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Vibrio parahaemolyticus ExsE is requisite for initial adhesion and subsequent type III secretion system 1-dependent autophagy in HeLa cells

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Vibrio parahaemolyticus pandemic serotype O3 : K6 causes acute gastroenteritis, wound infections and septicaemia in humans. This organism encodes two type III secretion systems (T3SS1 and T3SS2); host-cell cytotoxicity has been attributed to T3SS1. Synthesis and secretion of T3SS1 proteins is positively regulated by ExsA, which is presumptively regulated by the ExsCDE pathway, similar to *Pseudomonas aeruginosa*. Herein we deleted the putative *exsE* from *V. parahaemolyticus* and found constitutive expression of the T3SS1 in broth culture as expected. More importantly, however, in a cell culture model, the Δ*exsE* strain was unable to induce cytotoxicity, as measured by release of lactate dehydrogenase (LDH), or autophagy, as measured by LC3 conversion. This is markedly different from *P. aeruginosa*, where deletion of *exsE* has no effect on host-cell cytolysis. Swarming and cytoadhesion were reduced for the deletion mutant and could be recovered along with T3SS1-induced HeLa cell cytotoxicity by *in cis* expression of *exsE* in the Δ*exsE* strain. Loss of adhesion and swarming motility was associated with the loss of flagella biogenesis in the *exsE*-deficient strain. Mouse mortality was unaffected by the deletion of *exsE* compared with a wild-type control, suggesting that additional adhesins are important for intoxication *in vivo*. Based on these data, we conclude that ExsE contributes to the negative regulation of T3SS1 and, in addition, contributes to regulation of an adherence phenotype that is requisite for translocation of effector proteins into HeLa cells.

INTRODUCTION

Vibrio parahaemolyticus, a Gram-negative, halophilic bacterial pathogen, can cause acute gastroenteritis following consumption of raw or undercooked seafood (Daniels *et al.*, 2000; Hlady & Klontz, 1996; Morris & Black, 1985; Tison & Kelly, 1984). Wound infections and septicaemia can also result from *V. parahaemolyticus* exposure, with immune-compromised individuals being at highest risk (Daniels *et al.*, 2000; Hlady & Klontz, 1996; Mertens *et al.*, 1979; Qadri *et al.*, 2003; Ryan, 1976). Pathogenicity of the pandemic *V. parahaemolyticus* serotype O3 : K6 has been attributed to several factors including thermostable direct haemolysin (TDH) (Nishibuchi *et al.*, 1992), TDH-related haemolysin (Xu *et al.*, 1994) and two distinct type III secretion systems (T3SSs) (Park *et al.*, 2004; Piñeyro *et al.*, 2010). Contact with host cells will induce synthesis of T3SS1 proteins, as has been demonstrated *in vitro* and *in vivo* by multiple independent investigations (Burdette *et al.*, 2009; Hiyoshi *et al.*, 2010; Ono *et al.*, 2006; Park *et al.*, 2004; Zhou *et al.*, 2009). Importantly, it is unclear how host cell contact signals upregulate T3SS1 expression.

T3SSs were first discovered in *Yersinia* (Michiels *et al.*, 1990; Salmond & Reeves, 1993), and later found in many Gram-negative pathogens including *Escherichia coli*, and *Pseudomonas*, *Shigella*, *Salmonella* and *Vibrio* strains (Coburn *et al.*, 2007). T3SSs are structurally conserved and are composed of three parts: (i) a basal body that spans the periplasmic space; (ii) a needle-like complex that extends from the bacterial surface; and (iii) the translocation apparatus that forms a channel through the target cell membrane. This specialized molecular machine translocates effector proteins into the eukaryotic target cell where they are able to sabotage host cellular processes and modulate the target cell environment.

V. parahaemolyticus T3SS1 is phylogenetically related to the Ysc family of T3SS injectisomes and shares many common characteristics with both *Yersinia* and *Pseudomonas aeruginosa* T3SSs (Makino *et al.*, 2003; Troisfontaines & Cornelis, 2005). Induction of the T3SS in *P. aeruginosa*

Abbreviations: LDH, lactate dehydrogenase; qPCR, quantitative RT-PCR; TEM, transmission electron microscopy; T3SS, type III secretion system.

Four supplementary figures are available with the online version of this paper.

is, in most cases, primarily dependent on contact with a eukaryotic host cell, although there are potential secondary inducing signals such as temperature, metabolic stress and environmental stimuli (Frank, 1997; Hornef *et al.*, 2000; Urbanowski *et al.*, 2007; Vallis *et al.*, 1999). *P. aeruginosa* T3SS gene transcription is controlled by an AraC-like transcriptional activator, ExsA, which acts as a positive regulator and is coupled to secretion competency by the regulatory cascade ExsCDE (Yahr & Wolfgang, 2006). ExsD is an 'anti-activator' that binds ExsA and prevents transcriptional regulation (McCaw *et al.*, 2002) while ExsC is an 'anti-anti-activator' that binds ExsD to permit promoter binding activity of ExsA (Dasgupta *et al.*, 2004; Hovey & Frank, 1995). ExsC also acts as a chaperone for the protein ExsE (Rietsch *et al.*, 2005; Urbanowski *et al.*, 2007) and under non-inducing conditions the ExsC–ExsE protein interaction prevents ExsC from binding to ExsD, thereby preventing high-level expression of the T3SS. Upon host cell contact, ExsE is exported via a T3SS apparatus allowing ExsC to bind ExsD, thereby freeing the transcriptional regulator ExsA to upregulate T3SS expression. This model is based on the presence of low-level, constitutive expression of the T3SS.

Regulation of T3SS1 in *V. parahaemolyticus* is also controlled in a manner similar to *P. aeruginosa* (Kodama *et al.*, 2010; Zhou *et al.*, 2010a; Zhou *et al.*, 2008). Beyond the interaction of the proximal regulators ExsA, ExsD and ExsC, however, little is known about the induction of T3SS1 other than studies showing that gene expression can be induced with host cell contact and when *V. parahaemolyticus* is cultured under specific conditions (Gode-Potratz *et al.*, 2010; Zhou *et al.*, 2008). Herein we further examine the applicability of the *P. aeruginosa* regulatory model in the control of T3SS1 expression in *V. parahaemolyticus* using a host cell contact model.

Kodama *et al.* (2010) recently demonstrated that deletion of the putative *V. parahaemolyticus exsE* (*vp1702*) results in constitutive high-level T3SS1 protein synthesis under non-inducing conditions, as would be predicted from the *P. aeruginosa* model (Rietsch *et al.*, 2005). In the present study, we confirmed that deletion of *exsE* leads to constitutive protein synthesis under non-inducing conditions, and we also showed that this deletion does not negatively affect the T3SS1 regulon or its secretory function in the presence of HeLa cells. Despite successful secretory function, however, we found that deletion of *exsE* results in a loss of both cytolysis and induction of autophagy in HeLa cell culture; these phenotypes could be recovered by *in cis* complementation of *exsE*. Cytoadherence was also reduced in our mutant indicating that *V. parahaemolyticus* ExsE is involved with regulation of adhesion that may be requisite for successful translocation of effector proteins. Mouse mortality was unaffected compared with the wild-type strain, suggesting that either multiple adhesins are present to allow sufficient cell–cell contact during *in vivo* infection or adhesion required *in vitro* is simply unnecessary to affect *in vivo* host cell intoxication by T3SS1.

