Subcellular localisation of lipoproteins of *Vibrio vulnificus* by the identification of outer membrane vesicles components

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Abstract *Vibrio vulnificus*, a Gram-negative halophilic bacterium, is an opportunistic human pathogen that is responsible for the majority of seafood-associated deaths worldwide. Lipoproteins are important components of the bacterial cell envelope and have been shown to be involved in a wide variety of cellular processes. Little is known about the localisation or transport mechanism of lipoproteins in *V. vulnificus*. To assess the localisation of lipoproteins in *V. vulnificus*, we tested two established techniques for

the rapid separation of membrane-associated proteins: detergent extraction with Sarkosyl and outer membrane vesicles (OMVs) preparation. The results showed that Sarkosyl extraction was not useful for the separation of lipoproteins from the different membranes of *V. vulnificus*. On the other hand, we confirmed that OMVs produced by *V. vulnificus* contained lipoproteins from the outer but not the inner membrane. Analysis of the OMVs components confirmed the localisation of several well-known lipoproteins to membranes that were different from expected, based on their predicted functions. Using this technique, we found that Asp at position +2 of mature lipoproteins can function as an inner membrane retention signal in *V. vulnificus*. Interestingly, the *Escherichia coli* “+2 rule” does not apply to the *V. vulnificus* lipoprotein IlpA (G2D) mutant, as a Ser to Asp mutation at position +2 of IlpA did not affect its outer membrane localisation. Furthermore, an IlpA tether-mRFP chimeric lipoprotein and its G2D mutant also behaved like IlpA. Together, these results suggest that the sorting rule of lipoproteins in *V. vulnificus* might be different from that in *E. coli*.

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Introduction

Vibrio vulnificus, a Gram-negative halophilic bacterium, is ubiquitous in estuaries and coastal environments and can cause human illness, known as vibriosis (Baker-Austin and Oliver 2017). This serious human pathogen is responsible for the majority of seafood-associated deaths worldwide (Jones and Oliver 2009) and for vibriosis in different species of aquatic animals (Amaro et al. 2015). Treatment of infected humans and aquatic animals is becoming increasingly challenging as *V. vulnificus* has begun to develop antibiotic resistance (Heng et al. 2017). Many aspects of its biology, genomics, virulence and epidemiology still need to be fully elucidated.

Bacterial lipoproteins are a subset of membrane proteins with their membrane association mediated by a lipid moiety attached to the N-terminal Cysteine residue of the mature protein (Braun and Wu 1994; Nakayama et al. 2012). Lipoproteins are typically essential components of the Gram-negative cell envelope and important virulence factors (Kovacs-Simon et al. 2011). Most lipoproteins in *Escherichia coli* and other *Enterobacteriaceae* are found on the periplasmic side of the inner or outer membranes. They are transferred from the inner membrane (IM) to the outer membrane (OM) by the Lol (localization of lipoproteins) machinery (Okuda and Tokuda 2011). Localisation is determined by the amino acid immediately following the lipidated Cys, in the +2 position (Yamaguchi et al. 1988). The “+2 rule” for *E. coli* lipoprotein sorting states that Asp at the +2 position acts as an IM retention signal, while any other amino acid in that position results in OM localisation. Results from a variety of recent studies now suggest that the surface exposure of lipoproteins in Gram-negative bacteria is a widespread phenomenon (Konovalova and Silhavy 2015; Wilson and Bernstein 2016). Unfortunately, few of the surface lipoprotein secretion mechanisms have been studied in detail.

V. vulnificus is predicted to encode ~ 100 lipoproteins, based on algorithms that search for cysteine-containing ‘lipobox’ motifs in genome sequences and other criteria (Babu et al. 2006; <http://www.mrc-lmb.cam.ac.uk/genomes/dolop/compngen.shtml>). Interestingly, a reported surface lipoprotein, immunogenic lipoprotein A (IlpA), has dual functions as an adhesin and a major immunostimulant in *V. vulnificus*-host cell interactions (Goo et al. 2007; Lee

et al. 2010). However, until recently, little work had been carried out on the other lipoproteins in *V. vulnificus*. The Lol pathway components LolA-E and the lipoprotein maturation enzymes (Lgt, Lsp, and Lnt) (Sutcliffe et al. 2012) are found in *V. vulnificus* genome. The detailed mechanism of lipoprotein biogenesis, including lipoprotein maturation and transport to the OM, remains elusive. Developing a technique to assess localisation of lipoproteins is an important step for the study of the lipoprotein transport mechanism in *V. vulnificus* and potentially for vaccine development against this bacterial infection.

