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Research Letter – Pathogens & Pathogenicity

Characterisation of the *E. coli* and *Salmonella qseC* and *qseE* mutants reveals a metabolic rather than adrenergic receptor role

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One sentence summary: *Escherichia coli* and *Salmonella* are the most studied stress hormone-responsive bacteria inhabiting the human body, and the question we are asking is if these bacteria use the QseC and QseE proteins to listen in on their host's stress?
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Abstract

Catecholamine stress hormones (norepinephrine, epinephrine, and dopamine) are signals that have been shown to be used as environmental cues, which affect the growth and virulence of normal microbiota as well as pathogenic bacteria. It has been reported that *Escherichia coli* and *Salmonella* use the two-component system proteins QseC and QseE to recognise catecholamines and so act as bacterial adrenergic receptors. In this study, we mutated the *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium genes encoding QseC and QseE and found that this did not block stress hormone responsiveness in either species. Motility, biofilm formation, and analysis of virulence of the mutants using two infection models were similar to the wild-type strains. The main differences in phenotypes of the *qseC* and *qseE* mutants were responses to changes in temperature and growth in different media particularly with respect to salt, carbon, and nitrogen salt sources. In this physiological respect, it was also found that the phenotypes of the *qseC* and *qseE* mutants differed between *E. coli* and *Salmonella*. These findings collectively suggest that QseC and QseE are not essential for *E. coli* and *Salmonella* to respond to stress hormones and that the proteins may be playing a role in regulating metabolism.

Keywords: stress, catecholamines, *E. coli*, *Salmonella*, QseC, QseE

Introduction

in vivo growth of commensal and pathogenic bacteria and regulation of their gene expression are known to be altered by changes in the environmental conditions in their host, such as the nutrients present, oxygen tension, and iron availability (Ratledge and Dover 2000). However, it is now also realised that stress experienced by the host can have a profound impact on the behaviour of its endogenous microbiota (Borre et al. 2014, Sandrini et al. 2015). In part, it is believed that catecholamine hormones released under stress increase the risk of developing an infection because of their role in reducing immune function (Reiche et al. 2004, Glaser and Kiecolt-Glaser 2005). However, it is now clear that infectious bacteria are able to directly respond to stress hormones (Sandrini et al. 2015) and the field of Microbial Endocrinology proposes that through their long cohabitation with animals and plants, microbes have evolved systems to detect and respond to their host's hormones (Lyte 2004, Freestone et al. 2008, Freestone 2013). Microbial endocrinology studies have largely focused on the interaction of enteric bacteria with those hormones most associated with stress (Freestone 2013). This is because the gut is highly innervated by the enteric nervous system, which has close connections to the central nervous system. The catecholamine norepinephrine (NE) is released into the gut from sympathetic nerve fibers terminating in the gut wall, and about half of NE synthesised within the mammalian body is produced intestinally creating a catecholamine-rich environment (Furness 2006). Within eukaryotic systems, NE and epinephrine (Epi) bring about their physiological effects *via* specific binding to adrenergic receptors (Costa et al. 2000, Goldstein et al. 2003, Furness 2006). While it is clear prokaryotes are responsive to catecholamines, genome sequence analyses show they do not possess obvious homologues of the eukaryotic adrenergic receptors (Freestone 2013). However, it has been proposed that the bacterial two-component system (TCS) sensor kinases QseC and QseE can act as receptors for NE and Epi in enteric prokaryotes (Clarke et al. 2006; Reading et al. 2009), although other studies have suggested these TCS respond to a variety of stimuli (Karovolos et al. 2013) including those related to metabolism (Hadjifrangiskou et al. 2011, Weigel et al. 2015). Further, mutation studies have indicated that QseC and QseE are dispensable for NE-induced effects on bacterial pathogenesis (Pullinger et al. 2010a, Sharma and Casey 2014a,b). In *Yersinia enterocolitica*, QseC is not present yet this species still responds to NE (Freestone et al. 2007). QseC and QseE phenotypes have also been associated with iron as catecholamines bind Fe and contain a cat-

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echol moiety similar to that in bacterial siderophores (Freestone et al. 2000, Burton et al. 2002, 2003, Bearson et al. 2010, Karovolos et al. 2013). It is plausible that this may explain why NE augments the virulence of enteric pathogens in experimental infection studies, where iron is a key requirement for bacterial replication. There is, therefore, considerable disparity in the literature between phenotypes observed for QseC and QseE mutants. In an attempt to reconcile this we investigated the phenotypes of *E. coli* and *Salmonella* mutants lacking *qseC* and *qseE*.

Material and methods

Bacterial strains

Enterohaemorrhagic *E. coli* O157:H7 85–170 nal^R is a spontaneous nalidixic acid resistant *stx1*- and *stx2*-negative derivative of strain 84–289 (Vlisidou et al. 2004). *Escherichia coli* 85–170 nal^R mutants lacking *qseC* or *qseE* were generated by λRed recombinase-mediated integration of linear PCR products containing the pKD4-encoded kanamycin resistance cassette as described previously (Pullinger et al. 2010a). Following homologous recombination, the kanamycin resistance cassette was excised by expression of the flippase recombinase and the strains cured of the plasmid for its expression as described (Pullinger et al. 2010a). Mutants were verified to contain unmarked deletions of the expected size by PCR with primers flanking the target genes. *Salmonella enterica* serovar Typhimurium strain ST4/74 is the parent of the widely used SL1344 *his* auxotroph and its Δ*qseC* and Δ*qseE,* mutants were also constructed as described previously (Pullinger et al. 2010a). Wild-type *E. coli* and *Salmonella* and Δ*qseC* and Δ*qseE* mutants were routinely cultured in Luria–Bertani (LB) medium or serum-SAPI (Freestone et al. 1999).

Catecholamine growth and antagonism assays

Catecholamines L(-)-Norepinephrine-(+)-bitartrate salt (NE), dopamine hydrochloride (Dop), and Epinephrine hydrochloride (Epi) were purchased from Sigma, Poole, UK. To test growth responsiveness to the catecholamines, overnight bacterial cultures grown in LB were serially diluted to 50–100 CFU/ml in serum-SAPI medium (low initial numbers of bacteria are intended to mimic the likely infectious doses occurring *in vivo*). A serum-based medium was also used to approximate the growth-restrictive *in vivo* conditions within a mammalian host (Freestone et al. 1999, 2002). Bacterial growth induction by catecholamines is concentration-dependent (Freestone et al. 2008), and so we first compared catecholamine growth responsiveness over the 1–500 μM range (data not shown). The dose response profiles of the *E. coli* and *Salmonella* Δ*qseC* and Δ*qseE* mutants were similar to their wild-type parent, and for *E. coli* the level of catecholamine needed for a 2 log-fold increase in growth relative to controls was for NE and Dop 20 μM, and Epi 50 μM. For *Salmonella*, the NE level was 20 μM, for Dop 30 μM, and for Epi 50 μM. We, therefore, used 50 μM catecholamine-levels for the growth response assays. Phentolamine antagonism of NE-growth induction assays used a fixed concentration of 50 μM NE and concentrations of phentolamine up to 300 μM (Freestone et al. 2007). For all assays in serum-SAPI, cultures were incubated statically at 37°C in a 5% CO_2 humidified incubator for 18 h; final growth levels were determined by serial dilution in PBS and plating onto Luria agar. All catecholamine responsiveness assays were carried out in at least duplicate, and all experiments performed on at least three occasions.

