FEMS MICROBIOLOGY ECOLOGY

RESEARCH ARTICLE

Persistence of *Escherichia coli* in freshwater periphyton: biofilm-forming capacity as a selective advantage

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Received 5 August 2011; revised 27 October 2011; accepted 28 October 2011.
Final version published online 30 November 2011.

DOI: 10.1111/j.1574-6941.2011.01244.x

Editor: Gary King

Keywords
environmentally persistent *Escherichia coli*; biofilm; periphyton; curli protein; shiga-like toxin-producing *Escherichia coli*.

Abstract

Recent research has shown that *Escherichia coli* can persist in aquatic environments, although the characteristics that contribute to their survival remain poorly understood. This study examines periphytic *E. coli* populations that were continuously present in three temperate freshwater lakes from June to October 2008 in numbers ranging from 2 to 2×10^2 CFU 100 cm^{-2}. A crystal violet assay revealed that all tested periphytic *E. coli* isolates were superior biofilm formers and they formed, on average, 2.5 times as much biofilm as *E. coli* isolated from humans, 4.5 times as much biofilm as shiga-like toxin-producing *E. coli*, and 7.5 times as much biofilm as bovine *E. coli* isolates. Repetitive extragenic palindromic polymerase chain reaction (REP-PCR) DNA fingerprinting analysis demonstrated the genetically diverse nature of the periphytic isolates, with genetic similarity between strains ranging from 40% to 86%. Additionally, the role of curli fibers in biofilm formation was investigated by comparing biofilm formation with curli expression under optimal conditions, although little correlation ($R^2 = 0.095$, $P = 0.005$) was found. The high mean biofilm-forming capacity observed in *E. coli* isolated from the periphyton suggests that selective pressures may favor *E. coli* capable of forming biofilms in freshwater environments.

Introduction

Escherichia coli are commonly used in testing recreational water for the presence of fecal contamination (USEPA, 1986; Tallon *et al.*, 2005; Ishii & Sadowsky, 2008; Standridge, 2008). To be considered a reliable fecal indicator, an organism should be unable to replicate in the environment and have similar persistence to fecal pathogens (Ishii & Sadowsky, 2008). The widespread use of *E. coli* as a fecal indicator is because of its supposed fulfillment of these criteria along with a strong correlation of disease incidence in swimmers with the presence of *E. coli* in recreational waters (Ishii & Sadowsky, 2008; Marion *et al.*, 2010).

Recently, some evidence has come to light suggesting the existence of environmentally persistent *E. coli* populations (Fujioka *et al.*, 1999; Power *et al.*, 2005; Ishii *et al.*, 2007; Ksoll *et al.*, 2007; Ishii & Sadowsky, 2008). These purportedly autochthonous bacteria have been isolated from a number of sources, including soil, sand, temperate lake water, algae, and freshwater periphyton (Byappanahalli *et al.*, 2003; Power *et al.*, 2005; Ksoll *et al.*, 2007; Walk *et al.*, 2007; Ishii & Sadowsky, 2008; Brennan *et al.*, 2010). In the case of the periphytic *E. coli*, strains isolated from shoreline rocks were shown to persist throughout the winter and could not be identified by comparing their DNA fingerprint to a large library of local animal and sewage sources (Ksoll *et al.*, 2007). Furthermore, a high degree of similarity was found between the DNA fingerprints of the periphytic *E. coli* and several planktonic *E. coli* isolates of a previously unidentified source (Ksoll *et al.*, 2007).

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The existence of environmentally persistent *E. coli* and their potential contribution to the water column places the reliability of *E. coli* as a fecal indicator under doubt. *Escherichia coli* released from the periphyton may artificially inflate bacterial counts and errantly indicate fecal contamination where there is none. Ishii & Sadowsky (2008) end their review of the topic by posing some key questions concerning the ecology of these naturalized strains. These questions all center on the main issue: why do these naturalized strains persist and multiply in the environment while other *E. coli* die off?

Walk *et al.* (2007) examined the genetic diversity of environmental *E. coli* isolates sampled from six freshwater beaches using sequence-based techniques. Although the genetic diversity of these isolates was found to be reasonably large, certain genotypes were recovered multiple times from different sites (Walk *et al.*, 2007). Walk *et al.* (2007) suggest that these persistent genotypes may be the result of selective pressures from the environment. It seems likely that these particular genotypes would be closely associated with one or more phenotypic characteristics that facilitate the establishment of environmentally persistent *E. coli* populations.

As biofilm formation is associated with stress tolerance, the ability of *E. coli* to form biofilms in the environment may represent a survivorship strategy. If this was the case, the low-nutrient environment of temperate freshwater lakes would apply selective pressures that favor strains capable of forming mature biofilms under temperate conditions. To confirm this hypothesis, the biofilm-forming capacity of periphytic *E. coli* isolates and *E. coli* isolates from other sources could be compared. Reisner *et al.* (2006) have demonstrated that the ability to form mature biofilms is not universal among human *E. coli* isolates. Instead, human isolates tend to exhibit a wide range of biofilm-forming competence that is often dependent on the growth media (Reisner *et al.*, 2006). If environmental persistence is dependent on biofilm formation, then periphytic *E. coli* isolates should exhibit higher mean biofilm-forming ability and decreased intragroup variation in biofilm-forming ability when compared with *E. coli* isolated from humans and other animal sources.

Escherichia coli biofilm formation is controlled by a number of factors, including several surface proteins such as conjugative pili, type-1 fimbriae, and curli (Van Houdt & Michiels, 2005). Amyloid curli fibers are a mediating factor in the attachment stage of biofilm formation (Barnhart & Chapman, 2006) and have been shown to be a common determinant of adhesion in environmental isolates in a small sample population (Castonguay *et al.*, 2006). Additionally, Carter *et al.* (2011) have demonstrated how curli-positive *E. coli* O157 variants grow better under nutrient limited conditions than their curli-negative counterparts. If environmentally persistent *E. coli* isolates can be shown to have uniformly high curli production, it might indicate that the curli-mediated attachment phase largely determines whether a strain will be successful in colonizing the periphyton and persisting in the environment.