There are two commonly used techniques for subcellular fractionation of membrane-associated proteins: detergent extraction with Sarkosyl and outer membrane vesicles (OMVs) preparation. Sarkosyl extraction is widely used to separate the OM and IM systems and localise membrane-associated proteins in Gram-negative bacteria. Membrane proteins can be obtained by selective solubilisation of IM components from crude membrane fractions by detergent treatment. This technique was applied in *Vibrio cholerae* to determine lipoprotein localisations (Bose and Taylor 2005; Morris et al. 2008). OMVs are small, spherical bilayered vesicles released into the extracellular environment from the OM of Gram-negative bacteria (Beveridge 1999). OMVs can perform a variety of functions including acquisition of nutrients, elimination of competing organisms, cell-to-cell signaling, aid bacterial survival, and modulation of host immune response, and are considered a novel secretion system (Lee et al. 2008; Jan 2017). Recent proteomic analyses of Gram-negative bacterial OMVs indicate that, compared with cellular proteins, OMVs are enriched with proteins associated with the extracellular region, outer membrane, and periplasm, and are depleted for inner membrane proteins (Lee et al. 2016). The localisation of membrane associated proteins, including lipoproteins, in *Borrelia burgdorferi* (Skare et al. 1995; Schulze and Zückert 2006) and *E. coli* (Mori et al. 2010) has been achieved by analysing the components of whole cells and OMV fractions.

In this study, we aimed to develop a technique to assess the localisation of lipoproteins in *V. vulnificus*. We achieved this by testing two techniques commonly used for the separation of membrane associated proteins, Sarkosyl extraction and OMVs preparation. We then used the OMVs technique to determine if the “+2 rule” sorting rule from *E. coli* can be applied to *V.*

vulnificus. Our results show that the sorting rule of lipoproteins in *V. vulnificus* might be different from that of *E. coli*.

Materials and methods

Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 1. *V. vulnificus* ATCC 27562 was cultured in Luria–Bertani (LB) broth or on LB agar at 30 °C. To prepare OMVs, *V. vulnificus* was cultured overnight in LB broth as a starter culture. Overnight cell cultures were inoculated (1:100, v/v) into fresh LB medium and allowed to grow to late exponential phase (OD₆₀₀ = 1.0). When appropriate, antibiotics were

added at the following concentrations: chloramphenicol (17 µg/ml), and polymyxin E (12.5 µg/ml). *E. coli* DH5α and SM10 λ pir were cultured as described above except ampicillin (100 µg/ml) was added when needed.

Construction of plasmids

All plasmids used in this study are derivatives of pSCT32 (Zhang et al. 2017) and were generated using ClonExpress One Step Cloning Kit (Vazyme Biotech). The expression of the recombinant proteins was driven by an IPTG-inducible promoter. All proteins were modified with a C-terminal His₆-tag to facilitate subsequent immunoblot analysis and purification. Both vector and insert DNA fragments were amplified using Phusion (Thermo Scientific) high-fidelity DNA

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or references
Strains		
<i>V. vulnificus</i>		
ATCC 27562	Type strain	Lab collection
<i>E. coli</i>		
DH5α	<i>supE44lacU169(lacZM15)hsdR17 recA lendA1gyrA96 thi-1 relA1</i>	Lab collection
SM10		Simon et al. (1983)
Plasmids		
pSCT32	<i>thi thr leu tonA lacY supE recA</i> [RP4-2-Tc::Mu] λ pirR6K Km ^r	Yan-Jiao et al. (2017)
pSCT24	<i>ilpA</i> in pSCT32	This study
pSCT25	<i>ompU</i> in pSCT32	This study
pSCT33	<i>wza</i> in pSCT32	This study
pSCT34	<i>wza</i> -(T2D) in pSCT32	This study
pSCT35	<i>ilpA</i> -(G2D) in pSCT32	This study
pSCT36	<i>mfp</i> in pSCT32	This study
pSCT40	<i>lolB</i> in pSCT32	This study
pSCT41	<i>lolE</i> in pSCT32	This study
pSCT43	<i>lsp</i> in pSCT32	This study
pSCT46	<i>lptE</i> in pSCT32	This study
pSCT47	<i>lptE</i> -(G2D) in pSCT32	This study
pSCT48	<i>bamD</i> in pSCT32	This study
pSCT49	<i>ilpA32-mrfp</i> fusion in pSCT32	This study
pSCT50	<i>ilpA32-mrfp</i> -(G2D) fusion in pSCT32	This study
pSCT109	<i>AOT11_RS11575</i> in pSCT32	This study

polymerase to perform recombination cloning. The vector fragment was amplified from pSCT32 with the pSCTNdeI-rev and Linker His-fwd primers. Protein genes (*ompU*, *ilpA*, *wza*, *lsp*, *bamD*, *lolB*, *lptE*, *lolE*, *mfp* and *AOT11_RS11575*) were amplified from *V. vulnificus* ATCC 27562 genomic DNA with gene-specific primers (Table 2). The *V. vulnificus* IlpA signal peptide was inserted to replace the original signal peptide of the LolB protein for improved expression. After ligation, the products were transfected to *E. coli* competent cells. The bacterial colonies were collected and confirmed by DNA sequencing.

