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FEATURE ARTICLE

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Evolutionary patterns of *Escherichia coli* growth in seawater determined with a Host to Coast Environmental Laboratory Analog

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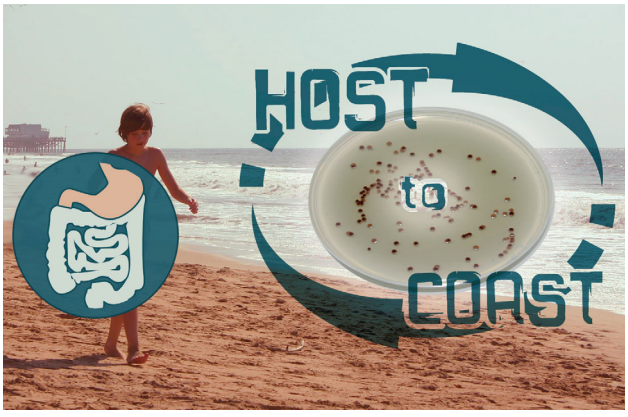
ABSTRACT: The present study investigated evolutionary patterns of *Escherichia coli* growth and survival in transmission through the coastal ecosystem between human host and seawater using a novel 'Host to Coast' Laboratory Analog simulating an 11 d sequence cycling through the pH and temperature conditions of the small intestine, colon, sewer, seawater, human stomach, and back into the small intestine. Although historically *E. coli* has been assumed to die off rapidly in seawater, a few instances of *E. coli* growth have been observed in environmental fresh water, soils, and nutrient-enriched tropical seawater, suggesting the potential for evolving greater viability in such environments. This study investigated whether *E. coli* can evolve increased capacity for growth and survival in seawater through selective exposures to singular abiotic factors such as the high alkalinity or low temperatures approximating coastal aquatic conditions, and also whether adaptation to cycling pH can lead to increased growth fitness in a human host. Specialists and generalists were examined to reveal patterns of trade-offs in the multi-stress environment of the Analog. Dramatic increases in fitness revealed in the Host to Coast Analog due to evolutionary exposure to alkalinity and cycling pH suggested that these factors are critically important in the evolutionary ecology of *E. coli*. Evolving the capacity for seawater survival and growth, combined with increased capability to infect human hosts, could facilitate *E. coli* infection from seawater exposure. Results suggest implications for wastewater management in order to prevent superior strains of pathogenic *E. coli* from evolving.

KEY WORDS: Experimental evolution · *Escherichia coli* · Evolving pathogens · Seawater, Water quality management · Human host · Microbial ecology · Multi-stress



Host to Coast Environmental Laboratory Analog illustrated with images of coastline, host, and colonies of evolving *Escherichia coli* bacterium.

Illustration/Photo: Shane Hunter Hughes

INTRODUCTION

Escherichia coli in the environment

Over millions of years the enteric bacterium *Escherichia coli* has evolved to life inside human and other mammalian hosts in an environment characterized by warm temperatures and acidic or neutral pH. Wide-scale attention to the serious dangers of pathogenic *E. coli* to health has focused largely on its transmission through cattle and has led to its gradual reduction in ground beef (Naugle et al. 2006). However, pathogenic *E. coli* is now finding its way into recreational waters (Dziuban et al. 2006), which may represent an important new trend in transmission. Rapid urban development in coastal regions has resulted in increased volumes of sewage discharge and urban

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runoff to the coastal ocean (Noble et al. 2003, Ahn et al. 2005), with various strains of *E. coli* being released to the marine bathing water. *E. coli* have long been assumed to die off rapidly in seawater and to lack the ability to grow and proliferate in the ocean (Savageau 1983), so their presence has been interpreted and used to indicate a recent introduction, i.e. sewage spill. However, studies have also shown that *E. coli* can grow in nutrient-enriched seawater (Jannasch 1968, Lopez-Torres et al. 1988), as might occur within highly eutrophic regions of a river mouth, in near-shore marine or lake sediments (Solo-Gabriele et al. 2000, Ishii et al. 2006), or possibly around sewage plumes. While beach closures have relied upon levels of *E. coli* detected as a mere indicator of dangers, there should be a growing concern, spurred by increasingly frequent outbreaks reported by the Centers for Disease Control and Prevention (CDC-USA) (Dziuban et al. 2006), about the potential spread of pathogenic strains of the *E. coli* itself through our coastal ecosystem. Those concerns prompted the present study. Could this organism evolve increased growth and survival in seawater through selective exposure to novel abiotic factors of high alkalinity or low temperatures found in the modern sewer or marine environment? In tropical freshwater environments, *E. coli* have been found in the absence of known fecal sources (Jimenez et al. 1989). Since a different strain was similarly tested and quickly died off, Jimenez et al. (1989) suggested the possibility that the natural isolate may have become genetically adapted to that environment. More recently, Ishii et al. (2006) reported evidence of naturalized autochthonous *E. coli* growing in temperate soil. The present study sought to establish a laboratory analog method to determine the correlated evolutionary role that various environmental factors can play in *E. coli*'s potential evolution of increased fitness for the coastal environments of seawater and human host. Experimental lineages of this bacterium adapted to the environmental factors of temperature (Bennett et al. 1992) and pH (Hughes et al. 2007a,b) were previously derived and available for this purpose.

The successful colonization of bacteria must be adapted so that they survive transmission through the human gut's extreme fluctuating acidity, which ranges approximately between pH 2 and 7 (Nugent et al. 2001). A swimmer, ingesting as few as 10 cells, may be infected by *Escherichia coli* with its highly evolved acid tolerance, while *Salmonella*, with an acid tolerance limited to a pH of only 3.0, requires over 10 000 cells to produce a similar infection (Audia et al. 2001). This superior infective ability makes *E. coli* a potentially more dangerous environmental pathogen than *Salmonella* or other less acid-tolerant waterborne bacteria. Studies with non-pathogenic *E. coli* show that highly significant increases of fitness in respect to acid tolerance can happen in less than 1000 generations (Hughes et al. 2007a,b). Pathogenic and non-pathogenic strains of *E. coli* have similar resistance to acidity (Lin et al. 1996), so the experimental evolutionary study of non-pathogenic strains may be helpful in predicting the patterns one may observe in the natural evolution of pathogenic *E. coli* in the environment. During alkaline stress the peptidoglycan layer of Gram-negative bacteria, weakened by high pH, may be less capable of protecting the cytoplasmic membrane of *E. coli* (Mendonca et al. 1994). Using the methods of experimental evolution, we recently discovered that long-term evolutionary exposure to alkaline pH 7.8, near that of the ocean or sewers, can promote the evolution of bacterial lines with increased resistance and higher fitness toward alkalinity and also some surprising instances of correlated increased fitness in respect to acidity (Hughes et al. 2007a,b).

