterica* serovar Typhimurium is attenuated in intestinal colonization but elicits colitis in streptomycin-treated mice. *Infect. Immun.* 77:2568–2575.
 28. Jones BD, Ghori N, Falkow S. 1994. *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *J. Exp. Med.* 180:15–23.
 29. Kopp EB, Medzhitov R. 1999. The Toll-receptor family and control of innate immunity. *Curr. Opin. Immunol.* 11:13–18.
 30. Kuhn HM, Meier-Dieter U, Mayer H. 1988. ECA, the enterobacterial common antigen. *FEMS Microbiol. Rev.* 4:195–222.
 31. Lawlor MS, Hsu J, Rick PD, Miller VL. 2005. Identification of *Klebsiella pneumoniae* virulence determinants using an intranasal infection model. *Mol. Microbiol.* 58:1054–1073.
 32. Lew HC, Nikaido H, Makela PH. 1978. Biosynthesis of uridine diphosphate N-acetylmannosaminuronic acid in *rff* mutants of *Salmonella typhimurium*. *J. Bacteriol.* 136:227–233.
 33. Licht TR, et al. 1996. Role of lipopolysaccharide in colonization of the mouse intestine by *Salmonella typhimurium* studied by in situ hybridization. *Infect. Immun.* 64:3811–3817.
 34. Lindgren SW, Stojiljkovic I, Heffron F. 1996. Macrophage killing is an essential virulence mechanism of *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. U. S. A.* 93:4197–4201.
 35. Loppnow H, Brade H, Rietschel ET, Flad HD. 1994. Induction of cytokines in mononuclear and vascular cells by endotoxin and other bacterial products. *Methods Enzymol.* 236:3–10.
 36. Matsui H, et al. 2003. Oral immunization with ATP-dependent protease-deficient mutants protects mice against subsequent oral challenge with virulent *Salmonella enterica* serovar typhimurium. *Infect. Immun.* 71: 30–39.
 37. Mayer H, Schmidt G. 1979. Chemistry and biology of the enterobacterial common antigen (ECA). *Curr. Top. Microbiol. Immunol.* 85:99–153.
 38. Medzhitov R, Janeway C Jr. 2000. Innate immune recognition: mechanisms and pathways. *Immunol. Rev.* 173:89–97.
 39. Meier U, Mayer H. 1985. Genetic location of genes encoding enterobacterial common antigen. *J. Bacteriol.* 163:756–762.
 40. Miller SI, Kukral AM, Mekalanos JJ. 1989. A two-component regulatory system (phoP phoQ) controls *Salmonella typhimurium* virulence. *Proc. Natl. Acad. Sci. U. S. A.* 86:5054–5058.
 41. Monack DM, Bouley DM, Falkow S. 2004. *Salmonella typhimurium* persists within macrophages in the mesenteric lymph nodes of chronically infected Nramp1 +/+ mice and can be reactivated by IFN γ neutralization. *J. Exp. Med.* 199:231–241.
 42. Nelson DL, Kennedy EP. 1972. Transport of magnesium by a repressible and a nonrepressible system in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* 69:1091–1093.
 43. Nevala JJ, Stocker BA, Laux DC, Cohen PS. 1985. Colonization of the mouse intestine by an avirulent *Salmonella typhimurium* strain and its lipopolysaccharide-defective mutants. *Infect. Immun.* 50:152–159.
 44. O'Brien AD, Taylor BA, Rosenstreich DL. 1984. Genetic control of natural resistance to *Salmonella typhimurium* in mice during the late phase of infection. *J. Immunol.* 133:3313–3318.
 45. O'Callaghan D, Maskell D, Liew FY, Easmon CS, Dougan G. 1988. Characterization of aromatic- and purine-dependent *Salmonella typhimurium*: attention, persistence, and ability to induce protective immunity in BALB/c mice. *Infect. Immun.* 56:419–423.