The *IlpA32-mrfp* fusion constructs, in which monomeric red fluorescence protein (*mrfp*) was fused to the truncated lipoprotein IlpA tether peptide IlpA32, were generated by sequence overlap extension PCR (SOEPCR). Fragments containing the IlpA tether peptide IlpA32, amplified from *V. vulnificus* ATCC 27562 genomic DNA with IlpA-fwd and IlpA32-rev, and *mrfp* gene, amplified from pRJS1077 (Schulze et al. 2010) with IlpA32-mRFP-fwd and mRFP-rev, were fused using IlpA-fwd and mRFP-rev. The amplicons were then ligated with the pSCT32 vector fragment and transformed into *E. coli*.

The +2 site mutation was introduced into lipoproteins Wza, LptE, IlpA and IlpA32-mRFP with primer pairs Wza T2D-fwd and Wza T2D-rev, LptE G2D-fwd and LptE G2D-rev, IlpA G2D-fwd and IlpA G2D-rev, using the QuikChange-II XL site-directed mutagenesis kit (Stratagene), resulting in plasmids pSCT34, pSCT47, pSCT35 and pSCT50 respectively.

All plasmids were verified by PCR and DNA sequencing (Sangon Biotech., Shanghai, China). The resulting plasmids were transformed into *E. coli* SM10 λ pir and transferred into *V. vulnificus* ATCC 27562 by conjugation. The exconjugants were selected on LB agar containing chloramphenicol and polymyxin E.

Preparation of antiserum of OmpU and IlpA

The recombinant form of the OmpU (rOmpU) protein was overexpressed in *E. coli* BL21(DE3) carrying pSCT25 by adding IPTG at a concentration of 0.1 mM and purified using the HisPur Cobalt affinity column as directed by the manufacturer (Thermo Scientific). The recombinant form of IlpA (rIlpA) protein was overexpressed in *E. coli* BL21 (DE3) carrying plasmid pSCT24 and purified in the same manner as

the rOmpU protein. Purified rOmpU or rIlpA (each 120 μ g) was used to immunise Kunming mice (4-week-old, female) for the production of polyclonal antisera. The antisera against OmpU or IlpA were obtained from the immunised mice 1 week after the third immunisation and used for immunoblot analysis.

Cellular fractionation

V. vulnificus ATCC 27562 cells were obtained by centrifuging the bacterial cultures at 10,000 $\times$ g for 15 min at 4 °C. Cells were washed with 10 mM Tris–Cl (pH 8.0). Washed cells were resuspended and broken by sonication. Unbroken cells and debris were removed by centrifugation (10,000 $\times$ g, 15 min, 4 °C). Fractionation of whole-cell lysates into total membrane (TM), outer membrane (OM), and inner membrane (IM) fractions was accomplished by the method of Bose and Taylor (2005). Protein estimation was carried out using the BCA protein assay kit (Pierce) with bovine serum albumin as a standard.

Purification of *V. vulnificus* OMVs

OMVs were prepared from exponential phase cultures of strain ATCC 27562 as previously described with minor modifications (Wai et al. 2003). Briefly, culture supernatants were obtained by centrifuging the bacterial cultures at 10,000 $\times$ g for 15 min at 4 °C. The supernatant was collected and filtered through 0.22 μ m pore-size membrane filters (Millipore) to remove remaining bacterial cells. The supernatants were then centrifuged at 150,000 $\times$ g for 4 °C in a 45 Ti rotor (Beckman Instruments, Inc.). The resulting pellets, which contained the OMVs, were resuspended in PBS (pH 7.6). Protein estimation was carried out using the BCA protein assay kit (Pierce).

Transmission electron microscopy

The purified OMVs were adsorbed onto carbon-coated nickel 400-mesh grids. The grids were allowed to dry, stained with 2% phosphotungstic acid (pH 7.0) for 5 min, and washed with PBS. The morphology of the OMVs was examined under a Hitachi TEM System HC-1 at 80 kV.

