The Outer Surface Lipoprotein VolA Mediates Utilization of Exogenous Lipids by *Vibrio cholerae*

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ABSTRACT **Previous work from our laboratory showed that the Gram-negative aquatic pathogen *Vibrio cholerae* can take up a much wider repertoire of fatty acids than other Gram-negative organisms. The current work elaborated on the ability of *V. cholerae* to exploit an even more diverse pool of lipid nutrients from its environment. We have demonstrated that the bacterium can use lysophosphatidylcholine as a metabolite for growth. Using a combination of thin-layer chromatography and mass spectrometry, we also showed that lysophosphatidylcholine-derived fatty acid moieties can be used for remodeling the *V. cholerae* membrane architecture. Furthermore, we have identified a lysophospholipase, VolA (*Vibrio* outer membrane lysophospholipase A), required for these activities. The enzyme is well conserved in *Vibrio* species, is coexpressed with the outer membrane fatty acid transporter FadL, is one of very few surface-exposed lipoprotein enzymes to be identified in Gram-negative bacteria and the first instance of a surface lipoprotein phospholipase. We propose a model whereby the bacterium efficiently couples the liberation of fatty acid from lysophosphatidylcholine to its subsequent metabolic uptake. An expanded ability to scavenge diverse environmental lipids at the bacterial surface increases overall bacterial fitness and promotes homeoviscous adaptation through membrane remodeling.**

IMPORTANCE **Our understanding of how bacteria utilize environmental lipid sources has been limited to lipids such as fatty acids and cholesterol. This narrow scope may be attributed to both the intricate nature of lipid uptake mechanisms and the diversity of lipid substrates encountered within an ecological niche. By examining the ability of the pathogen *Vibrio cholerae* to utilize exogenous lipids, we uncovered a surface-exposed lipoprotein (VolA) that is required for processing the prevalent host lipid lysophosphatidylcholine. VolA functions as a lipase liberating a fatty acid from exogenous lysophospholipids. The freed fatty acid is then transported into the cell, serving as a carbon source, or shunted into phospholipid synthesis for membrane assembly. A limited number of surface-exposed lipoproteins have been found in Gram-negative organisms, and few have enzymatic function. This work highlights the ability of bacteria to exploit exogenous lipids for both maintenance of the membrane and carbon source acquisition.**

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Gram-negative bacteria have developed several mechanisms for scavenging nutrients and other important metabolites from their surroundings. One such mechanism, the fatty acid uptake pathway, allows Gram-negatives to import long-chain fatty acids (LCFAs) through their cell wall via the outer membrane transporter FadL and ligate them to coenzyme A using the fatty acyl coenzyme A ligase FadD. After this ligation event, the activated fatty acids are destined for energy production or membrane construction. Exogenous fatty acids can serve as an energy source through the degradative β-oxidation pathway, which yields numerous activated carrier molecules for metabolic gain. Alternatively, the steep energy requirements for fatty acid biosynthesis can be circumvented by shunting the imported fatty acid into the membrane for phospholipid assembly (1).

Previous research by our laboratory has shown that the Gram-negative human pathogen *Vibrio cholerae* is capable of utilizing diverse fatty acids from the surrounding environment, including very-long-chain fatty acids with multiple unsaturations (2). This is in contrast to other Gram-negative organisms; *Escherichia coli* cannot metabolize fatty acids that are longer than 20 carbons nor process those fatty acids with equally high degrees of unsaturation (3–5). This difference in substrate profiles between *V. cholerae* and other Gram-negatives may be attributed to *V. cholerae*'s multiple homologs of FadL and FadD. A previous study from our laboratory determined that the bile secreted in the small intestine caused variation in the phospholipid profile of *V. cholerae* when analyzed via thin-layer chromatography and mass spectrometry (2). All of the major phospholipids—including phosphatidylglycerol, phosphatidylethanolamine, and cardiolipin (see Fig. S1A in the supplemental material)—showed an altered acylation profile. Further experimentation revealed that the fatty acid component of bile specifically caused the altered migratory pattern. The ability of

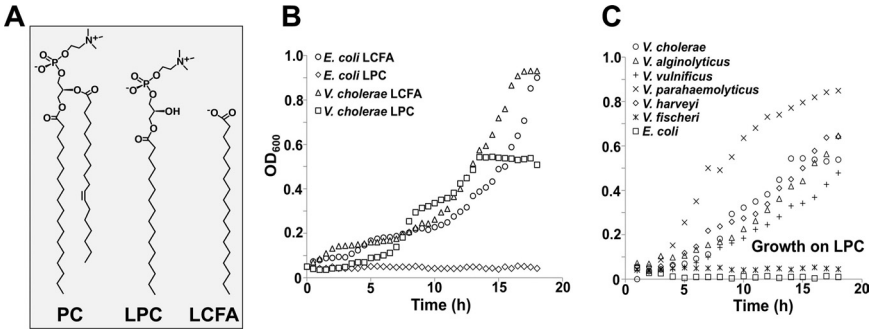

FIG 1 Growth of *E. coli* and *Vibrio* species on LPC or LCFA. (A) Chemical structures of PC (phosphatidylcholine), LPC (lysophosphatidylcholine), and an LCFA (long-chain fatty acid). (B) Both *E. coli* and *V. cholerae* can utilize LCFA as the sole carbon source for growth, while only *V. cholerae* can utilize LPC. (C) All *Vibrio* species tested were able to use LPC as the sole carbon source for growth, with the exception of *V. fischeri*.

V. cholerae to remodel its membrane phospholipids using exogenous lipid sources is an important advantage. This is further emphasized by the fact that environmental fatty acids found in aquatic sediment were also found to cause migratory shifts (2). Altering the phospholipid profile in this manner can have dramatic effects on fitness via homeoviscous adaptation and reduced energetic requirements for membrane biogenesis.

In their respective niches, Gram-negatives are in contact with diverse lipid chemical species, more than fatty acids alone, which can serve as potential carbon sources. *V. cholerae* is immersed in lipids when colonizing the human intestine and when living in an aquatic environment (6). For example, copepods, which constitute a common animal reservoir for *V. cholerae*, are known to synthesize phospholipids with very-long-chain fatty acyl tails, including phosphatidylcholine with attached docosapentaenoic ($C_{22:5}$) and docosahexaenoic ($C_{22:6}$) acids (7). The lumen of the human intestine has significant amounts of lysophosphatidylcholine (LPC), derived from lecithinase activity on biliary and dietary sources of phosphatidylcholine (8, 9). Given that *V. cholerae* has been shown to remodel its membrane architecture using exogenous lipids and that the bacteria have consistent exposure to these alternative lipids, *V. cholerae* might have evolved mechanisms to utilize these alternate sources of acyl chains for membrane remodeling as well.

