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Phenotypic Diversity Caused by Differential RpoS Activity among Environmental *Escherichia coli* Isolates▽†

Sarah M. Chiang,[1] Tao Dong,[1]‡ Thomas A. Edge,[2] and Herb E. Schellhorn[1]*

Department of Biology, McMaster University, Life Sciences Building, 1280 Main St. West, Hamilton, Ontario, Canada L8S 4K1,[1] *and National Water Research Institute, Environment Canada, 867 Lakeshore Road, P.O. Box 5050, Burlington, Ontario, Canada L7R 4A6*[2]

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Enteric bacteria deposited into the environment by animal hosts are subject to diverse selective pressures. These pressures may act on phenotypic differences in bacterial populations and select adaptive mutations for survival in stress. As a model to study phenotypic diversity in environmental bacteria, we examined mutations of the stress response sigma factor, RpoS, in environmental *Escherichia coli* isolates. A total of 2,040 isolates from urban beaches and nearby fecal pollution sources on Lake Ontario (Canada) were screened for RpoS function by examining growth on succinate and catalase activity, two RpoS-dependent phenotypes. The *rpoS* sequence was determined for 45 isolates, including all candidate RpoS mutants, and of these, six isolates were confirmed as mutants with the complete loss of RpoS function. Similarly to laboratory strains, the RpoS expression of these environmental isolates was stationary phase dependent. However, the expression of RpoS regulon members KatE and AppA had differing levels of expression in several environmental isolates compared to those in laboratory strains. Furthermore, after plating *rpoS*$^+$ isolates on succinate, RpoS mutants could be readily selected from environmental *E. coli*. Naturally isolated and succinate-selected RpoS mutants had lower generation times on poor carbon sources and lower stress resistance than their *rpoS*$^+$ isogenic parental strains. These results show that RpoS mutants are present in the environment (with a frequency of 0.003 among isolates) and that, similarly to laboratory and pathogenic strains, growth on poor carbon sources selects for *rpoS* mutations in environmental *E. coli*. RpoS selection may be an important determinant of phenotypic diversification and, hence, the survival of *E. coli* in the environment.

The fecal bacterial contamination of fresh waters is a recurring issue for public beaches surrounding the Great Lakes of North America (57, 79). Direct fecal deposits (20, 78), as well as input from streams (60) and land runoffs (5), often result in high levels of bacteria in both water and sediment. Populations of *Escherichia coli* can persist for long periods outside a host organism and possibly multiply in soil (34). Although fecal deposits are implicated as the primary source of *E. coli* distribution, the environment likely selects for some stress-tolerant *E. coli* strains (4, 35). These "naturalized" *E. coli* populations add a newly recognized complexity to the contamination of fresh waters (11, 34, 80). The persistence and possible multiplication of *E. coli* in the environment may allow for the selection of adaptive mutations comparable to that of long-term laboratory *E. coli* cultures (82, 84). In particular for environmental *E. coli* populations, the selection of adaptive mutations may be central for the survival of cells in adverse conditions (24).

E. coli isolated from different hosts can possess high genotypic diversity (63). Spontaneous mutations are estimated to occur at 5.4×10^{-10} mutations per base pair per replication (19), and the selection of some mutations by unique environmental conditions results in niche-specific adaptation (37, 74). The mutation rate itself is dependent on the environment and likely is important in evolution (7, 29). For laboratory *E. coli* K-12, adaptive mutations are frequently fixed in populations during starvation in long-term batch cultures (28, 84, 85). One such mutation is within the stationary-phase sigma factor gene *rpoS* (13). The loss of RpoS, however, reduces mutagenesis in aging colonies (7) and the variability of adaptation in an evolving *E. coli* population (71). As seen with the Lac system, RpoS is required for point mutations and gene amplification in stationary phase (52), likely in part due to the RpoS regulation of error-prone DNA polymerase IV (47). Greater evolutionary potential can result in a more enduring population, despite a possible short-term disadvantage in competitive fitness (81).

RpoS (σ^s), which is present in *E. coli* and many proteobacteria (14), regulates stationary-phase and stress-related genes (51, 64). Interestingly, RpoS mutants have shorter doubling times on poor carbon sources and in long-term culture (13, 61). The fixation of *rpoS* mutations in a population provides enhanced fitness in nutrient-poor conditions but reduced stress resistance (21, 42, 62), as in low pH (70) or osmotic upshift (31). *E. coli* growth in a glucose-limited chemostat environment selects for multiple *rpoS* genotype subpopulations (54), which can lead to divergence under environmental selection (24). The *rpoS* gene can further be regarded as polymorphic (39, 55, 61), and this polymorphism influences the trade-off between self preservation and nutritional competence (SPANC) (22, 26). Phenotypic diversity observed in clinical

* Corresponding author. Mailing address: Life Sciences Building, Rm. 433, McMaster University, 1280 Main St. West, Hamilton, Ontario, Canada L8S 4K1. Phone: (905) 525-9140, ext. 27316. Fax: (905) 522-6066. E-mail: schell@mcmaster.ca.

‡ Present address: Department of Microbiology and Molecular Genetics, Harvard Medical School, Armenise 411, 200 Longwood Ave., Boston, MA 02115.