Our research examines the biofilm-forming ability and curli expression of *E. coli* that persist in the periphyton of temperate freshwater lakes. We hypothesized that the ability to form mature biofilms is the key factor that allows strains to persist in the environment and that the ability to express curli is the main controlling factor in environmental biofilm formation. To determine this, the biofilm-forming ability and curli expression of periphytic *E. coli* isolates were compared with human, bovine, and shiga-like toxin-producing *E. coli* (STEC) isolates. Additionally, Repetitive extragenic palindromic polymerase chain reaction (REP-PCR) was used to compare the genetic diversity of periphytic *E. coli* with the genetic diversity of *E. coli* isolated from other sources.

Materials and methods

Enumeration and isolation of periphytic *E. coli*

Periphytic samples were taken from Boulevard Lake (48°27′34″N and 89°12′26″W), Chippewa Park Beach (48°20′6″N and 89°11′52″W), and Billy Lake (48°46′39″N and 88°37′43″W), three water bodies in the Thunder Bay District of Northwestern Ontario. Boulevard Lake is a man-made widening of the Current River, while Chippewa Park Beach is a protected bay on Lake Superior. Both locations are close to the Thunder Bay city center and are used frequently by swimmers. Billy Lake, on the other hand, is a small, undeveloped boreal lake near Dorion, Ontario, about 70 km east of Thunder Bay.

At each site, a rock submerged 30–40 cm below the water's surface was selected. Prior to removal from water, the rock was gently shaken 10 times to remove sediment from its surface. The rock was placed on shore with the surface to be sampled facing up. A sterile, square rubber template delineating an area of 10 cm × 10 cm was placed on the rock and used to quantify the substratum surface. Material was scraped from within the area marked by the template using a sterile spatula and suspended in 50 mL of sterile phosphate-buffered saline (PBS; 8.00 g NaCl, 0.20 g KCl, 1.44 g Na_2HPO_4, KH_2PO_4, 1 L distilled H_2O, pH adjusted to 7.4). Nine rocks were selected from each site for enumeration at each of the four sampling time-points on 12 June, 20 July, 23 September, and from 26–27 October 2008.

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For enumeration, 5 mL of the periphyton sample suspended in PBS was drawn through a sterile 47-mm mixed cellulose ester filter (Fisherbrand water testing membrane filter, pore size 0.45 μm; Thermo Fisher Scientific, Whitby, ON, Canada) using a sterile pneumatic pump funnel filtration apparatus. The filter was placed face-up on membrane fecal coliform agar (Becton, Dickinson, and Co., Sparkton, MD) and incubated overnight at 44.5 °C. Blue colonies growing on the filter were selected and streaked on CHROMagar *E. coli* agar (Dalynn Biologicals, Calgary, Alberta, Canada) and incubated at 37 °C overnight. Blue colonies were presumptively identified as *E. coli* and used to compute the overall population at that time point. Two to three presumptive *E. coli* isolates were randomly selected and isolated from each rock sample at each time point, and a total of about 80 periphytic *E. coli* isolates were isolated from each site. The isolates were maintained in trypticase soy broth (TSB) (Becton, Dickinson, and Co.) supplemented with 25% glycerol (v/v) and stored at −80 °C.

Seventeen random isolates were selected for biofilm and curli protein analysis from the samples collected at Boulevard Lake (four isolates chosen), Chippewa Park Beach (seven isolates), and Billy Lake (seven isolates). These isolates tested positive on CHROMagar *E. coli* agar medium and were subjected to IMViC (indole, methyl red, Vogues–Proskauer, and citrate) testing to confirm their identity as *E. coli*. All 17 isolates were confirmed to be *E. coli* and were stored for further experimentation.

Enumeration of heterotrophic periphyton bacteria

In addition to *E. coli*, heterotrophic background bacteria were enumerated from periphyton samples. Periphyton samples collected in the manner described previously were serially diluted and spread-plated on R2A agar (Becton, Dickinson, and Co.) in triplicate. The plates were incubated for 48 h at 30 °C, whereupon the total number of bacteria was counted and recorded.

Human and bovine *E. coli* isolates

Eighteen human *E. coli* isolates were obtained from fecal samples provided by six individuals. The fecal samples were serially diluted and filtered through sterile 47-mm mixed cellulose ester using a sterile pneumatic pump funnel filtration apparatus. The filter was placed face-up on Differential Coliform agar (Oxoid Ltd, Basingstoke, Hampshire, UK) and incubated at 45 °C overnight. Positive, blue colonies were selected, streaked onto differential coliform agar, and incubated at 37 °C overnight. Isolated colonies were confirmed as *E. coli* through IMViC testing and maintained as described for the environmental samples.

Eighteen bovine isolates were obtained from fecal samples taken from 10 dairy cows (Valley Centre Farms, Thunder Bay District, ON, Canada) using the same procedure described for the human isolates.

Shiga-like toxin-producing *E. coli* strains

C. Gyles at the University of Guelph provided 28 isolates to comprise the STEC grouping (Gyles *et al.*, 1998). Of these isolates, 10 representatives were from serotypes O157:H7 and O157:H- (four of bovine and seven of human origin), seven representatives were from serotypes O111:H8 and O111:H- (four of bovine and three of human origin), and 11 representatives were from serotypes O26:H11 and O26:H- (five of bovine origin and six of human origin).