Currently, while some steps in the transport of fatty acids into the bacterial cell are well characterized—such as those performed by FadL and FadD—other steps—such as how *V. cholerae* might utilize alternative lipid sources in conjunction with the fatty acid transport pathway—are enigmatic. This work reports the ability of *V. cholerae* El Tor O1 to utilize LPC for nutrition and for remodeling of cell wall phospholipids. We have identified a putative surface-exposed lipoprotein, VolA, that is responsible for this activity, and we suggest a mechanism for its function. VolA mediates the unusual lipid utilization characteristics of *V. cholerae* and represents a newly discovered member of undercharacterized surface-exposed lipoprotein enzymes of Gram-negative bacteria.

RESULTS

***Vibrio cholerae* can use lysophosphatidylcholine as the sole carbon source.** In order to determine if *V. cholerae* El Tor O1 could utilize lysophospholipids, bacteria (strains are listed in Table S1 in the supplemental material) were grown in defined minimal medium with either 0.2% glucose, 2 mM stearoyl ($C_{18:0}$)-lysophosphatidylcholine (LPC), or 2 mM stearic acid, serving as the sole carbon source (Fig. 1; see also Fig. S2). When *E. coli* and *V. cholerae* were grown with LCFAs, no difference in growth could be detected. However, only *V. cholerae* was able to utilize LPC. The maximal optical density at 600 nm (OD_{600}) of *V. cholerae* grown on LPC was approximately half that of the LCFA-grown cultures (Fig. 1B); the slower growth on LPC is likely due to the rate-limiting step of release of the fatty acid from the LPC molecule. Similar results were obtained when the level of growth was measured by determining CFU of viable cells on agar plates (see Fig. S3).

After observing this phenotype in *V. cholerae* El Tor, we expanded our testing to other *Vibrio* species, including *V. alginolyticus*, *V. fischeri*, *V. harveyi*, *V. parahaemolyticus*, and *V. vulnificus*. Bacteria were grown under conditions similar to those of the initial experiments, with either 0.2% glucose (see Fig. S2) or 2 mM LPC (Fig. 1C). A defined salt-rich minimal medium was used for growth of *V. fischeri* and *V. harveyi* strains. Interestingly, when grown with 2 mM LPC as the sole source of carbon, all strains were able to grow, with the exception of *E. coli* and *V. fischeri* (Fig. 1C).

Bioinformatic analysis of *Vibrio* strains reveals a putative phospholipase in an operon with a FadL homolog. Since the ability to use LPC as the sole carbon source was found in multiple species of *Vibrio* and not in others, a comparative genome search was performed using the String 9.0 database (http://string-db.org). In all species capable of growth on LPC, an additional gene (*vca0863* in *V. cholerae*) was found directly downstream of a FadL homolog (gene *vca0862* in *V. cholerae*) (see Fig. S4 in the supplemental material). A similar genetic organization was conspicuously absent from the *V. fischeri* genome. Because only those bacterial strains that contained this putative operon were capable of growth on LPC, it suggested that *vca0863* might be involved in the LPC growth phenotype. Interestingly, VcA0863 is annotated as a putative lipase (Uniprot Q9KL83 [http://www.uniprot.org]; GenBank accession no. AAF96761.1 [http://ncbi.nlm.nih.gov]) and has an N-terminal lipoprotein signal sequence. VcA0863 shows strong homology to pfam12262, having the known lipase amino acid sequence motif Gly-Xaa-Ser-Xaa-Gly (10). This motif, containing a catalytic serine, has been identified in lipases derived from other aquatic organisms (10) and could potentially act in a similar manner in *V. cholerae*. A nonaspartate residue is present in the +2 position after the signal peptide cleavage site, and based upon rules governing lipoprotein sorting and localization, the protein should be localized to the outer membrane (11).

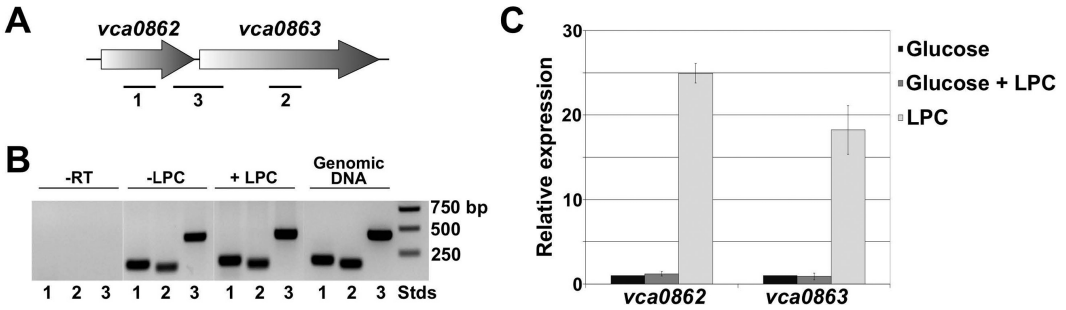

FIG 2 Organization and expression of *vca0862* and *vca0863*. (A) The genetic organization of *vca0862* (FadL) and *vca0863* (VolA) in *V. cholerae*, along with primer extension locations used in RT-PCR. (B) RT-PCR of *vca0862* and *vca0863* show that the genes are cotranscribed and basal levels of expression are independent of the presence of LPC. A genomic DNA template was used to confirm the amplified product sizes, and cDNA without reverse transcriptase (-RT) was used as the negative control verifying DNA-free RNA. (C) qRT-PCR data of the expression of *vca0862* and *vca0863* grown with glucose and/or LPC. When growth was in minimal medium containing LPC as the sole carbon source, expression of *vca0862* and *vca0863* increased 25- and 18-fold, respectively, compared to expression for a glucose-grown control.

The genes *vca0862* and *vca0863* are cotranscribed in *V. cholerae*. The intergenic region between the genes *vca0862* and *vca0863* in all *Vibrio* species was between 16 and 23 bp. To confirm that the FadL homolog (encoded by *vca0862*) and the phospholipase (encoded by *vca0863*) are encoded in an operon, RNA was purified from wild-type *V. cholerae* grown in minimal medium supplemented with glucose or glucose and LPC. cDNA was generated and amplified by primers designed for internal regions of *vca0862*, *vca0863*, or a region spanning 400 bases centered on the intergenic region (Fig. 2A). Genomic DNA from wild-type *V. cholerae* was used as a positive control. PCR products were observed for the internal regions of the individual genes under both conditions: a 200-bp band representing *vca0862* and a 165-bp band representing *vca0863* (Fig. 2B). The presence of LPC in the growth medium was not required for gene transcription compared to the glucose control. A 430-bp band, generated from the intergene-spanning primers, confirms that *vca0862* and *vca0863* are cotranscribed.

