A Single Nucleotide Polymorphism in \textit{lptG} Increases Tolerance to Bile Salts, Acid, and Staining of Calcofluor-Binding Polysaccharides in \textit{Salmonella enterica} Serovar Typhimurium E40

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The outer membrane of \textit{Salmonella enterica} plays an important role in combating stress encountered in the environment and hosts. The transport and insertion of lipopolysaccharides (LPS) into the outer membrane involves lipopolysaccharide transport proteins (LptA-F) and mutations in the genes encoding for these proteins are often lethal or result in the transport of atypical LPS that can alter stress tolerance in bacteria. During studies of heterogeneity in bile salts tolerance, \textit{S. enterica} serovar Typhimurium E40 was segregated into bile salts tolerant and sensitive cells by screening for growth in TSB with 10% bile salts. An isolate (E40V) with a bile salts MIC > 20% was selected for further characterization. Whole-genome sequencing of E40 and E40V using Illumina and PacBio SMRT technologies revealed a non-synonymous single nucleotide polymorphism (SNP) in \textit{lptG}. Leucine at residue 26 in E40 was substituted with proline in E40V. In addition to growth in the presence of 10% bile salts, E40V was susceptible to novobiocin while E40 was not. Transcriptional analysis of E40 and E40V, in the absence of bile salts, revealed significantly greater ($p < 0.05$) levels of transcript in three genes in E40V; \textit{yjbE} (encoding for an extracellular polymeric substance production protein), \textit{yciE} (encoding for a putative stress response protein), and an uncharacterized gene annotated as an acid shock protein precursor (ASPP). No transcripts of genes were present at a greater level in E40 compared to E40V. Corresponding with the greater level of these transcripts, E40V had greater survival at pH 3.35 and staining of Calcofluor-binding polysaccharide (CBPS). To confirm the SNP in \textit{lptG} was associated with these phenotypes, strain E40E was engineered from E40 to encode for the variant form of LptG (L26P). E40E exhibited the same differences in gene transcripts and phenotypes as E40V, including susceptibility to novobiocin, confirming the SNP was responsible for these differences.

\textbf{Keywords:} \textit{lptG}, LPS, SNP, bile salts, acid tolerance, extracellular polymeric substance, \textit{S. enterica}
INTRODUCTION

Salmonella enterica is an important human pathogen that is responsible for an estimated 21 million cases of typhoid fever, 100 million cases of gastroenteritis, and 350,000 deaths annually (Crump et al., 2004; Majowicz et al., 2010). S. enterica is associated with a range of animals and usually transmitted to humans by contaminated water and foods (Bäumler, 1997; Groisman and Ochman, 1997; Mahon et al., 1997; Thrëfall, 2002; Isaacs et al., 2005; Lan et al., 2009; Newell et al., 2010; Painter et al., 2013; Dechet et al., 2014). Most serovars cause self-limiting gastroenteritis in humans while Typhi strains can cause persistent infections, partly through formation of biofilms within the bile rich gall bladder (Lai et al., 1992; Crawford et al., 2010; Gonzalez-Escobedo et al., 2011; Gonzalez-Escobedo and Gunn, 2013; Gunn et al., 2014). The success of S. enterica as a pathogen is due in part to the level of diversity within the species. S. enterica contains six subspecies that can be further divided into more than 2,000 serovars (Bäumler, 1997; Groisman and Ochman, 1997; Lan et al., 2009). Within individual Salmonella serovars, genetic differences can provide advantages such as antibiotic resistance or antigenic variation, that can enhance Salmonella fitness during infection (Piddock et al., 1998; Giraud et al., 1999; Bonifield and Hughes, 2003; Levy et al., 2004; Aldridge et al., 2006; Le et al., 2007; Stewart and Cookson, 2014).

Salmonella encounters an array of environmental assaults during transmission and following ingestion, including low pH in the stomach and bile secreted from the gall bladder. These stresses can be mitigated through activation of general stress regulons, RpoS (Hengge-Aronis, 2002; Battesti et al., 2011) and RpoH (Guo and Gross, 2014; Roncarati and Scarlato, 2017), as well as stress specific pathways such as the PhoPQ operon (Foster and Hall, 1990; Spector and Kenyon, 2012), the marRAB operon (Prouty et al., 2004a), and the acrAB operon (Nikaido et al., 2008). The outer membrane (OM), including lipopolysaccharides (LPS), and extracellular polymeric substance (EPS) are central to protection from environmental conditions and signaling pathways (Nikaido, 2003; Flemming and Wingender, 2010; Zhang et al., 2013; Flemming, 2016).

Extracellular polymeric substance is a complex mixture of proteins, polysaccharides, and additional components that form a protective layer around the cell and promote biofilm formation. In contrast to the multicomponent EPS, Salmonella LPS is a defined extracellular structure comprised of membrane embedded Lipid A, core oligosaccharides, and the O-antigen polysaccharide. The production and export of LPS is important for virulence (Zhang et al., 2013). Rough mutants, which produce LPS without O-antigen, have reduced virulence, but can yield persisters since O-antigen elicits a strong immune response (Chart et al., 1991; Freudenberg et al., 2008).