Experimental evolution

Experimental evolution is a particularly well-suited method for effectively examining bacterial adaptation to abiotic environments, since such environments are easy to regulate and control in a laboratory, and multiple replicate populations of bacteria can be exposed simultaneously to identical conditions. Evolutionary experiments are usually conducted through 3 possible approaches: artificial truncation selection, laboratory culling selection, or laboratory natural selection (Rose et al. 1990). To maximize the realism of the evolutionary patterns evolved, this study specifically utilized laboratory natural selection, exposing the microbe to a novel environment in which intra-population competition alone determines which traits are favored (Bennett 2003). Whatever traits naturally play a role will be selected through competition within the given environment in much the same way evolutionary selection might occur naturally, but eliminating the complication of interspecies competition.

Many experimental evolution studies have investigated beneficial adaptation directly within the experimental selection environments, and results of direct fitness gains have been well demonstrated with microbial populations. However, the present study utilizes a more unusual approach of measuring correlated fitness, also referred to as preadaptation or exaptation, within novel environmental conditions. Such an approach is especially advantageous for this investigation, since the multi-stress environment of seawater does not permit the growth of *E. coli* necessary for evolution in that aquatic environment to occur directly. This limitation was navigated here by evolving the

bacteria under less complicated and more tractable environmental stresses, in which evolutionary propagation could proceed. The lineages of genotypes evolved by these various environmental forces could then be measured for fitness in the more complex seawater conditions, implicating specific environmental forces for their potential to cause correlated adaptation to the novel environments of the coastal ecosystem. Fortunately, in addition to further characterizing more complex evolutionary patterns for extending the study of evolution itself, this method would also be informative for investigating important environmental questions, such as whether long-term exposure to alkaline sewage systems might evolve pathogenic *E. coli* that could survive and proliferate in seawater. As the field of experimental evolution progresses, such correlated studies may prove increasingly important as researchers search responsibly for previously unforeseen evolutionary effects.

Experimentally derived bacterial lineages adapted for 2000 generations to acid, alkaline, and cycling pH were used in this experiment, along with similarly derived lineages adapted to low and high temperature (Table 1). Derivation of each group involves daily transfer batch-culturing propagating 6.67 cell divisions within the defined pH or thermal conditions, i.e. constant pH 5.3 (at 37°C) for acid media (see Hughes et al. 2007a,b), precisely limiting glucose to control the number of the generations (Lenski et al. 1991). To calibrate and expand the Analog system to reflect the natural world, environmental *Escherichia coli* isolates (Table 1) taken from an urban sewer and coastal seawater were also included. This study bridges the disciplines of experimental evolution and aquatic microbial ecology, extending experimental evolutionary methods into multi-stress complexities more closely approximating the real world, while at the same time contributing a useful aquatic microbiological method for empirically estimating natural evolutionary patterns with laboratory control.

Purpose of the Host to Coast Analog

A laboratory experiment was developed to provide a controlled test of the relative importance of different evolutionary factors evolving increased growth and survival of *Escherichia coli* within the coastal ecosystem. The present experiment was not intended to evolve bacteria within the Host to Coast Analog itself, but rather, was designed to test the hypothesis that prior adaptation to alkaline, acid, cycling environmental pH, or high or low temperature would lead to increased survival, growth, and fitness in seawater and a human host. This Host to Coast Analog involved a much more realistically complex, sequential combination of multi-stress environments than had ever previously been used to measure evolutionary patterns. Specifically, the laboratory analog simulated an 11 d sequence cycling through the pH and temperature conditions of the small intestine, colon, sewer, seawater, human stomach, and back into the small intestine; development of this (henceforth termed 'Host to Coast Environmental Laboratory Analog'; Table 2) was designed for a balance of tractability and realism, through iteratively approximating parameters of pH, temperature, and media to natural environmental levels, as closely as could be afforded within the constraints of utilizing the media and methods typically employed for microbial evolutionary experimentation with *E. coli* (see Lenski et al. 1991, Bennett et al. 1992, and Hughes et al. 2007a,b). For experimental control, other abiotic factors such as light radiation (Fujioka et al. 1981) or biotic factors such as protozoan grazing (Barcina et al. 1992) were excluded from the scope of this analog design. Although models can always be improved, to our knowledge, the Host to Coast Analog described here represented the first successful attempt to link natural-selection-based experimental evolution to the microbial ecological pattern of transmission through the coastal ecosystem between human host and seawater, with repeatable controlled laboratory-based methods.

Host Analog design

Although it may be experimentally challenging, it is possible to estimate pathogen behavior under controlled conditions that approximate gastric conditions (Tamplin 2005). An earlier attempt by other researchers to model bacteria survival in a stomach, with a pH decrease immediately below 2.0, could find no

Table 1. *Escherichia coli*. Experimental bacterial lines. Terminology of study: terminology used in this article to refer to each *E. coli* Line; Reference name: official archival name for reference to stored *E. coli* Lines; Source: first published article to refer to each *E. coli* Line. –: not previously published

Terminology of study	Reference name	Source
Acid	AFB739 5+1	Hughes et al. (2007a)
Alkaline	AFB757 8+1	Hughes et al. (2007a)
Cycled pH	AFB763 E+1	Hughes et al. (2007b)
14°C	AFB1170 14A/+	J. Schlumbohm (unpubl.)
42°C	REL2142 42+1	Bennett et al. (1992)
Host Isolate	AFB1258 S10	–
Coast Isolate	AFB1260 E13	–
Ancestor/Control	REL1206 A-	Lenski et al. (1991)

Table 2. Host to Coast Environmental Laboratory Analog. Experimental conditions of temperature, pH, and culture medium, as sequenced in the Analog. Seawater (see Table 3 for characterization) was sampled from Balboa Pier in March 2006 and filtered; Acid Cycle (see Fig. 1 and 'Host Analog design' section for details) involves gradual acidification in the stomach and gradual neutralization near the pancreatic duct in the duodenum. LB: Luria-Bertani broth; DM: Davis Minimal Media

