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Stress resistance in naturalised waste water *E. coli* strains

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The authors recently demonstrated that naturalised strains of *Escherichia coli* exist in municipal waste water, characterised by (*a*) biomarker patterns in intergenic regions distinct from human and animal *E. coli* strains and (*b*) an insertion element (*IS30*) located in the *uspC–flhDC* intergenic region of the genome. Remarkably, these strains are naturally adapted to survival and growth in waste water and differentially survive the treatment process. The authors sought to explore the adaptive mechanisms used by these strains for survival. A serial stress experiment (nutrient deprivation and osmotic stress followed by chlorine treatment) was performed and survival was measured using culture. Waste water strains were shown to be approximately 100 times more resistant to chlorine treatment than a wild-type human faecal strain. Naturalised waste water strains were also more robust at producing biofilms – an adaptive strategy for surviving environmental stressors. Since biofilm formation has been linked to increased motility, the authors examined the expression of the flagellar regulator gene, *flhDC*, under serial stress conditions. Chlorine was a potent inducer of *flhDC* expression in waste water strains. The results demonstrate that waste water strains possess adaptive genotypic/phenotypic properties related to their survival in waste water and challenge the understanding of treatment reduction based on *E. coli* as an indicator of treatment performance.

Introduction

Escherichia coli has been one of the most studied microorganisms (Tenaillon *et al*., 2010) since its discovery in 1885 and has been used as a water quality indicator for faecal contamination for years. It has been generally assumed that this bacterium is restricted to the gastrointestinal system of warm-blooded animals, but several studies have found that *E. coli* can survive and replicate outside the gastrointestinal tract of animals in a non-host environment (water/sand/sediment) (Anderson *et al*., 2005; Byappanahalli *et al*., 2012; Chandrasekaran *et al*., 2015; Kon *et al*., 2007; Power *et al*., 2005; Solo-Gabriele *et al*., 2000; Tymensen *et al*., 2015; Winfield and Groisman, 2003). Survival and colonisation in both the animal host and non-host habitats are mediated by adaptations to nutrient availability, temperature, pH, osmolarity and the presence of competitive microflora. In bacteria, survival and adaptation in different environments is necessary for their continued evolutionary success.

Previously, the authors characterised the occurrence of host-specific strains of *E. coli* in a variety of human and animal isolates by using logic regression analysis of intergenic deoxyribonucleic acid (DNA) sequences (Zhi *et al*., 2015, 2016a). The authors applied these novel bioinformatic tools to understand *E. coli* population genetics better in non-host environments, such as waste water, leading to the discovery of naturalised strains of *E. coli* in municipal waste water (Zhi *et al*., 2016b). Remarkably, these naturalised populations of *E. coli* appear adapted to survive in the waste water environment. These strains are characterised by distinct genetic single-nucleotide polymorphism biomarker patterns in intergenic regions (ITGRs) that are different from those seen in human and animal strains of *E. coli* (Zhi *et al*., 2016b). Moreover, the majority of these naturalised waste water *E. coli* strains also possess a distinct genetic signature in which an insertion sequence (*IS30*) is specifically located in the *uspC–flhDC* IGR (*uspC–IS30–flhDC*) of the genome (Zhi *et al*., 2016b). These strains are easily identified in waste water by using a polymerase chain reaction (PCR) assay targeting this genetic locus (Zhi *et al*., 2016b). This genetic signature was not observed in (*a*) *E. coli* libraries originating from a wide range of animals, including humans; (*b*) whole-genome sequence databases for *E. coli* from humans or animals; or (*c*) in any *E. coli* DNA sequence data represented in GenBank. To date, these naturalised waste water *E. coli* strains have been found in all waste water treatment plants (WWTPs) that the authors have tested in Alberta (n = 10), Canada, suggesting that they are widely disseminated and implying that some strains of *E. coli* appear to have evolved adaptations for survival and growth in a waste water environment. These findings have challenged the understanding of waste water treatment in the context of using faecal indicator bacteria, such as *E. coli*, as a measure of treatment performance, demonstrating that bacteria in the sewage influent may be genetically different from those in the WWTP effluent.

Given the relative abundance of these naturalised *E. coli* strains in sewage/waste water and their widespread distribution throughout WWTPs in Alberta, the genetic and phenotypic properties of these strains likely allow for their survival and persistence in a stressful

waste water environment. A wide repertoire of genes/pathways are known to regulate the stress response in *E. coli*, and these include (*a*) the generalised stress response (RpoS), which facilitates survival against nutrient derivation, and oxidative and osmotic stressors (Battesti *et al*., 2011; White *et al*., 2011); (*b*) the universal stress proteins, known to be important for resistance to oxidative stress, ultraviolet (UV) radiation and antibiotics (Gustavsson *et al*., 2002; Nachin *et al*., 2005); and (*c*) the more recently discovered locus of heat resistance – a genomic island encoding up to 16 different proteins associated with heat shock, cell envelop maintenance and turnover of misfolded proteins (Mercer *et al*., 2015) – important for repairing cellular damage. Surprisingly, naturalised waste water strains of *E. coli* possess all these stress-adaptive mechanisms (Zhi *et al*., 2016b), which in themselves, are reflective of the processes used to treat waste water (i.e. osmotic shock, oxidative stress (hydrogen peroxide (H_2O_2), chlorine), UV treatment, nutrient deprivation/competition), suggesting an evolutionary adaptation of *E. coli* to this non-host environment.

A further adaptation appears to be the site-specific insertion of the *IS30* element between the *uspC* and *flhDC* genes. Insertion elements located in IGRs have been shown to modify expression of certain genes (Barker *et al*., 2004). As outlined earlier, the *uspC* gene plays an important role in promoting resistance to oxidative and UV damage through DNA repair (Gustavsson *et al*., 2002; Kvint *et al*., 2003; Nachin *et al*., 2005), suggesting that the *IS30* element may play a role in upregulating *uspC* expression during UV treatment of waste water. The *flhDC* gene encodes the master regulator for flagellar biosynthesis, acting as an activator for the expression of bacterial flagellar proteins (Anderson *et al*., 2010). Flagella promote bacterial motility, which, in turn, is positively associated with biofilm formation (Wood *et al*., 2006). Biofilm formation is an important strategy used by bacteria to survive unfavourable environmental conditions, and the formation of biofilms has been shown to promote resistance to chlorine, UV radiation (UVC), oxidative damage and even predation (Bianco *et al*., 2006; Elasri and Miller, 1999; Ryu and Beuchat, 2005; Vogeleer *et al*., 2014) – important microbial reduction strategies used during waste water treatment. Consequently, the *uspC–IS30–flhDC* polymorphism may play an important adaptive strategy in these naturalised bacterial populations towards colonisation and survival in tertiary treated waste water.

