**Project Title:** Evaluation of a Modified *E. coli* O157:H7 Strain for Use as a Vaccine for Reducing Prevalence and Shedding of *Escherichia coli* O157:H7 in cattle

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## Background

Cattle are considered to be an important reservoir for *E. coli* O157:H7 (7). *E. coli* O157:H7 resides in the intestine of cattle, which intermittently shed this pathogen in their feces (4). Fecal contamination of meats during processing of cattle causes significant economic losses to the cattle industry and accounts for numerous outbreaks of severe diarrheal illness in humans (9). Moreover, release of *E. coli* O157:H7-contaminated manure into the environment carries a potential risk for contaminating ground water, fruits and vegetables. Several disease outbreaks have resulted from consumption of vegetables (5), fruits/fruit juices (3), and drinking water (1) which presumably become contaminated with *E. coli* O157:H7- containing manure. Reducing valence and shedding of *E. coli* O157:H7 in cattle, therefore, may prove an effective pre-harvest strategy for reducing post-harvest contamination of meats and for protecting water and agricultural products from *E. coli* O157:H7 contamination.

The objectives of the study were to use a modified strain of *E. coli* O157:H7 as a vaccine against *E. coli* O157:H7.

## Methodology

Bacterial strains, plasmids, and growth conditions.
Strains and plasmids used in this study are listed in Table 1. All *E. coli* strains were propagated on Luria-Bertani (LB) agar at 37°C. 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 minimal Eagles medium (DMEM) was purchased from Invitrogen, Carlsbad, CA. Media were supplemented, when required, with selective antibiotics at the following concentrations: ampicillin, 50 µg/ml; kanamycin, 50 µg/ml.

Primer design, PCR amplification, and DNA sequencing.
Primers for PCR amplification of nucleotide sequences of *E. coli* O157:H7 strain 86-24 are listed in Table 2. These primer sequences were selected from the published sequence of *E. coli* O157:H7 EDL933 (10). Primers were synthesized by Integrated DNA Technologies (Coralville, IA). PCR amplifications were performed in 50 µl containing 5 µl of DNA (0.2 µg) and 0.3 µM each of forward and reverse primers. AmpliTaq Gold (PE Biosystems, Foster City, CA.) or Failsafe PCR Kits (Epicenter Technologies, Madison, WI.) were used to amplify DNA fragments < 2.0-kb or > 2.0-kb, respectively, according to the instructions provided by the manufacturer. PCR amplified products were purified by using either the Qiagen PCR Purification Kit or by agarose gel electrophoresis followed by DNA extraction using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).

Construction of the modified vaccine strain:
We used the allelic replacement method for constructing the hha-sepB deletion mutant of *E. coli* O157:H7 that we had proposed to use as a vaccine. To generate an in-frame deletion of sepB, a 1.5 kb sequence located upstream (US) and a 1.5 kb sequence located downstream (DS) of



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sepB were amplified by PCR from *E. coli* O157:H7 strain 86-24 by PCR using primers listed in Table 2. These fragments were cloned in pCRXL TOPO TA cloning vector (InVitrogen, Carlsbad, CA) to generate plasmid pSM281 and pSM282, respectively. The 1.5 kb DS fragment was isolated from pSM282 and cloned at the 3' end of the 1.5 kb US fragment present in pSM281 to construct plasmid pSM284. The 3 kb US-DS fragment of pSM284 was isolated using XbaI and cloned at the XbaI site of the temperature-sensitive plasmid pAM450 to generate a recombinant plasmid pSM302. Plasmid pSM302 was electroporated into *E. coli* O157:H7 strain 86-24 Δstx2 Δlac Δhha and the new strain 86-24 Δstx2 Δlac/pSM302 was cultured under growth conditions, as described previously (12), that facilitated deletion of the chromosomal copy of sepB. The deletion of sepB was confirmed by PCR using appropriate primers (Table 2).

