

# Restrictive Streptomycin Resistance Mutations Decrease the Formation of Attaching and Effacing Lesions in *Escherichia coli* O157:H7 Strains

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**Streptomycin binds to the bacterial ribosome and disrupts protein synthesis by promoting misreading of mRNA. Restrictive mutations on the ribosomal subunit protein S12 confer a streptomycin resistance (Str<sup>r</sup>) phenotype and concomitantly increase the accuracy of the decoding process and decrease the rate of translation. Spontaneous Str<sup>r</sup> mutants of *Escherichia coli* O157:H7 have been generated for *in vivo* studies to promote colonization and to provide a selective marker for this pathogen. The locus of enterocyte effacement (LEE) of *E. coli* O157:H7 encodes a type III secretion system (T3SS), which is required for attaching and effacing to the intestinal epithelium. In this study, we observed decreases in both the expression and secretion levels of the T3SS translocated proteins EspA and EspB in *E. coli* O157:H7 Str<sup>r</sup> restrictive mutants, which have K42T or K42I mutations in S12. However, mildly restrictive (K87R) and nonrestrictive (K42R) mutants showed slight or indistinguishable changes in EspA and EspB secretion. Adherence and actin staining assays indicated that restrictive mutations compromised the formation of attaching and effacing lesions in *E. coli* O157:H7. Therefore, we suggest that *E. coli* O157:H7 strains selected for Str<sup>r</sup> should be thoroughly characterized before *in vivo* and *in vitro* experiments that assay for LEE-directed phenotypes and that strains carrying nonrestrictive mutations such as K42R make better surrogates of wild-type strains than those carrying restrictive mutations.**

Streptomycin (Str), produced by the actinobacterium *Streptomyces griseus*, was the first discovered antibiotic of the aminoglycosides class (1). Str is bactericidal and targets the ribosome, resulting in misreading of mRNA. Several biochemical and structural studies have demonstrated that this miscoding occurs at the step of ternary complex (EF-Tu-GTP-amino acid tRNA) binding to the ribosomal A site (2). In the presence of Str, the forward rate constant of the next kinetic step, GTPase activation and hydrolysis, is indistinguishable for both cognate or near-cognate ternary complexes, resulting in reduced accuracy of amino acid-tRNA selection (2–4).

Streptomycin-resistant (Str<sup>r</sup>) bacteria were discovered shortly after clinical introduction of the antibiotic (5, 6). Spontaneous mutations conferring Str<sup>r</sup> in *E. coli* and other bacteria are typically found within the *rpsL*-encoded 30S ribosomal subunit protein S12 (7, 8), which is involved in the inspection of codon-anticodon pairings in the A site (9). Str<sup>r</sup>-conferring mutations within S12 are divided into two classes: restrictive and nonrestrictive (7). Mutations conferring restrictive decoding accuracy, including K42T (9) and K42N (10), decrease the rate of translation (7, 9, 11, 12) and increase cell doubling time (9, 13–16). Nonrestrictive mutations, such as K42R (10), are similar to wild-type strains in terms of translational accuracy and rate of peptide elongation (7). “Mildly restrictive” mutations have also been described (17).

Spontaneous Str<sup>r</sup> mutants of the food-borne pathogen enterohemorrhagic *E. coli* O157:H7 have been used for genetic (18) and animal colonization (19–22) studies. *E. coli* O157:H7 causes serious diseases, including hemorrhagic colitis and hemolytic-uremic syndrome (HUS) (23). Many of the key virulence factors of O157:H7 are encoded within the locus of enterocyte effacement (LEE), which is also found in both enteropathogenic *E. coli* (EPEC) (24, 25) and in the mouse pathogen *Citrobacter rodentium* (26). The LEE facilitates the intimate adherence between bacteria and epithelial cells and directs the effacement of microvilli, which

collectively is called the attaching and effacing (A/E) phenotype (27). The protein products encoded by the LEE include an outer membrane protein (intimin), structural components of a type III secretion system (T3SS), and secreted effector proteins (28). During A/E lesion formation, LEE-encoded EspA (*E. coli*-secreted protein A) assembles into the T3SS syringe, forming the physical conduit between the bacterium and the eukaryotic cell surface (29). At the distal end of this conduit, EspB and EspD make 3- to 5-nm pores in the host epithelial cell membrane, completing a channel for protein translocation into host cells (30, 31). A protein designated the translocated intimin receptor (Tir) inserts within the host membrane and binds to intimin, mediating the intimate attachment of bacteria to the epithelium (32, 33). Subsequently, the cytoplasmic domains of Tir can trigger a marked rearrangement of the host actin cytoskeleton, resulting in the formation of a pedestal structure beneath the adherent bacterium (34, 35).

