The protein RpoS is responsible for mediating cell survival during the stationary phase by conferring cell resistance to various stressors and has been linked to biofilm formation. In this study, the role of the rpoS gene in *Escherichia coli* O157:H7 biofilm formation and survival in water was investigated. Confocal scanning laser microscopy of biofilms established on coverslips revealed a nutrient-dependent role of rpoS in biofilm formation, where the biofilm biomass volume of the rpoS mutant was 2.4- to 7.5-fold the size of its rpoS+ wild-type counterpart in minimal growth medium. The enhanced biofilm formation of the rpoS mutant did not, however, translate to increased survival in sterile double-distilled water (ddH2O), filter-sterilized lake water, or unfiltered lake water. The rpoS mutant had an overall reduction of 3.10 and 5.30 log10 in sterile ddH2O and filter-sterilized lake water, respectively, while only minor reductions of 0.53 and 0.61 log10 in viable counts were observed for the wild-type form in the two media over a 13-day period, respectively. However, the survival rates of the detached biofilm-derived rpoS+ and rpoS mutant cells were comparable. Under the competitive stress conditions of unfiltered lake water, the advantage conferred by the presence of rpoS was lost, and both the wild-type and knockout forms displayed similar declines in viable counts. These results suggest that rpoS does have an influence on both biofilm formation and survival of *E. coli* O157:H7 and that the advantage conferred by rpoS is contingent on the environmental conditions.

In aquatic environments, bacteria are often protected from biofilms. Biofilms are complex communities of bacteria attached to the substratum and enclosed in an extracellular matrix (ECM) of exopolysaccharides and, to a lesser degree, proteins, nucleic acids, and other exogenous substances (14, 16). This protective aggregation allows the bacteria to withstand challenges, such as antibiotics, biocides, phagocytosis, and desiccation, better than their planktonic counterparts (13, 17, 31, 37, 39, 40, 42). Given the enhanced survival of biofilm cells, the biofilm state is often recognized as the preferred state of existence for bacteria under many environmental conditions (26, 29, 61).

The increased resistance of biofilm cells to various assaults has been attributed to five potential interacting factors: the inhibited penetration of the biofilm, decreased metabolism due to nutrient deprivation in the biofilm interior, a heterogeneous display of phenotypes induced by variable conditions throughout the biofilm, the production of persistor cells, and cross-protection through the activity of a general stress response mechanism (36, 49, 50). One gene that is often implicated in biofilm formation and is responsible for mediating gene expression during periods of stress, starvation, and stationary-phase growth is rpoS, the stationary-phase sigma factor gene (32, 34, 35, 38). RpoS (σ5), the gene product of rpoS, mediates the binding of RNA polymerase to the promoter of rpoS-dependent genes, thereby facilitating their transcription (33, 34, 35). RpoS is reportedly responsible for the regulation of approximately 10% of the *Escherichia coli* genome, with over 140 genes upregulated by RpoS during periods of stress or stationary-phase growth (56).

Given the high cell density of biofilms and the corresponding potential for stress due to low oxygen and nutrient deprivation (44), it is not surprising that rpoS and numerous rpoS-dependent genes have been reported as differentially expressed between the planktonic and biofilm states (4, 20, 46, 48). Additionally, a number of studies have found rpoS to impact biofilm formation by *E. coli* K-12 under various conditions (1, 12, 30, 48). While these studies have highlighted the importance of rpoS in biofilm formation, their findings have lacked uniformity.

Little is known about the role of RpoS in the formation of biofilms by *E. coli* O157:H7. Even studies that broadly investigated differential gene expression between planktonic and biofilm states in pathogenic *E. coli* have been limited. A proteomic study by Trémoulet et al. (51) of a nonpathogenic *E. coli* O157:H7 derivative revealed differential protein expression between planktonic and biofilm cells. Of the 17 proteins with increased expression in biofilm cells, those with known functions could be roughly categorized as proteins responsible for general metabolism (including an enzyme from the tricarboxylic acid [TCA] cycle), transport, or DNA binding (51). Interestingly, one of the periplasmic binding proteins was speculated to be responsible for the transport of amino acids, and one of the DNA binding proteins is known to protect nonspecific DNA from oxidative damage. These findings are indicative of a change in the global gene regulation of *E. coli* O157:H7 from the planktonic to the biofilm state and are not incongruent with the potential for σ5 involvement—a notion requiring further pursuit.
A persistent paradox of rpoS studies has been that under certain starvation conditions, mutations in the rpoS gene can actually confer an advantage. Numerous rpoS mutants have demonstrated increased fitness and survival over wild-type strains in planktonic cultures incubated for a prolonged period after stationary-phase growth has been achieved (23, 25, 58, 59, 60). The fitness advantage of these rpoS mutants may be provided by an increased capacity to scavenge for amino acids, which are utilized by the TCA cycle as sources of energy (25). In fact, E. coli strains with reversible rpoS mutations can employ succinate, a TCA cycle intermediate, as the sole source of carbon, whereas wild-type E. coli strains do not have the same capability (9, 10). Therefore, mutations in rpoS may be selected for studies under low-nutrient conditions, thus allowing the surviving cells to more efficiently capture and utilize the amino acids and other nutrients released by dead cells.

