Intracellular d-Serine Accumulation Promotes Genetic Diversity via Modulated Induction of RecA in Enterohemorrhagic Escherichia coli

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ABSTRACT
We recently discovered that exposure of enterohemorrhagic Escherichia coli (EHEC) to d-serine resulted in accumulation of this unusual amino acid, induction of the SOS regulon, and downregulation of the type III secretion system that is essential for efficient colonization of the host. Here, we have investigated the physiological relevance of this elevated SOS response, which is of particular interest given the presence of Stx toxin-carrying lysogenic prophages on the EHEC chromosome that are activated during the SOS response. We found that RecA elevation in response to d-serine, while being significant, was heterogeneous and not capable of activating stx expression or stx phage transduction to a nonlysogenic recipient. This “SOS-like response” was, however, capable of increasing the mutation frequency associated with low-level RecA activity, thus promoting genetic diversity. Furthermore, this response was entirely dependent on RecA and enhanced in the presence of a DNA-damaging agent, indicating a functional SOS response, but did not result in observable cleavage of the LexA repressor alone, indicating a controlled mechanism of induction. This work demonstrates that environmental factors not usually associated with DNA damage are capable of promoting an SOS-like response. We propose that this modulated induction of RecA allows EHEC to adapt to environmental insults such as d-serine while avoiding unwanted phage-induced lysis.

IMPORTANCE
The SOS response is a global stress network that is triggered by the presence of DNA damage due to breakage or stalled replication forks. Activation of the SOS response can trigger the replication of lytic bacteriophages and promote genetic diversification through error-prone polymerases. We have demonstrated that the host-associated metabolite d-serine contributes to Escherichia coli niche specification and accumulates inside cells that cannot catabolize it. This results in a modulated activation of the SOS antirepressor RecA that is insufficient to trigger lytic bacteriophage but capable of increasing the SOS-associated mutation frequency. These findings describe how relevant signals not normally associated with DNA damage can hijack the SOS response, promoting diversity as E. coli strains adapt while avoiding unwanted phage lysis.

The bacterial SOS response is a global network of DNA repair proteins that is triggered in the presence of DNA replication inhibition, DNA strand breaks, or DNA-damaging agents such as antibiotics, UV light, reactive oxygen species, and pressure (1, 2). The SOS regulon is under the control of the LexA repressor and consists of >40 genes in Escherichia coli (1, 3–5). Upon DNA damage, the LexA antirepressor RecA is activated by forming a filament structure with single-stranded DNA and promotes autocatalytic cleavage of LexA, thus relieving the repression of SOS-regulated genes (6–9). A second mechanism of SOS induction has also been described that involves the two-component sensor DpiAB, which activates the SOS network in response to β-lactam antibiotics (10). This induction is independent of DNA damage but instead is in response to replication inhibition by DpiA overproduction and results in transient halting of replication, allowing bacteria to survive otherwise lethal doses of antibiotics. Induction of the SOS response can also have further effects on the cell, including activation of the lytic life cycle of lysogenic bacteriophages (11, 12). Temperate lysogenic prophages are carried on bacterial chromosomes and are repressed against replication by the cI phage repressor, a structural homologue of LexA. During the SOS response, activated RecA can also cleave cI, forcing the phage out of latency and thus resulting in lysis of the host cell (13–15). Furthermore, bacteriophages also often carry virulence genes and therefore can act as shuttles of horizontal gene transfer once activated (16). Enterohemorrhagic E. coli (EHEC) is a Gram-negative food-borne pathogen associated with severe diarrheal illness in humans worldwide (17). The Centers for Disease Control and Prevention estimates >260,000 EHEC infections each year in the United States alone, with the common O157:H7 serotype being responsible for ~36% of these (www.cdc.gov). The natural host for EHEC is ruminants, particularly cattle, where the organism asymptptomatically colonizes the recto-anal junction (18, 19), which acts as a reservoir for indirect transmission to the human host through contamination of meat, fresh produce, and water sources. Cattle feces have been reported to harbor as many as 10⁶ CFU of EHEC/g, and the infectious dose of EHEC in humans can be extremely low (10 to 100 bacteria of the most common O157:H7 serotype) (20). Disease progression of EHEC infections can range...
from mild diarrhea to hemorrhagic colitis and, in extreme cases, the potentially life-threatening hemolytic-uremic syndrome.

EHEC pathogenesis is mediated by a number of virulence factors that are carried primarily by horizontally acquired genomic elements (21, 22). The locus of enterocyte effacement is a large pathogenicity island containing five polycistronic operons that are responsible for the formation of a type III secretion system (T3SS), which facilitates attachment to host cells and translocation of numerous effector proteins into the host cell cytosol (23–25). This complex process ultimately results in the modulation of diverse host signaling pathways, leading to rearrangement of the host cell actin cytoskeleton around the bacterial site of attachment, forming a raised pedestal upon which the EHEC cell resides that is known as an attaching and effacing lesion (26). The second major virulence factor carried by EHEC is the Shiga-like toxin (Stx), of which there are a number of variants that are carried by lambdoid bacteriophages (27). Stx is an Aβ-type cytotoxin composed of the enzymatically active A subunit noncovalently linked to the B unit pentamer (28). The B subunit recognizes the glycolipid Gb3 receptor on Paneth cells of the intestinal epithelium, as well as kidney epithelial cells, leading to internalization of the active A subunit and cleavage of the 28S rRNA subunit, thus inhibiting protein synthesis and inducing apoptosis (19, 29). Stx is known to be the causative agent of hemolytic-uremic syndrome in infected patients (30).