In the current study, we isolated 7 unique spontaneous Str<sup>r</sup> *E. coli* O157:H7 mutants that can be classified as restrictive, mildly restrictive, or nonrestrictive. We observed a striking decrease in EspA and EspB secretion levels with the restrictive mutants as well as compromised adherence and A/E lesion formation *in vitro*. We propose that restrictive Str<sup>r</sup> mutations in *E. coli* O157:H7 may impact *in vivo* and *in vitro* phenotypes that depend upon a functional T3SS.

Received 4 April 2013 Returned for modification 25 May 2013

Accepted 14 June 2013

Published ahead of print 24 June 2013

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.00709-13>.

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doi:10.1128/AAC.00709-13

TABLE 1 Strains used in this study

Strain	Relevant characteristics	Reference
Sakai	<i>E. coli</i> O157:H7; <i>stx</i> <sub>1</sub> <sup>+</sup> <i>stx</i> <sub>2</sub> <sup>+</sup>	W. Zhang <sup>a</sup>
Sakai-strR	Spontaneous Str <sup>r</sup> mutant of Sakai	This study
EDL933	<i>E. coli</i> O157:H7; <i>stx</i> <sub>1</sub> <sup>+</sup> <i>stx</i> <sub>2</sub> <sup>+</sup>	68
EDL933-strR1	Spontaneous Str <sup>r</sup> mutant of EDL933	This study
EDL933-strR2	Spontaneous Str <sup>r</sup> mutant of EDL933	This study
EDL933-strR3	Spontaneous Str <sup>r</sup> mutant of EDL933	This study
PA4	<i>E. coli</i> O157:H7; <i>stx</i> <sub>1</sub> <sup>+</sup> <i>stx</i> <sub>2</sub> <sup>+</sup>	40
PA4-strR	Spontaneous Str <sup>r</sup> mutant of PA4	This study
PA5	<i>E. coli</i> O157:H7; <i>stx</i> <sub>1</sub> <sup>+</sup> <i>stx</i> <sub>2</sub> <sup>+</sup>	40
PA5-strR	Spontaneous Str <sup>r</sup> mutant of PA5	This study
PA11	<i>E. coli</i> O157:H7; <i>stx</i> <sub>2</sub> <sup>+</sup>	40
PA11-strR	Spontaneous Str <sup>r</sup> mutant of PA11	This study

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## MATERIALS AND METHODS

**Bacterial strains and culture media.** The bacterial strains and plasmids used in this study are described in Table 1. Strains were routinely grown in liquid or solid lysogeny broth medium (LB) (36, 37). All stocks were maintained at  $-80^{\circ}\text{C}$  in 10% glycerol. Str<sup>r</sup> mutants of *E. coli* O157:H7 strains were screened by plating overnight cultures of the wild-type strains on LB agar supplemented with Str (100  $\mu\text{g}/\text{ml}$ ). Putative Str<sup>r</sup> colonies were streaked on the same medium for purification. In addition to the prototypical *E. coli* O157:H7 strains EDL933 (38) and Sakai (39), three previously characterized O157:H7 clinical isolates obtained from the Pennsylvania Department of Health (40) were selected for comparative purposes.

**Analysis of secreted and intracellular proteins.** Quantification of secreted EspA and EspB was performed as previously reported (41). *E. coli* O157:H7 strains were incubated statically at  $37^{\circ}\text{C}$  for 8 h in both high-glucose Dulbecco's modified Eagle's medium (DMEM; with 4.5 g/liter glucose and L-glutamine and without sodium pyruvate; Cellgro, Manassas, VA) and low-glucose DMEM (with 1 g/liter glucose L-glutamine and sodium pyruvate; Gibco, Carlsbad, CA). Upon harvesting, no significant differences in cell density were observed between the wild type and mutants. Prior to precipitation, 2  $\mu\text{g}/\text{ml}$  bovine serum albumin (BSA) was added to the supernatants as a control for the efficiency of the protein precipitation. BSA was visualized using a Coomassie brilliant blue staining. After SDS-PAGE, the gel was photographed using a transilluminator (UVP, Upland, CA). All experiments were conducted with at least two biological replicates.

For total intracellular protein, 2 ml of each culture was centrifuged (16,000  $\times g$ , 2 min,  $20^{\circ}\text{C}$ ), and the pellet was resuspended in 100  $\mu\text{l}$  of 1 $\times$  Laemmli buffer (12 mM Tris-Cl [pH 6.8], 0.4% SDS, 2% glycerol, 1%  $\beta$ -mercaptoethanol, and 0.002% bromophenol blue). To lyse the bacterial cells, the samples were incubated at  $100^{\circ}\text{C}$  for 10 min. The Western blot method referenced above was used to visualize secreted EspB and EspA and was also used to detect and quantify the intracellular proteins. RNA polymerase (Pol) was used as an internal control and was detected using mouse anti-RNA Pol  $\alpha$  (Santa Cruz Biotechnology, Santa Cruz, CA), followed by IRDye 800CW goat anti-mouse IgG (Licor, Lincoln, NE). The relative intracellular levels of EspA or EspB (in arbitrary units) from EDL933 derivatives (Esp<sub>mutant</sub>), compared with the wild-type strain (Esp<sub>WT</sub>), were calculated as follows: (Esp<sub>mutant</sub>/RNA Pol<sub>mutant</sub>)/(Esp<sub>WT</sub>/RNA Pol<sub>WT</sub>). The experiments were performed in three biological replicates.

