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Evaluation of *hha* and *hha sepB* mutant strains of *Escherichia coli* O157:H7 as bacterins for reducing *E. coli* O157:H7 shedding in cattle☆

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ABSTRACT

Escherichia coli O157:H7 colonizes cattle intestines by using the locus of enterocyte effacement (*LEE*)-encoded proteins. The induction of systemic immune response against *LEE*-encoded proteins, therefore, will prove effective in reducing *E. coli* O157:H7 colonization in cattle. The previous studies have demonstrated that a *hha* (encodes for a hemolysin expression modulating protein) deletion enhances expression of *LEE*-encoded proteins and a *sepB* (encodes an ATPase required for the secretion of *LEE*-encoded proteins) deletion results in intracellular accumulation of LEE proteins. In this study, we demonstrate the efficacy of the *hha* and *hha sepB* deletion mutants as bacterins for reducing fecal shedding of *E. coli* O157:H7 in experimentally inoculated weaned calves. The weaned calves were injected intramuscularly with the bacterins containing 10^9 heat-killed cells of the *hha*$^+$ wild-type or *hha* or *hha sepB* isogenic mutants, and boosted with the same doses 2- and 4-weeks later. The evaluation of the immune response two weeks after the last booster immunization revealed that the calves vaccinated with the *hha* mutant bacterin had higher antibody titers against LEE proteins compared to the titers for these antibodies in the calves vaccinated with the *hha sepB* mutant or *hha*$^+$ wild-type bacterins. Following oral inoculations with 10^{10} CFU of the wild-type *E. coli* O157:H7, the greater numbers of calves in the group vaccinated with the *hha* or *hha sepB* mutant bacterins stopped shedding the inoculum strain within a few days after the inoculations compared to the group of calves vaccinated with the *hha*$^+$ wild-type bacterin or PBS sham vaccine. Thus, the use of bacterins prepared from the *hha* and *hha sepB* mutants for reducing colonization of *E. coli* O157:H7 in cattle could represent a potentially important pre-harvest strategy to enhance post-harvest safety of bovine food products, water and produce.

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1. Introduction

Escherichia coli O157:H7 are Shiga toxin-producing zoonotic pathogens capable of causing gastroenteritis of varied severity in humans of all age groups [1]. Adult cattle are asymptomatic carriers of *E. coli* O157:H7 and these animals serve as major sources for this pathogen [2]. The major sites for *E. coli* O157:H7 colonization of bovine intestines include terminal rectum and cecum [3–5]. Cattle colonized with *E. coli* O157:H7 excrete these bacteria in their feces for variable durations and magnitudes, which in turn increases the risk of hide contamination and contamination of carcasses during meat processing operations [6]. The intimate adherence of *E. coli* O157:H7 bacteria to epithelial cells results in the production of characteristic attaching and effacing (A/E) lesions [3,7,8].

A 43-kb pathogenicity island termed the locus of enterocyte effacement (*LEE*) encodes factors essential for the formation of A/E lesions on intestinal epithelial cells [9–11]. *LEE*, which contains five major operons (*LEE*1 to *LEE*5), encodes for a type III secretion system (T3SS), translocated intimin receptor (Tir), intimin, and secreted proteins EspA, EspB, EspD [12,13]. The protein EspA, an integral component of T3SS, forms tubular structures at the bacterial cell surface for delivering Esp proteins and Tir into intestinal epithelial cells [14]. Tir is inserted into the epithelial cell membrane where it interacts with intimin (an auto-transporter) located on the bacterial cell surface to facilitate intimate adherence between bacterial and epithelial cells [15,16]. The proteins secreted by T3SS cause cytoskeletal changes in epithelial cells resulting in the production of A/E lesions [17]. Several genetic factors regulate *LEE* expression by directly or indirectly affecting the expression of Ler, *LEE1*-encoded positive regulator of *LEE* [18]. In recent reports, we demonstrated that a deletion of *hha* resulted in an increased transcription of *LEE*

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[19] and allowed increased adherence of the mutant *E. coli* O157:H7 strain to HEp-2 cells [20]

Numerous studies have demonstrated the requirement for *LEE*-encoded proteins in adherence and colonization by *E. coli* O157:H7 of epithelial cells using *in vitro* adherence assays and animal models. For example, the *sepB* deletion mutants of *E. coli* O157:H7 are unable to secrete the *LEE*-encoded adherence factors and are thus compromised in their ability to adhere to epithelial cells and to colonize animal intestines [11,12,21]. SepB, which is encoded by the *LEE3* operon, functions as an ATPase to generate energy for the T3SS to secrete LEE proteins [12]. In another study, calves inoculated with a library of signature-tagged mutants of *E. coli* O157:H7 showed that 13 of 59 mutants impaired in their ability to colonize bovine gastrointestinal tracts had these mutations clustered in the genes encoded by *LEE* [21]. Similarly, the inoculation of calves, rabbits, or pigs with *E. coli* O157:H7 mutants disrupted or deleted in genes *eae*, *espA*, *espB*, or *tir* or various combinations of these genes abolished or reduced the ability of these mutants to colonize intestines of these animals [7,17,22–28]. The importance of *LEE*-encoded factors in intestinal colonization was also demonstrated in animals that could no longer be colonized or were colonized at reduced levels by *E. coli* O157:H7 because of the presence of circulating or mucosal antibodies (produced either by vaccination or maternally acquired) to one or more *LEE*-encoded proteins [29–32].

Epidemiological surveys conducted over the past several years indicate that *E. coli* O157:H7 is ubiquitous at the herd level in both dairy and beef cattle operations but prevalence of these bacteria in individual animals or animals in pens varies greatly [33–36]. Thus, application of strategies for reducing pre-harvest prevalence of *E. coli* O157:H7 at the farm is important for enhancing safety of bovine food products and mitigating economic impact of *E. coli* O157:H7 caused disease outbreaks on the public health system and the food industry [37].