METHODS

Strains and growth conditions. *V. parahaemolyticus* strain NY-4 (serotype O3 : K6) was used as the wild-type strain for these experiments (Table 1) (Zhou *et al.*, 2009). Bacteria were cultured at 37 °C in Luria–Bertani (LB) medium (Difco) supplemented with antibiotics where appropriate and 2.5 % (w/v) NaCl (LBS) for *V. parahaemolyticus* strains. HeLa cells were maintained at 37 °C, 5 % CO_2 in DMEM (Thermo Scientific) supplemented with 10 % (v/v) fetal bovine serum (FBS) (Thermo Scientific).

Construction of deletion mutants. Deletion mutants were constructed using a method of allelic exchange described previously (Milton *et al.*, 1996). For Δ*exsE*, primers vp1702-1F, vp1702-1Rre, vp1702-2Fre and vp1702-2R were used to amplify regions flanking the *exsE* gene (Table 2). The resulting amplicons were digested with *Xba*I and ligated together. Primers vp1702-1F and vp1702-2R were then used to amplify the full-length fragment minus the deleted sequence. This amplicon was digested with *Xho*I and *Sph*I followed by ligation into the suicide vector pDM4 (digested with the same) and electroporated into *E. coli* S17 λ*pir*. For Δ*lfgE*, primers lfgE-1F, lfgE-1R, lfgE-2F and lfgE-2R were used to amplify regions flanking the *lfgE* gene (Table 2). The resulting amplicons were used as template in a splicing by overlap extension PCR to amplify the full-length amplicon. This amplicon was digested with *Xho*I and *Sac*I followed by ligation into the suicide vector pDM4 (digested with the same) and electroporated into *E. coli* S17 λ*pir*. Constructs were introduced to NY-4 by conjugation and positive insertions were selected on LBS agar supplemented with 34 μg chloramphenicol ml^{-1}. To complete allelic exchange, cells were cultured in the presence of 5 % (w/v) sucrose. Chloramphenicol-sensitive, sucrose-resistant colonies were then screened for the gene deletion by PCR and confirmed by sequencing using primers delta-exsEseq-up and delta-exsEseq-down or lfgE-F and lfgE-R, respectively (Table 2).

Complementation. To complement the *exsE* deletion, we generated a gene fusion consisting of the ExsA-dependent promoter sequence upstream of *vp1668* with the full-length *exsE* cloned into the suicide vector, pKN8 (Table 1). A control strain was also constructed consisting of the promoter sequence only. The gene fusion was constructed by amplifying approximately 220 bp DNA fragment upstream of *vp1668* using primers P1668F-*Xba*I and P1668R-*Bam*HI and the full-length *exsE* using primers vp1702F-*Bam*HI and vp1702R-*Bgl*II (Table 2). Amplicons were digested with *Bam*HI and ligated, and the resulting product was amplified by PCR using primers P1668F-*Xba*I and vp1702R-*Bgl*II. To generate the promoter-only control, primers P1668F-*Xba*I and P1668R-*Bgl*II (Table 2) were used to amplify the promoter sequence alone. The gene fusion and promoter sequence amplicons were digested with *Xba*I and *Bgl*II, and cloned into pKN8 digested with the same enzymes, resulting in the plasmids pKN8-P1668-*exsE* and pKN8-P1668 (Table 1). The plasmids were transformed into *E. coli* S17 λ*pir* by electroporation, resulting in strains S17pKN8-P1668-*exsE* and S17pKN8-P1668, respectively (Table 1). pKN8-P1668-*exsE* and pKN8-P1668 were introduced into *V. parahaemolyticus* NY-4Δ*exsE* by conjugation, resulting in the single cross-over strains Δ*exsE*::pKN8-P1668-*exsE* (*in cis* complemented strain) and Δ*exsE*::pKN8-P1668 (*in cis* negative control), respectively, where each strain harbours a chromosomal insertion of the respective suicide vector upstream of *vp1668* in the P1668 promoter region (Table 1). PCR was used to confirm these insertions (data not shown). *In trans* expression of *exsE* using the expression vector pMMB207 (Morales *et al.*, 1991) was also used for this study but resulted in an overexpression of ExsE and subsequent repression of T3SS1 resulting in failure to complement the cytolysis phenotype (Fig. S1, available with the online version of this paper).

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
***E. coli* strains**		
S17 *λpir*	*thi pro hsdR hsdM*$^+$ *recA* RP-4-2-TC::MU-Km::Tn7 *λpir*	Milton *et al.* (1992)
S17pKN8-P1668-*exsE*	S17 containing P1668-*exsE* fusion gene located on plasmid pKN8	This study
S17pKN8-P1668	S17 containing P1668 located on plasmid pKN8	This study
***V. parahaemolyticus* strains**		
NY-4	Clinical isolate O3:K6	
NY-4 : p*exsA*	Wild-type strain containing *exsA* located in plasmid pMMB207	Zhou *et al.* (2008)
Δ*exsE*	*exsE* deletion mutant	This study
ΔexsE::pKN8-P1668-e*xsE*	Δ*exsE* complemented with chromosomal insertion of pKN8-P1668-*exsE*	This study
ΔexsE::pKN8-P1668	Δ*exsE* complemented with chromosomal insertion of pKN8-P1668	This study
NY-4 : pP1668-*exsE-his*	Wild-type strain containing P1668-*exsE-His* gene fusion located in plasmid pMMB207	This study
Δ*exsE*:pP1668-*exsE-his*	Δ*exsE* containing P1668-*exsE-His* gene fusion located in plasmid pMMB207	This study
Δ*vcrD*	T3SS-deficient mutant	Zhou *et al.* (2009)
Δ*exsA*	*exsA* deletion mutant	
Δ*exsD*	*exsD* deletion mutant	Zhou *et al.* (2008)
Δ*lfgE*	Lateral flagella-deficient mutant	This study
Plasmids		
pDM4	Suicide vector with *ori* R6K *sacB* Cm^r	Milton *et al.* (1996)
pKN8	Suicide vector with *ori* R6K Cm^r *mob*$^+$ (RP4) *lacZYA*	Falker *et al.* (2005)
pKN8-P1668-*exsE*	P1668-*exsE* gene fusion located on plasmid pKN8	This study
pKN8-P1668	Promoter P1668 located on plasmid pKN8	This study
pMMB207	RSF1010 derivative with IncQ Cm^r P*tac oriT*	Morales *et al.* (1991)
pP1668-*exsE-His*	P1668-*exsE* gene fusion located on plasmid pMMB207	This study