The purpose of this study was to explore the potential correlation between the presence of the *uspC–IS30–flhDC* ITGR polymorphism observed in naturalised waste water *E. coli* strains and the adaptive phenotypes of motility, biofilm formation and chlorine resistance as strategies that promote the survival of naturalised *E. coli* in a waste water environment.

Methods

Serial stress survival experiment

Two naturalised *uspC–IS30–flhDC* marker-positive waste water *E. coli* isolates (WW10 and WW63) and two *uspC–IS30–flhDC* marker-negative wild-type *E. coli* isolates (H51 and H54) isolated from human faeces were used in the serial stress experiment. The authors' previous paper describes the isolation and characterisation of these naturalised waste water strains (Zhi *et al*., 2016b). All *E. coli* strains were cultured in tryptic soy broth (TSB) for 24 h at 37°C, after which the culture was diluted to 1:10 by using sterile distilled water and incubated for another 24 h at room temperature (nutrient deprivation/osmotic stress). The cultures were then treated with chlorine by adding 0·8% bleach to the culture. The mixture was shaken for 2 min and free chlorine was measured using a ChemMets kit (ChemMetrics, Midland, Virginia). The bleach volume added to the culture was adjusted until the free residual chlorine reached 0·3–0·5 parts per million (ppm). The culture was allowed to incubate for 5 min at room temperature and 10% sodium thiosulfate was added to neutralise the free chlorine. Free chlorine was measured again to ensure that it had been neutralised. The survivability of the *E. coli* strains was immediately determined based on culture methods (see in the following paragraph). Triplicate samples were used for this experiment.

Bacterial cell counts were performed at three time points: after the initial 24 h culture in TSB; after 24 h incubation of the 1:10 dilution in distilled water under room temperature; and immediately after chlorine treatment. A tenfold serial dilution was made for each culture, and 100 µl of each dilution was plated on LB agar plates and incubated at 35°C overnight and colony counting was performed 24 h later.

flhDC gene expression studies

For measurement of *flhDC* expression, *E. coli* cultures were collected for ribonucleic acid (RNA) extraction at the same three time points as described earlier for the serial stress experiment. RNA extraction was performed using RNAprotect Bacteria Reagent (Qiagen, Inc., Valencia, California) and RNeasy Mini Kit (Qiagen, Inc.). Specifically, one volume of bacterial culture was mixed with two volumes of the RNA protection reagent. The mixture was incubated for 5 min at room temperature and centrifuged at 6300***g*** for 10 min to collect the pellet. The pellet was then used for RNA extraction by using the RNeasy Mini Kit according to the manufacturer's instructions. RNA was treated with Optizyme deoxyribonuclease I (Fisher Scientific, Waltham, Massachusetts) for DNA removal.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed to measure the expression of the *flhDC* gene by using the TaqMan RNA-to-Ct 1-Step Kit (Thermo Fisher Scientific, Waltham, Massachusetts). All reactions were carried out in a 20 µl volume which contained 200 ng of RNA template, 10 µl of TaqMan RT-PCR Master Mix, 0·5 µl of TaqMan RT Enzyme Mix, 0·9 µM of each primer, 0·25 µM of TaqMan probe and molecular biology-degree water. The primers used are listed in Table 1.

The RT-qPCR conditions were as follows: a holding stage at 48°C for 15 min for reverse transcription, another holding stage at 95°C for 10 min for the activation of enzymes and 40 cycles of

Table 1. PCR primers used in this study

Target	Primers and probes	Primer and probe sequence (5′–3′)	Reference
rpoA	rpoA-F	GGCTTGACGATTTCGACATC	Sharma and Bearson (2013)
	rpoA-R	GGTGAGAGTTCAGGGCAAAG	
	rpoA-P	Fam–TGAAGTTATTCTTACCTTGAATAAATCTGGCATTG–Tamra	
flhDC	flhDC-F	ACAACATTAGCGGCACTGAC	Sharma and Bearson (2013)
	flhDC-R	AGAGTAATCGTCTGGTGGCTG	
	flhDC-P	Fam–AAACGGAAGTGACAAACCAGCTGATTG–Tamra	

Fam, 6-carboxyfluorescein; Tamra, tetramethylrhodamine

denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. The expression of *flhDC* in two *uspC–IS30–flhDC*-positive waste water strains (WW10 and WW63) was compared to the expression of the *flhDC* in wild-type human faecal strain H51. The expression data of *flhDC* were normalised to endogenous levels of the housekeeping gene *rpoA*.

Bacterial biofilm formation assay

Eighteen *E. coli* strains were used in this portion of the study. Ten were naturalised waste water *E. coli* strains (*uspC–IS30–flhDC* positive) originating from four different WWTPs in Alberta, Canada. Eight of them were human *E. coli* strains (*uspC–IS30–flhDC* negative) isolated from different patient faecal swabs submitted to the Edmonton site of the Alberta Provincial Laboratory for Public Health (ProvLab) for routine microbiological testing. Human faecal sampling adhered to all ethics requirements (file number Pro00005478_CLS3 at the University of Alberta). All strains were confirmed as *E. coli* by biochemical analysis using a Vitek bacterial identification system (BioMerieux Canada Inc., St Laurent, Canada) according to the manufacturer's instructions and protocols at ProvLab.

Biofilm formation was evaluated as previously described by O'Toole (2011) with some modifications. Briefly, strains of *E. coli* were grown in TSB overnight at 35°C. The optical densities of the cultures were adjusted to $O_{D600\,nm}$ = 1·0, and 100 µl of the cultures was added into microtitre plates (CoStar 3595, New York, USA) to test for their ability to form biofilms. The lid of the microtitre plate was replaced with a sterile 96-well PCR plate (Greiner Bio-One, Frickenhausen, Germany) by inserting the PCR plate wells into the wells of the culture microtitre plate to help biofilm binding. The plate was incubated at 35°C for 24 h. The culture media was then discarded. For each well, 125 µl of 0·1% crystal violet was added to stain the biofilm and the whole plate was washed with distilled water four times to remove excess cells and dye. Biofilm formation was quantified by adding 125 µl of 30% acetic acid to dissolve the crystal violet adhering to biofilms on the PCR plate and the absorbance at 550 nm was measured using a microplate fluorometer (Fluostar Omega, Thermo Fisher, Whitby, Ontario, Canada).