Quantitative reverse transcription-PCR (qRT-PCR) was performed to determine the expression profiles of *vca0862* and *vca0863* in wild-type bacteria grown in the presence of glucose, LPC, or glucose and LPC together. The relative expression of both genes was measured as a ratio compared to expression for the glucose-only control. Results showed that there was no statistically significant difference in the expression of *vca0862* and *vca0863* when LPC was added to the glucose control (Fig. 2C). However, when the strain was grown with LPC as the sole carbon source, expression of the genes increased dramatically, with that of *vca0862* increasing ~25-fold and that of *vca0863* increasing ~18-fold compared to expression for the glucose control. Analysis of the *vca0862* promoter region using a footprinting algorithm (12) revealed binding sites for both cAMP receptor protein (CRP) and FadR. *E. coli* CRP is a transcriptional activator that binds to DNA in a complex with cyclic AMP (cAMP); in the presence of glucose, levels of cAMP are decreased and CRP does not bind to the DNA (13, 14). FadR is a repressor of many fatty acid metabolism genes, including *fadL*, and is inhibited by binding coenzyme A (CoA)-activated fatty acids (15). Thus, in the presence of glucose, both genes could be downregulated due to a lack of active CRP; this is consistent with the upregulation that is observed in cultures grown with LPC as the sole carbon source. Additionally, for growth in succinate (see Fig. S5 in the supplemental material), this downregulation is abrogated, and growth with both succinate in the presence of LPC and LPC only shows an upregulation of *vca0862* and *vca0863* compared to findings for a succinate-only control. This upregulation in the presence of LPC could also be due in part to a lack of downregulation by FadR because of its inhibition by LPC-derived fatty acids.

***vca0863* is necessary for utilization of lysophosphatidylcholine.** After identification of an operon containing a phospholipase and an outer membrane fatty acid transporter, we investigated whether bacterial growth on LPC was dependent upon expression of *vca0863*. When a *vca0863*-deficient *V. cholerae* strain was incubated with LPC as the sole carbon source, we found that it failed to grow (Fig. 3A). Growth on LPC is restored when *vca0863* was expressed in *trans* from a low-copy-number vector (pVcA0863). However, the doubling time for the complemented strain was decreased ~3-fold compared to that for the wild type and may arise from increased release of LCFA from LPC by the lipase activity.

Although *E. coli* K-12 is unable to grow on LPC, heterologous expression of VcA0863 in *E. coli* conferred growth on LPC (Fig. 3B). However, expression of VcA0863 in which the amino acid glycine at position 20 is replaced with an aspartate residue (VcA0863-G20D) does not support growth. Based upon the rules of lipoprotein sorting, replacement of the glycine residue with an aspartic acid in the +2 position after the lipoprotein signal sequence would result in mislocalization of VcA0863 to the inner membrane (11). These results suggest that expression of VcA0863 is sufficient for the bacterium to utilize lysophospholipids from the environment and that the protein must be localized to the outer membrane.

Strains expressing *vca0863* modify their membrane phospholipids using the fatty acyl moiety of lysophosphatidylcholine. Previously, we reported that *V. cholerae* underwent substantial membrane remodeling by incorporating long-chain fatty acids from its surrounding environment. Here, we asked if membrane remodeling also occurred in the presence of lysophospholipids. Cultures of wild-type *V. cholerae* El Tor O1, the *vca0863* mutant strain, and the complemented mutant were grown in defined minimal medium containing 2% glucose and $^{32}P_i$ in the presence of LCFA or LPC. Wild-type *E. coli* K-12 was included as a negative control (see Fig. S1B in the supplemental material). ^{32}P–labeled phospholipids were extracted and analyzed by thin-layer chromatography (TLC) (Fig. 4A). By using commercially

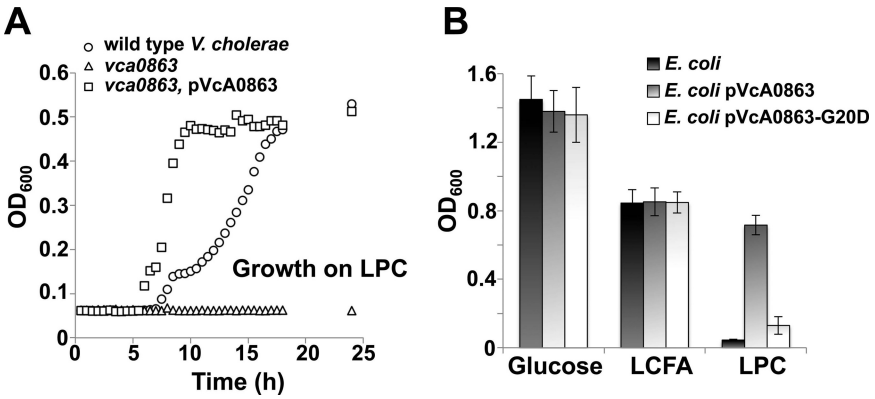

FIG 3 Expression of *vca0863* is required for utilization of LPC as the sole carbon source. (A) Growth of wild-type *V. cholerae*, *vca0863* transposon mutant, and the complemented mutant on LPC. (B) Growth of wild-type *E. coli* and *E. coli* containing either pVcA0863 or pVcA0863-G20D on glucose, LCFA, or LPC.

available exogenous lipid sources with acyl chains that cannot be generated by the bacteria *de novo*, we can monitor the ability of the bacteria to utilize the exogenous lipid in the phospholipid biosynthesis pathway by TLC. Here, we grew cultures in the presence of a mixed sample of either long-chain fatty acids or LPC, predominantly consisting of 18:2 and 18:3 acyl chains; *V. cholerae* does not synthesize either of these chains *de novo* (16). When all three strains were grown in LCFA and 2% glucose, an upwards shift in mobility could be observed in all three of the major phospholipids (Fig. 4A), consistent with incorporation of the exogenous LCFAs into phospholipids. When the cultures were grown with glucose and LPC, only strains expressing *vca0863* showed the same upward shift in mobility. The lack of a shift in the mobility pattern in wild-type *E. coli* and the *vca0863* transposon mutant is consistent with a requirement for VcA0863 expression to liberate the fatty acid from the LPC molecule.