^{55}Fe Transferrin iron and ^{3}H-norepinephrine uptake

To investigate if QseC or QseE were involved in the mechanism by which catecholamines remove Fe from serum transferrin, *Salmonella*, and *E. coli* parental cultures and their *qseC* and *qseE* mutants were inoculated at 10 000 CFU/ml into serum-SAPI supplemented with 1×10^5 cpm of ^{55}Fe-transferrin +/− 50 μM NE. The high inoculum was used to ensure nonsupplemented controls grew similarly to NE-induced cultures. Bacteria were incubated at 37°C in a 5% CO_2 humidified incubator for 18 h, then enumerated for growth, and harvested for radiolabel counting as described previously (Freestone et al. 2000). Bacterial ^{55}Fe incorporation was measured using Emulsifier safe scintillant (Canberra-Packard, UK), and counted in the tritium channel of a Minaxi Tri-Carb 400 scintillation counter (Canberra-Packard). For [^{3}H] norepinephrine uptake measurement cultures were grown overnight in Luria broth, and harvested by centrifugation at 4000 rpm for 15 min. Bacteria were washed once with 50 mM SAPI-Tris pH 7.5 and the bacterial cultures normalised to OD_{600} 1.0. The cultures were incubated in duplicate in 3 ml of 50 mM SAPI-Tris pH7.5 supplemented with ^{3}H-NE (1×10^5 cpm/ml) at 37°C and measured for ^{3}H-NE uptake after 90 min incubation (time courses of ^{3}H-NE uptake in all the strains in this study indicated this is the optimum incubation time for maximal ^{3}H-NE incorporation). After incubation bacteria were harvested by centrifugation at 13 500 rpm for 5 min and washed once in SAPI-Tris pH 7.5; and bacterial pellets resuspended in 100 μl of PBS, and 2 ml of OptiPhase Safe scintillation fluid added (Ultima Gold 6013326, PerkinElmer). [^{3}H]-NE internalisation was measured using scintillation counting as described for ^{55}Fe incorporation.

Motility and biofilm formation

Bacterial motility assays were performed using Dulbecco's Modified Eagles Medium (DMEM) with 0.3% agar using previous methodologies (O'Toole and Kolter 1998, Merighi et al. 2009). Exponential phase bacteria grown in DMEM were stab-inoculated into 0.3% agar DMEM plates containing 50 μM additions of NE, Epi, Dop, or water (control). Plates were incubated statically at 37°C for 18 h, and motility assessed qualitatively by measuring the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation. For analysis of biofilm formation, wild-type *Salmonella*, and *E. coli* and their Δ*qseC* and Δ*qseE* mutants were grown overnight in LB, and diluted into 1:1000 in DMEM or serum-SAPI +/− 50 μM catecholamine. Controls comprised culture media only. Cultures were incubated statically at 37°C for 18 h, then measured for growth, culture supernatants removed, and the plates washed twice with PBS to remove nonattached bacteria. After drying at 50°C for 20 min cultures were stained with 0.2% (w/v) crystal violet and attachment measured at 595 nm using an ELISA LT-4500 plate reader as previously described (Freestone et al. 2012).

Analysis of virulence-associated phenotypes

The human epithelial Caco-2 cell line was used to study virulence-associated phenotypes of wild-type *E. coli* O157:H7 and *Salmonella* Typhimurium and their Δ*qseC* and Δ*qseE* mutants. Bacteria were grown to exponential phase in DMEM, optical densities of cultures were measured at 595 nm, and then diluted to give an OD595 nm value of 0.1. Cultures were then further diluted in 10-fold steps to reach a multiplicity of infection (MOI) of 100:1 for *Salmonella* strains, and a MOI of 50:1 for *E. coli* strains Precise cell densities of the inoculated cultures were determined by plate counts on Luria