DNA extraction

Individual *E. coli* strains were streaked onto Luria Bertani agar from frozen stock and incubated at 37 °C overnight. Individual colonies were selected and subsequently grown in Luria Bertani broth overnight at 37 °C with shaking at 150 r.p.m. After growth, 1 mL of inoculum was centrifuged for 3 min at 21 000 ***g*** to pellet the bacteria. The supernatant was removed, and 800 μL of XS lysis buffer (1% w/v potassium ethyl xanthogenate, 100 mM Tris–HCl, 20 mM EDTA, 1% w/v SDS, 800 mM ammonium acetate) and 1 μL of 5 μg μL^{-1} RNase (Promega, Madison, WI) were added to each sample (adapted from Tillett & Neilan, 2000). The samples were subsequently re-suspended and incubated for 1 h at 37 °C. Following incubation, the samples were placed in a 70 °C water bath for 15 min, mixed, and placed on ice for 30 min to precipitate cell debris. The cell debris was removed by centrifuging for 10 min at 21 000 ***g***. The supernatant was transferred to a new, sterile tube. The DNA in the supernatant was precipitated by adding 750 μL of isopropyl alcohol and then incubating at room temperature for 10 min with regular inversions. The precipitated DNA was isolated by centrifuging at 21 000 ***g*** for 10 min and removing the supernatant. The DNA was washed with 750 μL of 70% ethanol and stored in 100 μL of sterile, distilled, deionized water.

REP-PCR

Specially designed REP primers (Sigma-Aldrich, St. Louis, MO) were used to target REP sequences within the bacterial genome (de Bruijn, 1992) The sequences between the

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REP elements were amplified through PCR and separated using gel electrophoresis, generating unique banding patterns that could be used to compare the diversity of isolates.

Each polymerase chain reaction had a final volume of 25 μL containing 2.5 mM $MgCl_2$, 1× Taq buffer (Fermentas, Burlington, ON, Canada), 0.2 mM of each deoxynucleoside triphosphates, 1 μM Primer REP-2I (5′;-ICGICTTATCIGGCCTAC-3′), 1 μM Primer REP-IR (5′-IIIICGICGICATCIGGG-3′), 1.25 units *Taq* polymerase, and 1 μL of DNA from extraction. The PCR parameters include an initial denaturation step of 6 min at 95 °C followed by 30 cycles of denaturation (1 min at 94 °C), annealing (1 min at 40 °C), and extension (8 min at 65 °C), with a final extension step of 16 min at 65 °C. The final PCR products were held at 4 °C (de Bruijn, 1992).

After amplification, the individual bands were separated using agarose gel electrophoresis (2% w/v agarose, 50 mL 1× TAE buffer) with 5 μL of 10 mg mL^{-1} ethidium bromide (1 μg mL^{-1}) for visualization under UV light. Five μL of 6× Fermentas loading dye was added to each reaction tube, giving a final volume of 30 μL. The first and last wells of the gel were loaded with 6 μL of 0.1 μg μL^{-1} 1 kb plus DNA ladder (Fermentas). The remaining wells were loaded with 10 μL of sample. The PCR samples were separated by gel electrophoresis at 100 V for 60 min. Upon finishing, the bands were visualized using a Chemi Genius Bio-imaging system (Syngene, Frederick, MD). An image of the gel was saved and imported into the Informatix Fingerprinting II software (Bio-Rad Laboratories Inc., Hercules, CA) for analysis.

Analysis of diversity

To analyze the diversity of *E. coli* isolates, a dendrogram was created using Informatrix Fingerprinting II. For consistency, 15 lane gels were used and the images were normalized based on 1 kb Plus DNA ladder external standards such that individual samples from separate agarose gels could be compared. After final band assignment, a cluster analysis comparison was made (Pearson's correlation; UPGMA) to generate a dendrogram based on percent similarity between samples. The K-12 *E. coli* type strain (MG1655 or ATCC 700926) was used as a standard to test the degree of variability of the REP-PCR profile between gels. Six separately run gels were used to create REP-PCR profiles for the K-12 strain. The similarity of these profiles ranged from 90% to 98% similar (data not shown). Our results are similar to previous studies (Ishii *et al.*, 2007) that considered *E. coli* isolates with REP-PCR DNA fingerprints showing percent similarity > 92% to be the same strain.

Biofilm assay

To quantify biofilm-forming ability, individual *E. coli* isolates were grown in polystyrene wells and subjected to a crystal violet assay (adapted from Jackson *et al.*, 2002). Cells that adhere to the polystyrene can be considered biofilm cells and will retain a crystal violet stain while planktonic cells and excess dye are washed off through rinsing. The dye can be recovered from the stained biofilm cells and quantified using spectrophotometric methods. The resulting absorbance is directly related to the amount of cells adhered to the polystyrene and thus is indicative of the isolate's biofilm-forming ability.

Biofilm inocula were created by streaking frozen stock on Luria Bertani agar (15% agar w/v; Becton, Dickinson, and Co.) and incubating overnight at 37 °C. An isolated colony was used to inoculate 30 mL of Luria Bertani broth (Becton, Dickinson, and Co.), which was incubated at 37 °C overnight with shaking at 150 r.p.m. The resulting cell suspension was pelleted down through centrifugation at 2450 ***g*** and then washed through disposal of the supernatant and re-suspension in 10 mL of sterile PBS. The wash step was repeated twice more before the cells were re-suspended to an optical density ($OD_{600\ nm}$) of 1.00 ± 0.05 (approximately 1×10^9 CFU mL^{-1}) in a minimal salt media with 0.04% glucose [MSM; 1.249 mM KH_2PO_4, 3.73 mM K_2HPO_4, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.02 mM $FeSO_4 \cdot 7H_2O$, 1.4 mM NH_4Cl, 0.04% glucose (w/v)]. The OD1 cells were diluted to a cell concentration of roughly 1×10^6 CFU mL^{-1} by adding 10 μL of the inoculum to 190 μL of MSM in eight replicate wells in a 96-well polystyrene flat-bottomed microtitre plate (Castar, Corning, NY). Inoculated plates were incubated at 22 °C for 48 h with shaking at 25 r.p.m.

Following incubation, the wells were rinsed three times with sterile, double distilled water. After drying, the replicate wells were stained with 200 μL of 0.1% crystal violet solution (Harleco, Gibbstow, NJ) for 10 min. Excess stain and any remaining planktonic cells were washed away by rinsing three times with sterile, double distilled water. The wells were allowed to dry, and the stain was subsequently re-solubilized by de-staining with 200 μL of a 20% acetone and 80% ethanol solution for 10 min. One hundred and fifty microliters of the re-solubilized stain was then transferred to a fresh microtiter plate, where the concentration of dye could be quantified by measuring the absorbance at 600 nm using a FluoStar Optima automated absorbance reader (BMG Labtech, Offenberg, Germany).