The survival advantage conferred to rpoS mutants in long-term stationary-phase growth and the resistance of biofilm cells to a variety of stressors implicate both rpoS and biofilm formation in the survival of pathogenic E. coli under conditions of nutrient starvation. Of concern is the survival of pathogenic E. coli for extended periods of time under the comparatively nutrient-deprived conditions of untreated well water, bottled water, municipal drinking water, natural water bodies, and water distribution systems (24, 52, 53, 54). As such, further investigation into the role of rpoS in the survival of E. coli O157:H7 biofilm cells in both natural and treated water samples is required.

This study evaluated the biofilm-forming capabilities of wild-type and rpoS knockout strains of E. coli O157:H7 under different culture conditions in an effort to elucidate the role of rpoS in E. coli O157:H7 biofilm formation and to help reconcile the differences in the current literature. Additionally, the role of rpoS in the survival of established E. coli O157:H7 biofilm cells in both filter-sterilized and unfiltered lake water samples was investigated.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** The bacterial strains used in this study are summarized in Table 1. The E. coli O157:H7 H32 strain we employed was obtained from C. Gyles at the University of Guelph (Guelph, ON, Canada) and corresponds to the strain identification number EC920004 from this culture collection. H32 is a bovine isolate and possesses genes for Shiga-like toxins I and II (55). It also contains the eae gene for intimin, the virulence factor responsible for mediating the attachment of E. coli to colonic epithelial cells (22, 28). The stock cultures for all strains were prepared in tryptic soy broth (TSB) (BD Difco, Mississauga, ON, Canada) supplemented with 25% (vol/vol) glycerol and maintained at 80°C (55). All strains were recovered overnight at 37°C on tryptic soy agar (TSA) with the exception of the strains bearing plasmid pKD46, which were recovered at 30°C. Following recovery, the strains were subcultured in antibiotic selective medium overnight prior to the preparation of cells for experimentation. Antibiotics were added as required for the maintenance of plasmids or selection of chromosomally labeled strains at concentrations of 100 μg ampicillin, 20 μg gentamicin, and/or 50 μg kanamycin per ml of medium. For all molecular manipulations, E. coli strains were routinely cultured in Luria-Bertani Miller broth (LB) (BD Difco) at 37°C.

**gfp labeling of strain H32.** The H32 strain was labeled with a gfp-aph(3’I) (gfp-kan) cassette, excised from the pJBA29 plasmid, using the EZ-Tn5 pMOD(MCS) construction vector (Epicentre Biotechnologies, Madison, WI). The gfp-kan cassette was excised from the pJBA29 plasmid using KpnI digestion (NEB, Ipswich, MA) and ligated to the EZ-Tn5(gfp-kan) plasmid containing the EZ-Tn5(gfp-kan) transposon. The pMOD-EZTn5(gfp-kan) plasmid was then electroporated into E. coli strain JM109 using an optimized standard protocol (47) and extracted using a Promega Wizard Plus SV miniprep DNA purification system.

The EZTn5(gfp-kan) transposon cassette was liberated from the pMOD-EZTn5(gfp-kan) plasmid through PshA1 digestion (NEB, Ipswich, MA) and gel purified. The transposase reaction mixture was prepared with slight modifications to the recommended protocol (Epicentre Biotechnologies), namely, reducing the amount of enzyme solution in an effort to prevent arching. Electroporation was used to introduce the transposon cassette to strain H32, thus facilitating transposition. Screening on an LB plate with ampicillin (LBA plate) supplemented with both ampicillin and kanamycin was used in conjunction with the isolation of colonies displaying green fluorescence. Successful transposition was confirmed and identification of the insertion site was performed through genomic sequencing (MOBIX Lab, Hamilton, ON, Canada) using the primers.