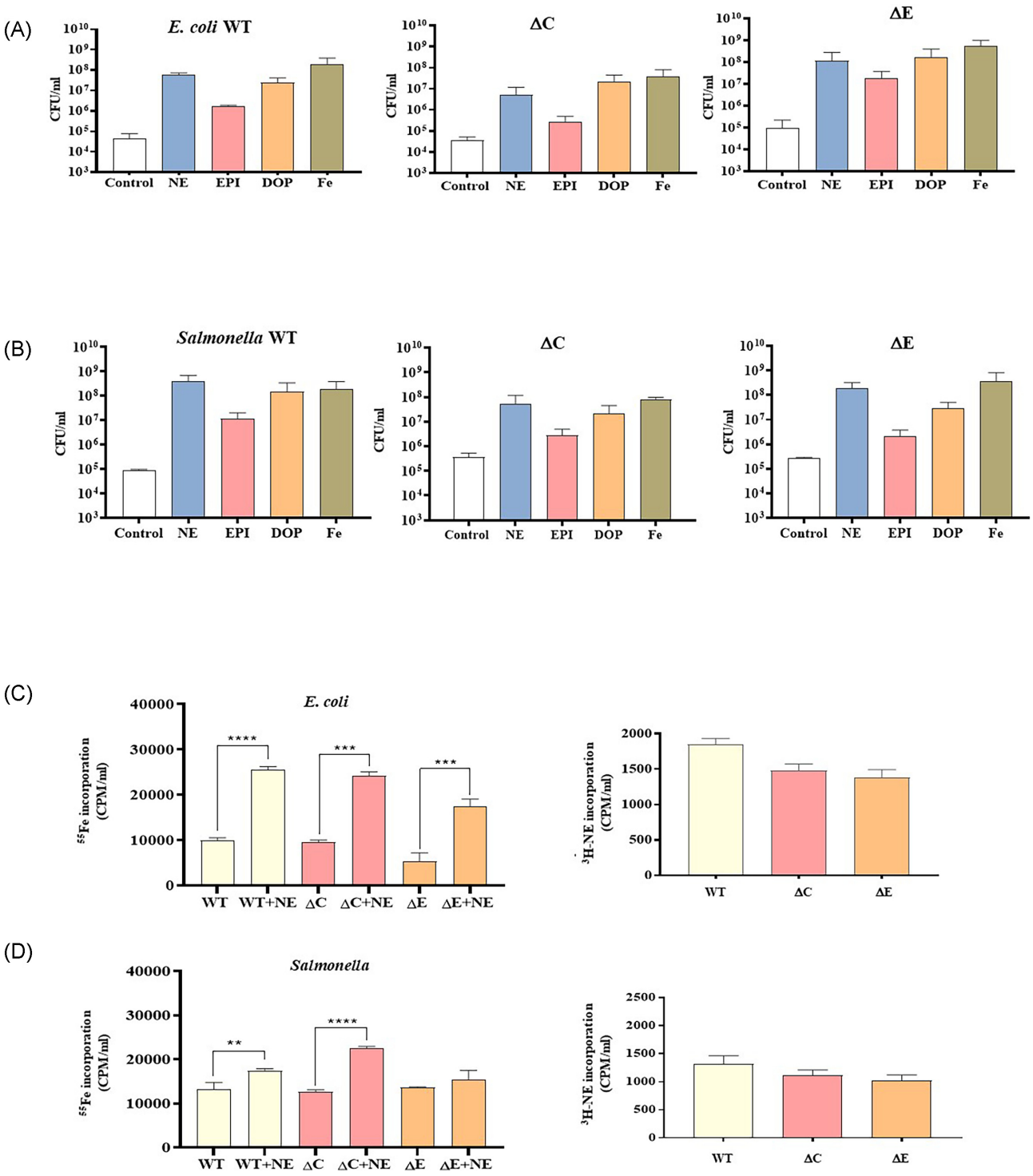

Figure 1. Growth induction of wild-type *E. coli* and *Salmonella* and their Δ*qseC* and Δ*qseE* mutants in serum-SAPI medium +/− catecholamines. Overnight cultures of wild-type *E. coli* **(A)** or *Salmonella* **(B)** and their Δ*qseC* and Δ*qseE* mutants were diluted to around 100 CFU/ml into serum-SAPI with no additions (negative control) or with the catecholamines shown (all at 50 μM), and grown for 18 h statically at 37°C. Iron in the form of ferric nitrate (50 μM) was used as a positive growth control. After incubation, bacteria were enumerated by serial dilution and growth on LA plates. Values shown represent the means and standard deviations of triplicate viable count determinations from triplicate cultures ($n = 3$). To measure incorporation of ^{55}Fe from ^{55}Fe-labelled transferrin into wild-type strains and Δ*qseC* and Δ*qseE* mutants (left hand in **(C)** and **(D)**), bacteria were incubated for 18 h at 37°C in serum-SAPI supplemented with 2.1×10^5 cpm of ^{55}Fe-labelled transferrin in the presence or absence of 50 μM of NE as described in Materials and methods. Bacterial uptake of radioactively labelled iron [^{55}Fe] by wild-type and mutant strains were measured using scintillation counting. To measure ^{3}H-NE internalisation of wild-type strains and Δ*qseC* and Δ*qseE* mutant cultures were incubated statically at 37°C in duplicate in 3 ml of 50 mM SAPI-Tris pH7.5 supplemented with ^{3}H-NE (1×10^5 cpm/ml) and measured for ^{3}H-NE uptake after 90 min incubation as described in Materials and methods (right hand in (C) and (D)). One way ANOVA was used to calculate the level of significance. The assay was performed in triplicate on three independent occasions. Key: WT, wild-type; ΔC, Δ*qseC* mutant; ΔE, and Δ*qseE* mutant; asterisks indicate statistical significance of * $P < .05$, ** $P < .01$, and *** $P < .001$.

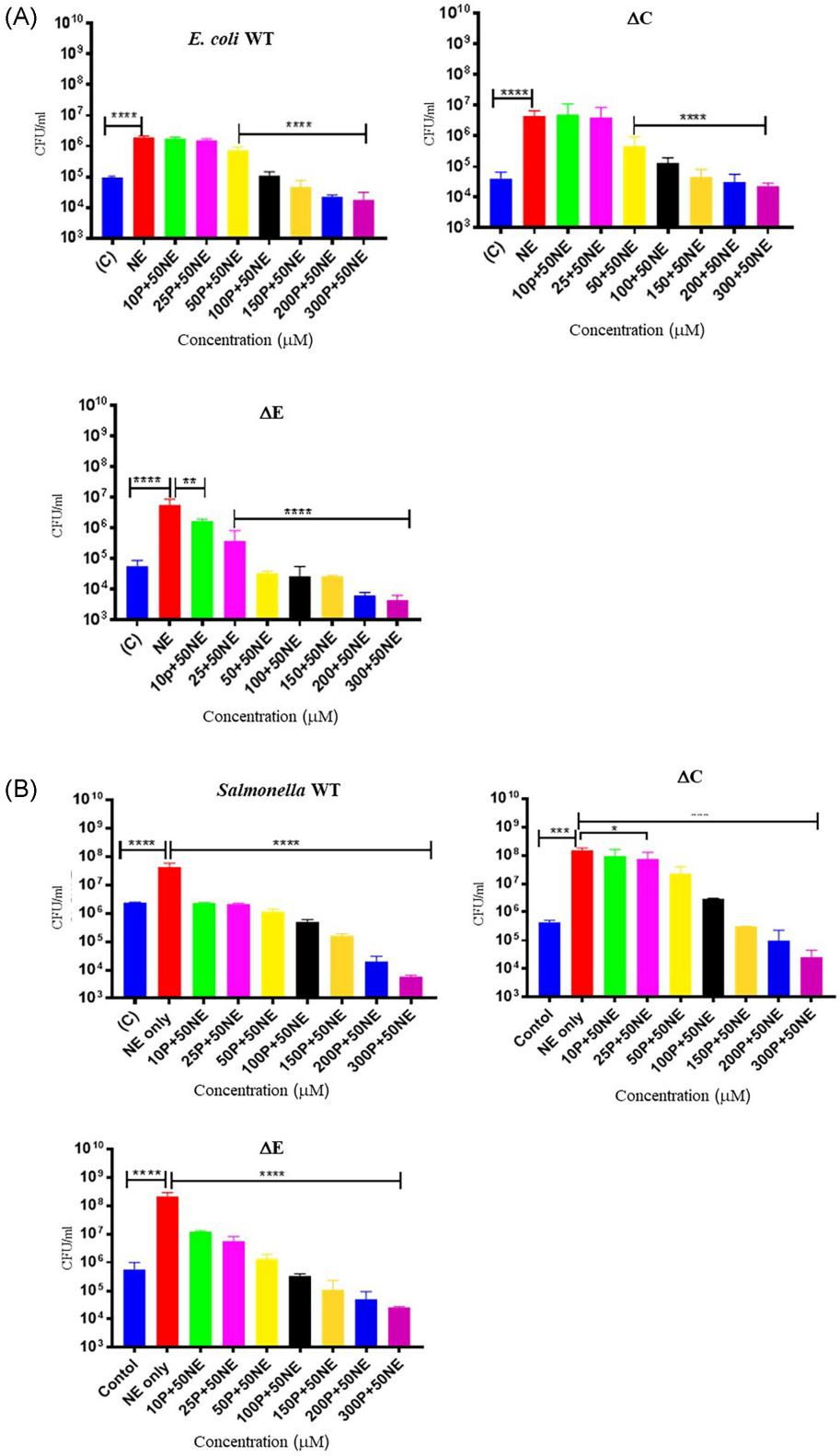

Figure 2. Adrenergic antagonist inhibition of NE growth induction of wild-type *E. coli* and *Salmonella* and their Δ*qseC* and Δ*qseE* mutants Overnight cultures of wild-type *E. coli* **(A)** or *Salmonella* **(B)** and their Δ*qseC* and Δ*qseE* mutants were diluted to around 100 CFU/ml into serum-SAPI without (control) or with 50 μM NE plus concentrations of the phentolamine shown (10–300 μM) and grown for 18 h statically at 37°C. Note phentolamine by itself was not toxic at 300 μM (data not shown). Cultures were then enumerated for viable cells as described for Fig. 1. Values represent the means and standard deviations of triplicate viable count determinations from triplicate cultures ($n = 3$). Key: lines show data comparisons. Asterisks indicate statistical significance of * $P < .05$, ** $P < .01$, and *** $P < .001$.