**Growth curve and plating assay.** To profile the bacterial growth in high-glucose DMEM, overnight cultures of each strain were diluted to an optical density at 600 nm (OD<sub>600</sub>) of 0.05 and incubated statically at  $37^{\circ}\text{C}$  for 8 h. The OD<sub>600</sub> was taken over the time course to profile the changes in cell densities. Measurements between 20 min and 2.5 h postinoculation were used to calculate the bacterial growth rates during the logarithmic

phase. The experiments were done in three biological replicates. Cultures were sampled at the end of incubation, and the CFU/ml was determined by plating serial dilutions onto LB agar. Each plating assay was performed in two biological replicates with two experimental replicates.

**Sequencing of the *rpsL* gene.** Identification of *rpsL* mutations in the genomes of O157:H7 isolates was accomplished by PCR amplification using the primer pair rpsL-L and rpsL-R (see Table S1 in the supplemental material), followed by DNA sequencing at the Penn State Genomics Core Facility (University Park, PA).

**Cell culture and adherence assays.** HeLa cells were maintained in T75 flasks containing high-glucose DMEM at  $37^{\circ}\text{C}$  and 5% CO<sub>2</sub>. DMEM was supplemented with 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA) and 1% antibiotic/antimycotic (Life Technologies, Carlsbad, CA). For adherence assays, 24-well plates were seeded with 10<sup>5</sup> HeLa cells per well and incubated as described above until monolayers were confluent. Before use, HeLa cells were washed twice with 1 ml of sterile phosphate-buffered saline (PBS, pH 7.4). Cells were then replenished with DMEM containing no additives. Bacteria were grown in LB broth overnight at  $37^{\circ}\text{C}$ , and Str<sup>r</sup> mutants were grown in LB with the addition of Str. A 1:25 dilution of the overnight culture was made, and bacteria were grown to the logarithmic phase. Triplicate replicates of each strain were infected with a multiplicity of infection (MOI) of 10. Inoculum inputs were verified by dot plating onto LB plates containing selective antibiotics. Infected monolayers were incubated for 2 h at  $37^{\circ}\text{C}$  and 5% CO<sub>2</sub>. Non-adherent bacteria were removed by three consecutive 1-ml PBS washes. Monolayers were then incubated with 200  $\mu\text{l}$  of PBS containing 0.1% Triton X-100 until monolayers detached. Recovered bacteria were then serially diluted and plated via drop plate method onto LB plates containing selective antibiotic. Results are from three independent experiments. The percentage of bacterial recovery was calculated as the recovered cell density divided by that of the inocula and then normalized against that of the respective wild-type strain. In order to estimate the bacterial cell density after 2 h of incubation in high-glucose DMEM, EDL933 and its derivatives were grown under the same conditions but in the absence of HeLa cells, and the CFU/ml was determined in a plating assay. This experiment was performed with two biological replicates, each with two technical repeats.

**Fluorescent actin staining assays.** Fluorescent actin staining assays (FAS) were performed as previously described (42), using low-glucose DMEM supplemented with 10% FBS during infection. The medium was changed 3 h postinoculation, and an additional 3 h of incubation was allowed for the actin rearrangement to occur. After staining, pedestal formation was quantified as the number of HeLa cells with pedestals divided by the total number of HeLa cells within one field. The experiments were run with four biological replicates, and a total of 9 fields (2 or 3 in each replicate experiment) were counted for each strain. In order to estimate the bacterial cell density before the medium change, we inoculated *E. coli* O157:H7 strains into the same medium in the absence of HeLa cells. After 3 h of incubation, each culture was sampled and plated on LB agar. This experiment was performed with two biological replicates, each with two technical repeats.

**RNA preparation and reverse transcription-qPCR.** *E. coli* cultures were grown under similar conditions as described previously for the protein secretion assays, and the total RNA was harvested after an 8-h static incubation in high-glucose DMEM, using the mirVana miRNA isolation kit (Life Technologies, Carlsbad, CA). The protocol from the manufacturer was generally followed, with one exception: RLT buffer (Qiagen, Valencia, CA) plus 1%  $\beta$ -mercaptoethanol was used instead of the original lysis/binding solution for higher bacterial cell lysis efficiency. Reverse transcription (RT) was performed using the ThermoScript RT-PCR system (Invitrogen, Grand Island, NY) and the reverse primers espA-R, espB-R, and rrsA-R (see Table S1 in the supplemental material). Quantitative PCR (qPCR) was used to quantify the transcription levels of *espA*, *espB*, and the internal control, *rrsA*. Each reaction mixture (20  $\mu\text{l}$ ) contained 10  $\mu\text{l}$  PerfeCta SYBR green FastMix for iQ (Quanta, Gaithersburg,