### Table 1: Characteristics of bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>Strains</td>
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<tr>
<td><em>Escherichia coli</em> O157:H7</td>
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<td></td>
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<tr>
<td>H32</td>
<td>Wild-type bovine isolate</td>
<td>28, 54</td>
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<tr>
<td>H32-gfp</td>
<td>Labeled with <em>aph(3’I)</em> and gfp through random Tn5 insertion; KANr*</td>
<td>This study</td>
</tr>
<tr>
<td>H32-gfpΔrpoS:gm</td>
<td>Labeled with <em>aph(3’I)</em> and gfp, rpoS disrupted with <em>accC1</em>; KAN’ <em>Gm</em>†</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli JM109</td>
<td>Cloning host</td>
<td>Promega</td>
</tr>
<tr>
<td>Plasmids</td>
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<td></td>
</tr>
<tr>
<td>pBAD29</td>
<td>gfp and <em>aph(3’I)</em> ((gfp-kan) cassette) template vector; KAN’ Ampr*</td>
<td>2</td>
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<tr>
<td>pMOD-2(MCS)</td>
<td>Construction vector; Amp’ (bla)</td>
<td>Epicentre Biotechnologies</td>
</tr>
<tr>
<td>pMOD(gfp-kan)</td>
<td>Source of EZTn5(gfp-kan) transposon, template for (gfp-kan) cassette; KAN’ Amp’</td>
<td>This study</td>
</tr>
<tr>
<td>pKD46</td>
<td>λ Red recombinase system (P*&lt;srt<em>gam-bet-exo</em>&gt;, temp-sensitive [oriR101 repA101(Ts)]; Amp’ (bla)</td>
<td><em>E. coli</em> Genetic Stock Center, New Haven, CT; 15</td>
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<tr>
<td>pGEM T-Easy</td>
<td>Cloning host, lacZ insertion site; Amp’ (bla)</td>
<td>Promega</td>
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<td>pGEM-rpoS</td>
<td>Construction vector rpoS-flanked linear DNA fragments; Amp’</td>
<td>This study</td>
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<td>p34S-Gm</td>
<td>Template for <em>accC1</em>; Gm’</td>
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<tr>
<td>pGEMΔrpoS:gm</td>
<td>Source of ΔrpoS:gm cassette for disruption of <em>rpoS</em>; Amp’ Gm’</td>
<td>This study</td>
</tr>
</tbody>
</table>

* KAN’, kanamycin resistance.
† Gm’, gentamicin resistance.
‡ Amp’, ampicillin resistance.

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SqFPl (5′-GCGAGACCTTACGCGACCTAGCAAC) and SqRP (5′-AGGCC AATATGCGGAAACCCGAGGAA) oriented outward from the multiple cloning site (Epigenic Biotechnologies, Madison, WI).

Construction of an rpoS-deficient strain by lambda Red recombination. Lambda Red recombination was employed to generate an rpoS-deficient strain of gfp-labeled H32, using a method modified from the original protocol described by Datsenko and Wanner (15). The linear fragment rpoS::gm served as the substrate for homologous recombination and the simultaneous gene disruption in H32-gfp. The cassette was constructed to bear approximately 500 bp of rpoS sequences flanking a gentamicin resistance determinant (accC1) using the pGEM T-Easy cloning vector (Promega). The target rpoS region was amplified from the H32 chromosome using a 50-μM PCR mixture containing 20 to 100 ng of extracted H32 genomic DNA, 0.2 μM each rpoS-F (5′-TTATGAGTTCGAA ATACGCTGAAG) and rpoS-R primer (5′-TTACTTCGGGAAACACCG CCTCCG), 1 unit of Taq DNA polymerase, and other PCR components as described by the manufacturer (Fermentas, Burlington, ON, Canada). The PCR consisted of 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C and a final extension for 10 min at 72°C (PCR Sprint thermal cycler; Thermo Fisher Scientific). The amplified 993-bp rpoS gene was ligated with the pGEM T-easy vector and inserted with accC1, a gentamicin resistance gene at a site 549 bp downstream of the start codon. The insertion of accC1 into rpoS was facilitated through digestion with Eco721, generating the plasmid pGEMrpoS::gm. The plasmid was subsequently chemically transformed into strain JM109 using a TransformAid bacterial transformation kit (Fermentas). Successful insertion of rpoS was confirmed through digestion of the construct with EcoRI, as well as through sequencing from the pGEM T-Easy multiple-cloning site using the universal primers SP6 and T7.