Table 2 Oligonucleotides used in this study

Name	Sequence (5'–3')	Description
pSCT NdeI-rev	CATATGGTCTGTTTCCTGTGTG	Vector cloning forward primer
LinkerHis-fwd	CCCGGGGGTTCAGGAGCTCATCATCATCACCATCACTAAG	Vector cloning reverse primer
OmpU-fwd	CACACAGGAAACAGACCATATGAAAAAGACATTAATCGCACTTTC	<i>ompU</i> gene cloning forward primer
OmpU-rev	GCTCCTGAACCCCCGGGGAAGTCGTAACGTAGACCTAG	<i>ompU</i> gene cloning reverse primer
IlpA-fwd	CACACAGGAAACAGACCATATGAAATTTAGCCTCAAAGG	<i>ilpA</i> gene cloning forward primer
IlpA-rev	GCTCCTGAACCCCCGGGCCAGCCTTTTACTACGCC	<i>ilpA</i> gene cloning reverse primer
Wza-fwd	CACACAGGAAACAGACCATATGCTTTCAAAGCGTTAGAAAG	<i>wza</i> gene cloning forward primer
Wza-rev	GCTCCTGAACCCCCGGGCCAGCTTTTAAACGCGTAG	<i>wza</i> gene cloning reverse primer
Lsp-fwd	CACACAGGAAACAGACCATATGAGCGGTAAAGCGCAG	<i>lsp</i> gene cloning forward primer
Lsp-rev	GCTCCTGAACCCCCGGGCTTCTCTTCTTCTTTTCCG	<i>lsp</i> gene cloning reverse primer
BamD-fwd	CACACAGGAAACAGACCATATGAGCGGTAAAGCGCAG	<i>bamD</i> gene cloning forward primer
BamD-rev	GCTCCTGAACCCCCGGGCTTCTCTTCTTCTTTTCCG	<i>bamD</i> gene cloning reverse primer
LolB-fwd	CACACAGGAAACAGACCATATGCTCTTGCCGCTGTTATG	<i>lolB</i> gene cloning forward primer
LolB-rev	GCTCCTGAACCCCCGGGTTTAGCGTCCACTTACTCAC	<i>lolB</i> gene cloning reverse primer
LptE-fwd	CACACAGGAAACAGACCATATGCGTTTTACGTCCGTG	<i>lptE</i> gene cloning forward primer
LptE-rev	GCTCCTGAACCCCCGGGCTGATTACTTGAGTTGACGG	<i>lptE</i> gene cloning reverse primer
MFP-fwd	CACACAGGAAACAGACCATATGCGCAAACCCAGATAAC	<i>mfp</i> gene cloning forward primer
MFP-rev	GCTCCTGAACCCCCGGGAATGCCCTCCTCGCGTAC	<i>mfp</i> gene cloning reverse primer
LolE-fwd	CACACAGGAAACAGACCATATGATTTCTTCTTGCGG	<i>lolE</i> gene cloning forward primer
LolE-rev	GCTCCTGAACCCCCGGGTTTACTGCTCAGCACGGC	<i>lolE</i> gene cloning reverse primer
IlpA32-rev	TTTGCTCGTGTAACGGC	IlpA tether truncation (to aa 32) reverse primer
IlpA32-mRFP-fwd	GCCGTTGACACGAGCAAAGACGTCATCAAGGAGTTCATG	IlpA tether-mRFP fusion cloning forward primer
mRFP-rev	GCTCCTGAACCCCCGGGGCGCCGGTGAGTGCGG	<i>mrfp</i> gene cloning reverse primer
Wza T2D-fwd	GCCGGTTGTGATGTACCCGGCTCACATCTATCC	Wza tether T2D forward mutagenic primer
Wza T2D-rev	CCGGGTACATCACAACCGCAAGCAAGGC	Wza tether T2D reverse mutagenic primer
LptE G2D-fwd	GTCTGCATGTGATTTTCATTTACGTGGTGAGTACTC	LptE tether T2D forward mutagenic primer
LptE G2D-rev	ATGAAAATCACATGCAGACAACATGGC	LptE tether T2D reverse mutagenic primer
IlpA G2D-fwd	AGCAGGTTGTGACGAAAAAGCCGTTGACACG	IlpA tether T2D forward mutagenic primer
IlpA G2D-rev	GCTTTTTCGTCACAACCTGCTAATACCAATGTTG	IlpA tether T2D reverse mutagenic primer
RS11575-fwd	CACACAGGAAACAGACCATATGTGTAACCTACTCAAATCGTC	<i>RS11575</i> gene cloning forward primer
RS11575-rev	GCTCCTGAACCCCCGGGACGCATCGTATCGACTTG	<i>RS11575</i> gene cloning reverse primer