Because the stx genes are carried by lambdoid bacteriophages, induction is triggered by the bacterial SOS response (13–15). For this reason, antibiotic treatment of EHEC infections is not recommended, as induction of stx-carrying phages would result in lysis of the host cell and release of the Stx toxin (27). We recently reported that D-serine, a host metabolite found in abundance at extraintestinal sites such as the urinary tract, was capable of independently repressing the T3SS and triggering transcription of the SOS regulon in EHEC O157:H7 (31). This D-serine-mediated “SOS response” is dependent on the intracellular accumulation of this amino acid by E. coli isolates that cannot catabolize it, such as EHEC, because of a lack of functional D-serine catabolic genes. In this work, we have explored the impact of the D-serine-induced SOS response on EHEC, in particular, the importance of this response for stx production. We found that levels of RecA reached by accumulation of D-serine resulted in a modulated induction of RecA that was lower than that of exposure to subinhibitory concentrations of mitomycin C (MMC). D-Serine did not result in activation of lytic stx phages but was found to induce SOS-mediated mutations caused by error-prone polymerase expression, thus resulting in genetic diversity and adaptation of exposed populations. These results demonstrate a novel method of SOS regulation and generation of genetic diversity in response to an environmentally relevant stress signal that can be encountered by EHEC.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are described in Table S1 in the supplemental material (32, 33). For liquid culture, single colonies from a pure culture on an LB agar plate were inoculated into 5 ml of LB broth and grown overnight at 37°C and 200 rpm. For growth and reporter assays, 1/100 dilution of an overnight culture was used to inoculate prewarmed minimum essential medium (MEM)-HEPES (Sigma, St. Louis, MO; catalog no. m7278) and samples were cultured at 37°C with shaking at 200 rpm. Antibiotics were used where indicated (100 μg/ml ampicillin; 30 μg/ml chloramphenicol; 40 μg/ml kanamycin; 12 μg/ml tetracycline; 100 μg/ml rifampin). D-Amino acids and MMC were used at the concentrations indicated. All of the chemicals used were purchased from Sigma.

**Generation of single gene deletions.** A nonpolar recA deletion mutant was generated by the lambda Red-mediated method (34). Briefly, the kanamycin resistance cassette from pKD4 was amplified by PCR with primers flanked by 50 bp directly homologous to flanking sequences immediately up- and downstream of the gene of interest (forward, CAAACATATGGCATATCCCGATTAAAAGGAGCTGAGCAGCTG; reverse, TAAAAAGCAAAAGGGCGCACGATTACCCGGCATGACAGGAGTAAAAGGAGCTGAGCAGCTG). The PCR product was purified by phenol-chloroform extraction and ethanol precipitated, DpnII treated, and transformed into electrocompetent TVU93-0 carrying pKD46 at a concentration of 1 μg/50 μl of cells. TVU93-0 carrying pKD46 was cultured in SOB medium at 30°C and induced with 10 mM arabinose prior to electroporation. After recovery for 2 h at 37°C in SOC medium, the reaction mixture was plated on LB agar containing 40 μg/ml kanamycin and grown at 37°C for 16 h. Phenotypically positive colonies were checked for positive gene knockouts by PCR with primers located up- and downstream of the gene of interest (forward, TGACAGGATGAAAAAGGCT; reverse, ATTCTGTACCGGATACCTCTC). Positive deletions were confirmed by isolation on kanamycin plates and subsequent sequencing. Removal of kanamycin resistance was performed by temperature selection with pCP20 (34).

**In vitro GFP reporter fusion assays of recA and stx transcription.** To assay the promoter activities of individual genes, bacterial strains of interest were transformed with plasmids carrying fusions of specific promoter regions with green fluorescent protein (GFP) (preC-ΔGFp or stxβ-ΔGFP) (35). Transformed strains were used to inoculate 10 ml of MEM-HEPES for growth analysis and cultured at 37°C with shaking (200 rpm). Samples were taken at regular intervals for measurement of population cell density (optical density at 600 nm [OD600]) and fluorescence indicative of promoter activity. Population GFP expression was measured by transferring 200-μl aliquots to a black 96-well plate for GFP fluorescence measurement (excitation at 485 nm, emission at 500 nm) with a FLUOstar Optima Fluorescence Plate Reader (BMG Labtech, United Kingdom). Background fluorescence was accounted for by measuring fluorescence from cells carrying promoterless reporter plasmids and subtracting these values from corresponding data. GraphPad Prism 5.0 (GraphPad, San Diego, CA) was used to either plot scatter curves of GFP expression or generate a standard curve of OD600 versus fluorescence and obtain values at specific OD600s for quantification and direct comparison of samples. The data are presented as the mean value ± the standard error of the mean (SEM) of at least three biological replicates.