Western blot analysis. Cells were harvested by centrifugation and the pellet was resuspended into SDS loading buffer and boiled for 5 min. Secreted proteins were collected from filtered supernatant by trichloroacetic acid precipitation (Zhou *et al.*, 2008). Protein was loaded for each sample and separated using SDS-PAGE on pre-cast 4–20 % gels (Bio-Rad) and then transferred to PVDF membrane using a mini-PROTEAN tetra system following the manufacturer's recommendations (Bio-Rad). Membranes were incubated with primary antibody (see below) for 2 h at room temperature or overnight at 4 °C, washed five times for 2 min each with PBS containing 0.05 % (v/v) Tween 20 (PBS-T) and incubated with either anti-mouse-DyLight 800 or anti-rabbit-DyLight 800 conjugate (1 : 10 000, Thermo Scientific) for 1 h at room temperature. Blots were scanned and images were obtained using the Odyssey Infrared Imaging system (LI-COR). Primary antibodies used at a 1 : 5000 dilution were anti-Vp1656 (Zhou *et al.*, 2008), anti-DnaK (Zhou *et al.*, 2010a) and anti-LC3 (Novus Biologicals).

RNA isolation and reverse transcriptase (RT)-PCR. After 4 h infection, total RNA was isolated by using a RiboPure-Bacteria kit (Ambion). Reverse transcription was performed using 500 ng RNA, 200 ng random hexamers and Superscript III (Invitrogen). PCR was performed according to the manufacturer's instructions (1 U Master *Taq* polymerase, 200 μM each of the four dNTPs and 1 μM each primer). Primer pairs for amplification of cDNA used for analysis of gene expression are listed in Table 2. Cycling parameters were identical for all primer sets: one cycle of 94 °C for 4 min; 30 cycles of 95 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min; and a final incubation at 72 °C for 5 min. Amplicons were separated using 1 % (w/v) agarose gel electrophoresis and visualized using MultiImage light cabinet and AlphaEase FC software (Alpha Innotech).

Lactate dehydrogenase (LDH)-release assay. Growth media was removed from 70–80 % confluent HeLa cell monolayers and replaced with DMEM supplemented with 1 % (v/v) FBS prior to infection at an m.o.i. of 100. After infection, cell cultures were centrifuged briefly and a 50 μl aliquot of the supernatant was removed for LDH-release assay using CytoTox 96 non-radioactive cytotoxicity assay performed as per the manufacturer's instructions (Promega).

Swarming assay. Semi-solid swarming motility agar was prepared based on the method described by Niu *et al.* (2005) using antibiotic-free LBS broth with the addition of 0.5 % (w/v) agar, 0.04 % (v/v) sterile Tween 80 and supplemented with 1 mM IPTG. A single isolated colony was selected for each strain listed and inoculated to a freshly prepared swarm agar plate and incubated at 37 °C for 8 h. Images were obtained using MultiImage light cabinet and AlphaEase FC software. The assay was repeated three times with similar results and representative photographs are shown here.

Adhesion assay. Strains cultured overnight in LBS broth were used to infect HeLa cell monolayers cultured on four-well glass slides (Nunc) in DMEM supplemented with 10 % (v/v) FBS at an m.o.i. ~100. After a 30 min incubation, slides were washed five times with sterile PBS and stained using Diff-Quik (Siemens). Slides were fixed with coverslips and images were obtained using an Axio Imager system with a 63× oil objective (Zeiss). For each biological replicate, a minimum of 20 HeLa cells were observed for each strain per well and the number of attached bacteria were enumerated by direct count.

quantitative RT-PCR (qPCR). HeLa cell monolayers were infected at an m.o.i. of 100. Over the course of infection, cells were harvested at the indicated time points and total RNA was isolated using a RiboPure-Bacteria kit (Ambion). cDNA was prepared by reverse-transcription using 2 μg RNA, 200 ng random hexamers and Superscript III (Invitrogen). qPCR was performed using SsoFast EvaGreen reagent according to the manufacturer's instructions (Bio-Rad). Primer pairs

Table 2. Primers used in this study

Primer	Sequence (5′–3′)
vp1702-1F	AGGTTACTCGAGTCCCCACGGACTGAGCGCAT
vp1702-1Rre	AGGATATCTAGAAAGACACCTAAACTC
vp1702-1Fre	AGGATATCTAGAACACAAGTTATCACGCAC
vp1702-2R	GAGTTTAGGTGTCTTACACAAGTTATCACGCAC
delta-exsEseq up	TGGCCGAAGAGACGCCTGCT
delta-exsEseq down	GGGGATCGGGCGTGAAACGC
lfgE-1F	AGGATAAACTCGAGTTCGCTACCGTGTATCAAAAAAC
lfgE-1R	CTATCCATGCAGAACTCCTAAAAAGACCTCTGATTTGCTT
lfgE-2F	AAGCAAATCAGAGGTCTTTTTAGGAGTTCTGCATGGATAG
lfgE-2R	AGTTAGTCTAGACGTGCCGACAACGCGCACAC
lfgE-F	CTTGGCTTAAAGCCGGGCAA
lfgE-R	CGCGTTGATGCAGAGTTTGTGAC
P1668F-*Xba*I	AGGATATCTAGAAATACTCATTCACTTGCACTC
P1668R-*Bam*HI	AGTTAGGGATCCAATGTAAAAAATATGCGCAATG
P1668R-*Bgl*II	AGTTAGAGATCTAATGTAAAAAATATGCGCAATG
vp1702F-*Bam*HI	AGGATAGGATCCATGTCTAATGACATCCAATC
vp1702R-*Bgl*II	AGTTAGAGATCTTTAATGGTGATGGTGATGGTGCCTTTCGCTTCGAGCAA
1656-up	AGGATAGAATTCAGGAGATATACCATGTTGGATAAAATTGGTGGAAC
1656-down	AGTTAGTCTAGATTAATGGTGATGGTGATGGTGCACTGTCGGGATAGATGCGC
1680–600-up	TCTACGTTCCATAAAGGCCAC
1680–600-down	CAACATCGCGTGACCAATGG
1686–600-up	ATTCTAAATGAAGGCAAACTCAGC
1686–600-down	GTTTAAATCCGTACTTGCGAGC
SecYF	TGGTGCTCTTGAGCGTGCATC
SecYR	CCTTGTTGACGCTTCGCGTAG
qExsA-F	TCCGTCAGCTTCCACTCTTT
qExsA-R	CTCGGGCTTGTTTTCTTTTG
qExsE-F	AACGTTTCAAGGTCGCAAAG
qExsE-R	TACCTTTCGCTTCGAGCAAT
qLafA-F	CGCAGCTATCACTGACGGTA
qLafA-R	TCCATGATACGGCCTTTAGC
qSecY-F	ACTGGCTCAGTGGTTTGGTC
qSecY-R	GGGTACGAATGCACCAGACT

used for analysis of gene expression are listed in the last eight rows of Table 2. Cycling parameters were identical for all primer sets: one cycle of 95 °C for 30 s; 38 cycles of 95 °C for 1 s, 52 °C for 5 s and 72 °C for 15 s. Reactions were performed using the CFX 96 real-time PCR system (Bio-Rad) and relative expression levels were calculated using the $\Delta\Delta C_t$ method with *secY* as the control transcript.

