Biochimica et Biophysica Acta 1848 (2015) 742–751

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

The YhhN protein of *Legionella pneumophila* is a Lysoplasmalogenase

Marianne S. Jurkowitz [a,*], Aalapi Patel [a], Lai-Chu Wu [a,b], Annalise Krautwater [a], Douglas R. Pfeiffer [a], Charles E. Bell [a,c]

[a] Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, OH 43210, USA
[b] The Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210, USA
[c] Department of Chemistry and Biochemistry, The Ohio State University, Columbus, OH 43210, USA

ARTICLE INFO

Article history:
Received 17 June 2014
Received in revised form 25 October 2014
Accepted 10 November 2014
Available online 16 November 2014

Keywords:
Plasmalogen/lysoplasmalogen
Lysoplasmalogenase
YhhN gene Lpg1991
Vinyl ether bond
Legionella pneumophila
Lysophospholipase

ABSTRACT

Lysoplasmalogenase catalyzes hydrolytic cleavage of the vinyl-ether bond of lysoplasmalogen to yield fatty aldehyde and glycerophospho-ethanolamine or glycerophospho-choline. We recently purified lysoplasmalogenase from rat liver microsomes and identified the protein as TMEM86B, an integral membrane protein that is a member of the YhhN family found in numerous species of eukaryotes and bacteria. To test the hypothesis that bacterial YhhN proteins also function as lysoplasmalogenase enzymes, we cloned the Lpg1991 gene of *Legionella pneumophila,* which encodes a 216 amino acid YhhN protein (LpYhhN), and expressed it in *Escherichia coli* as a C-terminal-GFP-His8-fusion. Membranes were solubilized and the fusion protein was purified by nickel-affinity chromatography, cleaved with Tobacco Etch Virus protease, and subjected to a reverse nickel column to purify the un-tagged LpYhhN. Both the fusion protein and un-tagged LpYhhN exhibit robust lysoplasmalogenase activity, cleaving the vinyl-ether bond of lysoplasmalogen with a V_{max} of 12 μmol/min/mg protein and a K_m of 45 μM. LpYhhN has no activity on diradyl plasmalogen, 1-alkenyl-glycerol, and monoacylglycerophospho-ethanolamine or monoacylglycerophospho-choline; the pH optimum is 6.5–7.0. These properties are very similar to mammalian TMEM86B. Sequence analysis suggests that YhhN proteins contain eight transmembrane helices, an N-in/C-in topology, and about 5 highly conserved amino acid residues that may form an active site. This work is the first to demonstrate a function for a bacterial YhhN protein, as a vinyl ether bond hydrolase specific for lysoplasmalogen. Since *L. pneumophila* does not contain endogenous plasmalogens, we hypothesize that LpYhhN may serve to protect the bacterium from lysis by lysoplasmalogen derived from plasmalogens of the host.

Published by Elsevier B.V.

1. Introduction

Plasmalogens (1-alk-1′-enyl- 2- acyl-*sn*-glycero-3-phospho-ethanolamine or –choline) are glycerophospholipids that differ from the common diacylglycerophospholipids in having a vinyl-ether linkage at *sn*-1 [1]. These membrane lipids are widely distributed in vertebrate and invertebrate animal species [2] and in anaerobic bacteria, but absent in aerobic and facultative anaerobic bacteria, fungi, and plants [1]. In mammals, plasmalogens constitute 4–32% of the total phospholipid mass, depending on the cell and tissue type. They are particularly high in neural tissue, heart, lung, and circulating immune cells.

The presence of the vinyl-ether bond confers unique physical and chemical properties to plasmalogens and to the membranes that contain them [3]. Plasmalogens pack more closely with one another, which leads to decreases in membrane fluidity and passive ion permeability [4]. Plasmalogens also have a higher propensity to form an inverse hexagonal phase, which is important for membrane fusion events [5]. As the vinyl ether bond is particularly sensitive to non-enzymatic oxidative cleavage [6,7], plasmalogens may help to protect other membrane lipid and protein components from oxidative damage [8]. The acyl chain at *sn-2* of plasmalogens is typically enriched in bioactive polyunsaturated fatty acids (PUFA), such as arachidonic acid (AA) and docosahexaenoic acid (DHA). Plasmalogens thus serve as reservoirs of PUFA [9] and are enriched in lipid-raft domains of cell membranes, which are hubs of signal transduction [10].