As shown in Figure 1, lipid A is ligated to the core oligosaccharide in the cytosol and transported to the periplasmic face of the inner membrane (IM) by the ABC transporter MsbA (Kalyneh et al., 2014; Whitfield and Trent, 2014; Okuda et al., 2016). Independently, O-antigen monomers are transported through the inner membrane and polymerized into complete O-antigen polysaccharides, which are then ligated to the core oligosaccharide in the periplasmic space. Fully assembled LPS is transferred through the periplasmic space and the OM by the lipopolysaccharide transport proteins (LptA-G). LptG and LptF form a transmembrane complex in the IM associated with a homodimer of the ATP-binding protein LptB. LPS that is anchored to the periplasmic face of the IM enters the LptGF transmembrane domain and is shuttled using ATP hydrolysis through the periplasmic space by LptA and LptC, which complex with LptFG and form a link to the OM LptDE complex. LPS is then transferred through the large beta barrel structure within LptD to reach the OM (Sperandeo et al., 2007; Ruiz et al., 2008; Narita and Tokuda, 2009; Dong et al., 2014, 2017; Okuda et al., 2016; Luo et al., 2017; Sherman et al., 2018).

Salmonella serovars were screened for the ability to grow in TSB with 10% bile salts. This screen identified a S. enterica Typhimurium E40 variant (E40V) that was significantly more tolerant to bile salts (MIC > 20%) than a majority of the cells in the E40 culture as well as other serovars. Analysis of E40 and E40V in the absence of bile salts found a greater steady state level of transcripts in three genes in E40V: yjbe (an extracellular polymeric substance production gene), yciE (a putative stress response gene), and Acid Shock Protein Precursor. The greater level of transcripts for these genes was associated with phenotypes that matched their predicted functions. DNA sequencing and site directed mutagenesis confirmed the SNP in lptG was involved in the difference in the level of three transcripts and novobiocin sensitivity.

MATERIALS AND METHODS

Bacterial Strains, Growth, and Storage

The strains and plasmids used in this study are listed in Table 1. Frozen stock cultures were stored in Luria Bertani (LB) broth with 15% glycerol at −70°C. Working stock cultures were cultured on LB agar (Becton Dickinson, Sparks, MD, United States), incubated at 37°C for 20–24 h, and maintained at 4°C for up to 1 month. Overnight cultures were grown from a single colony in 5 ml of tryptic soy broth (TSB; Becton Dickinson) at 37°C for 20–24 h. For bile salts tolerance screening, TSB with 10% bile salts was inoculated with inocula ranging from 10^5 to 10^6 CFU/ml. For survival and regrowth experiments, TSB with 10% bile salts was inoculated with approximately 10^6 CFU/ml and numbers of CFU/ml determined periodically during static incubation at 37°C.

Isolation of Bile Salts Tolerant and Sensitive Cells

Bile salts (Sigma Aldrich, St. Louis, MO, United States) were an equal mixture of sodium cholate and sodium deoxycholate. Bile salts added to TSB were sterilized by filtration (0.22 µm-pore filter). A diagram of the isolation of bile salts tolerant cells is shown in Figures 2A,B. Overnight cultures were diluted in 0.01 M phosphate-buffered saline (PBS; pH 7.4), plated on tryptic soy agar (TSA; Becton Dickinson), and incubated at 37°C for 24 h. Individual colonies (288 per trial) were inoculated.

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in 200 µl of TSB in 96-well plates (Becton Dickinson) and incubated at 37°C for 24 h. Cells from each well were diluted and inoculated into TSB with 10% bile salts at a final concentration of approximately 10^5 CFU/ml (Figure 2A). Plates were incubated at 37°C for 48 h, and growth was monitored by measuring optical density (OD_{600nm}) every hour using a Bioscreen C (Growth Curves USA, Piscataway, NJ, United States). Wells exhibiting a change in OD_{600nm} greater than 0.1 units from the baseline reading were considered positive for growth. Cells from growth-positive and growth-negative wells were recovered and stored at −70°C.

**Survival in Bile Salts**

Survival in TSB with 10% bile salts was monitored by inoculating approximately 10^6 cells of E40 or E40V and determining the number of CFU/ml with LB agar plates after 3, 16, and 20 h of incubation at 37°C.

**Antibiotic Susceptibility Testing**

Cells from overnight cultures were spread with a swab on LB agar plates to create lawns. Discs of Whatman filter paper containing ampicillin (10 µg/disc), novobiocin (0.5 µg/disc), or polymyxin B (1 µg/disc) were placed on the swabbed plates. Plates were incubated at 37°C for 18 h, and zones of growth inhibition were measured.

**Pulsed-Field Gel Electrophoresis**

Exponential phase cells were harvested, washed with 1 M NaCl and 10 mM Tris–HCl, and suspended in a 1% low melting agarose (Gibco Brl, Gaithersburg, MD, United States). Cells were lysed and washed as previously described (Tamplin et al., 1996; Buchrieser et al., 1997). Genomic DNA in agarose plugs was digested with XbaI (New England Biolabs, Ipswich, MA, United States) at 37°C for 20 h. High molecular weight fragments were resolved using a 1.3% ultrapure agarose gel (J. T. Baker, Philipsburg, NJ, United States) in a CHEF-DR II pulsed-field system (Bio-Rad Laboratories, Richmond, CA, United States). Electrophoretic parameters were 6 V/cm for 22 h at 15°C with switching times from...
FIGURE 2 | Separation and screening of bile salts tolerant and sensitive strains. (A) A stock culture of strain E40 was grown in TSB and then diluted and plated on LB agar to obtain single colonies. Individual colonies were inoculated into separate wells of a 96-well plate containing TSB and incubated overnight at 37°C. Growth from each well was diluted to approximately 10⁵ CFU/ml and used to inoculate separate wells of a Bioscreen plate containing TSB with 10% bile salts. Growth was monitored by optical density at 600 nm during incubation at 37°C for 48 h. (B) A representative sample of optical density measurements from growth-positive and -negative wells are shown. Growth varied in the length of lag phase, slope of exponential phase, and maximum optical density. (C) Cells from growth-positive wells in (B) were transferred to wells of a new plate with TSB and 10% bile salts. The lag, exponential, and stationary phases of growth were consistent among bile salts tolerant cells.