The biofilm-forming capacity of each isolate was also tested after growth in MSM at 37 °C, TSB at 22 °C, and TSB at 37 °C to optimize experimental conditions.

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Curli assay

Curli proteins have been demonstrated to stain red in the presence of the diazo dye Congo red (Barnhart & Chapman, 2006). The expression of curli can be qualitatively determined by growing bacteria on media containing Congo red and observing whether the colonies stain red. To quantify curli production, bacteria grown on Congo red containing media can be lysed and the amount of Congo red released can be measured through spectrophotometric methods (adapted from Gophna *et al.*, 2001). The resulting absorbance reading correlates directly with the amount of curli expressed by the bacteria. Curli production of each experimental strain was analyzed qualitatively and quantitatively.

In the qualitative analysis, each strain was grown on MSM agar supplemented with 0.02% Congo red for 72 h at 22 °C. The media and temperature were selected to match the growth conditions used in the crystal violet assay. The strains were grown for 72 h to ensure that a sufficient amount of Congo red was taken up by curli-positive colonies. Following growth, the presence or absence of Congo red in the bacterial colonies was determined visually with or without the use of a stereo microscope (40× magnification). Strains that appeared red were presumed curli positive, while strains that remained white were labeled curli negative.

To complete the quantitative curli assay, bacteria need to be grown on solid medium containing Congo red and suspended to an OD of 1. To accomplish this, isolates need to be grown in a sufficient enough quantity that they can be scraped off the agar plate and suspended in PBS at a desired OD. Colonies grown on MSM agar do not reach sufficient biomass for this assay. As a substitute, isolates were grown on trypticase soy agar (TSA) containing 0.02% Congo red.

Individual *E. coli* isolates were grown on TSA (Becton, Dickinson, and Co.) containing 0.02% Congo red (Harleco) at 22 °C for 72 h. Environmental *E. coli* isolate 1A was used as a positive control, and STEC isolate H32 was used as a negative control. Following incubation, isolates were suspended to an optical density of $1.00_{600\ \mathrm{nm}} \pm 0.05$ in PBS in triplicate. A 1.00-mL aliquot of the inoculum was transferred to a microcentrifigue tube and pelleted down by centrifugation at 3100 ***g*** for 5 min.

To properly lyse the cells, the supernatant was removed and the pellet re-suspended in 100 μL of buffer (10 mM EDTA, 25 mM Tris–HCl, adjusted to pH 7.4). Following this, the cell suspension was mixed with 200 μL of the lysis solution (1 M NaOH, 1% SDS) and placed in a 70 °C water bath for 5 min. After heating, the sample was sonicated by exposure to ten 1-s bursts from a bench-top sonicator equipped with a microtip (50% Duty Cycle and 6 Output Control; Branson Sonic Power Company, Danbury, CT) and subsequently placed in the water bath for another 5 min. Cell debris was removed by centrifuging at 21 000 ***g*** for 2 min and by recovering only the supernatant.

To determine the concentration of Congo red in the solution, 250 μL of the supernatant was diluted to 750 μL with PBS in a disposable plastic cuvette (Fisher Scientific, Whitby, ON; 10 mm light path), whereupon the optical density at 488 nm was measured using a bench-top spectrophotometer (Thermoscientific, West Palm Beach, FL).

Statistics

A two-way analysis of variance (ANOVA) was used to analyze differences in environmental *E. coli* cell densities over time. To optimize experimental conditions, a one-way ANOVA with a *post hoc* Tukey test was used to compare the mean biofilm formation of all isolates in four different conditions. A one-way ANOVA with a *post hoc* Scheffé test was used to compare mean biofilm formation and mean curli expression between source groupings because of the uneven sample sizes. A linear regression analysis was used to assess whether there is any correlation between biofilm formation and curli expression. All analyses were conducted using PASW STATISTICS 18 (IBM SPSS Inc.). ANOVA assay results were considered significantly different at the α level of 0.05. Figures were generated using PASW STATISTICS 18. Error bars represent 95% confidence intervals. Genetic diversity of the isolates was examined by generating a dendrogram using Fingerprinting II Informatrix software (Bio-Rad Laboratories Inc.).

Results and discussion

Enumeration of periphytic *E. coli*

From June through October, 2008, cell densities of *E. coli* residing in the epilithic periphyton of Boulevard Lake, Chippewa Park Beach, and Billy Lake were monitored (Fig. 1a). Cell densities were not found to vary significantly between sampling sites ($P = 0.795$) or between sampling dates ($P = 0.282$), despite the change of water temperature throughout the sampling period (average temperatures on June 12, July 20, September 23, and October 27 were 14.3, 19.7, 19.7, and 5.5 °C, respectively). Periphytic *E. coli* populations peaked in July at Chippewa Park Beach, in September at Boulevard Lake, and remained relatively constant throughout all four sampling time-points in Billy Lake. *Escherichia coli* populations in Boulevard Lake and Chippewa Park Beach peaked at $> 1 \times 10^2$ CFU 100 cm^{-2} in September and

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July, respectively, and reduced to < 10 CFU 100 cm^{-2} in October. Periphytic *E. coli* cell densities at Billy Lake peaked in June at 50 CFU 100 cm^{-2} but remained relatively constant, never falling below 10 CFU 100 cm^{-2}.

Heterotrophic background bacteria were also collected from the periphyton at each sampling time-point and enumerated (Fig. 1b). These heterotrophic populations remained relatively stable throughout the sampling period, fluctuating from 1×10^7 to 1×10^9 CFU 100 cm^{-2}. The relatively low *E. coli* cell densities compared with the heterotrophic cell densities demonstrate that *E. coli* only comprise a very small component of the total periphytic bacterial population.

Finding a stable *E. coli* population in the periphyton of temperate freshwater lakes would be puzzling without taking recent research into consideration. Under the traditional view, *E. coli* can only be present in the environment when there is fecal contamination, and any substrate colonization by *E. coli* can only be transient (Ishii & Sadowsky, 2008). It is unlikely that all the three boreal lakes were continuously contaminated by fecal materials during the sampling period.