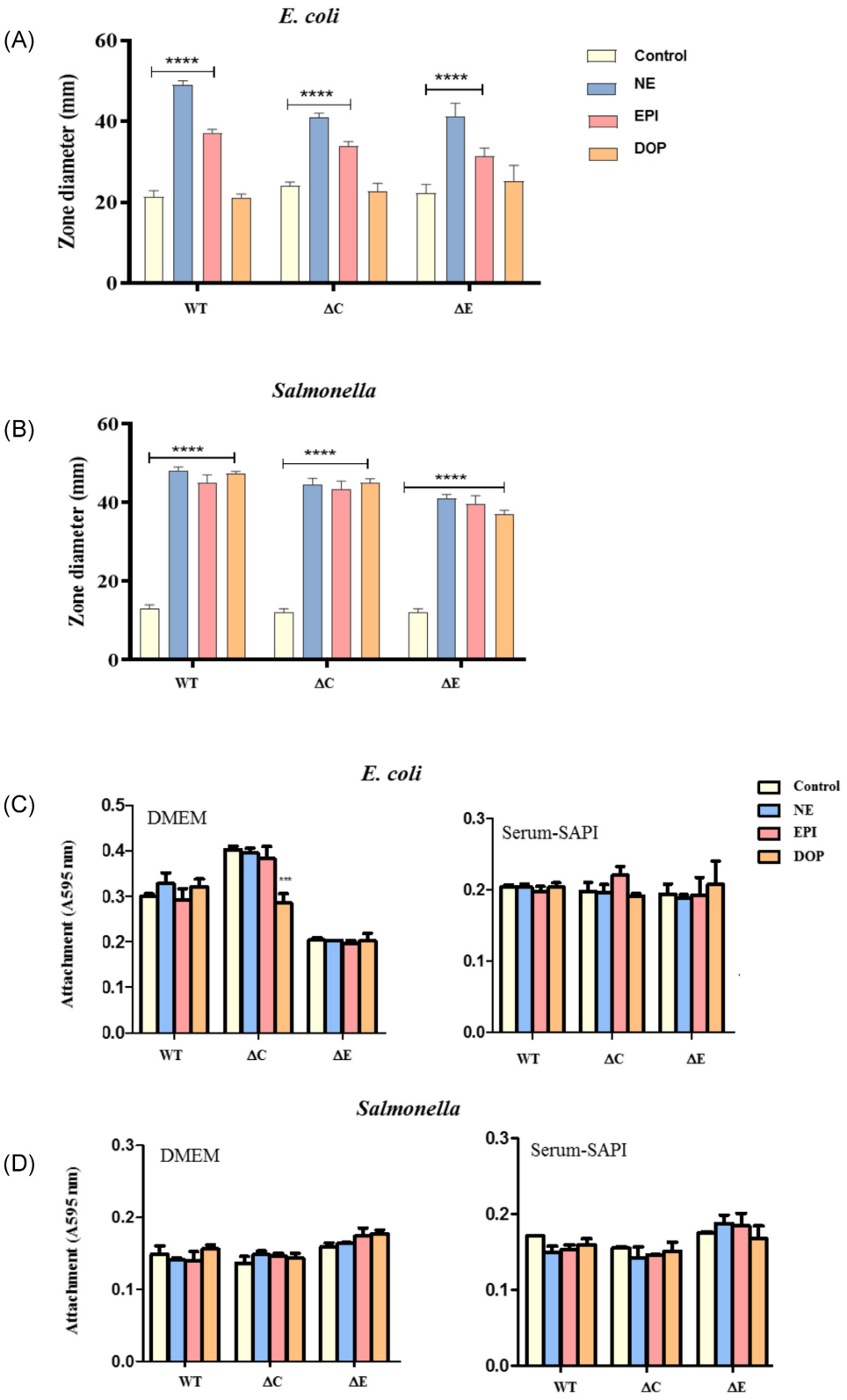

Figure 3. Effect of catecholamines on the motility and biofilm formation of wild-type *E. coli* and *Salmonella* and their Δ*qseC* and Δ*qseE* mutants The histograms in **(A)** and **(B)** show the motility in soft agar of wild-type *E. coli* (A) and *Salmonella* (B) and their Δ*qseC* and Δ*qseE* mutants in the absence and presence of catecholamines (50 μM, NE, Dop, or Epi) as described in Materials and methods; ($n = 3$). **(C)** and **(D)** show biofilm formation of *E. coli* and *Salmonella* Δ*qseC or* Δ*qseE* mutants in DMEM and serum-SAPI. Overnight cultures of wild-type *E. coli* (C) or *Salmonella* (D) and their Δ*qseC or* Δ*qseE* mutants were diluted 1:1000 into DMEM or serum-SAPI without and with 50 μM, NE, Dop, or Epi and incubated for 18 h statically at 37°C; attachment was measured using crystal violet staining. Culture values were corrected for staining due to media only. Data represent means and SEM of four biological replicates. Key: lines show comparisons. Asterisks and the symbols * indicate statistical significance of * $P < .05$, ** $P < .01$; and *** $P < .001$.