In mammals, lysoplasmalogens are formed from plasmalogens by the action of calcium-independent PLA_2 ($iPLA_2$) enzymes, which cleave the acyl chain at *sn-2* to release a free fatty acid [11]. A number of iPLA2 enzymes, including those from alveolar cells [12] and macrophages [13],

Abbreviations: AA, arachidonic acid; ADH, alcohol dehydrogenase; BSA, bovine serum albumin; DHA, docosahexaenoic acid; DTT, dithiothreitol; GFP, Green Fluorescent Protein; HDL, high-density lipoproteins; His, histidine; NADH, nicotinamide adenine dinucleotide; KP_i, potassium phosphate buffer; PBS, phosphate-buffered saline; $iPLA_2$, calcium-independent phospholipase A_2; SDS, sodium dodecyl sulfate; TMEM86B, transmembrane protein 86B

* Corresponding author at: Department of Molecular and Cellular Biochemistry, 1645 Neil Avenue, Room 310 Hamilton Hall,Ohio State University Medical School, The Ohio State University, Columbus, OH 43210, USA. Tel.: +1 614 885 8182.
E-mail address: jurkowitz.1@osu.edu (M.S. Jurkowitz).

http://dx.doi.org/10.1016/j.bbamem.2014.11.011
0005-2736/Published by Elsevier B.V.

are selective for plasmalogen substrates, and activated by extracellular signals [11]. At submicellar concentrations, lysoplasmalogens affect the dynamics of cell membranes by increasing membrane fluidity [14]. They have specific effects on several transmembrane proteins, such as inhibition of cholinergic signal transduction [15], inhibition of Na-K-ATPase [16], and activation of cAMP-dependent protein kinase C [17]. Lysoplasmalogens also serve as self antigens that stimulate invariant natural killer T cells in the thymus [18]. Due to their amphiphilic nature, lysoplasmalogens can lyse cell membranes if present at high enough levels [19,20]. Thus, lysoplasmalogens are normally maintained at very low levels in cells [21].

Lysoplasmalogens can be converted back into plasmalogens by a coenzyme A-independent transacylase, which re-acylates *sn-2* in a process referred to as "remodeling" [22]. Alternatively, lysoplasmalogens can be further broken down by phospholipases C and D [23,24], or by lysoplasmalogenase [25]. Lysoplasmalogenase (EC 3.3.2.2; EC 3.3.2.5) catalyzes hydrolysis of the vinyl ether linkage at *sn-1* to release a fatty aldehyde and glycerophospho-ethanolamine (or choline) [25–28]. The enzyme is highly specific for glycerophospholipids containing a vinyl-ether linkage at *sn-1* and a hydroxyl (lyso) at *sn-2*, and acts with nearly equal efficiency on ethanolamine and choline classes [28]. Lysoplasmalogenase has been proposed to maintain lysoplasmalogen at low levels, to prevent toxicity [27,28].

In previous studies, we purified lysoplasmalogenase from rat liver microsomes and identified the gene as TMEM86B [27,28], which encodes a 226 amino acid protein with eight predicted transmembrane helices and previously unknown function [28]. TMEM86B is a member of the larger YhhN family of proteins which are present in 138 species of eukaryotes and 1205 species of bacteria, including many that are human pathogens. Interestingly, many of the bacteria that encode a YhhN family protein do not contain endogenous plasmalogens. Anaerobic bacteria contain relatively high amounts of plasmalogens, which are synthesized by a pathway that is very different from the aerobic pathway used in eukaryotes. However, aerobic and facultative anaerobic bacteria have lost the capacity to synthesize plasmalogens and thus do not contain them [1].

Given that the mammalian YhhN protein is a lysoplasmalogenase, it seemed paradoxical to us that many of the aerobic bacteria that encode a YhhN gene, such as *Legionella pneumophila and Mycobacterium tuberculosis*, do not contain endogenous plasmalogens. It thus seems likely that either the bacterial YhhN proteins hydrolyze other types of lipids or else act on lysoplasmalogens derived from host cells. *L. pneumophila* is a Gram-negative intracellular pathogen that is the cause of Legionnaire's disease, a severe form of pneumonia [29]. The bacteria replicate inside single cell protozoa, such as amoeba. When contaminated water droplets contact human lung the bacteria enter into and replicate inside lung macrophages and alveolar cells. To aid their survival, the bacteria orchestrate the development of a specialized membrane-bound compartment, the Legionella containing vacuole (LCV), which protects the bacteria from lysosomal degradation and facilitates nutrient uptake [30,31]. The LCV membrane is derived in part from membrane lipids of the host plasma membrane and endoplasmic reticulum [32]. The processes involved in LCV membrane development and in the interchange of biomolecules between bacteria and the host, are not well understood but may involve the action of bacterial phospholipase enzymes acting on host phospholipids. Indeed, *L. pneumophila* encodes at least 14 different phospholipase A enzymes, some of which have activity on lysophospholipids. These phospholipases have been studied using ester-linked substrates [33], but plasmalogen or lysoplasmalogen substrates have not been tested.