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TABLE 1. Summary of source types for the 2,040 environmental *E. coli* isolates used in this study

Type	No. of isolates
Beach water	971
Beach sand	617
Canada Goose (*Branta canadensis*) dropping	131
Sewage plant final effluent	84
Untreated combined sewer overflow	68
Dog (*Canis lupus familiaris*) dropping	65
Cat (*Felis catus*) dropping	48
Gull (*Larus delawarensis*) dropping	38
Mallard duck (*Anas platyrhynchos*) dropping	18

TABLE 2. *rpoS* and *fliA* oligonucleotides used in this study for ORF amplification

Oligonucleotide	ORF	Sequence
ML-08-145	*rpoS*	5′-CAACAAGAAGTGAAGGCGGG-3′
ML-08-4514	*rpoS*	5′-CTTGCATTTTGAAATTCGTTACA-3′
rpoS754	*rpoS*	5′-GATGACGATATGAAGCAGAG-3′
ML-08-4515	*rpoS*	5′-TTAACGACCATTCTCGGTTTTAC-3′
ML-08-5873	*rpoS*	5′-GGTGCAATCTCCAGCCG-3′
ML-08-5874	*rpoS*	5′-GGAGAATCGTGGCTTAGTCAG-3′
rpoSrv834	*rpoS*	5′-TAACATCAAACGAATCGACC-3′
ML-08-3246	*fliA*	5′-ACCTGTAACCCCCAAATAAC-3′
ML-08-3247	*fliA*	5′-CAATGGGTCTGGCTGTG-3′

isolates was attributable, at least in part, to distinct RpoS levels among isolates and its effect on SPANC (49). RpoS mutants have been identified among laboratory (36, 75) and pathogenic (17) *E. coli* strains, and the ancestral *rpoS* sequence of laboratory *E. coli* K-12 likely possessed an amber mutation at codon 33 (2). Nine of 31 tested *E. coli* strains from the ECOR collection, a standard reference collection of *E. coli* from hosts (63), carried deleterious *rpoS* mutations (25). We have previously found that poor carbon sources, such as succinate, readily select for the loss of RpoS function in both laboratory (13) and pathogenic strains (17) at a frequency of 10^{-8} mutants per cell plated. By extension, stressful environmental conditions, such as poor carbon and nutrient sources, may select for RpoS mutants in environmental *E. coli* populations.

Adaptive mutations within the *rpoS* gene are selected in laboratory and pathogenic *E. coli* strains in nutrient-poor conditions or during growth on nonpreferred carbon sources (13, 17, 61, 84). Because *E. coli* can be found in a wide range of environments (10, 34, 56), including putative poor carbon environments, we postulated that RpoS mutants are present, and may be selected, in environmental *E. coli* populations. Recent work using the ECOR strains found RpoS mutants in 29% of natural isolates (25). In this study, the frequency of RpoS mutants among environmental *E. coli* isolates was determined, as well as the ability to select for RpoS mutants from environmental *E. coli* on a poor carbon source. Differential RpoS expression is examined as a mechanism for generating phenotypic diversity in environmental *E. coli*. The RpoS sigma factor is a useful model because it is well-studied, is known to be lost by selection in nutrient-poor conditions, and as a global regulator has phenotypic effects that can be readily assessed in the laboratory.

MATERIALS AND METHODS

Bacterial strains. Laboratory *Escherichia coli* K-12 strains used in this study were MG1655 and the precise *rpoS* deletion of MG1655, which was constructed previously (64). A total of 2,040 environmental *E. coli* isolates were collected from urban beaches (water and sand samples) and nearby fecal pollution sources (wastewater effluents and animal fecal droppings) in the cities of Hamilton and Toronto on Lake Ontario (Canada). The cities of Hamilton and Toronto, Ontario, are large urban centers along the shoreline at the western end of Lake Ontario. The area has a temperate climate, with summer temperatures reaching more than 30°C at times, and ice cover in the nearshore areas during the winter months. *E. coli* isolates were obtained from water and sand samples at Bayfront Park and Burlington Beaches around Hamilton (43°15′ N, 79°51′ W) and at Kew and Centre Island Beaches in Toronto (43°40′ N, 79°24′ W). *E. coli* isolates from wastewater effluent samples were obtained from the final effluents of sewage treatment plants in Hamilton and Toronto. *E. coli* isolates were obtained from fresh fecal droppings of dogs (*Canis lupus familiaris*) and cats (*Felis catus*) at Hamilton and Toronto animal shelters. Isolates also were obtained from fresh fecal droppings of Canada Goose (*Branta canadensis*), gulls (*Larus delawarensis*), and Mallard ducks (*Anas platyrhynchos*) around Hamilton and Toronto beaches. The *E. coli* isolates were obtained by previously described methods (13), and their environmental sources are summarized in Table 1. A full table of individual isolates is provided in Table S1 in the supplemental material.

Media and growth conditions. Environmental isolates were replica plated into sterile 96-well microplates containing 200 μl/well of Luria-Bertani (LB) medium (58). The microplates were incubated overnight without shaking at 37°C. Prior to experiments, isolates were replica plated from the microplates containing LB medium into sterile microplates containing 200 μl/well of 1× M9 salts (58) for the purpose of minimizing nutrient carryover from the LB-rich media. The isolates in 1× M9 salts were used immediately in replica plate experiments on solid medium. For testing growth on tricarboxylic acid (TCA) intermediates and weak acids, isolates were replicated onto 0.5% (wt/vol) glucose, 1% succinate, 1% fumarate, 1% citrate, 1% α-ketoglutarate, 1% acetate, 1% (vol/vol) lactate, 0.5% formate, or 0.5% proprionate M9 minimal media (pH 7) (65). For sequencing and immunoblot analyses, isolates were streaked onto solid LB medium and incubated overnight at 37°C. Single colony isolates were used in all experiments. For calculating generation times, environmental isolates were grown at 37°C with shaking to an optical density at 600 nm (OD_{600}) of ~1 in 0.5% (wt/vol) glucose M9 minimal medium and subcultured to an OD_{600} of ~0.03 in 0.5% glucose, 1% succinate, or 1% fumarate M9 minimal medium (pH 7). Growth was monitored spectrophotometrically (OD_{600}), and the generation time was calculated (17).

RpoS-dependent phenotype tests. (i) Growth on succinate minimal medium. Environmental isolates were replica plated onto 1% succinate M9 minimal medium (65) and incubated overnight at 37°C. Isolates were observed for growth after 24 and 48 h of incubation. Isolate patches with substantial growth after 24 h at 37°C were considered to grow well on succinate (designated Suc^{++}). As a control for nonselective growth on minimal medium, isolates were replica plated onto 0.5% glucose M9 minimal medium (65).