SDS-PAGE and immunoblot analysis

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualised by Coomassie blue staining. For immunoblots, proteins were electrophoretically transferred to a PVDF membrane (Millipore) using a Transblot semi-dry transfer cell (Bio-Rad). Membranes were blocked and incubated with antibodies in 5% dry milk, 20 mM Tris-500 mM NaCl, 0.05% Tween-20. The antibodies used were mouse polyclonal antisera against OmpU (dilution, 1:20,000) and IlpA (dilution, 1:10,000). The secondary antibody was goat-anti-mouse IgG (H+L) (Sigma). Proteins overexpressed in *V. vulnificus* such as Wza, BamD, Lsp, LptE, MFP, LolB, and LolE, which were tagged with a hexahistidine epitope tag, were detected directly with a nickel-activated HisProbe-HRP conjugate (Pierce). SuperSignal West Dura substrate (Pierce) was used for the chemiluminescent detection of HRP-labeled proteins.

Results

Sarkosyl extraction cannot be used for localisation of lipoproteins in *V. vulnificus*

Sarkosyl has been shown to be useful for dividing crude envelope samples from *V. cholerae* into distinct inner and outer membrane protein profiles (Lohia et al. 1984). More recently, this technique has been used to localise lipoproteins in *V. cholerae* (Bose and Taylor 2005; Morris et al. 2008). Therefore, we first attempted to investigate the subcellular localisation of lipoproteins in *V. vulnificus* using a Sarkosyl-based membrane fractionation protocol (Bose and Taylor, 2005).

V. vulnificus strain ATCC 27562, which expressed a C-terminal His₆-tag Wza protein, was grown to OD₆₀₀ 2.0. The bacteria were harvested and subcellular fractionation was performed as described in materials and methods. The TM, OM and IM fractions were isolated from the strain and run on an SDS-PAGE gel. To verify the correct lipoprotein localisation, one known OM protein, OmpU, and two known OM lipoproteins, Wza and IlpA, were identified by immunoblotting analysis. The His₆-tagged Wza was identified using an anti-His tag reagent HisProbe, while OmpU and IlpA were identified using specific

Fig. 1 Localization of proteins by Sarkosyl extraction. *V. vulnificus* cells harboring pSCT33 were grown in LB medium to OD₆₀₀ = 1 as described in materials and methods. The bacteria were harvested and subcellular fractionation was performed. Total membrane (TM), outer membrane (OM) and inner membrane (IM) were isolated from the *V. vulnificus* cell and immunoblotting analysis of whole cell (WC), TM, OM and IM samples were performed as described in the [Materials and methods](#) section. OmpU an outer membrane protein, IlpA an OM lipoprotein, Wza an OM lipoprotein

antisera (Fig. 1). All three proteins, Wza, OmpU and IlpA, were clearly detected in the whole cell samples and the TM sample, indicating that these three proteins are membrane-associated proteins. A lack of OmpU in the IM fraction and an enrichment of OmpU in the OM fraction indicated that these samples are enriched specifically for OM proteins. However, as determined by Western blotting (Fig. 1), the two known OM lipoproteins, IlpA and Wza, were detected predominantly in the IM, rather than the OM fraction. These results were not consistent with previously published protein functional and localisation studies (Goo et al. 2007; Wright et al. 2001), which indicated that Sarkosyl-based membrane fractionation method is not suitable for separation and localisation of lipoproteins in *V. vulnificus*.

V. vulnificus produces OMVs with OM lipoprotein

Lipoproteins in *B. burgdorferi* (Skare et al. 1995; Dowdell et al. 2017) and *E. coli* (Moriel et al. 2010) have been localised by analysis of the components of OMVs. However, proteomic analyses of Gram-negative bacterial OMVs indicate that bacterial OMVs still carried IM components (Lee et al. 2016). Therefore, we investigated whether OMVs of *V. vulnificus* can be used to localise lipoproteins.

First we isolated OMVs from exponential phase cultures of *V. vulnificus* strain ATCC 27562. Vesicle

Fig. 2 *V. vulnificus* produces OMVs carrying OM lipoprotein. **a** Transmission electron micrograph (TEM) of negatively stained *V. vulnificus* strain ATCC 27562 cultured in logarithmic phase of growth shows the presence of vesicles (indicated by arrows). Bar = 1 μ m. **b** TEM of negatively stained OMVs purified from culture broth of *V. vulnificus* strain ATCC 27562. Two types of OMVs are seen in the photographs. Most vesicles have a single membrane, but occasionally vesicles with two membranes are observed (black arrow). Both vesicle chain-like