Mouse intrapulmonary infections. Mice were housed in micro-isolator cages and allowed *ad libitum* access to food and water. *In vivo* infections were performed as described by Piñeyro *et al.* (2010) with minor modifications. Briefly, 4–5-month-old C57-B6 mice ($n=8$ per strain) weighing 20–35 g were sedated with 150–200 mg Avertin kg^{-1} administered intraperitoneally and were restrained ventrally. An inoculum of $\sim 10^5$ c.f.u. bacteria was delivered via gavage tube just proximal to the larynx to induce aspiration. Mice were monitored every hour for 12 h and every 3 h thereafter for mortality; moribund animals were euthanized immediately with time of death documented. Lungs were collected following death or euthanasia, homogenized in sterile PBS and an aliquot was plated to thiocitrate bile salts (TCBS) agar. Recovered bacteria were strain confirmed by PCR. Animal challenge experiments conformed to a protocol approved by the Washington State University Institutional Animal Care and Use Committee.

Statistical analysis. Tests for significance were performed using NCSS (v.7.1.19; www.ncss.com/ncss.html) and the resulting data were plotted using Sigma Plot (v.11.0; www.sigmaplot.com/). One-way ANOVA using the Dunnett's two-sided multiple-comparison test versus control group was employed for LDH release, LC3 conversion, cytoadherence assays and qPCR assays. For *in vivo* mortality, Kaplan–Meier curves were plotted and statistical significance between strains was determined using the log-rank test. For all tests, $P=0.05$ was selected for determining significance.

RESULTS

ExsE is not required for T3SS1 synthesis or secretion

Vp1656 (a putative PopD orthologue) is synthesized in an ExsA-dependent manner and is secreted in a T3SS1-dependent manner (Ono *et al.*, 2006; Zhou *et al.*, 2008). Thus, Vp1656 serves as a simple marker for T3SS1-dependent secretion. We infected HeLa cell monolayers with wild-type NY-4, Δ*vcrD*, Δ*exsE*, Δ*exsE*::pKN8-P1668-*exsE*

and Δ*exsE*::pKN8-P1668 strains for 4 h before collecting whole-cell lysates and cell-free culture supernatants. All strains synthesized Vp1656 (Fig. 1a, pellet) and only the T3SS1-deficient Δ*vcrD* strain failed to secrete Vp1656 (Fig. 1a, supernatant). We also performed RT-PCR for three T3SS1 genes (*vp1656*, *vp1680* and *vp1686*) and a housekeeping gene (*secY*) following HeLa cell infection and found that transcripts were produced in all strains (Fig. 1b) although with a potential reduction in transcript levels for the Δ*vcrD* strain. Consequently, deletion of *exsE* has no apparent impact on the synthesis of T3SS1 proteins or the T3SS1-dependent secretion of Vp1656 when *V. parahaemolyticus* is co-cultured with HeLa cells.

ExsE is required for *in vitro* cytotoxicity

Epithelial cells infected with wild-type NY-4 undergo rapid cytolysis *in vitro* through a T3SS1-dependent mechanism that includes rounding and lysis (Burdette *et al.*, 2008; Zhou *et al.*, 2009). Based on the secretion and RT-PCR results (see above), we hypothesized that HeLa cells infected with Δ*exsE* would undergo cytolysis equivalent to the wild-type strain (rounding followed by lysis). Unexpectedly, the HeLa cell monolayers infected with the Δ*exsE* strain remained visibly unaffected (no rounding) up to 8 h post-infection (data not shown). LDH release after 4 h of infection was measured using NY-4 and Δ*vcrD* as positive and negative controls, respectively. LDH release from Δ*exsE* infected cells was reduced significantly compared to NY-4 and the phenotype was recovered by *in cis* complementation while the negative insertion control (Δ*exsE*::pKN8-P1668) did not induce cytolysis (Fig. 2). Consequently, ExsE appears to be requisite for T3SS1-dependent cell lysis.

ExsE is required for induction of host cell autophagy

Our results show that deletion of *exsE* results in unimpaired T3SS1 secretion (Fig. 1), but loss of T3SS1-dependent host cell cytolysis (Fig. 2). Consequently, we hypothesized that deletion of *exsE* results in a loss of effector protein translocation. To test this, we measured the induction of autophagy for infected HeLa cells. Conversion of cytosolic LC3-I to vesicle-bound LC3-II can be used to measure induction of autophagy (Kabeya *et al.*, 2000) and this assay has been used to assess induction of autophagy by *V. parahaemolyticus* (Burdette *et al.*, 2008; Zhou *et al.*, 2010b). Furthermore, VopQ (Vp1680) is necessary and sufficient to induce autophagy in HeLa cells infected with *V. parahaemolyticus* and VopQ is translocated in a T3SS1-dependent manner (Burdette *et al.*, 2009). Thus, loss of autophagy would indicate probable failure to translocate VopQ. For this experiment, NY-4 served as a positive control while Δ*vcrD* and uninfected cells served as negative controls. Deletion of *exsE* abrogated LC3-I to LC3-II conversion to a level equivalent to a T3SS1 knockout (Δ*vcrD*, Fig. 3). Complementation of *exsE* recovered the