DNA Sequencing and Analysis
E40 and E40V were sequenced by Illumina and PacBio SMRT sequencing. For Illumina sequencing, E40 and E40V cultures were individually inoculated into LB broth directly from frozen stock cultures. Following incubation overnight at 37°C, cells were harvested by centrifugation. DNA was prepared using MasterPure Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, United States). RNA was removed by the addition of RNase A (Thermo Fisher Scientific, Waltham, MA, United States) and incubation for 30 min at 37°C. The manufacturer’s protocol was modified to include a step to precipitate DNA by overnight incubation at 70% ethanol at −20°C. DNA was submitted to the University of Wisconsin-Madison Biotechnology Center for sequencing. DNA concentration was verified using the Qubit® dsDNA HS Assay Kit (Life Technologies, Grand Island, NY, United States) and prepared with a TruSeq Nano DNA LT Library Prep Kit (Illumina Inc., San Diego, CA, United States) with minor modifications. Samples were sheared using a Covaris M220 Ultrasonicator (Covaris Inc., Woburn, MA, United States), and size selected for an average insert size of 550 bp using SPRI bead-based size exclusion. The quality and quantity of the finished libraries were assessed using an Agilent High Sensitivity DNA kit and Qubit® dsDNA HS Assay Kit, respectively. Libraries were standardized to 2 nM and paired-end 250 bp sequencing was performed using an Illumina MiSeq Sequencer and a MiSeq 500 bp (v2) sequencing cartridge. Images were analyzed using the standard Illumina Pipeline, version 1.8.2.

Genomic DNA for PacBio sequencing was purified from overnight cultures that were harvested by centrifugation and washed four times with sterile 10% glycerol. DNA from washed cell pellets was purified by CTAB DNA extraction (William et al., 2004). Standard Pacific Biosciences large insert library preparation was performed at the UW-Milwaukee Great Lakes Genomics Center. DNA was fragmented to approximately 20 kb using Covaris G tubes (Covaris, Woburn, MA, United States). Fragmented DNA was enzymatically repaired and ligated to a PacBio adapter to form the SMRTbell Template. Templates larger than 10 kb were size selected by BluePippin (Sage Science, Beverly, MA, United States). Templates were annealed to sequencing primer, bound to polymerase (P6) and then PacBio Mag – Beads and SMRT cell, and sequenced using C4 chemistry.

Genome assemblies were created from PacBio reads using SPAdes (Bankevich et al., 2012). The resulting contigs underwent iterative improvement using Pilon (Walker et al., 2014) utilizing short-read Illumina data that was corrected using the BayesHammer module (Nikolenko et al., 2013) included in SPAdes 3.11.1. Annotations were generated using Rapid Annotation Using Subsystem Technology (RAST) (Aziz et al., 2008; Overbeek et al., 2014; Brettin et al., 2015).
Comparisons between E40 and E40V were performed using Mauve (Darling et al., 2010), BLAST (Madden, 2002), MUSCLE (Edgar, 2004), and IGV (Robinson et al., 2012). Regions containing differences in the E40 and E40V assemblies were amplified by PCR and sequenced by Sanger sequencing (Sanger et al., 1977) at the UW-Madison Biotechnology Center. Primers used are shown in Supplementary Table 1.

mRNA Analysis
RNA was purified using standard Trizol purification (Rio et al., 2010) from cells grown to OD600nm = 0.3 in TSB (pH 7.3). Total RNA was submitted to the University of Wisconsin-Madison Biotechnology Center and verified for purity and integrity via the NanoDrop2000 Spectrophotometer and Agilent 2100 BioAnalyzer, respectively. Samples that met the Illumina sample input guidelines were prepared according to the TruSeq® RNA Sample Preparation Guide (Rev. C, May 2012). For each sample, 2 µg of total RNA underwent ribosomal RNA (rRNA) reduction using the EpiCentre Ribo-Zero™ rRNA Removal (Gram-Negative Bacteria) kit (EpiCentre Inc., Madison, WI, United States). The rRNA-depleted samples were purified by ethanol precipitation and quantified on the Qubit fluorimeter with the Qubit® RNA Assay Kit (Invitrogen, Carlsbad, CA, United States). rRNA-depleted samples (60 ng) were fragmented, using divalent cations under elevated temperature, and immediately reverse transcribed into double-stranded (ds) cDNA using SuperScript II Reverse Transcriptase (Invitrogen) and random primers. The ds cDNA was purified with paramagnetic beads, end-repaired by T4 DNA polymerase and Klenow DNA Polymerase, and phosphorylated by T4 polynucleotide kinase. The blunt ended cDNA was purified with paramagnetic beads and then incubated with Klenow DNA Polymerase to add an “A” base (Adenine) to the 3’ end of the blunt phosphorylated DNA fragments. DNA fragments were ligated to Illumina adapters, which have a single “T” base (Thymine) overhang at their 3’ end. The adapter-ligated products were again purified with paramagnetic beads. Products of the ligation were PCR-amplified with Phusion DNA polymerase and Klenow DNA Polymerase using Illumina’s genomic DNA primer set targeting (Edgar, 2004), and IGV (Robinson et al., 2012). Regions containing differences in the E40 and E40V assemblies were amplified by PCR and sequenced by Sanger sequencing (Sanger et al., 1977) at the UW-Madison Biotechnology Center. Primers used are shown in Supplementary Table 1.