To test the hypothesis that *L. pneumophila* contains a lysophospholipase that is active on lysoplasmalogens, we cloned the Lpg1991 gene that encodes a YhhN family protein (hereafter referred to as LpYhhN), and expressed it as a GFP fusion protein in *Escherichia coli*. Both the fusion protein and the LpYhhN protein released after protease treatment exhibit abundant levels of lysoplasmalogenase activity. The substrate specificity and physical-chemical properties of the LpYhhN fusion protein and the purified LpYhhN protein are very similar to those of mammalian TMEM86B. This work is to our knowledge the first to demonstrate a biochemical activity for a bacterial YhhN protein. The work is also the first to identify a bacterial lysophospholipase that catalyzes hydrolysis of the vinyl ether bond of lysoplasmalogen.

2. Experimental procedures

2.1. Chemicals and materials

Porcine brain ethanolamine lysoplasmalogen (850095), Porcine heart L-α phosphatidylcholine (840052), 1-O-1′-(Z)-octadecenyl-2-hydroxy-*sn*-glycero-3-phosphocholine (852465), 1-(1′-(Z)-octadecenyl)–*sn*-glycerol (791459), sphingomyelin, and lysophosphatidic acid were from Avanti Polar lipid, Inc (Alabaster, AL). Bovine brain phosphatidyl ethanolamine (356) was from Doosan-Serdary Research Laboratories (Ontario, Canada). Yeast alcohol dehydrogenase (LS001070) was from Worthington Biochemical Corporation (Lakewood, N.J.). TRIS base, KCl, NaCl, HEPES, mannitol and sucrose were reagent grade from SigmaAldrich Chemical Co. (St. Louis, MO). Dodecyl maltoside was from Inalco Biochemicals (San Luis Obispo, CA). The nickel affinity columns were from GE Healthcare (His trap TM HP 17-524701). The FPLC system was from GE-Healthcare Bio-Science Corp. (Piscataway, NJ). The Amicon Ultra Centrifugal filter units (4 ml) (10,000 molecular weight cutoff) were from Millipore Corporation (Temecula, CA). The AcTEV Protease kit was from Invitrogen, (12575-015); this AcTEV protease contains a poly Histidine tag at its N-terminus.

2.2. Construction of the plasmid for LpYhhN expression

The LpYhhN gene encoding the LpYhhN protein was PCR amplified with Pfu Turbo DNA polymerase (Stratagene) from genomic DNA from *L. pneumophila* strain ATCC 33152D-5 Philadelphia-1. The forward primer was 5′-GCGCCATATGACTTATTCTTTTTCCAAACC-3′ and the reverse primer was 5′-GCGCGGATCCATCCCCTTCTTCATATAATCTG-3′ (*Nde*I and *Bam*HI restriction sites are underlined). The resulting PCR product was purified (Qiagen PCR purification kit), and digested with *Nde*I and *Bam*HI restriction enzymes (New England Biolabs). After a second PCR purification step, the digested PCR product was ligated into the pWaldo-GFPd vector, also digested with *Nde*I and *Bam*HI, using the Quick Ligation kit (New England Biolabs), and transformed into chemically competent DH5α *E. coli* cells. DNA sequencing of the resulting plasmid revealed that the gene was identical to LpYhhN (GenBank accession number AAU28060.1) with the exception of a single nucleotide position resulting in a GAA codon instead of AAA at amino acid position 214, which results in a K214E substitution in the protein. The resulting plasmid expressed the LpYhhN protein as a C-terminal GFP-8xHis fusion with an intervening site for cleavage by TEV protease [34]. After cleavage with TEV protease the protein will have an extra GSENLYFQ sequence at the C-terminus (Fig. 1).