Since *LEE*-encoded proteins are critical in gastrointestinal colonization of cattle by *E. coli* O157:H7, several studies have reported evaluations of vaccines containing one or more *LEE*-encoded proteins to reduce intestinal colonization in cattle experimentally or naturally infected with *E. coli* O157:H7 [31,38–41]. However, only one such *LEE*-based vaccine, called EconicheTM, has been granted a full license for use in cattle feedlots in Canada (Bioniche Life Sciences, Inc., Belleville, Ontario, Canada). The EconicheTM vaccine is a protein cocktail prepared by concentrating T3SS-secreted proteins from culture supernatants of *E. coli* O157:H7. Three dose regimens (50 μg of secreted proteins per dose) of this vaccine had been tested for its effectiveness in reducing *E. coli* O157:H7 shedding in cattle housed under experimental conditions and in feedlots. Cattle housed under experimental conditions showed enhanced immune response to several constituent secreted proteins of the cocktail that correlated with a significant reduction in the duration and magnitude of fecal shedding of *E. coli* O157:H7 [40]. Similarly, a significant reduction in the prevalence of *E. coli* O157:H7 was observed in vaccinated versus unvaccinated feedlot cattle [41]. A non-*LEE* based vaccine containing siderophore receptors and porins of *E. coli* outer membranes had also been shown to be effective in reducing shedding of *E. coli* O157:H7 in feedlot cattle [42].

In this study, we constructed *hha* and *hha sepB* deletion mutants of a *hha*$^+$ wild-type strain of *E. coli* O157:H7 and determined the effects of these mutations on the secretion of *LEE*-encoded proteins Tir, EspA, EspB and intimin. We used these mutants as heat-killed preparations, referred to as bacterins hereafter, for intramuscular immunization of cattle and determined the impact of these vaccinations on fecal shedding of *E. coli* O157:H7 relative to that in cattle vaccinated either with a *hha*$^+$ wild-type bacterin or a PBS sham vaccine. In addition, we determined relative titers of circulating IgG antibodies in vaccinated cattle to assess the relative importance of various *LEE*-encoded proteins in inducing immune responses effective in reducing fecal shedding of *E. coli* O157:H7.

2. Methods

2.1. Bacterial strains, plasmids, and growth conditions

Table 1 lists the bacterial strains and plasmids used in this study. All *E. coli* strains were propagated at 37 °C in Luria–Bertani broth (LB) or LB containing 1.5% agar. For liquid cultures, colonies from LB agar plates were inoculated into LB broth and incubated at 37 °C, unless stated otherwise, on a shaker at 200 rpm. Dulbecco's Modified Eagle Medium (DMEM) or M9+ Medium (minimal salts plus 1% casamino acids) were used for the optimal production of *LEE* proteins in the liquid cultures [43]. Media were supplemented, when required, with selective antibiotics at the following concentrations: ampicillin 50 μg/ml; kanamycin 50 μg/ml; and streptomycin100 μg/ml.

2.2. Primer design and PCR amplification

The primers used in this study are listed in Table 1. These primer sequences were selected from the published sequence of *E. coli* O157:H7 EDL933 genome [44], and were synthesized by Integrated DNA Technologies (Coralville, IA). The PCR mixture contained 5 μl of DNA (0.1 μg), 0.3 μM each of forward and reverse primers, 15 mM Tris–HCl (pH 8.0), 50 mM KCl, 3.0 mM $MgCl_2$, 200 μM of each of the four dNTPs (Roche Diagnostic Corporation, Indianapolis, IN), and 1.25 units of AmpliTaq Gold polymerase (PE Biosystems, Foster City, CA). The PCR amplification was performed by heating the PCR mixture to 95 °C for 10 min followed by 40 cycles of heating (94 °C for 30 s), annealing (55 °C for 30 s), extension (72 °C for 60 s per 1000 bp amplification), and termination (72 °C for 5 min). The PCR amplified products were resolved by the standard agarose gel electrophoresis. The DNA fragments of the predicted sizes were extracted from the agarose gel by a QIAquick Gel Extraction Kit according to the manufacturer's instructions (Qiagen, Valencia, CA), and concentrations of purified DNA fragments were determined using a spectrophotometer (NanoDrop Technologies, Wilmington, DE).

2.3. Construction of hha and hha sepB deletion mutants

The *hha* deletion mutant of *E. coli* O157:H7 strain 86-24 Δstx2 (Table 1) was constructed by using plasmid pSM122 and the allelic replacement procedure described in a previous study [19]. We used the same allelic replacement procedure to delete *sepB* in *E. coli* O157:H7 strain 86-24 Δstx2 Δ*hha* (Table 1). To delete *sepB*, we PCR amplified a DNA fragment containing 1500-bp upstream (US-fragment) of the start codon and another fragment containing 1500-bp downstream (DS-fragment) of the stop codon of *sepB* by using the primers VS545/VS546 and VS547/VS548, respectively. The US- and DS-fragments were cloned into pCR-XL/TOP10 cloning system to generate plasmids pSM281 and pSM282, respectively, according to manufacturer's instructions (Invitrogen Corporation, Carlsbad, CA). The US-fragment of pSM281 was recovered by using the restriction endonucleases *Bam*HI (restriction site present in pCR-XL multiple cloning site immediate upstream of 5′ end of US-fragment) and *Sal*I (restriction site created at the 3′ end of US-fragment by primer VS546). The *Bam*HI and *Sal*I US-fragment was then cloned at the corresponding *Bam*HI (vector) and *Sal*I restriction (created by the primer VS548 at the 5′ end of DS-fragment of pSM282) sites to generate the plasmid pSM284 (Table 1). The 3-kb fragment containing both the US- and DS-fragments in tandem (5′ US-DS 3′) was then isolated from pSM284 using *Xba*I, restriction site created by the primers VS547 and VS545 at the 5′ and 3′

Table 1
Bacterial strains, plasmids[a], and primers.