Day of Analog	Environment simulated	Temperature (°C)	pH	Culture medium
Day 1 (Host)	Proximal→distal small intestine	37	6.8→7.7	LB
Day 2 (Host)	Right colon Left colon	37	7.0	DM
Days 3 and 4 ($Coast_{Transition}$)	Sewer system	20	6.8→8.2	LB
Days 5[a] to 9 (Coast)	Seawater	14	8.2	Seawater
Day 10[b] (Host)	Stomach acidification→ intestine neutralization	37	Acid Cycle 6.8→2.0 2.0→6.8	LB +HCL +$NaHCO_3$
Day 11[c] (Host)	Proximal→distal small intestine	37	6.8→7.7	LB

[a]First, [b]second and [c]third relative fitness assay

culturable cells (Beumer et al. 1992). Our choice of an initial stomach pH of 6.7 for the Analog Acid Cycle (see Fig. 1), which is typical immediately after meal intake rather than at fasting when pH is below 2.0 (Dressman et al. 1990, Russell et al. 1993), was made to avoid such a total loss of culturable bacteria and it is also a more realistic pH model condition for bacteria that are ingested with food. A 2 h acid cycle exposure time used in the Analog was modeled after stomach acid physiologies of young healthy adults, who require an average time of 127 ± 13 min (mean ± SE) for 50% of their stomach contents to empty (Clarkston et al. 1997). Gradual acidification and alkalization with shaking, combined with additional time for repopulation growth during the small intestine simulation, were used to achieve a similarity to human physiology and to yield successful measurements of fitness, which would otherwise have been impossible.

Coast Analog design

The Coast Analog includes both transitional sewer (Table 2: $Coast_{Transition}$) and seawater components, simulating the transmission of *Escherichia coli* from human host through a sewer into the coastal seawater prior to re-entry back into the host environment. It was modeled to include some aspects of bacterial pre-shock which typically occur in the multiple stresses of environmental microbial transmission (see Troussellier et al. 1998), and often involve the general anti-stress response encoded by the *rpoS* gene (Loewen & Hengge-Aronis 1994). For example, under natural circumstances it is rare that exponential phase bacteria are released directly into seawater, but instead they are typically in stationary phase in their transit through wastewater (Gauthier et al. 1993), which was emulated in the Host to Coast Analog by bringing the *E. coli* to stationary phase by nutrient starvation before the sewer simulation and again during the sewer simulation prior to transfer to seawater. *E. coli* are least sensitive to the hyperosmotic shock of seawater when they are in stationary phase (Troussellier et al. 1998), so the realism of survival increases from stationary phase within environmental sewage or seawater was present in the Analog. Other design challenges were addressed, such as biofilm formations

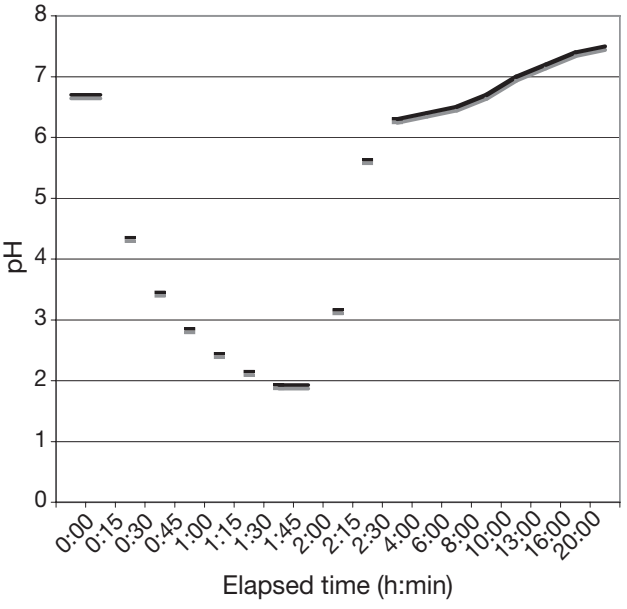

Fig. 1. pH of media versus time elapsed during the acid cycle of the Host Analog. Short discontinuous horizontal bars indicate rapidly shifted pH levels accompanying 0.9 ml additions of 0.15 M HCl acid at times 0:15, 0:30, 0:45, 1:00, 1:15, and 1:30 h during acidification and 0.5 ml additions of 7.5% Solution $NaHCO_3$ base at times 2:00, 2:15, and 2:30 h during neutralization. Continuous lines indicate sustained pH levels of flasks, beginning with a mixture of 9.6 ml of Luria-Bertani broth (LB) media and 0.4 ml seawater containing the competing bacteria, at time = 0:00 h, pH = 6.7; time = 1:30 h, pH = 1.93; and time = 2:30 h, pH = 6.3, then gradually increasing to 7.5 at elapsed time = 20:00 h

that were found in sewer conditions at lower temperatures (≤14°C) and prevented accurate census. This problem was avoided by using a temperature of 20°C, which simulated a warmer region sewer component. Another design issue was the transition from the sewer to the costal seawater, which required 0.8 ml of the sewer Luria-Bertani broth (LB) to ensure countable numbers and to provide nutrients depleted by seawater filtration. This nutrient addition, which simulated the environmental factor of sewer leakage, also promotes *E. coli* growth in seawater (Jannasch 1968, Lopez-Torres et al. 1988). Therefore, the amount was precisely gauged to avoid growth in the Ancestor/Control population (see Fig. 2) while still allowing realistic persistence of populations in the Coastal Isolate Line.