Determination of the level of type III secretion in hha-sepB mutant strain. Bacterial strains were grown in Hepes-buffered DMEM medium that has been shown to enhance the production of locus of enterocyte effacement (LEE)-encoded proteins (2). After about 7 h of growth, cultures were centrifuged at 5000 × g. Culture supernatants and bacterial cell pellets were processed by the following procedures.

- Culture supernatants were filtered through 0.45 µm membranes, filtrates were concentrated using an ultrafiltration device, and concentrated protein preparations were analyzed by SDS-PAGE.
- The bacterial cell pellet recovered after centrifugation was resuspended in PBS. An aliquot of the cell suspension (adjusted to an OD600 2.0) was mixed with SDS buffer and heated in 100°C water bath for 5 min to produce a total cell lysate. The heated preparation was centrifuged briefly and the cell lysate was analyzed by SDS-PAGE.
- SDS-PAGE gel was stained using the GelCode Blue Stain Reagent (Pierce, Rockford, IL) for 1 hour. The gel was destained in water to visualize protein bands.

Demonstration of immune response to the modified vaccine strain of E. coli O157:H7:
Eight weaned calves were divided into four groups of two calves each. Blood samples were taken from the jugular artery to determine the presence of anti-O157:H7 antibodies in these calves before vaccination. Calves in group 1, which served as the control, were injected with a 2-ml of saline. Calves in group 2, 3, and 4 were administered heat-killed vaccines prepared from the non-vaccine parent strain, hha-mutant strain, or hha-sepB mutant strain, respectively. All injections were administered subcutaneously in the neck muscles. These animals were revaccinated at week 2 and 4 post initial vaccination. Blood samples were collected following each vaccination for monitoring development of immune response against purified Tir and EspB (LEE-encoded proteins) as well as against culture supernatants and cell lysates.

Purification of Tir and EspB:
A 483-bp fragment encoding carboxy-terminal portion of Tir was PCR amplified from strain 86-24 by using primers VS336/VS624. The amplified fragment was digested with XhoI and EcoRI and cloned in expression vector pGEX-4T-3 (Amersham Pharmacia Biotech Inc., Piscataway, NJ) to generate an in-frame translational fusion of Tir with the 3□ end of the sequence encoding glutathione-S-transferase (GST). The plasmid containing GST-tir fusion (pSM335) was introduced into E. coli BL21 and GST-Tir fusion protein was purified by using the procedure described previously (12).

For EspB purification, a 530-bp DNA fragment encoding EspB was PCR amplified from strain 86-24 using primers VS559/VS560, digested with BamHI and SalI and cloned in pQE30 (Qiagen Inc., Valencia, CA) to produce a plasmid pSM371, which was transformed into *E. coli* Omnimax2T (InVitrogen, Carlsbad, CA). The EspB protein was purified as 6X-His-EspB fusion from *E. coli* Omnimax2T containing pSM371 by following manufacturer's instructions (Qiagen Inc. Valencia,



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CA). The purified protein preparations were analyzed on a sodium dodecyl sulfate (SDS) -12% polyacrylamide gel to determine their molecular weights and purity. The conditions for polyacrylamide gel electrophoresis (PAGE), staining and destaining of gels were as described in the manual for the Mini-PROTEAN II electrophoresis cell (Bio-Rad, Hercules, CA.). Fusion protein concentrations were estimated by using BioRad protein assay kit (Bio-Rad, Hercules, CA). Purified proteins were stored at −20°C in PBS containing 50% glycerol.

Determining the effectiveness of vaccine strains to protect calves from colonization by E. coli O157:H7:

Following vaccination, gelatin capsules containing 1-ml (109 bacterial cells) of *E. coli* O157:H7 strain 86-24 was orally administered to each calf. Feces were collected everyday for two weeks. Feces were diluted 1:10 fold in PBS, centrifuged briefly at 500 × g to remove large food particles, and 10-fold serial dilutions of the supernatant fluid were plated on sorbitol McConkey agar containing 100 µg/ ml of streptomycin. Following 24-48 h incubation, sorbitol-negative O157:H7 colonies were enumerated on these plates. Identity of these colonies as O157:H7 was verified by testing randomly selected colonies from these plates by a slide agglutination using O157:H7 specific antiserum.