(ii) Catalase test. Environmental isolates were replica plated onto solid LB medium without antibiotics. Plates were incubated overnight at 37°C and tested for the presence of catalase by the addition of 5 μl of 30% hydrogen peroxide (H_2O_2) onto the patches. Isolates were recorded as catalase positive or negative, where catalase-negative strains had a severe lag in bubbling time after the addition of H_2O_2 (13).

Selection for loss of RpoS activity on succinate minimal media. Selection for the loss of RpoS activity by growth with succinate as the sole carbon source was performed by growing single colonies of environmental isolates in LB medium at 37°C and 200 rpm overnight. Cultures were washed by centrifugation in 1× M9 salts, and cells (~10^9) were plated on succinate minimal plates (13). After 48 to 72 h of incubation at 37°C, fast-growing mutants (Suc^{++}) could be observed. Single Suc^{++} mutant colonies then were serially streaked onto LB solid medium for purity. Suc^{++} mutants were tested for catalase activity and succinate growth as described above. The loss of RpoS activity in selected mutants was confirmed by the sequencing of the *rpoS* gene.

PCR amplification and sequencing of *rpoS* and *fliA* genes. Whole-colony PCR amplicons of *rpoS* and *fliA* genes from several isolates were sequenced. Single colonies were picked and boiled in 10 μl of sterile deionized/distilled water (ddH_2O) at 95°C for 5 min. A 2-μl aliquot then was transferred to the PCR reagent mix. The primers used for *rpoS* and *fliA* open reading frame (ORF) amplification are listed in Table 2 (synthesized by MOBIX Laboratory, McMaster University, Hamilton, Ontario, Canada). PCR was performed with Fer-

mentas *Pfu* DNA polymerase (Fermentas, Inc., Burlington, Ontario, Canada). PCR conditions consisted of an initial denaturation step at 95°C for 2 min, 30 cycles of 30 s at 95°C, 30 s at 58°C, and 2.5 min at 72°C, and a terminal extension step at 72°C for 5 min. All PCR products were purified using a NucleoSpin Extract II kit (Machery-Nagel GmbH & Co., Inc., Bethlehem, PA) and visualized on a 1% agarose gel for quantification prior to sequencing. Samples were sequenced by MOBIX Laboratory and analyzed with Sequence Scanner, version 1.0 (Applied Biosystems, Inc., Streetsville, Ontario, Canada). Sequencing was performed on both strands of the PCR product.

Analyses of *rpoS* and *fliA* sequences in environmental *E. coli* isolates. The *rpoS* and *fliA* ORF sequences were edited using Sequence Scanner, version 1.0 (Applied Biosystems, Inc., Streetsville, Ontario, Canada) and aligned with Clustal X (IUB DNA weight matrix) (46). The *rpoS* sequences were compared to the consensus sequence, which is a composite sequence composed of the most frequent (modal) base at each nucleotide position among isolate and laboratory K-12 *rpoS* sequences. The *rpoS* sequences were constructed into a dendrogram using the neighbor-joining method (46). The dendrogram was bootstrapped 1,000 times, and it was visualized with MEGA 4.0 (73) as previously described (14). The *fliA* sequences were compared to the laboratory K-12 *fliA* sequence.

Motility assay. Individual colonies of bacteria were stabbed into 200 μl of 0.15% LB agar in a sterile 96-well microplate. The microplate was incubated at 37°C overnight without shaking prior to observation.

Native PAGE analysis of catalase activity. Overnight *E. coli* cultures in LB liquid medium were sampled and centrifuged at 4,000 × *g* for 15 min. Samples were washed with 50 mM potassium phosphate buffer (pH 7.0) and sonicated (17). Five micrograms of protein was separated on 10% nondenaturing polyacrylamide gels and stained for catalase using horseradish peroxidase and diaminobenzidine (30).

Immunoblot analyses of RpoS protein levels. Overnight *E. coli* cultures in LB liquid medium were subcultured 1:10,000 into 50 ml of LB and incubated at 37°C with shaking at 200 rpm. Culture samples were taken at exponential phase (OD_{600} of 0.3), early stationary phase (OD_{600} of 1.5), and 24 h after subculture. Chloramphenicol was immediately added to the samples to a final concentration of 150 μg/ml to stop protein synthesis, and samples were centrifuged at 12,000 × *g* for 5 min. The supernatant was removed by pipette, and pellets were resuspended in SDS loading buffer (125 mM Tris-Cl, pH 6.8; 2.5% β-mercaptoethanol; 8.7% glycerol; 1% SDS; 0.01% bromophenol blue) for a final cell concentration equivalent to an OD_{600} of 1.0. Resuspended pellets were placed in boiling water for 5 min.

Ten microliters of protein samples was resolved on 10% SDS polyacrylamide stacking gels. A second gel was stained for protein with 0.1% Coomassie blue dye to ensure equal protein loading. Resolved proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Inc., Billerica, MA), and the membrane was incubated for 1 h at room temperature in 5% milk made in TBS-T (87 mM NaCl; 10 mM Tris-Cl, pH 8; 0.05% Tween 20). The blocking buffer was replaced with a 1:10,000 dilution of primary antibody (anti-σ^s antibody; Neoclone, Inc., Madison, WI; or anti-AppA; a gift from C.W. Forsberg, University of Guelph), and the membrane was left overnight with gentle shaking at 4°C. After washing with TBS-T, the membrane then was placed for 1 h at room temperature with shaking in a 1:3,000 dilution of the secondary anti-mouse antibody for the σ^s membrane or anti-rabbit antibody for the AppA membrane (Bio-Rad Laboratories, Inc., Mississauga, Ontario, Canada). After washing again with TBS-T, the secondary antibody fluorophore was activated in ECL staining solution (1:1 detection reagent mixture; Amersham GE Healthcare, Inc., Baie d'Urfe, Quebec, Canada) for 1 min prior to exposure on Amersham Hyperfilm ECL for 10 s to 1 min.

