Biochemical characterization of the enterotoxigenic *Escherichia coli* LeoA protein

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Enterotoxigenic *Escherichia coli* (ETEC) causes enterotoxin-induced diarrhoea and significant mortality. The molecular mechanisms underlying how the heat-labile enterotoxin (LT) is secreted during infection are poorly understood. ETEC produce outer-membrane vesicles (OMVs) containing LT that are endocytosed into host cells. Although OMV production and protein content may be a regulated component of ETEC pathogenesis, how LT loading into OMVs is regulated is unknown. The LeoA protein plays a role in secreting LT from the bacterial periplasm. To begin to understand the function of LeoA and its role in ETEC H10407 pathogenesis, a site-directed mutant lacking the putative GTP-binding domain was constructed. The ability of wild-type and mutant LeoA to hydrolyse GTP *in vitro* was quantified. This domain was found to be responsible for GTP binding; it is important to LeoA's function in LT secretion, and may play a modest role in the formation and protein content of OMVs. Deletion of *leoA* reduced the abundance of OmpX in outer-membrane protein preparations and of LT in OMVs. Immunoprecipitation experiments revealed that LeoA interacts directly with OmpA, but that the GTP-binding domain is non-essential for this interaction. Deletion of *leoA* rendered ETEC H10407 non-motile, through apparent periplasmic accumulation of FliC.

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INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) causes enterotoxin-induced diarrhoea and significant morbidity and mortality in humans and livestock (Berberov *et al.*, 2004). ETEC was first characterized as the causative agent of travellers' diarrhoea in separate studies of military personnel in Aden (Rowe *et al.*, 1970) and Vietnam (DuPont *et al.*, 1971). The prototypical human strain, H10407 (O78:H11), expresses two different surface pili that serve as colonization factors (Cheney & Boedeker, 1983). ETEC express several enterotoxins whose combined activities induce water and electrolyte loss from the intestine of infected subjects (Nataro & Kaper, 1998). The heat-labile enterotoxin (LT) is structurally and functionally similar to the cholera toxin of *Vibrio cholerae*, inducing activation of adenylate cyclase in the host to alter normal electrolyte transport and cause diarrhoea (Spangler, 1992).

Despite its role as a major ETEC virulence determinant, only recently has significant effort been put into understanding how LT is secreted from the bacterium during infection (Tauschek *et al.*, 2002). Earlier efforts failed to identify a protein secretory pathway, leading to the belief

that ETEC release LT only during lysis (Wai *et al.*, 1995). It was later realized that a type II secretion system, homologous to that used by *V. cholerae* to transport cholera toxin, is used to transport LT (Tauschek *et al.*, 2002). As LT does not remain in the ETEC periplasm, but is instead found on the cell exterior (Horstman & Kuehn, 2000), the involvement of outer-membrane vesicles (OMVs) in providing a point of display for LT was then recognized (Horstman & Kuehn, 2002).

Gram-negative bacteria produce OMVs (Kesty *et al.*, 2004), which in pathogenic strains are used to secrete virulence genes and proteins. Shiga toxins are delivered from *E. coli* O157:H7 into the host epithelium through OMVs (Kolling & Matthews, 1999) and *Helicobacter pylori* releases the VacA toxin through OMV budding (Fiocca *et al.*, 1999; Ilver *et al.*, 2004). In addition to transporting virulence proteins, OMVs serve an important role in disseminating bacterial DNA. *Pseudomonas aeruginosa* OMV production increases in response to bacterial stress, and DNA isolated from OMVs can be expressed in other pathogenic isolates, implicating OMVs as an important mechanism of horizontal gene transfer (Kadurugamuwa & Beveridge, 1997). LT is highly enriched in ETEC vesicles (Horstman & Kuehn, 2000), whose endocytosis is dependent on cholesterol-rich lipid rafts found on the host epithelial surface (Kesty *et al.*, 2004). OMVs derived from ETEC, but not from non-pathogenic *E. coli* strains, associate with host cells in a time- and receptor-dependent manner (Kesty

Abbreviations: ETEC, enterotoxigenic *Escherichia coli*; LT, heat-labile enterotoxin; OMP, outer-membrane protein; OMV, outer-membrane vesicle; wt, wild-type.

et al., 2004), suggesting that OMV production and protein content may be a regulated component of ETEC pathogenesis. Release of OMVs also enhances survival during bacterial stress or during accumulation of misfolded proteins (McBroom & Kuehn, 2007).