Acid Challenge
TSB (pH 7.8) was inoculated 1:500 from overnight cultures. Cultures were grown at 37°C with shaking (100 rpm) to OD600nm = 0.2. Cells were diluted and inoculated in LB pH 3.0, which resulted in a final challenge pH of 3.35. Cultures were incubated statically at 37°C for 6 h. Samples were serially diluted in 0.1% peptone (pH 7.0), plated on LB agar, incubated overnight at 37°C, and the number of colony-forming units enumerated.

Detection of CBPS
Tryptic Soy Agar (MP Biomedicals, Solon, OH, United States) plates containing 1% calcilflour white were spotted with 2 µl of overnight culture. Plates were incubated at 37°C for 3 days. Calcilflour incorporation was visualized with long-wave UV light and images recorded using an Olympus E-PM1 camera (Richter et al., 2014; Hawkins et al., 2017). Staining was quantified using the Fiji distribution of ImageJ (Schindelin et al., 2012; Schneider et al., 2012).

Extraction and LPS Examination
Cells from overnight cultures grown in LB were normalized to OD600nm = 1.0 and collected by centrifugation. LPS was extracted with hot aqueous-phenol as described previously (Davis Jr. and Goldberg, 2012). Extracted LPS was separated on a Tris-Tricine gel (Lesse et al., 1990) and visualized using a Pierce Silver Stain kit (Thermo Fisher Scientific, Waltham, MA, United States). Quantification of band intensity was performed using the Fiji distribution of ImageJ (Schindelin et al., 2012; Schneider et al., 2012).

Engineering of E40E
Strain E40E was engineered using No-SCAR genome editing (Reisch and Prather, 2015). pKDsAgRNA:LptG was generated using CPEC (Reisch and Prather, 2017). The repair template (Supplementary Table 1) was designed to introduce the desired SNP as well as an additional synonymous SNP to reduce cas9 targeting (Table 2). This change also created a Bsr1 (NEB) cut site, which was used to identify recombinants. One out of 72 recombinants screened was positive by Bsr1 digestion and was confirmed to have the desired modification by Sanger sequencing (Sanger et al., 1977).

| Strain | LptG position |
|--------|---------------|
|        |         |
| E40    | amino acids  |
|        | nucleotides  |
|        | TTC         |
|        | ATG         |
|        | CTG         |
|        | GTG         |
|        | TCG         |
| E40V   | amino acids  |
|        | nucleotides  |
|        | TTC         |
|        | ATG         |
|        | CGG         |
|        | GTG         |
|        | TCG         |
| E40E   | amino acids  |
|        | nucleotides  |
|        | TTC         |
|        | ATG         |
|        | CCA         |
|        | GTG         |
|        | TCG         |
**Statistical Analysis**

The number of independent biological replicates conducted is presented within individual figure legends. Standard error is shown. Alignments of RNA sequencing reads were created using Rsubread (Liao et al., 2013) and statistical comparisons were performed using DESeq2 using the nbinomWaldTest (Love et al., 2014). For RNAseq experiments, Benjamini-Hochberg adjusted p values <0.05 were considered significant. RT-qPCR data were analyzed (Pfaffl, 2001) with two reference genes. P values were determined using the student's t test (2-tailed).

**RESULTS**

**Heterogeneity in *S. enterica* Growth in TSB With 10% Bile Salts**

Two hundred and eighty-eight colonies from *S. Typhimurium* culture E40 were propagated overnight, diluted to approximately 10^3 CFU/ml, and inoculated in TSB with 10% bile salts (Figure 2A). Growth was monitored by optical density (OD_{600nm}) of individual wells over 48 h. Fifty six percent of the wells had growth that varied in the length of lag phase, slope of exponential phase, and maximal optical density achieved (Figure 2B). Cells from growth-positive wells retained their ability to grow in the presence of 10% bile salts but had a shorter lag phase and more uniform growth than the initial growth curves (Figure 2C). The frequencies of growth-positive colonies from *S. enterica* Typhimurium strain M-09-0001A-1 (42%) and *S. enterica* Enteriditis strain FRick671 (64%), were similar to *S. enterica* Typhimurium E40 (56%). In contrast, *S. enterica* Tennessee strain 4539H had a nearly 100-fold lower frequency of growth positive colonies (0.7%). In comparison to *S. enterica* Typhimurium strain E40 (MIC 8%), strain E40V had an especially high bile salts MIC (>20%), which was the highest concentration of bile salts that could be dissolved in TSB without formation of precipitate.

