INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC) causes enterotoxin-induced diarrhoea and significant 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 et al., 2004).

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.

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 ΔleoA mutants.** We acquired from Dr James Fleckenstein (University of Tennessee Health Science Center) an ETEC H10407 ΔleoA 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-334, 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 ampiclon containing the GTP-binding site substitution (leoAΔG). 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-LeoAAG 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 μg sample of purified His-LeoA or His-LeoAAG was added to 1 ml reaction mixtures preincubated for 10 min at 22 °C that contained 1 x 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

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**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.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>DNA sequence (5’–3’)</th>
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<tbody>
<tr>
<td>PRH534 BamHI leoA f</td>
<td>GCTGGATCCATGAGAAGCTTACG</td>
</tr>
<tr>
<td>PRH535 XhoI leoA r</td>
<td>GCTGCTTGGAGATTGTATG</td>
</tr>
<tr>
<td>PRH538 XhoI leoA f</td>
<td>GCTGCTTGGAGATTGTATG</td>
</tr>
<tr>
<td>PRH539 BglII leoA r</td>
<td>GCTGCTTGGAGATTGTATG</td>
</tr>
<tr>
<td>PRH557 SOE leoA r</td>
<td>GCTGCTTGGAGATTGTATG</td>
</tr>
<tr>
<td>PRH558 SOE leoA f</td>
<td>GCTGCTTGGAGATTGTATG</td>
</tr>
<tr>
<td>PRH633 EcoRI ompA f</td>
<td>GCTGCTTGGAGATTGTATG</td>
</tr>
<tr>
<td>PRH634 XhoI ompA r</td>
<td>GCTGCTTGGAGATTGTATG</td>
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<tr>
<td>PRH635 XhoI ompA f</td>
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<tr>
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<td>GCTGCTTGGAGATTGTATG</td>
</tr>
<tr>
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</tr>
<tr>
<td>PRH638 EcoRI+2 ompA f</td>
<td>GCTGCTTGGAGATTGTATG</td>
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</tbody>
</table>

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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 \(\mu\)g ml\(^{-1}\)). After incubation on ice for 10 min, cell pellets were subjected to repeated freeze–thaw cycles. After centrifugation (16000 \(g\), 15 min, 4 \(^o\)C), clarified lysates were frozen at \(-80\) \(^o\)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 \textit{et al.} (1983). Subcultures (10 ml) from frozen bacterial stocks were grown overnight in SOB at 37 \(^o\)C with vigorous aeration and pelleted by centrifugation (16000 \(g\), 15 min, 4 \(^o\)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 (10000 \(g\), 10 min, 4 \(^o\)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 (38000 \(g\), 1 h, 4 \(^o\)C) to pellet the membrane proteins. The membrane protein pellet was washed with PBS, recentrifuged, and resuspended in sterile water. A 25 \(\mu\)g 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 \(^o\)C for 15 h with shaking (150 r.p.m.). Vesicles were harvested from the supernatant according to the method of Kesty \textit{et al.} (2004). After incubation, cells were pelleted by centrifugation (10000 \(g\), 10 min, 4 \(^o\)C) and the supernatant was decanted and passed through a 0.22 \(\mu\)m-pore-size filter. Vesicles were collected by ultracentrifugation (150000 \(g\), 3 h, 4 \(^o\)C), washed with PBS, resuspended in 100 \(\mu\)l m-qH\(_2\)O, and stored at 4 \(^o\)C for 800 \(g\), 10 min, 4 \(^o\)C) to pellet the membrane proteins. The membrane protein pellet was washed with PBS, recentrifuged, and resuspended in sterile water. A 25 \(\mu\)g sample of protein was mixed with SDS-loading buffer and separated by 10% SDS-PAGE.

**Immunoprecipitation of FLAG-LeoA.** ETEC expressing FLAG-LeoA, FLAG-LeoA\(^{\Delta\text{AG}}\) or a FLAG epitope control were subcultured 1:50 into 10 ml LB, grown for 3 h at 37 \(^o\)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 \(^o\)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 \(^o\)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 \(\mu\)m NanoEase (Waters), and separated using a C18 RP PepMap 75 \(\mu\)m 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]-X\(_4\)-G-K-[ST], LeoA appears to contain an Era (\textit{E. coli} Ras-like protein)-like domain, found in several essential bacterial GTPases involved in ribosome assembly (Inoue \textit{et al.}, 2003). We also detected significant homology between LeoA and an endoplasmic reticulum-localized GTPase IMAP8 (immunity-associated protein 8) from eukaryotes. Significant homology was also observed to Sar1p (secretion-associated and Ras-related protein), a GTPase from \textit{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 \textit{et al.}, 2002)]. Sar1 is believed to be an ancestral member of...
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.