Statistics

Statistical analysis of the data for the *flhDC* expression test and the biofilm formation test was performed using R software version 3.0.0. For the biofilm formation test, an unpaired Student's *t*-test was performed. For the *flhDC* gene expression test, a paired Student's *t*-test was performed. A significance value of $p < 0{\cdot}1$ was deemed relevant.

Results

E. coli survivability under stressed conditions

Four *E. coli* strains were analysed for survivability against serial stress conditions and included the following: (*a*) H51 – a human wild-type faecal strain lacking both a generalised stress response (RpoS) and the *uspC–IS30–flhDC* biomarker; (*b*) H54 – a human faecal strain possessing a strong RpoS generalised stress response but lacking the *uspC–IS30–flhDC* biomarker; and (*c*) WW10 and WW63 – naturalised waste water strains possessing strong RpoS activity and the *uspC–IS30–flhDC* biomarker. The strains were grown in TSB for 24 h, diluted to 1:10 in distilled water for 24 h at room temperature (mimicking nutrient deprivation and osmotic stressors) and subsequently treated with chlorine bleach. All four *E. coli* strains displayed similar replicative potential in TSB, reaching levels of ~10^9 colony-forming units/ml after 24 h (Table 2). When TSB cultures were diluted to 1:10 in distilled water and incubated for 24 h, no decline in survivability was observed among the four *E. coli* strains. However, survivability among the strains was significantly affected by treatment with chlorine. A 4·1 $\log_{10}$ mean reduction in bacterial cell counts was observed for the H51 negative control strain, whereas for the human H54 strain and the naturalised waste water *E. coli* strains (WW10 and WW63), bacterial concentrations were reduced only by ~2 $\log_{10}$, suggesting differential survival among *E. coli* strains in response to chlorine treatment (Table 2).

Motility and biofilm formation

To understand phenotypic alterations associated with differential resistance to chlorine better, phenotypic characteristics such as biofilm formation and motility (as determined by *flhDC* gene expression assays) were examined as survival phenotypes for *E. coli* in the non-host environment. Biofilm formation was assessed among eight human faecal strains (not possessing the *uspC-uspC–IS30–flhDC* marker) and ten naturalised waste water strains possessing the *uspC–IS30–flhDC* marker. The biofilm-forming capacity of the naturalised waste water strains was significantly greater (p value = 0·04) than that of the human faecal strains, although a large variation was observed within each of the groups (Figure 1). Biofilm formation, as measured by crystal violet absorption to the polysaccharide matrix, was 3·2 times greater in

Table 2. Survival of human and naturalised waste water *E. coli* strains after nutrient deprivation/osmotic stress and chlorine treatment

E. coli strains	*E. coli* source	Control	Treatment			
			Nutrient deprivation/osmotic stress[a]		Chlorine treatment[b]	
		E. coli numbers after 24 h culture in TSB:[c] ml^{-1}	*E. coli* numbers after osmotic stress:[c] ml^{-1}	log_{10} reduction after osmotic stress[c]	*E. coli* numbers after chlorine treatment:[c] ml^{-1}	log_{10} reduction after chlorine treatment[c]
H51	Human (RpoS negative/*uspC–IS30–flhDC* negative, control strain)	$2{\cdot}0 \pm 0{\cdot}28 \times 10^9$	$3{\cdot}2 \pm 0{\cdot}62 \times 10^9$	$-0{\cdot}20 \pm 0{\cdot}15$[d]	$3{\cdot}3 \pm 0{\cdot}8 \times 10^5$	$4{\cdot}1 \pm 0{\cdot}3$[e]
H54	Human (RpoS positive *uspC–IS30–flhDC* negative, control strain)	$1{\cdot}1 \pm 0{\cdot}19 \times 10^9$	$3{\cdot}0 \pm 0{\cdot}27 \times 10^9$	$-0{\cdot}46 \pm 0{\cdot}05$[d]	$2{\cdot}2 \pm 0{\cdot}12 \times 10^7$	$2{\cdot}1 \pm 0{\cdot}10$[e,f]
WW10	Waste water	$1{\cdot}0 \pm 0{\cdot}09 \times 10^9$	$2{\cdot}1 \pm 0{\cdot}15 \times 10^9$	$-0{\cdot}32 \pm 0{\cdot}06$[d]	$1{\cdot}7 \pm 0{\cdot}15 \times 10^7$	$2{\cdot}1 \pm 0{\cdot}03$[e,f]
WW63	Waste water	$9{\cdot}3 \pm 1{\cdot}5 \times 10^8$	$8{\cdot}8 \pm 1{\cdot}0 \times 10^8$	$-0{\cdot}01 \pm 0{\cdot}10$[d]	$5{\cdot}6 \pm 0{\cdot}91 \times 10^6$	$2{\cdot}2 \pm 0{\cdot}13$[e,f]

[a] Nutrient deprivation/osmotic shock performed by diluting TSB cultures to 1:10 in distilled water and incubating for 24 h at room temperature
[b] Cells treated with 0·3–0·5 ppm residual-free chlorine for 5 min
[c] *E. coli* concentrations and log_{10} reductions are presented as mean ± standard error ($n = 3$)
[d] No significant reduction (p value > 0·05) in *E. coli* log_{10} survival when comparing bacterial levels between TSB control cultures and nutrient deprivation/osmotic stress treatment
[e] Significant reduction (p value < 0·05) in *E. coli* log_{10} survival when comparing bacterial levels between nutrient deprivation/osmotic stress and chlorine treatment
[f] Significant difference in log_{10} survival outcomes between the strain indicated and the negative control human strain (H51 strain lacking the generalised RpoS stress response)

waste water strains than human strains (i.e. mean absorbance values of waste water strains of 0·295 ± 0·27, compared to 0·092 ± 0·09 of human faecal strains). However, not all waste water strains had a high biofilm-forming capacity. For example, the waste water strain WW53, although possessing the *uspC–IS30–flhDC* marker, had a lower biofilm-forming capacity than those of most of the human strains.