2.3. Overexpression of LpYhhN fusion protein

The plasmid encoding the LpYhhN-GFP fusion protein was transformed into C43 (DE3) cells, and grown in 6 × 1 L cultures in 75% Luria broth/25% Terrific broth (TB) containing 50 μg/ml kanamycin, in 3 L baffled flasks at 170 rpm and 37 °C. When the OD_{600} reached 0.3–

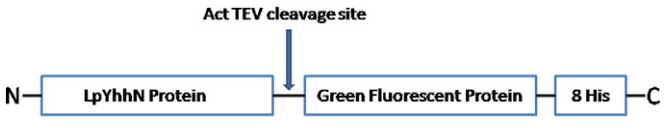

Fig. 1. Cartoon of the LpYhhN-TEV-GFP-8xHis fusion protein.

0.5, the temperature was lowered to 30 °C and expression was induced with 0.7 mM isopropyl-B-D-thiogalactoside (IPTG). Cells were shaken at 170 rpm at 30 °C overnight (17 h), cooled to 2 °C on ice, and harvested by centrifugation at 8000 ×*g* for 10 min. Cells were washed once in 10 times the pellet volume at 0 °C in Buffer A (150 mM NaCl, 15 mM Tris–HCl, 15 mM NaH_2PO_4, 0.75 mM DTT, pH 7.9), and stored as pellets at −80 °C.

2.4. Cell lysis and subcellular fractionation

The cell pellet (about 8 gm wet weight) from each 1 L of culture was suspended in 40 ml Buffer A, to which 100 U/ml DNase I and 1 mM $MgCl_2$ were added. Cells were passed through a French press twice at 15,000 p.s.i. at 4 °C. The resulting lysates were centrifuged at 20,000 ×*g* at 4 °C for 20 min to remove the unbroken cells. The supernatants containing the membranes were centrifuged at 130,000 ×*g* at 4 °C for 55 min. The translucent pellets, which contained 28 mg of protein as determined by Bradford assay [35], were resuspended in 4 ml of Buffer A using a dounce homogenizer. About 220 μl of DDM (20% stock in water) was added dropwise to a final concentration of 1.0%, and the suspensions were stirred occasionally for 30 min. The solubilized membranes were centrifuged at 130,000 ×*g* for 1 h to remove insoluble material.

2.5. Partial purification of the LpYhhN fusion protein from 1 L of cell culture

A 1 ml nickel column (GE Healthcare) charged with 0.1 M nickel sulfate was equilibrated in Buffer B (300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, 0.1% DDM, pH 7.9). 10 ml of solubilized membranes were loaded onto the column at 0.2 ml/min at 2 °C. The column was washed with 15 ml of Buffer B at 0.2 ml/min, and the protein was eluted by a gradient from 10 to 300 mM imidazole in Buffer B over 20 ml at 0.14 ml/min. One-milliliter fractions were assayed for lysoplasmalogenase activity and total protein. Fractions containing activity were concentrated using Amicon centrifugal filter units (10 kDa MWCO) that had been equilibrated with Buffer A containing 0.1% DDM. Portions of the concentrated fractions were stored at −20 °C after an equal volume of glycerol was added, to stabilize the activity at – 20 °C [28]. Other portions were treated with TEV protease to release the LpYhhN from the fusion protein, as described below.

2.6. Purification of un-tagged LpYhhN from the LpYhhN-GFP fusion protein

The preparation and partial purification of LpYhhN-GFP fusion protein from 1 L of cells described above was scaled up to process protein from 3 L of culture. The cells were lysed by French press, membranes were isolated by differential centrifugation and solubilized as described above. The 120,000 ×*g* supernatant containing solubilized membranes was applied to a 5 ml nickel column and the fusion protein was eluted with an imidazole gradient. Three enriched 1.5 ml fractions from the nickel column were pooled and concentrated to 800 μl. For cleavage with TEV protease, the 4 ml reaction included 1.24 mg LpYhhN-GFP protein from the nickel column, 1.0 mM DTT, 0.1% DDM, 50 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, and 1167 U of TEV protease (Invitrogen 12575-015). The reaction contents were gently mixed for 36 h at 0 °C. Aliquots were retained for activity and gel analyses. The remainder (3500 μl) was loaded on to a 1 ml nickel column at 0.02 ml per minute, and the flow through containing un-tagged LpYhhN protein was concentrated. Aliquots were made 50% with glycerol and stored in small aliquots at −20 °C for kinetic analyses. Other aliquots were treated with 5 x loading buffer for analyses on 11% PAGE gels. The GFP-His protein and the TEV protease (with His tag) were eluted late in a 20 ml gradient of 10 mM – 300 mM imidazole in buffer B.