MATERIALS AND METHODS

Culture techniques. All experimental groups were run with 6-fold independent replication. The culture media included unbuffered Luria-Bertani broth (LB); LB with added 150 mmol HCl and 7.5% $NaHCO_3$; buffered pH 7.0 Davis minimal media (DM); and filtered seawater (see 'Seawater collection, processing, and characterization' below) (Fig. 1), at controlled temperature and pH, as described in Lenski et al. (1991) and Hughes et al. (2007a,b), respectively. Cultures were propagated within 50 ml Erlenmeyer flasks in 120 rpm shaking incubators (New Brunswick Models G25 and G25-KC). To avoid discrepancies between viability and culturability of potentially viable-but-non-culturable cells (see Rozen & Belkin 2001), all population enumerations were based on culturable cells counted on solid agar plates. Population counts of the 2 competitors were differentially enumerated through colony growth on tetrazolium-arabinose (TA) plates (in which a 2 l batch contains 2 ml of 5% triphenyltetrazolium-chloride, 20 g arabinose, 20 g tryptone, 2 g yeast extract, 10 g sodium chloride, and 32 g agar in 2 l distilled water), on which the neutral marker for arabinose-utilizing Ara+ evolved strains displays white colonies that can easily be distinguished by their differing coloration compared to the Ara– ancestral strain that grows red colonies (see Lenski et al. 1991).

Origin of laboratory-derived lines of *Escherichia coli*. Evolved lines of *E. coli* examined in this study (Table 1) were founded from a common ancestor that was already well adapted to general laboratory conditions after 2000 generations of lab propagation (Lenski et al. 1991). pH evolution lines (constant temperature 37°C) included an acid line evolved for 2000 generations at a constant pH of 5.3 and an alkaline line evolved for 2000 generations at a constant pH of 7.8 (see Hughes et al. 2007a), as well as a cycled pH line evolved for 2000 generations of continually alternating pH (from 5.3 to 7.8 on the first day and then from 7.8 to 5.3 on the subsequent day; see Hughes et al. 2007b). The 2 thermal evolution lines (constant pH 7.0) included a lineage evolved for 2000 generations at a constant temperature of 14°C and another at 42°C (Bennett et al. 1992).

Origin of natural isolate lines of *Escherichia coli*. By including comparative analysis of natural isolates versus the control bacterium that is also the experimental ancestor, we sought to better characterize the effective calibration of this system for real world relevancy. Two natural isolates of *E. coli* were obtained from human waste and from seawater samples, to represent 'Host' and 'Coast', respectively. The natural Host isolate was obtained from the primary sewage effluent from the Irvine Ranch Water District, California, USA, on February 13, 2006, and the natural Coast isolate was obtained from surface seawater collection off the end of Balboa Pier, Newport Beach, California, USA, on January 21, 2005. These natural Host and Coast strains were isolated on mTEC agar (Difco) and confirmed to be excellent matches to *E. coli* by API 20-E biochemical substrate tests (bioMerieux). These natural isolates were positive (+) for the arabinose marker, enabling their quantification by identical methods to that of the experimental evolution lines.

Seawater collection, processing, and characterization. Seawater was collected by 15-gallon carboy from the coastal Pacific Ocean near Balboa Pier. The homogeneously mixed water sample was filtered through tangential flow filtration (Pall Filtron LV Centramate TFF System) with a 0.22 µm pore sized cartridge to remove bacteria and other large plankton. Filtrates were stored at –20°C and thawed for 2 d at 5°C with shaking for 20 min prior to use. Analysis of seawater characteristics was conducted via high temperature combustion using the Shimadzu TOC-V in the shore-based laboratory at the University of California, Santa Barbara (Table 3) (see methods of Carlson et al. 2004). Other seawater characterizations were conducted at the University of California, Irvine (Table 3). Sea surface temperature was measured on site at Balboa Pier using an infrared gun (Raynger-ST, Raytek). Sample splits were analyzed for chlorophyll fluorescence (YSI 6025, YSI Incorporated) and conductivity (Model 162A, Thermo Orion); conductivity was subsequently converted to salinity using the practical salinity scale. Aliquots (1.0 ml) of each sample were analyzed for fecal indicating bacteria (FIB) by 1:10 dilution into Butterfield's phosphate buffer solution (Hardy Diagnostics), followed by analysis using Colilert-18 (for total coliform and *Escherichia coli*) and Enterolert (for enterococci bacteria) tests (IDEXX), using 96-well quantitrays (Edberg et al. 1988). These tests yield the

concentration of FIB in units of most probable number (MPN) of bacteria per 100 ml of sample. Measurement of both filtered and unfiltered seawater is shown for comparison (Table 3), with the dissolved organic carbon (DOC) of the filtered seawater 35% less, dissolved organic nitrates (DON) lowered by 40%, and phosphate reduced by 46% from that of the unfiltered levels. Salinity was not measurably affected by filtration, while the pH dropped from 8.27 to 8.16 during filtration.

Measurements of fitness. Relative survival and growth fitness of various pH and thermal evolutionary lines were measured through direct competition with the common Ancestor/Control, which serves as either an ancestor (more specifically an Ara– clone of the Ara+ ancestor), as a control or both. In the case of the laboratory-evolved lines, these measurements characterized evolutionary fitness changes in addition to serving as a control baseline for comparison. For natural Host and Coast Isolates, it served purely as a control baseline for transitive comparison to the evolved lines. The relative fitness of an experimentally evolved line was calculated from the ratio of the logarithm of population growth doublings achieved by the experimental competitor compared to that of the Ancestor/Control (Lenski et al. 1991). A relative fitness significantly greater than 1.0 signifies improved evolutionary fitness or superiority to the ancestor, while a relative fitness significantly less than 1.0 denotes an evolved loss in fitness compared to the ancestor (Lenski et al. 1991). Since *Escherichia coli* does not typically divide and grow in seawater, the relative fitness measurement for this phase of the Analog was necessarily modified to detect mere persistence, substituting linear ratios of survival in place of the logarithmic doubling function, and is referred to as relative survival fitness. Quantitative interpretations remained otherwise synonymous, with superiority of the experimental line over the ancestor evident when relative survival fitness measured greater than 1.0 and vice versa. Counting the mixed competitors via plating was facilitated by a distinguishable marker of the colony color when grown on TA plates, which was facilitated by the differential (– or +) capacity for arabinose utilization present between the Ancestor/Control and all of the experimental lines. This arabinose marker has been shown to be neutral for fitness effects in many environments, including temperature and pH (Bennett et al. 1992, Hughes et al. 2007a). These platings of competitive census were conducted on all possible tractable and relevant days, which were limited exclusively yet effectively to only those points before and after Coast Analog and before and after the Host Analog.