Survival assays. (i) Acid resistance. Overnight cultures of environmental isolates in LB were subcultured to ~10^5 cells into LB medium adjusted to pH 2.5 with hydrochloric acid. Cultures then were incubated at 37°C with shaking at 200 rpm for 2 h and serially plated onto LB plates to determine the final CFU/ml (76). Percent survival was calculated as (final CFU/ml)/(initial CFU/ml) × 100.

(ii) H_2O_2 resistance. Overnight cultures of environmental isolates in LB were washed with 0.9% NaCl and subcultured to ~10^7 cells into LB medium containing 15 mM H_2O_2. Cultures then were incubated at 37°C with shaking at 200 rpm, and CFU/ml was determined over time by serially plating onto LB plates (18, 45). The percent survival was calculated as (final CFU/ml)/(initial CFU/ml) × 100.

Nucleotide sequence accession numbers. Sequences were deposited at GenBank (3) with accession numbers JN191237 to JN191281 for *rpoS*, JN191282 to JN191289 for *fliA*, and JN191290 to JN191292 for succinate-selected RpoS mutants.

RESULTS

Environmental *E. coli* isolates. A collection of 2,040 diverse environmental *E. coli* isolates from urban beaches and nearby fecal pollution sources in the cities of Hamilton and Toronto were used for this study. These isolates were collected between April and September 2004 and between May and June 2005 from beach water, foreshore beach sand, sewage treatment plant final effluent, untreated sewage from combined sewer overflow storage tanks, and fresh fecal droppings from dogs, cats, gulls, ducks, and Canada Goose (Table 1). These isolates were used as a representative collection of *E. coli* isolated from environmental sources outside a host organism.

Screen for RpoS activity using two RpoS-dependent phenotypes, growth on succinate and catalase activity. RpoS activity of *E. coli* isolates were assessed using two RpoS-dependent phenotypes, growth on succinate and catalase activity, to identify the prevalence of environmental RpoS mutants.

RpoS mutants of laboratory *E. coli* exhibit better growth on poor carbon than wild-type cells (13, 41). After replica plating environmental *E. coli* isolates onto media with succinate as the sole carbon source, 93 isolates had significant growth after 24 h of incubation (Suc^{++}), which is similar to that of an *rpoS* mutant control. Seven Suc^{++} isolates also were catalase negative, as determined by a catalase test. The Suc^{++} isolates were from beach water (45/971), beach sand (31/617), untreated sewage (6/68), sewage treatment plant final effluent (4/84), and fresh animal fecal droppings (7/300). The largest percentage of Suc^{++} isolates, therefore, was from untreated sewage (8.8%).

RpoS regulates one of two primary catalase genes, *katE*, in laboratory *E. coli* K-12 (67), and consequently an RpoS mutant colony has reduced bubbling compared to that of an RpoS-positive colony with the addition of hydrogen peroxide. Of the 2,040 isolates, 38 isolates were determined to have reduced catalase activity. These isolates were from beach water (14/971), beach sand (11/617), animal fecal droppings (5/300), sewage treatment plant final effluent (5/84), and untreated sewage (3/68). Similarly to the Suc^{++} phenotype, the largest percentages of catalase-negative isolates were from untreated sewage (4.4%) and final effluent (5.6%).

***rpoS* alleles confirm presence of RpoS mutants among environmental *E. coli* isolates.** The *rpoS* genes of 45 environmental *E. coli* isolates, including all isolates that were both Suc^{++} and catalase deficient, as well as representatives of isolates with only one RpoS mutant phenotype, were sequenced to confirm the presence of RpoS mutants. A dendrogram of the 45 *rpoS* sequences with the isolate sources can be found in Fig. S1 in the supplemental material.

Eight nonsynonymous and 81 distinct synonymous mutations were identified among the isolates (see Table S2 in the supplemental material). A nonsynonymous mutation at codon 33, where glutamine (CAG) is replaced with glutamic acid (GAG), is characteristic of non-K-12 strains (2, 72), and not surprisingly, it was found in all environmental *E. coli rpoS* sequences determined in this study. Three other nonsynonymous mutations resulted in an amino acid change but yielded a putatively functional RpoS protein, as these mutations had no other notable impact on the RpoS protein. More specifically, isolate ABC01 from beach sand had lysine (AAG) replace glutamic acid (GAG) at codon 122; EKF07 from beach

TABLE 3. Identified mutations in the *rpoS* gene that predict a nonfunctional RpoS protein in environmental *E. coli* isolates

Isolate	Source type	Type of mutation	Effect of mutation on amino acid sequence	Location (nucleotides)	*rpoS* accession no.
ECE12	Untreated combined sewer overflow	5-bp deletion	Frameshift, short protein	255–259	JN191271
BNB03[a]	Beach sand	1-bp deletion	Frameshift, short protein	378	JN191252
AZB07	Beach sand	G→A transition	TGG→TAG (amber stop codon)	443	JN191250
ASC02	Sewage treatment plant final effluent	1,329-bp insertion (IS*10* transposase) and 9-bp duplication	Short protein	776	JN191249

[a] Isolates BNB04 (JN191253) and BNB07 (JN191254), from beach sand, possessed the same *rpoS* mutation as isolate BNB03.

water had tyrosine (TAC) replace asparagine (AAC) at codon 124; and ECH01 from beach water had serine (TCC) replace threonine (ACC) at codon 298. The remaining four nonsynonymous mutations resulted in a nonfunctional RpoS protein. ECE12 isolated from untreated sewage had a 5-bp deletion (nucleotides 255 to 259); BNB03, BNB04, and BNB07 from beach sand had a 1-bp deletion (nucleotide 378); AZB07 from beach sand had a G→A transition (nucleotide 443), resulting in an amber stop codon; and ASC02 from sewage treatment plant final effluent had a 1,329-bp insertion, which has 100% identity to a putative IS*10* transposase of *E. coli* O111:H$^-$ strain 11128 (NCBI; February 2011) and a characteristic surrounding 9-bp DNA repeat (43) (Table 3).