How the protein content of bacterial OMVs is regulated is unknown. Vesiculation may be a directed process as observed in eukaryotes, or, more likely, deformation of the bacterial envelope due to particular localization of the protein secretion machinery may focus secretion loci (Kesty *et al.*, 2004). The *leoA* (labile enterotoxin output) gene was discovered in a mutational screen designed to find ETEC genes that confer the ability to invade intestinal cells *in vitro*. An in-frame deletion in *leoA* resulted in diminished LT secretion and a lack of fluid accumulation in a rabbit ileal loop model of infection, apparently due to a failure to secrete LT from the bacterial periplasm (Fleckenstein *et al.*, 2000). *E. coli* possessing the pathogenicity island in which *leoA* is encoded secrete LT, while *E. coli* lacking the pathogenicity island retain LT in the periplasm (Fleckenstein *et al.*, 2000). LeoA is homologous to proteins from bacterial secretion apparatuses, including EpsE from *V. cholerae*, which is required for cholera toxin secretion (Fleckenstein *et al.*, 2000). In this study, we present data from experiments designed to characterize the biochemical activities of LeoA, the role of its GTP-binding domain in LT secretion, and its interaction with ETEC outer-membrane proteins.

METHODS

Construction and acquisition of Δ *leoA* mutants. We acquired from Dr James Fleckenstein (University of Tennessee Health Science Center) an ETEC H10407 Δ *leoA* strain (Fleckenstein *et al.*, 2000). A site-directed mutant was constructed through overlap PCR (Warrens *et al.*, 1997), in which the putative GTP-binding site (GAFSDGKT) of LeoA was replaced with an inert sequence (GAGAGAGA). PRH-534,

and PRH-557 and PRH-535 and PRH-558 (see Table 1 for oligonucleotide sequences), were combined in separate PCR reactions with ETEC H10407 genomic DNA. Products from these reactions were purified, combined in a third PCR, and amplified with PRH-534 and PRH-535 to generate a *leoA* amplicon containing the GTP-binding site substitution (*leoA* Δ G). This PCR product was cloned into PCR2.1-TOPO and then subcloned into pFLAG-CTC for complementation studies and into pET28a for protein purification.

Protein purification. Standard molecular biology techniques were employed to subclone *leoA* into pET-28a. This construct was expressed in *E. coli* BL21(DE3). A 250 ml culture was grown to an OD₆₀₀ of 0.4, when IPTG was added to 1 mM. After 3 h additional growth, cells were pelleted and resuspended in sonication buffer (5 mM imidazole, 250 mM NaCl, 20 mM Tris/HCl pH 7.9). Cells were sonicated and centrifuged to clarify the supernatant. The supernatant was added to packed pre-equilibrated Ni-NTA agarose and incubated for 1 h at 4 °C. The slurry was poured into a disposable Poly-Prep chromatography column, washed five times with wash buffer (60 mM imidazole, 250 mM NaCl, 20 mM Tris/HCl pH 7.9). His-LeoA was eluted in 1 ml fractions of wash buffer supplemented with increasing concentrations of imidazole and analysed by 10 % SDS-PAGE. Fractions containing His-LeoA were dialysed into storage buffer (20 mM Tris/HCl pH 7.0, 20 mM NaCl, 1 mM DTT, 5 % glycerol) using a Slide-A-Lyser dialysis cassette (Pierce). The His-LeoA Δ G mutant was purified under denaturing (6 M guanidine.HCl), but otherwise identical conditions, and refolded by stepwise dialysis into storage buffer.

Quantification of GTPase activity. The Enzchek Phosphate assay kit (Molecular Probes) was utilized according the manufacturer's specifications. A 1 μ g sample of purified His-LeoA or His-LeoA Δ G was added to 1 ml reaction mixtures preincubated for 10 min at 22 °C that contained 1 $\times$ reaction buffer, MESG substrate and 1 U purine nucleoside phosphorylase. Absorbance at 360 nm was monitored as a function of time.

Quantification of LT secretion. Subcultures from frozen bacterial stocks were grown overnight in CYE-glucose medium at 37 °C with vigorous aeration and pelleted by centrifugation at 16 000 g for 15 min. The resulting clarified bacterial supernatants were frozen at –80 °C. To measure LT retained in the periplasm, bacterial pellets were washed twice in 1 ml cold phosphate-buffered saline (PBS) and

Table 1. Oligonucleotides utilized in this study

Oligonucleotide name and purpose are indicated. Restriction sites used for cloning are designated in italics. The mutagenic primer region utilized in overlap PCR is shown in bold.