morphotype (indicated by white arrows) and free vesicles were observed in the purified vesicle fraction. Bar = 500 nm. **c** *V. vulnificus* whole-cell lysates (WC) and purified OMVs were separated by SDS-PAGE and the gel was stained with Coomassie blue. **d** Immunoblotting analysis of protein contents in WC and OMVs fractions from *V. vulnificus*. The OMVs contain bacterial proteins localized to the outer membrane. *IlpA* an OM lipoprotein, *Lsp* an IM signal peptidase, *OmpU* an OM protein

production by *V. vulnificus* was confirmed by transmission electron microscopy (Fig. 2a, b). We observed free vesicles and vesicle chain-like morphotypes with diameters ranging from 30 to 250 nm. Most vesicles had a single membrane, but occasionally vesicles with two membranes were observed. Subsequently, purified OMVs were subjected to SDS-PAGE and immunoblotting. *OmpU* and *IlpA* were used again as OM markers for detection of OMVs. The known IM protein *Lsp*, the dedicated lipoprotein signal peptidase, was used as a control to assess the purity of the OMV preparation, as it should be absent from the OMV preparation. The immunoreactive bands of *OmpU* and *IlpA* were observed in both whole cell and OMVs samples (Fig. 2d). There was no IM marker protein *Lsp* found in the OMV samples, indicating that the presence of *OmpU* and *IlpA* in the

OMVs was not a consequence of contamination with IM proteins during the secretion of *V. vulnificus* OMVs. Thus, immunoblot analyses confirmed that the purified OMVs were derived from the OM of *V. vulnificus* and carried OM lipoproteins.

Localisation of lipoproteins by isolation of OMVs in *V. vulnificus*

In order to further confirm the association of the OM lipoproteins with OMVs, we expanded our study to include five additional proteins, whose functions and localisations are conserved across Gram-negative bacteria. It was reported that OMVs released by normally growing *E. coli* contain a low concentration of lipoproteins (Wensink and Witholt 1981). To improve the sensitivity of protein detection, we

decided to overexpress lipoproteins in *V. vulnificus*. Three known *V. vulnificus* OM lipoproteins, BamD (Onufryk et al. 2005; Malinverni et al. 2006), LolB (Tanaka et al. 2001) and LptE (Wu et al. 2006), a known IM protein LolE (the Lol pathway ABC transporter component; Yakushi et al. 2000) and two predicted IM lipoproteins, membrane fusion protein (MFP) and AOT11_RS11575, were overexpressed with a C-terminal His₆-tag in strain ATCC 27562.

OMVs were isolated from exponential (6 h) phase cultures of *V. vulnificus* cells harbouring different protein overexpression plasmids. *V. vulnificus* whole-cell lysates and purified OMVs were separated by SDS-PAGE, then immunoblotting was used to screen for the His₆-tagged proteins. The three known OM lipoproteins, BamD (Fig. 3a), LolB (Fig. 3b) and LptE (Fig. 3c), were detected in the OMVs samples. While the predicted IM lipoprotein MFP (Fig. 3e), AOT11_RS11575 (Fig. 3f) and the IM marker protein

LolE (Fig. 3d) were almost undetectable in the OMVs samples. Taken together, these results demonstrated that OM lipoproteins but not IM lipoproteins are present in the *V. vulnificus* OMVs preparations.

The “+2 rule” does not apply to *V. vulnificus* lipoprotein IlpA (G2D) mutant

In *E. coli*, lipoprotein localisation follows the so called “+2 rule”, where an N-terminal Asp+2 is predictive of IM localisation, while any other residue at that position stipulates OM localisation (Yamaguchi et al. 1988). Of the nearly 100 predicted lipoproteins from the *V. vulnificus* YJ016 genome, only two have an Asp+2 at the N-terminus (Babu et al. 2006). Because we expect a greater proportion of lipoproteins to be localised to the IM, we wondered if the *E. coli* “+2 rule” was applicable to *V. vulnificus*. To assess if the N-terminal Asp+2 plays a crucial role in localisation

Fig. 3 Localisation of lipoproteins and Lol gene by isolation of OMVs in *V. vulnificus*. *V. vulnificus* cells harbouring different protein over-expression plasmids were grown in LB medium. OMVs were isolated from exponential phase cultures as described in materials and methods. *V. vulnificus* whole-cell lysates and purified OMVs were separated by SDS-PAGE. The expression of His-tagged lipoprotein BamD (a), LolB (b), LptE (c), Lol pathway ABC transporter component LolE (d), predicted IM lipoprotein MFP (e) and AOT11_RS11575 (f) were detected by Western blotting with Hisprobe. Samples were normalized to the OM protein OmpU