**Strains E40 and E40V Differ by a Single Nucleotide Polymorphism (SNP)**

Strains E40 and E40V were both serotyped and confirmed as serovar Typhimurium. Additionally, pulsed-field gel electrophoresis of XbaI digests (Supplementary Figure 1) did not show any discernible chromosome differences between E40 and E40V. Results from serotyping and pulsed-field gel electrophoresis ruled out that the culture was a mixture of different serovars or Typhimurium strains. To determine if genetic differences accounted for the discrepancy in bile salts tolerance, paired-end Illumina and PacBio sequencing of E40 and E40V was performed. Illumina sequencing resulted in 114× and 113× coverage of E40 and E40V, respectively (Supplementary Table 4A). PacBio sequencing resulted in average read lengths of 8,020 and 8,908 bp, with coverage of 189× and 164× for E40 and E40V, respectively (Supplementary Table 4B). Assembly of PacBio reads for E40 and E40V both resulted in two contigs, representing the chromosome and one plasmid. These contigs were polished using paired-end Illumina reads (Supplementary Table 4C). The E40 chromosome assembly (4,890,368 bp) was 30 bp longer than the E40V chromosome assembly (4,890,338 bp). The plasmid assemblies for both E40 and E40V were identical (94,009 bp). The E40 (NCBI accession number CP038432-CP038433) and E40V (NCBI accession number CP038434-CP038435) chromosome and plasmid assemblies were compared using a combination of Mauve (Darling et al., 2010), BLAST (Madden, 2002), MUSCLE (Edgar, 2004), and IGV (Robinson et al., 2012). Six regions were identified outside of rRNA and tRNA regions with nucleotide differences, ranging from individual SNPs to a 4-codon deletion. Sanger sequencing was used to reexamine the putative differences. Five of the six differences were not confirmed by Sanger sequencing and were considered assembly errors (Supplementary Table 5). These assembly errors accounted for 16 bp of the 30 bp difference between the E40 and E40V chromosome assemblies, and the remaining difference in assembly length was attributed to putative assembly errors in rRNA and tRNA regions. Analysis of the assembly errors revealed that a majority were either in genes that had homologs elsewhere in the genome or were in repetitive regions. The remaining difference was a non-synonymous SNP in the LPS transport gene *lptG* that changed amino acid 26 from a leucine to a proline (p.leu26pro) (Table 2). Analysis of *Salmonella* genomes in the National Center for Biotechnology Information (NCBI) database did not find a match to the E40V *lptG* sequence.

**E40 and E40V Were Present in the Parent Culture**

To assess if the SNP found in E40V was present in the parent culture prior to exposure to 10% bile salts, Illumina reads used for transcriptional analysis were analyzed for the presence of the SNP. Reads (726) from E40 replicates were identified that aligned to *lptG*. Two of these reads, each from separate replicates, contained the E40V SNP, indicating the E40V variant was present before exposure to bile salts.

**LtpG (p.leu26pro) Was Responsible for the Observed Phenotypic Differences**

To determine if the SNP in *lptG* was responsible for the observed difference in bile salts tolerance, strain E40 was engineered to create strain E40E. Strain E40E encoded for LtpG with a proline at position 26, matching the E40V *lptG* sequence (Table 2). Similar to E40V, strain E40E grew in TSB with 10% bile salts while E40 did not when inoculations ranged from 10^3 to 10^5 CFU/ml. We hypothesized that LtpG (p.leu26pro) would increase viability during initial exposure to bile salts; however, the survival of E40V (0.27 ± 0.05%) was significantly lower (p = 0.01) after 3 h in TSB with 10% bile salts than E40 (0.81 ± 0.11%) (Figure 3A). After 16 hours in TSB with 10% bile salts, E40V (6.6 × 10^7 CFU/ml) had significantly more growth (p = 7 × 10^-6) than E40 (3.3 × 10^4 CFU/ml) (Figure 2B). At 20 h, E40 and E40V had equivalent numbers of CFU/ml when inoculated with CFU/ml (Figure 3C). Adaptive responses to bile salts can increase resistance to antibiotics (Prouty et al., 2004a) and differences in susceptibility to hydrophobic antibiotics indicate differences in
the outer membrane composition or LPS modifications (Delcour, 2010). The hydrophobic antibiotic novobiocin created a zone of inhibition in E40V (12.6 ± 0.5 mm); whereas there was no zone of inhibition with E40 (p < 0.01) (Table 3). Like E40V, the engineered strain E40E was inhibited by novobiocin (11.1 ± 0.3 mm). Polymyxin B and ampicillin were also inhibitory but the zones of inhibition were not significantly different between strains.

Lipopolysaccharides was extracted and visualized by electrophoresis and silver staining to determine if LptG p.leu26pro affected the makeup and quantities of LPS components. In general, the LPS profiles were similar although quantification of the gray scale values of the imaged gel indicated minor differences between strain E40 and strains E40V and E40E. From pixels 1,000–15,000, strains E40E and E40V had peaks and a declining plateau with gray values ranging from 140 to 120 while the peaks and plateau from strain E40 remained nearly constant with gray values around 120 (Figure 4).

**Steady State Levels of yjbE, yciE, and Acid Shock Protein Precursor (ASPP) Transcripts Were Greater in E40V and E40E**

To determine if there were transcriptional differences that contributed to the difference in bile salts tolerance, RNAseq was used to examine exponential-phase cultures of E40 and E40V grown in TSB with no bile salts. Analysis was conducted in the absence of bile salts since the initial separation of E40 and E40V (separate colonies Figure 2A) was conducted in the absence of bile salts. Three genes were identified as differentially expressed in E40 and E40V (Table 4). yjbE (involved in EPS production), yciE (a putative stress response gene), and ASPP (promotes acid tolerance) were expressed at log₂ values of 2.0, 3.0, and 1.2 greater in E40V than E40, respectively. No transcripts were present at a significantly greater level in E40 compared to E40V. Greater steady state levels of transcripts were confirmed by RT-qPCR using independently extracted RNA samples, with log₂ values of 2.6, 1.1, and 0.4 for yjbE, yciE, and ASPP, respectively (Table 4). The role of the LptG SNP (p.leu26pro) in the differences in transcript levels of these three genes was confirmed by RT-qPCR using RNA isolated from E40E. Like E40V, there were greater steady state levels of yjbE, yciE, and ASPP mRNA in E40E compared to E40 with log₂ values of 1.6, 2.6, and 0.34, respectively (p = 1.5×10⁻⁵, p = 6.6×10⁻⁴, and p = 0.1; respectively). These