Comparison of mutational frequencies of the *rpoS* and *fliA* genes. Among the environmental *E. coli* isolates, there were 81 distinct synonymous mutations within the *rpoS* gene (see Table S2 in the supplemental material), for a frequency of 1.8×10^{-3} synonymous mutations per base per isolate. The *rpoS-mutS* region of *E. coli*, as in other enteric bacteria, is an area of high genetic variation and putative high recombination (9, 32, 55). Therefore, we chose a gene of length similar to that of *rpoS*, the flagellar sigma factor *fliA*, to determine if the frequency of synonymous mutations in *rpoS* was unique among other genes in environmental *E. coli*.

The *fliA* gene was sequenced for eight isolates of different catalase activity and succinate growth. These isolates were one RpoS mutant (ECE12); two *rpoS*$^+$ (according to *rpoS* sequencing), catalase-negative isolates; three *rpoS*$^+$, Suc^{++} isolates; and two comparison isolates. All isolates carried synonymous mutations from K-12 *fliA*, and one nonsynonymous mutation was identified in ABB10 (see Table S3 in the supplemental material). A motility assay in 0.15% LB agar indicated that ABB10 had reduced motility (data not shown). Among the eight isolates, 12 synonymous mutations were identified in 720 nucleotides (see Table S3), for a frequency of 2.1×10^{-3} synonymous mutations per base per isolate. Therefore, the number of synonymous mutations in the *fliA* gene was comparable to that in the *rpoS* gene, with a frequency of 10^{-3} synonymous mutations per base per isolate.

RpoS protein levels in environmental *E. coli* isolates. In laboratory *E. coli*, RpoS protein levels increase during entry into stationary phase (38). For environmental isolates, RpoS expression may be altered through phenotypic diversification and environmental selection. To examine if RpoS in environmental isolates has stationary-phase expression similar to that of laboratory strains, we performed immunoblots on six isolates at exponential phase, early stationary phase, and 24 h after subculture. These isolates were three isolates with a functional RpoS phenotype (ABB02, ECA03, and ECA08) and three isolates with the *rpoS*$^+$, Suc^{++} phenotype (ECF09, ECG09, and ECC08). All isolates grew well in rich media (Fig. 1a).

Like the control *E. coli* K-12 strain, RpoS expression was stationary phase dependent in all environmental *E. coli* isolates tested (Fig. 1b). The expression of RpoS from early stationary phase to 24 h postinoculation decreased in environmental isolates, although the overall expression of RpoS was isolate dependent. ABB02, ECA03, and ECG09 had more RpoS protein at early stationary phase than the other three isolates. Importantly, RpoS expression was not decreased in *rpoS*$^+$, Suc^{++} isolates.

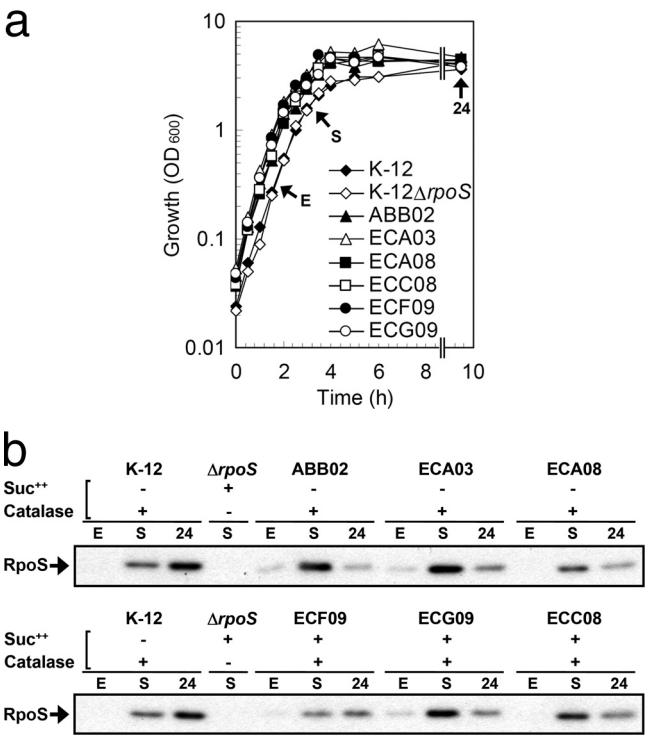

FIG. 1. (a) Growth of environmental isolates in rich media. ABB02 from beach sand, ECA03 from beach water, and ECA08 from beach water have RpoS-positive catalase and succinate growth phenotypes. ECF09 from untreated combined sewer overflow sewage, ECG09 from beach water, and ECC08 from beach water have RpoS-negative, Suc^{++} phenotypes but are catalase positive. Samplings are indicated as exponential phase (E), early stationary phase (S), and 24 h postinoculation (24). (b) Immunoblot detection of RpoS protein levels in environmental *E. coli* isolates.

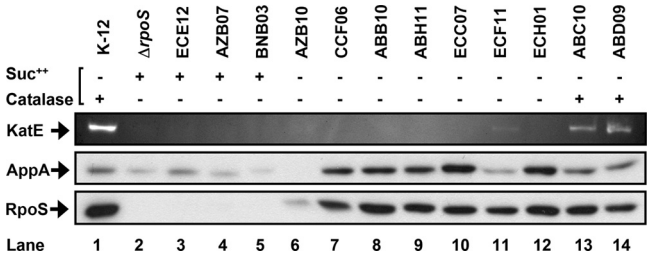

FIG. 2. RpoS-dependent KatE and AppA levels compared to those of RpoS in catalase-deficient environmental *E. coli* isolates in stationary phase. The upper gel shows 5 μg of protein run on nature PAGE and stained for catalase. The middle and lower panels show 4 μg of protein run on SDS-PAGE, and AppA and RpoS proteins were detected by immunoblotting. Isolates ECE12, AZB07, and BNB03 are confirmed RpoS mutants. Isolates ABC10 and ABD09 are catalase positive.

RpoS-dependent protein expression. The RpoS sigma factor regulates a large portion of the *E. coli* genome (44, 64, 77). To examine the effect of RpoS on regulon genes in environmental *E. coli*, the protein levels from two RpoS-dependent genes, *katE* (68) and *appA* (45), were determined (Fig. 2).