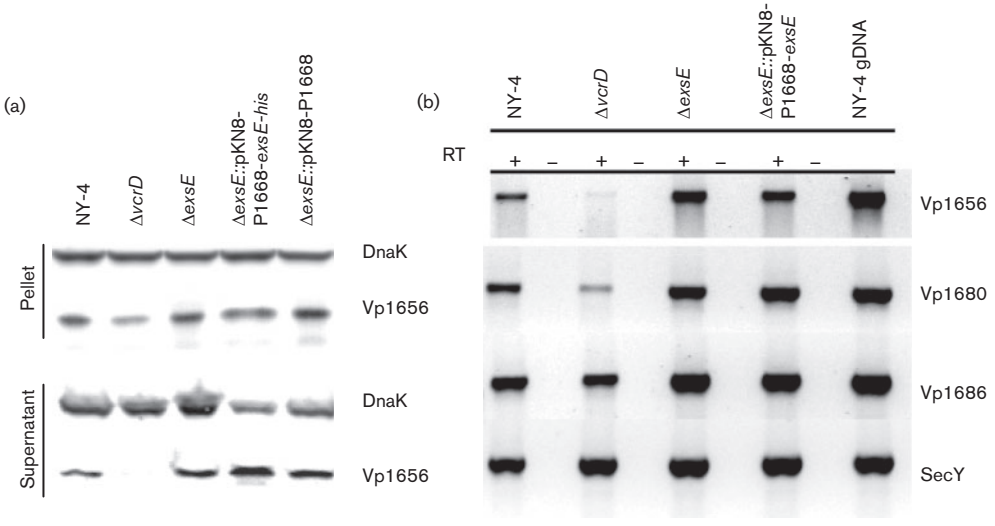

Fig. 1. Deletion of *exsE* does not negatively affect synthesis or secretion of Vp1656. (a) The indicated strains were used to infect HeLa cells cultured in DMEM supplemented with 10 % (v/v) FBS at an m.o.i. of 100 for 4 h followed by centrifugation to separate cell pellet and supernatant fractions. Cell-associated and secreted proteins obtained from whole-cell lysate and trichloroacetic acid-precipitated cell-free supernatant, respectively, were separated using 4–20 % SDS-PAGE, transferred to PVDF and probed using anti-Vp1656 and anti-DnaK antibodies. DnaK was used as a loading control. This experiment was replicated three times and a representative blot is shown here. (b) RT-PCR was used to detect mRNA transcripts for a subset of T3SS1 genes after infection. RT+ reactions included reverse transcriptase; RT− reactions contained no reverse transcriptase. The latter indicated no evidence for contaminating DNA in the reaction.

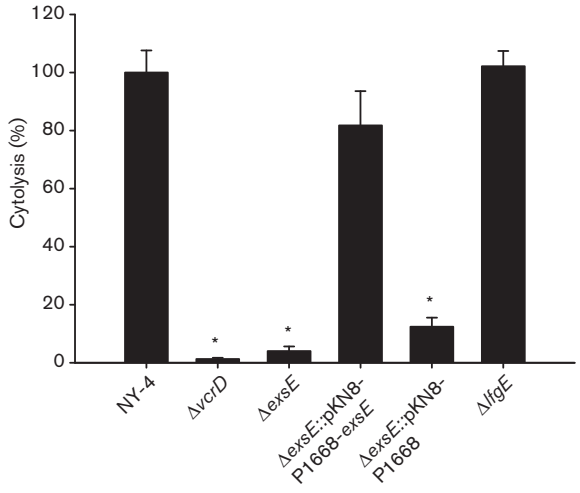

Fig. 2. Deletion of *exsE* results in loss of HeLa cell cytotoxicity. The indicated strains were used to infect HeLa cells cultured in DMEM supplemented with 1 % FBS (v/v) at an m.o.i. of 100 for 4 h and assayed for T3SS1-dependent cytotoxicity. Percentage cytotoxicity was calculated based on the release of LDH relative to the uninfected control (0 %) and wild-type NY-4 strain (100 %). The reported values represent the mean ± SEM for three independent replicates. Asterisks, statistically significant difference in mean value compared with wild-type NY-4 by one-way ANOVA ($P<0.05$).

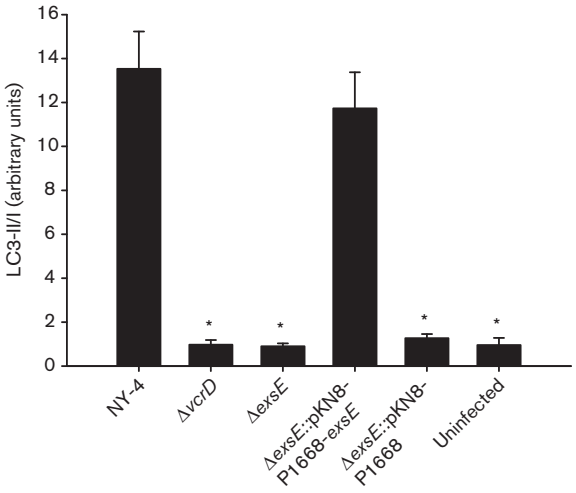

Fig. 3. Deletion of *exsE* results in loss of LC3 conversion. The indicated strains were used to infect HeLa cell monolayers at an m.o.i. of 100. Whole-cell lysates were separated using 4–20 % SDS-PAGE, transferred to a membrane and probed using anti-LC3 and anti-actin antibody. Densitometry was used to determine relative LC3-II accumulation and to calculate the LC3-II/I ratio. The reported values represent the mean ± SEM for three independent experiments. Asterisks, statistically significant difference in mean value compared with wild-type NY-4 by one-way ANOVA ($P<0.05$).

autophagy phenotype, indicating that ExsE is necessary for successful translocation of VopQ.

ExsE is required for adhesion to HeLa cells and swarming motility

Loss of translocation could result from a defective translocon apparatus or from some other change in requisite cell–cell interaction. We tested for changes in host cell adhesion (Fig. 4a) and found that Δ*exsE* exhibits a significant reduction in cytoadherence compared with NY-4 (Fig. 4b). This phenotype is recovered by *in cis* complementation indicating that ExsE is required for *V. parahaemolyticus* adherence to HeLa cells.

Lateral flagella plays a role in adherence to HeLa cells and these structures are required for a swarming phenotype (Park *et al.*, 2005; Shinoda & Okamoto, 1977). Consequently, we hypothesized that deletion of *exsE* results in a loss of lateral flagella synthesis and subsequent swarming and adhesion phenotypes due to unrestricted ExsA activity (Gode-Potratz *et al.*, 2010). To test this hypothesis we first measured swarming on semi solid agar media and found that deletion of *exsE* results in the loss of a swarming phenotype (Fig. 5) consistent with a loss of functional lateral flagella in the Δ*exsE* strain. Next we tested the hypothesis that the loss of functional lateral flagella could be responsible for lost adhesion by the Δ*exsE* strain (Fig. 4) using qPCR to measure levels of *exsA*, *exsE* and *lafA* transcripts relative to the control transcript *secY*. Time points of 0, 2 and 6 h post-infection were chosen to reflect pre-infection, peak infection (>90 % cell rounding and/or cytolysis) and post-infection (100 % cytolysis) periods. Our results show that stationary phase bacteria cultured in LBS broth have relatively low level *exsA*, *exsE* and *lafA* gene transcripts in both the wild-type and Δ*exsE* prior to infection ($t=0$). Both strains show a statistically significant increase in *exsA* and *exsE* transcript at 2 h followed by a reduction at 6 h. As expected, wild-type *lafA* transcript was relatively low at 0 h and then increased significantly at 6 h, consistent with expression of *lafA* after contact with a solid surface. Surprisingly, this pattern was also observed in the Δ*exsE* mutant that lacked a swarming phenotype after 8 h (Fig. 5). Visualization of individual Δ*exsE* cells using transmission electron microscopy (TEM) confirmed the loss of lateral flagella synthesis (Fig. S2). In addition, a lateral flagella-deficient mutant (Δ*lfgE*) was negative for swarming (data not shown) and deficient in lateral flagella (Fig. S2). This mutant also had reduced cytoadhesion compared with NY-4 (Fig. 4), although not as reduced as the Δ*exsE* strain ($P=0.005$). Despite the reduced level of cytoadhesion, the Δ*lfgE* strain was still cytotoxic (Fig. 2). These data are consistent with (1) lateral flagella playing a role in cytoadhesion, (2) a potential role for ExsE in lateral flagella biogenesis and (3) only partial cytoadherence being necessary for host-cell intoxication.