TABLE 2 Point mutations identified in *E. coli* O157:H7 Str<sup>r</sup> strains

Strain	S12 alteration <sup>a</sup>		Phenotype
	Site 42	Site 87	
Wild type <sup>b</sup>	AAA(Lys)	AAA(Lys)	
EDL933-strR1	<u>AGA</u> (Arg)		Nonrestrictive
EDL933-strR2	<u>ACA</u> (Thr)		Restrictive
EDL933-strR3	<u>AAC</u> (Asn)		Restrictive
Sakai-strR	<u>ACA</u> (Thr)		Restrictive
PA4-strR	<u>ACA</u> (Thr)		Restrictive
PA5-strR	<u>ACA</u> (Thr)		Restrictive
PA11-strR		<u>AGA</u> (Arg)	Mildly restrictive

<sup>a</sup> The mutated bases are underlined.

<sup>b</sup> The sequences of the wild-type allele of *rpsL* were the same in all *E. coli* O157:H7 strains included in this study.

MD), forward and reverse primers (*espA*-F/R and *espB*-F/R or *rrsA*-F/R, 0.1 μM [see Table S1]), and 2.5 μl of the cDNA as the template. When necessary, the cDNA templates were diluted 100-fold prior to qPCR, so that the resulting  $C_T$  (threshold cycle) was in the linear range of the assay. The relative mRNA levels were then determined as follows:  $C_T$  of *Esp* -  $C_T$  of *RrsA*. Each qPCR assay was run in triplicate, and extracted *E. coli* O157:H7 Sakai genome DNA (positive control), as well as no-template (negative-control) reactions were included. A total of three biological replicates were performed. A no-reverse transcriptase control was conducted for each RNA sample.

**Statistical analysis.** Statistical analyses (Student's *t* test, one-way analysis of variance [ANOVA] and Tukey's multiple-comparison test) were conducted using the Minitab 16.2.0 F-test, within Microsoft Excel, to determine equal variance of the means.

## RESULTS

**The nature of *rpsL* mutations in Str<sup>r</sup> strains affects *EspA*/*EspB* secretion.** To determine whether Str<sup>r</sup>-conferring mutations in the S12 subunit affect the production of virulence proteins, we isolated spontaneous mutants of the prototypical strain *E. coli* O157:H7 EDL933 and screened them for changes in secreted levels of the T3SS proteins *EspA* and *EspB*. Most Str<sup>r</sup> mutants had reduced secretion levels of *EspA* and *EspB* compared to the Str<sup>s</sup> wild-type strain. We confirmed that our observation was reproducible by streaking our laboratory stock of EDL933 for isolated colonies, generating new Str<sup>r</sup> mutants from these isolates, and comparing *EspA* and *EspB* secretion levels between the new wild-type and Str<sup>r</sup> strains (data not shown).

Next, we selected three spontaneous Str<sup>r</sup> mutants of *E. coli* O157:H7 EDL933 (Table 1) and characterized them by using a combination of Western blotting and DNA sequencing of *rpsL*. Three different mutations were identified: the isolates designated EDL933-strR1, -strR2, and -strR3 contained K42R, K42T, and

K42N mutations in *rpsL*, respectively (Table 2). Secretion levels of *EspA*/*B* from EDL933-strR1 were indistinguishable from that from the wild-type strain; however, secretion levels for both EDL933-strR2 and -strR3 were dramatically reduced (Fig. 1). These phenotypes were observed in both high-glucose (Fig. 1) and low-glucose (data not shown) DMEM.

In order to determine whether the observed defects in secretion were specific to the EDL933 strain, we isolated Str<sup>r</sup> derivatives from a second prototypical O157:H7 strain, Sakai (39), and from three clinical isolates previously designated PA4, PA5, and PA11 (40) (Table 1). Three isolates (Sakai-strR, PA4-strR, and PA5-strR) carried the K42T mutation in S12 (Table 2), and all three showed reduced *EspA* and *EspB* secretion levels compared to their respective Str<sup>s</sup> wild-type strains (Fig. 1). The isolate PA11-strR, containing a K87R mutation previously defined as "mildly restrictive" (10, 17, 43), exhibited only a slight reduction of *EspA* and *EspB* compared to the wild-type strain (Fig. 1).

**Restrictive Str<sup>r</sup> mutations alter the bacterial growth rate.** It was previously reported that restrictive mutations negatively impact the growth rate of *E. coli* (13–16). Hence, we compared the growth profiles of the three EDL933 derivatives with the wild type in high-glucose DMEM (see Fig. S1 in the supplemental material). There were no obvious differences in the lag times between EDL933, EDL933-strR1, EDL933-strR2, and EDL933-strR3; however, the doubling times during logarithmic growth were  $63.0 \pm 0.8$ ,  $63.3 \pm 2.4$ ,  $68.5 \pm 2.7$ , and  $72.9 \pm 0.8$  (mean  $\pm$  standard deviation) minutes, respectively. The OD<sub>600</sub> of the two restrictive mutants, EDL933-strR2 and -strR3, were consistently below those of EDL933 and EDL933-strR1 for the first 5 h; however, no differences in cell density were observed between these strains 6 h postinoculation.