Table 3. Seawater analyses for characterization of source and filtered seawater used in the Analog. Source seawater was gathered from surface water near the end of Balboa Pier on March 21, 2006, and its characteristics measured by single sample analysis. Filtered seawater was processed from the source seawater by filtering through a 0.22 µm tangential flow system. It was then frozen, thawed, and used for the Coast Analog. Mean ± 95% confidence interval (CI) is based on a sample size of 5 for each characteristic analyzed and a significance level of $\alpha = 0.05$. UCSB and UCI: analyses performed at University of California, Santa Barbara and University of California, Irvine, respectively (see 'Materials and methods: Seawater collection, processing, and characterization'). DOC: dissolved organic carbon; TN: total nitrogen; DON: dissolved organic nitrogen; FIB: fecal indicating bacteria; TC: total coliform; EC: *Escherichia coli*; ENT: enterococci; MPN: most probable number; –: eliminated by filtration

Characteristic	Source seawater	Filtered seawater (mean ± 95% CI)
UCSB		
DOC (µM)	170.207	110.689 ± 5.994
TN (µM)	17.960	10.810 ± 1.325
DON (µM)	14.091	7.301 ± 1.202
Phosphate (µM)	0.689	0.371 ± 0.024
Nitrite (µM)	0.222	0.068 ± 0.016
Nitrite + Nitrate (µM)	2.862	2.798 ± 0.037
Ammonia (µM)	1.006	0.712 ± 0.126
UCI		
pH	8.270	8.162 ± 0.007
Salinity (ppt)	32.700	32.700 ± 0.048
Chlorophyll (µg l^{-1})	30.000	–
FIB:TC (MPN per 100 ml)	216.000	–
FIB:EC (MPN per 100 ml)	30.000	–
FIB:ENT (MPN per 100 ml)	10.000	–

Analog sequence Day 1 (Host). Day 1 of the Analog sequence approximated the 37°C temperature of a human host along with a pH in the proximal small intestine of 6.8, climbing gradually to 7.7 at the distal end (Sasaki et al. 1997). This variation in pH was efficiently achieved by simply inoculating the bacteria into unbuffered LB medium and allowing it to gradually rise in pH from metabolites of cellular growth produced during the day in the 37°C shaking incubator (verified with Fisher Accumet Model 15 pH meter).

Analog sequence Day 2 (Host). Day 2 of the Analog placed the *Escherichia coli* in the colon, with a temperature of 37°C and pH of 7.0 (as averaged between the right and left colon measurements of pH 6.8 and 7.2 made by Sasaki et al. 1997). A total of 0.1 ml was transferred from Day 1 flasks into 9.9 ml of buffered pH 7.0 DM, incubated at 37°C. DM was substituted for LB to simulate the poor nutrient condition within the colon.

Analog sequence Days 3 and 4 (Coast). Days 3 and 4 of the Analog simulated a sewer, with a temperature of 20°C and pH increasing gradually from 6.8 to 8.2, as may be typical for sewer systems attempting to match their outlet pH. A total of 0.1 ml was transferred from

Day 2 flasks into 9.9 ml of unbuffered LB and then the alkalinity was allowed to rise from accumulation of metabolites during 2 d of prolonged incubation.

Analog sequence Days 5 to 9 (Coast). Days 5 through 9 of the Analog represented 5 days submerged in coastal seawater during the month of March, when Southern California coastal seawater temperatures averaged near 14°C and pH was near 8.2 (Table 3) in the source seawater obtained in mid-March of 2006 for this experiment. Such rainy-season months correlate with high wastewater spillage. Five days in seawater at 14°C causes *Escherichia coli* to exhibit a dramatically higher death rate compared to lower temperatures (Vasconcelos & Swartz 1976), so this temperature was optimal for detecting differential survival of the bacterial lines.

Transition into the seawater initiated the beginning of the relative survival fitness competition between the common Ancestor/Control line and each experimental line (Plating 1). These measurements commenced by transferring 0.4 ml from the Ancestor/Control line culture and 0.4 ml of an experimental line at the end of Day 4, for a total of 0.8 ml, into 9.2 ml of the seawater media, in which it was incubated for 5 d. Then, 0.4 ml from the seawater flasks was transferred into 9.6 ml of LB and final seawater survival counts were obtained by a second TA plating (Plating 2).

Analog sequence Day 10 Acid Cycle (Host). Day 10 of the Analog represented transmission from 14°C seawater into a human host at 37°C, through the acidity cycle of the stomach's secretion of HCl and passage into neutralization in the small intestine through $NaHCO_3$ secretion at the duodenum (Montrose 2001). Final seawater survival counts also served as the initial counts for the growth competition in the Host component of the Analog (Plating 2). Acidification and alkalization sequence (see Fig. 1) was carried out gradually through 6 stepwise 0.9 ml additions of 150 mmol HCl, the human physiological concentration of cephalic phase parietal cell secretion (Guyton & Hall 2000), administered directly into fresh bacteria-containing LB flasks (120 rpm shaking) at 15 min intervals to reduce pH from 6.7 down to slightly below 2.0 over the course of 1.5 h. The flasks remained at this low pH for 30 min before commencing neutralization by 3 stepwise 0.5 ml additions of 7.5% $NaHCO_3$ administered at 15 min intervals to increase pH from below 2.0 to 6.3 over the course of a further 45 min. During the extreme acidity phase of this cycle, culturable bacterial counts decreased to levels undetectable by plating, so the remaining bacteria were allowed to repopulate competitively through the remainder of the 20 h acid cycle of the Host Analog (Table 3), as the unbuffered LB media continued to rise gradually from pH 6.3 to 7.5 through build up of alkalizing cellular metabolites.

Analog sequence Day 11 (Host). The rising pH of Day 11 was analogous to the physiology of a gradual transport between the proximal and distal end of the small intestine (pH 6.5 to 7.5) (Nugent et al. 2001). After this competitive repopulation day, a third TA plating served as the final measurement of bacterial growth in the host. This marked the end of the completed sequence of the Host to Coast Analog (Plating 3).

Statistics. Mean fitness values of all lines were based on 6 replicates for each assay. Significance of evolved relative fitness means were analyzed by *t*-distributions compared to a null hypothesis value of 1, representing fitness equal to the Ancestor/Control. Conservative 2-tailed probabilities were employed consistently, with the number of independent replications used to establish the degrees of freedom, which were df = n – 1 = 5 in every case, except for seawater analysis which used df = n – 1 = 4. In all tests, $\alpha = 0.05$ was used to delineate significance. The test for similarity between mean fitness of different lines evolved in the same evolutionary conditions was analyzed using the Tukey-Kramer test for comparison of means. The fitness measurements produced in the various conditions of the Host to Coast Analog tested only *a priori* hypotheses.