H32-gfp was transformed with the pKD46 plasmid using electroporation, producing the recombinant-proficient strain H32-gfp/pKD46. Overnight cultures of H32-gfp/pKD46 were used to inoculate 30 ml of LB Amp50 to an optical density at 600 nm (OD600) of 0.1 and incubated at 30°C until an OD600 of 0.4 to 0.6 was achieved with 10 ml t-arabinose for induction of the recombinase system. Ten-milliliter portions of the cell culture were harvested and washed once in ice-cold sterile double-distilled water (ddH2O) and thinned in 10% glycerol. Following the last wash, cells were resuspended in 50 μl 10% glycerol and mixed with 100 ng to 1 μg of the linear rpoS::gm fragment. After 1 min of incubation on ice, electroporation was performed using an Eppendorf electroporator 2510 and 100-μl cuvettes with a 1-mm gap per the manufacturer’s instructions (Eppendorf, Mississauga, ON, Canada). The cells were recovered in 1 ml cuvettes with a 1-mm gap per the manufacturer’s instructions and 100-μl volumes of LB at 37°C for 1.5 h. The transformants were selected for on LBA Amp50 plates. The aforementioned cultures were used to inoculate 50 ml of LB for each strain, supplemented with the appropriate antibiotic. The aforementioned cultures were used to inoculate 200 ml of LB and MSMG for each strain, without antibiotics, to an OD600 of 0.5 to 1.0. The aforementioned cultures were used to inoculate 50 ml of LB for each strain, supplemented with gentamicin (10 μg/ml) and kanamycin (25 μg/ml) and screened through colony PCR using the primers MidrpoS-F (5′-CCGGTTATGGGAACTGCGTTCGTTCT) and MidrpoS-R (5′-TCAAGCTCATTACAACTGGAAGTATGATGATCC) (Molecular Probes, Eugene, OR) for 5 min. All three strains were stained to ensure comparable levels of fluorescence. Unadhered cells and excess dye were removed by submerging the coverslips once in sterile ddH2O. The coverslips were placed, sterile side up, over a depression slide filled with sterile ddH2O and sealed with nail polish. The slide was prepared for microscopy by swabbing the sterile side of the coverslip with 10% sodium hypochlorite, ddH2O, 85% ethanol, and ddH2O, in succession.

The biofilm cells were visualized on an Olympus Fluoview FV300 confocal scanning laser microscope using an ×60 Plan Apo 1.4 numerical-aperture oil immersion objective lens and a 10-mW 488-nm argon laser (Olympus, Tokyo, Japan). The detection of SYTO 9 fluorescence was performed using a band pass emission from 510 to 530 nm (DM570 dichroic mirror, FVX-BA 510–530 filter set; Olympus). Each image stack (Z-stack) was analyzed for biofilm biovolume using a phobia laser imaging processor run through the MatLab 7.0.4 platform (MatLab, Natick, MA). Biovolume is defined as volume of biomass per unit substratum area (μm²/μm³). Representative Z-stacks were compiled into single images using ImagePro (Media Cybernetics, Silver Spring, MD).

Survival of biofilm cells in lake water samples. The survival of biofilm cells was assessed in sterile ddH2O, filter-sterilized lake water, and unfiltered lake water. Lake water samples were obtained in early spring from Boulevard Lake (48°27′30″N, 89°11′41″W), a man-made lake formed by the damming of the lower Current River (Thunder Bay, ON, Canada). However, the water temperature of Boulevard Lake is normally between 15 and 25°C during the summer months. Water was collected in sterile 500-ml Nalgene bottles at a depth of 15 to 30 cm, 1.5 m from shore. The Nalgene bottles were held beneath the surface, uncapped and inverted to fill, and recapped underwater. The samples were transported on ice and stored at 4°C until required for use. Filter-sterilized lake water was obtained by performing vacuum filtration on the collected lake water samples using a sterile 0.45-μm-pore-size mixed cellulose ester filter, followed by syringe filtration through a sterile nylon 0.22-μm-pore-size filter (Fisher Scientific Canada, Ottawa, ON). Filter-sterilized lake water was used immediately following filtration. The chemical and biochemical properties of the unfiltered lake water were analyzed by the Lakehead University Centre for Analytical Service (LUCAS) and are reported as follows (in mg/liter): 4.0 dissolved organic carbon, 0.6 K, 3.41 Mg, 4.04 Na, 13.71 Ca, 6.12 Cl−, 0.284 NO3−, 0.299 Al, 0.013 Ba, 0.005 Cu, 0.562 Fe, 0.0109 Mn, 1.775, 0.027 SO42−, 0.005 Sr, and 6.60 Zn. The levels of As, Be, Cd, Cr, Ni, NO2−, Pb, and PO43− were below the detection limits (<0.005 mg/liter, with the exception of PO43−, which had a detection...
RESULTS