**Intracellular expression of *EspA* and *EspB* in *E. coli* O157:H7 EDL933 and its Str<sup>r</sup> mutants.** We next asked whether defects in *EspA* and *EspB* secretion were mediated at the translational and/or transcriptional levels. To test the first hypothesis, we prepared whole-cell extracts of EDL933 and the three mutants after 8 h of incubation, when all cultures achieved the same optical density (see Fig. S1). Western blot analysis showed that the intracellular levels of *EspB* from EDL933-strR2 and -strR3 and of *EspA* from all three mutants were significantly lower than that seen with the wild-type strain ( $P < 0.05$ , Student's *t* test) (Fig. 2A). The reduction in *EspA*/*B* accumulation was greater for EDL933-strR2 and -strR3 than for EDL933-strR1.

It is well recognized that restrictive Str<sup>r</sup> mutations lower the rate of protein synthesis (7, 9, 11, 12). However, a variety of proteins have also been identified that directly or indirectly impact transcription of LEE-encoded proteins (44). Therefore, our next question was whether the defects in *EspA*/*B* accumulation were

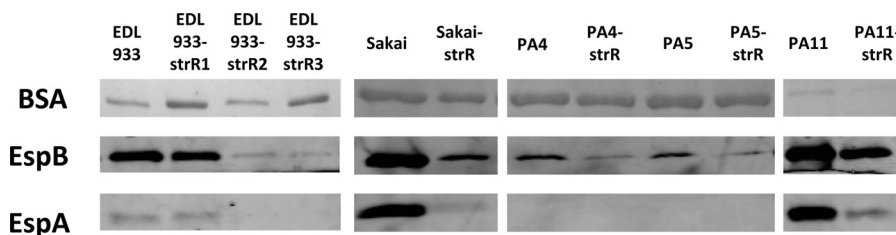
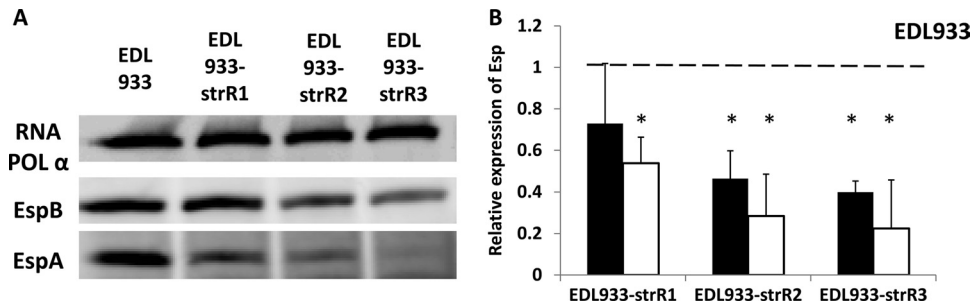


FIG 1 Secretion of *EspA* and *EspB* from *E. coli* O157:H7 strains and their respective Str<sup>r</sup> mutants. Total secreted protein was collected after 8 h of incubation in high-glucose DMEM and separated by SDS-PAGE using a 4-to-20% linear gradient polyacrylamide gel. BSA was detected by Coomassie blue staining. *EspB* and *EspA* were detected by Western blotting.



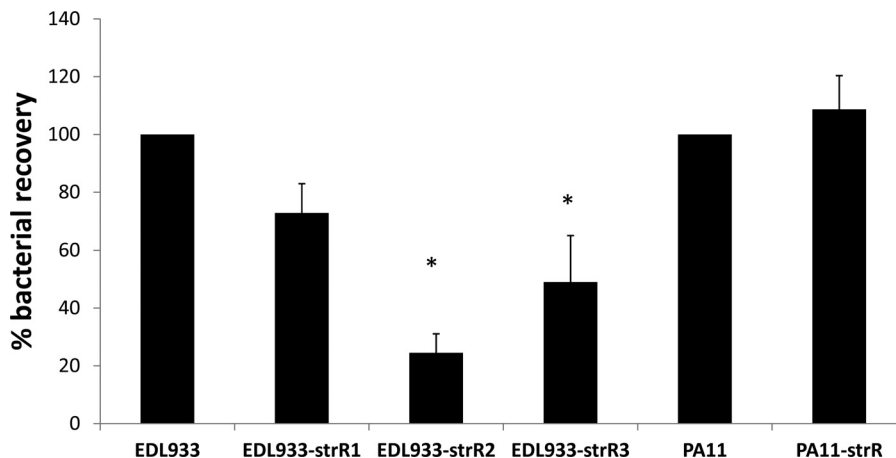
**FIG 2** The effect of Str<sup>r</sup> mutations on EspA and EspB expression. (A) Representative Western blots of RNA Pol subunit  $\alpha$ , EspB, and EspA of *E. coli* O157:H7 EDL933 and its mutants. (B) The intensities of Esp protein bands (closed boxes, EspB; open boxes, EspA) were analyzed using the Odyssey application software. The relative Esp expression levels of the derivative strains compared to wild-type EDL933 were calculated as described in the text. \*, significantly less EspA or EspB was recovered from the whole-cell extract ( $P < 0.05$ , Student's *t* test). All results for Esp quantification are averages from three biological repeats, and error bars represent 1 standard deviation.