Strain or plasmid	Genotype and description	Source or reference
***E. coli* strains**		
E. coli O157:H7 strain 86-24	*stx2*$^+$	[56]
Strain 86-24 Δ*stx2*	86-24 deleted of *stx2*	[19]
Strain 86-24 Δ*stx2* Δ*hha*	86-24 deleted of *stx2* and *hha*	This study
Strain 86-24 Δstx2 Δ*hha* Δ*sepB*	86-24 deleted *stx2*, *hha*, and *sepB*	This study
TOP 10	endA1 recA1 hsdR17 (r_K^- m_K^-) supE44	Invitrogen
Plasmids		
pCR-XL	Cloning vector	Invitrogen
pAM450	Temperature-sensitive allelic replacement vector	[25]
pSM122	pAM450 derivative used for deleting *hha*	[19]
pSM281	1.5-kb sequence located upstream of *sepB* (escN) cloned in pCR-XL	This study
pSM282	1.5-kb sequence located downstream of *sepB* (escN) cloned in pCR-XL	This study
pSM284	1.5-kb sequence of pSM282 cloned downstream of 1.5-kb sequence of pSM281	This study
pSM302	3-kb sequence of pSM284 containing upstream and downstream sequences of *sepB* (escN) cloned in pAM450	This study
Primers used for PCR amplification		
Primer	Nucleotide sequence (5′–3′)[b]	Location (bp)[c]
VS501	ACGTTTGGCTTATTGGCTCTG	4676296–4676276
VS545	GCGTCTAGAGCGCGCAGGCGTTATTGACCC	4677526–4677546
VS546	GCGGTCGACTTACCGTTCCTAATACTTTAAGTTCG	4676018–4676043
VS547	GCGTCTAGACGCAACATGTGTATATCAATATGGAC	4673159–4673184
VS548	GCGGTCGACGTATGTTGGACAGAATTTATCTATTC	4674675–4674650
VS561	CACCAGCGATGCACAATATGCCTG	4674507–4674530

[a] The detailed descriptions of bacterial strains and plasmids listed in this table is provided under Section 2.
[b] The nucleotide sequences of primers used in this study were selected from the published complete genome sequence of *E. coli* O157:H7 strain EDL933 with the accession number AE005174.2.
[c] Location refers to the position of primer sequence in the genome of EDL933.

ends, respectively, and cloned into the *Xba*I site of a temperature-sensitive plasmid pAM450 (Table 1) to generate a recombinant plasmid pSM302. The plasmid pSM302 was electroporated into *E. coli* O157:H7 strain 86-24 Δ*stx2* Δ*hha*, and the new strain 86-24 Δ*stx2* Δ*hha*/pSM302 was cultured under growth conditions, as described previously [19], that facilitated deletion of the chromosomal copy of *sepB*. The deletion of *sepB* was confirmed by PCR using the primers VS501 and VS561 (Table 1).

2.4. Precipitation and quantification of secreted proteins and preparation of whole-cell lysates

The bacterial strains were grown in M9+ or DMEM media at 37 °C overnight and diluted 1:500 into fresh M9 or DMEM media and incubated at 37 °C for 6 h. The cultures were adjusted to $A_{600\,nm}$ of 1.0 and centrifuged at 10,000 × *g* for 10 min. The cell pellets were resuspended in 1 ml of TBS (0.1 M Tris, 0.154 M NaCl, pH 7.4) and stored at −20 °C until used for making the whole-cell lysates. The culture supernatants were mixed with tricholoroacetic acid (TCA) to a final concentration of 10%, incubated overnight at 4 °C, and centrifuged for 30 min (10,000 × *g*). After discarding supernatants, the TCA pellets were resuspended in 0.1 M TBS and then stored at −20 °C. The protein content of resuspended TCA precipitates was estimated from $A_{280\,nm}$ measurements of these samples.

2.5. SDS–PAGE and Western blotting

Two-volumes of TBS-suspended cell pellets or TCA precipitated proteins, prepared from the cultures adjusted to $A_{600\,nm}$ of 1.0 as described above, were mixed with one-volume of SDS sample buffer (Invitrogen Corporation, Carlsbad, CA) and incubated at 95 °C for 5 min. The boiled samples were loaded into the wells of a 10–20% Tris–Tricine (Invitrogen Corporation, Carlsbad, CA) or 4–20% Tris-buffered (Biorad Laboratories, Hercules, CA) SDS–polyacrylamide gels. The gels were subjected to electrophoresis using a SDS–PAGE running buffer (Invitrogen Corporation, Carlsbad, CA) at a constant voltage to resolve proteins present in the whole-cell lysates or TCA-precipitates of culture supernatants. The gels were either stained with Coomassie brilliant blue to visualize the presence of total proteins or used for transferring the proteins to nitrocellulose membranes (Schleicher and Schuell, Inc., Keene, NH) using a standard western blotting procedure.

2.6. Detection of T3SS secreted LEE proteins

The nitrocellulose membranes, following the western blotting of proteins, were secured and processed in a multichannel miniblotter according to the manufacturer's instructions (Immunetics, Boston, MA). The unbound sites on the membranes were blocked by adding 50 μl of TBS-T (TBS containing 0.05% Tween 20) [29] to each well for 5 min. The blocking step was repeated for a total of 3 × 5 min. For detecting the presence of *LEE*-encoded proteins in whole-cell lysates or TCA-precipitated culture supernatants of the *hha*$^+$ wild-type and *hha* and *hha sepB* mutant strains, 50 μl of rabbit antibodies (diluted 1:5000 in TBS-T) prepared against purified EspA, EspB (kindly provided by Dr. Jorge Giron, University of Florida, Gainesville, FL), Tir and intimin (kindly provided by Dr. Alison O'Brien, Uniformed Services University of the Health Sciences, Bethesda, MD) were added to individual tracks for detecting EspA, EspB, Tir, and intimin. After 60 min of incubation at the room temperature, the unbound antibodies were washed off by adding 50 μl of TBS-T to each track and letting it incubate for 5 min. The washing was repeated for two additional times. Fifty μl of biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) diluted in 0.4% Block Ace in TBS-T (Serotec, Oxford, UK) was added to each well. Following incubation at the room temperature for 30 min, the membranes were washed with TBS-T (3 × 5 min), and removed from the blotter. The membranes were developed using Vector ABC staining kit according to the manufacturer's instructions (Vector Laboratories, Inc., Burlingame, CA).