Deletion of *exsE* does not negatively affect pathogenicity *in vivo*

Based on our *in vitro* cytolysis (Fig. 2) and cytoadherence results (Fig. 4), we hypothesized that our deletion mutant

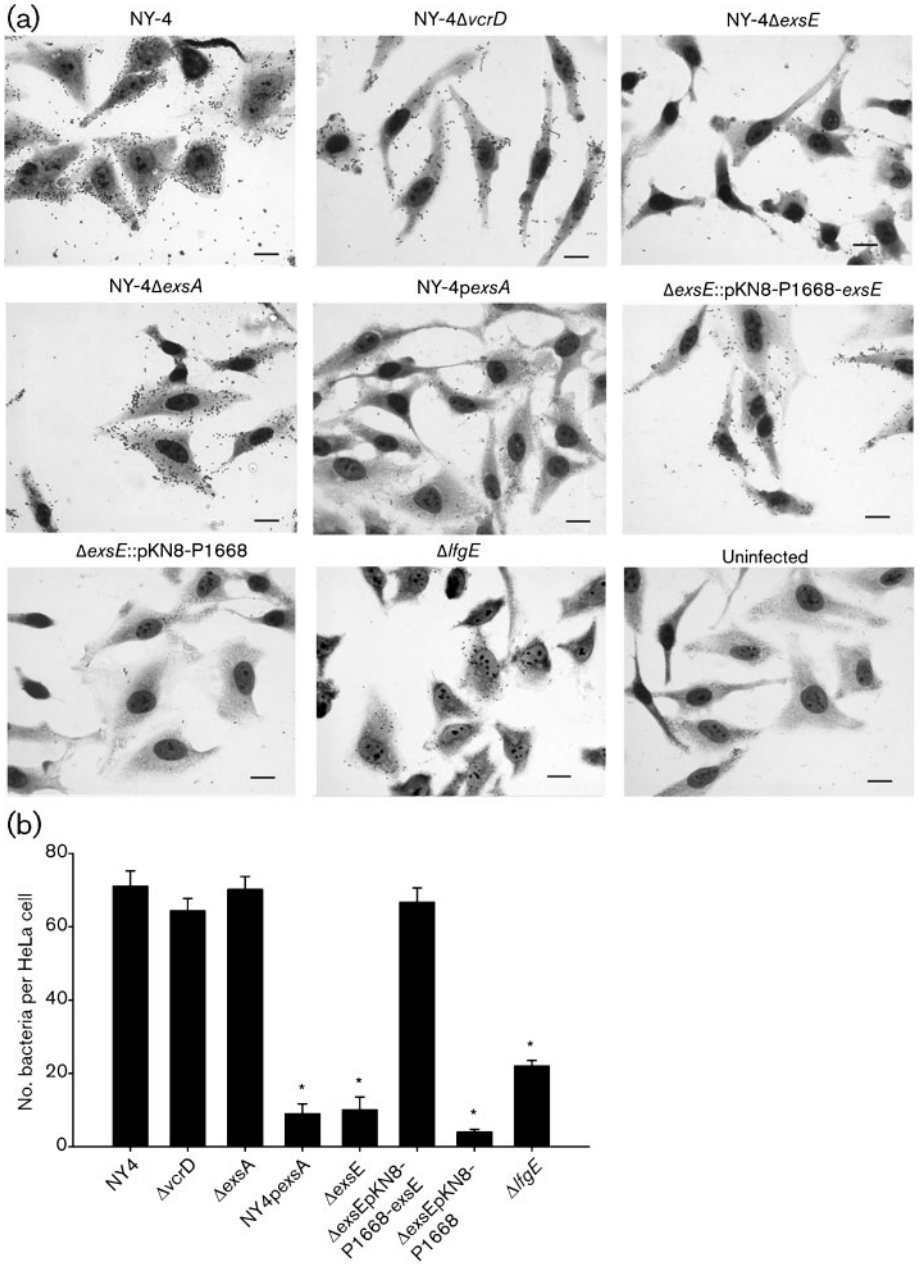

Fig. 4. Deletion of *exsE* results in loss of adhesion. The indicated strains were used to infect HeLa cell monolayers for 30 min. Slides were washed to remove non-adherent bacteria and stained using Diff-Quik. (a) Representative fields for each strain are shown here (63×). Bar, 20 μm. (b) Attached bacteria were enumerated by direct count and the reported values represent the mean ± SEM for three biological replicates. Asterisks, statistically significant difference in mean value compared with wild-type NY-4 by one-way ANOVA ($P<0.05$).

would be attenuated for an intrapulmonary infection model (Piñeyro *et al.*, 2010). Our results show no reduction in mortality compared with the wild-type strain, indicating that loss of *in vitro* cytoadhesion does not prevent T3SS1-dependent mortality in a mouse-challenge model (Fig. 6).

DISCUSSION

Many T3SSs use a regulatory cascade involving a T3SS protein at the top of the cascade leading to the activation of an AraC-like transcriptional activator at the bottom. Examples of this coupled secretion model include members of the Ysc family

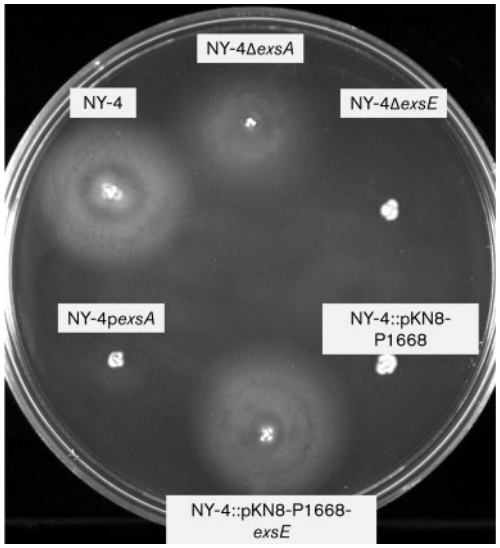

Fig. 5. Deletion of *exsE* results in a non-swarming phenotype. Swarm agar was inoculated with the indicated strains and incubated at 37 °C for 8 h. *In cis* complementation of *exsE* recovered the swarming phenotype. The assay was repeated three times with similar results and a representative photograph is shown here.