**TABLE 3 | Zones of inhibition of S. enterica strains E40, E40V and E40E with the following antibiotics: novobiocin (1.5 µg/disc), polymyxin B (3 µg/disc), and ampicillin (10 µg/disc).**

| Strain | Novobiocin | Polymyxin B | Ampicillin |
|--------|------------|-------------|------------|
| E40    | 0.0        | 12.2 +/- 0.3| 25.8 +/- 0.3|
| E40V   | 12.6 +/- 0.5| 12.9 +/- 0.1| 26.0 +/- 0.8|
| E40E   | 11.1 +/- 0.3| 12.4 +/- 0.2| ND         |

*aE40 significantly different from E40V and E40E (p < 0.01), n=4.

Values are in mm +/- the standard error.

*ND, not determined.*
results confirm that the differences in the level of these gene transcripts in E40V were linked to the SNP in lptG.

**LptG (p.leu26pro) Had Greater Acid Tolerance and Calcofluor-Binding Polysaccharides**

Based on the differences in steady state level of transcripts, additional phenotypic characterization of E40, E40V, and E40E was performed. Both E40V and E40E showed greater levels of transcript for the gene encoding for the ASPP, which is implicated in acid tolerance (Table 4). Acid challenge (pH 3.35) of E40, E40V, and E40E found that the strains E40V (1.8 ± 0.5%) and E40E (2.25 ± 0.4%) had statistically greater survival (p = 0.02 and p = 0.003, respectively) than E40 (0.3% ± 0.09%) (Figure 5). Likewise, transcriptional analyses found a greater quantity of yjbE transcript; therefore, calcofluor-binding polysaccharides (CBPS) were visualized and quantified using the fluorescent polysaccharide stain calcofluor white. Visual examination showed E40V and E40E colonies had a brighter and wider band of fluorescence than E40 colonies. Measurement of inverted gray value saturation showed values of E40 colonies (0.0069 ± 8.7×10⁻⁵) were significantly less than E40V (0.0073 ± 1.1×10⁻⁴) and E40E (0.0076 ± 1.6×10⁻⁴) (Figure 6). Characterization of E40 and E40V identified three distinguishing phenotypes (growth in TSB with 10% bile salts, acid tolerance, and staining of CBPS). Strain E40E (p.leu26pro) had the same phenotypes as E40V demonstrating that the SNP in lptG was linked to the observed phenotypes.

![FIGURE 4](image-url) LPS profiles and scans from E40, E40V, and E40E. (A) Cells were harvested, the LPS extracted with hot aqueous phenol, separated in a Tris-Tricine gel, and visualized by silver staining. (B) The LPS profiles were scanned using ImageJ and bands assessed by gray scale.
**DISCUSSION**

*Salmonella enterica* is a pervasive pathogen that encounters a variety of unfavorable conditions in the environment and hosts. *Salmonella* has multiple stress tolerance pathways, but the outer membrane is an important first line of defense against environmental stressors. This study demonstrated that a SNP (p.leu26pro) in the gene encoding for lipopolysaccharide transport protein G (*lptG*) found in strain E40V increased tolerance to two important stressors (bile salts and acid) that *Salmonella* encounters during human infection. The SNP also increased staining of CBPS by calcofluor. Although strain E40V was able to grow in the presence of 10% bile salts within 48 h, it increased staining of CBPS by calcofluor. Although strain E40V was able to grow in the presence of 10% bile salts within 48 h, it had lower initial viability in TSB with 10% bile salts compared to E40 (Figure 3A). E40 did not grow in 10% bile salts when inoculated at 10⁵ CFU/ml but did when inoculated at 10⁹ CFU/ml (Figure 3C). The survival curve of E40V was characterized by a die-off followed by the outgrowth of a few survivors that is characteristic of the “phoenix phenomenon” that has been described previously (Mellefont et al., 2005). These findings suggest that E40V was not more tolerant to bile salts but rather a few survivors had adapted in a manner that permitted their growth. A possible contributing factor to these observations was E40V had greater steady state level of transcripts from three genes (*yjbE, yciE*, and a gene encoding for acid shock precursor protein) in comparison to strain E40 that corresponded with the observed phenotypes in E40V. The ability of E40V to survive and grow in TSB with 10% bile salts could be driven by the protein products of these genes. The mechanism by which LptG p.leu26pro gives rise to an increase in these three transcripts remains unresolved and is the subject of ongoing research.