2.7. Lipid substrates

Aqueous suspensions of lipid substrates were prepared about every 3 days from stock solutions stored in chloroform/methanol 2/1 (vol/vol) at −20 °C as previously described [28]. For a portion of the experiments shown in Fig. 6 (as indicated in the legend), the choline lysoplasmalogen substrates were prepared in liposomes at 3 different molar ratios of the lysoplasmalogen to the diradyl phospholipids. Chloroform/methanolic solutions of choline lysoplasmalogen, bovine brain glycerophosphoethanolamine, and 1-oleoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine were mixed in the molar ratios of 5%, 47.5%, 47.5%, respectively, 10%, 45%, 45%, respectively, and 20%, 40%, 40%, respectively. The mixtures were dried as a thin film in glass conical tubes under Nitrogen gas. 70 mM glycyl glycine-NaOH (pH 7.1) was added to make aqueous suspensions of between 0.5 and 2 mM with respect to the choline lysoplasmalogen. The mixtures were vortexed and sonicated four times for 15 seconds. The liposomes, which were turbid, were maintained at 2 °C.

2.8. Coupled enzyme assay for lysoplasmalogenase activity (Assay 1)

A coupled enzyme assay using yeast alcohol dehydrogenase (ADH) was performed as described previously [27,36,37]. Briefly, the fatty aldehyde that is released by hydrolysis of lysoplasmalogen is reduced to an alcohol by ADH in a reaction that is coupled to oxidation of NADH to NAD^+, which is monitored by loss of signal at 340 nm for NADH. The standard reaction mixture (0.52 ml) contained 70 mM glycylglycine-NaOH (pH 7.1), 1 mM DTT, 0.20 mM NADH, 0.52 mg (130 IU) of yeast alcohol dehydrogenase, 220 nmol of lysoplasmalogen substrate, and between 1 and 20 μg of LpYhhN enzyme, depending on the specific activity of the fraction. For determining the pH optimum, the buffers in the coupled enzyme assay included 80 mM glycyl-glycine (NaOH) for pH 6.3 to 7.4, or 80 mM Tris–HCl for pH 7.2 – 8.5. A unit of lysoplasmalogenase activity is defined as one nmol/min.

2.9. Lipid extraction, TLC, and phosphorus analysis assay for lysoplasmalogenase activity (Assay 2)

The TLC assay was performed as described previously [27]. Briefly, a separate reaction mixture was prepared for each time point in conical glass test tubes with ground glass stoppers. The 2 ml reaction mixtures were the same as described above for the coupled enzyme assay, except that alcohol dehydrogenase and NADH were omitted, and fatty acid-free BSA was included (0.43 mg/ml). The reactions were stopped after 5 and 10 min by addition of 12 ml of chloroform/methanol (2/1, v/v) [38]. Following vigorous mixing, the lipid and aqueous phases were separated, and lysoplasmalogen (lower phase) and glycerophosphoethanolamine (upper phase) were each concentrated and run on TLC plates to separate and identify the lipids or water soluble components [39]. The glycerophosphoethanolamine and lysoplasmalogen spots were quantified by phosphorus assay [40].

2.10. SDS–PAGE analysis of the partially purified proteins

Sufficient 5× loading buffer was added to each purified sample to obtain final concentrations of 40 mM Tris–HCl pH 6.8, 10% glycerol, 2.4% SDS, 50 mM dithiothreitol, and 0.001% bromophenol blue. Samples were incubated at 40 °C for 30 min prior to loading onto the gel. In the experiment of Fig. 5, the samples in lanes 1 and 3 were incubated at 40 °C for 30 min, and the samples in lanes 2 and 4 were heated to 95 °C for 4 min. The fractions were analyzed using 11% or 12% gels using Tris–glycine buffer and Laemmli procedure [41]. GFP-containing protein fragments were visualized under UV-light using a Biorad imager. Protein bands were visualized with Coomassie stain. Protein concentrations were determined by the Bradford procedure [35].

3. Results

3.1. Overview of YhhN family proteins

The current Pfam database, version 27.0 [42], lists 1531 different protein sequences, from 1341 different species, that are considered to be YhhN family proteins based on sequence homology. A total of 1322 of these are from bacteria (1205 species) and 206 are from eukaryotes (138 species). Humans and most other mammals for which complete sequence information is available have two YhhN family proteins, which are TMEM86B (known to encode lysoplasmalogenase) and TMEM86A (unknown function) in human. Among model organisms, YhhN family proteins are found in *Mus musculus* (2 proteins), *Xenopus laevis* (2 proteins), *Drosophila melanogaster* (2 proteins), and *Caenorhabditis elegans* (1 protein). Fungi tend to have only one YhhN family member. The great majority of bacterial species that encode a YhhN protein encode only one.