due to decreased synthesis of one or more of these regulators. Using qPCR, we determined that the relative transcription levels ( $C_T$  of Esp -  $C_T$  of RrsA) for *espA* were  $15.25 \pm 1.28$  (mean  $\pm$  standard deviation),  $14.70 \pm 1.03$ ,  $15.29 \pm 1.02$ , and  $14.64 \pm 1.12$  for EDL933, EDL933-strR1, -strR2, and -strR3, respectively. In the case of *espB*, the values were  $12.40 \pm 0.15$ ,  $13.08 \pm 1.14$ ,  $11.43 \pm 2.02$ , and  $12.70 \pm 2.36$ , respectively. Although slight differences in *espA/B* transcription levels were seen among EDL933 and its mutants, the differences were not significant ( $P = 0.833$  for *espA*,  $P = 0.665$  for *espB* [one-way ANOVA]). This experiment supported a model where translational but not transcriptional defects are primarily responsible for the reductions in EspA/B accumulation described above.

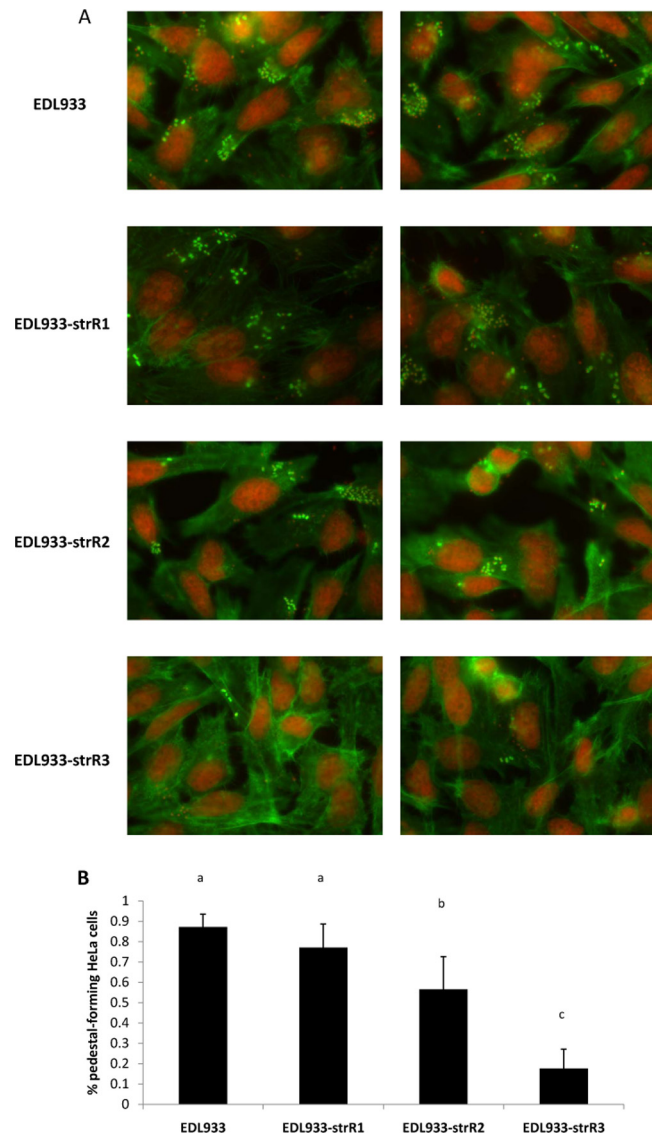
**Effect of Str<sup>r</sup> mutation on adherence of *E. coli* O157:H7 to HeLa cells.** EspA and EspB are critical for T3SS-mediated attachment to human cell lines (45). Therefore, we next hypothesized that our Str<sup>r</sup> strains would have reduced abilities to adhere to HeLa cells compared to the wild type. Adherence of EDL933-strR1, EDL933-strR2, and EDL933-strR3 was decreased by 27%, 76%, and 51% from the wild-type EDL933, respectively (Fig. 3); the values for EDL933-strR2 and -strR3 were significantly de-

creased ( $P < 0.05$ , one-way ANOVA with Tukey's test). Hence, the decrease in adherence correlated with reduced EspA/B secretion levels (Fig. 1). Plate counts taken 2 h postinoculation for the wild-type strain, EDL933-strR1, -strR2, and -strR3 were estimated to be  $(1.50 \pm 0.33) \times 10^7$  (mean  $\pm$  standard deviation),  $(1.49 \pm 0.27) \times 10^7$ ,  $(1.05 \pm 0.17) \times 10^7$ , and  $(0.54 \pm 0.23) \times 10^7$  CFU/ml, respectively. Adherence levels were comparable for the mildly restrictive strain PA11-strR and its wild-type control ( $P = 0.50$ , Student's *t* test) (Fig. 3).