## Findings

We have previously reported that *E. coli* O157:H7 strain 86-24 lacking hha is upregulated in the expression of genes encoding type III secretion system and virulence proteins secreted by the type III system (12). We also demonstrated that hha mutant O157:H7 exhibited increased adherence to Hep-2 cells in tissue culture assays (11). These results lead us to propose that the introduction of a sepB deletion, which had been shown to reduce secretion of several type III secreted proteins in *E. coli* O157:H7 and related enteropathogenic *E. coli* strains (6), would allow intracellular accumulation of large quantities of type III secreted proteins in 86-24 Δhha strain. We introduced sepB deletion in 86-24 Δhha by allelic replacement method and demonstrated by PCR that 86-24 Δhha ΔsepB mutant had undergone a sepB-specific DNA sequence deletion. As shown in Fig. 1, two mutant isolates (lanes 1 and 2) produced a fragment of 2.45 kb compared to the 4 kb DNA fragment produced by the sepB-positive strain 86-24 Δhha (lane 4). Next, we compared the level of type III secretion in 86-24 Δhha ΔsepB relative to the wild-type 86-24 and the 86-24 Δhha strains. As shown in Fig. 2, SDS-PAGE analysis of culture supernatants revealed that 86-24 Δhha ΔsepB (lane 3) had lower level of type III secretion for many LEE-encoded proteins compared to 86-24 Δhha (lane 2). On the other hand, the parent 86-24 strain harboring hha and sepB contained very low levels of these proteins in culture supernatants (lane 1). The three strains (parent 86-24, 86-24 Δhha, and 86-24 Δhha ΔsepB) were heat-treated to produce heat-killed bacterin preparations that were used for vaccinating weaned calves (3-4 month old Holsteins). Six weeks following vaccination, calves were orally inoculated with approximately 1010 cfu of *E. coli* O157:H7 strain 86-24. Feces from these calves were collected for 14-days and cultured on streptomycin containing sorbitol McConkey agar plates. Results of these fecal cultures are shown in Table 3 and are also described graphically in Fig. 3. Two calves that were vaccinated with saline only (negative control calves) continued shedding *E. coli* O157:H7 in their feces on an average of 5.3 × 104 cfu/g of feces for the first 6 days and 1.75 × 102 cfu/g for days 7 through 14. The negative control calf number 1 shed O157:H7 in its feces for every day of the 14-day sampling period except for no shedding detected on day 7, 11, 13 and 14 and the calf number 2 had no detectable levels of O157:H7 in feces at day 8 and 9 and this calf had to be euthanized at day 14 because of displaced abomassum. Calves 3 and 4 that were vaccinated with the 86-24 bacterin continued shedding O157:H7 following the challenge on an average of 1.1 × 104 cfu/g of feces during the 14 day sampling period except for no shedding observed on days 3 and 14 for calf 3 and day 14 for calf 4. Calves 5 and 6 that received 86-24 Δhha bacterin shed on an average of 2.3 × 102 cfu/g for the first 2 days of sampling following the challenge and thereafter had no detectable levels of O157:H7 shedding. Calves 7 and 8 that were vaccinated



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with bacterin derived from 86-24 Δhha ΔsepB shed an average of 1.55 × 102 cfu/g on day 1 post challenge and had no detectable shedding during the remainder of the sampling period. These results indicate that bacterins derived from 86-24 Δhha and 86-24 Δhha ΔsepB were effective in inhibiting colonization of calf intestinal by O157:H7. The reduced colonization in vaccinated animals was reflected by the significant reduction in the shedding of O157:H7 in the feces of these calves. Vaccination of calves 3 and 4 with the bacterin derived from wild-type 86-24 had no effect on the shedding of O157:H7 suggesting that this bacterin lacked sufficient levels of protective antigens needed to be present intracellularly or on bacterial cell surface or at both cellular locations to effectively induce an immune response to block O157:H7 colonization. This conclusion is supported in part by the reduced secretion of type III secreted proteins observed in the wild-type 86-24 strain harboring hha (lane 1, Fig. 1). On the other hand, increased protection against *E. coli* O157:H7 colonization conferred by the bacterins prepared from strains lacking hha or both hha and sepB indicates that the increased expression of type III secreted proteins and perhaps unidentified proteins is responsible for enhanced immunity against *E. coli* O157:H7. At this point, however, we do not have the serological data to show what specific antibodies were induced in sera of calves that were administered bacterins derived from 86-24 Δhha and 86-254 Δhha ΔsepB. However, we are currently screening sera of immunized calves to identify antibodies presumably involved in protection against *E. coli* O157:H7.