To determine if bacterial motility, and consequently biofilm formation, could be indirectly altered by the *IS30* element in naturalised waste water *E. coli* strains (WW10 and WW63), the expressions of the flagellar regulator gene, *flhDC*, were compared between the human faecal strain H51 and the two *uspC–IS30–flhDC*-positive waste water strains (WW10 and WW63) under the serial stress treatments described earlier. Relative expression levels between the housekeeping gene *rpoA* and *flhDC* were used as a measure of gene transcription, since relative expression controlled for cell death associated with decreased survivability of H51 to chlorine. The results demonstrate that there was no significant difference in *flhDC* expression between wild-type strain H51 and *uspC–IS30–flhDC*-positive strains WW10 or WW63 after 24 h of TSB culture followed by nutrient deprivation/osmotic stress (Figure 2, p value > 0·1). However, a significant difference (p value < 0·1) in *flhDC* expression between the wild-type human strain H51 and the WW10 (p value = 0·06) and WW63 strains (p value = 0·08) was observed after chlorine treatment. The relative expressions of *flhDC* of WW10 and WW64 increased 3·6- and 2·6-fold compared to the H51 human strain, respectively, after chlorine exposure.

Discussion

Several studies have demonstrated that although influent sewage is largely composed of human and animal faecal wastes, the waste water matrix itself possesses a very unique microbiome (Wang *et al*., 2012). Thus, although faecal bacteria, such as *E. coli*, may comprise a major component of the microbial population entering the WWTP, certain strains of *E. coli* appear to have evolved to become naturalised populations that survive and replicate in this non-host environment (Zhi *et al*., 2016b). The fact that the *uspC–IS30–flhDC* marker is highly specific to waste water (Zhi *et al*., 2016b) and is geographically distributed throughout WWTPs in Alberta, Canada, suggests that this genetic polymorphism may play a functional role in the survival of these naturalised strains in the waste water environment.

To understand better the potential role of this marker in the adaptive selection and evolution of naturalised *E. coli* populations in waste water, the survival of *uspC–IS30–flhDC*-positive and *uspC–IS30–flhDC*-negative *E. coli* isolates was examined after serial stress conditions. In addition, other phenotypic outcomes related to stress, including bacterial motility and biofilm formation, were also examined, both of which have been shown to be important for *E. coli* survival in a non-host environment. Barker *et al*. (2004) observed that the placement of an insertion sequence known as *IS5* upstream of *flhDC* in *E. coli* activated transcription of *flhDC* by potentially releasing transcriptional repression, leading to increased flagellar synthesis and bacterial motility, an effect known to promote biofilm formation in bacteria (O'Toole and Kolter, 1998). Sharma and Bearson (2013) demonstrated that differential regulation of *flhDC* influenced biofilm formation, and Wood *et al*. (2006) demonstrated that bacterial motility was positively related to biofilm-forming capacity. Bacterial flagellar expression is highly complex, involving more than 40 genes (McCarter, 2006), but it was hypothesised that naturalised waste water strains may increase

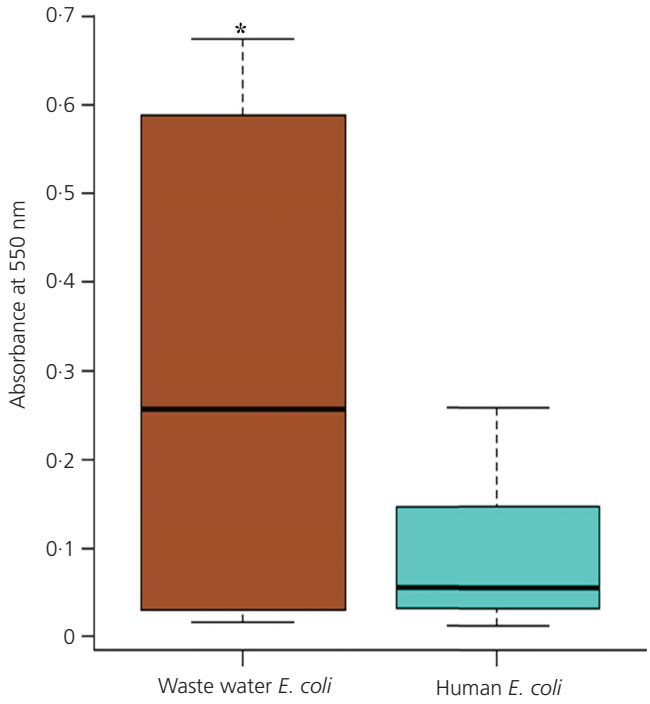

Figure 1. Comparison of biofilm-formation capacity in ten *uspC–IS30–flhDC*-positive waste water strains and eight *uspC–IS30–flhDC*-negative human faecal *E. coli* strains. Absorbance at 550 nm was used as a measure of biofilm formation capacity in biofilm formation assay. The result demonstrated that the biofilm-forming capacity of the naturalised waste water strains (*uspC–IS30–flhDC* positive) was significantly greater (asterisk represents p value < 0·1) than that of the human faecal strains (*uspC–IS30–flhDC* negative). For the box and whisker plots, the median values are represented by a thick line, the box represents the 25th and 75th percentiles and the whiskers are the upper and lower values

levels of *flhDC* expression in response to environmental stressors (i.e. chlorine), resulting in increased production of biofilms. It has been previously shown that biofilm-producing *E. coli* strains are more resistant to chlorine (De Beer *et al*., 1994; Ryu and Beuchat, 2005) and that biofilm formation is important for protecting bacteria in harsh non-host environments (Stewart *et al*., 2013). Ryu and Beuchat (2005) demonstrated that the viability of an *E. coli* O157 strain was completely unaffected by chlorine doses as high as 10 mg/l chlorine for 10 min at 12°C, compared to control strains in which an 8 $\log_{10}$ inactivation was observed. Remarkably, when this same strain was allowed to form biofilms on a solid support (i.e. stainless steel coupon), no decline in viability was observed with a chlorine dose of 50 mg/l for 5 min at 22°C, compared to the control strain, where a 6 $\log_{10}$ inactivation was observed under the same treatment conditions.

To examine these potential survival mechanisms, one human strain (*uspC–IS30–flhDC* negative) and two waste water strains (*uspC–IS30–flhDC* positive) were subjected to nutrient deprivation/osmotic shock and chlorine treatment and the expression of *flhDC* was examined. The waste water strains had significantly greater relative expression of *flhDC* compared to the human strain after chlorine treatment, suggesting that chlorine treatment is a potent inducer of *flhDC* expression in naturalised strains. In addition, biofilm production across ten naturalised waste water strains (all possessing the *uspC–IS30–flhDC* marker) was greater than that observed for eight human faecal strains lacking the *uspC–IS30–flhDC* marker, suggesting that both motility and biofilm formation may be important strategies for survival of these strains in a waste water matrix.