Fig. 4 “+2 rule” cannot apply to *V. vulnificus* IlpA (G2D) mutant. **a** A schematic overview of lipoproteins and their tether peptide “+2” site mutant, as well as the fusion proteins created from IlpA and mRFP. Tether peptides are shown in one-letter amino acid codes. Amino acid positions relative to the acyl-modified Cys residue (defined as +1) are shown above the wt peptide sequence. His₆-tagged lipoprotein and their “+2” site mutants, IlpA and IlpA (G2D) (**b**), LptE and LptE (G2D) (**c**), Wza and Wza (T2D) (**d**), IlpA32-mRFP and IlpA32-mRFP

(G2D) (**e**), were expressed in *V. vulnificus*, respectively. *V. vulnificus* cells harboring different protein over-expression plasmids were grown in LB medium. OMVs were isolated from exponential phase cultures as described in materials and methods. *V. vulnificus* whole-cell lysates and purified OMVs were analyzed by SDS-PAGE and immunoblotting. His₆-tagged lipoprotein was detected with Hisprobe. OmpU were detected with the antisera. Samples were normalized to the OM protein OmpU

of lipoproteins in *V. vulnificus*, the second residues in three mature OM lipoproteins, Wza, LptE, and IlpA were mutated to aspartates.

Wza, LptE, and IlpA tether peptide mutants (Fig. 4a) were generated by modifying existing Wza, LptE, and IlpA expression plasmids through oligo-mediated site-directed mutagenesis as described in the Materials and Methods. The resulting plasmids were used to transform *V. vulnificus* strain ATCC 27562. The localisation of +2 site mutants was identified by immunoblot analysis of the OMVs. While overexpressed wild type Wza and LptE were found in the

OMVs preparations, the Wza (T2D) and LptE (G2D) mutants were mislocalised, being almost undetectable in the OMVs samples (Fig. 4b, c). This could indicate that Asp at position +2 functions as an IM retention signal in *V. vulnificus*. However, similar to the wild type IlpA protein, the IlpA (G2D) mutant was localised predominantly to the OM, suggesting the “+2 rule” from *E. coli* does not always hold true for *V. vulnificus*.

In order to confirm that the lipoprotein sorting signal is present in the N-terminal tether residues of IlpA, we constructed an IlpA32-mRFP chimeric

lipoprotein, comprising the signal peptide, 10 N-terminal residues of the mature region of IlpA and a reporter protein, mRFP (Fig. 4a). The resulting fusion lipoprotein was well expressed and located in the outer membrane of *V. vulnificus* (Fig. 4e). The mRFP reporter fusion to the wild type N-terminal lipopeptide behaved like the wild type IlpA lipoprotein, showing that the N-terminus of lipoprotein IlpA is sufficient for proper OM localisation, which is consistent with other reports in *E. coli* and *B. burgdorferi* (Ghrayeb and Inouye 1984; Schulze and Zückert 2006). The IlpA32-mRFP fusion lipoprotein retained its predominantly OM location when +2 site Gly was mutated to Asp (Fig. 4d). This result confirmed the localisation pattern of the IlpA (G2D) mutant and showed that the C-terminal globular protein region does not affect the final destinations of lipoproteins.

Together, these experiments demonstrated that while Asp can serve as the canonical IM retention signal in *V. vulnificus*, the ‘+2 rule’ does not apply to all *V. vulnificus* lipoproteins.

Discussion

The established techniques developed for separating membrane-associated proteins in bacteria include sucrose density gradient centrifugation, detergent extraction of the crude envelope and OMVs preparation. The sucrose density gradient centrifugation technique has been commonly used in studies of Gram-negative bacteria. However, in some cases, a clean separation of the outer and inner membranes was not achieved by this technique. For example, incomplete separation of the two membranes by this technique were found in *V. cholerae*, which might due to a tighter linking of the inner and outer membranes than in other Gram-negative bacteria (Kelley and Parker 1981; Lohia et al. 1984).

Techniques based on selective solubilisation by detergents are widely used for separating OM and IM associated proteins, with the detergent-extracted outer membrane sample having lower phospholipid content (Lugtenberg and Van Alphen 1983; Lohia et al. 1984). In the current study, we could not detect the OM lipoprotein markers, IlpA and Wza, in Sarkosyl-extracted OM fractions of *V. vulnificus*. Detergent treatment of OM preparations might result in the loss of lipoproteins together with the phospholipids.

Therefore, Sarkosyl-based membrane fractionation is not suitable for separation and localisation of lipoproteins in *V. vulnificus*, although it worked in *V. cholerae* (Bose and Taylor 2005; Morris et al. 2008).