2.7. Detection of antibodies against LEE-encoded proteins in sera of vaccinated animals

The western-blotted nitrocellulose membranes containing transferred TCA-precipitated proteins (described above) were used

for determining anti-intimin, anti-Tir, anti-EspA, and anti-EspB titers in the sera prepared from the blood collected at pre- and four weeks post-vaccination of calves with bacterins. These blots were processed as described above with the exception that the diluted pre- and post-immune sera (100 μl of 1:100, 1:500, and 1:1000 dilution in TBS-T) were used as a source of primary antibodies and the biotinylated goat anti-bovine IgG was used as a secondary antibody. After washing (3 × 5 min), the membranes were developed using Vector ABC staining kit (Vector Laboratories, Inc., Burlingame, CA), and the intensities of the colored bands produced on the membranes were determined on ChemiDoc Imager (BioRad) using a QuanityOne software.

2.8. Preparation of bacterins

The *hha*$^+$ wild-type and *hha* and *hha sepB* mutant strains of *E. coli* O157:H7) were propagated overnight in LB broth containing 100 μg per ml of streptomycin in a shaker-incubator (37 °C, 200 rpm). The overnight culture of each strain was diluted 1:100 in DMEM and incubated at 37 °C for 6 h (200 rpm). The cultures were centrifuged at 5000 × *g* for 10 min, supernatants removed and the cell pellets resuspended in phosphate-buffered saline (PBS) to one-tenth the volume of the original culture. An aliquot of the PBS-bacterial cell suspension was diluted (10-fold serial dilutions), plated on LB agar containing streptomycin, and incubated overnight at 37 °C. The number of colonies that grew on these plates provided total viable cell counts of these bacterial suspensions. The remainder of the PBS suspension was incubated in a heated water-bath (80 °C) for 30 min to kill bacterial cells. One hundred μl aliquots of heated cell suspensions were plated on LB agar containing streptomycin and incubated overnight at 37 °C to confirm that the heated cells were not viable. Based on the viable cell counts, the remainder of the heat-killed suspension was adjusted to about 10^9 cells per ml before storing at −4 °C.

2.9. Vaccination of calves and detection of antibodies directed against LEE-encoded proteins in vaccinated calves

The weaned male calves (8–10 weeks in age) were purchased from local farms in Iowa and were housed in accordance with the guidelines of the American Association for Laboratory Animal Care. The calves were housed in pairs in climate-controlled BL-2 barns and were allowed to acclimate for 2-weeks to the animal housing environment prior to experimentation. The calves were fed twice daily with both the pelleted feed and alfalfa hay cubes in amounts (16% crude protein, 2.5% crude fat, 8% fiber) equal to 1% of their body weights, and water was offered ad libitum. All animal protocols were approved by the National Animal Disease Center Animal Care and Use Committee. In each of the three vaccine trials conducted, eight calves were divided into four groups of two calves each. The blood samples were taken from the jugular vein as preinoculum samples. While the calves in the group 1 were injected with 1-ml of phosphate-buffered saline (PBS), the calves in groups 2, 3, and 4 were injected with the heat-killed vaccines (containing 10^9 cells/ml), called bacterins, prepared from the *hha*$^+$ wild-type, or *hha*, or *hha sepB* mutant strains, respectively. The use of bacterins at 10^9 cells per ml in the current study was based on the previously published reports describing the effectiveness of an *E. coli* J-5 bacterin for its protective effects in calves from mastitis and endotoxin-caused diseases [45,46]. All injections were administered intramuscularly in the neck using an 18-Gauge needle attached to a syringe. All animals were revaccinated at week 2 and 4 post initial vaccination. The blood samples were collected two weeks post-second vaccination and the sera prepared from these blood samples were used for measuring the IgG titers against intimin, Tir, EspA, and EspB by immunoblotting as described above.

2.10. Determination of fecal shedding of E. coli O157:H7 in vaccinated calves

Two weeks after the third booster dose of the bacterins, all calves were administered gelatin capsules containing 1-ml (10^{10} CFU) of a culture of a wild-type *E. coli* O157:H7 (strain 86-24) and a few feed pellets. A balling gun was used for delivering the capsules into the calf pharynx. The feces were collected everyday for two weeks by the rectal palpation of calves. Ten-fold serial dilutions of feces were prepared in PBS and 100 μl aliquots plated on duplicate sets of Sorbitol MacConkey agar containing 100 μg/ml of streptomycin. After incubating plates at 37 °C for 24 h, white, sorbitol-negative colonies were counted for determining viable colony forming units (CFU) of *E. coli* O157:H7 in feces. Several colonies were randomly picked and tested for agglutination with an *E. coli* O157:H7-specific antiserum (Oxoid, Unipath Ltd., Ogdensburg, NY). The sensitivity of detection was 10^2 bacterial CFU/g of feces.

2.11. Statistics

The significance of the differences in the outcome variables, such as the numbers of animals shedding or the duration of shedding of *E. coli* O157:H7 in feces among animals vaccinated with different bacterins, was determined using an Unpaired Student *t*-test with a two-tailed *P* value estimation or the one-way ANOVA (Kruskal–Wallis test with Dunn's Multiple Comparison). The differences in the outcome variables were considered significant if the *P* values were less than 0.05.