We performed liquid chromatography/electrospray ionization (ESI)-tandem mass spectrometry on lipids extracted from wild-

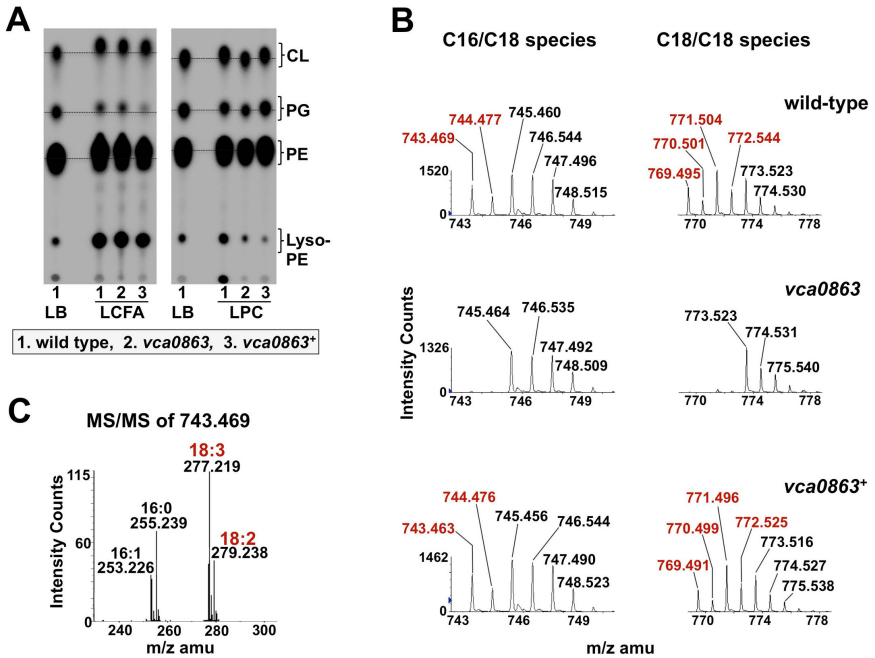

FIG 4 Incorporation of LPC-derived fatty acids into membrane phospholipids of *V. cholerae*. (A) TLC of LCFA- and LPC-grown cultures of *V. cholerae*. *V. cholerae* strains, including the wild type, the *vca0863* transposon mutant, and the *vca0863*-complemented mutant, were grown in the presence of 2 mM LCFA or LPC containing 18:2 and 18:3 unsaturated carbon chains that *V. cholerae* cannot synthesize *de novo*. All three strains show a shift in mobility for each of the major phospholipids when treated with LCFA, as expected. When treated with the LPC mix, only strains expressing *vca0863* (wild type and complemented mutant) showed a similar shift, indicating that *vca0863* is required for generating LPC-derived fatty acid. The dashed line has been included for comparison on mobility shifts. (B) Phosphatidylglycerol (PG) was isolated from wild-type, *vca0863* mutant, and *vca0863* mutant complement strains of *V. cholerae* and analyzed by liquid chromatography/ESI-mass spectrometry. Strains that express *vca0863* showed a unique set of peaks (shown in red) corresponding to weights of PG that have acyl chains with unsaturations matching those in the LPC mix. These peaks were absent from the *vca0863* mutant. (C) Tandem mass spectrometry (MS/MS) of the PG peak, with an *m*/*z* of 743.469 found only in *vca0863*-expressing strains. MS/MS showed that $C_{18:2}$ and $C_{18:3}$ acyl chains that originate with exogenous LPC are incorporated into the *V. cholerae* membrane.

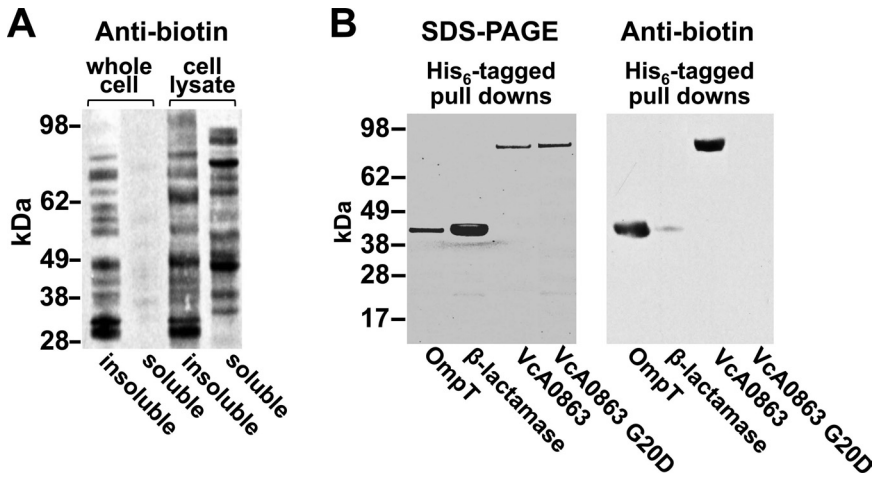

FIG 5 Biotin labeling of surface-exposed VcA0863. (A) Antibiotin Western blot of soluble and insoluble protein fractions from either whole *V. cholerae* cells or cell lysates labeled with NHS-LC-LC-biotin. (B) SDS-PAGE and antibiotin Western blot of His-tagged VcA0863, β-lactamase, OmpT, and mislocalized VcA0863 affinity purified from cell extracts of biotin-labeled whole cells. Protein bands at ~81 kDa (VcA0863-His_6 and mislocalized VcA0863-His_6) and ~41 kDa (β-lactamase-His_6 and OmpT-His_6) in size were observed in SDS-PAGE, establishing that all proteins were running according to their molecular masses. The antibiotin Western blot showed two proteins, an 81-kDa band representing wild-type VcA0863 and a 41-kDa band representing OmpT. The fact that VcA0863 was labeled with biotin strongly indicates that VcA0863 is surface exposed. OmpT was also labeled due to its exposure on the surface of the cell. Little or no signal could be detected for either β-lactamase or mislocalized VcA0863 due to lack of surface exposure.

type *V. cholerae*, *vca0863 V. cholerae*, and the complemented mutant. Each of the major phospholipids was analyzed. Analyses of spectra revealed distinct differences between the *vca0863* transposon mutant and the wild-type and complemented strains. Specifically, results from the Lipid Maps Structure Database prediction algorithm (http://www.lipidmaps.org/) showed several phospholipid species corresponding to phospholipids containing $C_{18:2}$ and $C_{18:3}$ that were present only in spectra for wild-type or complemented *V. cholerae* grown in the presence of $C_{18:2/18:3}$-LPC (Fig. 4; see also Fig. S6 and S7 in the supplemental material). Masses of phospholipid species specific to LPC growth are highlighted red in Fig. 4B (see also Fig. S6 and S7). A phosphatidylglycerol $[M-H]^{1-}$ ion peak with mass of 743.469 (Fig. 4B) in the wild-type or complemented strain was selected for tandem mass spectrometry. Collision-induced dissociation of the *m*/*z* 743.469 ion revealed two phosphatidylglycerol species containing $C_{18:2}$/$C_{16:1}$ or $C_{18:3}$/$C_{16:0}$, confirmed by the presence of peaks at *m*/*z* 277.219 and 279.238, which correspond to $C_{18:3}$ and $C_{18:2}$ acyl chains, respectively (Fig. 4C). Similar phenotypes were observed for phosphatidylethanolamine and cardiolipin species, indicating that lipids derived from LPC can be used both for nutrition and for remodeling of all of the membrane phospholipids (see Fig. S6 and S7).