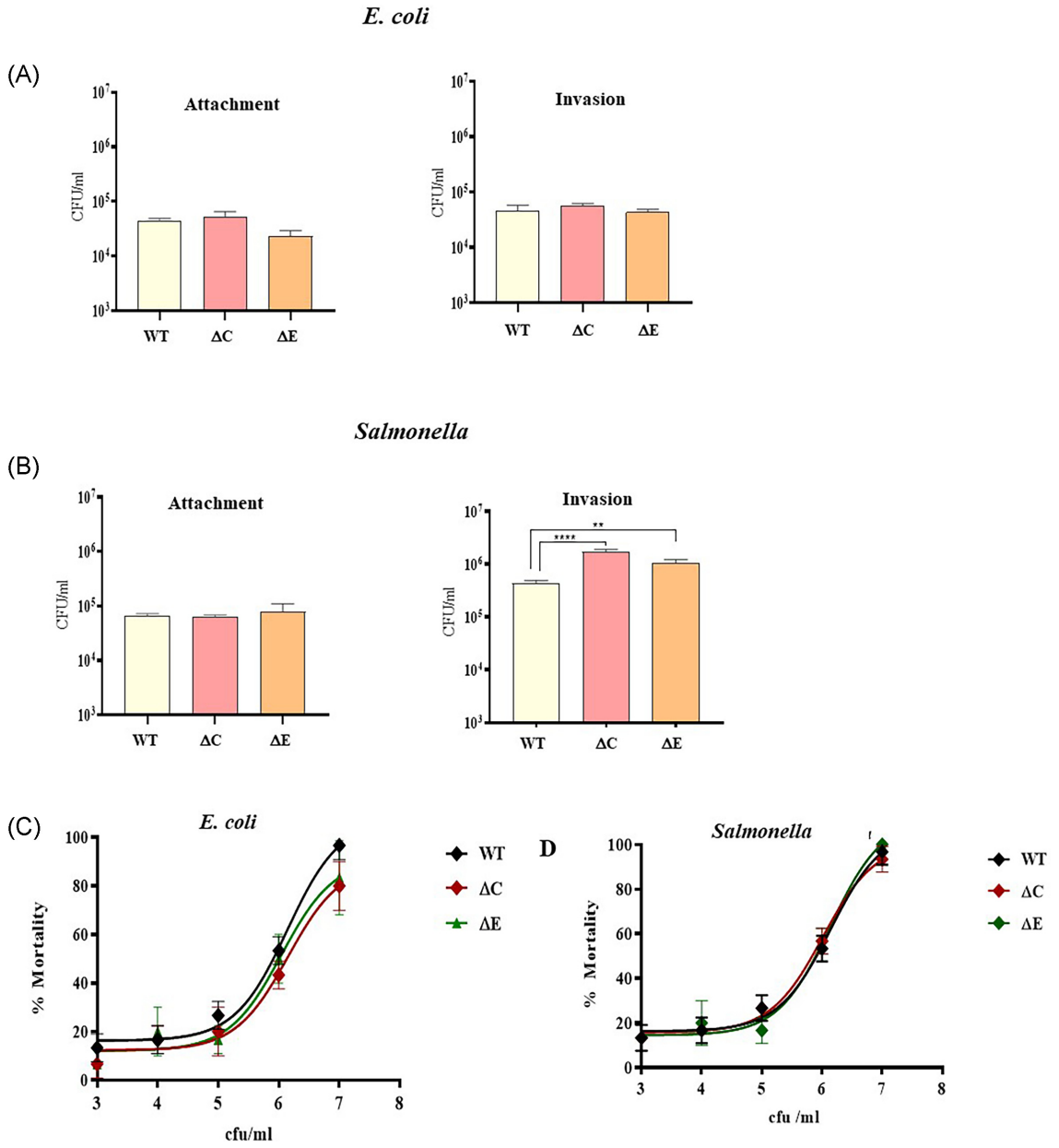

Figure 4. Virulence-associated phenotypes of wild-type *E. coli* and *Salmonella* Δ*qseC* and their Δ*qseE* mutants **(A)** and **(B)** show the attachment to, and invasion of, Caco-2 cells by wild-type (WT) *E. coli* (A) and *Salmonella* (B) and their Δ*qseC* and Δ*qseE* mutants (ΔC and ΔE) after 3 h incubation at 37°C; $n = 3$. Bacterial numbers were quantified as described in Materials and methods. **(C)** (*E. coli*) and **(D)** (*Salmonella*) show the virulence (LD50) of the wild-type and Δ*qseC* and Δ*qseE* mutant strains in a *Galleria* model of infection ($n = 3$). Data are expressed as % survival, and are representative results of at least three independent experiments.

agar of the culture dilutions. Inoculated Caco-2 cultures were incubated for 3 h at 37°C in a humidified, 5% CO_2 incubator, and nonadherent bacteria removed by washing twice with PBS, after which the cells were lysed with 1% Triton X-100. The lysed cultures (containing attached and intracellular bacteria) were serially diluted with PBS, and dilutions plated on Luria agar. To measure bacteria invading the Caco-2 cells, after infection the Caco-2 culture was washed twice with DMEM, and treated with 40 μg/ml gentamicin in DMEM for 1 h to kill extracellular bacteria. Cells were then washed and lysed with 1% Triton X-100; internalised bacteria were enumerated by serial dilution and plating onto Luria agar.

The virulence of *Salmonella* and *E. coli* wild-type strains and their Δ*qseC* and Δ*qseE* mutants was also assessed using a *Galleria mellonella* infection model (Tsai et al. 2016). The inoculation route was via intra-haemo-coelic injection through the last left pro-leg using a sterilised 26S gauge 10 μl Hamilton syringe (Cotter et al. 2000). To compare the virulence of *E. coli* and *Salmonella* wild-type and mutant strains and to determine the 50% lethal dose (LD_{50}), larvae were injected with bacterial inocula diluted into PBS buffer over the 10^3, 10^4, 10^5, 10^6, and 10^7 CFU range. Precise levels of bacteria inoculated were determined by plating serial dilutions of the inoculum onto Luria agar. Controls to determine the impact of the tissue trauma of the injection on the viability of the larvae used an equivalent volume of PBS only. Larvae were incubated at 37°C in the dark and scored regularly for up to 5 days for melanisation and viability using reflex to physical contact.

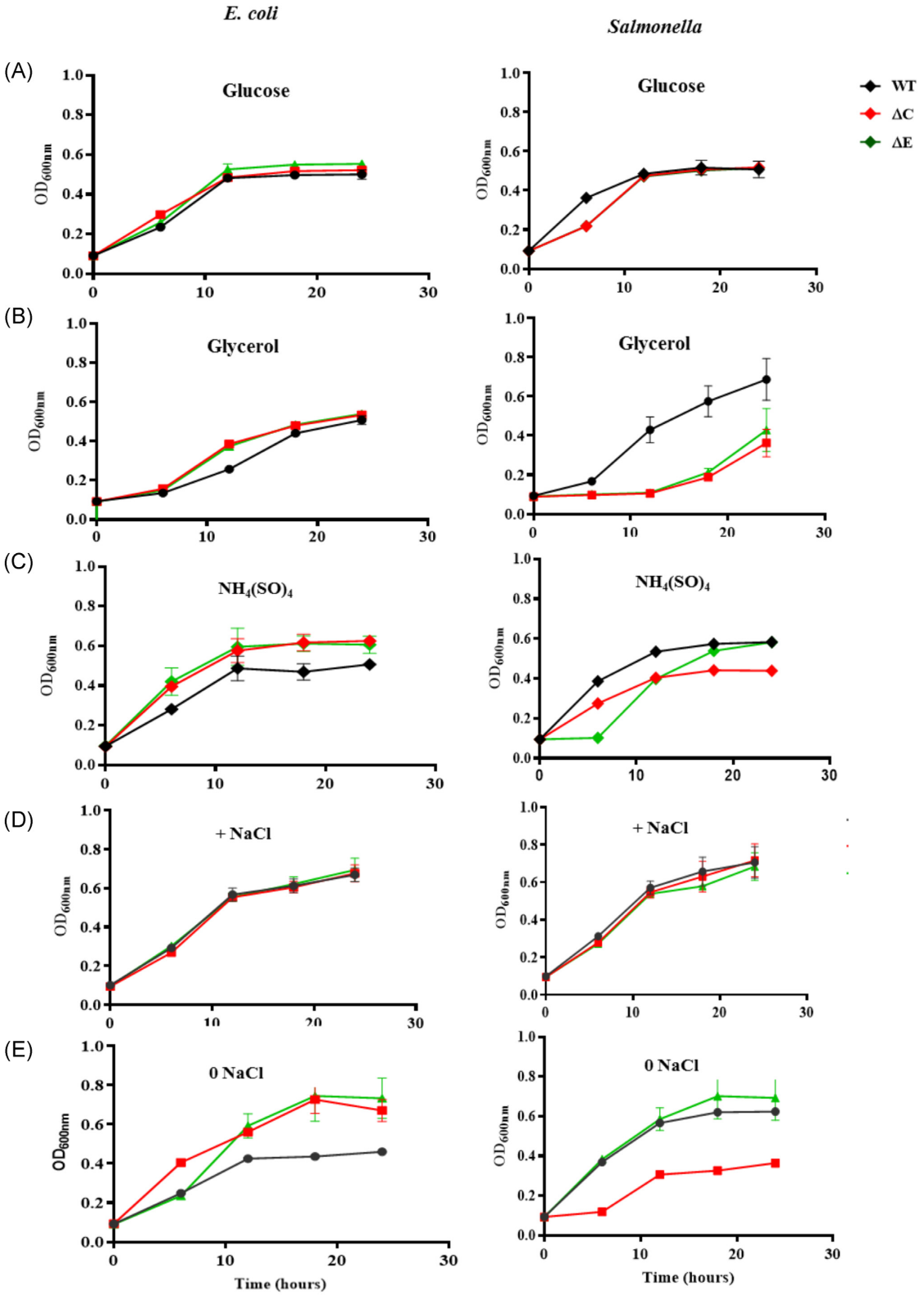

Figure 5. Growth of wild-type *E. coli* and *Salmonella* and their Δ*qseC* and Δ*qseE* mutants in C or N modified M9 medium. Overnight cultures of wild-type *E. coli* or *Salmonella* and their Δ*qseC* and Δ*qseE* mutants were diluted 1:1000 in M9 medium supplemented with 0.4% glucose **(A)**, 0.4% glycerol **(B)** or 8.55 mM of ammonium sulphate instead of ammonium chloride **(C)**. To measure the growth kinetics of *E. coli* and *Salmonella* Δ*qseC* and Δ*qseE* mutants in high and low osmotic strength media, cultures were diluted 1:100 in M9 medium with 0.4% glucose as carbon source with either 0.6 M NaCl **(D)** or no (0) M NaCl **(E)**. Growth kinetics were measured at 37°C over 24 h in a Varioscan spectrophotometer. Values shown represent the means and standard deviations of values from triplicate cultures ($n = 3$).