**qRT-PCR.** Quantitative reverse transcriptase PCR (qRT-PCR) was performed with KAPA SYBR FAST Universal master mix (KAPA Biosystems, Woburn, MA) and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Total RNA was extracted with an RNeasy kit (Qiagen) in accordance with the manufacturer’s specifications. qRT-PCR analysis was carried out in a one-step reaction, i.e., cDNA synthesis, followed by qRT-PCR in accordance with the manufacturer’s specifications. Individual reaction mixtures were prepared in technical triplicate, and each gene was analyzed in biological triplicate. The gspA housekeeping gene was used as a calibrator for the analysis. qRT-PCRs were carried out with the Eco real-time PCR system (Illumina, San Diego, CA), and data were analyzed by the 2^{-ΔΔCt} method (36).

** Immunoblot analysis of whole-cell lysate.** Levels of RecA and LexA production were determined by immunoblotting of whole-cell lysates with anti-RecA and anti-LexA antibodies (Abcam, Cambridge, United Kingdom). Samples were taken from growth cultures at specific time points, normalized by OD600 and lysed with BugBuster Protein Extraction buffer (Merck, New Jersey) before separation by SDS-PAGE and transfer to a nitrocellulose membrane with the Novex system (Invitrogen, April 2016 Volume 195 Number 7 jb.asm.org).
Carlsbad, CA). Membranes were blocked with phosphate-buffered saline–Tween 20 (PBST) containing 5% skim milk powder. Anti-RecA and anti-LeXa antibodies were used at 1/4,000 and 1/5,000, respectively, in PBST containing 1% skim milk powder to probe membranes. Anti-DnaK antibody was used at 1/10,000 to detect equal protein loading between lanes. Comparison of protein levels from immunoblot experiments was carried out with ImageJ. Experiments were performed in triplicate to confirm the results.

Fluorescence microscopy. Strains of interest carrying the fluorescent recA-GFP reporter plasmid were cultured to an OD_{600} of 0.6 and mixed 1:1 with 4% paraformaldehyde at room temperature for 20 min. Twenty microliters of each sample was dotted onto a single well each of a multilwell microscope slide and allowed to dry. Slides were coverslipped mounted with Dako fluorescent mounting fluid (Invitrogen). Slides were left to set and imaged on a Leica DM18 microscope (Leica Microsystems). Cells were imaged at ×100 magnification with oil immersion, and images were analyzed with Leica Application Suite X. To compare recA-GFP induction of the wild-type and d-serine-treated cultures directly, the exposure times were normalized between the samples compared. Cells fluorescing above the background level of wild-type cells were considered induced for recA expression. MMC-induced samples were adjusted for GFP exposure time accordingly, given the strong induction of the reporter. GFP fluorescence was measured at an excitation wavelength of 488 nm. Experiments were carried out in triplicate, with at least five random fields of views analyzed in each replicate.

Phage transduction assay. To assay the production and transduction of phages in response to d-serine or MMC, we used E. coli strain JP10819, which contains pFP27 of EHEC with the stx gene being replaced with a tetracycline resistance marker (37). JP10819 was cultured in LB at 37°C until an OD_{600} of ~0.25 was reached. d-Serine or MMC was added at the concentrations indicated. These cultures were grown at 32°C with slow shaking (80 rpm) for 4 h and then left on the bench overnight at room temperature. Phage lysate was filtered through a 0.2-µm filter and serially diluted in phage buffer (100 mM NaCl, 0.5 M Tris [pH 7.8], 1 mM MgSO_4, 4 mM CaCl_2). A culture of MG1655 was grown in LB at 37°C until an OD_{600} of ~1.4 was reached, at which point CaCl_2 was added to a final concentration of 10 mM. A 100-µl volume of each serially diluted phage lysate was added to a 1-ml aliquot of MG1655 with CaCl_2 before static incubation at 37°C for 30 min. A 3-ml volume of soft LB agar (0.75%) agar was added to these cultures and poured over LB agar plates (1.5%) containing 6 µg/ml tetracycline and 1.7 mM sodium citrate. Plates were incubated at 37°C overnight. Colonies were counted and multiplied by the appropriate dilution factor to calculate the relative transduction frequency. Each colony was interpreted as a single phage transduction event.

GraphPad Prism 5.0 was used to generate figures and perform analysis. Mean data (± the SEM) were used to calculate statistical significance with a Student t test. Mutation assays were performed on at least five independent occasions.

RESULTS

Intracellular accumulation of d-serine increases RecA production in EHEC. Previous work by our group identified d-serine as an important niche-specific signal that is involved in the regulation of the infection process by repressing the EHEC T3SS essential for colonization of the host (31). Transcriptomic analysis showed that exposure to millimolar concentrations of d-serine (similar to those found in the urinary tract) also resulted in up-regulation of the SOS response regulon, and this was dependent on d-serine accumulation independently of T3SS repression. To verify the role of d-serine accumulation in SOS induction, we performed qRT-PCR analysis of total RNA extracted from EHEC TUV93-0 cells cultured in MEM-HEPES (conditions known to strongly induce T3SS expression) alone or supplemented with 1 mM d-serine. d-Serine accumulation resulted in a >5-fold increase (P ≤ 0.001) in recA transcription compared to that of the wild type (Fig. 1). As a control, EHEC was transformed with a plasmid expressing D-serine deaminase from UPEC CFT073 (psdA), allowing this strain to catabolize d-serine. Accordingly, the levels of recA were reduced to those of the wild type, indicating that d-serine accumulation is required for SOS induction.