## Implications

Results of fecal monitoring showed that the vaccinated calves stopped shedding *E. coli* O157:H7 after 1 – 2 days of oral infection while the unvaccinated controls continued shedding O157:H7 for most of the test period. This research provides preliminary but useful information pertaining to the development of a vaccine strain for reducing *E. coli* O157:H7 colonization in cattle in controlled studies and provides a basis for evaluating the effectiveness of this vaccine in reducing *E. coli* O157:H7 contamination in cattle herds in natural settings.

TABLE 1. Bacterial strains and plasmids[a]

| Strain or plasmid | Relevant genotype and description | Source or reference |
|---|---|---|
| *E.coli* O157:H7 Δ*stx2* Δ*lac* | 86-24 deleted of *stx2* and *lac* operon | (12) |
| *E. coli* O157:H7 Δ*stx2* Δ*lac* Δ*hha* | 86-24 Δ*stx2* Δ*lac* deleted of *hha* | (12) |
| *E. coli* O157:H7 Δ*stx2* Δ*lac* Δ*hha* Δ*sepB* | 86-24 deleted of *sepB* | This study |
| *E. coli* TOP 10 | *endA1 recA1 hsdR17*($r_K^-$ $m_K^-$) *supE44* $\phi$*80*d*lacZ*Δ*M15*Δ(*lacZYA-argF*) | InVitrogen |
| *E. coli* BL21 | $F^-$ *ompT hsdS*($r_B^-$ $m_B^-$) *gal dcm* | Amersham Biosciences |
| *E. coli* Omnimax2T | F´ {*proAB*$^+$ *lacI*$^q$ *lacZ* Δ*M15Tn* (Tet$^R$) Δ(*mrr-hds*RMS-*mcr*BC) | InVitrogen |
| Plasmids | | |
| pCRXL | Cloning vector | Invitrogen |
| pAM450 | Suicide vector | (8) |
| pGEX-4T-3 | Protein expression vector | Amersham Biosciences |
| pSM281 | pCRXL containing 1.5 kb sequence located 5' of *sepB* (US sequence) | This study |
| pSM282 | pCRXL containing 1.5 kb sequence located 3' of *sepB* (DS sequence) | This study |
| pSM284 | pCRXL containing 3 kb US-DS fragment (constructed by ligating 1.5 kb DS sequence of pSM282 at the 3' end of 1.5 kb US sequence present in pSM281) | This study |
| pSM302 | pAM450 containing 3 kb fragment from pSM284 | This study |
| pSM335 | pGEX-4T-3 carrying a 483 bp 3 ' sequence of Tir for expressing GST-Tir fusion protein | This study |
| pSM371 | pQE30 carrying *espB* for expressing 6X-His-EspB | This study |

[a] Detailed description of bacterial strains and plasmids listed in this table is provided under material and methods section.