While knocking out or knocking down Lpt pathway proteins is deleterious or even lethal to bacteria (Ruiz et al., 2008; Chimalakonda et al., 2011), alterations of Lpt pathway proteins can mitigate other mutations or increase stress tolerance. In *Burkholderia cenocepacia*, LPS is modified with the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N), which confers resistance to antimicrobial peptides. Knocking out the pathway for L-Ara4N modification generates nonviable cells; however, a SNP in *lptG* restores viability by allowing the export of LPS that does not have the L-Ara4N modification (Hamad et al., 2012). Alteration of LptG in *Pseudomonas aeruginosa* increases susceptibility to some classes of antibiotics, while inducing hypersensitivity to other antibiotics (Harrison et al., 2019). In *Salmonella*, single codon deletions and SNPs in *lptC* and *lptE* increase tolerance to bile salts. Analysis of LPS profiles of the *lptC* and *lptE* mutants found differences in polysaccharide chain length, compared to wild type LPS (Hernández et al., 2012). These studies demonstrate that SNPs in *lpt* genes result in minor changes to Lpt proteins that transport LPS molecules with structural variations that alter the chemical composition or charge of the outer membrane that can impact permeability and stress tolerance.

Analysis of DNA sequence data obtained from multiple sequencing technologies (PacBio, Illumina, and Sanger) identified a single non-synonymous SNP in *lptG* that changed position 26 from a leucine (E40) to a proline (E40V). Phenotypic characterization revealed that this SNP had pleiotropic effects. As has been shown with *lptC* and *lptE*, the mutation in *lptG* found in E40V increased tolerance to bile salts. Additionally, E40V had increased acid tolerance (Figure 5) and staining of calcofluor-binding polysaccharides (CBPS) (Figure 6), when compared to E40. RT-qPCR analysis of RNA from exponential-phase cells showed a greater level of transcripts from three genes (*yciE, yjbE*, and ASPP) in E40V and E40E, compared to E40 (Table 4). The predicted function of these genes corresponded

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**TABLE 4** | Strain E40V genes with greater steady state levels of mRNA than strain E40.

| Gene | Function | RNAseq | RT-qPCR^a |
|------|----------|--------|-----------|
|      |          | log₂-fold change^b | p value^c | log₂-fold change^b | p value^c |
| yjbE | EPS^2 production | 2.0 | 4.8 × 10⁻⁷ | 2.6 | 2.6 × 10⁻⁴ |
| yciE | putative stress response protein | 3.0 | 1.1 × 10⁻⁵ | 1.1 | 1.3 × 10⁻³ |
| ASPP | acid tolerance | 1.2 | 2.8 × 10⁻² | 0.4 | 6.8 × 10⁻² |

^a Reverse transcriptase quantitative PCR.

^b Average log₂-fold change from three biological replicates, values are levels in E40V compared to E40.

^c Benjamini-Hochberg adjusted (Benjamini and Hochberg, 1995). Calculated using student’s t-test.

^d Extracellular polymeric substance.

^e Acid shock protein precursor.

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**FIGURE 5** | Survival of strains E40, E40V, and E40E at pH 3.0. Strains were inoculated into TSB at pH 3.0 and the number of CFU/ml determined after 6 h of incubation at 37°C. Percent survival was determined using the number of CFU/ml following inoculation and after 6 h of incubation. The average values from four trials are shown. Error bars are the standard error of the means. *p = 0.02 between E40 and E40V. **p = 0.003 between E40 and E40E.
with the observed phenotypic differences between E40 and the LptG variants E40V and E40E.

Other studies found that changes in Lpt pathway proteins can alter the LPS present in a strain (Hernández et al., 2012; Bertani et al., 2018). Analysis of LPS from E40, E40V, and E40E using a similar method found only minor differences in the staining intensity of LPS O antigen side chains (Figure 4). Changes to LPS can alter stress tolerance and resistance to antibiotics (Delcour, 2010) similar to the decline in CFU/ml of E40V in bile salts containing medium (Figure 3) and the sensitivity to novobiocin (Table 3). It is unlikely that minor differences in O antigens alter permeability to novobiocin (Sheng et al., 2008); whereas, LPS that is unmodified by PhoPQ allows novobiocin to pass through the membrane (Nobre et al., 2015).

It is most likely that LptG (p.leu26pro) resulted in the transport of unmodified or modified LPS that resulted in a membrane permeable to bile salts and novobiocin (Bertani et al., 2018). The sensitivity of E40V and E40E to novobiocin demonstrated the outer membranes differed from E40 and that this difference permitted novobiocin permeability which is typically prevented by PhoPQ-modified LPS (Nobre et al., 2015). The proline in LptG (p.leu26pro) is seven residues away from a series of lysine residues in transmembrane alpha helices that are critical for LPS binding (Dong et al., 2017; Bertani et al., 2018). Proline can disrupt alpha helices (Richardson, 1981) but it is unlikely that LptG is completely inactivated by proline since it is essential, but it could influence the type of LPS transported, possibly by altering selectivity. One of the more interesting phenotypes associated with the SNP in lptG was the observed differences in the transcripts of three genes in E40V. It is unknown how the SNP resulted in differences in the expression or stability of these transcripts but changes in membrane permeability could contribute to the observed difference.