To demonstrate that elevation of RecA production was specific to d-serine, whole-cell lysates of EHEC TUV93-0 cultured in MEM-HEPES with or without supplementation with various D-amino acids at 1 mM were probed by immunoblotting with anti-RecA antibody. d-Serine resulted in a >6-fold increase in RecA production, whereas the variation in response to other D-amino acids was more modest, with little variation either above or below the wild-type level (see Fig. S1 in the supplemental material). As a control, whole-cell lysates were also probed with anti-DnaK antibody to demonstrate normalized total protein levels and equal loading of the lanes. This indicates that this SOS response is unique to d-serine, as was the repression of the EHEC T3SS (31).
D-Serine-induced RecA production does not activate lysogenic stx phages. Given that D-serine accumulation induced the SOS response, we next addressed the physiological relevance of this. First, we investigated whether D-serine is capable of inducing the activation of lytic phages through the SOS response. This is an important question, as the stx genes of EHEC are carried by horizontally acquired lysogenic phages (21, 22). We profiled the growth of EHEC strain Sakai (stx mutant) in MEM-HEPES alone and with three concentrations of D-serine (0.02, 0.2, and 1 mM) or MMC (0.05, 0.5, and 2 μg/ml) added after 2 h at 37°C with shaking. Addition of D-serine at any of the concentrations tested had no effect on the growth rate of EHEC under these conditions (Fig. 2A). At 0.05 μg/ml, MMC also did not affect growth, and this was therefore considered a subinhibitory concentration of MMC under these conditions (Fig. 2A). At 2 μg/ml, MMC conversely resulted in a dramatic drop in OD, indicating phage-mediated lysis of the bacterial population and SOS response. At 0.5 μg/ml, MMC resulted in partial lysis of the population (Fig. 2B).

Next, we addressed the effects of D-serine on stx expression directly. To facilitate this, we employed a reporter plasmid containing the stx1 promoter region fused to GFP. Transformed strains were cultured in MEM-HEPES for 2 h before induction with a range of D-serine or MMC concentrations. SOS induction by D-serine was insufficient to alter stx expression levels at all of the concentrations tested (Fig. 2C). Exposure to MMC at lytic concentrations resulted in a sharp increase in stx expression between 3 and 5 h postinduction, the point at which the cell population begins mass lysis due to the SOS response (Fig. 2B). This corresponds to an ~7.5-fold increase in stx expression above the wild-type background at 2 μg/ml MMC (P ≤ 0.001). Accordingly, the partially lytic concentration of MMC (0.5 μg/ml) resulted in only a limited, albeit significant, increase in stx expression (P ≤ 0.001). No significant increase in stx transcription was observed at subinhibitory MMC concentrations.

At lytic concentrations of MMC, expression of the SOS response results in activation of dormant lysogenic phages and transduction of packaged phage particles to recipient cells via lysis of the donor cell. To assay the effect of D-serine or MMC on phage transduction, we used E. coli strain JP10819, a derivative of MG1655 containing a modified Φ27 prophage (37). Φ27 normally carries stx, but in this construct, it has been replaced with a tetracycline resistance marker (tetA), which is used to select for successful recipients of the activated phage. Lysates of JP10819 induced with either D-serine or MMC at various concentrations...
were used to transduce wild-type MG1655, resulting in tetracycline-resistant recipients. In agreement with the growth assays, ω-serine at concentrations of up to 1 mM had no effect on the transduction of the φP27 phage from JP10819 (Fig. 2C). MMC induction resulted in an increased number of phage transductants at lytic concentrations, with the highest number of events occurring at 2 μg/ml MMC (P ≤ 0.001) and decreasing accordingly. Subinhibitory MMC concentrations resulted in only a modest increase in the phage titer over the uninduced wild-type level. Collectively, these data indicate that known ω-serine levels encountered in the host cannot activate the transduction of lysogenic phages or the expression of stx, despite being able to induce the transcription of recA and the SOS regulon.