 46. Ohl ME, Miller SI. 2001. *Salmonella*: a model for bacterial pathogenesis. *Annu. Rev. Med.* 52:259–274.
 47. Pegues DA, Hohmann EL, Miller SI. 1995. *Salmonella*, including *S. typhi*, p. 785–809. In Blaser MJ (ed.), *Infections of the gastrointestinal tract*. Raven Press, New York, NY.
 48. Rabsch W, Tschape H, Baumler AJ. 2001. Non-typhoidal salmonellosis: emerging problems. *Microbes Infect.* 3:237–247.
 49. Ramos-Morales F, Prieto AI, Beuzon CR, Holden DW, Casadesus J. 2003. Role for *Salmonella enterica* enterobacterial common antigen in bile resistance and virulence. *J. Bacteriol.* 185:5328–5332.
 50. Rick PD, Mayer H, Neumeyer BA, Wolski S, Bitter-Suermann D. 1985. Biosynthesis of enterobacterial common antigen. *J. Bacteriol.* 162: 495–503.
 51. Rick PD, et al. 1998. Characterization of the lipid-carrier involved in the synthesis of enterobacterial common antigen (ECA) and identification of a novel phosphoglyceride in a mutant of *Salmonella typhimurium* defective in ECA synthesis. *Glycobiology* 8:557–567.
 52. Rick PD, et al. 1995. Enterobacterial common antigen and capsular polysaccharides, p. 104–112. In Neidhardt FC, et al. (ed.), *Escherichia coli and Salmonella: cellular and molecular biology*. ASM Press, Washington, DC.

53. Rietschel ET, et al. 1994. Bacterial endotoxin: molecular relationships of structure to activity and function. *FASEB J.* 8:217–225.
54. Rodriguez-Morales O, et al. 2006. *Salmonella enterica* serovar Typhimurium ompS1 and ompS2 mutants are attenuated for virulence in mice. *Infect. Immun.* 74:1398–1402.
55. Rose RE. 1988. The nucleotide sequence of pACYC184. *Nucleic Acids Res.* 16:355.
56. Schletter J, Heine H, Ulmer AJ, Rietschel ET. 1995. Molecular mechanisms of endotoxin activity. *Arch. Microbiol.* 164:383–389.
57. Sydenham M, et al. 2000. *Salmonella enterica* serovar typhimurium surA mutants are attenuated and effective live oral vaccines. *Infect. Immun.* 68:1109–1115.
58. Tauxe RV. 1996. An update on *Salmonella*. *Health Environ. Digest* 10:1–4.
59. Toguchi A, Siano M, Burkart M, Harshey RM. 2000. Genetics of swarming motility in *Salmonella enterica* serovar typhimurium: critical role for lipopolysaccharide. *J. Bacteriol.* 182:6308–6321.
60. Tsai CM, Frasch CE. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* 119:115–119.
61. Valtonen MV, Larinkari UM, Plosila M, Valtonen VV, Makela PH. 1976. Effect of the enterobacterial common antigen on mouse virulence of *Salmonella typhimurium*. *Infect. Immun.* 13:1601–1605.
62. Wray C, Sojka WJ. 1978. Experimental *Salmonella typhimurium* infection in calves. *Res. Vet. Sci.* 25:139–143.
63. Yamamoto T, et al. 2001. Disruption of the genes for ClpXP protease in *Salmonella enterica* serovar Typhimurium results in persistent infection in mice, and development of persistence requires endogenous gamma interferon and tumor necrosis factor alpha. *Infect. Immun.* 69:3164–3174.
64. Zhang L, Radziejewska-Lebrecht J, Krajewska-Pietrasik D, Toivanen P, Skurnik M. 1997. Molecular and chemical characterization of the lipopolysaccharide O-antigen and its role in the virulence of *Yersinia enterocolitica* serotype O:8. *Mol. Microbiol.* 23:63–76.