RESEARCH LETTER

Translocation of *Vibrio parahaemolyticus* across an *in vitro* M cell model

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Abstract

Consumption of *Vibrio parahaemolyticus* via contaminated shellfish results in inflammatory gastroenteritis characterised by severe diarrhoea, nausea and stomach cramps. This study investigated the translocation of *V. parahaemolyticus* across a Peyer's patch M cell-like Caco-2/Raji B co-culture model system, as M cells represent a primary site of infection for many pathogenic bacteria. *Vibrio parahaemolyticus* translocated across co-culture monolayers in higher numbers as compared to Caco-2 monolayers. Moreover, the bacteria induced a greater disruption of the transepithelial resistance in M cell-like co-cultures than in Caco-2 monocultures. Virulence factors associated with this pathogen include two type three secretion systems (TTSS-1 and TTSS-2). TTSS-1 had no effect on translocation efficiency, with TTSS-2 exhibiting a modest enhancing effect. ERK activity was required for optimal translocation 1 h postinfection, however, neither ERK nor the JNK and p38 MAPK were required at 2 h pi. Additionally, TER disruption in response to bacterial infection occurred independently of the TTSS and MAPK activation. It was concluded that *V. parahaemolyticus* causes TER disruption of M cell-like co-cultures and translocates in high numbers across the M cell-like co-culture monolayer. These data implicate M cells as important sites for *V. parahaemolyticus* invasion across the intestinal epithelium during infection.

Introduction

The human gastrointestinal pathogen *Vibrio parahaemolyticus* is a Gram-negative bacterium whose natural habitat is marine and estuarine sediment (Daniels *et al.*, 2000; Makino *et al.*, 2003). Infection is characterised by severe gastroenteritis following consumption of contaminated, uncooked shellfish. Infection of the host epithelium by *V. parahaemolyticus* is associated with the presence of two haemolysins and two type three secretion systems, namely TTSS-1 and TTSS-2. While TTSS-1 is involved in the cytotoxic effects of the bacterium, TTSS-2 is responsible for bacterial enterotoxicity (Park *et al.*, 2004a, b).

The intestinal monolayer is an important defensive barrier following the consumption of contaminated seafood (Catalioto *et al.*, 2011). Disruption of this barrier results in an increase in intestinal permeability and has been associated with a number of different disease states, including inflammatory bowel disease, ulcerative colitis and various diarrhoeal diseases caused by gastrointestinal pathogens (Strauman *et al.*, 2010; Salim & Soderholm, 2011). Specialised sampling sites found along the length of the intestinal tract, such as the M cells of the follicle-associated epithelium (FAE) found overlaying the intestinal Peyer's patches, facilitate the delivery of foreign material across the intestine *via* active transport. The M cell transport system appears to be the key to the pathogenesis of certain bacterial and viral diseases (Neutra *et al.*, 1996; Siebers & Finlay, 1996). To study the M cell phenotype *in vitro,* three Caco-2:B lymphocyte co-culture models have been developed with slightly different constructions (Kerneis *et al.*, 1997; Gullberg *et al.*, 2000; des Rieux *et al.*, 2007). The Caco-2/Raji B construct of Gullberg *et al.*, with some modifications, was utilised in

this study to investigate the transport of *V. parahaemolyticus* across the intestinal epithelium.

Previous studies using *Salmonella enterica* and *Escherichia coli* demonstrated the role of the TTSS in translocation across the co-culture model. *Salmonella enterica* serovar Typhimurium translocated across Caco-2 monolayers in reduced numbers compared to numbers translocating across the co-culture model (Martinez-Argudo & Jepson, 2008). Mutation of either the SPI-1 or SPI-2 secretion systems did not attenuate the ability of the bacteria to translocate across the co-culture model. In contrast, enteropathogenic *E. coli* (EPEC) translocate across both Caco-2 monolayers and co-culture models in comparably low numbers (Martinez-Argudo *et al.*, 2007). Mutation of the TTSS resulted in increased numbers of translocated bacteria suggesting that, in this instance, the TTSS play an inhibitory role. Studies have demonstrated that viable *Vibrio cholerae* is transported across rabbit intestinal M cells (Owen *et al.*, 1986). *Vibrio cholerae* were also translocated across an M cell-like model, and translocation was enhanced 100- to 1000-fold by cholera toxin binding to the GM1 receptor (Blanco & DiRita, 2006). The *V. cholerae* strains employed did not possess TTSS, while *V. parahaemolyticus* does not possess cholera toxin. Therefore, we expected differences in the interaction of each *Vibrio* species with M cells. This study aimed to investigate the translocation of *V. parahaemolyticus* and the role of the TTSS in the transport of the bacterium across co-culture models *in vitro*. The effects of *V. parahaemolyticus* on the MAPK signalling pathways were also investigated as the bacteria interfere with the MAPK cascades in Caco-2 cells in a TTSS-1-dependent manner (Matlawska-Wasowska *et al.*, 2010).