For confirmed RpoS mutants ECE12, AZB07, and BNB03, RpoS protein could not be detected by immunoblotting (Fig. 2, lanes 3 to 5). KatE expression also was low, comparable to that of the K-12Δ*rpoS* strain. For all other isolates tested, RpoS protein levels were similar, with the exception of lower RpoS levels in isolate AZB10 from beach sand (Fig. 2, lane 6). Isolate ECF11 from bird fecal droppings had minimal KatE expression (Fig. 2, lane 11), and for the *rpoS*$^+$, catalase-deficient isolates, KatE expression was too low for detection, even though RpoS was present (Fig. 2, lanes 7 to 10 and 12). As *rpoS*$^+$ environmental isolate controls, isolates ABC10 and ABD09 from animal feces and untreated sewage, respectively, expressed both RpoS and KatE similarly to K-12 (Fig. 2, lanes 13 and 14).

Despite the presence of RpoS as determined by immunoblotting, KatE expression is low in several isolates, which may be the result of impaired RpoS activity. To determine if RpoS activity is impaired, the levels of a second RpoS-dependent protein, AppA (45), were examined and compared to the levels of KatE and RpoS. As expected, the RpoS mutant isolates had lower AppA expression. The *rpoS*$^+$, catalase-deficient isolates had greater AppA expression than K-12, with the exceptions of AZB10 and ECF11. AZB10, with lower overall RpoS expression, had undetectable levels of AppA (Fig. 2, lane 6), and ECF11, despite the presence of RpoS, had lower AppA levels (Fig. 2, lane 11).

Selection of RpoS mutants from environmental *E. coli* on succinate. In this study, we identified six null RpoS mutants in 2,040 environmental isolates (0.3% among isolates). Using laboratory and pathogenic *E. coli*, we have previously reported that RpoS mutants are readily selected on poor carbon sources (13, 17). To determine if RpoS in environmental *E. coli* isolates can be similarly subject to environmental selection, we plated three *rpoS*$^+$ environmental isolates (ECA01, ECD03, and ECF01) on succinate minimal media.

After 2 to 3 days of incubation at 37°C on succinate minimal media, larger colonies (Suc^{++}) were visible at a frequency of approximately 10^{-8}, as seen with laboratory and pathogenic strains (13, 17). One independent mutant from each isolate was tested and confirmed to be an RpoS mutant by sequencing the *rpoS* ORF. Single null mutations were identified within the *rpoS* ORF of each succinate-selected mutant (Table 4).

Characterization of RpoS mutants. For environmental *E. coli* outside a host, *rpoS* mutations may be selected under adverse conditions and lead to phenotypic diversity. The loss of RpoS increases the ability of *E. coli* to grow on nonpreferred carbon sources (13, 17, 23) and concomitantly decreases survival in stress (24, 61, 76). Therefore, to examine the RpoS mutant phenotype of environmental *E. coli* populations, naturally isolated RpoS mutant AZB07 and succinate-selected RpoS mutant ECA01Suc^{++} were tested for their abilities to grow on poor carbon sources as well as for their resistance to acidic pH and to oxidative stress.

For growth on the poor carbon sources succinate and fumarate, RpoS mutants had lower generation times than the K-12 control and ECA01Suc^{++} isogenic parental strain, ECA01 (Table 5). More specifically, RpoS mutants had approximately 18× faster growth on succinate and 6× faster growth on fumarate. As seen in other studies (13, 17), the difference in generation time was not apparent for growth with the preferred carbon source, glucose.

For stress resistance, RpoS mutants were less resistant to both acidic pH (Fig. 3a) and exposure to H_2O_2 (Fig. 3b). Functional RpoS was essential for cells to withstand low pH, as less than 0.0001% of RpoS mutant cells were able to retain viability. Interestingly, while the K-12 control had just 31% survival after 2 h at pH 2.5, ECA01 showed an increase in numbers of CFU/ml, which suggests a strong resistance in this environmental isolate to acidic conditions. During exposure to H_2O_2, RpoS mutants lost viability more rapidly than RpoS-positive cells. These results are in agreement with previous research using laboratory *E. coli* strains (31, 70).

DISCUSSION

Bacterial adaptation depends on complex regulatory systems that may control the differential expression of hundreds of genes. Although such adaptation has been extensively examined in laboratory strains over the last two decades, the role of adaptation in natural *E. coli* isolates has received little atten-

TABLE 4. Mutations within the *rpoS* gene of succinate-selected mutants of environmental isolates

Succinate mutant	Selection source	Type of mutation	Effect of mutation on amino acid sequence	Location (amino acids)	*rpoS* accession no.
ECA01Suc^{++}	ECA01	96-bp deletion	32-amino-acid deletion	54–85	JN191290
ECD03Suc^{++}	ECD03	G→A transition	TGG→TAG (amber stop codon)	148	JN191291
ECF01 Suc^{++}	ECF01	Adenosine insertion	Frameshift, short protein	32	JN191292

TABLE 5. Growth of environmental *E. coli* RpoS mutants on glucose or on the poor carbon source succinate or fumarate[a]

Substrate	Generation time (min) of strain:				
	K-12	K-12Δ*rpoS*	ECA01	ECA01Suc^{++}	AZB07
Glucose	93.0 ± 2.1	85.1 ± 3.5	79.5 ± 3.8	78.1 ± 8.8	76.3 ± 1.0
Succinate	1,347.1 ± 62.9	141.9 ± 5.1	2,403.0 ± 105.2	90.8 ± 5.9	75.4 ± 2.7
Fumarate	832.1 ± 100.2	129.6 ± 13.3	537.4 ± 68.5	81.1 ± 7.5	118.9 ± 1.2

[a] ECA01Suc^{++} is a succinate-selected RpoS mutant from ECA01, and AZB07 is an RpoS mutant isolated from beach sand. Values are means ± standard errors of the means, where $n = 3$.