Production of H32-gfp and H32-gfpΔrpoS::gm. Successful transposition of H32 with (gfp-kan) producing the fluorescently labeled strain H32-gfp was tentatively established through the isolation of kanamycin-resistant colonies exhibiting green fluorescence under UV transillumination. The insertion of (gfp-kan) in the H32 genome was confirmed through DNA sequencing using the primers SqFP and SqRP, oriented outward from (gfp-kan). BLAST results of the sequences (about 640 bases) generated by both primers revealed sequences similar to a putative E. coli regulatory protein, with 97% and 93% DNA sequence identities for the SqFP and SqRP sequence results, respectively. The lack of complete sequence similarity can be attributed to miscalled bases in the genomic sequencing reaction and the genomic differences between H32 and the E. coli strains in the GenBank database. The function of the regulatory protein is unknown or inferred in all BLAST sequence results except in E. coli O157:H7 strain EC4115 (accession no. CP001164), where it is tentatively identified through protein homology as UhpC, a nonessential regulatory protein involved in sugar phosphate transport (accession no. NC_011353.1[445971 to 447275, complement]). The API 20E test results revealed that H32 and H32-gfp have identical profiles and that E. coli is a very good identification (identification ≥ 99%) for these strains (profile no. 5144132). The API 20E results confirm that H32-gfp represents an E. coli strain that is indistinguishable from H32 in terms of fundamental biochemical activities. The stability of the (gfp-kan) label was confirmed through the observation of uninhibited growth by H32-gfp on TSA supplemented with 50 μg/ml kanamycin, relative to TSA, following 6 transfers on nonselective media. Additionally, H32-gfp fluoresced equally under UV illumination over the 6-day transfer period.

H32-gfp was used as the target for rpoS disruption, resulting in the production of a gfp-labeled rpoS strain of E. coli, H32-gfpΔrpoS::gm. The use of fluorescently labeled wild-type and rpoS mutant strains allowed for the differentiation of E. coli from background bacteria in subsequent survival experiments in lake water samples. The H32-gfpΔrpoS::gm strain was generated through lambda Red recombination and confirmed through PCR. The amplification of a 1,953-bp product using the primers FullrpoS-F and FullrpoS-R on the H32-gfpΔrpoS::gm insertion mutant showed the disruption of rpoS by the 848-bp gentamicin resistance determinant, whereas PCR on wild-type rpoS yielded the expected 1,106-bp amplicon. DNA sequencing of the 1,953-bp fragment revealed an insertion of the gentamicin resistance gene (accC1) in rpoS.

Comparative growth curves. The planktonic growth curves for E. coli O157:H7 strains H32, H32-gfp, and H32-gfpΔrpoS::gm are shown in Fig. 1. Growth curves were generated at 37°C in both LB and MSMG. As expected, there was a statistically significant increase in cell density (CFU/ml) over time for all strains in both media (P < 0.001), as both curves exhibit the typical stages of exponential- and stationary-phase growth. All three strains exhibited slower growth in minimal media than in rich media, as stationary phase was reached after 6 h for all strains in LB but not until 10 h in MSMG. The viable cell counts for all strains were approximately 1 log CFU/ml higher in rich media than in minimal media.

In the LB medium, the growth curves of the two rpoS+ strains were not significantly different from each other (P = 0.928). When the growth curve of the rpoS mutant was compared to those of the rpoS+ strains, the density of the rpoS mutant was significantly lower than that of the H32 (P = 0.002) and H32-gfp (P = 0.004) strains at only 6 h, the entry point to stationary phase. The cell densities of the rpoS mutant, H32, and H32-gfp at 6 h were 1.27 × 10^9 ± 0.21 × 10^9, 2.27 × 10^9 ± 0.21 × 10^9, and 2.14 × 10^9 ± 0.08 × 10^9 CFU/ml, respectively. No significant differences in growth were revealed between any of the strains in MSMG (P = 0.898). The gfp labeling did not have an apparent influence on the planktonic growth of H32-gfp relative to H32 in either media.

Biofilm formation of the E. coli strains. The qualitative visualization of H32, H32-gfp, and H32-gfpΔrpoS::gm biofilms using confocal scanning light microscopy (CSLM) after 24 h revealed that the development of biofilms for the three H32 strains was growth medium dependent (Fig. 2). In observing H32, H32-gfp, and H32-gfpΔrpoS::gm in TSB, most fields of view under CSLM
contained sparse single cells or pairs of adhered cells. In MSMG (at both 22°C and 37°C), H32 and H32-gfp demonstrated small clusters of cells and larger aggregations of cells (microcolonies) interspersed with single cells and noncolonized areas. In contrast, H32-gfp/H9004 rpoS::gm exhibited an extremely dense uniform mat of cells that appeared to be 2 to 3 layers of cells deep. Given the enhanced biofilm growth of all three strains in MSMG, this medium was selected to promote optimal biofilm formation. In addition, 22°C was chosen for all subsequent experiments to mimic environmental conditions.