D-Serine-induced RecA production is sufficient to generate mutation-dependent genetic diversity at the population level. Three DNA polymerases are induced as part of the SOS response in E. coli, high-fidelity PolB, error-prone DinB, and Pol V (UmuDC) (1). We previously demonstrated by transcriptome sequencing that multiple members of the SOS regulon were induced by ω-serine accumulation in EHEC, including dinB, umuDC, and polB (31). The enzymes encoded by these genes are key members of the DNA repair process, but it is known that error-prone polymerase activity can lead to mutations in the population and that this can occur spontaneously in stationary phase or in response to antibiotic treatment, even at subinhibitory concentrations (38–40). We thus hypothesized that the SOS-like response induced by ω-serine could be capable of promoting genetic diversity through this mechanism. Cultures of EHEC TUV93-0 in MEM-HEPES were induced with either 1 mM ω-serine or sublytic MMC and allowed to grow for a further 2 or 8 h before being harvested by centrifugation. TUV93-0 has had the stx₁- and stx₂-carrying prophages deleted, and therefore it will not lyse upon SOS activation, allowing investigation of the ω-serine- and MMC-induced SOS response over the full course of growth experiments. Incubation times of 2 and 8 h postinduction were chosen to represent the exponential and stationary phases on the basis of the growth experiments described in Fig. 2. Bacteria obtained in the exponential phase had no indication of the appearance of a higher Rif resistance to rifampin (Rifr) in EHEC after SOS induction by 1 mM ω-serine and 0.05 μg/ml MMC was assayed. EHEC cell cultures were harvested and plated on rifampin-containing LB plates in exponential phase at 2 h postinduction (A) and in stationary phase at 8 h postinduction (B). The dotted line indicates the background level of spontaneous Rifr emergence in uninduced EHEC cells, to which all other conditions were compared. The Rifr frequency after each incubation is relative to the number of CFU present in each culture. As a control, EHEC/pdsdA, which does not accumulate ω-serine, was also assayed for the emergence of Rifr. Error bars represent the SEM calculated from at least five biological replicates. *, P ≤ 0.05 (compared to the wild-type control).

FIG 3 Increased SOS-like mutation frequency after ω-serine accumulation. The emergence of resistance to rifampin (Rifr) in EHEC after SOS induction by 1 mM ω-serine and 0.05 μg/ml MMC was assayed. EHEC cell cultures were harvested and plated on rifampin-containing LB plates in exponential phase at 2 h postinduction (A) and in stationary phase at 8 h postinduction (B). The dotted line indicates the background level of spontaneous Rifr emergence in uninduced EHEC cells, to which all other conditions were compared. The Rifr frequency after each incubation is relative to the number of CFU present in each culture. As a control, EHEC/pdsdA, which does not accumulate ω-serine, was also assayed for the emergence of Rifr. Error bars represent the SEM calculated from at least five biological replicates. *, P ≤ 0.05 (compared to the wild-type control).

D-Serine induces a modulated level of RecA production. Given that ω-serine was capable of causing an increase in the mutation frequency normally associated with SOS, we next addressed the extent of recA induction under these various conditions in order to gain mechanistic insights into the phenomenon. Using EHEC/precA-GFP, we compared ω-serine- and MMC-induced transcription of recA. At 1 mM, ω-serine significantly (∼3-fold; P ≤ 0.05) increased recA transcription at the mid to late exponential phase (OD₆₀₀ of 0.8), in agreement with our previous findings. This induction, however, was markedly lower than the transcription of recA in response to even subinhibitory concentrations of MMC (Fig. 4A). At 0.05 μg/ml, MMC significantly increased recA (∼13-fold; P ≤ 0.001) severalfold above that of the wild type at the mid- to late exponential phase. The effect of a lytic concentration of MMC (2 μg/ml) was even more pronounced, activating recA transcription to a level that is 28-fold higher than that of the wild type (Fig. 4B). Whole-cell lysate samples were taken from
growth assays at 0, 2, and 5 h postinduction with either d-serine or MMC and probed for RecA levels by immunoblotting. At time zero, the levels of RecA in all of the samples were comparable. By 2 h postinduction, an increase in RecA was observed at higher d-serine concentrations (see Fig. S3 in the supplemental material). In agreement with transcriptional reporter data, RecA production by MMC was much more pronounced over all of the concentrations tested. At 5 h postinduction, RecA levels in response to d-serine did not continue to increase; however, prolonged MMC exposure resulted in a large increase in RecA production at all of the concentrations tested (see Fig. S2). Levels of DnaK were also probed at each time point to control for protein loading effects.

The SOS response is known to be a stochastic process, and within a population, a small percentage of the cells in a living culture can be spontaneously induced (42, 43). To determine if the low-level SOS response is indeed representative of the population or rather a sporadic occurrence in a small number of cells, we visualized EHEC/precA–GFP cell populations left untreated or treated with 1 mM d-serine or 2.0 μg/ml MMC. Bacterial cells were imaged by phase-contrast microscopy, and recA induction was determined by detection of GFP-expressing cells. The merged channel indicates the level of recA-GFP induction per cell. Representative images are displayed for each condition, and data are summarized below as the percentage of the population expressing enhanced recA-GFP activity (green bars, cells induced for recA-GFP expression; gray bars, cells uninduced for recA-GFP expression). Experiments were performed in triplicate, and the results are shown as the mean ± the SEM. WT, wild type. (C) Immunoblot analysis of RecA, LexA, and DnaK levels in whole-cell lysates of EHEC cultured in MEM-HEPES with or without SOS induction. An EHEC ΔrecA mutant was used as a negative control for SOS induction. The concentrations of d-serine and MMC used to induce the SOS response are indicated above the immunoblots. DnaK levels were used to assess equal protein loading of samples. Experiments were performed at least in triplicate to confirm the results.