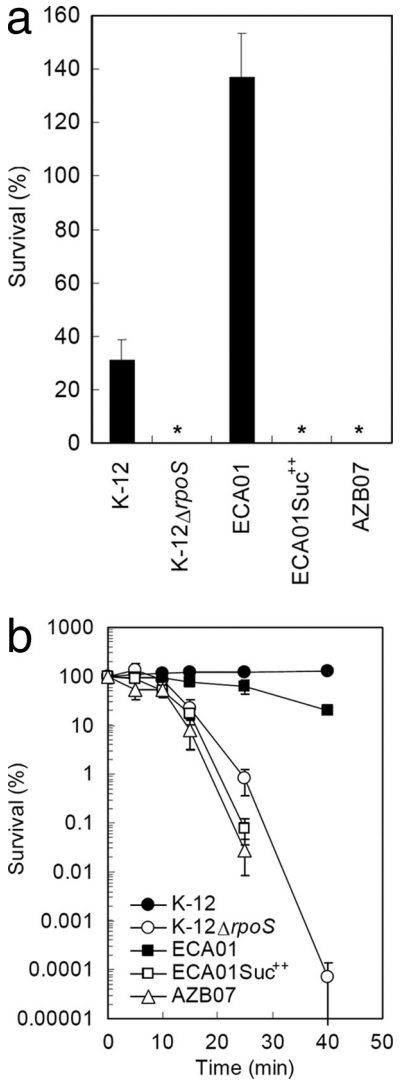

FIG. 3. (a) Survival of environmental isolates in acidic conditions. LB medium (pH 2.5) was inoculated with ~10^5 cells and incubated at 37°C with shaking for 2 h. Percent survival was calculated as (final CFU/ml)/(initial CFU/ml) × 100, and values of <0.0001% are indicated with an asterisk. ECA01Suc^{++} is a succinate-selected RpoS mutant from ECA01, and AZB07 is an RpoS mutant isolated from beach sand. The experiment was done in triplicate, and values represent means ± standard errors of the means. (b) Survival of environmental isolates in oxidative stress. LB medium with 15 mM H_2O_2 was inoculated with ~10^7 cells, and the percent survival was calculated over time. At 40 min, ECA01Suc^{++} and AZB07 had <0.0001% survival. ECA01Suc^{++} is a succinate-selected RpoS mutant from ECA01, and AZB07 is an RpoS mutant isolated from beach sand. Experiments were done in triplicate, and values represent means ± SEM.

tion. In this study, we examined the RpoS regulon as a model of phenotypic diversity among a large collection of environmental *E. coli* isolates. This represents the first broad survey of RpoS activity in *E. coli* isolates collected from the environment. These strains underwent minimal laboratory handling prior to testing and storage, thus they represent a unique collection that can be examined for natural phenotypic diversity. RpoS loss-of-function mutants previously have been identified in several contexts, including laboratory (2, 36, 75) and pathogenic (17) *E. coli* strains and during long-term culture (25, 61, 83) and growth on poor carbon sources (13), suggesting that such mutants are selected for under some circumstances. Because diverse environmental conditions where *E. coli* can be found may mimic these conditions, we wished to determine the frequency of RpoS mutants in environmental *E. coli* isolates.

Two RpoS-dependent phenotype tests, growth on succinate and catalase activity, were used initially in this study to provide an indirect assessment of RpoS activity, since these can be readily adapted to colony screening. The highest percentage of Suc^{++} isolates and of catalase-negative isolates were found in human fecal sources, which may indicate a favorable selective environment. By screening for these phenotypes among the 2,040 environmental isolates, and the subsequent sequencing of the *rpoS* gene in candidate mutant strains, six isolates were identified to carry deleterious *rpoS* mutations. These six isolates, as well as one *rpoS*$^+$ isolate (EKF07, from beach water), had a consistent RpoS mutant phenotype for both succinate growth and catalase activity. The beach water isolate (EKF07) may have been attenuated by a nonsynonymous mutation at codon 124, where tyrosine (TAC) replaced asparagine (AAC). Considering the spontaneous mutation rate of 5.4×10^{-10} mutations per base pair per replication in *E. coli* (19), the relatively high abundance of RpoS mutants among environmental *E. coli* isolates (0.3%) suggests that certain natural environments favor the selective growth of RpoS loss-of-function mutants.

Thirty-one natural isolates from the ECOR collection, a genotypically diverse set of natural *E. coli* strains from animal and human hosts (63), recently was examined for RpoS expression (25). Interestingly, RpoS mutants were found at a substantially higher frequency (0.29) among the ECOR collection compared to that among the environmental isolates studied here (0.003). Because the ECOR collection was compiled largely based on genotypic diversity (63), the sequence variability of the *rpoS* gene in these strains may not be surprising. ECOR strains with elevated RpoS expression also accumulate *rpoS* mutations under nutrient limitation (25). The long-term storage of the ECOR strains, as well as the diverse hosts from

which these strains were isolated, may have contributed to a higher observed frequency of RpoS mutants in the ECOR collection than in the environmental isolates examined in this study.

Of the RpoS mutants identified among the environmental isolates, three RpoS mutants (BNB03, BNB04, and BNB07) taken from the same beach sand sample possessed identical *rpoS* mutations. These beach sand isolates may have resulted from clonal growth in a natural environment, as the multiplication of *E. coli* in sediment has previously been shown to reach a high cell density of 10^5 CFU/g nonsterile soil (34). Alternatively, clones from the same fecal deposit may have persisted in the soil. One naturally isolated RpoS mutant (AZB07) from beach sand also was identified to carry the same G→A transition, resulting in an amber stop codon, as a later succinate-selected RpoS mutant (ECD03 Suc^{++}). The selection of this G→A transition in both AZB07 and ECD03 Suc^{++} is not surprising, given that G/C-to-A/T transitions in bacteria are more common than A/T to G/C (33, 50). The same *rpoS* mutation in the naturally isolated and the succinate-selected RpoS mutant strains also suggests that growth on succinate can select for mutations that mimic mutations that are naturally selected. Finally, to our knowledge, the presence of a transposon element within the *rpoS* gene rendering a nonfunctional RpoS protein, as was found in isolate ASC02 from sewage treatment plant final effluent, has not been reported previously for environmental *E. coli* strains. Unfortunately, using the six RpoS mutants identified, it is not possible to determine a common stimulus that selects for RpoS mutants, as the isolates came from several independent sources.