All of the YhhN family proteins tend to be from 200 to 240 amino acids in length and contain about eight transmembrane helices, as predicted from hydropathy plot analysis [43,44]. Fig. 2 shows a sequence alignment of six different YhhN proteins, including four from bacteria (*E. coli*, *Mycobacterium tuberculosis*, *L. pneumophila*, and *Burkholderia multivorans*), and TMEM86A and TMEM86B from human. All of these proteins, except for the one from *E. coli*, are predicted to have their N-and C-termini facing the cytoplasm. The core regions of these proteins exhibit about 30% pairwise sequence identity with one another. TMEM86A and TMEM86B from humans are 39% identical over the regions from amino acids 25–223 and 15–230, respectively.

Rather strikingly, the sequence alignment reveals a number of highly conserved amino acid residues of the types that are capable of performing active site chemistry. In TMEM86B, these residues are Lys-53, Asp-88, His-108, Ser-182, and Tyr-206. All of these residues are located on predicted transmembrane helices and most are embedded within noticeably conserved regions of the protein. In an alignment of 24 YhhN family proteins from version 17.0 of the PANDIT database [46], Gly-174, Ser-182, and Tyr-206 near the C-terminal end of TMEM86B are all completely invariant. Lys-53 is highly conserved but also present as Gln (2 proteins), Arg (2 proteins), or Leu (1 protein). Asp-88 is present in 23 proteins as Asp and in one protein as His. His-108 is highly conserved but also present as Gln (4 proteins), Tyr (1 protein), and Pro (1 protein). In summary, sequence analysis of the YhhN family indicates a protein of about 220 amino acids, 8 transmembrane helices, an N-in, C-in topology, and about 5 highly conserved amino acids that could form an active site.

3.2. Expression and purification of YhhN protein from L. pneumophila

The goal of this work was to clone, express, and purify a bacterial homolog of TMEM86B to determine whether other members of the YhhN-