Biofilm growth in MSMG, sampled at 24, 48, and 72 h and analyzed using PHLIP, confirmed that the rpoS knockout produces biofilms of greater biovolume than both of the rpoS+ strains at 24 and 48 h (P < 0.001) (Fig. 3). At 72 h, the biovolume of the rpoS mutant was 2.4-fold and 2.7-fold the size of the H32-gfp (P = 0.07) and H32 (P = 0.01) strains, respectively. The maximal biofilm biovolume occurred after 48 h for H32-gfpΔrpoS::gm and was enhanced approximately 7.5-fold relative to either of rpoS+ H32 or H32-gfp strain (P < 0.001). H32-gfp displayed a slight increase in biofilm formation at 72 h compared to 48 h, and H32 exhibited comparable biofilm formation (from 5.3 × 10^3 to 7.4 × 10^3 μm^3/mm^2) on all 3 days. Overall, H32 and H32-gfp formed biofilms with similar biovolumes, suggesting that the labeling procedure did not impact the biofilm-forming capabilities of H32-gfp.

Survival of attached and detached biofilm cells in water samples. The total numbers of culturable H32-gfp biofilm cells, including both attached and detached biofilm cells, were significantly higher than that of the H32-gfpΔrpoS::gm strain at the end of the 13-day period, with P values of 0.021, <0.001, and 0.019 in sterile distilled water, filter-sterilized lake water, and unfiltered lake water, respectively (Fig. 4).

Quantification of the attached cells alone (using the plate counting method) showed greater overall survival of H32-gfp than of H32-gfpΔrpoS::gm in sterile ddH_2O (P < 0.001), filter-sterilized lake water (P < 0.001), and unfiltered lake water (P = 0.019). The rpoS mutant displayed markedly reduced culturable cell numbers relative to H32-gfp at all time points other than at day 0 (P <

FIG 1. Planktonic growth of E. coli O157:H7 strains H32 (●), H32-gfp (○), and H32-gfpΔrpoS::gm (▼) in a rich medium (LB) (top graph) and a minimal medium (MSMG) (bottom graph) at 37°C over 24 h. Bars indicate standard deviations at a 95% confidence level.

FIG 2. Representative CSLM images of biofilm cells of E. coli O157:H7 strains H32, H32-gfp, and H32-gfpΔrpoS::gm (KO) after 24 h of growth in TSB or MSMG. The biofilms were cultivated in TSB at 37°C (left column), MSMG at 37°C (middle column), and MSMG at 22°C (right column). All three E. coli strains were stained with SYTO 9 and visualized with a 60X objective lens.

FIG 3. Biofilm development of E. coli O157:H7 strain H32 (●), H32-gfp (○), and H32-gfpΔrpoS::gm (▼) in MSMG at 22°C. All three E. coli strains were stained with SYTO 9. Bars indicate standard deviations at a 95% confidence level.
The *rpoS* mutant had an overall reduction of 3.10 and 5.30 log_{10} in sterile ddH2O and filter-sterilized lake water, respectively, whereas H32-*gfp* maintained its survival over time. In unfiltered lake water, the numbers of attached cells recovered from biofilms of both the *rpoS* mutant and *rpoS*/*H11001* strains decreased significantly between days 0 and 13 (*P* < 0.001), but the *rpoS*/*H11001* strain still held a survival advantage over the *rpoS* mutant at 13 days, with a *P* value of 0.007 (Fig. 4).

Plate counting of the detached biofilm cells in the sterile ddH2O and filter-sterilized lake water showed minor decreases (about 1 log_{10} reduction) for both the *rpoS* mutant and *rpoS*/*H11001* strains between days 3 and 13. In addition, the declining rates of H32-*gfp* and H32-*gfp*Δ*rpoS::gm* were also similar (Fig. 4). In the untreated lake water samples, the culturable cell density of H32-*gfp* was about 2 log_{10} higher than that of H32-*gfp*Δ*rpoS::gm* on day 3. However, the H32-*gfp* cell density dropped sharply from 7.29 to 4.43 log_{10} CFU/ml between days 3 and 6 and further decreased to 3.67 log_{10} CFU/ml on day 13. For the *rpoS* mutant, it decreased steadily from 5.26 to 2.93 log_{10} CFU/ml between days 3 and 13.

**Visualization of *E. coli* biofilm cells in water samples.** Qualitative visualizations of the H32-*gfp* (Fig. 5A) and H32-*gfp*Δ*rpoS::gm* (Fig. 5B) biofilms over a 13-day period were performed in sterile distilled water, filter-sterilized lake water, and unfiltered lake water. Multiple techniques were employed to visualize the O157:H7 cells and/or the background cells, including just the green-fluorescing *E. coli* cells (no stain [NS]), the total number of cells including background (SYTO 9 stain [S9]), and the total cell viability (Live/Dead stain [LD]).