To determine whether these specific alternations were associated with increased survival, culture-based methods were used as means of quantifying the recovery of stressed bacteria. Four *E. coli* strains were assayed for this portion of the study. One human strain, H51 (also used in the *flhDC* expression studies), lacked an RpoS stress response, whereas another human strain, H54, possessed a vigorous RpoS stress response – a cellular response shown to be important for environment survival in *E. coli* (White *et al*., 2011). Two waste water strains, each possessing the *uspC–IS30–flhDC* marker and having a strong RpoS response, were also selected for evaluation. Both waste water strains as well as the human H54 wild-type strain were shown to be highly resistant to chlorine, displaying 100 times more resistance to chlorine compared to the human faecal strain H51. Interestingly, there was no difference in survivability between the human wild-type H54 strain and the naturalised waste water strains against chlorine treatment and, consequently, there does not appear to be a direct association between chlorine tolerance and the presence or absence of the *uspC–IS30–flhDC* marker, even though biofilm production and *flhDC* expression were enhanced in these strains. Nevertheless, biofilm formation and motility are not the only strategies utilised by bacteria to survive chlorine stress. Several inducible genes/regulators are also produced in response to exposure to reactive chlorine species in *E. coli* (Gray *et al*., 2013; Parker *et al*., 2013), the mechanisms of which can lead to increased intracellular glutathione levels (Chesney *et al*., 1996; Saby *et al*., 1999) as well as other antioxidants such as catalases (Dukan and Touati, 1996), methionine sulfoxide reductase (Rosen *et al*., 2009) and even oxidative repair mechanisms (Gray *et al*., 2013). All waste water-naturalised strains of *E. coli* have been previously shown to display a strong RpoS response (Zhi *et al*., 2016b), but regulation of chlorine resistance mechanisms in these *E. coli* strains is unknown.

In addition to possessing alternative chlorine stress-adaptive mechanisms, *E. coli* can also enter into a viable but non-culturable state (VBNC) – a common survival strategy used by *E. coli* to deal with adverse environmental conditions (Trevors, 2011) such as chlorine treatment (Oliver *et al*., 2005). During transition into a VBNC state, bacteria condense cellular material, often becoming much smaller, yet their cellular structures remain intact, and they become more resistant to inactivation or treatment (E *et al*., 2015; Zhang *et al*., 2015). PCR assays incorporating propidium monoazide (PMA) have been used to evaluate VBNC

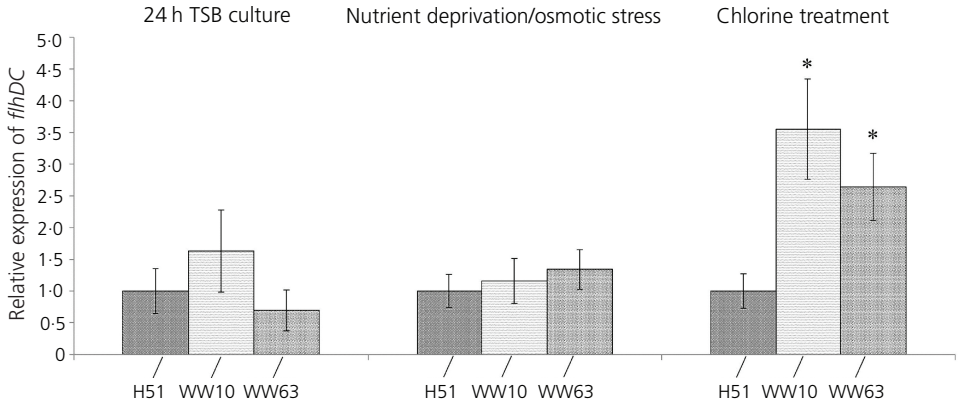

Figure 2. Expression of *flhDC* in response to nutrient deprivation/osmotic shock and chlorine treatment. Relative expression of *flhDC* was normalised to the expression of the reference gene, *rpoA*. The expressions of *flhDC* were compared between *uspC–IS30–flhDC*-negative *E. coli* strain H51 and the two *uspC–IS30–flhDC*-positive strains WW10 and WW63 after 24 h TSB culture, nutrient deprivation/osmotic stress and chlorine treatment. The results demonstrate that there was no significant difference in *flhDC* expression between wild-type strain H51 and *uspC–IS30–flhDC*-positive strains WW10 or WW63 after 24 h TSB culture or after nutrient deprivation/osmotic stress (p value > 0·1). However, a significant difference (p value < 0·1 (asterisk)) in *flhDC* expression between the wild-type human strain H51 and the WW10 (p value = 0·06) or WW63 (p value = 0·08) after chlorine treatment was observed. The relative expressions of *flhDC* of WW10 and WW64 increased 3·6- and 2·6-fold compared to that of the H51 human strain, respectively

survival under stress conditions (Nocker *et al*., 2006; van Frankenhuyzen *et al*., 2011; Xiao *et al*., 2013). PMA binds to DNA, preventing PCR amplification of target sequences, but can penetrate only cells in which the cellular membrane of the bacteria has been compromised (Nocker *et al*., 2006). It is conceivable that although similar survival outcomes were observed between the human H54 strain and the naturalised waste water *E. coli* strains based on cell culture, that significant differences in overall survival may have existed if VBNC was also are accounted for in the authors' studies, similar to that observed by Oliver *et al*. (2005).

The fact that naturalised waste water strains of *E. coli* displayed chlorine tolerance in the authors' serial stress experiments corroborates their previous finding that a substantially greater proportion of the culturable *E. coli* population found in chlorine-treated waste water were shown to carry the *uspC–IS30–flhDC* marker (59%) compared to the *E. coli* population in untreated waste water (5%) (Zhi *et al*., 2016b). This finding also corroborates the authors' previous findings of differential survival of naturalised waste water *E. coli* strains across the waste water treatment process (Zhi *et al*., 2016b), providing further circumstantial evidence of the potential important role that *uspC–IS30–flhDC* may play in survival in a waste water matrix. However, a definitive understanding of the role of the *uspC–IS30–flhDC* locus as an adaptive genotype for survival of naturalised *E. coli* populations in waste water requires the generation of isogenic mutants in which this locus is altered or eliminated using site-directed mutagenesis.