The *rpoS-mutS* region of *E. coli* strains is hypervariable (32) and undergoes frequent recombination (9, 15). The *rpoS* gene is polymorphic in *E. coli* (6, 27), as well as in other gammaproteobacteria (39). Despite the reported high sequence variability of the *rpoS* gene, we found that the frequency of synonymous mutations in the *fliA* gene, encoding another nonessential sigma factor, was comparable to that of the *rpoS* gene. At nucleotide position 519 of *fliA*, all sequenced environmental isolates carried guanosine instead of adenosine (found in the *fliA* K-12 strain), strongly suggesting that guanosine at this position is ancestral. Isolate ABB10 from untreated sewage, which carried a Ser→Asn mutation at codon 176, also had decreased motility, which suggests that this site is functionally important for motility. The high and comparable number of mutations identified in *rpoS* and *fliA* is consistent with the theory that many of these mutations are neutral under natural selection (40). Studies on codon bias, a determinant of synonymous mutation rates in enterobacteria (69), and its effect on selection for *rpoS* mutations in environmental *E. coli* would be of future interest. Putatively, synonymous mutations in *rpoS* accumulate under low environmental selection, while under strong selection, such as succinate minimal media, single *rpoS* null mutations become fixed in *E. coli* populations.

As noted above, RpoS regulates the expression of the catalase gene, *katE* (67), and using the catalase test (see Materials and Methods), environmental isolates were screened for RpoS activity. A total of 38 isolates showed reduced bubbling, indicating lower catalase expression, yet only six of these were RpoS mutants. Previous studies have found that mutations in the *rpoS* allele result in intermediate bubbling (84). In two $rpoS^+$, catalase-negative isolates, ECH01 and EKF07, threonine is replaced with serine (amino acid 198) and asparagine is replaced with tyrosine (amino acid 124), respectively (see Table S2 in the supplemental material), which indicates the functional importance of these amino acids at these sites. However, the remaining $rpoS^+$, catalase-negative isolates possessed only synonymous *rpoS* mutations. Environmental *E. coli* strains may have adaptive modifications in the expression of regulon genes that are independent of those found in RpoS. RpoS regulon composition, compared between species, is diverse and largely species specific (14, 66), but the extent of RpoS regulon plasticity between strains of the same species is not known. By examining the expression of a second RpoS-dependent protein, AppA (45), and the expression of RpoS in several $rpoS^+$, catalase-negative isolates, it was determined that only one isolate (AZB10 from beach sand; Fig. 2, lane 6) had reduced RpoS expression, resulting in lower regulon expression. The remaining $rpoS^+$, catalase-negative isolates exhibited little to no KatE expression, although RpoS and RpoS-dependent AppA were present (Fig. 2, lanes 7 to 12). This indicates that the expression of even prototypical RpoS regulon members is strongly influenced by factors in environmental *E. coli* isolates. Mutations to RpoS posttranslational factors, such as to Crl (8), also may contribute to differential regulon expression. Interestingly, the AppA expression of $rpoS^+$, catalase-negative isolates, with the exception of isolate AZB10 from beach sand and isolate ECF11 from bird fecal droppings, had higher AppA levels than the laboratory control (Fig. 2, lanes 7 to 10 and 12). A decrease in the expression of one RpoS regulon member may allow for more transcriptional recruitment of RpoS to other dependent promoters. Additional work on RpoS regulon expression is needed to validate this possibility.

Mutations in *rpoS* can be selected from laboratory and pathogenic *E. coli* strains grown with succinate as the sole carbon source (13, 17). In this study, we show that *rpoS* mutants also can be readily selected from environmental *E. coli* when grown on succinate (Table 4) and have enhanced growth on poor carbon sources (Table 5). Because RpoS mutants were found in 0.3% of isolates, there likely are natural environments that also favor RpoS mutant growth. Of course, it is not clear from this study when *rpoS* mutations accrue, during passage through the gut or after deposition in the environment, and what natural environments select for RpoS mutants. *E. coli* can adapt to carbon source and oxygen availability through changes in metabolism (48), and the loss of RpoS previously has been shown to induce the expression of TCA cycle genes (64). RpoS mutants may have an adaptive advantage during nutrient-limited growth in the natural environment as well as in some host environments, such as in the urinary tract, where the TCA cycle is required for infection *in vivo* (1). On the other hand, fermentation in the gut and the production of short-chain fatty acids, such as acetate, proprionate, and butyrate (16, 53), may select for isolates with functional RpoS protein, as RpoS is important in acid resistance (Fig. 3b). The environmental isolates used in this study exhibit differential abilities to utilize carbon sources, as growth on a range of TCA cycle intermediates or on weak acids present in the gut is strain dependent (data not shown). Metabolic demands on *E. coli* in the host (1, 12, 59) and in the environment may be important determinants of RpoS selection. Further study is required to

identify natural conditions that select for *rpoS* mutations and diverse metabolic capabilities of environmental *E. coli*.

In conclusion, RpoS is an alternative sigma factor that is known to be subject to environmental selection (13, 41). As such, mutations in the *rpoS* gene lead to phenotypic diversity in environmental *E. coli* isolates. Previous work has focused on *rpoS* mutations in laboratory and pathogenic *E. coli* (13, 17), yet the diverse conditions that environmental *E. coli* experience may have high selective pressures. Indeed, environmental RpoS mutants were determined to exist at a frequency as high as 0.003, and RpoS mutants from environmental *E. coli* isolates could be selected when grown on a poor carbon source. The natural selection of RpoS mutants outside a host may, therefore, be an important determinant in environmental *E. coli* adaptation and survival.

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