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TABLE 2. Primers used for PCR and sequencing

| Primer | Nucleotide sequence (5' – 3') | Position[a] |
|---|---|---|
| VS336 | CACTCGAGTTAGACGAAACGATGGGATCC | 19954-19980 |
| VS360 | TTAGACATTTACCTGGTTATAAATAACC | 29580-29554 |
| VS545 | GCGTCTAGAGCGCGCAGGCGTTATTGACCC | 28587-28567 |
| VS546 | GCGGTCGACTTACCGTTCCTAATACTTTAAGTTCG | 27056-27083 |
| VS547 | GCGTCTAGACGCAACATGTGTATATCAATATGGAC | 24200-24226 |
| VS548 | GCGGTCGACGTATGTTGGACAGAATTTATCTATTC | 25717-25691 |
| VS559 | GCGGGATCCATGAATACTATTGATAATACTCAAG | 12005-11981 |
| VS560 | GCGGTCGACTTACCCAGCTAAGCGACCCG | 11067-11090 |
| VS624 | GCGAGATCTACAAGCGCACGTACGGTAGAG | 20424-20404 |

[a] Position of the primer sequence represents the location in the published sequence deposited under the accession number AF071034 at NCBI. Underlined sequences GGATCC, GTCGAC, and TCTAGA represent restriction sites *Bam*HI, *Sal*I, and *Xba*I, respectively



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## Table 3. Determination of O157:H7 shedding in vaccinated calves

| | | CFU/g feces on day[1] | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CALF# | Vaccine[2] | 5/4/06<br>1 | 5/5/06<br>2 | 5/6/06<br>3 | 5/7/06<br>4 | 5/8/06<br>5 | 5/9/06<br>6 | 5/10/06<br>7 | 5/11/06<br>8 | 5/12/06<br>9 | 5/13/06<br>10 | 5/14/06<br>11 | 5/15/06<br>12 | 5/16/06<br>13 | 5/17/06<br>14 |
| 1 | PBS | 5.0E+01 | 2.6E+04 | 8.9E+04 | 2.6E+04 | 1.4E+05 | 4.8E+03 | ND | 2.0E+02 | 1.00E+02 | NS[4] | ND | 3.00E+02 | ND | ND |
| 2 | PBS | 1.2E+05 | 1.2E+04 | 4.0E+02 | 2.2E+05 | 8.1E+03 | 9.0E+02 | 2.5E+02 | ND | ND | NS | 5.00E+01 | 5.00E+01 | 1.00E+02 | CE[5] |
| 3 | Bacterin 1 | 7.0E+03 | 1.0E+02 | ND | 2.8E+03 | 6.5E+02 | 7.0E+03 | 2.6E+03 | 3.7E+03 | 5.86E+04 | NS | 9.00E+02 | 2.50E+02 | 1.00E+02 | ND |
| 4 | Bacterin 1 | 7.7E+03 | 2.4E+04 | 7.0E+02 | 1.7E+03 | 2.5E+02 | 8.0E+02 | ND | 1.3E+04 | 1.18E+04 | NS | 1.50E+03 | 5.00E+02 | 1.10E+03 | 9.50E+02 |
| 5 | Bacterin 2 | 4.0E+02 | 1.0E+02 | ND[3] | ND | ND | ND | ND | ND | ND | NS | ND | ND | ND | ND |
| 6 | Bacterin 2 | 2.0E+02 | ND | ND | ND | ND | ND | ND | ND | ND | NS | ND | ND | ND | ND |
| 7 | Bacterin 3 | 3.0E+02 | ND | ND | ND | ND | ND | ND | ND | ND | NS | ND | ND | ND | ND |
| 8 | Bacterin 3 | 1.0E+01 | ND | ND | ND | ND | ND | ND | ND | ND | NS | ND | ND | ND | ND |

[1] CFU were obtained by plating fecal dilutions prepared in PBS (phosphate-buffered saline) on streptomcyin sorbitol McConkey plates.