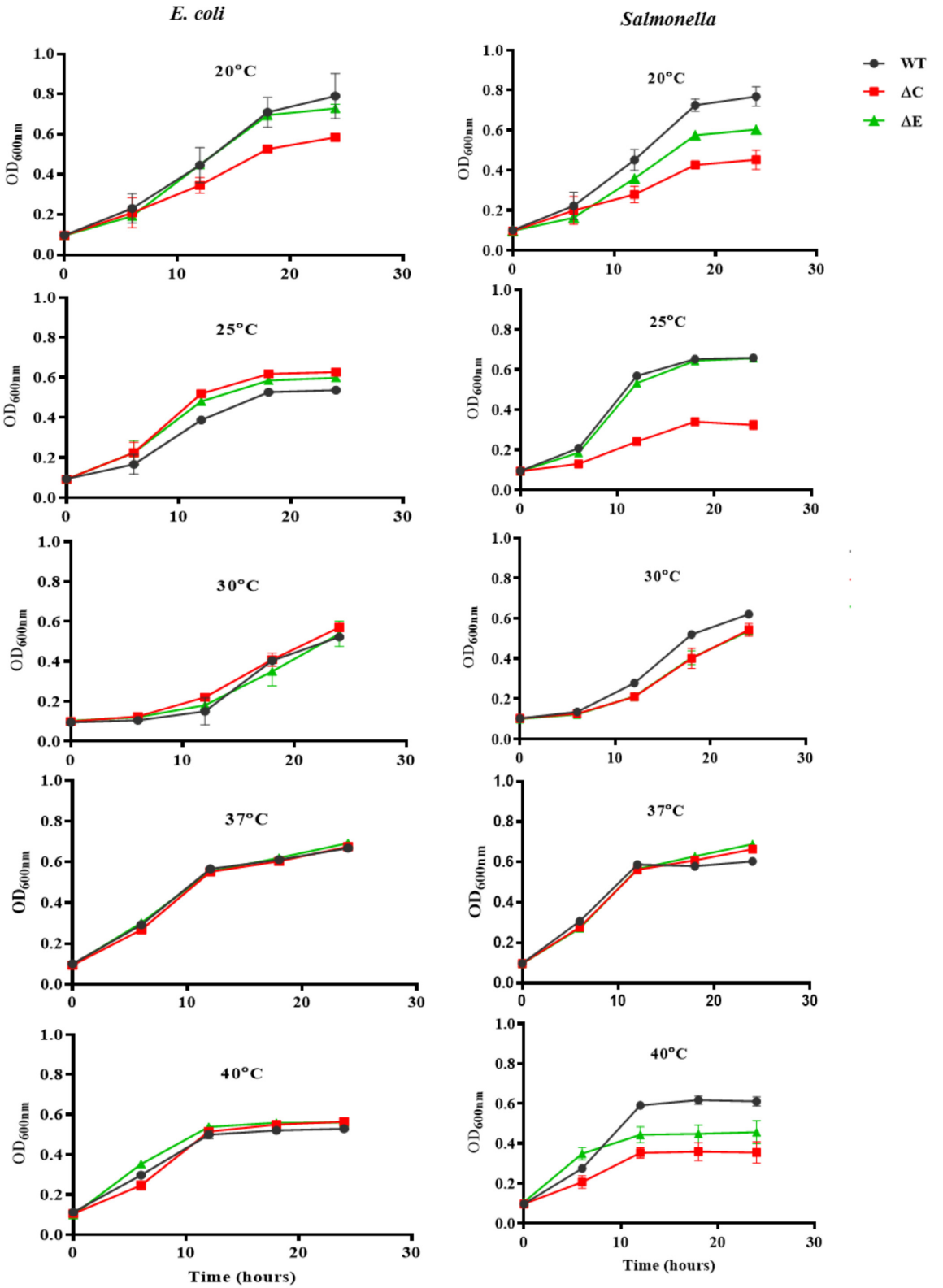

Figure 6. Temperature sensitivity of wild-type *E. coli* and *Salmonella* and their Δ*qseC* and Δ*qseE* mutants Overnight cultures of wild-type *E. coli* or *Salmonella* and their Δ*qseC or* Δ*qseE* mutants were diluted 1:1000 in M9 medium supplemented with 0.4% glucose, and growth kinetics over the 20–40°C range measured for 24 h in a Varioscan spectrophotometer. Values represent the means and standard deviations of values from triplicate cultures ($n = 3$).

Growth responses to different nutrient sources

Overnight cultures grown in LB were diluted (1:1000) into M9 minimal medium with 0.4% glucose or 0.4% glycerol as carbon source. QseE has been shown to recognise sulphate, and so we examined the effect of a different nitrogen salt source, the ammonium chloride in M9 medium was replaced with ammonium sulphate (Reading et al. 2009). To test the effect of osmotic stress conditions on growth, M9 medium with 0.4% glucose was supplemented with no NaCl or 0.6M NaCl. Optical density of the time course cultures were measured at 600 nm every 15 min for 24 h using a Varioskan spectrophotometer (Transgalactic Ltd, UK). Growth curves shown were derived from triplicate time courses using three independent cultures. All experiments were performed in duplicate or triplicate on at least three separate occasions. Graphs were plotted using Graph Pad Prism. Where appropriate, statistical analysis was carried out using One-way ANOVA (Analysis of Variance) Tukey`s multiple comparison and two-way ANOVA with Dunnet's *post hoc* tests. Statistical significance is indicated by a *P* value of less than .05.

Results and discussion

Catecholamine responsiveness is not affected by loss of QseC or QseE

It has been proposed by Sperandio and co-workers that the *E. coli* O157:H7 QseC and QseE proteins are bacterial adrenergic catecholamine receptors that autophosphorylate on recognition of NE and Epi, then activate a cognate response regulator that enhances expression of genes associated with growth, motility and virulence (Sperandio et al. 2003, Clarke et al. 2006, Reading et al. 2009, Moreira et al. 2012). Others have in contrast reported that growth and motility of an *E. coli* O157:H7 *qseBC* mutant was comparable to that of the parent in the presence or absence of NE (Sharma and Casey 2014a). Further, while Moreira et al. (2016) reported a role for QseC in NE-induced growth, motility, and Type III secretion system expression in S. Typhimurium, Pullinger et al. (2010a) reported that *qseC*, *-E*, and *-CE* mutants respond to NE at wild-type levels. In the current study, to further analyze the adrenergic receptor role of QseC and QseE, the growth response to different catecholamines of wild-type *E. coli* and *Salmonella* was compared to their isogenic Δ*qseC* and Δ*qseE* mutants. In Fig. 1(A) and (B), it can be seen that the Δ*qseC* and Δ*qseE* mutants of both species exhibited a similar ability to respond to the levels of NE and Dop (50 μM) as their parent. The reduced response of all strains to Epi may be because that this catecholamine is not normally found within the gut (Furness 2006) and enteric bacteria are therefore less likely to encounter it.