Materials and methods

All chemicals and reagents were obtained from Sigma unless otherwise stated.

Vibrio parahaemolyticus strains

Vibrio parahaemolyticus, RIMD2210633, O3:K6 serotype (Makino *et al.*, 2003) was utilised as the parental strain for mutant construction and as the wild-type (wt) strain. ΔTTSS-1 possessed a mutation in the *vscN1* gene encoding the ATPase for TTSS-1, and ΔTTSS-2 possessed a deletion in the *vscN2* gene encoding the ATPase for TTSS-2 (Matlawska-Wasowska *et al.*, 2010).

Bacterial culture

Vibrio parahaemolyticus was grown at 37 °C in Luria–Bertani medium (10.0 g L^{-1} tryptone, 5.0 g L^{-1} yeast extract, 10.0 g L^{-1} sodium chloride) supplemented with 3% (w/v) NaCl (LBN) and the addition of 1.5% (w/v) agar where appropriate.

Eukaryotic cell culture

The Caco-2 cell line (86010202) and the human Burkitt's lymphoma B cell line, Raji (85011429), were obtained from the European Collection of Animal Cell Cultures, Salisbury, UK. Caco-2 cells were maintained in DMEM supplemented with 10% foetal bovine serum (FBS), Pen-Strep (100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin) and 1% nonessential amino acids. Raji B cells were maintained in RPMI supplemented with 10% FBS, Pen-Strep and 1% nonessential amino acids. Both Caco-2 and Raji cells were used between passages 1–10. Medium was changed every other day.

Co-culture induction

Caco-2 cells were seeded onto the apical surface of Matrigel™ Basement Membrane Matrix (Becton Dickinson, Bedford, MA)-coated Transwell® inserts (12 mm diameter, 3.0 μm pore size, polyester; Corning, Costar) at a density of 300 000 cells per filter and grown for 21 days at 37 °C/5% CO_2, until fully differentiated. Medium was replaced every other day. Raji B cells (resuspended in RPMI : DMEM 1 : 2) were added to the basolateral compartment of 14- to 16-day-old Caco-2 cell monolayers at a density of 500 000 cells per well and maintained for 5–6 days. Transepithelial resistance (TER) was monitored throughout this period as a measure of monolayer integrity. TER was measured using the EVOM meter and STX2 electrode set (World Precision Instruments, UK).

Particle transport

Carboxylated latex particles, with mean diameters of 0.5 and 1.0 μm (Molecular Probes) and labelled with FITC and Nile red, respectively, were used in particle transport studies. Latex particles were suspended in Hank's balanced salt solution (HBSS) supplemented with 5.5 M glucose and buffered to pH 7.4 with 25 mM HEPES, such that each monolayer was exposed to 2.5×10^8 of 0.5 and 1.0-μm particles. After equilibration, the HBSS on the donor apical side of the monolayer was replaced with prewarmed particle suspension. Particle transport was studied after a 2-h period by receiver basolateral chamber sampling. After establishing standard curves, the number of particles transported across cell monolayers was enumerated by a Dako CyAn ADP flow cytometer (Beckman Coulter).

Preparation of bacteria for infection studies

Bacteria were grown to mid-log phase in LBN at 37 °C with agitation. The bacteria were washed with PBS, and OD_{600} values were measured to determine bacterial numbers (O'Boyle *et al.*, 2013).