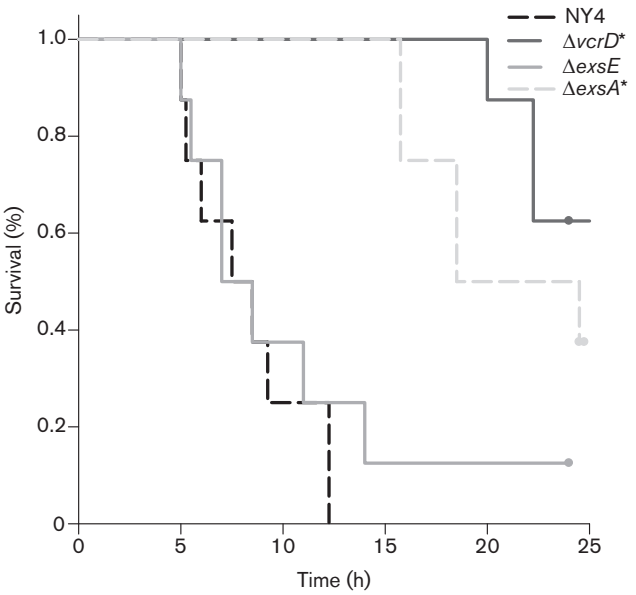

Fig. 6. Deletion of *exsE* does not affect *in vivo* lethality. Indicated strains were used to infect C57-B6 mice as described by Piñeyro *et al.* (2010). Mice were observed over the course of 24 h and mortality was recorded. Kaplan–Meier survival plots for all strains are shown here; asterisks represent values with a statistically significant difference in mortality compared with wild-type NY-4 by log-rank analysis.

of T3SSs found in *P. aeruginosa*, *Yersinia* and *Aeromonas*. In *P. aeruginosa*, ExsE indirectly regulates expression of T3SS proteins by binding ExsC, allowing ExsD to bind ExsA and prevent transcription of T3SS genes. *In silico* evaluation of the *V. parahaemolyticus* O3 : K6 genome revealed probable orthologues for *exsA*, *exsC* and *exsD* based on sequence identity (40, 34 and 30 %, respectively). Zhou *et al.* (2010a, 2008) verified that *exsA*, *exsC* and *exsD* are functional orthologues of their *P. aeruginosa* counterparts. No *exsE* homologue has been identified by sequence similarity.

ExsE is a small (81 aa) and highly charged protein that is secreted in a T3SS-dependent manner (Rietsch *et al.*, 2005; Urbanowski *et al.*, 2007). For *P. aeruginosa*, the protein is translocated into the host cell but has no defined function once inside the cell, suggesting that its major function involves regulation of T3SS expression through interaction with ExsC. Deletion of this gene leads to constitutive expression of the T3SS operon in *P. aeruginosa* and subsequent T3SS-dependent cytopathic effect when co-cultured with A549 cells (Rietsch *et al.*, 2005). Expression of an *exsE* allele resulting in an ExsE secretion and translocation-deficient mutant showed significant reduction in T3SS-dependent cytotoxicity and loss of T3SS gene expression when exposed to host cells (Urbanowski *et al.*, 2007). Based on synteny of T3SS genes found in *P. aeruginosa* and *V. parahaemolyticus* T3SSs, we and others (Kodama *et al.*, 2010) hypothesized that *vp1702* encodes the functional orthologue of ExsE. *vp1702* is similar to *P. aeruginosa exsE* in that it encodes a small (103 aa, 11.6 kDa), highly charged protein and the gene sequence is located immediately downstream of *vp1701* (*exsC*), suggesting conserved synteny with *P. aeruginosa*.

We created the *vp1702*-deletion mutant by gene knockout and found constitutive T3SS1 protein synthesis and secretion in broth culture (Fig. S3) similar to findings by Kodama *et al.* (2010), and this is analogous to the function of ExsE in *P. aeruginosa*. Overexpression of ExsE in wild-type NY-4 using a plasmid-based expression vector repressed T3SS1 upregulation, which is also consistent with the role of ExsE as a negative regulator of T3SS1 (Fig. S1). It was therefore unexpected to find that the *V. parahaemolyticus* Δ*exsE* strain was not cytotoxic compared with the highly cytotoxic wild-type strain (Fig. 2).

Two important *V. parahaemolyticus* effector proteins have been described, VopQ and VopS, that are responsible for inducing autophagy, activating MAPK signalling pathways, and actin destabilization in epithelial cells (Broberg *et al.*, 2010; Burdette *et al.*, 2009; Casselli *et al.*, 2008; Matlawska-Wasowska *et al.*, 2010; Yarbrough *et al.*, 2009). Autophagy and cytoskeleton destabilization phenotypes are readily observed through Western blot analysis that shows conversion of LC3-I to LC3-II, or by visualization of cell rounding, respectively. Both effectors require T3SS1-dependent translocation to induce these phenotypes and therefore they are ideal markers for measuring T3SS1-dependent translocation. Deletion of *exsE* does not affect T3SS1-dependent secretion of our marker protein Vp1656 (Fig. 1a), and

transcription of *vopQ* and *vopS* appears to be unaffected (Fig. 1b). Nevertheless, deletion of *exsE* eliminates cell lysis (Fig. 2), cell rounding (data not shown) and LC3 conversion (Fig. 3), indicating that ExsE is required for translocation of *V. parahaemolyticus* effector proteins.

There are several possibilities for why we see normal T3SS1 induction during HeLa cell infection (Fig. 1) but apparent loss of effector translocation. Firstly, ExsE may be a structural component of the T3SS1 needle or translocon. Presumably, only a minimally formed needle structure is sufficient to secrete proteins into the extracellular milieu. V-tip proteins are found in the Ysc family of T3SSs (reviewed by Sato & Frank, 2011) but there is no apparent sequence homology with Vp1702. Another possibility is that ExsE may act as a chaperone required for effective stabilization of an unidentified protein needed for translocon functionality, but we have no evidence for this role. VopQ is necessary and sufficient to induce autophagy (Broberg *et al.*, 2010; Burdette *et al.*, 2009) and thus it is unlikely that ExsE is functionally partnered with this protein. In any case, the basal body and at least a portion of the needle complex are apparently assembled, as shown by the secretion of Vp1656 into cell culture supernatant. This leaves the possibility that ExsE impacts other factors that are required for successful HeLa-cell cytolysis.

Synthesis of lateral flagella, encoded by the *laf* regulon in *V. parahaemolyticus*, is associated with swarming motility, adherence and cytotoxicity (Belas & Colwell, 1982a; Belas & Colwell, 1982b; Gode-Potratz *et al.*, 2011; McCarter, 2004; Park *et al.*, 2005). Cross-talk between bacterial T3SSs and flagellar systems is a common phenomenon (Bleves *et al.*, 2002; Diaz *et al.*, 2011; Eichelberg & Galán, 2000; Iyoda *et al.*, 2006; Soscia *et al.*, 2007). *In trans* expression of ExsA in *V. parahaemolyticus* reduces *laf* gene expression and swarming motility through an undefined mechanism (Gode-Potratz *et al.*, 2010) but does not negatively affect cytolysis (Zhou *et al.*, 2008). Thus the reduction in swarming (Fig. 5) and adhesion observed with Δ*exsE* in this study (Fig. 4) may simply be a result of unmitigated basal level ExsA activity leading to suppression of the *laf* regulon. We tested this hypothesis using the NY-4 : p*exsA* strain and found reduced swarming motility (Fig. 5) and cytoadhesion (Fig. 4) as expected. Deletion of *exsA*, however, had no effect on either cytoadhesion or swarming phenotypes (Figs 4 and 5, respectively). It is interesting that our NY-4 : p*exsA* strain also retains wild-type level cytolysis despite a significant decrease in cytoadhesion. We speculate that this is due to the artificially high level of ExsA in NY-4 : p*exsA* prior to infection, which probably results in an abnormal abundance of assembled and functional T3SS1 components (including both structural and effector proteins) such that only minimal cell–cell contact is necessary for successful translocation of effector proteins. This is in contrast with the wild-type and Δ*exsE* strains that require intimate host cell contact to both upregulate *exsA* expression (Fig. 7) and subsequently translocate effectors to the host cell.