The *vca0863* gene product is a surface-exposed lipoprotein. In the course of this study, we observed that a *fadL* mutant of *E. coli* failed to grow using LPC as the sole carbon source when heterologously expressing *vca0863* (see Fig. S8 in the supplemental material). Because FadL is responsible for the uptake of LCFAs, it is reasonable to assume that the breakdown of LPC occurs prior to entry into the cell. This indicates that VcA0863 may be surface exposed. To confirm localization of VcA0863, whole bacteria were labeled with an amine-reactive biotin (*N*-hydroxysuccinimide [NHS]-long-chain [LC]-LC biotin), and the labeled proteins were visualized using a streptavidin horseradish peroxidase conjugate (17). The large size and polar nature of the biotin compound prevent passage across the outer membrane, allowing selective labeling of surface-exposed proteins. When whole cells were labeled, only minimal signal could be detected in the soluble fraction, indicating that the biotin compound failed to significantly penetrate the outer membrane (Fig. 5A).

To determine if VcA0863 was surface exposed, a *V. cholerae* El Tor O1 *vca0863*-deficient strain was transformed with vectors expressing His-tagged variants of either *vca0863* or *vca0863* encoding the G20D replacement (*vca0863*-G20D). Cells were also separately transformed with vectors containing an *E. coli*-derived β-lactamase (encoded by *ampC*) (18), serving as a negative control, or *V. cholerae ompT*, an outer membrane β-barrel acting as a positive control (19). After labeling the *V. cholerae* strains with NHS-LC-LC biotin, cells were harvested and lysed, and polyhistidine tagged proteins were isolated using cobalt affinity resin and analyzed via Western blotting (Fig. 5B). On the antibiotin blot, controls reflected the surface specificity of the biotin labeling. For example, surface-exposed OmpT was efficiently biotinylated and produced a strong signal. Periplasmic β-lactamase, however, showed minimal labeling even though the protein is overexpressed, suggesting that NHS-LC-LC biotin did not cross the outer membrane to a significant degree. Also detected on the antibiotin blot was a prominent 83-kDa band representing VcA0863, indicating that it is surface exposed. No signal could be detected for the mislocalized VcA0863-G20D mutant.

In addition to using surface biotinylation, we independently confirmed that VcA0863 is surface exposed through the use of immunogold electron microscopy. Wild-type *E. coli* K-12 or wild-type *V. cholerae* El Tor expressing VcA0863 were incubated with polyclonal anti-VcA0863 antibodies followed by gold-conjugated goat anti-rabbit antibodies. The labeled whole cells were then analyzed via electron microscopy. Both *E. coli* and *V. cholerae* cells expressing VcA0863 showed a considerable number of gold particles associated with the cell surface in comparison to strains lacking VcA0863, which showed no surface labeling (Fig. 6). Together

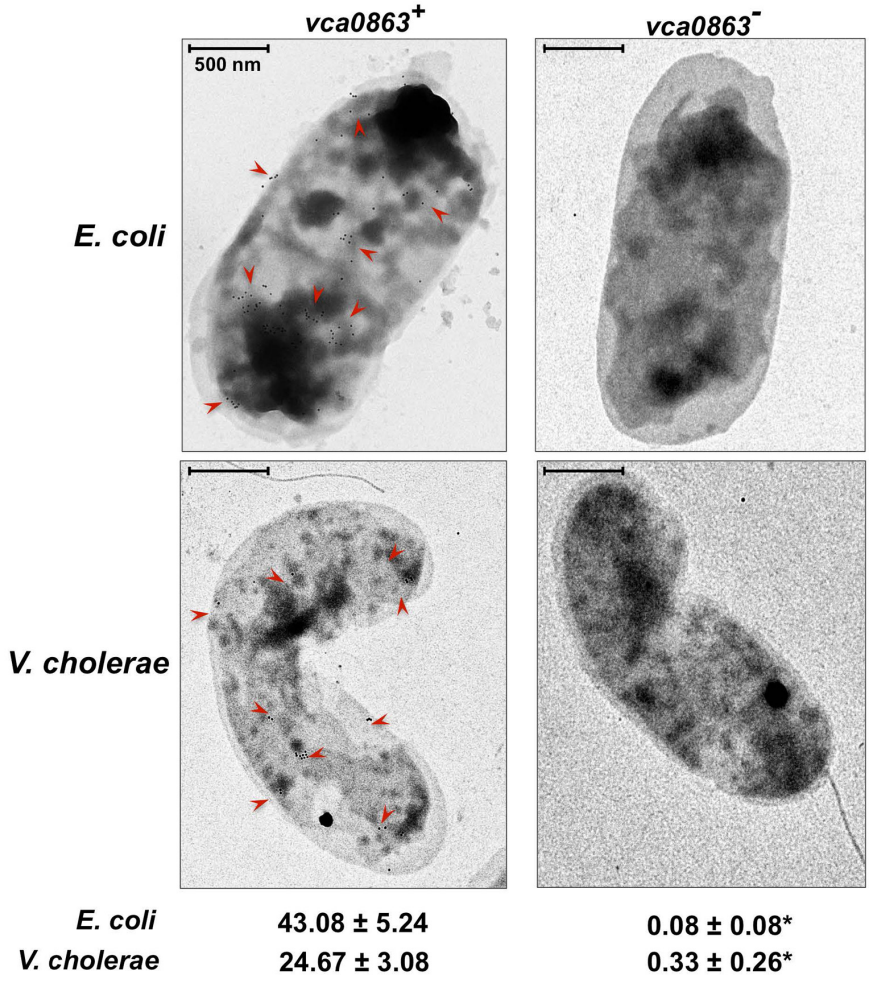

FIG 6 Immunogold electron micrographs of *E. coli* and *V. cholerae* strains expressing *vca0863*. Gold particles (examples are denoted with a red arrow) indicate the presence of surface-exposed VcA0863. Both *E. coli* expressing *vca0863* from the plasmid and wild-type *V. cholerae* showed gold particles associated with the surface of the cell, indicating that VcA0863 is exposed to the surface in both strains. Wild-type *E. coli* or the *V. cholerae vca0863* mutant failed to display any associated gold particles on the bacterial surface. Mean gold particle counts are reported with standard errors ($n > 10$); statistical significance was observed between results for strains expressing and not expressing *vca0863*. *, $P < 0.0005$, Mann-Whitney U test.

with the surface biotinylation of VcA0863, these data indicate a clear surface localization of the lipase. Given that VcA0863 has a lipase domain, is required for growth on lysophospholipids, and is localized to the bacterial surface, we have named the *vca0863* gene product VolA for *Vibrio* outer membrane lysophospholipase A.