3. Results

3.1. T3SS-mediated secretion of LEE-encoded proteins is enhanced in hha and reduced in sepB deletion mutants

Since previous studies have shown that a *hha* deletion results in an increased *LEE* transcription [19] and a *sepB* deletion abrogates T3SS activity [43], we wanted to determine relative activities of T3SS in the *hha* and *hha sepB* mutants by monitoring the levels of *LEE*-encoded proteins secreted into the bacterial culture supernatants. The total protein content of a TCA-precipitated culture supernatant of the *hha* mutant was about 2-fold higher (3.3 mg/ml) than that of the *hha*$^+$ wild-type (1.6 mg/ml) and 1.5-fold higher than the *hha sepB* mutant (2.1 mg/ml). As shown in the Coomassie blue stained SDS–polyacrylamide gel (Fig. 1A), TCA-precipitates of the *hha* mutant culture supernatant contained several proteins that were readily visible (lane 2) compared to only a few barely detectable but identical protein bands in the TCA-precipitated culture supernatants of the *hha*$^+$ wild-type (lane 1) or the *hha sepB* mutant (lane 3) strains, respectively. Four of these protein bands appeared to approximate the masses predicted for EspA (21 kDa), EspB (34 kDa), Tir (62 kDa), and intimin (102 kDa) of *E. coli* O157:H7. The protein profiles of the whole-cell lysates prepared from the *hha*$^+$ wild-type (lane 1), *hha* mutant (lane 2), and *hha sepB* mutant (lane 3) strains were similar (Fig. 1B). The probing of the nitrocellulose membranes carrying immobilized TCA-precipitated proteins with the antibodies specific for *LEE*-encoded EspA, EspB, Tir, and intimin, showed intense bands (Fig. 2) corresponding to these proteins in the *hha* mutant (middle panel), two of the four bands (EspA and EspB) in the *hha*$^+$ wild-type (left panel), and none of the four proteins in the *hha sepB* mutant strains (right panel).

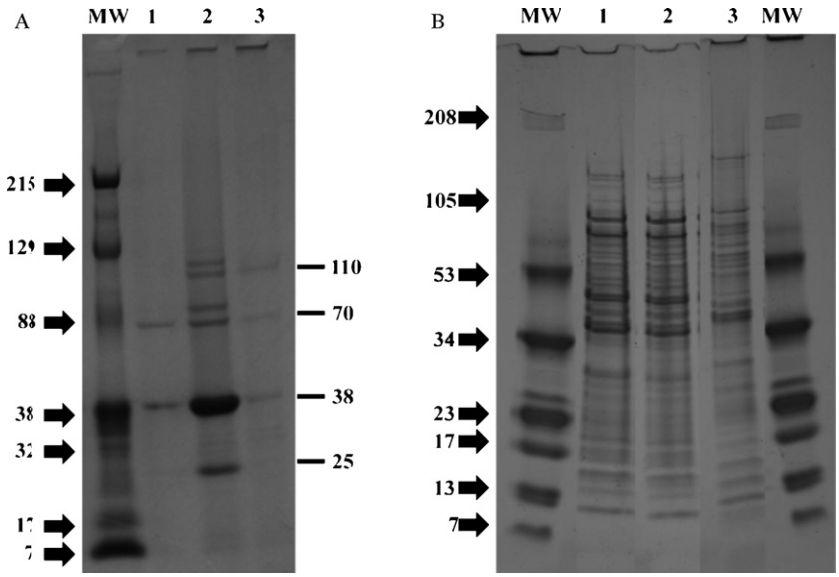

Fig. 1. Determination of the effects of *hha* and *sepB* deletions on T3SS-mediated protein secretion. Trichloroacetic acid precipitated culture supernatants (A) and whole-cell lysates (B) were resolved by SDS–PAGE and gels were stained with Coomassie brilliant blue to visualize protein bands. The numbers and relative abundances of protein bands are shown in lane 1 (*hha*$^+$ wild-type strain), lane 2 (*hha* deletion mutant), and lane 3 (*hha sepB* deletion mutant). Lanes labeled MW, represent protein molecular mass standards in kilodaltons (kDa), indicated on left. The approximate molecular masses of secreted proteins are indicated on the right side of (A).

3.2. sepB deletion enhances intracellular accumulation of LEE-encoded proteins

Since *sepB* deletion has been shown to compromise T3SS-mediated secretion of several *LEE*-encoded proteins [12], we next examined whether a *sepB* deletion would result in an increased intracellular accumulation of *LEE*-encoded proteins. As shown in Fig. 3, the western blot analysis of the whole-cell lysates showed the presence of bands corresponding to intimin, Tir, EspB and EspA in the *hha*$^+$ wild-type, and *hha* and *hha sepB* mutant strains (Fig. 3). However, a visual examination of this gel showed higher amounts of these four proteins in the *hha* and *hha sepB* mutants than in the *hha*$^+$ wild-type strain. Several low-molecular weight proteins, which could either represent truncated or degraded species, were also detected in the lanes corresponding to intimin, Tir, and EspB in blots prepared from the wild-type and the two mutant strains.