Oligonucleotide	DNA sequence (5'–3')
PRH534 BamHI <i>leoA</i> f	G ₂ C ₂ GGATCCATG ₂ A ₂ CA ₂ T ₂ CA ₃ CAGT ₂ C
PRH535 XhoI <i>leoA</i> r	G ₂ C ₂ CTCGAGCTATCTG ₂ CAGTATG ₂ T ₄ C
PRH538 XhoI <i>leoA</i> f	G ₂ C ₂ CTCGAGATG ₂ A ₂ CA ₂ T ₂ CA ₃ CAGT ₂ C
PRH539 BglII <i>leoA</i> r	G ₂ C ₂ AGATCTCTATCTG ₂ CAGTATG ₂ T ₄ C
PRH557 SOE <i>leoA</i> r	C ₂ ATGCG ₂ CGATA ₂ CGCT ₂ C ₂ AC₂GC₂TC₂AC₂TC₂GC₅ A ₂ CAGAGCA ₂ TACG
PRH558 SOE <i>leoA</i> f	CGTAT ₂ GCTCTGT ₂ G ₅ CG₂AG₂TG₂AG₂CG₂TG₂A₂GCGT₂ATCGC₂GCATG₂
PRH633 EcoRI <i>ompA</i> f	G ₂ C ₂ GAATTCATGA ₅ GACAGC
PRH634 XhoI <i>ompA</i> r	G ₂ C ₂ CTCGAGAGC ₂ TGCG ₂ CTGAG
PRH635 XhoI <i>ompA</i> f	G ₂ C ₂ CTCGAGATGA ₅ GACAGC
PRH636 KpnI <i>ompA</i> r	G ₂ C ₂ GGTACCAGC ₂ TGCG ₂ CTGAG
PRH637 EcoRI + 1 <i>ompA</i> f	G ₂ C ₂ GAATTCATGA ₅ GACAGC
PRH638 EcoRI + 2 <i>ompA</i> f	G ₂ C ₂ GAATTCATGA ₅ GACAGC

resuspended in an equal volume of buffer containing 25 mM Tris/HCl (pH 8.0), 10 mM EDTA, lysozyme (3 mg ml⁻¹), polymyxin B (2 mg ml⁻¹), and phenylmethylsulfonyl fluoride (8 µg ml⁻¹). After incubation on ice for 10 min, cell pellets were subjected to repeated freeze-thaw cycles. After centrifugation (16 000 g, 15 min, 4 °C), clarified lysates were frozen at -80 °C until used in LT assays. LT quantification was performed with a mixed-ganglioside ELISA (Horstman & Kuehn, 2002).

Outer-membrane protein (OMP) isolation. OMPs were isolated as described by Achtman *et al.* (1983). Subcultures (10 ml) from frozen bacterial stocks were grown overnight in SOB at 37 °C with vigorous aeration and pelleted by centrifugation (16 000 g, 15 min, 4 °C). Bacterial pellets were washed twice in 1.5 ml 10 mM Tris/HCl (pH 8.0), resuspended a third time in this buffer, and lysed by sonication. Whole cells were removed by centrifugation (10 000 g, 10 min, 4 °C), the supernatants were aspirated into ultratubes, and Sarkosyl was added to 2% (w/v). This mixture was incubated at room temperature for 30 min and then centrifuged (38 000 g, 1 h, 4 °C) to pellet the membrane proteins. The membrane protein pellet was washed with PBS, recentrifuged, and resuspended in sterile water. A 25 µg sample of protein was mixed with SDS-loading buffer and separated by 10% SDS-PAGE.

Electron microscopy analysis of OMVs. OMVs were prepared by inoculating tryptic soy broth (TSB) with ETEC strains and incubating at 37 °C for 15 h with shaking (150 r.p.m.). Vesicles were harvested from the supernatant according to the method of Kesty *et al.* (2004). After incubation, cells were pelleted by centrifugation (10 000 g, 10 min, 4 °C) and the supernatant was decanted and passed through a 0.22 µm-pore-size filter. Vesicles were collected by ultracentrifugation (150 000 g, 3 h, 4 °C), washed with PBS, resuspended in 100 µl mqH₂O, and stored at 4 °C. Samples (10 µl) were placed on 300-mesh carbon-coated copper grids, allowed to settle on the film for 5 min, then the excess was removed and the grids were stained for 5 min with 1% uranyl acetate. After drying, grids were viewed in a JEOL 1200EX transmission electron microscope and 10 random fields per grid were imaged for final vesicle counts. The protein content of OMVs was also analysed by 10% SDS-PAGE.

Immunoprecipitation of FLAG-LeoA. ETEC expressing FLAG-LeoA, FLAG-LeoAΔG or a FLAG epitope control were subcultured 1:50 into 10 ml LB, grown for 3 h at 37 °C, and pelleted by centrifugation. After washing with PBS, bacteria were resuspended in PBS containing 1% DMSO and incubated for 30 min at room temperature. Saturated Tris was added to 100 mM and incubation was continued for 10 min. Cells were pelleted by centrifugation, resuspended in lysis buffer (1% Triton X-100, 10 mM Tris/HCl, pH 7.0, 1 mM PMSF), and sonicated. Following centrifugation to remove insoluble material, the supernatant was incubated for 2 h at 4 °C with 5 µg mouse-anti-FLAG antibody with gentle rotation. Then 25 µl protein G-Sepharose beads (pre-blocked with 1% BSA) was added and the mixture was incubated overnight at 4 °C with gentle rotation. Beads were pelleted by centrifugation (2000 g, 1 min, 4 °C), washed three times with 1 ml lysis buffer. The final wash was removed with a 25-gauge needle, the pellet was resuspended in SDS sample buffer, and samples were analysed by 10% SDS-PAGE.