DISCUSSION

This study demonstrates an unusual form of lipid utilization in the Gram-negative bacterium *Vibrio cholerae*. In the course of its life cycle, *V. cholerae* experiences a variety of environments, from its planktonic existence in the ocean, living as aggregates associated with animal reservoirs in aquatic ecosystems, to infecting a human host. Concentrations of nutrients, ions, and salts vary significantly between these conditions, as does the presence of antimicrobial agents; because of this, *V. cholerae* has developed specific systems to adapt itself for survival. One such system is the fatty acid uptake pathway, used to incorporate fatty acids from the environment directly into various metabolic pathways. Previous experiments from our laboratory have shown that *V. cholerae* is capable of using very-long-chain fatty acids from the environment both as a carbon source and to remodel its membrane lipids; these fatty acids have not been shown to be used by other Gram-negatives. This distinct uptake profile could be due to an uncommon evolution of multiple homologs of FadL and FadD found only in *V. cholerae* and some related species. Elaborating on the utilization of unconventional lipids by *V. cholerae*, the current work highlights the ability of *V. cholerae* to process lysophosphatidylcholine (LPC) for use in various metabolic pathways. The ability to use LPC from its surroundings has advantages for *V. cholerae* fitness in terms of nutrient acquisition and environmental acclimatization; LPC-derived fatty acids can be used as a nutrient source, reducing the energetic needs of the cell, but they can also be used to remodel phospholipids. By using environmental lipids to remodel the membrane architecture of the cell, *V. cholerae* could respond to membrane stress through homeoviscous adaptation, a mechanism by which bacteria can regulate their membrane fluidity when exposed to a new environment.

LPC and its progenitor phospholipids are commonly found in both aquatic environments and in human host. In the ocean, *V. cholerae* associates with a variety of organisms, including zoo-

plankton (20, 21), chironomid egg masses (22), flora (23), protozoa (24, 25), mollusks (26), and fish (27). Phospholipids and lysophospholipids are present in and on these various reservoirs (28–31). Secretion of a lecithinase in *V. cholerae* (32) could free the lyso derivative from phosphatidylcholine in the environment. The ability of *V. cholerae* to use LPC as a carbon source provides a fitness advantage in nutrient-poor aquatic environments. Furthermore, the ability to scavenge lysophospholipids may reach beyond the usage of LPC. Other environmental lipids present in appreciable amounts could also be processed by VolA or VolA-like proteins.

Once *V. cholerae* infects a human host, it is consistently exposed to phospholipids and their derivatives. Through human pancreatic phospholipase A2 activity for both dietary and bile-sourced phospholipids, the human intestine has significant levels of lysophospholipids, with LPC as the predominant lipid (33). *V. cholerae* can modify its membrane lipids using the fatty acid freed from LPC in a manner similar to that of exogenous fatty acid remodeling. By breaking down the LPC that is present and altering its own membrane phospholipids to resemble the host, *V. cholerae* could protect itself against membrane stress. More importantly, LPC has been shown to be involved in several proinflammatory signaling pathways (34, 35), generation of reactive oxygen species (36), and indirect antimicrobial effects on Gram-negatives (37, 38). A secondary effect of this utilization may be to reduce the local concentration of LPC, thus circumventing host defenses and reducing bacterial clearance.

The importance of lipid utilization in bacteria is not fully understood. While fatty acid uptake has been investigated, few data are available concerning alternate lipid sources. This study is just one example of how other lipids might be used via undiscovered enzymes that work in conjunction with established uptake pathways. Other lipids may play similar roles in nutrition and membrane remodeling in a manner similar to fatty acids and LPC. Cholesterol, for example, has been shown to have significant effects on membrane strength when incorporated into the human pathogen *Helicobacter pylori* (39); alternatively, it plays a role in nutrition in *Mycobacterium tuberculosis* (40). It is unclear if lipid uptake plays as substantial a role in other organisms besides Gram-negatives. Uptake of fatty acids has been observed in Gram-positives (41), but it is unknown if the mechanism of uptake is similar to that of Gram-negatives.

We propose a model for the mechanism of LPC utilization (Fig. 7). In this model, VolA acts on LPC prior to its passage through FadL, supported by the fact that growth on LPC as the sole carbon source is dependent on expression of FadL (see Fig. S8 in the supplemental material). This model is particularly intriguing because there are currently only a few surface-exposed lipoproteins in Gram-negative bacteria: TraTp (42), an F sex factor, WZA_{K30} (43), a multimeric pore-forming complex involved in K30 capsular polysaccharide expression, and CsgG (44), a member of the secretion apparatus of curli fibers in the *Enterobacteriaceae*. Additionally, the most abundant protein in *E. coli*, Lpp, was recently identified by the Silhavy group as being expressed as a surface-exposed lipoprotein (17). Lpp has been shown to have two forms, existing in an ~1:2 ratio: a peptidoglycan-bound form and a "free" form. Cowles et al. (17) demonstrated that nearly all of the free form of Lpp is localized to the surface of the outer membrane of *E. coli*. While these are several examples of surface-exposed lipoproteins, there is a noticeable lack of discovered lipoprotein

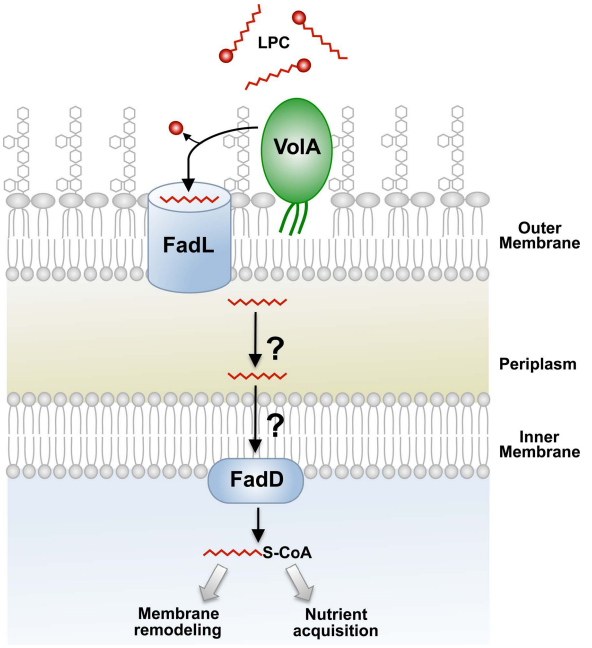

FIG 7 Proposed model for VolA-dependent utilization of lysophospholipids. Exogenous LPC is initially cleaved by the surface-exposed lipoprotein VolA (VcA0863). One of the *V. cholerae* homologs of FadL (encoded by *vc1042*, *vc1043*, or *vca0862*) (see Fig. S4 in the supplemental material) can then transport the free fatty acid. Following transport across the periplasm and the inner membrane, the fatty acid is converted to an acyl-CoA by FadD. The activated fatty acid can be utilized for phospholipid biosynthesis or used as a carbon source for the bacterial cell.

enzymes. Pullulanase, a starch-debranching lipoprotein that is exported to the outer surface of *Klebsiella* species and subsequently released into the growth medium, is one of the only known examples of a surface lipoprotein with enzymatic function (45). The protein described in this work is an instance of a surface-exposed lipase illuminating how lipoproteins can play a role in extracellular enzymatic mechanisms.