RESULTS

Growth fitness in the Host

Fitness in the Host component of the Analog was measured for each of the experimental lines through direct competitions with the common Ancestor/Control bacterium (Table 4). These measurements pro-

Table 4. Host Analog relative growth fitness (mean ± SE, based on 6 replicate tests for each evolved or natural isolate experimental line) of experimental lines tested in competition with the ancestral control bacterium in the laboratory environment of the Host Analog on Days 10 and 11. Two-tailed probabilities were calculated using the *t*-distribution with df = n – 1 = 5; the null hypothesis was that the mean fitness equaled 1

Experimental *E. coli* Line	Relative growth fitness	2-tailed probability
Acid	0.382 ± 0.076	<0.001
Alkaline	0.997 ± 0.028	0.920
Cycled pH	1.186 ± 0.019	<0.001
14°C	1.154 ± 0.036	0.008
42°C	0.556 ± 0.014	<0.001
Host Isolate	2.117 ± 0.096	<0.001
Coast Isolate	1.712 ± 0.051	<0.001

vided a transitive comparison between each of the experimental lines for their relative performance in environments simulating some of the stresses experienced in a human gastrointestinal tract. Population counts for each competitor and the common Ancestor/Control were enumerated once before the Host Analog acidification in the simulation of the stomach on Day 10 and again after the neutralization on Day 11 of the simulated day of passage through the small intestine. Interpretation of the measurements shown in Table 4 pivot around a relative growth fitness value of 1.0, with values significantly higher than 1.0 indicating increased fitness and values lower than 1.0 indicating a loss of fitness, relative to the Ancestor/Control. The Acid Line (Table 4), with a long-term evolutionary exposure to the factor of constant acidity, had a fitness loss of 62%, with a fitness of only 0.382, in the Host Analog environment. Such a loss in fitness was not the case with the Alkaline Line, which had no significant change in fitness. The Cycled pH Line evolved a fitness gain of 19% (1.186) in the Host Analog and the 14°C Line gained 15% (1.154) fitness, while the 42°C Line actually lost 44% (0.556) fitness. The natural Host Isolate Line had the highest fitness in this environment, with 112% better fitness compared to the Ancestor/Control. The natural Coast Isolate Line also had a very high fitness, at 71% higher level than the Ancestor/Control. Although these natural isolate line fitness performances are not relative to their ancestor, and therefore do not measure their actual evolution, they do provide a useful comparison to all of the other lines by transitive comparison to the Ancestor/Control, which functions here as a common control among all lines measured and thus allows characterization of both experimental and natural strains to be considered.

Table 5. Coast Analog relative survival fitness (mean ± SE, based on 6 replicate tests for each evolved or natural isolate experimental line) of experimental lines tested in competition with the ancestral control bacterium in the laboratory environment of the Coast Analog. Two-tailed probabilities were calculated using the t-distribution with df = n – 1 = 5; the null hypothesis was that the mean fitness equaled 1

Experimental *E. coli* Line	Relative survival fitness	2-tailed probability
Acid	1.144 ± 0.279	0.263
Alkaline	2.909 ± 0.469	0.010
Cycled pH	0.996 ± 0.020	0.866
14°C	1.050 ± 0.083	0.570
42°C	1.124 ± 0.030	0.010
Host Isolate	0.213 ± 0.013	<0.001
Coast Isolate	1.457 ± 0.029	<0.001

Survival fitness in the Coast

The Coast Analog tested the relative survival fitness for exposure to 5 d in seawater at 14°C, measured by comparative counts on Days 5 and 10. Measurements shown in Table 5 were calculated with linear population ratios to measure survival fitness, rather than the logarithmic ratios used to measure growth fitness, since the absolute populations often decreased between the beginning and end points of the competition (Fig. 2). None of the Acid, Cycled pH, or 14°C Lines showed statistically significant changes in relative seawater survival fitness. However, the 42°C Line demonstrated a 12% gain and, most noteworthy, the Alkaline Line showed an extremely high significant 191% gain in seawater survival fitness. For an environmental comparison, the natural Host Isolate showed 79% lower survival fitness and the natural Coast Isolate had 46% higher survival fitness relative to the common Ancestor/Control bacterium.

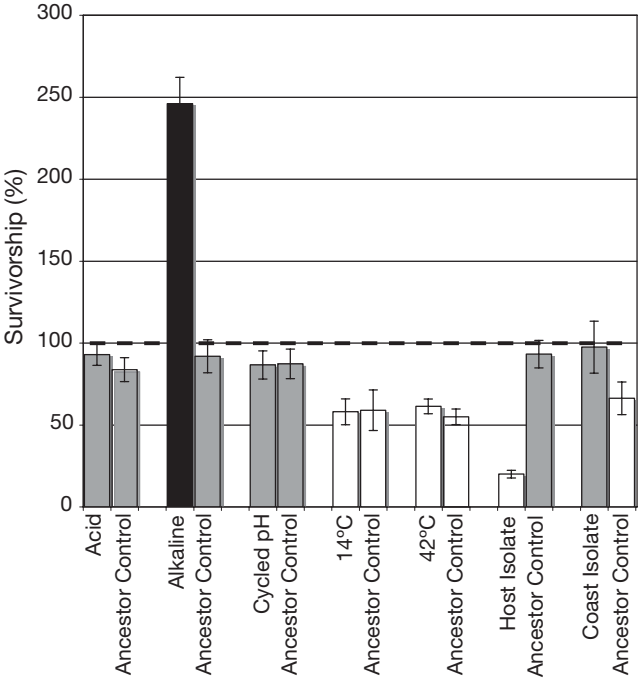

Fig. 2. Absolute survivorship percentage after 5 d exposure to Host Analog filtered seawater (characterization analysis in Table 3) is shown for each experimental line (Acid, Alkaline, pH Cycled, 14°C, 42°C, Host Isolate, and Coast Isolate) paired alongside the survivorship of the matched Ancestor/Control with which it competed in the same flask. Survivorship values significantly higher than 100% (dashed line) indicate population growth (black bar); those not significantly different from 100% indicate population persistence (gray bars); and survivorship values significantly less than 100% indicate population decline (white bars)