Co-immunoprecipitation. BL21(DE3) cells were co-transformed with plasmids expressing FLAG- and His-tagged forms of LeoA, LeoAΔG and OmpA. Immunoprecipitations were conducted with protein G-Sepharose as described above. Mock immunoprecipitations of cultures expressing only a single construct were conducted to demonstrate antibody specificity.

Bacterial two-hybrid assay. ETEC *leoA*, *leoAΔG* and *ompA* were subcloned into the BacterioMatch two-hybrid system vector kit bait

(pBT) and target (pTRG) plasmids (Stratagene). Plasmids were co-transformed into XL1 Blue MRF [kanamycin-resistant (Kan^r)] and selected on LB agar plates containing the following antibiotics (µg ml⁻¹): kanamycin (50), chloramphenicol (25), tetracycline (10) and carbenicillin (0 to 500). Positive protein-protein interactions in the assay turn on the *bla* reporter gene, yielding a transformant resistant to carbenicillin (Cb^r). Transformation controls were performed on plates lacking Cb. Interaction controls were performed by cotransforming with empty pBT and pTRG vectors and with vectors expressing an unrelated fusion protein (*E. coli* O157:H7 EspG).

Periplasmic protein isolation and motility assays. ETEC strains were subcultured 1:50 into 5 ml LB, grown for 3 h at 37 °C, and pelleted by centrifugation. The bacterial pellet was washed with 10 ml PBS and the centrifugation was repeated. The supernatant was decanted and the pellet was resuspended in 1 ml 50 mM Tris pH 7.0, 20% sucrose, 10 mM EDTA, 0.25 mg lysozyme ml⁻¹. The suspension was incubated at room temperature for 10 min and centrifuged at 8000 g, 10 min, 4 °C. The supernatant was retained as the periplasmic fraction. Bacterial motility was evaluated by inoculating with a needle semisolid agar tubes containing BBL motility test medium (Beckton Dickinson), as described by the manufacturer.

Protein identification by LC-ESI-MS/MS. Bands excised from protein gels were digested in-gel with trypsin (Promega) at 37 °C overnight. The tryptic peptide solution was transferred to a microcentrifuge tube, extracted with 1% formic acid (Sigma-Aldrich), 2% acetonitrile (ACN; Fisher) in water, followed by an extraction with 50% ACN/water. Both extracts were combined with the tryptic peptide solution, concentrated in a SpeedVac and suspended in 1% formic acid, 2% ACN. Peptide analysis was performed using LC-ESI-MS/MS. The peptides were desalted in-line and concentrated with an RP-Trap Symmetry300 C18 column, 5 µm NanoEase (Waters), and separated using a C18 RP PepMap 75 µm capillary column on a CapLC (Waters). Spectra were obtained in the positive-ion mode with a Q-TOF micro mass spectrometer (Micromass), deconvoluted, and analysed using the MassLynx software 4.0 (Micromass). Peptide matching and protein searches were performed using the ProteinLynx Global Server v 2.1 (Micromass) against the NCBI nr v20060912 database.

RESULTS AND DISCUSSION

LeoA is homologous to eubacterial and eukaryotic GTPases

We performed a PSI-BLAST search to identify important functional domains and homologues of LeoA (Table 2). We identified a region of LeoA (sequence GAFSDGKT) corresponding exactly to the GTP-binding consensus sequence of [AG]-X₄-G-K-[ST]. LeoA appears to contain an Era (*E. coli* Ras-like protein)-like domain, found in several essential bacterial GTPases involved in ribosome assembly (Inoue *et al.*, 2003). We also detected significant homology between LeoA and an endoplasmic reticulum-localized GTPase IMA8 (immunity-associated protein 8) from eukaryotes. Significant homology was also observed to Sar1p (secretion-associated and Ras-related protein), a GTPase from *Anabaena variabilis*, a heterocyst-forming cyanobacterium. In eukaryotes, Sar1 mediates the transport of proteins from the endoplasmic reticulum to the Golgi apparatus via the coat protein complex II [COPII (Miller *et al.*, 2002)]. Sar1 is believed to be an ancestral member of