It is currently unknown how the protein identified in this study is transported to the surface of *V. cholerae*. VolA is likely shuttled through the Lol system (46), especially since mutation of the +2 residue after the lipoprotein signal sequence can disrupt localization (Fig. 5). Since the Lol transport machinery ends at the periplasmic face of the outer membrane, how VolA reaches the outer surface of the cell (Fig. 7) is unknown. The fact that VolA can be properly expressed in *E. coli*, combined with the recent discovery that Lpp is also surface exposed, indicates that both *E. coli* and *V. cholerae* express the machinery required to transport lipoproteins to the outer surface. The easily observed growth phenotype associated with the utilization of lysophospholipids presents an opportunity to identify additional machinery required for the transport of VolA to the bacterial surface.

The current study continues to highlight the importance of lipid utilization by Gram-negative bacteria. *Vibrio* species may have evolved this adaptation as an efficient means for both maintenance of the membrane and carbon source acquisition. The ability to exploit exogenous lipids in different environmental reservoirs would allow the bacterium to adopt a phospholipid profile that reflects the fatty acid composition of its surrounding environment, perhaps as a mechanism of homeoviscous adaptation to

survive membrane stress. The roles that VolA and membrane remodeling play in the adaptation of *V. cholerae* to diverse environments are currently under investigation.

MATERIALS AND METHODS

A description of all bacteria strains, plasmids, and oligonucleotides can be found in Table S1 in the supplemental material. Methods for the purification and characterization of phospholipids, electron microscopy, biotinylation of proteins, and the generation of polyclonal antibodies are all described in the supplemental Materials and Methods. Methods describing recombinant DNA and RNA techniques and the generation of complementation plasmids can also be found in the supplemental Materials and Methods.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00305-13/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, PDF file, 0.6 MB.
Figure S2, PDF file, 0.3 MB.
Figure S3, PDF file, 0.3 MB.
Figure S4, PDF file, 0.9 MB.
Figure S5, PDF file, 0.2 MB.
Figure S6, PDF file, 0.5 MB.
Figure S7, PDF file, 0.9 MB.
Figure S8, PDF file, 0.3 MB.
Table S1, DOCX file, 0.1 MB.

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REFERENCES

1. **Campbell JW, Morgan-Kiss RM, Cronan JE, Jr..** 2003. A new *Escherichia coli* metabolic competency: growth on fatty acids by a novel anaerobic beta-oxidation pathway. Mol. Microbiol. **47**:793–805.
2. **Giles DK, Hankins JV, Guan Z, Trent MS.** 2011. Remodelling of the *Vibrio cholerae* membrane by incorporation of exogenous fatty acids from host and aquatic environments. Mol. Microbiol. **79**:716–728.
3. **Nunn WD, Simons RW.** 1978. Transport of long-chain fatty acids by *Escherichia coli*: mapping and characterization of mutants in the fadL gene. Proc. Natl. Acad. Sci. U. S. A. **75**:3377–3381.
4. **Overath P, Raufuss EM.** 1967. The induction of the enzymes of fatty acid degradation in *Escherichia coli*. Biochem. Biophys. Res. Commun. **29**: 28–33.
5. **Esfahani M, Barnes EM, Jr, Wakil SJ.** 1969. Control of fatty acid composition in phospholipids of *Escherichia coli*: response to fatty acid supplements in a fatty acid auxotroph. Proc. Natl. Acad. Sci. U. S. A. **64**: 1057–1064.
6. **Lipp EK, Huq A, Colwell RR.** 2002. Effects of global climate on infectious disease: the cholera model. Clin. Microbiol. Rev. **15**:757–770.
7. **Kattner G, Hagen W.** 2009. Lipids in marine copepods: latitudinal characteristics and perspective to global warming, p 257–280. *In* Arts M, Brett M, Kainz M (ed), Lipids in aquatic ecosystems. Springer, Dordrecht, The Netherlands.
8. **Braun A, Treede I, Gotthardt D, Tietje A, Zahn A, Ruhwald R, Schoenfeld U, Welsch T, Kienle P, Erben G, Lehmann WD, Fuellekrug J, Stremmel W, Ehehalt R.** 2009. Alterations of phospholipid concentration and species composition of the intestinal mucus barrier in ulcerative colitis: a clue to pathogenesis. Inflamm. Bowel Dis. **15**:1705–1720.
9. **Ansell GB, Spanner S.** 1982. Phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine, p 1–50. *In* Neuberger A, Van Deenen LLM (ed), Phospholipids. Elsevier, Philadelphia, PA.
10. **Chuang YC, Chiou SF, Su JH, Wu ML, Chang MC.** 1997. Molecular analysis and expression of the extracellular lipase of Aeromonas hydrophila MCC-2. Microbiology **143**:803–812.
11. **Okuda S, Tokuda H.** 2011. Lipoprotein sorting in bacteria. Annu. Rev. Microbiol. **65**:239–259.
12. **Munch R, Hiller K, Grote A, Scheer M, Klein J, Schobert M, Jahn D.** 2005. Virtual footprint and PRODORIC: an integrative framework for regulon prediction in prokaryotes. Bioinformatics **21**:4187–4189.
13. **Kolb A, Busby S, Buc H, Garges S, Adhya S.** 1993. Transcriptional regulation by cAMP and its receptor protein. Annu. Rev. Biochem. **62**: 749–795.
14. **Zubay G, Schwartz D, Beckwith J.** 1970. Mechanism of activation of catabolite-sensitive genes: a positive control system. Proc. Natl. Acad. Sci. U. S. A. **66**:104–110.
15. **DiRusso CC, Metzger AK, Heimert TL.** 1993. Regulation of transcription of genes required for fatty acid transport and unsaturated fatty acid biosynthesis in *Escherichia coli* by FadR. Mol. Microbiol. **7**:311–322.
16. **Raziuddin SR.** 1976. Effect of growth temperature and culture age on the lipid composition of *Vibrio cholerae* 569B (Inaba). J. Gen. Microbiol. **94**: 367–372.
17. **Cowles CE, Li Y, Semmelhack MF, Cristea IM, Silhavy TJ.** 2011. The free and bound forms of Lpp occupy distinct subcellular locations in *Escherichia coli*. Mol. Microbiol. **79**:1168–1181.
18. **Boman HG, Eriksson-Grennberg KG, Foldes J, Lindstrom EB.** 1967. The regulation and possible evolution of a penicillinase-like enzyme in *Escherichia coli*, p 366–372. *In* Koningsberger VV, Bosch L (ed), Regulation of nucleic acid and protein biosynthesis, BBA Library, vol. **10**. Elsevier, Amsterdam, The Netherlands.
19. **Miller VL, Mekalanos JJ.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170**:2575–2583.
20. **Huq A, Small EB, West PA, Huq MI, Rahman R, Colwell RR.** 1983. Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. Appl. Environ. Microbiol. **45**:275–283.
21. **Huq A, Colwell RR, Rahman R, Ali A, Chowdhury MA, Parveen S, Sack DA, Russek-Cohen E.** 1990. Detection of *Vibrio cholerae* O1 in the aquatic environment by fluorescent-monoclonal antibody and culture methods. Appl. Environ. Microbiol. **56**:2370–2373.
22. **Broza M, Halpern M.** 2001. Pathogen reservoirs. Chironomid egg masses and *Vibrio cholerae*. Nature **412**:40. http://dx.doi.org/10.1038/35083694.
23. **Islam MS, Drasar BS, Sack RB.** 1994. The aquatic flora and fauna as reservoirs of *Vibrio cholerae*: a review. J. Diarrhoeal Dis. Res. **12**:87–96.
24. **Abd H, Saeed A, Weintraub A, Nair GB, Sandström G.** 2007. Vibrio cholerae O1 strains are facultative intracellular bacteria, able to survive and multiply symbiotically inside the aquatic free-living amoeba *Acanthamoeba castellanii*. FEMS Microbiol. Ecol. **60**:33–39.
25. **Abd H, Weintraub A, Sandström G.** 2005. Intracellular survival and replication of *Vibrio cholerae* O139 in aquatic free-living amoebae. Environ. Microbiol. **7**:1003–1008.
26. **Marino A, Crisafi G, Maugeri TL, Nostro A, Alonzo V.** 1999. Uptake and retention of *Vibrio cholerae* non-O1, *Salmonella typhi*, *Escherichia coli* and *Vibrio harvey* by mussels in seawater. New Microbiol. **22**:129–138.
27. **Senderovich Y, Izhaki I, Halpern M.** 2010. Fish as reservoirs and vectors of *Vibrio cholerae*. PLoS One **5**:e8607. http://dx.doi.org/10.1371/journal.pone.0008607.
28. **Kariotoglou DM, Mastronicolis SK.** 2003. Sphingophosphonolipid molecular species from edible mollusks and a jellyfish. Comp. Biochem. Physiol. B Biochem. Mol. Biol. **136**:27–44.
29. **Bhaskar N, Kinami T, Miyashita K, Park SB, Endo Y, Fujimoto K.** 2004. Occurrence of conjugated polyenoic fatty acids in seaweeds from the Indian Ocean. Z. Naturforsch. C **59**:310–314.
30. **Ohman MD.** 1997. On the determination of zooplankton lipid content and the occurrence of gelatinous copepods. J. Plankton Res. **19**: 1235–1250.
31. **Ghioni C, Bell JG, Sargent JR.** 1996. Polyunsaturated fatty acids in neutral lipids and phospholipids of some freshwater insects. Comp. Biochem. Physiol. B **114**:161–170.
32. **Fiore AE, Michalski JM, Russell RG, Sears CL, Kaper JB.** 1997. Cloning, characterization, and chromosomal mapping of a phospholipase (lecithinase) produced by *Vibrio cholerae*. Infect. Immun. **65**:3112–3117.
33. **Froehlich F, Gonvers JJ, Fried M.** 1995. Role of nutrient fat and cholecystokinin in regulation of gallbladder emptying in man. Dig. Dis. Sci. **40**:529–533.
34. **Nishi E, Kume N, Ueno Y, Ochi H, Moriwaki H, Kita T.** 1998. Lyso-