**yciE** is in an operon with catalase **katN** and **yciGF** that contains a putative RpoS-regulated promoter (Robbe-Saule et al., 2001). The **yciGFE-katN** operon is upregulated in response to bile, likely in an RpoS independent manner (Prouty et al., 2004b); however, the greater level of **yciE** detected in these studies was in the absence of bile salts in exponential-phase cells. **yciE** is annotated as a putative DNA damage stress response gene and bile salts can cause DNA damage (Merritt and Donaldson, 2009); therefore, YciE might contribute to bile salts tolerance noted in E40V. The second gene that was expressed at a greater level in E40V and E40E was **yjbE**. This gene is part of the EPS production operon **yjbEFGH** and partially regulated by the RcsCDB phosphorelay pathway. Due to differences in stability, **yjbE** mRNA is often detected in greater amounts than **yjbFGH** mRNAs (Ferrières et al., 2007). The differences in mRNA stability could mean that the entire yjb operon was upregulated in E40V and E40E, but only **yjbE** mRNA was detected. Upregulation of this operon likely explains the increase in staining of CBPS in E40V and E40E in comparison to E40. Alternatively, LptG (p.leu26pro) might export atypical LPS that enhances staining of CBPS. Unlike **yciE** and **yjbE**, the gene encoding for ASPP is mostly uncharacterized and is described in NCBI as required for adaptive acid tolerance. Increased expression of ASPP is likely responsible for the observed increase in acid tolerance in E40V and E40E. ASPP expression is in part controlled by RpoN (nitrogen limitation sigma factor, $\sigma^{54}$) in an indirect manner (Bono et al., 2017).
The regulation of \( yciE, yjbE, \) and ASPP has been partially attributed to RpoS, Rcs phosphorelay, and RpoN, respectively. Changes in the cellular envelope could trigger intracellular stress and RpoS production as well as the Rcs phosphorelay signaling (Majdalani and Gottesman, 2005) that would account for the greater levels of \( yciE \) or \( yjbE \). However, an increase in RpoS or Rcs should result in more extensive transcriptional differences than were observed. Similarly, regulation of ASPP by RpoN would also cause additional transcriptional differences that were not observed (Bono et al., 2017). Interestingly, the regulation of \( yciE, yjbE, \) and ASPP has only been partially attributed to the above-mentioned regulators, and the regulation of \( yciE \) in response to bile salts is likely independent of RpoS (Prouty et al., 2004b). It is possible that a yet to be described effector could be responsible for the upregulation of these genes in E40V. Although \( yciE, yjbE, \) and ASPP are not positioned close enough in the chromosome to be within the same operon, they may share an undescribed regulatory motif that allows for coregulation. Further elucidation of the relationship between the SNP in \( lptG \) and differences in the level of these three transcripts will be a subject of future work.

Engineering the substitution of proline for leucine at LptG position 26 resulted in all of the phenotypes reported for E40V confirming the role of the SNP in the observed phenotypes. While mutations in Lpt pathway proteins that enhance bile salts tolerance can be selected for in experimental systems, there may not be a strong selective pressure for these mutations in hosts or natural environments. During infection, \( \text{Salmonella} \) encounters bile concentrations ranging from 0.2 to 2% in the small intestine to as high as 8% in the gall bladder (Gunn, 2000). However, the E40V variant \( lptG \) sequence was not identified in the NCBI database that contains a large number of \( \text{Salmonella} \) genomes indicating this form of LptG is not beneficial in natural populations or may even be deleterious.

Clonal and near clonal \( \text{Salmonella} \) populations can contain phenotypically distinct subpopulations, created through a variety of mechanisms including inversions of promoter sequences (Andrewes, 1922; Stocker, 1949; Bonifield and Hughes, 2003; Aldridge et al., 2006; Stewart and Cookson, 2014), SNPs (Piddock et al., 1998; Giraud et al., 1999; Levy et al., 2004; Le et al., 2007; Hernández et al., 2012), and heritable methylation patterns (Broadbent et al., 2010; Cota et al., 2015; Cota and Casadesús, 2016). Next Generation Sequencing (NGS) technologies make genetic and epigenetic (Pirone-Davies et al., 2015) comparisons possible, and these analyses can be combined with traditional bench techniques. While NGS approaches have clear advantages, they can also have limitations. Our assemblies produced using both PacBio and Illumina reads, with a total coverage nearing 300x, contained multiple errors that were rectified by Sanger sequencing. Many of the errors were in genes that had closely related homologs elsewhere in the genome or were in repetitive nucleotide regions. The error rate achieved in our assemblies was likely acceptable for many applications; however, the results from this study demonstrate that a single base pair change can have broad phenotypic effects. Comparative analyses using assembled genomes should take into account the possibility of assembly errors.

\( \text{Salmonella enterica} \) utilizes phenotypic heterogeneity to enhance fitness for a varied hosts and environments. In this study, it was demonstrated that a SNP in the LPS transport gene \( lptG \) resulted in transcriptional differences in three genes that were linked to identified phenotypes. Additionally, the SNP resulted in an outer membrane that rendered strains E40V and E40E susceptible to novobiocin. Future studies will focus on deciphering the mechanism driving the observed differences in the levels of \( yjbE, yciE, \) and ASPP transcripts.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, CP038432; https://www.ncbi.nlm.nih.gov/genbank/, CP038433; https://www.ncbi.nlm.nih.gov/genbank/, CP038434; https://www.ncbi.nlm.nih.gov/genbank/, CP038435.

**AUTHOR CONTRIBUTIONS**

CK and AW developed the concept of the study. ES and TW assembled the DNA sequence data into draft genomes, conducted genome comparisons, and analyzed RNAseq data. TW, JG, and AS performed the experiments and conducted statistical analyses. TW and CK drafted the manuscript. All authors have read and approved the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.671453/full#supplementary-material

Supplementary Figure 1 | Comparison of Xba1 digests of \( S. \) enterica serovar Typhimurium strains E40 and E40V by pulsed field electrophoresis. A lambda ladder was used as a molecular size marker (kb).
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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