Table 2. PSI-BLAST identification of ETEC LeoA homologues

Protein	Organism	Annotation	Identity (%)	E value
Cog1159	<i>Pseudomonas fluorescens</i>	GTPase	46	1×10^{-157}
HP0731	<i>Helicobacter pylori</i>	Predicted coding region	30	1×10^{-137}
JHP0668	<i>Helicobacter pylori</i>	Hypothetical protein	30	1×10^{-135}
CE2856	<i>Corynebacterium efficiens</i>	Hypothetical protein	16	1×10^{-103}
YP_154925	<i>Idiomarina loihiensis</i>	Predicted GTPase	15	1×10^{-95}
Sar1p	<i>Anabaena variabilis</i>	GTPase Sar1	19	3×10^{-77}

the Ras superfamily (Jekely, 2003) and is able to complement prokaryotic GTPases (Hartzell, 1997). While it is intriguing to speculate that the loading of protein cargo into bacterial OMVs may be in some way akin to GTPase-mediated control of protein sorting in eukaryotic secretory pathways, experiments from the Kuehn laboratory suggest that a 'cargo tag' is not required for protein sorting into bacterial vesicles (Kesty *et al.*, 2004). Differential localization of type II secretion machinery may instead determine secretion loci (Kesty *et al.*, 2004). Fleckenstein *et al.* (2000) also noted similarity to EpsE, the *V. cholerae* member of type II/type IV secretion NTPases that provide energy for bacterial protein secretion (Camberg & Sandkvist, 2005). EpsE is known to have both a cytoplasmic (Abendroth *et al.*, 2005) and inner-membrane association (Camberg & Sandkvist, 2005), dependent upon the expression of other Eps proteins.

LeoA is a GTPase

To quantify the ability of LeoA to hydrolyse GTP *in vitro*, we overexpressed and purified His-tagged forms of LeoA and a LeoA mutant (LeoAΔG) in which the putative GTP-binding site (GAFSDGKT) was replaced, through overlap PCR (Warrens *et al.*, 1997), with an inert sequence

(GAGAGAGA). These constructs were subcloned into pET28a (C-terminal His₆-tag) and expressed in *E. coli* BL21(DE3). We purified His-LeoA through Ni-NTA resin under native conditions (Fig. 1a). Because of reduced solubility in BL21(DE3) lysate, His-LeoAΔG was purified in the presence of 6 M guanidine hydrochloride and refolded via stepwise dialysis. To serve as a refolding control in subsequent biochemical assays, we also purified His-LeoA under denaturing conditions.

To monitor *in vitro* GTP hydrolysis by His-LeoA and His-LeoAΔG, 1 μg purified protein was added to the Enzchek Phosphate assay kit (Molecular Probes), which provides a sensitive GTPase assay system used previously to study the activity of a related family of GTPases (Zhuang *et al.*, 2005). GTP hydrolysis was assayed every 15 s for 5 min by measuring the change in absorbance at 360 nm (Fig. 1b). For His-LeoA, A_{360} changed linearly with respect to time (Fig. 1b; open triangles). However, addition of 1 μg His-LeoAΔG did not result in efficient GTP hydrolysis, compared to His-LeoA (Fig. 1b; open squares). To exclude the possibility that this observation was due to the denaturing purification protocol employed for His-LeoAΔG, we analysed the activity of His-LeoA purified under denaturing conditions (Fig. 1b; filled triangles). This protein behaved indistinguishably from native His-LeoA.

Fig. 1. LeoA is a GTPase. (a) Purification of His-LeoA. *leoA* was cloned into pET28a, expressed in *E. coli* BL21(DE3), purified by passage over pre-equilibrated Ni-NTA resin (Qiagen), and eluted with 300 mM imidazole. Shown is a Coomassie-stained 10% SDS-polyacrylamide gel of the induced bacterial lysate, purified His-LeoA and His-LeoAΔG. S, Size standards. (b) Quantification of LeoA GTPase activity. GTP hydrolysis was monitored by measuring the change A_{360} vs time, following the addition of 1 μg LeoA proteins. The Enzchek Phosphate assay was performed according to the manufacturer's instructions (Invitrogen). Error bars (not shown if smaller than symbols) represent the standard deviation of three independent experiments. ○, Buffer; □, His-LeoAΔG; △, His-LeoA native; ▲, His-LeoA refolded.

This observation suggests that the deficient GTP hydrolysis observed with His-LeoAΔG is due solely to the site-directed mutation, and not the purification method. Overall, these data suggest that LeoA is a functional GTPase.

Deletion of *leoA* alters OMP composition

To determine the role of LeoA in maintaining OMP composition, we isolated OMPs from H10407 possessing or lacking LeoA and assessed changes in OMP composition by SDS-PAGE. We first complemented the $\Delta leoA$ strain with plasmids expressing FLAG-tagged versions of LeoA (*pleoA*-FLAG) and *leoA*ΔG (*pleoA*ΔG-FLAG) and verified proper expression by Western blotting (Fig. 2a). OMPs were isolated and prepared by ultracentrifugation from lysates obtained from 10 ml bacterial subcultures; 25 µg of protein was analysed by 10 % SDS-PAGE (Fig. 2b). Notably, a protein of ~16 kDa was present in greatly reduced abundance in the $\Delta leoA$ strain, relative to wild-type (wt) H10407. Complementation with either LeoA-FLAG or LeoAΔG-FLAG restored protein abundance to wt levels. We excised this band and determined through mass spectrometry that this protein is OmpX.