There is, however, a growing body of research dealing with *E. coli* that persist in the environment long after their original input. A continuous, stable presence of *E. coli* has been demonstrated in soil, river sediments, and freshwater periphyton (Byappanhalli *et al.*, 2003; Ksoll *et al.*, 2007; Brennan *et al.*, 2010). Additionally, many isolates from a population persisting in the epilithic periphyton of Lake Superior have been demonstrated to be genetically distinct from *E. coli* isolated from any local external source (Ksoll *et al.*, 2007). While we cannot fully confirm that our isolates are naturalized (i.e. genetically distinct from all external *E. coli* populations), we have demonstrated a continuous, stable *E. coli* population in the periphyton of three boreal water bodies.

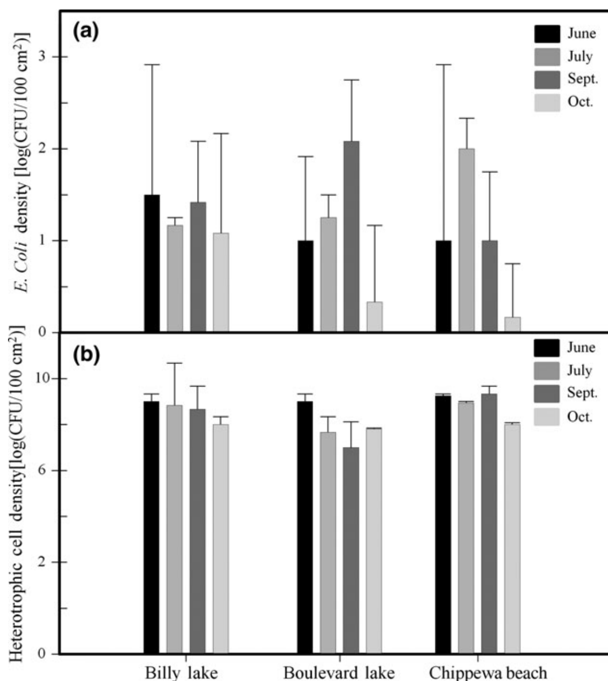

Fig. 1. Mean (a) *Escherichia coli* and (b) heterotrophic cell densities in periphyton samples taken from Billy Lake, Boulevard Lake, and Chippewa Park from June until October, 2008.

Genetic diversity of *E. coli* isolates

Based on a cluster analysis of the REP-PCR profiles, the 81 *E. coli* isolates were highly diverse, with similarity levels ranging from 14% to 98% (Fig. 2). Despite their genetic diversity, the isolates could be divided into six major clusters (Cluster I–VI) at about 40–50% similarity levels. The overall diversity of the periphytic *E. coli* isolates from the three sites was high. The Billy Lake and Chippewa Park Beach isolates were grouped within Clusters V and II, while the Boulevard Lake isolates were scattered among Clusters V and VI. With an exception of two to three isolates from Billy Lake and Chippewa Park Beach, most periphytic isolates were diverse with similarity levels between 40% and 86%.

Seventeen of the 18 human *E. coli* isolates fell into Cluster IV with similarity levels ranging from 40% to 98%. The genetic diversity of *E. coli* isolated from a single individual was low, while isolates taken from different individuals were diverse. The cow isolates were grouped together in Clusters I and III. Genetic differences between isolates from different clusters were large while *E. coli* grouping together in a single cluster possessed genetic similarity in the range of 47–98%.

Eight of 10 *E. coli* isolates from the O157 serogroup were grouped in Cluster II, with seven strains exhibiting genetic similarity > 90%. This clustering was independent from the source of the O157 isolate (i.e. isolated from humans or cows). This suggests a close genetic relationship among the majority of isolates from the O157 serogroup. Similar to the O157 strains, eight of the nine O26 strains were grouped in Cluster VI with more than 72% similarity. Serogroup O111 demonstrated the highest levels of genetic diversity among the three STEC serogroups in this study. The nine O111 strains were divided into three subgroups in Cluster VI with similarity levels ranging between 47% and 62%.

Optimal biofilm growth conditions

To optimize experimental protocol, the biofilm-forming capacity of *E. coli* isolates from different source groupings

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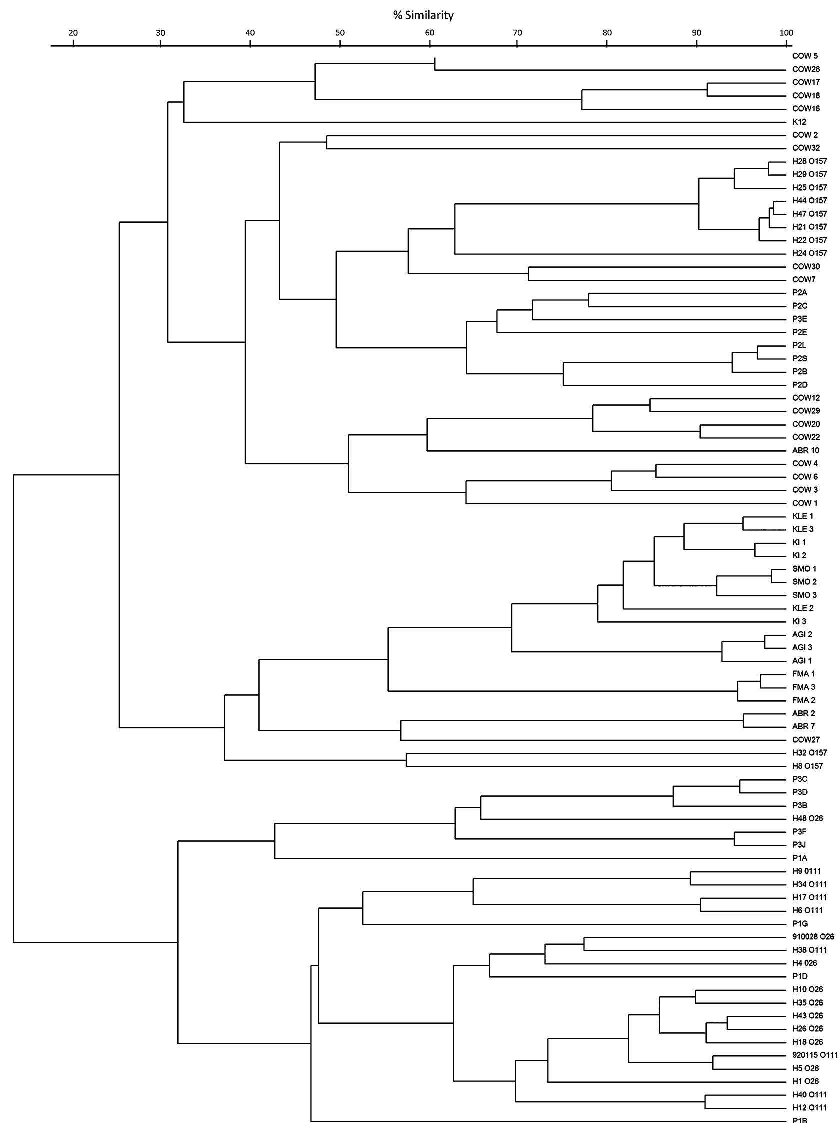