Next, we tested a lateral flagella-deficient mutant and found that cytoadhesion was reduced compared with NY-4, yet was significantly higher than Δ*exsE* (Fig. 4, $P=0.005$). Cytolysis was unaffected with Δ*lfgE* compared with NY-4, suggesting that the number of adherent bacteria, while reduced compared with the wild-type strain, was sufficient to intoxicate HeLa cells (Fig. 2). Nevertheless, while transcription of *lafA* was unaffected by deletion of *exsE* (Fig. 7), neither lateral nor polar flagella were observed by TEM (Fig. S2). We did find, however, that *lafA* was highly upregulated at 6 h for both wild-type and Δ*exsE* (Fig. 7), which is after the point when most, if not all, HeLa cells have been lysed by *V. parahaemolyticus* (Fig. 2). Thus, *lafA* appears to be upregulated post-infection. This is consistent with data from Gode-Potratz *et al.* (2010) who hypothesized that increased ExsA expression during host cell

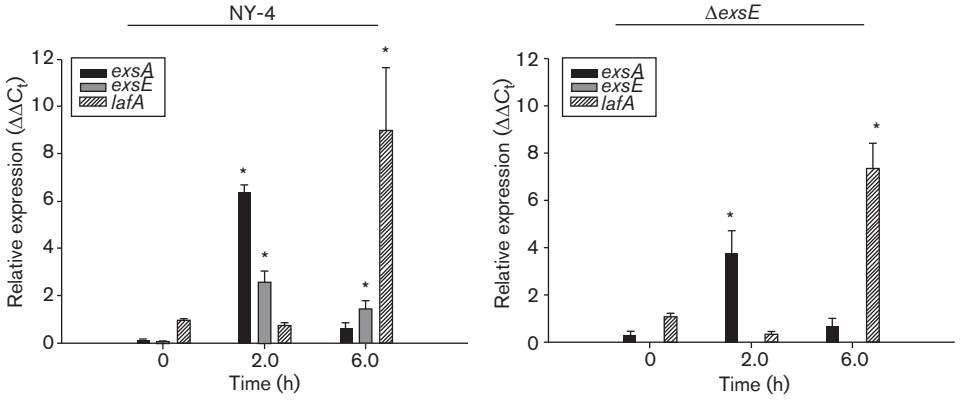

Fig. 7. Deletion of *exsE* does not affect relative transcription levels of *exsA* or *lafA*. HeLa cell monolayers were infected with either NY-4 or Δ*exsE* at an m.o.i. of 100. Total RNA was extracted and cDNA was synthesized for qPCR analysis as described in the main text. Reported values represent the mean ± SEM for relative expression level of four biological replicates at each time point. Asterisks, statistically significant difference in mean value compared with $t=0$ h by one-way ANOVA.

intoxication is coordinated with reduced swarming motility. After infection, swarming motility would be induced, presumably, to assist dispersal of *V. parahaemolyticus*. Deletion of *exsE* still prevents swarming with the agar plate assay and there were no observable lateral flagella, although this could be related to overexpression of ExsA in this assay [we did not measure mRNA levels for bacteria grown on agar, which enhances T3SS1 gene expression (Gode-Potratz *et al.*, 2011)]. The actual mechanism of how lateral flagella biogenesis is affected by ExsA remains unclear, though our results suggest it may occur post-translationally or at the protein level. Importantly, deletion of *exsE* dramatically impacts T3SS1-dependent cytolysis and this is consistent with a loss of adhesion (Fig. 4b), likely due in part to the loss of lateral flagella and possibly from loss of the polar flagella (Fig. S2). Additional adhesins might be affected by ExsE [e.g. MAM7 (Krachler & Orth, 2011)] or it is possible that ExsE itself must be translocated into the host cell to affect secure adhesion and subsequent translocation of effector proteins.

Kodama *et al.* (2010) reported that *vp1702* may encode a functional orthologue of *P. aeruginosa* ExsE and they showed that Vp1702 acts in a similar regulatory capacity for T3SS1 in *V. parahaemolyticus* as it does in *P. aeruginosa*. Kodama *et al.* (2010) measured synthesis and secretion using broth culture consisting of LBS with and without the addition of 5 mM $CaCl_2$, but they did not examine the effect of host cell co-culture and contact. In contrast, we found that while growth under high-calcium conditions (5 mM) results in an apparent decrease in T3SS1 synthesis, it does not block host cell cytolysis (Fig. S4). Zhou *et al.* (2008) showed previously that growth in LBS alone does not induce T3SS1 gene expression and we found the same results for this study (Figs 7 and S3). The difference between the current study and that of Kodama *et al.* may be due to use of different strains of *V. parahaemolyticus*, or due to reliance on LBS to test the induction of T3SS1; Luria Broth is a complex and undefined media that probably introduces a variety of confounding variables that vary between batches and manufacturers (Pavankumar *et al.*, 2012). Observations from the current study support findings with *P. aeruginosa* where treatment of A549 epithelial cells with calcimycin was used to increase intracellular calcium concentration, but this had no effect on expression of *exoS*, suggesting that cytoplasmic calcium concentrations may not play a significant role in effector induction (Cisz *et al.*, 2008). Alternatively, physical contact is necessary and sufficient to enhance expression of T3SS genes subsequent to cytotoxicity for both *V. parahaemolyticus* and *P. aeruginosa* (Gode-Potratz *et al.*, 2011; Vallis *et al.*, 1999). Unlike the LB broth model, host cell co-culture and contact appears to be an unambiguous induction model for T3SS synthesis and function.

The data presented herein provide further characterization of the putative ExsE of *V. parahaemolyticus*. Importantly, besides acting as a functional orthologue to ExsE in *P. aeruginosa*, we demonstrate that the putative ExsE from *V. parahaemolyticus* is a T3SS1-associated protein necessary for regulating cytoadhesion that is apparently required for the translocation of effector proteins and subsequent T3SS1-dependent cytolysis *in vitro*. To our knowledge, this is the first report of an association between the T3SS1-regulatory protein ExsE and a bacterial cytoadhesion phenotype. In addition, we report here for the first time expression kinetics for genes encoding the T3SS1 regulatory proteins ExsA and ExsE that support the coupled-secretory induction model. These data also suggest the presence of a host cell contact-dependent signal for upregulation of *exsA* expression that is independent of the ExsACDE pathway, and this signal has yet to be identified.

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