OmpX is a member of a protein family that may be important to virulence by neutralizing host defences. Kuehn and co-workers noted in their pioneering studies of LT secretion via vesicles that OmpX is present in ETEC outer membrane and vesicle preparations, that the abundance of OmpX differs as a function of the growth medium, and that this protein is not detected in preparations from non-pathogenic strains (Horstman & Kuehn, 2000). These authors also noted that OmpX is homologous to the *Yersinia* Ail protein, which has been

suggested to promote bacterial invasion (Miller *et al.*, 2001), and the OmpX adhesin of *Enterobacter cloacae*, which promotes invasiveness in rabbit ileal enterocytes (de Kort *et al.*, 1994). It remains to be determined why deletion of LeoA alters OmpX abundance in the outer membrane.

The GTPase domain of LeoA is essential to maximal LT secretion

To quantify the role of the GTPase domain of LeoA in LT secretion, we measured the concentration of LT in the periplasmic and supernatant fractions of ETEC possessing or lacking *leoA*. We determined that in wt H10407, a significant amount of LT was secreted into culture supernatants (Table 3; 199.7 ± 17.1 ng ml⁻¹), whereas in the $\Delta leoA$ strain, significantly less LT was detected (34.5 ± 4.3 ng ml⁻¹). Quantification of LT in the periplasmic fraction demonstrated a higher concentration in the $\Delta leoA$ strain, in agreement with previous reports (Fleckenstein *et al.*, 2000). When strains were complemented with plasmids expressing *leoA*, proper LT secretion was restored. However, in strains complemented with the *pleoA*ΔG-FLAG construct, LT secretion into culture supernatants did not return to wt levels. Surprisingly, wt ETEC transformed with *pleoA*ΔG-FLAG also displayed an LT secretion phenotype similar to the $\Delta leoA$ strain. Overall, these data suggest that the GTPase domain of LeoA is required for maximal secretion of LT from the H10407 periplasm and suggest that expression of LeoAΔG may exert a dominant-negative phenotype.

To determine if introduction of LeoA would alter LT secretion in ETEC that naturally lack the *leoA* gene, we transformed *pleoA*-FLAG into two other LT⁺ ETEC strains, 3030-2 (K88⁺ STa⁺ STb⁺ LT⁺), a porcine field

Fig. 2. Analysis of OMPs in ETEC strains either possessing or lacking LeoA. (a) Complementation of ETEC $\Delta leoA$ with *pleoA*-FLAG and *pleoA*ΔG-FLAG. Bacterial lysates from the parent and complemented ETEC $\Delta leoA$ strains were interrogated for the presence of FLAG-tagged LeoA and LeoAΔG by Western blotting using a mouse anti-FLAG antibody. (b) OMP composition. OMPs from the indicated strains were isolated and subjected to 10 % SDS-PAGE. Annotated bands were excised from gels and identified by mass spectrometry. (c) OMP composition. Protein content of OMPs isolated from the indicated strains was assessed by Coomassie G-250 staining of samples separated by 10 % SDS-PAGE. S, size standards.

Table 3. Quantification of LT secretion in ETEC strains possessing or lacking *leoA*

Strain	LT (ng ml ⁻¹)	
	Periplasmic	Supernatant
wt ETEC	15.2 ± 4.5	199.7 ± 17.1
ETECΔ <i>leoA</i>	27.0 ± 7.0*	34.5 ± 4.3†
wt ETEC/ <i>pleoA</i> -FLAG	32.3 ± 9.2*	124.7 ± 8.2†
ETECΔ <i>leoA</i> / <i>pleoA</i> -FLAG	24.8 ± 12.0*	126.3 ± 10.1†
wt ETEC/ <i>pleoA</i> ΔG-FLAG	20.5 ± 8.4	32.7 ± 5.7†
ETECΔ <i>leoA</i> / <i>pleoA</i> ΔG-FLAG	7.2 ± 4.4*	31.2 ± 6.4†

*Significantly different from wt; Student's *t*-test, *P* < 0.05.

†Significantly different from wt; Student's *t*-test, *P* < 0.005.

isolate (Erickson *et al.*, 1992), and 91.1283 (O6:STa⁺LT⁺), a pathogenic human isolate (Chobi DebRoy, personal communication). Whereas expression of *LeoA* in 3030-2 increased LT secretion by ~14 %, transformation of 91.1283 caused no detectable change.