Fig. 2. Relatedness of *Escherichia coli* isolates from various sources based on an analysis of REP-PCR DNA fingerprints using Pearson's correlation. Individual strains are identified based on a letter indicating source grouping (C, bovine; H, human; P, periphytic; O157, STEC serogroup O157; O111, STEC serogroup O111; O26, STEC serogroup O26) and a unique strain number. K12 represents the K-12 standard *E. coli* strain.

was measured under four different conditions. A total of 81 isolates were grown for 48 h in both MSM and TSB at 22 and 37 °C. After growth, the strains were subjected to a crystal violet assay and their total biofilm formation was measured using spectrophotometric methods (Table 1). A one-way ANOVA with a *post hoc* Tukey test revealed that the mean biofilm formation of all strains grown in MSM at 22 °C was significantly greater than the biofilm formation of strains grown in the other three conditions ($P < 0.001$). At 37 °C, the biofilm-forming ability of all *E. coli* strains was uniformly poor, with absorbance (600 nm) < 0.04 (Table 1). Additionally, individual strains cultured in MSM at 22 °C consistently formed more biofilm than the same strains cultured in TSB at 22 °C (data not shown). As a result, growth in MSM at 22 °C was selected as the optimal condition for this study. Strains grown in MSM at 22 °C formed approximately twice as much biofilm as strains grown in TSB at 22 °C and approximately 10 times as much biofilm as strains grown in either MSM or TSB at 37 °C. There was no significant difference in biofilm formation found between strains grown in MSM at 37 °C and strains grown in TSB at the same temperature ($P = 0.981$).

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Table 1. A comparison of biofilm formation in *Escherichia coli* from four different source groupings

	Biofilm-forming ability (A600)*			
	MSM 22 °C	MSM 37 °C	TSB 22 °C	TSB 37 °C
E. coli source grouping				
Periphytic [n=17]	0.906 ± 0.230	0.112 ± 0.017	0.584 ± .0733	0.0225 ± 0.0158
Human[n=18]	0.389 ± 0.455	0.0455 ± 0.0560	0.0901 ± 0.120	0.0406 ± 0.0457
Bovine [n=18]	0.120 ± 0.209	−0.00392 ± 0.0532	0.0638 ± 0.0931	0.0274 ± 0.0585
STEC [n=28]	0.213 ± 0.194	0.0229 ± 0.0439	0.146 ± 0.633	0.0442 ± 0.0742
Compiled [n = 81]	0.370 ± 0.423	0.0407 ± 0.0724	0.207 ± 0.507	0.0351 ± 0.0611

MSM, minimal salt medium with 0.04% glucose.
*Results are presented as the mean absorbance at 600 nm of all isolates from a specific grouping. One-way ANOVA testing reveals a significant difference between the biofilm formation of all strains grown in MSM at 22 °C when compared with strains grown in the other three conditions (P < 0.05).

These results suggest that temperature has a stronger influence than growth media in controlling *E. coli* biofilm formation.

Comparison of biofilm-forming abilities between different source groupings

The mean crystal violet assay results of each source grouping after growth in MSM at 22 °C were compared to determine whether there was any significant intergroup difference in biofilm-forming capacity (Fig. 3a). A one-way ANOVA and *post hoc* Scheffé test revealed that the periphytic isolates were significantly more competent at forming biofilms than isolates from any other source grouping (P < 0.001). Periphytic isolates formed, on average, approximately 2.5 times as much biofilm as human isolates, 4.5 times as much biofilm as STEC isolates, and 7.5 times as much biofilm as bovine isolates. Additionally, the periphytic isolates were clustered together with high biofilm-forming ability and small standard deviation. The biofilm-forming ability of human isolates varied more than two times as much as the biofilm-forming capacity of periphytic isolates. This increased biofilm-forming capacity and decreased intragroup variation in comparison with human isolates support the hypothesis that the ability to form biofilms is selected for in periphytic *E. coli* isolates.

Reisner *et al.* (2006) demonstrate how human isolates, including a variety of pathogenic strains, have incredibly diverse biofilm-forming ability. This is consistent with our results, which show a large spread in the ability of human and STEC isolates to form biofilms *in vitro*. The periphytic isolates, on the other hand, tend to cluster together, and all possess high biofilm-forming capacity. This occurs despite the large genetic diversity present in our periphytic isolates, demonstrated by our REP-PCR data. As the isolates comprising the periphytic grouping were collected from three distinct water bodies, it seems plausible that their similar environments (boreal freshwater lakes) would exert similar selective pressures that favored strong biofilm-forming *E. coli* strains. In this scenario, *E. coli* are deposited in the environment through fecal contamination and only those strains that possess superior biofilm-forming capacity would be able to persist in the periphyton, sediment, algal mats, sand, or some other substrata. If these biofilm populations persist for long enough, genetic changes may accumulate and result in a genetically unique, naturalized population. If true,