Chemical MAPK inhibitors

Inhibitors of the JNK (SP600125), p38 (SB203580) and ERK1/2 (PD98059) pathways were used at the following concentrations: 15 μM SP600125, 5 μM SB203580 and 40 μM PD98059. Inhibitors were added to the apical chamber of the transwell 2 h preinfection and maintained throughout the experiment.

Passage of bacteria across the intestinal epithelial monolayer

Cells were washed twice with PBS, and fresh DMEM minus Pen-Strep was added 2 h before infection. In all experiments, cells were infected at a multiplicity of infection (MOI) = 5. Bacteria were added to the apical chamber of the inserts, and monolayers were incubated at 37 °C for 1 h with agitation. Following incubation, all basolateral medium was removed and replaced with prewarmed DMEM minus Pen-Strep. The monolayers were incubated at 37 °C for a further hour with agitation, and the basolateral medium was again collected. The basolateral medium was serially diluted and plated on LBN. TER was measured at 0-, 1- and 2-h time points. All LBN plates were incubated at 37 °C overnight. Colony-forming units (CFU) were counted for each experimental parameter. Data were analysed using Graph Pad Prism® software.

Statistical analysis

All experiments were carried out three times in duplicate or triplicate, unless otherwise stated. Data are expressed as mean ± standard error of mean (SEM). Significances of differences between mean values were assessed using the two-tailed, unpaired Student's t-test or one-way ANOVA with Tukey *post hoc* test where appropriate, with significance set as $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ ***.

Results and discussion

V. parahaemolyticus translocates across the *in vitro* co-culture model

The passage of *V. parahaemolyticus* across the M cell-like co-culture model vs. Caco-2 monolayers was investigated. First, the translocation of fluorescently labelled particles across both the monolayers and co-cultures was investigated to confirm *in vitro* induction of the M-like phenotype in the latter. After 2 h, an 18- and 16-fold increase in 1- and 0.5-μm particle transcytosis, respectively, were observed in the co-cultures, when compared to transcytosis across the Caco-2 monolayer (Fig. 1a). These values

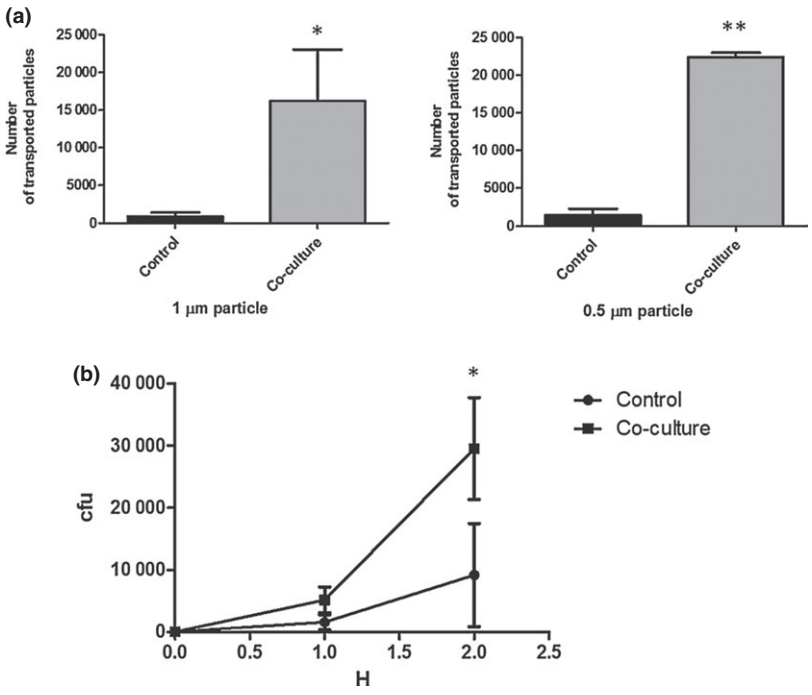

Fig. 1. Passage of *Vibrio parahaemolyticus* across the co-culture model vs. Caco-2 cell monolayers. The co-culture model was induced as described in the Materials and methods. Caco-2 monolayers grown on permeable supports were used as a control. Microparticles were added to the apical surface of the transwell, and basolateral medium was collected after 2 h to determine particle transcytosis. 1- or 0.5-μm particles were utilised (a). Cells were infected with wt bacteria at an MOI = 5. Basolateral medium was collected 1 and 2 h postinfection and the CFU determined (b). Two-tailed, unpaired, Student's *t*-tests were carried out with $P < 0.05$ *, $P < 0.01$ **.