LeoA is important to the formation of OMVs

To determine the role of *LeoA* in the formation of OMVs, we purified OMVs from ETEC either possessing or lacking *leoA* by ultracentrifugation and filtration (Kadurugamuwa & Beveridge, 1997) and quantified by electron microscopy the number of OMVs produced by each strain. Vesicle preparations from wt H10407 contained 15.2 ± 3.2 OMVs per grid, whereas Δ*leoA* produced 4.0 ± 1.8. Complementation of Δ*leoA* with *pleoA*-FLAG partially restored the production of OMVs (8.0 ± 3.4), whereas complementation with *pleoA*ΔG-FLAG actually reduced the number of OMVs observed (1.4 ± 3.1). Although gross morphological differences were not observed among OMVs produced by wt vs Δ*leoA*, it is possible that these OMVs may differ in their affinity for the electron microscopy grid.

We also sought to determine the extent to which OMV protein content differed among complemented strains by subjecting purified OMVs to SDS-PAGE. As shown in Fig. 2(c), the total protein content and distribution of specific proteins within OMVs differed substantially between wt and Δ*leoA*. Whereas complementation with *pleoA*-FLAG restored the wt OMV protein profile, complementation with *pleoA*ΔG-FLAG did not.

We also quantified the extent to which LT could be detected in OMVs purified from ETEC possessing or lacking *LeoA*. OMV-associated LT was found in wt H10407 (0.15 ng ml⁻¹), although at a lower concentration than reported from ETEC 2 (Horstman & Kuehn, 2000). We observed a slight reduction in LT concentration in OMVs isolated from Δ*leoA* and Δ*leoA*/*pleoA*ΔG-FLAG (0.08 and 0.07 ng ml⁻¹, respectively), but not from Δ*leoA*/*pleoA*-FLAG (0.13 ng ml⁻¹). These data suggest that *LeoA* may

play a modest role in OMV formation and that the GTP-binding domain is important to this process.

LeoA interacts with OmpA

To identify ETEC proteins that interact with *LeoA*, we expressed FLAG-tagged versions of *LeoA*, *LeoA*ΔG and a FLAG-epitope control in ETEC H10407. ETEC lysates were incubated with an anti-FLAG antibody, immunoprecipitated with protein G Sepharose, and analysed by SDS-PAGE. As shown in Fig. 3(a), *LeoA* could be immunoprecipitated from both the *pleoA*-FLAG and the *pleoA*ΔG-FLAG strains (top asterisk). An additional protein band immunoprecipitated from both the *pleoA*-FLAG and the *pleoA*ΔG-FLAG samples, but not in the FLAG-epitope control sample (bottom asterisk), was excised and identified by mass spectrometry as the outer-membrane protein OmpA.

OmpA is a major *E. coli* OMP with an important role in structural integrity and is believed to link physically the outer membrane with the peptidoglycan layer (Koebnik *et al.*, 2000). In *E. coli* K1, OmpA is associated with neonatal meningitis through invasion of endothelial cells (Prasadarao *et al.*, 1996). In *E. coli* O157:H7, OmpA plays a role in adherence to intestinal epithelial cells (Torres & Kaper, 2003) and is also believed to mediate stimulation of dendritic cells to produce cytokines (Torres *et al.*, 2006). Kuehn and co-workers demonstrated that ETEC OmpA is an outer-membrane component of native vesicles (Kesty *et al.*, 2004). They observed that ETEC lacking *LeoA* fail to grow in a deoxycholate resistance assay, suggesting a loss of membrane integrity (Kesty *et al.*, 2004). We observed that the Δ*leoA* strain also has a significantly reduced growth rate in rich media (data not shown). An interaction between *LeoA* and OmpA could account for these observations.

To begin to confirm the putative interaction between *LeoA* and OmpA, *ompA* was cloned into pET28a and pFLAG-CTC and coexpressed with His- and FLAG-tagged *LeoA* and *LeoA*ΔG in BL21(DE3) cells. Lysates were subjected to immunoprecipitation with an anti-His antibody and interrogated by anti-FLAG immunoblotting. FLAG-OmpA could only be immunoprecipitated by α-His antibodies when coincubated with either His-*LeoA* or His-*LeoA*ΔG (Fig. 3b, left). The converse experiment, in which anti-FLAG antibody was used to immunoprecipitate OmpA, also resulted in the enrichment of both His-*LeoA* and His-*LeoA*ΔG (Fig. 3b, right). Mock immunoprecipitations demonstrated that His-*LeoA* was not precipitated with anti-FLAG antibodies unless FLAG-OmpA was present. Similarly, FLAG-OmpA was not precipitated with anti-His antibodies in the absence of His-*LeoA*. Overall, these data support the notion that *LeoA* interacts with OmpA.