**Table 2. PSI-BLAST identification of ETEC LeoA homologues**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
<th>Annotation</th>
<th>Identity (%)</th>
<th>E value</th>
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<tr>
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<td>Pseudomonas fluorescens</td>
<td>GTPase</td>
<td>46</td>
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<td>HP0731</td>
<td>Helicobacter pylori</td>
<td>Predicted coding region</td>
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<td>1 \times 10^{-137}</td>
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<tr>
<td>JHP0668</td>
<td>Helicobacter pylori</td>
<td>Hypothetical protein</td>
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<td>1 \times 10^{-135}</td>
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<tr>
<td>CE2856</td>
<td>Corynebacterium efficiens</td>
<td>Hypothetical protein</td>
<td>16</td>
<td>1 \times 10^{-103}</td>
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<tr>
<td>YP_154925</td>
<td>Idiomarina loihiensis</td>
<td>Predicted GTPase</td>
<td>15</td>
<td>1 \times 10^{-95}</td>
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<tr>
<td>Sar1p</td>
<td>Anabaena variabilis</td>
<td>GTPase Sar1</td>
<td>19</td>
<td>3 \times 10^{-77}</td>
</tr>
</tbody>
</table>

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**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-LeoΔΔG 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 ΔleoA strain with plasmids expressing FLAG-tagged versions of LeoA (pleoA-FLAG) and leoAΔΔG (pleoAΔΔG-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 ΔleoA strain, relative to wild-type (wt) H10407. Complementation with either LeoA-FLAG or LeoAΔΔG-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 ΔleoA 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 ΔleoA 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 pleoAΔΔG-FLAG construct, LT secretion into culture supernatants did not return to wt levels. Surprisingly, wt ETEC transformed with pleoAΔΔG-FLAG also displayed an LT secretion phenotype similar to the ΔleoA 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 LeoAΔΔG 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
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, LeoΔAG 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 pLeoAΔAG-FLAG strains (top asterisk). An additional protein band immunoprecipitated from both the pLeoA-FLAG and the pLeoAΔAG-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., 1996). In *E. coli* K1, OmpA is associated with neonatal meningitis through invasion of endothelial cells (Prasad 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 ΔLeoA 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 LeoΔAG 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 z-His antibodies when coincubated with either His-LeoA or His-LeoΔAG (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-LeoΔAG (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

| 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 |
| ETECΔLeoA | 27.0±7.0* | 34.5±4.3† |
| wt ETEC/pleoA-FLAG | 32.3±9.2* | 124.7±8.2† |
| ETECΔLeoA/pleoA-FLAG | 24.8±12.0* | 126.3±10.1† |
| wt ETEC/pleoAΔAG-FLAG | 20.5±8.4 | 32.7±5.7† |
| ETECΔLeoA/pleoAΔAG-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.

isolates (Erickson et al., 1992), and 91.1283 (O6:STa¹ 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 ΔleoA produced 4.0±1.8. Complementation of ΔleoA with pleoA-FLAG partially restored the production of OMVs (8.0±3.4), whereas complementation with pleoAΔAG-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 ΔleoA, 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 ΔleoA. Whereas complementation with pleoA-FLAG restored the wt OMV protein profile, complementation with pleoAΔAG-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 ΔleoA and ΔleoA/pleoAΔAG-FLAG (0.08 and 0.07 ng ml⁻¹, respectively), but not from ΔleoA/pleoA-FLAG (0.13 ng ml⁻¹). These data suggest that LeoA may

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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 LeoA<sup>DG</sup> with OmpA conferred survival on selective medium (Fig. 3c, octants 2–5). The interaction between enteropathogenic <i>E. coli</i> 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 LeoA<sup>DG</sup>. 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 ΔLeoA, relative to wt and the complemented strain (ΔleoA/ 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 <i>E. coli</i> motility (Gomez-Gomez et al., 2007).

**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, LeoA<sup>DG</sup> 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 Cm ml<sup>−1</sup>) in the indicated plate octants: 1, sepD-pBT sepL-pTRG; 2, ompA-pBT leoA-pTRG; 3, ompA-pBT leoA<sup>DG</sup>-pTRG; 4, ompA-pTRG leoA-pBT; 5, ompA-pTRG leoA<sup>DG</sup>-pBT; 6, leoA-pBT leoA-pTRG; 7, leoA<sup>DG</sup>-pBT leoA<sup>DG</sup>-pTRG; 8, ompA-pBT ompA-pTRG.

**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 ΔleoA H10407 to Caco-2 cells (data not shown), when we evaluated bacterial motility, ΔleoA was significantly inhibited in its ability to swim through semisolid agar and complementation with p leoA-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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REFERENCES


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