phosphatidylcholine enhances cytokine-induced interferon gamma expression in human T lymphocytes. Circ. Res. **83**:508–515.
35. **Stock C, Schilling T, Schwab A, Eder C.** 2006. Lysophosphatidylcholine stimulates IL-1beta release from microglia via a P2X7 receptor-independent mechanism. J. Immunol. **177**:8560–8568.
36. **Schilling T, Eder C.** 2010. Importance of lipid rafts for lysophosphatidylcholine-induced caspase-1 activation and reactive oxygen species generation. Cell. Immunol. **265**:87–90.
37. **Yan JJ, Jung JS, Lee JE, Lee J, Huh SO, Kim HS, Jung KC, Cho JY, Nam JS, Suh HW, Kim YH, Song DK.** 2004. Therapeutic effects of lysophosphatidylcholine in experimental sepsis. Nat. Med. **10**:161–167.
38. **Steel HC, Cockeran R, Anderson R.** 2002. Platelet-activating factor and lyso-PAF possess direct antimicrobial properties *in vitro*. APMIS **110**: 158–164.
39. **Shimomura H, Hosoda K, Hayashi S, Yokota K, Hirai Y.** 2012. Phosphatidylethanolamine of Helicobacter pylori functions as a steroid-binding lipid in the assimilation of free cholesterol and 3beta-hydroxl steroids into the bacterial cell membrane. J. Bacteriol. **194**:2658–2667.
40. **Griffin JE, Pandey AK, Gilmore SA, Mizrahi V, McKinney JD, Bertozzi CR, Sassetti CM.** 2012. Cholesterol catabolism by Mycobacterium tuberculosis requires transcriptional and metabolic adaptations. Chem. Biol. **19**:218–227.
41. **Banchio C, Gramajo HC.** 1997. Medium- and long-chain fatty acid uptake and utilization by Streptomyces coelicolor A3(2): first characterization of a gram-positive bacterial system. Microbiology **143**:2439–2447.
42. **Manning PA, Beutin L, Achtman M.** 1980. Outer membrane of *Escherichia coli*: properties of the F sex factor *traT* protein which is involved in surface exclusion. J. Bacteriol. **142**:285–294.
43. **Drummelsmith J, Whitfield C.** 2000. Translocation of group 1 capsular polysaccharide to the surface of *Escherichia coli* requires a multimeric complex in the outer membrane. EMBO J. **19**:57–66.
44. **Robinson LS, Ashman EM, Hultgren SJ, Chapman MR.** 2006. Secretion of curli fibre subunits is mediated by the outer membrane-localized CsgG protein. Mol. Microbiol. **59**:870–881.
45. **Pugsley AP, Kornacker MG.** 1991. Secretion of the cell surface lipoprotein pullulanase in *Escherichia coli*. Cooperation or competition between the specific secretion pathway and the lipoprotein sorting pathway. J. Biol. Chem. **266**:13640–13645.
46. **Ruiz N, Kahne D, Silhavy TJ.** 2006. Advances in understanding bacterial outer-membrane biogenesis. Nat. Rev. Microbiol. **4**:57–66.