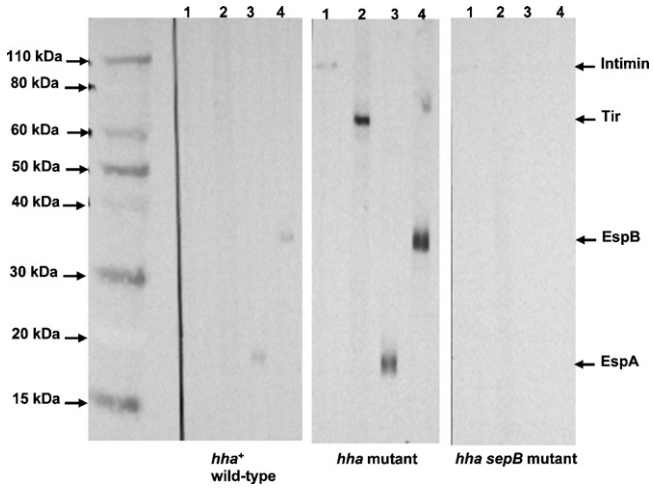

Fig. 2. Identification and determination of relative abundance of *LEE*-encoded proteins by Western blotting. Trichloroacetic acid precipitated culture supernatants prepared from *hha*$^+$ wild-type (left panel), *hha* mutant, (middle panel), and *hha sepB* mutant (right panel) strains were probed with rabbit anti-intimin (lane 1), anti-Tir (lane 2), anti-EspA (lane 3) and anti-EspB (lane 4). Lane labeled MW represents molecular mass standards in kilodaltons (kDa).

3.3. Vaccination enhanced immune response to LEE-encoded proteins

The animal sera prepared from the blood collected pre- or 4-weaks post-vaccination were analyzed by western blotting to determine the IgG titers for specific *LEE*-encoded proteins. As shown in Table 2, the group of calves administered the PBS sham vaccine showed no change in the median titers for IgG against intimin, Tir, EspA, and EspB between the pre- and post-vaccination sera. On the other hand, the post-vaccination sera of the calves vaccinated with the *hha*$^+$ wild-type or the *hha* or the *hha sepB* mutant bacterins had increased median titers for these four IgG antibodies with the exception that the IgG titers against EspA remained the same in the pre- and post-vaccination sera of the calves vaccinated with the *hha*$^+$ wild-type bacterin. The highest titers for the four antibodies were detected in the post-vaccination sera of the calves vaccinated with the *hha* mutant bacterin. The post-vaccination median titers for the anti-intimin IgG in the group of calves vaccinated with the *hha sepB* bacterin were 2-fold or 3-fold lower than the calves vaccinated with the *hha*$^+$ wild-type or the *hha*

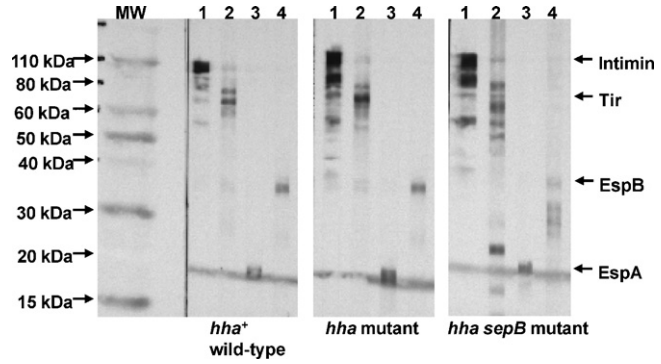

Fig. 3. Identification and determination of relative abundance of *LEE*-encoded proteins in whole-cell lysates by Western blotting. The whole- cell lysates prepared from *hha*$^+$ wild-type (left panel), *hha* mutant (middle panel), and *hha sepB* mutant (right panel) strains were probed with rabbit anti-intimin (lane 1), anti-Tir (lane 2), anti-EspA (lane 3) and anti-EspB (lane 4). Lane labeled MW, represent molecular mass standards in kilodaltons (kDa).

Table 2
Serum titers produced against *LEE*-encoded proteins in calves after vaccination.

Bacterins used	Median serum titers[a]							
	Anti-intimin		Anti-Tir		Anti-EspB		Anti-EspA	
	Prevac.[b]	Postvac.[b]	Prevac.	Postvac.	Prevac.	Postvac.	Prevac.	Postvac.
hha$^+$ wild-type	10	255	55	500	100	500	10	10
hha mutant	100	300	300	1000	100	1000	10	300
hha sepB mutant	10	100	100	500	100	750	55	100
PBS	100	100	100	100	100	100	55	55

[a] The median titers represent the reciprocals of the highest dilutions of serum yielding colored precipitates on blotted membranes.
[b] Pre-vaccination and 4-weeks post-vaccination.

mutant bacterins, respectively. The group of calves vaccinated with the *hha sepB* mutant bacterin also showed 1.5-fold lower anti-EspB IgG median titers than in the calves vaccinated with the *hha*$^+$ wild-type bacterin. The most notable observation was that the anti-EspA IgG titers were 30-fold or 10-fold higher in the calves vaccinated with the *hha* or the *hha sepB* mutant bacterins, respectively, compared to titers for this antibody in the calves vaccinated with the *hha*$^+$ wild-type bacterin.

3.4. Vaccination reduced the duration of shedding and the numbers of animals shedding E. coli O157:H7

The effects of vaccinations on the ability of *E. coli* O157:H7 to colonize and persist in the vaccinated calves were determined by comparing the mean number of calves that were positive for the presence of *E. coli* O157:H7 in their feces over a 2-week sampling period, and the number of days each calf continued to shed *E. coli* O157:H7 in feces. As shown in Fig. 4, significantly ($P < 0.05$) fewer calves were found positive among the calves vaccinated with the *hha* (mean, 2.4 calves) or the *hha sepB* (mean, 2 calves) mutant bacterins compared to the calves vaccinated with the *hha*$^+$ wild-type bacterin (mean, 4.6 calves). The numbers of calves shedding *E. coli* O157:H7 were lower (mean, 3.3 calves) in the group of calves vaccinated with the PBS sham than in the group vaccinated with the *hha*$^+$ wild-type bacterin (mean, 4.6), but this difference was not statistically significant ($P > 0.05$).