[2] vaccines used for immunizing calves; PBS = phosphate buffered saline; Bacterin 1 = *E. coli* O157:H7 86-24 (*hha*$^+$ *sepB*$^+$); Bacterin 2 = 86-24 Δ*hha*; Bacterin 3 = 86-24 Δ*hha* Δ*sepB*

[3] ND means no colonies were detected

[4] NS means no samples were collected on this day

[5] CE means calf euthanized



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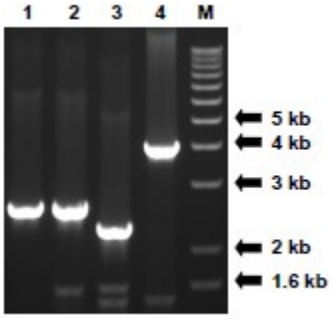


Figure 1. Confirmation of *sepB* deletion by PCR. Genomic DNA prepared from ampicillin-sensitive clones, which were recovered from strain 86-24 Δ*hha/*pSM302 following the allelic replacement procedure, were tested in PCR using primers VS360 and VS561. Amplified samples were analyzed by agarose gel electrophoresis and DNA bands visualized by staining the gel with ethidium bromide. Lanes 1 and 2 contain amplified samples from ampicillin-sensitive clones; lane 3, *sepB*-lacking pSM302 (negative control); lane 4, 86-24 Δ*hha* (positive control); lane M, DNA size markers.

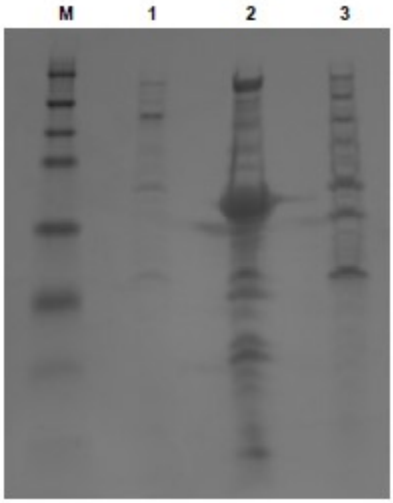


Figure 2. Relative levels of type III secretion in 86-24 and mutant strains. Concentrate culture supernants were resolved by SDS-PAGE and visualized following staining with GelBlue stain. Lane M, Molecular size markers; lane 1, 86-24 (parent strain); lane 2, 86-24 Δ*hha*, and lane 3, 86-24 Δ*hha* Δ*sepB*



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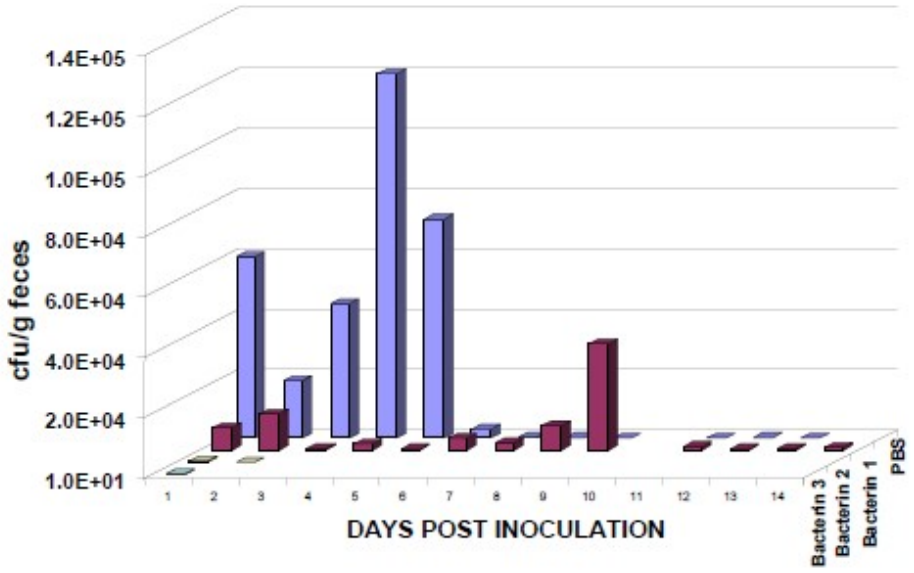


Figure 3. Graphic representation of the data from Table 3 showing O157:H7 shedding in vaccinated calves. PBS (phosphate-buffered saline); Bacterin 1 (parent strain 86-24); Bacterin 2 (Δ*hha* mutant); Bacterin 3 (Δ*hha*Δs*epB* mutant).



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