The observation that chlorine treatment selects for the survival of naturalised strains of waste water *E. coli* has some important implications for the water industry. Firstly, the US Environmental Protection Agency's microbiological alternate test procedure (US EPA, 2010) – a protocol used for evaluating the suitability of culture media on the recovery of chlorine-stressed faecal microbes such as *E. coli* – actually appears to select for survival and growth of naturalised *E. coli* populations in these samples. This suggests that the protocol may bias experimental outcomes towards the culture of naturalised waste water strains of *E. coli* as opposed to faecal strains of *E. coli*. Whether this has any impact on interpretation regarding the suitability of approved culture media for public health testing of faecal contamination of water remains to be determined. Also, since these approved culture media are used to evaluate waste water treatment performance, growth bias towards treatment-resistant naturalised strains may underestimate true inactivation efficiency against faecal strains, therefore acting as a conservative estimate of inactivation of bacteria and other pathogens derived from human faeces. As a corollary, the presence of naturalised treatment-resistant *E. coli* in waste water raises some concerns regarding the use of *E. coli* as a suitable *faecal* indicator of water quality. Secondly, the wide variation in the survivability of *E. coli* strains to chlorine raises some direct concerns for public health. It was observed as early as 1949 that certain populations of bacteria, including *E. coli*, have developed resistance to chlorine treatment (Allen and Brooks, 1949; Farkas-Himsley, 1964). Since that time, various mechanisms of chlorine resistance have been characterised in *E. coli* (Gray *et al*., 2013), and it is plausible that genetic determinants of chlorine tolerance from naturalised strains may act as reservoirs for horizontal transfer to pathogenic strains, or vice versa (i.e. virulence genes may transfer from pathogenic strains to naturalised waste water strains). Chlorine-tolerant *E. coli* generated from WWTPs located upstream of drinking water treatment plants may cause problems

if treatment at the drinking water plant is compromised and virulence maintained in chlorine-tolerant strains. Interestingly, it has been demonstrated that chlorine treatment can promote antibiotic resistance (Murray *et al*., 1984). Khan *et al*. (2016) observed that chlorine-tolerant strains of bacteria isolated from chlorinated drinking water systems were more resistant to tetracycline, sulfamethoxozole and amoxicillin, and Jia *et al*. (2015) observed that chlorine residual was an important parameter is driving antibiotic resistance in bacterial populations found in drinking water distribution systems, including *E. coli*. Given that antibiotic-resistant *E. coli* are relatively abundant in treated waste water (Iwane *et al*., 2001), as are *uspC–IS30–flhDC*-positive *E. coli* strains, it would be interesting to evaluate whether *uspC–IS30–flhDC*-positive *E. coli* are antibiotic resistant.

All naturalised waste water strains possess the *IS30* element in the *uspC–flhDC* intergenic locus, which the authors hypothesise to be a potentially important regulator of the *uspC* gene – a genetic element known to confer UV resistance (UVB) in *E. coli* (Gustavsson *et al*., 2002). Although nothing is known about how genetic variation at this locus (or its regulatory elements) may specifically alter sensitivity of *E. coli* to UV treatment, a diverse array of heritable UV resistance mechanisms have been described in *E. coli*. An understanding of how natural selection may lead to the evolution of UV resistance is important for the water industry. Some of the earliest studies examining the evolution of UV resistance were carried out by Witkin (1946), who exposed a single clone of *E. coli* to one round of low-pressure UVC radiation (only 0·1 mJ/cm^2) and observed that the survivor population had approximately 2·5 $\log_{10}$ greater resistance to UV than the parent strain. Similarly, Harris *et al*. (2009) examined the evolution of a single laboratory strain of *E. coli* exposed to over 20 iterative cycles of increasing ionising radiation and found that survivor strains were 2 $\log_{10}$ orders of magnitude more resistant to low-pressure UV inactivation than the parent strain was. They found that doses as high as 15 mJ/cm^2 induced a 4 $\log_{10}$ kill in radiation-resistant survivor strains compared to a 6 $\log_{10}$ inactivation of the parent strain. A fascinating study carried out by Alcantara-Diaz *et al*. (2004) demonstrated an even stronger evolutionary effect. In their study, 80 iterative cycles of increasing doses of low-pressure UVC exposure resulted in the evolution of an *E. coli* strain that was 4·5 $\log_{10}$ orders of magnitude more resistant to UVC than the parent strain is (1 $\log_{10}$ inactivation of the evolved strain compared to 5·5 $\log_{10}$ inactivation of the parent strain at 15 mJ/cm^2). A particularly interesting observation in this study was that the UV-resistant strain developed a mutator phenotype – a phenotype in which the organism dramatically increased its spontaneous mutation rate, leading to a hypermutable strain. Alcantara-Diaz *et al*. (2004) also found that multiple UV-resistant strains evolved (all of which had >3·5 $\log_{10}$ greater resistance to UVC than the parent strain at a 15 mJ/cm^2 dose), each with different genetic mutation events leading to increased resistance. Remarkably, this (2–4·5)$\log_{10}$ difference in evolution of UV resistance described in the studies mentioned earlier reflects the adaptive and vertically transmitted (parent to progeny) mutational properties of a *single* strain and does not encompass horizontal gene transfer (transformation, transduction, conjugation, cross-species genetic exchange) that may also regulate UV resistance in a population of *E. coli* undergoing natural selection (i.e. such as the mixed bacterial populations in a WWTP). These studies emphasise an important characteristic of microbial systems – that they are biologically robust and capable of rapidly evolving complex solutions to stressful situations.

In addition to the study of Alcantara-Diaz *et al*. (2004) and in terms of the development of *E. coli* resistance to UV doses relevant to water treatment standards, Chen *et al*. (2015) observed that a genetically modified UVC-resistant *E. coli* strain possessing the *radD*/*radA* genes was relatively unaffected by exposure to 10 mJ/cm^2 (i.e. highest dose tested). Gabriel (2015) observed that stress induction (acid treatment followed by desiccation stress) prior to UVC treatment increased the dose required to inactivate 1 $\log_{10}$ of the bacteria from 8·4 to 18·4 mJ/cm^2 for a food-borne *E. coli* O157 strain. Huang *et al*. (2016) observed that among a small sample of *E. coli* isolates (n = 5) collected from secondary treated waste water, considerable and variable resistance to low-pressure UV was observed, with doses as high as 40 mJ/cm^2, leading to *E. coli* inactivation levels between 2·9 $\log_{10}$ and 5·7 $\log_{10}$ among these isolates. Similarly, Anastasi *et al*. (2013) observed the selection of *E. coli* biotypes in response to UV irradiation in WWTPs in Australia, suggesting that some strains are better at surviving UV treatment than others are in a WWTP. Since naturalised waste water strains of *E. coli* are commonly found in UV-treated effluents (Zhi *et al*., 2016b), the authors hypothesise that these strains may also possess UV resistance characteristics important to the water industry.