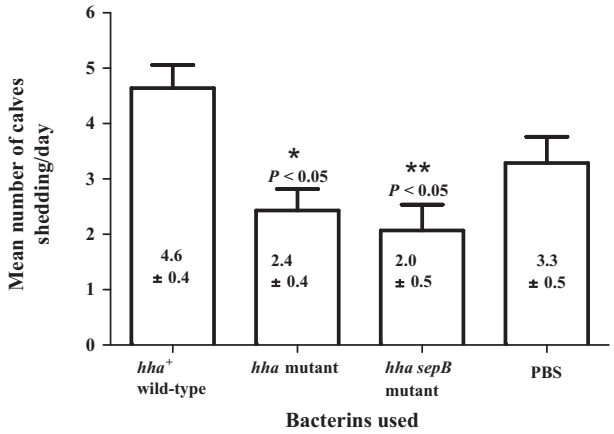

Fig. 4. Determination of numbers of calves shedding *E. coli* O157:H7 post-vaccination. The mean numbers of calves shedding detectable levels of *E. coli* O157:H7 after vaccination with a PBS sham vaccine or bacterins prepared from *hha*$^+$ wild-type or *hha* or *hha sepB* deletion mutants were estimated by averaging the numbers of calves shedding *E. coli* O157:H7 each day of the two weeks of fecal sampling period. The mean values for the numbers of calves shedding *E. coli* O15:H7 plus and minus the standard error of the means (SEM) are indicated in the corresponding bar. The significance of the variance ($P < 0.05$) of the means is shown as a single or double asterisk.

The evaluation of the duration of fecal shedding of *E. coli* O157:H7 revealed that the calves vaccinated with either the *hha* or the *hha sepB* mutant bacterin had 50% (3 of 6 calves in each group) of the calves stopped shedding detectable levels of *E. coli* O157:H7 within 1–2 days after oral inoculations with the wild-type *E. coli* O157:H7 (Fig. 5). The mean durations of fecal shedding for the calves vaccinated with the *hha* or the *hha sepB* mutant bacterins were 5.7 days ± 2.1 and 4.8 days ± 1.7, respectively. This reduction in the duration of fecal shedding in these two groups of vaccinated calves was statistically significant ($P < 0.05$) when compared to the duration of shedding in the calves vaccinated with the *hha*$^+$ wild-type bacterin (6 of 6 calves shedding for 9–14 days, with the mean duration of shedding lasting 10.8 days ± 0.75). Of the remaining three calves in the group vaccinated with the *hha* mutant bacterin, two calves were positive for fecal shedding for 8 and the third calf for 14 days of the 14-day fecal sampling period. Similarly, the remaining three calves in the group vaccinated with the *hha sepB* mutant bacterin continued shedding *E. coli* O157:H7 for 8–9 days. In the PBS sham vaccinated group, four calves remained positive for fecal shedding for 9–11 days (mean duration of shedding at 7.67 days ± 1.5) and the other two calves had detectable levels of *E. coli* O157H7 in their feces for 3 days. Thus, difference in the duration of fecal shedding in the PBS sham group was not statistically significant ($P \geq 0.5$) when compared to the duration of shedding in the group of calves vaccinated with the *hha*$^+$ wild-type bacterin.

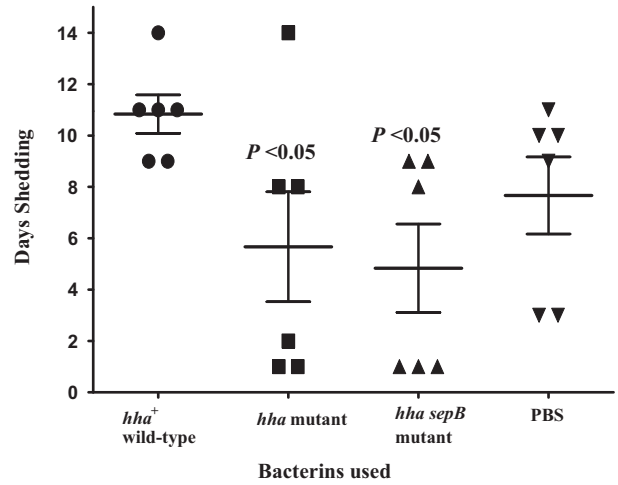

Fig. 5. Determination of the duration of fecal shedding of *E. coli* O157:H7 in vaccinated animals. The mean duration of fecal shedding in calves administered a PBS sham vaccine or bacterins prepared from *hha*$^+$ wild-type or *hha* or *hha sepB* deletion mutants was estimated by averaging the total number of days in a 2-week fecal sampling period (following oral inoculation with a live *E. coli* O157:H7) that the calves were determined positive for the presence of detectable levels (10^2 cfu/g feces) of the inoculum strain. The mean duration of shedding for each group of calves is shown as a horizontal bar intersected by a vertical bar representing the standard error of the means (SEM). The significance of the variance ($P < 0.05$) of the means is shown as a single or double asterisk above the SEM line.