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The SOS response is known to be a stochastic process, and within a population, a small percentage of the cells in a living culture can be spontaneously induced (42, 43). To determine if the low-level SOS response is indeed representative of the population or rather a sporadic occurrence in a small number of cells, we visualized EHEC/precA–GFP cells by fluorescence microscopy after treatment with both d-serine and MMC. d-Serine treatment resulted in heterogeneous induction of recA throughout the population, with >35% of the population observably increasing recA-GFP expression. This heterogeneity may explain how such a modest population-wide recA induction can affect the mutation frequency if the response is actually stronger but in a subpopulation of the cells. In contrast, a genuine SOS response induced by MMC results in nearly 100% of the population expressing recA-GFP to a much greater extent than with d-serine (Fig. 4B). Furthermore, MMC-treated cells were morphologically distinct, forming filamentous structures likely associated with inhibition of cell division, a phenomenon not observed in d-serine-treated cultures. These results collectively indicate that while d-serine is capable of inducing a significant increase in the transcription of the SOS regulon and the production of RecA, the dynamics and consequence of this response are quite separate from those of the classical SOS response to a DNA-damaging agent.

The finding that d-serine could activate the transcription of the SOS network but failed to induce stx expression or phage lysis suggested that this was an indirect consequence of d-serine accumulation in the cell and perhaps not a genuine SOS response to a DNA-damaging event. Indeed, prolonged exposure to MMC resulted in the continued elevation of the SOS network (see Fig. S3 in the supplemental material). The lack of effects on stx expression, as well as its heterogeneous nature, suggests that d-serine exposure does not have DNA-damaging effects. d-Serine was orig-
inally described as being bacteriostatic to susceptible strains by interfering with \( L \)-serine and pantothentic acid biosynthetic pathways (44). However, growth inhibition by \( D \)-serine is dependent on the culture conditions used and the transcriptional response described here is independent of growth defects. This raises the important question of whether this observed SOS response is indeed genuine. To begin to examine this, we investigated the dependency of this SOS activation on RecA. We generated a TUV93-0 \( recA \) (\( \Delta recA \)) deletion mutant and analyzed the pre\( C \)-GFP reporter activity in this genetic background, with the hypothesis being that if \( D \)-serine activated the SOS response by an indirect mechanism, \( recA \) promoter activity may still be observable. RecA was absolutely required for SOS induction by both \( D \)-serine and, as expected, MMC, confirming the dependency of these responses on RecA (see Fig. S4 in the supplemental material).

We next investigated whether \( D \)-serine accumulation leads to cleavage of the LexA repressor protein during the SOS response. Immunoblotting for LexA following exposure to 1 mM \( D \)-serine resulted in no observable decrease in the quantity of LexA protein detectable in whole-cell lysates (Fig. 4C). In contrast, subinhibitory (0.05 \( \mu \)g/ml MMC expression resulted in a \( \geq 2 \)-fold reduction in the quantity of LexA detectable in whole-cell lysates. Accordingly, a lytic concentration of MMC (2 \( \mu \)g/ml) completely abolished detectable levels of LexA in a \( recA \)-dependent manner (Fig. 4C). The lack of detectable LexA cleavage suggests two scenarios, i.e., (i) that the modesty of the \( D \)-serine-induced SOS response renders LexA degradation undetectable and (ii) that accumulation of \( D \)-serine activates RecA production by an undetermined mechanism and not a genuine SOS response.

SOS induction in a \( recA \)-dependent manner suggests that the canonical mechanism of induction but no observable LexA cleavage was seen in response to \( D \)-serine accumulation. To explore this further, we supplemented a sublytic concentration of MMC with high concentrations of \( D \)-serine in an attempt to increase \( recA \) or \( stx \) transcription above that achieved with MMC alone. Addition of either 1 or 5 mM \( D \)-serine to 0.05 \( \mu \)g/ml MMC increased \( recA \) transcription by 1.3- or 1.4-fold, respectively, over that achieved by exposure to 0.05 \( \mu \)g/ml MMC but was not as extreme as that of a lytic 2.0-\( \mu \)g/ml MMC concentration (~1.6-fold) (Fig. 5A). More strikingly, \( stx \) transcription was increased approximately 1.8- and 1.9-fold (\( P \leq 0.01 \)), respectively, in \( D \)-serine-supplemented assays but again was still not high enough to mirror the level of activation achieved by the lytic concentration of MMC (4.6-fold above the subinhibitory MMC concentration; \( P \leq 0.001 \)) (Fig. 5B). In accordance with these data, immunoblotting for LexA in whole-cell lysate from the combined treatment of MMC and \( D \)-serine resulted in enhanced degradation of detectable LexA (~2-fold) compared with MMC alone. However, as shown in Fig. 4, this result was obtainable only by treatment with a DNA-damaging agent (Fig. 5C). These results suggest that \( D \)-serine is capable of activating the transcription of the SOS regulon in an unusual but genuinely functional manner. However, the lack of DNA-damaging events in response to \( D \)-serine indicates a possible unique mechanism of transcriptional activation and a controlled increase in functional RecA within the cell.