The QseC protein of *Haemophilus influenzae* is activated by ferrous iron or zinc, and does not appear to respond to catecholamines alone (Steele et al. 2012). On the other hand, the QseC of *Aggregatibacter actinomycetemcomitans* is activated by a combination of a catecholamine and iron, but not by either individually (Weigel et al. 2015). It is, therefore, interesting that the only bacterial system found so far that when mutated blocks response to catecholamines are those involved in iron acquisition. Siderophore uptake or synthesis mutants of *E. coli* (*entA*, *entS*, or *tonB*) lose all catecholamine responsiveness (Burton et al. 2002, Freestone et al. 2003, Bearson et al. 2010). This is because the catecholamine acts as a pseudo-siderophore, removing transferrin, and lactoferrin Fe^{3+} passing it on to higher affinity ferric iron-binding siderophores such as enterobactin (Sandrini et al. 2010). Since catecholamines can remove Fe from transferrin and supply it to bacteria, we investigated if QseC and QseE played a role in this process. Using ^{55}Fe-loaded transferrin we compared uptake of ^{55}Fe from the transferrin by wild-type *E. coli* and *Salmonella* and their Δ*qseC* and Δ*qseE* mutants +/− 50μM NE (as this catecholamine had similar potency in all the strains). Figure 1(C) shows that for *E. coli* wild-type and mutant strains addition of NE increased ^{55}Fe internalisation of transferrin iron compared to un-supplemented controls ($P < .001$), though the magnitude of the ^{55}Fe uptake +/− NE was less for the Δ*qseE* mutant ($P < .01$). Although growth induction *via* NE was similar to wild-type, internalisation of the catecholamine (measured *via* uptake of ^{3}H-NE) was consistently slightly reduced for both mutants ($P < .01$). Figure 1(D) shows *Salmonella* wild-type and Δ*qseC* mutant were equally able to utilise the catecholamine to obtain increased levels of transferrin ^{55}Fe. However, the *Salmonella* Δ*qseE* mutant (Fig. 1D) while still able to extract and internalise ^{55}Fe from transferrin was not significantly advantaged by the presence of the NE ($P > .5$). In terms of ^{3}H-NE internalisation, as seen for *E. coli*, compared to wild-type, ^{3}H-NE levels were also slightly lower in the *Salmonella* Δ*qseE* and Δ*qseE* mutants ($P < .05$).

That an adrenergic receptor-antagonist can affect binding of NE to the *E. coli* QseC has been presented as evidence of it being an adrenergic receptor (Clarke et al. 2006, Weigel et al. 2015). Therefore, we also examined if an adrenergic antagonist could block NE growth induction in the *E. coli* and *Salmonella* Δ*qseC* and Δ*qseE* mutants. It was previously shown (Freestone et al. 2007) that beta adrenergic receptor antagonists did not affect catecholamine recognition by *E. coli*, *Salmonella*, or *Yersinia* and so the effects on NE growth induction were carried out using the alpha adrenergic receptor antagonist phentolamine. We used a fixed concentration of 50 μM NE and increasing concentrations of phentolamine up to 300 μM. The growth profiles in Fig. 2 shows that phentolamine was able to antagonise the growth-inducing effects of NE in wild-type *Salmonella*, *E. coli* and their Δ*qseC* and Δ*qseE* mutants in a similar dose-dependent manner ($P > .001$), suggesting that QseC and QseE are unlikely to be involved in the mechanism by which the antagonist blocks NE recognition. Freestone et al. (2007) showed that entry of ^{3}H-NE into wild-type *E. coli, Salmonella* and *Y. enterocolitica* could be blocked by phentolamine, while Sandrini et al. (2013) used ^{3}H-NE to show that NE entered *E. coli via* the outer-membrane porins OmpA and OmpC. Porin-mediated entry of catecholamines would, therefore, explain why in the current study an alpha antagonist was still able to block NE response in the absence of QseC and QseE, and why less NE was internalised in the Δ*qseC* and Δ*qseE* mutants. However, proteomic profiling of membrane proteins of wild-type *Salmonella*, *E. coli* and their Δ*qseC* and Δ*qseE* mutants did not reveal a reduction in the levels of the OmpA or OmpC porins (data not shown).

Motility and biofilm formation of *E. coli* and *Salmonella* Δ*qseC* and Δ*qseE* mutants

Some studies have reported that mutation of the *E. coli* and *Salmonella qseC* affects motility (Sperandio et al. 2003, Clarke et al. 2006, Bearson and Bearson 2008, 2010). In the current study, it was found that the motility of untreated *E. coli* and *Salmonella* Δ*qseC* and Δ*qseE* mutants were similar to their wild-type parent (Fig. 3A and B). Also, we saw that the motility of wild-type and Δ*qseC* and Δ*qseE* mutants were for both species similarly enhanced by the addition of NE and Epi compared to un-supplemented controls ($P < .001$). Although Dop had little effect on motility of *E. coli* and its mutants, it enhanced motility of all of the *Salmonella* strains. Sharma and Casey (2014a) also found that deleting *qseC* in *E. coli*

had no effect on motility and that motility of its *qseC* mutant was still enhanced by NE. Our data are consistent with those of Pullinger et al. (2010a) who reported no effect of loss of *qseC* or *qseE* on motility of S. Typhimurium in the presence of NE.

In terms of biofilm formation Fig. 3(C) and (D) show that the initial stage of the process, surface attachment, was not impaired by loss of QseC in *E. coli* or *Salmonella* in either DMEM or the host-mimicking serum-SAPI. We also found that were there no differences between mutant and wild-type strains in surface attachment with or without catecholamines. However, for the *E. coli* Δ*qseE* mutant, incubation in DMEM reduced attachment while in serum-SAPI attachment was unaffected, which was in marked contrast to the *Salmonella* Δ*qseE* mutant which showed a wild-type level of attachment in either media.

Virulence-associated phenotypes of *E. coli* and *Salmonella* lacking QseC or QseE

Assessing the ability of enteric bacteria to attach to, and invade intestinal epithelial cells is useful in distinguishing differences in virulence between strains (Izumikawa et al. 1998). It has been suggested that the QseC coordinates many aspects of virulence gene expression in enteropathogenic bacteria (Ribeiro et al. 2019). An *E. coli qseC* mutant was found to be attenuated for virulence using a rabbit infection model (Waldor and Sperandio 2007). Reading et al. (2007) reported that QseE is required for *E. coli* gut epithelial cell lesion formation. In *Salmonella enterica* serovar Dublin, the sensor kinase domains for all 31 TCS identified as present within the genome were mutated, and a calf infection model used to identify essential TCS. QseC was one of the five TCS identified as negatively selected for at all enteric and systemic sites sampled after dosing (Pullinger et al. 2010b). In the current study, a comparative analysis was carried out of the attachment and invasion capacity of *E. coli* and *Salmonella* wild-type and *qse* mutants using Caco-2 intestinal cells.