are in agreement with the reported value of 14-fold for similarly sized particles in former studies (Martinez-Argudo *et al.*, 2007). These data highlight the significant sampling ability of M-like cells, thereby confirming the successful induction of the co-culture model *in vitro*.

Examination of the transcytosis of *V. parahaemolyticus* indicated an increase in bacterial translocation across the co-cultures compared to the Caco-2 monolayers following 1 and 2 h of infection (Fig. 1b). One hour postinfection, transcytosed bacterial numbers were 3.3-fold higher in the co-cultures with a 3.2-fold difference between Caco-2 monolayers and co-cultures observed following 2 h of infection. Comparison of translocation of *V. parahaemolyticus* across the co-culture and control demonstrated a 5.7- and 5.8-fold increase, respectively, between the 1- and 2-h time points. The data indicate that while *V. parahaemolyticus* has the ability to translocate across both the control and co-culture models 1 h postinfection with a dramatic increase in translocation observed 2 h postinfection, it translocates across the co-culture model in significantly increased numbers when compared to passage across the Caco-2 monolayer. This suggests that bacteria are taken up by sampling by the M-like cells allowing for passage of bacteria across the co-culture in greater numbers than across the Caco-2 monolayer. Translocation of bacteria across both monolayers may also be occurring partly by an active invasion mechanism, and although this requires further investigation, it explains the relatively high number of bacteria translocated by Caco-2. Compared to viable bacteria, a severe reduction in transport of heat-killed *Salmonella* was previously observed (Martinez-Argudo & Jepson, 2008), suggesting a role for bacterial-directed invasion in the translocation process.

Late stage *V. parahaemolyticus* translocation occurs independently of MAPK activity

Previous studies have shown that *V. parahaemolyticus* activates the intracellular MAPK signalling pathways to exert its effects on host cells. As a result, we investigated the role of MAPK activation in the bacterial translocation across M cell-like co-cultures. Immunoblotting experiments demonstrated that the MAPK was endogenously activated in uninfected co-cultures and therefore no increased activation was observed upon infection with *V. parahaemolyticus* (data not shown). To determine whether the MAPK pathways are involved in bacterial translocation across the co-culture model, cells were pretreated with MAPK inhibitors (15 μM SP600125, 40 μM PD98059 and 5 μM SB203580, which inhibit the JNK, p38 and ERK pathways, respectively) 2 h prior to infection and maintained throughout the experiment. Co-cultures treated with SP600125, PD98059 and SB203580 displayed 1.2-, 6.6- and 2.0-fold decreases in translocation, respectively, 1 h postinfection (Fig. 2a). Two hour postinfection, co-cultures treated with SP600125 and PD98059 displayed a 1.3- and 1.7-fold decrease in translocation, respectively, while cells treated with SB203580 displayed a 1.8-fold increase in bacterial translocation (Fig. 2b). Statistical analysis of the data concludes that only the differences observed between untreated wt-infected co-cultures and those-treated with the ERK pathway inhibitor at 1 h postinfection are significant. The ERK signalling pathway is one of the most important in eukaryotic cells with roles in cell proliferation, differentiation and survival. PD98059 specifically inhibits the phosphorylation of ERK by inhibiting the activity of upstream MEK1/2, with limited off-target effects (Davies *et al.*, 2000). These data indicate that