**DISCUSSION**

The bacterial SOS response is a well-studied reaction to stresses that ultimately result in damage to DNA or halting of cellular replication. The system is ubiquitous in the bacterial kingdom and plays a fundamental role in the successful life cycle of all bacteria (1). Alternative mechanisms of SOS activation are less well understood, and specifically what role they play in the absence of direct DNA damage is a less-well-explored area. The finding that \( D \)-serine accumulation results in upregulation of the SOS regulon in EHEC was indicative of a stress that the bacteria must respond to in some way. However, the mechanism of SOS induction by \( D \)-serine was obscure, considering that the negative effects of this amino acid are known to be at the level of metabolism (44). Here we demonstrate that \( D \)-serine accumulation induces a functional but mechanistically distinct SOS response in EHEC at a tolerated low level capable of promoting genetic diversification.

Our results collectively indicate that the SOS regulon is tran-

**FIG 5** \( D \)-Serine (\( D \)-ser) accumulation induces a functional SOS-like response in EHEC. (A) The levels of \( recA \)-GFP (left) and \( stx \)-GFP (right) reporter expression were analyzed in cell cultures treated with 0.05 or 2.0 \( \mu \)g/ml MMC. The levels of reporter activity in response to a combination of a low MMC concentration supplemented with either 1 or 5 mM \( D \)-serine were also analyzed and compared to that of a low MMC concentration. Relative fluorescence units (RFU) were measured at 3 h postinduction (\( OD_{600} \)). (B) Immunoblot analysis of LexA and DnaK levels in whole-cell lysates of EHEC induced for an SOS response with \( D \)-serine, MMC, or both. The concentration of \( D \)-serine or MMC used to induce the SOS response is indicated above the immunoblots. DnaK levels were used to assess equal protein loading of samples. Experiments were performed at least in triplicate to confirm the results.
scriptionally activated in response to D-serine and results in increased production of RecA associated with such an event. This level of induction, however, is more modest than that observed after MMC exposure, even at a subinhibitory concentration. In contrast, MMC treatment resulted in both an increased SOS response and phage activation. Levels of stx activation in response to a subinhibitory MMC concentration, however, were not increased, likely representing the extent of DNA damage from this treatment. The fact that high concentrations of D-serine could induce the “SOS response” but not phage activation suggests either a level of induction too low to observe such side effects or an alternative mechanism that is independent of direct DNA damage. Cleavage of the SOS repressor LexA was not detectable after D-serine induction, suggesting a very controlled response and maintenance of normal LexA levels. Indeed, transcriptomic analysis of the response to D-serine has previously shown that the members of the SOS regulon upregulated include the cell division inhibitor sulA and the RecA destabilizers recA and dinI, as well as lexA itself (31, 45). This suggests that the response may be modulated in some way to maintain normal and controlled cellular replication. Importantly, the data have shown that D-serine exposure can actually enhance the SOS response and stx activation, as well as LexA cleavage, when used in combination with MMC, thus providing key evidence that this is in fact a functional SOS-like response but is activated in an unusual manner that is not specific to a DNA-damaging agent.

The toxicity of D-serine to bacteria is well known. E. coli isolates that carry an intact D-serine tolerance locus (dsdCXA) are capable of internalizing D-serine, regulating its uptake, and catabolizing it as a nutrient source into nontoxic pyruvate and ammonia (44, 46–49). The mechanism of toxicity for cells that do not carry functional dsdCXA has been reported to be bacteriostatic inhibition of cell growth due to interference with L-serine and pantothenate metabolic pathways (44). Several E. coli isolates carry a conserved truncation in dsdCXA that renders them susceptible to D-serine toxicity (48, 49). We previously reported that carriage of the genes encoding the T3SS was widespread in isolates with mutated dsdCXA, suggesting an incompatibility between EHEC virulence and D-serine tolerance (31). Indeed, D-serine represses the T3SS and therefore host cell attachment in EHEC, but D-serine is not found in abundance at sites normally colonized by EHEC. D-Serine is conversely very abundant at extraintestinal sites (such as the urinary tract) and is used by pathogens in this niche as a positive fitness trait and a regulator of virulence (50, 51). This led us to propose that EHEC (and similar pathotypes) has evolved to lose D-serine tolerance, thus limiting the possibility that it disseminates to an environment rich in D-serine, where its primary colonization factor would be redundant (31). More recently, we discovered a novel D-serine uptake system in E. coli. This system is highly conserved in all E. coli strains but is regulated differently in unique genetic backgrounds. EHEC counterintuitively uses this system to increase D-serine uptake in its presence but is capable of tolerating D-serine accumulation without actively breaking down this toxic metabolite. However, despite this apparent tolerance, expression of the SOS regulon persists, suggesting a mechanism of transcriptionally responding to this signal in order to quickly adapt to changing D-serine concentrations that may be encountered (31, 41). Furthermore, SOS activation can lead to an increased mutational frequency and, in turn, increase bacterial fitness after exposure to even subinhibitory concentrations of antibiotics, for example (38, 39, 52). Indeed, one study reported that ciprofloxacin treatment of E. coli could increase the mutation frequency by a factor of 10⁴ over the spontaneous rate (53). In this work, we found that D-serine accumulation could actually enhance the mutation rate of the population through RecA production and that this was dependent on D-serine accumulation in the cell. Interestingly, D-serine accumulation in a UPEC ΔsdA background is a much less tolerated event and exemplifies a fitness defect under these conditions (41). In the UPEC ΔsdA mutant, D-serine accumulation also results in a higher recA induction than that of EHEC, suggesting that this stress may account for the apparent adaptation to D-serine tolerance in EHEC. Recent studies have suggested that antibiotic-mediated mutations can not only increase resistance to antibiotics but also generally enhance the fitness of the affected bacterial cells (39, 52). It was also demonstrated that after rapid adaptation, SOS expression is actually decreased, limiting further unwanted mutagenic capability (52). This may explain why recA induction by D-serine is weaker in adapted EHEC than in a UPEC ΔsdA mutant that is more susceptible to toxic D-serine and is perhaps how EHEC has evolved to tolerate D-serine exposure and the associated induction of a stress response. We propose that this is to allow EHEC to continue monitoring and sampling its environment within the host for inhibitory D-serine by a mechanism that is unique to DNA damage or even spontaneous prophage induction and subsequently would result in a modulated transcriptional response to this stress.