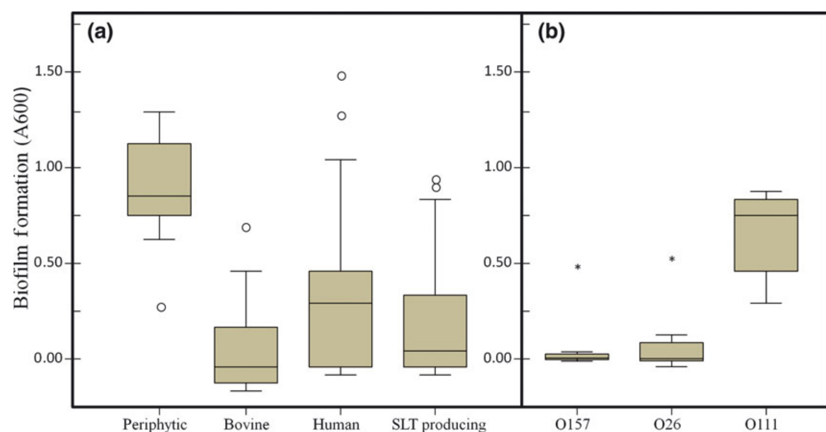

Fig. 3. Relative biofilm formation (expressed as absorbance at 600 nm) of *Escherichia coli* isolates grown in MSM supplemented with 0.04% glucose at 22 °C for 48 h. (a) Comparison of periphytic, human, bovine, and STEC biofilm formation. (b) Comparison of biofilm formation between three serogroups of STEC. Box plots show median, 25th and 75th percentiles, upper and lower fences, outliers, and extremes.

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this indicates that the ability to form biofilms is the determining factor in whether a strain will persist in the environment and become naturalized.

The bovine source grouping also clusters together with a trend toward relatively low biofilm-forming ability. Unlike the periphytic isolates, this likely does not indicate any adaptive significance. Rather, it likely represents the relatively small scope of our sampling site, as all bovine isolates were taken from 10 cows kept in the same barn of a small farm. It is also worth noting that several of the good biofilm-forming STEC isolates were obtained from cattle sources. This indicates that bovine *E. coli* isolates likely have much more variation in biofilm-forming ability than the data from our bovine source grouping suggest.

When grouped together as STEC, the O157, O111, and O26 serogroups, all exhibit a wide range of biofilm-forming abilities. This supports the findings of Reisner *et al.* (2006) who saw no significant increase in biofilm-forming capability for a variety of pathogenic strains when compared with nonpathogenic strains. When separated into serogroups (Fig. 3b), however, there is significant variability in biofilm-forming ability between serogroups. One-way ANOVA with a *post hoc* Scheffé test reveals that O111 isolates are significantly better biofilm formers than isolates from either the O157 ($P = 0.004$) or O26 serogroups ($P = 0.007$). We suggest that this clustering does not represent any adaptive significance. Instead, we propose that the uniformly poor biofilm-forming ability of the O26 and O157 isolates and the relatively uniformly good biofilm formation of the O111 isolates are merely phenotypic manifestations of low genetic diversity. This hypothesis is supported by our REP-PCR data, which demonstrate reduced genetic diversity in O26 and O157 isolates when compared with O111 isolates. Other studies (Whittam *et al.*, 1993; Noller *et al.*, 2003; Gilmour *et al.*, 2005) have examined multilocus sequence allelic variation between strains within a specific serotype of STEC. These independent studies all demonstrated a high degree of genetic relatedness within the O157:H7 and the O26:H11 serotypes, and a lower degree of genetic relatedness among the O111:H8 strains (Whittam *et al.*, 1993; Noller *et al.*, 2003; Gilmour *et al.*, 2005). This may explain the variation in biofilm-forming ability among STEC isolates. Isolates from the O157 and O26 serogroups exhibit significantly less intragroup variation in biofilm-forming ability than isolates from the O111 serogroup do.

Curli assay

Bacteria were grown both on MSM agar and on TSA containing 0.02% Congo red, and their resulting pigmentation was observed (Fig. 4). Following this, each experimental strain was subjected to a qualitative and quantitative Congo red curli assay.

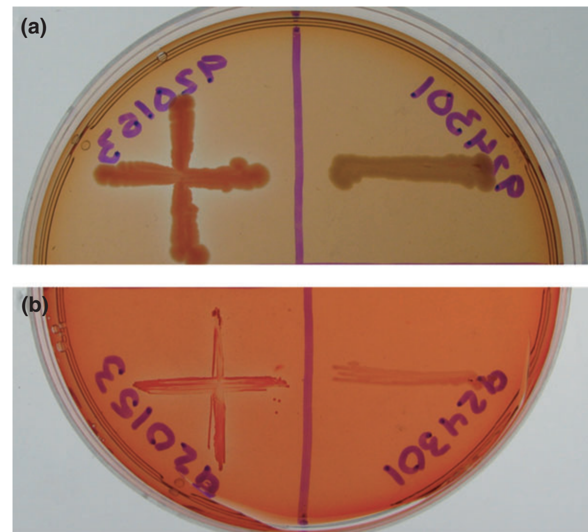

Fig. 4. Comparison of pigmentation in STEC isolates grown on (a) TSA supplemented with 0.02% Congo red and (b) MSM supplemented with 0.04% glucose, 15% agar, and 0.02% Congo red. Isolates grown in the '+' configuration are the same strain and are considered to be good curli formers. Isolates grown in the '−' configuration are the same strain and are considered to be poor curli formers.

The mean quantitative Congo red assay results of each source grouping were compared to determine whether there were any considerable intergroup differences in curli expression (Fig. 5a). A one-way ANOVA and *post hoc* Scheffé test revealed significant differences in curli production only between bovine and human isolates ($P = 0.023$).