4. Discussion

In this study, we described for the first time the potential application of bacterins for reducing the fecal shedding of *E. coli* O157:H7 in cattle. The premise for the development of the bacterins was to expose the immune system of cattle to multiple *LEE*-encoded proteins that are critical to the ability of *E. coli* O157:H7 to colonize cattle intestines, which in turn promote the long-term fecal shedding of these bacteria. The importance of *LEE*-encoded proteins as potential vaccine candidates has been evident in earlier studies that have used component vaccines containing either one or two *LEE*-encoded proteins or a combination of a *LEE*- and a non-*LEE* encoded proteins to immunize non-bovine animal species. For example, it has been demonstrated that the transfer of maternal anti-intimin antibodies to suckling piglets of sows immunized with a purified intimin, protected these piglets from *E. coli* O157:H7-caused disease compared to piglets that suckled on non-vaccinated sows [29]. Similarly, an oral feeding of a plant cell-based intimin vaccine to mice reduced the duration of shedding of *E. coli* O157:H7 in the feces of vaccinated animals [30]. Since these earlier studies, the component vaccines containing one or two *LEE*-encoded proteins or a combination of one *LEE*- and one non-*LEE*-encoded protein have been tested in experimental vaccinations of cattle for successfully inducing humoral immune responses to vaccine-specific proteins, but none of these studies showed a reduction in the fecal shedding of *E. coli* O157:H7 in vaccinated animals [47,48]. On the other hand, the vaccine preparations containing multiple *LEE*-encoded proteins have been found effective in inducing humoral immune responses to the *LEE*-encoded antigens contained in these vaccines, and in reducing the shedding of *E. coli* O157:H7 in the immunized cattle [31]. However, the preparation of these vaccines requires either the purification of individual *LEE*-encoded proteins using expression vector systems or a recovery of these proteins by an acid precipitation of culture supernatants of *E. coli* O157:H7. To circumvent the problem of purifying or precipitating proteins for use in the vaccine preparations, we used the *hha* deletion mutant as the basis for developing the bacterins because this mutant has been shown to express *LEE* at very high levels [19]. As demonstrated in the current study, the levels of several *LEE*-encoded proteins, especially EspA, EspB, intimin, and Tir, were higher in the cell-free supernatants and the whole-cell lysates of the *hha* deletion mutant indicating that these proteins represented major immunogens in the bacterins prepared from this mutant strain. This hypothesis is supported by the results showing that a parenteral administration of the *hha*-mutant based bacterins to cattle resulted in the highest increases of humoral immune responses to *LEE*-encoded proteins EspA, EspB, and Tir compared to the immune responses generated against these proteins in cattle administered the *hha*$^+$ wild-type bacterin or the PBS sham vaccine. Whether these humoral antibodies are transported to the intestinal mucosa to inhibit *E. coli* O157:H7 colonization or if the parenteral vaccination is also capable of inducing a mucosal immune response to the antigens present in the vaccine preparations, however, remains to be determined.

The increased immune response to *LEE*-encoded proteins in the calves vaccinated with the *hha* or *hha sepB* mutant bacterins reduced the numbers of animals shedding *E. coli* O157: H7 and the duration of fecal shedding of these bacteria in feces compared to the calves vaccinated with the *hha*$^+$ wild-type bacterin or with the PBS sham vaccine. One of the most striking findings of the vaccination was that an increase in the anti-EspA IgG titers was seen only in the calves vaccinated with the *hha* or *hha sepB* mutant bacterins, indicating that an increased immune response to EspA is critical for reducing the fecal shedding of *E. coli* O157:H7. Interestingly, the calves vaccinated with the *hha sepB* mutant bacterin showed 2 to 3-fold lower IgG titers against intimin, Tir, EspA, and EspB IgG compared to the calves vaccinated with the *hha* mutant bacterin. Since intimin, Tir, EspA, and EspB are considered important in inducing a humoral immune response in vaccinated animals [31,40,47–50], a compromised translocation of these proteins to the bacterial outer membranes of the *hha sepB* mutant may account for reduced IgG titers observed against these proteins in the sera of animals vaccinated with the *hha sepB* bacterin. The reduced translocation of these proteins is presumably responsible for a lowered magnitude of the immune response observed for these proteins in the calves vaccinated with the *hha sepB* bacterin. However, the *hha sepB* bacterin appears to be as effective as the *hha* mutant bacterin in terms of its ability to reduce the overall duration of fecal shedding and to reduce the numbers of animals shedding *E. coli* O157:H7. Although the genetic and molecular nature of the factors contributing to the effectiveness of the *hha sepB* bacterin are hitherto unknown, it is possible that the *hha sepB* double deletion causes quantitative and/or qualitative changes in some unknown bacterial cell surface proteins that could potentially induce an immune response comparable in effectiveness to the *hha* mutant bacterin. For example, a recent study had demonstrated that a parenteral administration of a bacterial cell surface siderophore receptor protein-based (SRP) vaccine induced humoral immune response in cattle to SRP, and induction of anti-SRP antibodies was effective in reducing the fecal shedding of *E. coli* O157:H7 in vaccinated versus un-vaccinated cattle [42,51,52].

Although an enhanced expression of the *LEE*-encoded proteins appears to be directly responsible for the induction of a systemic immune response to the LEE proteins in calves vaccinated with the bacterins prepared from the *hha* mutant, deleting *hha* and *stx2* in *E. coli* O157:H7 strain used for preparing these bacterins might also indirectly potentiate immunogenicity of these bacterins. For example, a previous study demonstrated that a *hha* deletion reduces the expression of *fliC*, the gene encoding flagellin, a major structural subunit of the flagella in *E. coli* O157:H7 [53]. Since the presence of flagellin in vaccine preparations has been suggested to impair the efficacy of these vaccines against *E. coli* O157:H7 due to the neutralization of TLR5 activation and an impairment of host innate immune response [54], the reduced expression of flagellin caused by the *hha* deletion would presumably facilitate an increased immune response in the animals vaccinated with the bacterins derived from the *hha* deletion mutants of *E. coli* O157:H7. These observations might explain why the greater numbers of animals in the group of calves vaccinated with the *hha*$^+$ wild-type bacterin were positive for fecal shedding compared to the PBS sham vaccinated group of calves. Similarly, deleting *stx2* in the bacterin strains that we have used in this study is advantageous as it has been demonstrated that the presence of *stx2* reduces lymphoproliferative immune response in cattle inoculated with the *stx2*-positive *E. coli* O157:H7 compared to cattle inoculated with the *stx2* deletion mutants [55].

Other potential advantages of the bacterins that we have described in this study are the ease and the low-cost of preparation compared to the vaccines that require purification and concentration of *LEE*-encoded or other proteins. In addition, novel bacterins could easily be constructed from specific mutants of *E. coli* O157:H7 for delivering large quantities of not only the *LEE*-encoded proteins but also other bacterial antigens to induce systemic and/or localized immune responses to multiple colonization factors for reducing *E. coli* O157 colonization of cattle intestines.

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