**Str<sup>r</sup> mutations affect actin reorganization.** The secretion of EspA (29) and EspB (30) are essential for the formation of A/E lesions on epithelial cells, which is observed as an accumulation of the host actin cytoskeleton beneath the bacterial attachment site (25, 46). To test whether different Str<sup>r</sup> mutations impacted actin polymerization, FAS assays were performed with *E. coli* O157:H7 EDL933 and its mutants (Fig. 4). Fluorescence microscopy revealed the formation of typical actin pedestals on HeLa cells upon infection (Fig. 4A). Actin polymerization was observed as a characteristic cup-like structure (bright green) underneath the bacterium (red). By quantifying the percentage of HeLa cells with at least one pedestal, per field, we observed that all 3 mutants were



**FIG 3** Relative adherence levels of *E. coli* O157:H7 strains and their respective Str<sup>r</sup> mutants. HeLa cells grown as monolayers were inoculated with bacteria to an MOI of 10 and incubated at 37°C and 5% CO<sub>2</sub> for 2 h. HeLa cells were lysed, and 10-fold serial dilutions were plated on LB agar to determine the CFU/ml. After calculating the percentages of bacterial recovery (final number of CFU per ml divided by the initial number of CFU/ml, times 100), the data from all the mutants were normalized against those for the parental wild-type strain. The averages from three biological replicates are presented, and the error bars represent 1 standard error. Asterisks indicate significantly different results from the respective wild type ( $P < 0.05$ , Student's *t* test).



**FIG 4** Str<sup>r</sup> mutations compromise A/E lesion formation of *E. coli* O157:H7 EDL933. A FAS assay was performed to assess the pedestal formation capabilities of EDL933 and its mutants. (A) Two fields for each strain are presented. Pedestals are bright green (actin) cups beneath the red bacteria. (B) Quantification of pedestal formation. The percentage of pedestal-forming cells per field was quantified. The result for each strain is an average from 9 fields (approximately 50 cells per field), and the error bar represents 1 standard deviation. Values assigned with different letters were significantly different ( $P < 0.05$ , one-way ANOVA with Tukey's multiple-comparison test).

defective in promoting actin polymerization (Fig. 4B). Compared to the wild-type strain, EDL933-strR1, -strR2, and -strR3 showed a 12%, 35%, and 80% reduction in pedestal formation, and the differences for EDL933-strR2 and -strR3 were statistically significant ( $P < 0.05$ , one-way ANOVA with Tukey's multiple comparison test). The decrease in A/E lesion formation was consistent with the reduction of EspA/B seen in the EDL933 Str<sup>r</sup> mutants (Fig. 1). In addition, after the first 3 h of incubation, the cell densities for the wild-type strain, EDL933-strR1, -strR2, and -strR3 were estimated to be  $(1.67 \pm 0.18) \times 10^7$  (mean  $\pm$  standard deviation),  $(1.89 \pm 0.64) \times 10^7$ ,  $(1.04 \pm 0.21) \times 10^7$ , and  $(0.72 \pm 0.23) \times 10^7$  CFU/ml, respectively.

## DISCUSSION

The Str-treated mouse model was proposed by Myhal et al. as a method for studying *E. coli* intestinal colonization (47). The antibiotic was reported to decrease the abundance of facultative anaerobes within the gut microflora, although another study has suggested that streptomycin treatment decreases colonization resistance through altering the pH and volatile fatty acid composition of the ecosystem (48). Str treatment has additionally been used to overcome colonization resistance when studying *E. coli* colonization in cattle (19–22), in mice (49–52), and for *in vivo* infection models of *Salmonella* (53, 54), *Aeromonas* (55), *Klebsiella* (56), and *Vibrio* (57) species. This experimental design remains common despite the earlier observations that restrictive Str<sup>r</sup> mutations compromise the fitness of *Salmonella* in mice (58, 59). These studies revealed that restrictive Str<sup>r</sup> mutants are out-competed 100- to 1,000-fold by the wild-type strain 4 days after coinjection intraperitoneally into mice at a 1:1 ratio. The investigators argued that this effect was due to the slight decrease in the growth rates of the mutants. In our study, we also observed 8.9% (K42T in S12) and 15.7% (K42N) decreases in the growth rates of Str<sup>r</sup> mutants of *E. coli* O157:H7 (see Fig. S1 in the supplemental material), but more importantly, we also observed reduced secretion of two critical components of the T3SS that are required for optimal colonization of cattle (60–62), humans (63), and pigs (64).