Much like the biofilm assay, the variation in curli expression within individual STEC serotypes is substantially lower than the overall variation of the STEC source grouping (Fig. 5b). A one-way ANOVA with a *post hoc* Scheffé test revealed that O111 isolates had significantly higher curli expression than O26 and O157 isolates ($P < 0.001$). Additionally, O111 isolates had greater intragroup variation than O26 or O157 isolates. This corroborates our hypothesis that the uniform, low biofilm-forming ability of O26 and O157 isolates does not have any adaptive significance and is only a result of the low genetic diversity present in these serogroups.

To determine whether there is any positive correlation between curli expression and biofilm formation, the crystal violet assay results of each individual isolate grown in TSB were compared with its TSA-Congo red assay results (Fig. 6). A linear regression analysis revealed an R^2 value of 0.095 ($P = 0.0052$), indicating little correlation between the biofilm-forming ability and curli expression of the bacteria. Many isolates, specifically those of bovine origin,

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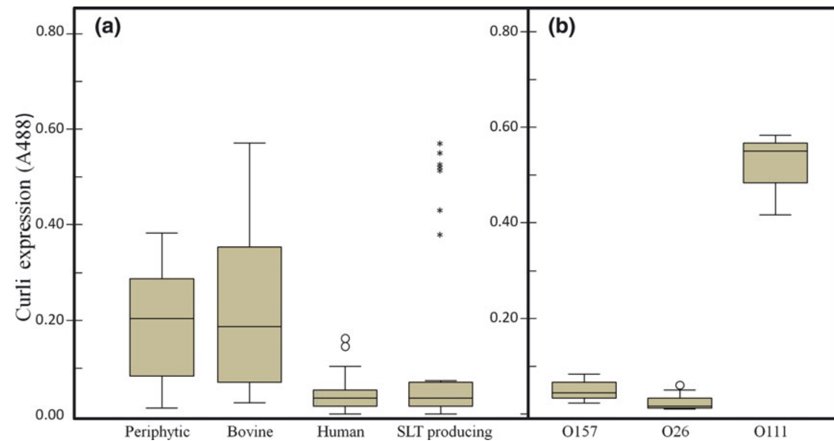

Fig. 5. Relative curli expression (expressed as absorbance at 488 nm) of *Escherichia coli* isolates grown on TSA supplemented with 0.02% Congo red at 22 °C for 48 h. (a) Comparison of periphytic, human, bovine, and STEC curli expression. (b) Comparison of curli expression between three serogroups of STEC. Box plots show median, 25th and 75th percentiles, upper and lower fences, outliers, and extremes.

Table 2. *Escherichia coli* curli expression and biofilm formation in MSM

	Percentage of isolates (%)	
	Biofilm positive*	Biofilm negative
Curli positive†	49.3	19.2
Curli negative	8.2	23.3

*Isolates were grown in MSM supplemented with 0.04% glucose at 22 °C for 48 h and subjected to a crystal violet assay. Those isolates that retained dye after de-staining ($A_{600\ nm} \geq 0.10$) were designated biofilm positive.

†Isolates were grown on MSM agar supplemented with 0.02% Congo red for 72 h. Colonies that appeared red were designated curli positive.

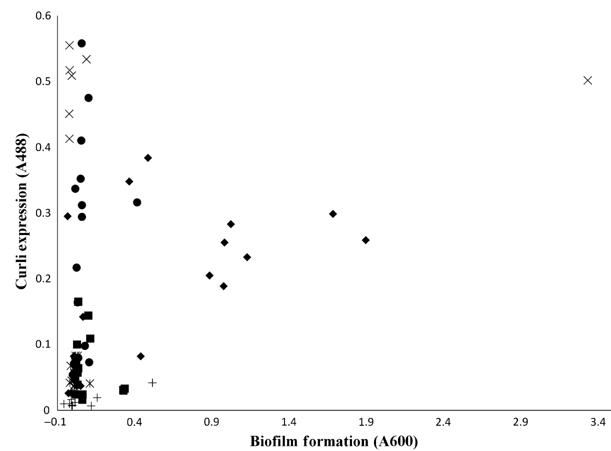

Fig. 6. Curli expression of *Escherichia coli* isolates grown on TSA supplemented with 0.02% Congo red at 22 °C for 48 h plotted against biofilm formation of *E. coli* isolates grown in TSB at 22 °C for 48 h. ● = Bovine isolates; ♦ = periphytic isolates; ■ = human isolates; × = O111 isolates; ⋇ = O157 isolates; + = O26 isolates.

demonstrated high levels of curli expression with poor biofilm formation. These results suggest that curli expression cannot be the sole mitigating factor in the persistence of *E. coli* in the periphyton. These results were corroborated by a qualitative curli assay (i.e. MSM agar–Congo red assay), which was completed under optimal biofilm-forming conditions (Table 2). The qualitative assay revealed that the expression of curli does not necessarily result in biofilm formation, as 19% of tested strains were curli positive but biofilm negative. Additionally, just over 8% of the tested strains demonstrated the ability to form biofilms with no curli expression (Table 2).

Castonguay *et al.* (2006) demonstrated, in a small sample size, that curli expression seems to be a determinant of adhesion in environmental isolates. Our results appear to contradict these findings, although it is possible that the poor biofilm formers with high curli expression were able to adhere to the substrate but lacked some other important factor required for growth and persistence. On the other hand, the presence of good biofilm formers with no curli expression indicates that curli is not required for biofilm formation. To better understand environmental biofilm formation, other factors such as conjugative pili and exopolysaccharide production should be investigated.

Our results, if nothing more, serve to highlight the complexity of *E. coli* biofilm formation in the environment. While it seems apparent that biofilm formation is required for persistence in the periphyton, the sampled periphytic *E. coli* strains were found to be both genetically diverse and diverse with respect to their production of curli protein. Further research into the physiological and genetical characteristics of environmentally persistent *E. coli* will be required to gain a full understanding of how these bacteria establish themselves and survive in the periphyton.

Acknowledgements

This work was supported by funding from the National Science and Engineering Research Council of Canada. We

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gratefully acknowledge Dr Carlton Gyles (University of Guelph) and the Valley Centre Farms (Thunder Bay, Ontario, Canada) for providing the STEC strains and cow manure samples used in this study, respectively.

Authors' contribution

S.M. and A.B. contributed equally to this work.

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