Factors that may activate stx expression are of particular importance when studying EHEC biology and potential intervention strategies. There are currently no recommended antibiotic treatments for Shiga-toxigenic E. coli infections, given the known repercussions of such procedures. Recently, there has been much interest in how niche-specific metabolites found in the host can regulate colonization by EHEC (54). This pathogen has evolved intricate mechanisms to specifically localize to the colonic epithelial surface by responding to numerous physiologically relevant signals such as host- and microbiota-derived nutrient sources, hormone-like signals, and short-chain fatty acids (55–58). Furthermore, an understanding of these mechanisms has led to the possibility of using such metabolites and small molecules as treatments themselves to reduce colonization (59–61). However, for an approach like this to work, it is important to understand fully the “off-target” effects of said treatment, such as induction of SOS-related genes. Xu et al. have shown that spontaneous stx activation results in the lysis of a small portion of the EHEC population, but this, in turn, promotes colonization by priming the epithelial surface for attachment of the nonlysed cells (62). Indeed, stx prophage induction has been associated with enhanced colonization of EHEC in vivo (63, 64). Furthermore, the T3SS of enteropathogenic E. coli has been shown to be under direct control of the SOS response (65). However, despite the fact that these studies link prophage induction and the SOS response with colonization by the T3SS, each case requires prophage activation or DNA damage. In contrast, this study highlights that D-serine does not result in phage expression, suggesting a different mechanism and role for this response.

How does D-serine accumulation induce the SOS regulon? Cases of unusual mechanisms leading to LexA derepression have been reported. In Bacillus subtilis, a transcription factor, ComK, is capable of activating recA transcription without displacing LexA itself (66). A second study has described an unusual mechanism of
LexA displacement in which the nucleoid-associated protein HU affects DNA topology close to the site of LexA binding, thus resulting in dissociation from its binding site (67). It has also been demonstrated experimentally that mutation of interaction sites between RecA and LexA can interrupt the cleavage of LexA while still allowing induction of RecA in response to DNA damage (68). The concept of LexA displacement is certainly intriguing, considering the lack of observable LexA cleavage in response to d-serine; however, the dependency of this response on RecA seems to suggest a more common route of activation that is possibly masked by the subtlety of the response. One could hypothesize that the bacteriostatic effects of d-serine in minimal medium could indirectly lead to SOS induction by halting cellular replication. Indeed, the DpiAB mechanism results in transient inhibition of replication via activation of the cell division inhibitor SulA in an SOS-dependent manner (10). However, we have observed growth tolerance of EHEC irrespective of sulA upregulation, suggesting that there are downstream factors that counteract the effects of increased SulA expression. What is most intriguing is that this tolerance appears to be unique to EHEC, given that a UPEC ΔsulA mutant is unable to tolerate d-serine accumulation and shows arrested growth under the same conditions (41). These results suggest EHEC-specific regulation that counteracts recA induction by d-serine.

Rapid adaptation to the environment is paramount to survival within a complex host environment where incoming threats are immediate but also long-term niche adaptation promotes a competitive advantage. EHEC isolates, while attracted specifically to the distal colon, have the capacity to at least come into contact with the urinary tract, and in some rare cases, have even been associated with urinary tract infections (69). Our data suggest, however, that in these cases, high concentrations of d-serine would not be a driver of stx expression and that other unknown factors contribute to this rare phenomenon. This ability to survive in an unusual environment may be a result of rapid adaptation to factors such as d-serine, but the uncommon nature of T3SS-mediated colonization of this niche may be due to the regulatory effects of d-serine and not its properties as a toxic carbon source (31). Taken together, these studies collectively suggest that d-serine may be a contributing factor in the evolution of E. coli niche specificity, depending on the individual’s genotypic and pathogenic properties.

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