We also studied the interaction between *LeoA* and OmpA in a bacterial two-hybrid assay system. In this assay, plasmids expressing fusion proteins to a DNA-binding

Fig. 3. LeoA interacts with OmpA. (a) Immunoprecipitation of FLAG-tagged LeoA from ETEC H10407. FLAG-tagged LeoA was immunoprecipitated from ETEC lysates and subjected to 10% SDS-PAGE. Bands selectively immunoprecipitated (indicated by asterisks) were identified by mass spectrometry as LeoA (upper) and OmpA (lower). (b) Co-immunoprecipitation experiments. His(H)- and FLAG(F)-tagged forms of LeoA, LeoAΔG and OmpA were coexpressed in BL21(DE3) cells. Bacterial lysates were immunoprecipitated with either anti-His or anti-FLAG antibodies and interrogated by immunoblotting. Shown are the input and immunoprecipitated samples from experiments performed with both anti-His (left) and anti-FLAG (right) antibodies. (c) Bacterial two-hybrid analysis of interactions between LeoA and OmpA. Co-transformants harbouring the indicated plasmids were plated on selective medium (LB + 300 μg Cb ml⁻¹) in the indicated plate octants: 1, *sepD*-pBT *sepL*-pTRG; 2, *ompA*-pBT *leoA*-pTRG; 3, *ompA*-pBT *leoAΔG*-pTRG; 4, *ompA*-pTRG *leoA*-pBT; 5, *ompA*-pTRG *leoAΔG*-pBT; 6, *leoA*-pBT *leoA*-pTRG; 7, *leoAΔG*-pBT *leoAΔG*-pTRG; 8, *ompA*-pBT *ompA*-pTRG.

domain and a transcriptional activation domain are co-transformed into reporter cells. Protein–protein interaction strength is assessed semiquantitatively by growth on selective media. Co-transformation of either LeoA or LeoAΔG with OmpA conferred survival on selective medium (Fig. 3c, octants 2–5). The interaction between enteropathogenic *E. coli* E2348/69 SepD–SepL was monitored as a positive control [Fig. 3c, octant 1; (Deng *et al.*, 2005)]. Reporter gene activation was also seen in cells that coexpressed reporter fusions to OmpA (Fig. 3c, octant 8). No growth was obtained in cells expressing only a single protein fusion, or with coexpression of LeoA constructs. No difference was observed in bacterial growth properties among strains transformed with LeoA or LeoAΔG. We

conclude from these data that the GTP-binding domain of LeoA appears nonessential in mediating binding to OmpA.

Deletion of *leoA* affects H10407 motility

We also interrogated the bacterial periplasm for evidence of proteins whose distribution might be affected by the presence or absence of LeoA. Significantly, a specific protein was highly increased in abundance in the periplasm of Δ*leoA*, relative to wt and the complemented strain (Δ*leoA*/pleoA-FLAG; Fig. 4a, asterisk). We identified this protein by mass spectrometry as FliC. FliC is the major component of the bacterial flagellum (Aldridge *et al.*, 2006), which has a proven role in *E. coli* motility (Gomez-Gomez *et al.*, 2007),

Fig. 4. LeoA deletion results in periplasmic accumulation of FliC and reduces bacterial motility. (a) Analysis of periplasmic proteins in ETEC strains either possessing or lacking LeoA. Periplasmic proteins from the indicated strains were isolated and subjected to 10% SDS-PAGE. The band indicated with an asterisk was excised and identified by mass spectrometry. (b) Bacterial motility assays. The indicated ETEC strains were inoculated into semisolid agar containing BBL motility test medium. Motility was evaluated 24 h post-inoculation.

host cell adherence (Giron *et al.*, 2002; Wright *et al.*, 2005), invasion (Parthasarathy *et al.*, 2007) and virulence (Giron *et al.*, 2002; Parthasarathy *et al.*, 2007).

While we did not observe significant differences in the adherence of wt and Δ leoA H10407 to Caco-2 cells (data not shown), when we evaluated bacterial motility, Δ leoA was significantly inhibited in its ability to swim through semisolid agar and complementation with pleoA-FLAG restored motility (Fig. 4b). Heterologous expression of LeoA in the porcine isolate 3030-2 modestly increased motility. Thus, in addition to roles in LT secretion and modulation of OMP composition, LeoA may in some way contribute to the formation of motility structures. However, it is unlikely that LeoA is generally important to ETEC motility, as the vast majority of characterized isolates are *leoA*⁻ (see below; Turner *et al.*, 2006). Our observations may instead result from the apparent pleiotropic nature of the *leoA* mutation on H10407 proteins associated with the periplasm or bacterial membranes.

It is interesting to speculate as to why LeoA appears to play a significant role in properties typically associated with H10407 virulence. The findings described here are of note because *leoA* is not typically found in ETEC isolates characterized to date. Turner *et al.* (2006) analysed the distribution of *leoA* by multilocus sequence typing of 209 different ETEC isolates and found that *leoA* was present in only 3 % of the tested strains. These authors speculate that because numerous isolates possessing LT do not encode *leoA*, this gene is not generally required for ETEC pathogenicity (Turner *et al.*, 2006). Ongoing and future experiments may elucidate how LeoA might contribute to the disease severity associated with H10407 and whether similar functions might be imparted by related virulence factors in isolates that have been less well characterized.

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