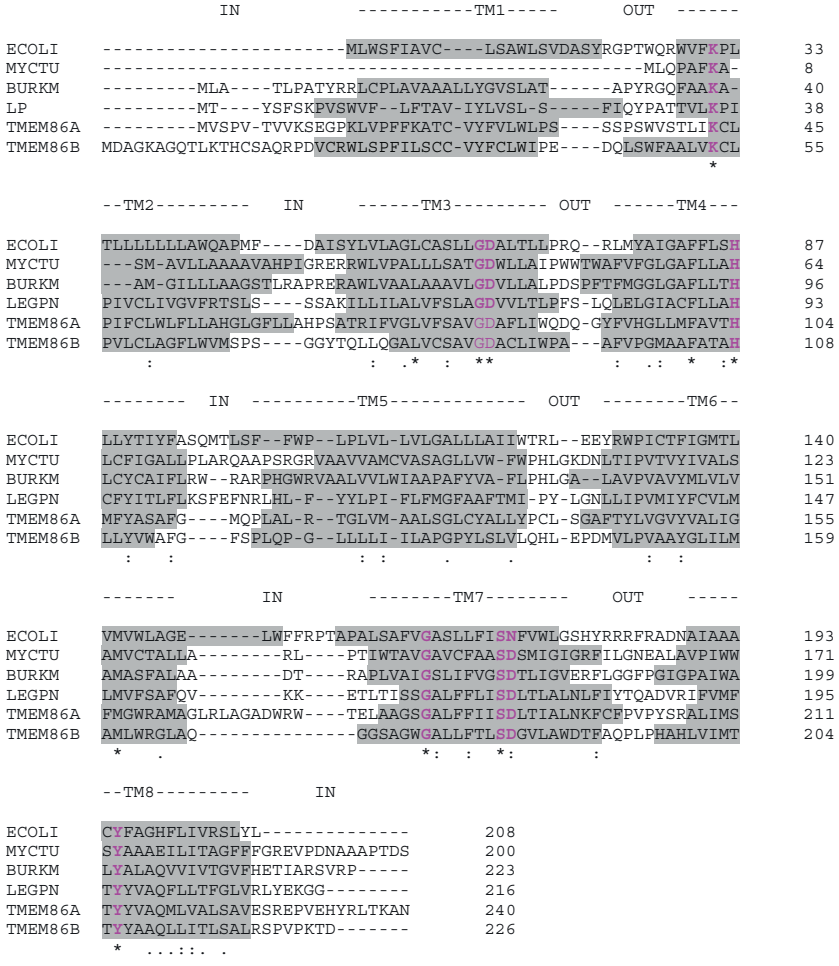

Fig. 2. Multiple-sequence alignment of YhhN proteins. The alignment was performed using Clustal Omega [45]. Sequences are, from top to bottom, *Escherichia coli* (ECOLI), *Mycobacterium tuberculosis* (MYCTU), *Burkolderia multivorans* (BURKM), *Legionella pneumophila* (LP), and *Homo sapiens* (TMEM86A and TMEM86B). The location of transmembrane helices, primarily as predicted by HMMTOP [44], are shaded in grey for each protein, with the consensus numbering of the helices and predicted In/Out topology of the loop regions indicated above the alignment. "In" refers to cytoplasmic side, and "out" refers to non-cytoplasmic side. The predicted topology of the *E. coli* protein is reversed relative to the others (it is predicted to be N-out, C-out). The shaded areas showing the predicted locations of helices TM1 and TM2 for TMEM86A, and TM3-TM5 of TMEM8B, used information from TMHMM [43], as the predictions from HMMTOP did not match the consensus. Highly conserved amino acids that are putative active site residues are indicated in magenta.

like family of proteins, particularly the more distant relatives from bacteria, are also lysoplasmalogenase enzymes. We chose the YhhN-like protein from *L. pneumophila* because of its importance as a human pathogen. The LpYhhN protein has 216 amino acids, eight predicted transmembrane helices, and 30% sequence homology with TMEM86B. All of the highly conserved putative active site residues indicated above are present as the consensus residue in LpYhhN. We cloned the gene into a pWaldo-GFP expression vector that has been shown to be useful for production of transmembrane proteins in *E. coli* [34]. PCR primers were designed to insert the gene between the *Nde*I and *Bam*HI restriction sites, to express the protein as a C-terminal GFP-8xHis fusion. The GFP allows each step of the protein expression and purification to be readily monitored by fluorescence, the 8xHis tag allows the LpYhhN fusion protein to be purified by nickel affinity chromatography, and the TEV site allows the tag to be removed with TEV protease.

The resulting plasmid was transformed into *E. coli* C43 (DE3) cells, which have been shown to be useful for expression of transmembrane proteins [47]. The cells were grown in 1 L cultures at 30 °C for 17 h post-induction. After re-suspending the cells in NaCl Tris–phosphate buffer (pH 7.9), the lysate prepared by French Press in the absence of detergent contained a total of 44,000 units (nmol/min) of lysoplasmalogenase activity, as determined using our standard enzyme-coupled assay that measures the fatty aldehyde released from choline lysoplasmalogen substrate [28]. Importantly, cells with an empty control vector grown under identical conditions yielded only 500 units of activity. Since the *E. coli* K12 strain, of which C43 is a derivative, encodes an endogenous YhhN family protein, it is possible that the basal level of activity is due to *E. coli* YhhN. In any case, the fact the induced cells contained dramatically higher levels of activity than the control cells provides strong evidence that the observed activity is due to LpYhhN-GFP.

Table 1 (Purification 1) shows a summary of a purification of LpYhhN-GFP from 1 L of *E. coli*. Briefly, the initial cell lysate was centrifuged at 20,000 ×*g* to remove unbroken cells, and the supernatant was centrifuged at 130,000 ×*g* to pellet the cell membranes. The resulting pellet fluoresced with bluish-green color under UV light, consistent with it containing high levels of LpYhhN-GFP. By contrast, a pellet prepared from cells grown with empty control vector did not fluoresce. Pelleted membranes were resuspended in buffer, DDM was added to 1%, and the suspension was centrifuged at 130,000 ×*g* to remove insoluble material. The supernatant, which was the material loaded onto the nickel column, contained 19,000 units of lysoplasmalogenase activity and 23.1 mg of total protein. At this stage, a faint band at 36 kDa was evident on a Coomassie-stained SDS–PAGE gel (Fig. 3B, lane 3).