Absolute survival count percentages, measuring actual population persistence and growth, are displayed in Fig. 2, with percentages significantly lower than 100% (white bar) indicating an inability to persist in seawater, percentages significantly higher than 100% (black bar) indicating actual growth in seawater, while no significant change (gray bar) represents persistence. Each line's survivorship percentage is paired adjacent to the survivorship percentage of its competing Ancestor/Control Line. Most notable was the 246% absolute survivorship of the Alkaline Line. This Alkaline Line displayed a 225% higher population count than the natural Host Isolate Line (calculated here: 1.46 – (–) 0.79 = 2.25, all relative to a baseline fitness of one, which may be expressed as 225%), but this large difference in relative survival fitness was not accompanied by any statistically significant (at $\alpha = 0.05$, Tukey-Kramer comparison test) difference between the absolute counts of their paired common ancestral competitors, demonstrating further validity to the dramatic comparison. More importantly this Alkaline Line was the only line that could actually grow during the 5 d seawater media exposure to the Coast Analog, demonstrating a 146% increase from its initial count.

Trade-off patterns

Defined here, a trade-off is a cost or loss of fitness in one environment accompanying a gain of fitness in another environment; a specialist displays trade-offs, while a generalist does not. In the Host Analog, only the Acid and 42°C Lines had trade-offs, while in the Coast Analog only the natural Host Isolate Line had trade-offs. This result characterized the Acid, 42°C, and Host Isolate Lines as specialists. The Alkaline, Cycled pH, 14°C, and Coast Isolate Lines were all Host to Coast generalists.

Pilot replication experiment

Although each bacterial line was independently tested in 6-fold replication, the large scope of this study prevented replication at the level of measuring multiple evolutionary lineages adapted to each environmental condition (e.g. high temperature). However, as a pilot experiment, 3 independently evolved Acid Lines were tested for analysis of consistency of response. All 3 of the lines (Acid +1 used in this experiment, Acid +2, and Acid +3) were statistically indistinguishable (Tukey-Kramer comparison $\alpha = 0.05$, $n = 6$ samples for each of 3 lines) in their relative fitness in both the Host and the Coast Analog.

DISCUSSION

The Host to Coast Analog contributed a novel interdisciplinary methodology through integrating experimental evolution with aquatic microbial ecology. Specific evolutionary patterns of growth and survival of *Escherichia coli* in our ecosystem are of particular concern, considering the importance of this bacterium to environmental health. While abiotic factors were the focus of this ecological Analog model, microbe-to-microbe interactions played a role in the measurement of relative fitness, since the evolved or natural isolate bacteria competed against a common Ancestor/Control bacterium directly for shared limited nutrients. Tests of evolutionary hypotheses regarding trade-offs, specialists, and generalists, were extended to encompass potential costs of adaptation in more realistically complex multi-stress environments than had ever been examined before. The results of this experiment support the hypotheses that long-term exposure to alkaline pH can permit the evolution of highly increased survival and growth in seawater, and that long-term cycling between acidic and alkaline environments, such as that increasingly occurring between humans and the oceans with mishandling of sewage, can increase growth fitness in an environment similar to the human gastrointestinal tract.

Growth fitness in the Host

Since the extreme pH of the Acid Cycle reduced populations to undetectable levels, populations were enumerated before the Host Analog stomach acidification of Day 10 and again only after the Day 11 simulation of passage through the small intestine. Repopulation during Day 11 from such a small population to densities exceeding millions of cells per ml, reflects the remarkable growth capacities of a bacterium that can colonize a host with as few as 10 cells (Audia et al. 2001) and simulated growth time and passage toward the colon, which is the niche where *Escherichia coli* are a highly successful competitor (Kaper et al. 2004). Among their main suggestions for future research on *E. coli* pathogenesis, Kaper et al. (2004) asked what factors allow commensal *E. coli* strains to colonize and survive so successfully in this niche and also what new strains may be likely to evolve. The Host Analog tested some environmental factors that affect such evolution.

Though counterintuitive, the Acid Line's correlated fitness loss of 62% in the acidic Host Analog environment agrees with Hughes et al. (2007b), who showed that evolution in constant acidity causes a correlated decrease of fitness in environments of fluctuating acidity. The Host Analog test fluctuated acidity (Fig. 1) to

reflect the physiology of passage through the stomach and intestines. Evolutionary exposure to fluctuating pH in the Cycled pH Line caused a correlated fitness gain of 19% in the Host Analog, as predicted based on our previous observation that long-term exposure to fluctuating pH causes increased fitness in cycling pH (Hughes et al. 2007b). Considering that the Host Analog was a warm environment at 37°C, it was an unexpected result that the cold 14°C Line gained 15% fitness while the hot 42°C Line lost 44% fitness. Not surprisingly, the natural Host Isolate *Escherichia coli* strain had the highest performance of relative growth fitness in the Host Analog, which may offer additional validity to the Analog as a simulation of the human host. A concerning pattern emerges, when considering that (1) combinations of cycling pH, constant alkalinity, and cold temperatures (as may occur when sewage is released into the ocean and transported back and forth to mammalian hosts) are factors that can cause *E. coli* to colonize and survive more successfully in this niche, and (2) the Coast Isolate was very successful in the Host Analog.

Escherichia coli survival during transmission into a human host is dependent upon resistance to pH damage, involving protein denaturing and depurination of DNA (Richard & Foster, 2004); 3 underlying mechanisms responsible for *E. coli* pH resistance have been described (Lin et al. 1995, Hersh et al. 1996 Castanie-Cornet et al. 1999, De Biase et al. 1999), with one mechanism involving a glutamate decarboxylase GAD system and another mechanism involving an arginine decarboxylase ARG system. In both of these mechanisms, in the presence of either glutamate or arginine, *E. coli* can reverse the electrical membrane potential, making the inside of the cell positively charged, a strategy used by various acidophiles in extremely low pH environments (Richard & Foster 2004). Involvement of the other mechanism, the RpoS system (Small et al. 1994), may be highly suspected, since in seawater there is a 1000-fold greater number of culturable *E. coli* containing a functional *rpoS* gene (Munro et al. 1995), and the *rpoS819* allele is known to confer a strong fitness advantage at basic pH while being disadvantageous under acidic conditions (Farrell & Finkel 2003). Evolution involving *rpoS* could potentially be partly responsible for the increased seawater survival, although many factors are known to affect the evolution of the RpoS system (for a short review see Ferenci 2008).