For H32-*gfp*Δ*rpoS::gm*, substantial reductions in green-fluorescing biofilm cells (NS, native fluorescence only) were observed in both the distilled water and the filter-sterilized lake water after 13 days of incubation (Fig. 5B), whereas the biofilm cell densities of H32-*gfp* in the sterile ddH2O and the filter-sterilized lake water samples remained similar over the 13-day period (Fig. 5A). This indicated that
more H32-gfpΔrpoS::gm biofilm cells had lost their viability (i.e., lost viability and hence the green fluorescence protein) in the water samples compared with the H32-gfp biofilm cells.

When compared to results from day 0, notable reductions of the SYTO 9-stained H32-gfpΔrpoS::gm biofilms were observed in both the distilled water and the filter-sterilized lake water samples on day 13. Because SYTO 9 is a DNA dye, it stains all the cells (including live, unculturable, and dead cells with partly degraded DNA) in a biofilm structure. Therefore, the reduction of the SYTO 9-stained H32-gfpΔrpoS::gm biofilm cells indicates that some rpoS mutant biofilm cells were released into the aqueous phase of the water samples. Furthermore, a comparison between the SYTO 9 staining and native fluorescence of the rpoS mutant H32 biofilm cells shows that there were more SYTO 9-stained cells than native fluorescent cells in both the sterile ddH₂O and the filter-sterilized lake water samples, implying that some rpoS mutant H32-gfp cells lost their fluorescence (i.e., death or loss of metabolic functions) in the biofilm matrix. However, this reduction of biofilm cells was not observed for the rpoS⁺ H32-gfp strain when treated under the same conditions as its rpoS⁻ counter-part. With the Live/Dead stain, a higher number of H32-gfpΔrpoS::gm biofilm cells appeared dead, stressed, or damaged (i.e., stained red) than the H32-gfp cells in both the sterile ddH₂O and the filter-sterilized lake water samples.

In unfiltered lake water, most fields of view were devoid of cells exhibiting native green fluorescence (NS) for both the rpoS mutant and rpoS⁺ strains and displayed considerable cell damage or mortality (red) under LD staining. A heterogeneous microbial community was evident in the unfiltered lake water, as biofilms with complex architecture with various different cell morphologies were visible using SYTO 9 staining. Live/Dead staining revealed that by 13 days, this community was largely composed of dead, stressed, or damaged cells interspersed with small clusters of active intact cells.

**DISCUSSION**

The vast majority of *E. coli* biofilm studies have been conducted on the laboratory strain *E. coli* K-12 in rich media. The concerns with extrapolating the findings of K-12 biofilm studies to *E. coli* O157:H7 strains are well documented (7, 11, 19, 41, 43, 45). The biofilm-forming capabilities of *E. coli* O157:H7 strains H32, H32-gfp, and H32-gfpΔrpoS::gm were investigated in this study. In corroboration with previous findings, *E. coli* O157:H7 biofilm formation was found to be influenced strongly by the media employed in cultivation (19). All three *E. coli* O157:H7 strains exhibited enhanced biofilm formation under low-nutrient conditions (MSMG) and an inability to establish appreciable biofilms in a rich medium (TSB).

The reports of the relative biofilm-forming capabilities of the wild-type and rpoS-deficient *E. coli* K-12 strains have been conflicting. When *E. coli* K-12 biofilms were established in flow cell systems fed by a rich medium, a mutation in rpoS inhibited biofilm formation (1, 30, 48). In contrast, when biofilms were established in a rich batch culture medium in microtiter plates, a mutation in rpoS enhanced biofilm formation (12). With the use of an O157:H7 strain, this study suggests that these inconsistent results may be partly attributable to differences in experimental design, the growth media used, and whether the biofilm samples were analyzed at the growth or survival phase.

Here we report a 2.4- to 7.5-fold increase in biofilm biovolume by an rpoS-deficient strain of *E. coli* O157:H7, relative to the wild-type strains, when grown under batch culture conditions in minimal medium (MSMG) (Fig. 3). However, no increase in the attachment of the rpoS mutant on the glass coverslip, relative to the rpoS⁺ strains, was observed in the nutrient-rich TSB medium. These results suggest that while RpoS may not affect biofilm formation when nutrients are readily available (e.g., rich medium or flow cell systems), it reduces biofilm formation under nutrient-limited conditions (e.g., minimal medium). Our findings are consistent with those of Corona-Izquierdo et al. (12), where an rpoS mutation enhances biofilm formation under nutrient-limited batch culture conditions and that the rpoS regulon is differentially expressed in minimal and rich media (21). Together, these results suggest that the expression of the rpoS-controlled genes, and con-
sequently of biofilm formation, is specific to nutrient availability. The exact role of rpoS in biofilm formation of E. coli O157:H7 under nutrient-limited conditions represents an area for further investigation.