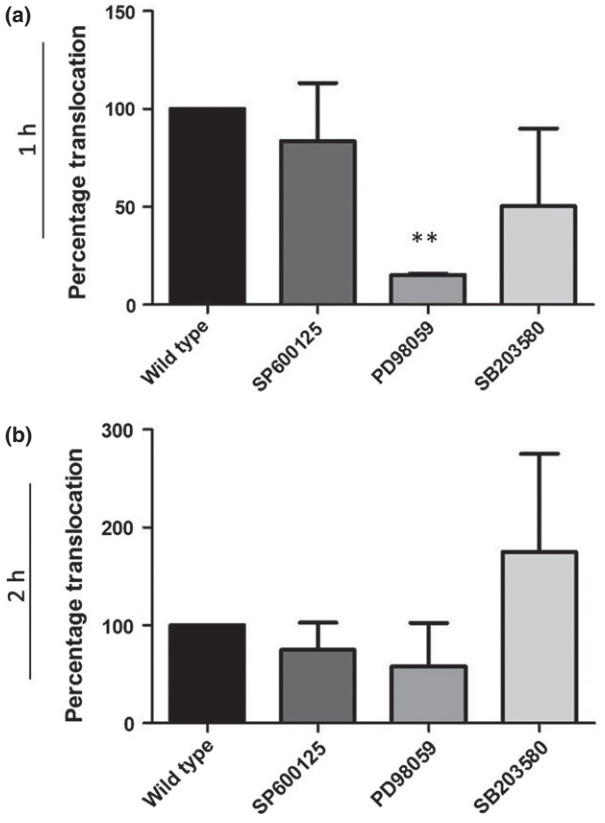

Fig. 2. *Vibrio parahaemolyticus* translocation occurs independently of MAPK activity during the later stages of infection. Co-cultures were pretreated with MAPK inhibitors 2 h prior to infection at concentrations of 15 μM SP600125, 40 μM PD98059 and 5 μM SB203580. Cells were consequently co-incubated with wt *V. parahaemolyticus* for 2 h at MOI = 5. Basolateral medium was collected and plated 1 h (a) and 2 h (b) postinfection. Average absolute values relating to 100% wt translocation in the absence of MAPK inhibition were 1.3×10^4 CFU per well at 1 h and 1.3×10^5 CFU per well 2 h postinfection. One-way ANOVA with Tukey *post hoc* test was carried out with $P < 0.01$ **.

ERK activity plays a role in the translocation of *V. parahaemolyticus* across the co-culture model during the early stages of infection.

The bacterial TTSS enhance, rather than inhibit, translocation across the co-culture model

Studies investigating enteropathogenic *E. coli* have demonstrated that the bacterial TTSS inhibit the translocation of the bacteria across co-cultures, therefore, the influence of *V. parahaemolyticus* TTSS on M cell-like co-culture translocation was investigated (Martinez-Argudo *et al.*, 2007). Individual single TTSS mutants were employed as previous studies have indicated that each TTSS delivers unique effectors into the host cell and each mediates unique effects on the host cell and *in vivo* (Park *et al.*, 2004a, b; Hiyoshi *et al.*, 2010; Matlawska-Wasowska *et al.*, 2010). The ΔTTSS-1 strain displayed a similar translocation efficiency as the wt. Following 1 and 2 h of co-incubation, the ΔTTSS-2 strain displayed a 2.6- and 1.6-fold decrease in translocation, respectively, compared to wt, although these decreases were not statistically significant (Fig. 3). These data demonstrate that the TTSS do not inhibit *V. parahaemolyticus* translocation. Instead the bacteria are transported across the M cell-like co-culture model independently of TTSS-1, while TTSS-2 has a modest enhancing effect on translocation at early stages of infection.

Disruption of the transepithelial resistance across the co-culture model coincides with *V. parahaemolyticus* transcytosis

To investigate whether *V. parahaemolyticus* translocates across the M cell-like co-culture model by disrupting the epithelial monolayer, the TER was measured in response to infection with wt, ΔTTSS-1 or ΔTTSS-2 bacteria. Measurement of the TER is one of the main ways to examine epithelial integrity *in vitro* (Terres *et al.*, 1998) as it represents the resistance to ion flow across the epithelial monolayer. Infection of the co-culture model with the wt bacteria resulted in a sharp decrease in TER 1 h postinfection with a further decrease observed 2 h postinfection (Fig. 4a). Similar decreases were detected for the ΔTTSS-1 and ΔTTSS-2 bacteria. Consequently, examination of the effects of *V. parahaemolyticus* on the TER of the M cell-like co-culture model indicates that the disruption occurs independently of either TTSS-1 or TTSS-2. Infection of the Caco-2 monolayer with wt bacteria also resulted in a decrease in TER (Fig. 4b). Comparison of these data indicates that *V. parahaemolyticus* infection results in an increase in TER disruption in co-culture models when compared to Caco-2 monolayers. Although not