Conclusion

E. coli is a highly adaptable microbe and a growing body of evidence suggests that this microbe is capable of colonising a variety of animal hosts and non-animal environmental niches, including waste water. The ability to colonise and survive in waste water suggests that natural selection has forced the evolution of certain strains to develop resistance mechanisms against the very water treatment processes typically used to inactivate these organisms. This paper, as well as others, raises concern about the evolution and emergence of treatment-resistant microbes in the water industry and the future impact that these agents may play in water quality, particularly in waste water reclamation, recreational water quality impacted by waste water discharge and potable/non-potable reuse of waste water systems.

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REFERENCES

Alcantara-Diaz D, Bfrena-Valle M and Serment-Guerrero J (2004) Divergent adaptation of *Escherichia coli* to cyclic ultraviolet light exposures. *Mutagenesis* **19(5)**: 349–354.

Allen LA and Brooks E (1949) Destruction of bacteria in sewage and other liquids by chlorine and by cyanogen chloride. *Journal of Hygiene (London)* **47(3)**: 320–336.

Anastasi EM, Wohlsen TD, Stratton HM and Katouli M (2013) Survival of *Escherichia coli* in two sewage treatment plants using UV irradiation and chlorination for disinfection. *Water Research* **47(17)**: 6670–6679.

Anderson KL, Whitlock JE and Harwood VJ (2005) Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. *Applied and Environmental Microbiology* **71(6)**: 3041–3048.

Anderson JK, Smith TG and Hoover TR (2010) Sense and sensibility: flagellum-mediated gene regulation. *Trends in Microbiology* **18(1)**: 30–37.

Barker CS, Prüss BM and Matsumura P (2004) Increased motility of *Escherichia coli* by insertion sequence element integration into the regulatory region of the *flhD* operon. *Journal of Bacteriology* **186(22)**: 7529–7537.

Battesti A, Majdalani N and Gottesman S (2011) The RpoS-mediated general stress response in *Escherichia coli*. *Annual Review of Microbiology* **65**: 189–213.

Bianco C, Imperlini E, Calogero R *et al.* (2006) Indole-3-acetic acid improves *Escherichia coli*'s defences to stress. *Archives of Microbiology* **185(5)**: 373–382.

Byappanahalli MN, Yan T, Hamilton MJ *et al.* (2012) The population structure of *Escherichia coli* isolated from subtropical and temperate soils. *Science of the Total Environment* **417–418**: 273–279.

Chandrasekaran R, Hamilton MJ, Wang P *et al.* (2015) Geographic isolation of *Escherichia coli* genotypes in sediments and water of the Seven Mile Creek – a constructed riverine watershed. *Science of the Total Environment* **538**: 78–85.

Chen SH, Byrne RT, Wood EA and Cox MM (2015) *Escherichia coli radD (yejH)* gene: a novel function involved in radiation resistance and double-strand break repair. *Molecular Microbiology* **95(5)**: 754–768.

Chesney JA, Eaton JW and Mahoney JR (1996) Bacterial glutathione: a sacrificial defense against chlorine compounds. *Journal of Bacteriology* **178(7)**: 2131–2135.

De Beer D, Srinivasan R and Stewart PS (1994) Direct measurement of chlorine penetration into biofilms during disinfection. *Applied and Environmental Microbiology* **60(12)**: 4339–4344.

Dukan S and Touati D (1996) Hypochlorous acid stress in *Escherichia coli*: resistance, DNA damage, and comparison with hydrogen peroxide stress. *Journal of Bacteriology* **178(21)**: 6145–6150.

E J, Jiang YT, Yan PF and Liang JP (2015) Biological changes of *Enterococcus faecalis* in the viable but nonculturable state. *Genetics and Molecular Research* **14(4)**: 14790–14801, http://dx.doi.org/10.4238/2015.November.18.44.

Elasri MO and Miller RV (1999) Study of the response of a biofilm bacterial community to UV radiation. *Applied and Environmental Microbiology* **65(5)**: 2025–2031.

Farkas-Himsley H (1964) Killing of chlorine-resistant bacteria by chlorine–bromine solutions. *Applied Microbiology* **12**: 1–6.

Gabriel AA (2015) Previous physiochemical stress exposures influence subsequent resistance of *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* to ultraviolet-C in coconut liquid endosperm beverage. *International Journal of Food Microbiology* **201**: 7–16.

Gray MJ, Wholey WY and Jakob U (2013) Bacterial responses to reactive chlorine species. *Annual Review of Microbiology* **67(1)**: 141–160.

Gustavsson N, Diez A and Nystrom T (2002) The universal stress protein paralogues of *Escherichia coli* are co-ordinately regulated and co-operate in the defence against DNA damage. *Molecular Microbiology* **43(1)**: 107–117.

Harris DR, Pollock SV, Wood EA *et al.* (2009) Directed evolution of ionizing radiation resistance in *Escherichia coli*. *Journal of Bacteriology* **191(16)**: 5240–5252.

Huang JJ, Xi J, Hu HY *et al.* (2016) UV light tolerance and reactivation potential of tetracycline-resistant bacteria from secondary effluents of a wastewater treatment plant. *Journal of Environmental Sciences* **41**: 146–153.

Iwane T, Urase T and Yamamoto K (2001) Possible impact of treated wastewater discharge on incidence of antibiotic resistant bacteria in river water. *Water Science and Technology* **43(2)**: 91–99.

Jia S, Shi P, Hu Q *et al.* (2015) Bacterial community shift drives antibiotic resistance promotion during drinking water chlorination. *Environmental Science & Technology* **49(20)**: 12271–12279.

Khan S, Beattie TK and Knapp CW (2016) Relationship between antibiotic- and disinfectant-resistance profiles in bacteria harvested from tap water. *Chemosphere* **152**: 132–141.

Kon T, Weir SC, Trevors JT *et al.* (2007) Microarray analysis of *Escherichia coli* strains from interstitial beach waters of Lake Huron (Canada). *Applied and Environmental Microbiology* **73(23)**: 7757–7758.

Kvint K, Nachin L, Diaz A and Nystrom T (2003) The bacterial universal stress protein: function and regulation. *Current Opinion in Microbiology* **6(2)**: 140–145.

McCarter LL (2006) Regulation of flagella. *Current Opinion in Microbiology* **9(2)**: 180–186.

Mercer RG, Zheng J, Garcia-Hernandez R *et al.* (2015) Genetic determinants of heat resistance in *Escherichia coli*. *Frontiers in Microbiology* **6**: 932.