Figure 4(A) and (B) show that relative to parental strains, adherence and invasion of the Caco-2 cells by the *E. coli* and *Salmonella* Δ*qseC* mutants was not reduced ($P = .1$). For *Salmonella*, removal of *qseE* had no effect on Caco-2 attachment, while there was a slight but reproducible reduction in attachment for the *E. coli* Δ*qseE* mutant relative to wild-type ($P < .05$). Since this mutant showed a reduced attachment to abiotic surfaces in DMEM (Fig. 3C), the medium in which the Caco-2 attachment assays were performed, the effect may be related to the medium. In terms of invasion, Fig. 4(A) and (B) show that relative to wild-type, removal of QseC or QseE did not affect Caco-2 cell invasion or survival within the host cells ($P < .05$). Merighi et al. (2009) also reported that a *Salmonella qseC* mutant did not exhibit attenuation for the invasion of mammalian epithelial cells, while Sharma and Casey (2014b) found that an *E. coli qseC* mutant outcompeted its parental strain in colonisation of cattle intestines. For *Salmonella* we also investigated its virulence using the J774 murine macrophage cell line used by Moreira et al. (2010) to assess the virulence of a *Salmonella qseC* mutant. In contrast, to their findings we found that the ability of the *Salmonella* Δ*qseC* and Δ*qseE* mutants to invade and survive within J774 macrophages for up to 4 h was no different to that of their parent ($P > .05$; data not shown).

We further evaluated if loss of QseC and QseE affected *E. coli* and *Salmonella* virulence using a *G. mellonella* infection model. The wax moth larvae have an immune system similar to the mammalian innate immune system, and the large numbers of tests possible mean results of such infection models are less subject to host background differences sometimes seen with mouse or rabbit infection models (Alghoribi et al. 2014, Ciesielczuk et al. 2015). The findings of this study (Fig. 4C and D) demonstrate that for *E. coli* loss of either QseC or QseE slightly impacted on their LD_{50} values ($P < .05$) while for *Salmonella* the virulence of the mutants and their parent were similar ($P = .5$). It has been claimed that QseC is a regulator of *Salmonella* virulence, including of the Type III secretion systems associated with induction of enteritis, yet the *Salmonella qseC* and *qseE* mutants described in the current study have been reported to elicit comparable secretory and inflammatory responses in a bovine ligated ileal loop model of infection as the wild-type parent (Pullinger et al. 2010a). The finding that a double *qseCE* mutant behaves similarly to the parent strain in this model argues against functional redundancy as an explanation for the absence of NE-induced phenotypes for the single mutants (Pullinger et al. 2010a).

Metabolism profiling

While it is clear that deleting the genes for QseC and QseE does not affect the ability of *E. coli* and *Salmonella* to respond to catecholamines, the biological question outstanding is what are these TCS proteins doing? Bacterial virulence is related to the capacity to grow within their host, which in turn is influenced by multiple environmental factors including nutrient availability, temperature, osmotic stress, pH, and oxygen levels (Nackerdien et al. 2008). It has been suggested that the *E. coli* QseC has a direct role in carbon metabolism (Hadjifrangiskou et al. 2011), while in *A. actinomycetemcomitans* QseC is involved in several metabolic and respiratory pathways (Weigel et al. 2015). Carbon is a particularly vital element among nutrient sources for bacteria, and its availability has been shown to play a major role in regulation of virulence especially for enteric pathogens (McGuckin et al. 2011). In the current study, there were found to be variations in behaviour of the *E. coli* Δ*qseE* mutant compared to wild-type that were media-related (Fig. 3C). We, therefore, examined the growth kinetics of wild-type *E. coli* and *Salmonella* and their mutants in M9 minimal medium containing different carbon or nitrogen salt sources (0.4% glucose or 0.4% glycerol, or the usual N source ammonium chloride replaced with an equivalent concentration of ammonium sulphate). Figure 5(A) shows that for *E. coli*, replacing glucose by glycerol did not impair growth for either mutant. However, for *Salmonella* (Fig. 5B) the glucose to glycerol switch markedly reduced the growth rate and final growth yield of the Δ*qseC* and Δ*qseE* mutants compared to their parent ($P < .001$). In terms of nitrogen source, Fig. 5(C) shows no impact on the *E. coli* strains when ammonium sulphate was the N source, while the *Salmonella* Δ*qseC* mutant displayed reduced growth ($P < .01$), and the Δ*qseE* mutant initially grew more slowly but reached a similar final biomass to its parent ($P < .05$). Hadjifrangiskou et al. (2011) used transcriptomics to show that the *E. coli* QseC sensed C and N nutrient sources; our data suggests that the *Salmonella* QseC protein might also be included in the nutrient sensing category of TCSs.

Two-component regulatory systems are important for bacteria to adapt to changes in their environment (Stock et al. 2000). He et al. (2018) showed the QseBC TCS of *Haemophilus parasuis* was involved in adaption to osmotic pressure. Therefore, we investigated the response of wild-type *E. coli* and *Salmonella* and their mutants to osmotic stress, such as growth in absence of an osmotic stabiliser (0 M NaCl) or in a higher level of salt (0.6M NaCl). Figure 5(D) and (E) show that the growth profiles in M9 medium in the presence or absence of NaCl were similar for wild-type *E. coli* and its Δ*qseC* and Δ*qseE* mutants. However, Fig. 5(E) shows for the

Salmonella qseC mutant there was a marked reduction in growth in low salt medium compared to wild-type ($P < .0001$).

We also examined growth and viability in other environments relevant to host habitats, such as high or low pH or low oxygen, but saw no differences in response to the environmental stress between *E. coli* and *Salmonella* wild-type and their Δ*qseC or* Δ*qseE* mutants. However, we did find differences in responsiveness to temperature. Figure 6 shows a 24-hour time course in M9 medium plus 0.4% glucose over the 20–40°C range. For *E. coli* the Δ*qseE* mutant showed a wild-type growth profile over all temperatures, while the Δ*qseC* grew less well at 20°C ($P < .01$) but displayed no growth defects at higher temperatures. The *Salmonella* Δ*qseC* mutant showed impaired growth relative to wild-type at all temperatures ($P < .001$) except 37°C, while the *Salmonella* Δ*qseE* mutant grew less well at 20 and 40°C compared to wild-type ($P < .001$). This suggests that in *Salmonella* QseC and QseE may play a role in regulating growth relative to changes in environmental temperature.

In conclusion, this report has shown that the published literature on the role of QseC and QseE is contradictory in terms of their role in stress hormone recognition. While it is clear from our work that QseC and QseE are not needed for *E. coli* and *Salmonella* to respond to catecholamines, the TCS were found to have other roles in *E. coli* and *Salmonella* that might explain some of the virulence and other phenotypes observed by others. Sensing environmental changes such as temperature and controlling metabolism are key housekeeping functions that when disrupted impact virulence, which would explain some of the attenuation that has been observed when the infectivity of *qseC* mutants have been studied (Clarke et al. 2006, Bearson and Bearson 2008). It is also interesting that in this study the phenotypic profiles observed in the Δ*qseC* and Δ*qseE* mutants differed between species, suggesting in *E. coli* and *Salmonella* that QseC and QseE may also be playing different physiological role(s). It is possible that differences in the specific nature of mutations, the strains used or laboratory protocols may underlie some of the disparities in the literate related to QseC- and QseE-dependent phenotypes. Although a considerable undertaking, deleting all or combinations of TCS sensor kinases in *E. coli* and *Salmonella* would determine if TCS other than QseC and QseE are involved in catecholamine recognition.

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