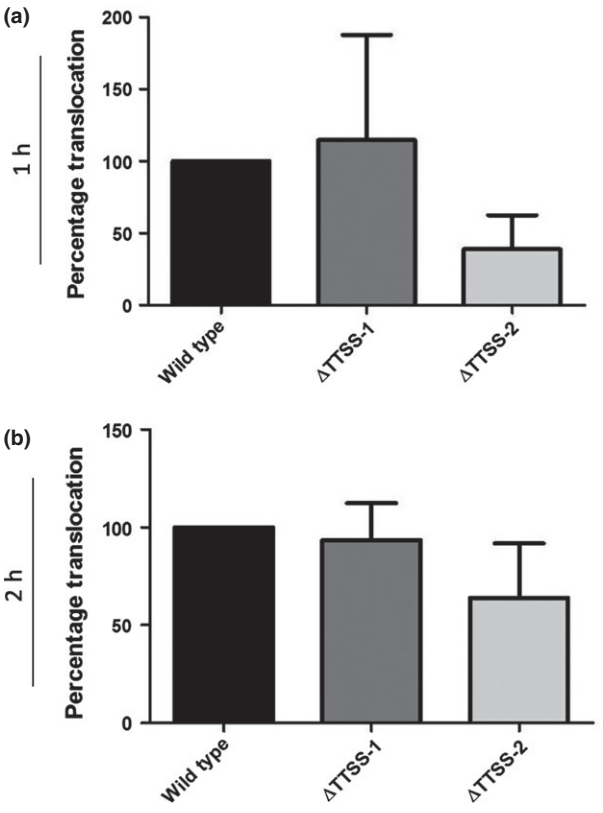

Fig. 3. Role of the TTSS in the passage of *Vibrio parahaemolyticus* across the co-culture model. Co-cultures were infected with wt bacteria and secretion system mutants at MOI = 5. Basolateral medium was collected and plated 1 h (a) and 2 h (b) postinfection. Results for the wt-infected co-cultures were taken as 100% (1.1×10^4 CFU per well at 1 h and 4.4×10^5 CFU per well 2 h postinfection). One-way ANOVA with Tukey *post hoc* test was performed.

statistically significant, the difference in TER decrease between Caco-2 and co-cultures was detected consistently. To determine whether MAPK activation has a role in the effects elicited by the bacteria on the co-culture, disruption of the TER in response to wt infection in the presence of MAPK inhibitors was examined. There was minimal difference between untreated co-cultures and co-cultures treated with the MAPK inhibitors (Fig. 4c). These nominal differences demonstrate that MAPK activation is not necessary for the disruption of the co-culture model in response to *V. parahaemolyticus* infection.

Comparison of Caco-2 monolayers with a co-culture M cell model in this study indicates that *V. parahaemolyticus* is translocated in increased numbers (threefold increase) across the co-culture model. In the intestine, Peyer's patch M cells actively endocytose bacteria and other foreign material for delivery to underlying