A wide variety of Gram-negative bacteria constitutively secrete OMVs during growth (Beveridge 1999; Jan 2017). OMVs are spherical, bilayered proteolipids and are composed of outer membrane proteins, lipopolysaccharide, outer membrane lipids, periplasmic proteins, cytoplasmic proteins, DNA, RNA and other factors associated with virulence (Lee et al. 2008). In some cases, OMVs also contain the components from the inner membrane and cytoplasm (Kulp and Kuehn 2010). The bacterial pathogens *V. cholerae* and *V. vulnificus* were reported to secrete OMVs (Chatterjee and Das 1967; Kim et al. 2010). Proteomic analysis of *V. cholerae* OMVs showed that of 90 identified proteins, 8% were IM associated and 6% were cytoplasmic proteins (Altindis et al. 2014). It was reported that OMVs released by normally growing *E. coli* contain very little lipoprotein (Wensink and Witholt 1981). Therefore, to verify our lipoprotein localisation method, we overexpressed and then identified the localisation of seven known *V. vulnificus* lipoproteins including two predicted IM lipoproteins, MFP and AOT11_RS11575. Our experimental data showed that *V. vulnificus* OMVs contained low amounts of IM proteins. Two overexpressed IM marker proteins, Lsp and LolE, were undetectable in the OMVs fractions. All lipoproteins included in this study were localised as expected based on their known functions; however, signal levels in OMVs fraction were weaker than those in whole cell samples after normalization with OmpU.

The *E. coli*-derived lipoprotein sorting rules seems to be conserved for bacteria in the family *Enterobacteriaceae* such as *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, *Yersinia pseudotuberculosis*, *Erwinia carotovora*, and *Klebsiella oxitoca* (Lewenza et al. 2006), but didn't fully extend to other γ -proteobacteria. Studies in *Pseudomonas aeruginosa* showed that an Asp at position +2 could function as an IM retention signal, but that Lys and Ser at position +3 and +4, respectively, were the innate IM signals of lipoprotein MexA (Narita and Tokuda 2007). Our experimental data in *V. vulnificus* showed that two predicted IM proteins, MFP and AOT11_RS11575, carrying the Asp+2 residue in the N-terminal tether, and two OM lipoprotein mutants,

Wza (T2D) and LptE (G2D), all located to the IM, which indicated that Asp+2 can function as an IM retention signal in *V. vulnificus*. However, a Gly to Asp mutation at position +2 of the OM lipoprotein IlpA tether did not affect its localisation to the OM. It was also found that, using mRFP as a localisation reporter, an IlpA tether-mRFP chimeric lipoprotein and its G2D mutant behaved like IlpA and localised to the OM. In *E. coli*, the presence of the His or Lys residues at position +3 lowered the stringency of IM retention by an Asp+2 signal (Gennity and Inouye 1991). When the residue at position +3 was Asp, Glu or Gln, lipoproteins with Asp+2 were strongly retained in spheroplasts even in the presence of a saturating concentration of LolA (Terada et al. 2001). However, in the *V. vulnificus* system, the IlpA (G2D) mutant and the IlpA32-mRFP (G2D) could be transported to the OM with Asp+2 and Glu+3 residues in their N-terminal tether. This led us to hypothesise that different residues in the tether sequence might govern the sorting of lipoproteins in *V. vulnificus*.

Interestingly, few putative IM lipoproteins with an Asp+2 at the N-terminus, could be found in the list of lipoproteins predicted by genome-wide computational analysis of the *V. vulnificus* genome (Babu et al. 2006). However, some IM-specific lipoproteins with a residue other than Asp at position +2, such as the membrane fusion proteins (MFPs), are present in the *V. vulnificus* lipoproteome (Fig. S1). The membrane fusion proteins (MFPs) are N-terminally anchored to the IM by either a lipid moiety or a helical signal anchor sequence and are a major component of resistance-nodulation-division (RND) 2-type multidrug efflux pumps (Paulsen et al. 1996) and should therefore be localized to the IM. However, none of the MFP lipoproteins in Fig. S1 have Asp at the position +2. These observations suggest that lipoprotein sorting signals differ between *V. vulnificus* and *E. coli*, a situation previously observed from the study of *P. aeruginosa* MexA (Narita and Tokuda 2007). The precise sorting rules of lipoproteins to the OM in *V. vulnificus* remain to be determined.

In conclusion, we have developed a convenient and precise technique to identify the membrane association of *V. vulnificus* lipoproteins, which might be applicable to other systems. Future studies will continue to define sorting rules for lipoproteins targeted to different membranes in *V. vulnificus*, and to test the involvement of the Lol machinery in the

secretion of surface lipoproteins. While the establishment of this technique will allow us to localise additional *V. vulnificus* lipoproteins, the finding that this species has novel sorting rules encourages us to fully elucidate the precise molecular mechanism of lipoprotein transport in *V. vulnificus*.

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Conflict of interest The authors declare that they have no conflict of interest.

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