Despite the fact that rpoS expression confers starvation-induced cross-protection against various stresses (38), numerous planktonic-state studies have reported a paradoxical advantage of rpoS mutations when under certain nutrient-limited conditions. When incubated for a prolonged period after the establishment of stationary-phase growth, rpoS-deficient E. coli strains have demonstrated increased fitness over wild-type strains (23, 25, 58, 59, 60). Conversely, under conditions of carbon starvation, the rpoS mutants have been shown to die off more rapidly than the wild-type strains during the late stationary phase (34). However, the role of rpoS in the survival of E. coli O157:H7 in biofilm has rarely been studied. This notion was further pursued in our investigation of the survival of biofilm cells for the rpoS+ and rpoS mutant E. coli O157:H7 strains in sterile distilled water, filter-sterilized lake water, and unfiltered lake water over a period of 13 days.

For both the attached and the total biofilm cells, the survival of the labeled wild-type (H32-gfp) strain was better in sterile distilled water and filter-sterilized lake water than in unfiltered lake water (Fig. 4). These findings are consistent with studies revealing that planktonic E. coli O157:H7 has better survival in autoclaved water and sterile filtered water than in untreated lake and well water (3, 52, 54). Predictably, decreased overall survival results from additive stress, where competition and predation from indigenous flora resulted in more significant reductions in E. coli O157:H7 than did the comparative nutrient deprivation in filter-sterilized lake water and both nutrient deprivation and osmotic stress in distilled water.

In contrast to the growth advantage conferred to rpoS mutants in the formation of biofilms in MSMG, rpoS deficiency was shown to reduce the survival of biofilm cells. Despite the substantial 3- to 5-log10 reduction in the culturability of the attached rpoS+ strain in sterile distilled water and filter-sterilized lake water, the decrease of the detached biofilm cells was relatively mild (0.90 to 1.23 log10) compared to that of the attached cells, implying that the RpoS protein is more important for the survival of attached cells than their planktonic counterparts. Presumably, the rpoS mutant was less equipped to respond to the stresses of the environment, coupled with the inherent stresses inside a biofilm, such as a high cell density and a low oxygen level (44). When differential Live/Dead staining was applied, the vast majority of the rpoS mutant cells exhibited uptake of the propidium iodide (red fluorescence), implying that these cells were either dead, stressed, or damaged. Notably, while the rpoS mutant exhibited decreased culturability of attached biofilm cells relative to the wild-type counterpart in all three water samples, the difference in survival between two strains was most pronounced in distilled water and filter-sterilized lake water but was not as notable in the unfiltered lake water samples (Fig. 4). These results suggest that while RpoS mitigates some of the stress imposed under various aquatic conditions, the advantage proffered by the general stress response is eventually overwhelmed when competition and predation pressures are introduced.

The decrease in the number of culturable cells, determined by plate counting, in the attached portion of the biofilm cells may be partly attributable to the loss of the biofilm cells into the aqueous phase. The substantial reduction of the attached rpoS mutant cells in the sterile ddH2O and filter-sterilized lake water relative to the rpoS+ cells (Fig. 5) indicates that a less stable biofilm was formed by the rpoS mutant H32 strain, despite a larger biofilm biomass being formed during the initial development phase. Therefore, this may explain why reports of the relative biofilm-forming capabilities of wild-type and rpoS-deficient E. coli K-12 strains have been conflicting in the literature. Depending on whether the final measurement is in the developmental or survival stage of the E. coli biofilm, the rpoS mutant may form larger or smaller biofilms than the rpoS+ strain.

The role of biofilms serving as a planktonic “cell factory” for both survival and proliferation, as proposed by Bester et al. (5, 6), coupled with the observation of a starvation-induced dispersal of Pseudomonas putida biofilms (27), has great implications for the dissemination of pathogenic E. coli in natural bodies of water and water distribution systems. The results of this study suggest a strong influence of nutrient availability on the role of rpoS in E. coli O157:H7 biofilm formation and survival, a finding that helps to reconcile inconsistencies found in the literature. Overall, E. coli O157:H7 exhibited a concerning propensity to form biofilms under low-nutrient conditions, similar to those found in natural environments, where rpoS disruption exaggerated this effect. Despite the establishment of smaller biofilms, the rpoS+ strain displayed enhanced survival in sterile distilled and treated lake water, likely through cross-protective mechanisms, before the effect was overwhelmed in untreated lake water. Altogether, this study suggests that the role of rpoS in E. coli O157:H7 biofilm formation and survival is governed not only by nutrient availability but also by other environmental stressors in water, such as competition and predation.

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