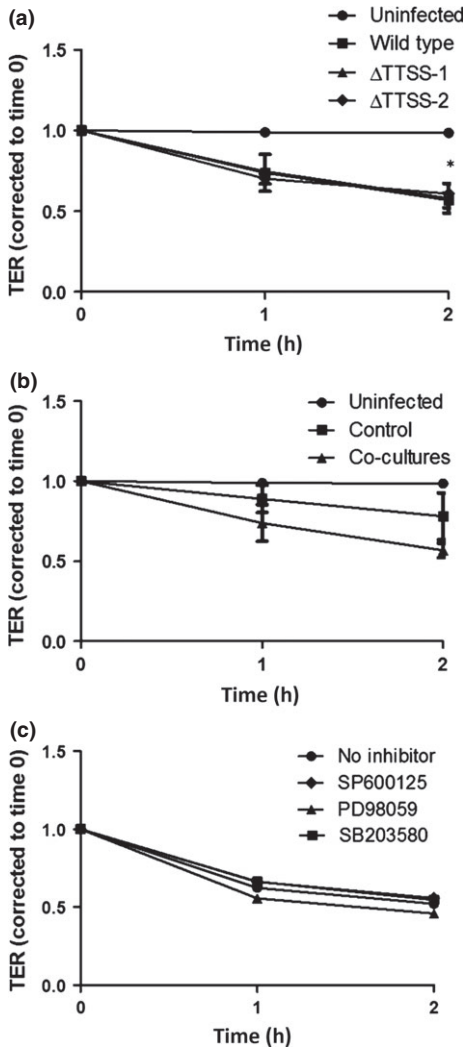

Fig. 4. Disruption of the transepithelial resistance in response to *Vibrio parahaemolyticus* infection. Co-cultures were infected with wt bacteria and secretion system mutants at MOI = 5. Uninfected co-cultures were used as a control (a). Caco-2 monolayers and co-culture models were infected with wt bacteria at MOI = 5 (b). Co-cultures were pretreated with chemical MAPK inhibitors 2 h prior to infection with wt bacteria (c). TER was measured 1 and 2 h postinfection. Basal TER readings were taken prior to infection with average co-culture TER readings of 520 Ohm.cm^2 and average Caco-2 monolayer TER readings of 657 Ohm.cm^2. Basal readings (time 0) were corrected to 1, and subsequent data were plotted as a ratio of time 0. One-way ANOVA with Tukey *post hoc* test was carried out with $P < 0.05$ *.

lymphocytes, and this intracellular translocation would be the principal explanation for the observed increases (Neutra *et al.*, 1996; Siebers & Finlay, 1996; Wong *et al.*, 2003; Jang *et al.*, 2004; Brayden *et al.*, 2005). Enhanced transport of other M cell tropic bacteria such as *Salmonella* across an *in vitro* co-culture model (Martinez-Argudo & Jepson, 2008) and invasion through murine Peyer's patches (Jones *et al.*, 1994) M cells has previously been documented. In the former instance, an upregulation of 9- to 40-fold higher translocation in co-cultures vs. controls was recorded. For *V. cholerae* possessing cholera toxin (*ctx*$^+$), a sixfold increase in bacterial translocation was observed between M cell-like and Caco-2 cells (Blanco & DiRita, 2006). While a direct comparison of the *V. cholerae* and *V. parahaemolyticus* data is not possible due to differing experimental conditions (e.g. moi = 80 and 5, respectively), the increase is similar between the species. The eightfold increase in *V. parahaemolyticus* translocation between the 1- and 2-h time points is also reflective of the situation in *V. cholerae,* where a 13-fold increase was observed. Interestingly, unlike the *ctx*$^+$ strain, *ctx*$^-$ *V. cholerae* did not cause a drop in TER, and furthermore, translocation was much reduced and did not increase between 1 and 2 h. We have shown here that translocation of *V. parahaemolyticus* coincides with TER disruption. The proteins responsible for the translocation and TER disruption upon *V. parahaemolyticus* infection of M-like cells remain to be identified, but as this *Vibrio* species does not possess cholera toxin, a different mechanism must be responsible. After 1 h of co-incubation, inhibition of the ERK signalling pathway and inactivation of TTSS-2 both reduced translocation of the bacteria across the co-culture model. However, during the later stages of infection, translocation was a TTSS-independent process that did not require MAPK activation. This is similar to the TTSS independence of *Salmonella* translocation across M cells (Martinez-Argudo & Jepson, 2008), but contrary to the translocation inhibition action of the *E. coli* TTSS (Martinez-Argudo *et al.*, 2007), illustrating the unique attributes of each TTSS and their specialisation to the pathogenicity of each bacterial species. In conclusion, translocation of *V. parahaemolyticus* across the co-culture M cell-like model occurs in significant numbers and coincides with TER disruption.

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Authors' contribution

R.F. and T.A. contributed equally to this work.

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