Survival fitness in the Coast

Few studies have measured the response of enteric bacteria under environmental conditions such as those encountered by bacteria in coastal waters (Troussellier et al. 1998). Raw sewage is often discharged into low-temperature marine environments, raising the question of whether *Escherichia coli* is able to adapt to and persist in this extreme environment (Winfield & Groisman 2003). The Coast Analog test begins to answer this question, as results of relative survival fitness values shown in Table 5 revealed increased fitness in seawater relative to the Ancestor/Control as indicated by values significantly higher than 1.

Although constant acid evolutionary lines lose substantial fitness in otherwise similar alkaline media (Hughes et al. 2007a), this was not the case for the Acid Line tested in the more complex seawater environment of the Coast Analog (Table 5). Cycled pH and cold 14°C evolutionary factors were found not to play a significant role in seawater survival fitness. Interestingly, the high temperature evolution of the 42°C Line did cause a significant 12% increase of relative survival fitness in the cold 14°C seawater. As predicted to characterize real-world relevance of the Analog system, in an inverse symmetry of performance in the Host Analog test, the Coast Analog test exhibited lower survival fitness for the natural Host Isolate Line and higher survival fitness for the natural Coast Isolate Line, when tested in seawater. Certainly, the most dramatic pattern of correlated relative survival fitness increase occurred in the Alkaline Line's highly significant and substantial gain of 191% evolved over the common ancestral control, far in excess over even the natural Coast Isolate. While it was predicted that alkalinity was potentially an important factor in seawater survival adaptation, such a large increase suggested it was a critical factor and perhaps even capable of enabling growth in seawater, posing a serious danger and calling into question the application of *Escherichia coli* as an indicator species.

Since relative survival fitness only measures a ratio between the experimental and ancestral control lines, it does not discern the extent to which lines are actually dying off or growing in a culture. Therefore, an additional measure of absolute survival was employed to ascertain the actual persistence and growth rates over the 5 d seawater exposure of the Coast Analog (Fig. 2), in which percentages significantly lower than 100% represent an eventual dead end for a bacterial line with an inability to persist in seawater; persistence near 100%, and percentages significantly higher than 100% indicate actual growth in seawater. These absolute bacterial population changes are shown in Fig. 2, with each line paired to its Ancestor/Control within a specific competitive match, i.e. competing within the same flask for limited resources. Fig. 2 shows 7 independent survival results for the Ancestor/Control and each competitive match-up would

presumably dictate different metabolic patterns of nutrient consumption leading to different population counts. For instance, the Ancestor/Control might produce lower populations when in the company of a competitor that would more effectively compete for nutrient resources or vice versa, and such patterns might be apparent in the Host and Coast Isolate Line competitions, respectively. However, excluding more resources from the Ancestor/Control may not translate to higher populations, i.e. the 14°C and 42°C Lines, if the experimental line is inefficient in yield. Furthermore, consideration of such microbe-to-microbe interactions may pale in comparison to another result, seen in the comparison between the highest and lowest performers in the Coast Analog. Surprisingly, the 225% population count difference between the Alkaline and natural Host Isolate Lines was not accompanied by any significant difference in the absolute counts of their common ancestral competitor, which may indicate improvement by metabolic efficiency rather than mere exclusion of nutrients from the competitor. However, such interesting considerations were dramatically eclipsed by the singular performance of the absolute survival counts of the Alkaline Line. This Alkaline Line demonstrated its vast superiority over all of the other experimental lines, including the natural Coast Isolate, by being the only line that could actually grow during the 5 d seawater media exposure in the Coast Analog. Its aggressive performance demonstrated an impressive 146% climb from its initial count.

Evolutionary conclusions

Previous studies examining trade-offs with this same bacterial system explored potential costs within the limited conditions of acidity and alkalinity for the pH lines (Hughes et al. 2007a,b) and at various temperatures for the thermal lines (Bennett & Lenski 1993, 2007). However, the present study extended the search for correlated trade-offs in the more complex and realistic multi-stress environmental conditions of the Host to Coast Analog. One of the more interesting results was the Acid Line's lack of trade-offs in seawater (Table 5), while this same line exhibited significant costs in other alkaline environments (Hughes et al. 2007a). The 42°C Line, which is a thermal generalist without tradeoffs in any previously tested thermal environment (e.g. 42°C = REL2051 in Bennett & Lenski 1993), was shown here to have significant trade-off costs of 44% in the more complex Host Analog (Table 4). By contrast, this 42°C Line had significant 12% fitness gains in this novel environment of the Coast Analog, which may be considered exaptation (Table 5). The Host Isolate was a Host specialist, exhibiting a fitness gain in the Host and a loss in the Coast Analogs. Since the 14°C Line gains in the Host Analog were not accompanied by a trade-off in the Coast Analog, it was a Host to Coast generalist. Likewise, yet reversed, the Alkaline Line was also a Host to Coast generalist, but with gains in the Coast and no trade-offs in the Host Analogs. Such a pattern might suggest the possibility that a more generalist physiology, without substantial costs of specialization, may be produced by natural seawater exposure, and continued laboratory research in this area may substantiate this pattern. Apparently, an evolutionary 'Jack of all trades' can be a master of many, although trade-offs might be revealed in more complex arenas.

Ecological implications

The evidence that alkaline pH adaptation also entailed increased survival and unprecedented growth of *Escherichia coli* in seawater underscored the dangers of environmental exposure to high pH in wastewater systems prior to release into marine waters. Furthermore, the finding that superior fitness for colonizing a human host was evolved by exposure to cycled fluctuation of pH and cold 14°C temperatures may help inform regulation of wastewater management to prevent evolution of superior strains of pathogenic *E. coli* possessing generalist physiologies that could facilitate increased growth in coastal seawater and infectiousness in human hosts. Such a fate may be forewarned by the more generalist type of physiology found in the Coast Isolate, and future directions for this research include the controlled experimental evolution of *E. coli* within the repeated cycling of the Host to Coast Analog and further tests of other natural isolate strains.

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