We analyzed 7 spontaneous Str<sup>r</sup> mutants from 5 *E. coli* O157:H7 strains and observed 4 different mutations in *rpsL* (Table 2). All mutations were located within either site 42 or 87. These were previously reported to be hot spots for Str<sup>r</sup> mutations (16), likely because they provide cells with higher levels of antibiotic resistance than other mutations. Based on previous studies (9, 10, 17), these 4 mutations could be divided into three groups: restrictive (K42T and K42N), mildly restrictive (K87R), and nonrestrictive (K42R) (Table 2). Mutations conferring restrictive decoding accuracy concomitantly decrease the rate of translation (7, 9, 11, 12), while the translational efficiency of nonrestrictive mutants is indistinguishable from the wild-type strain (7). In the current study, we observed a striking correspondence between the predicted translational efficiency and the EspA and EspB secretion levels. A dramatic reduction in secretion levels was observed in restrictive mutants (Sakai-strR, PA4-strR, PA5-strR, EDL933-strR2, and EDL933-strR3), a slight reduction with the mildly restrictive mutant (PA11-strR), and no difference in the nonrestrictive mutant (EDL933-strR1) (Fig. 1). Admittedly, this correspondence was observed when we used a small set of mutants, and the effects of Str<sup>r</sup> mutations on EspA/B secretion may vary depending upon the *E. coli* O157:H7 strain and specific Str<sup>r</sup>-conferring mechanism. We plan to address these questions in the future by designing more-comprehensive studies.

EspA is a major component in the T3SS syringe and is required for intimate physical interaction with host cells (29). EspB is translocated through the EspA filament (29) and forms pores in the host membrane. Both of these proteins are required for the translocation of Tir (65). Tir facilitates the bacterial intimate adherence and triggers the actin polymerization in the epithelia (34, 45). Reduced secretion of EspA and EspB in the restrictive mutants (EDL933-strR2 and -strR3) decreased bacterial adherence (Fig. 3), consistent with the previously reported study (66) in which *espA* and *espB* null mutants were used. We noticed that PA11-strR se-

creted markedly less EspA than the wild type (Fig. 1), although no significant differences in adherence to HeLa cells were observed. We noted that PA11-strR secretes EspA/B to levels that are comparable to wild-type EDL933 and higher than those seen with strains PA4 and PA5 (Fig. 1), suggesting that perhaps the amount of EspA/B secreted by PA11-strR is sufficient to promote optimal adherence in this assay. The reduced secretion of the two LEE-encoded proteins also had a remarkable impact on pedestal formation (Fig. 4). These observations collectively suggest a link between Str<sup>r</sup> mutations and the T3SS-related virulence potential of *E. coli* O157:H7. Hence, our results suggest that restrictive Str<sup>r</sup> mutants may not always be reliable surrogates for their wild-type parents, although this argument requires further *in vivo* experiments. Our work also highlights the fact that independently generated Str<sup>r</sup> strains may not be phenotypically equivalent.

The negative impact of restrictive mutations on bacterial growth has been previously reported (13–16), and we noticed growth differences as well in high-glucose DMEM during the first 5 h of incubation (see Fig. S1A in the supplemental material). The reduced growth rate of restrictive mutants likely contributed to the results in the adherence assay (Fig. 3) and FAS assay (Fig. 4), in which results were analyzed 2 and 3 h postinfection, respectively. Importantly, however, our results demonstrated for the first time that restrictive mutations also affect adherence and actin accumulation through decreased secretion of LEE-encoded proteins (Fig. 2). A variety of genes have been identified that regulate the transcription of *espA* and *espB*, yet the results from our qPCR experiments and Western blotting analysis of intracellular EspA and EspB (Fig. 2) strongly suggested that the secretion defect is primarily the result of a reduced rate of EspA and EspB protein synthesis. Why synthesis of EspA and EspB specifically is decreased needs to be further explored. The translation rate has been reported to impact the folding of protein domains (67), providing one mechanism worth investigating in the future.

In conclusion, our results clearly indicate that Str<sup>r</sup>-conferring mutations are not always neutral with regard to virulence phenotypes and may affect the progression of the colonization and pathogenesis of *E. coli* O157:H7. We suggest that the *rpsL* mutation imparting the Str<sup>r</sup> phenotype should be identified before using strains in assays that depend upon a functioning T3SS. Strains carrying nonrestrictive mutations, such as K42R, likely make better surrogates for the wild type than strains carrying restrictive mutations.

## ACKNOWLEDGMENTS

This work was funded by USDA-NIFA grant 2009-03611, by start-up funds through the Penn State University Department of Food Science and College of Agricultural Sciences, and by the Casida Development Professorship to E.G.D.

We thank the Penn State Genomics Core Facility (University Park, PA) for help in generating DNA sequence information. We thank Sarah Forester (Department of Food Science, the Penn State University) for help with equipment settings and James Kaper and Jane Michalski (University of Maryland, School of Medicine) for providing EspA and EspB antibodies.

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