Murray GE, Tobin RS, Junkins B and Kushner DJ (1984) Effect of chlorination on antibiotic resistance profiles of sewage-related bacteria. *Applied and Environmental Microbiology* **48(1)**: 73–77.

Nachin L, Nannmark U and Nystrom T (2005) Differential roles of the universal stress proteins of *Escherichia coli* in oxidative stress resistance, adhesion, and motility. *Journal of Bacteriology* **187(18)**: 6265–6272.

Nocker A, Cheung CY and Camper AK (2006) Comparison of propidum monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *Journal of Microbiological Methods* **67(2)**: 310–320.

Oliver JD, Dagher M and Linden K (2005) Induction of *Escherichia coli* and *Salmonella typhimurium* into the viable but nonculturable state following chlorination of wastewater. *Journal of Water Health* **3(3)**: 249–304.

O'Toole GA (2011) Microtiter dish biofilm formation assay. *Journal of Visualized Experiments* **47**: 2437.

O'Toole GA and Kolter R (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molecular Microbiology* **30(2)**: 295–304.

Parker BW, Schwessinger EA, Jakob U and Gray MJ (2013) The RclR protein is a reactive chlorine-specific transcription factor in *Escherichia coli*. *Journal of Biological Chemistry* **288(45)**: 32574–32584.

Power ML, Littlefield-Wyer J, Gordon DM, Veal DA and Slade MB (2005) Phenotypic and genotypic characterization of encapsulated *Escherichia coli* isolated from blooms in two Australian lakes. *Environmental Microbiology* **7(5)**: 631–640.

Rosen H, Klebanoff SJ, Wang Y *et al.* (2009) Methionine oxidation contributes to bacterial killing by the myeloperoxidase system of neutrophils. *Proceedings of the National Academy of Sciences of the United States of America* **106(44)**: 18686–18691.

Ryu JH and Beuchat LR (2005) Biofilm formation by *Escherichia coli* O157:H7 on stainless steel: effect of exopolysaccharide and curli production on its resistance to chlorine. *Applied and Environmental Microbiology* **71(1)**: 247–254.

Saby S, Leroy P and Block JC (1999) *Escherichia coli* resistance to chlorine and glutathione synthesis in response to oxygenation and starvation. *Applied and Environmental Microbiology* **65(12)**: 5600–5603.

Sharma VK and Bearson BL (2013) Hha controls *Escherichia coli* O157: H7 biofilm formation by differential regulation of global transcriptional regulators FlhDC and CsgD. *Applied and Environmental Microbiology* **79(7)**: 2384–2396.

Solo-Gabriele HM, Wolfert MA, Desmarais TR and Palmer CJ (2000) Sources of *Escherichia coli* in a coastal subtropical environment. *Applied and Environmental Microbiology* **66(1)**: 230–237.

Stewart EJ, Satorius AE, Younger JG and Solomon MJ (2013) Role of environmental and antibiotic stress on *Staphylococcus epidermidis* biofilm microstructure. *Langmuir* **29(23)**: 7017–7024.

Tenaillon O, Skurnik D, Picard B and Denamur E (2010) The population genetics of commensal *Escherichia coli*. *Nature Reviews Microbiology* **8(3)**: 207–217.

Trevors JT (2011) Viable but non-culturable (VBNC) bacteria: gene expression in planktonic and biofilm cells. *Journal of Microbiological Methods* **86(2)**: 266–273.

Tymensen LD, Pyrdok F, Coles D *et al.* (2015) Comparative accessory gene fingerprinting of surface water *Escherichia coli* reveals genetically diverse naturalized population. *Journal of Applied Microbiology* **119(1)**: 263–277.

US EPA (US Environmental Protection Agency) (2010) *EPA Microbiological Alternate Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, Wastewater and Sewage Sludge Monitoring Methods*. US EPA, Washington, DC, USA.

van Frankenhuyzen JK, Trevors JT, Lee H, Flemming CA and Habash MB (2011) Molecular pathogen detection in biosolids with a focus on quantitative PCR using propidium monazide for viable cell enumeration. *Journal of Microbiological Methods* **87(3)**: 263–272.

Vogeleer P, Tremblay YD, Mafu AA, Jacques M and Harel J (2014) Life on the outside: role of biofilms in environmental persistence of Shiga-toxin producing *Escherichia coli*. *Frontiers in Microbiology* **5**: 317.

Wang X, Hu M, Xia Y, Wen X and Ding K (2012) Pyrosequencing analysis of bacterial diversity in 14 wastewater treatment systems in China. *Applied and Environmental Microbiology* **78(19)**: 7042–7047.

White AP, Sibley KA, Sibley CD *et al.* (2011) Intergenic sequence comparison of *Escherichia coli* isolates reveals lifestyle adaptations but not host specificity. *Applied and Environmental Microbiology* **77(21)**: 7620–7632.

Winfield MD and Groisman EA (2003) Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. *Applied and Environmental Microbiology* **69(7)**: 3687–3694.

Witkin EM (1946) Inherited differences in sensitivity to radiation in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **32(3)**: 59–68.

Wood TK, Gonzalez Barrios AF, Herzberg M and Lee J (2006) Motility influences biofilm architecture in *Escherichia coli*. *Applied Microbiology and Biotechnology* **72(2)**: 361–367.

Xiao XL, Tain C, Yu YG and Wu H (2013) Detection of viable but nonculturable *Escherichia coli* O157:H7 using propidium monoazide treatments and qPCR. *Canadian Journal of Microbiology* **59(3)**: 157–163.

Zhang S, Ye C, Lin H, Lv L and Yu X (2015) UV disinfection induces a VBNC state in *Escherichia coli* and *Pseudomonas aeruginosa*. *Environmental Science & Technology* **49(3)**: 1721–1728.

Zhi S, Li Q, Yasui Y *et al.* (2015) Assessing host specificity of *Escherichia coli* using a supervised learning logic regression-based analysis of single nucleotide polymorphisms in intergenic regions. *Molecular Phylogenetics and Evolution* **92**: 72–81.

Zhi S, Li Q, Yasui Y, Banting G *et al.* (2016a) An evaluation of logic regression-based biomarker discovery across multiple intergenic regions for predicting host specificity in *Escherichia coli*. *Molecular Phylogenetics and Evolution* **103**: 133–142.

Zhi S, Banting G, Li Q *et al.* (2016b) Evidence of naturalized stress-tolerant strains of *Escherichia coli* in municipal wastewater treatment plants. *Applied and Environmental Microbiology* **82(18)**: 5505–5518.

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