After the nickel column, analysis of each 1 ml fraction for lysoplasmalogenase activity resulted in two overlapping peaks (Fig. 3A), centered on fractions 8–10 (Peak 1) and 11–14 (Peak 2). Fractions from each peak were pooled separately and concentrated. Peak 2 contained 1,445 units of activity, or 3.3% of the total from the lysate, and Peak 2 contained 1,621 units, which was 3.7% of the total. SDS–

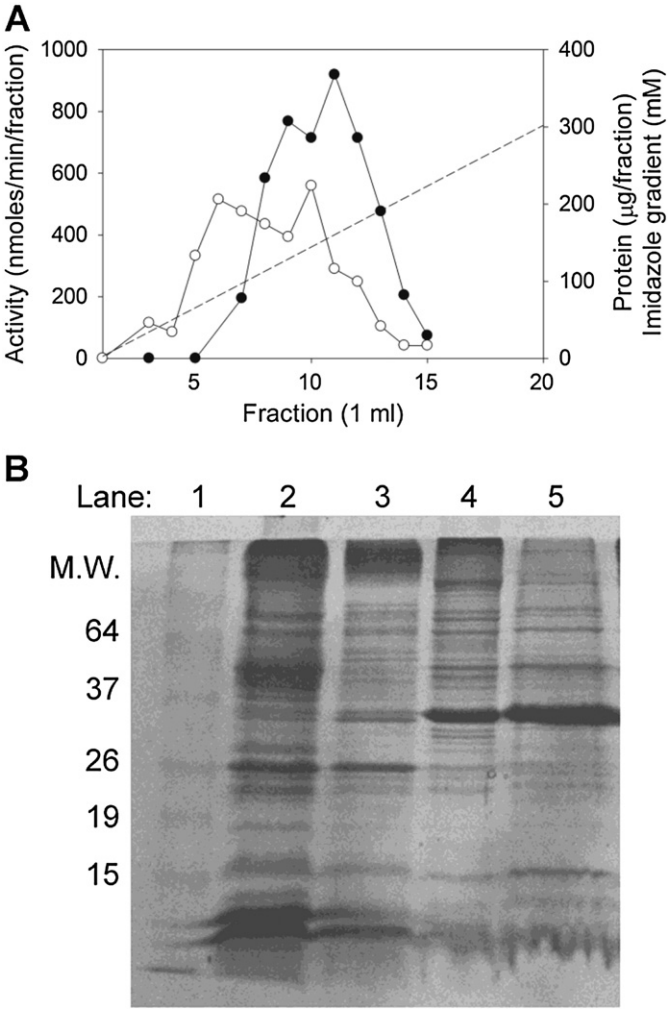

Fig. 3. Purification of LpYhhN-GFP by nickel chromatography. (A) Elution profile showing the lysoplasmalogenase activity (filled circles) and total protein (open circles) of each 1 ml fraction from the nickel column. (B) SDS–PAGE gel, 12%. Lane 1, MW standards; Lane 2, DDM treated 130,000 ×*g* insoluble pellet (4 units); Lane 3, solubilized membranes (130,000 ×*g* supernatant) (14 units); Lane 4, fractions 8–10 from the nickel column (37 units); Lane 5: fractions 11–14 from the nickel column (84 units). Each lane contained approximately 10 μg of total protein and the number of units indicated above in parentheses.

Table 1
Purification of LpYhhN-GFP from 1 L (Purification 1) and LpYhhN from 3 L (Purification 2) of *Escherichia coli* C43(DE3) cells.

Purification steps	Volume (ml)	Protein (mg/fraction)	Units (nmol/min/fraction)	Specific activity (nmol/min/mg)	Purification (fold)	Recovery (%)
Purification 1: starting with 1 L of cells						
1. Lysate from French press	35	800	44,000	55	1.0	100
2. 24,000 ×*g* supernatant (membranes and cytosol)	70	58	15,600	269	4.9	35.5
3. 130,000 ×*g* (1st) membrane pellet, solubilized with DDM	9.2	28.8	16,274	565	10.3	37
4. 130,000 ×*g* (2nd) pellet (insoluble membrane components)	2.8	4.86	1,419	292	5.3	3.2
5. 130,000 ×*g* (2nd) supernatant (solubilized membranes)	10	23.1	19,000	823	15	43
6a. His fractions 8–10	2.1	0.389	1,445	3,715	67.5	3.3
6b. His fractions 11–14	2.8	0.192	1,621	8,443	153.5	3.7
Purification 2: Starting with 3000 ml of cell culture (80,000 units)						
6. His fractions 17–19	4.5	1.2	5,230	4,350	79.2	6.5
7. TEV protease mixture	4.0	N.D.	4,800	N.D.	N.D.	N.D.
8. 2nd His column flow-through during enzyme addition, concentrated	3.5 concentrated to 900 μl	0.266	1,862	7,000	127.3	2.3

Lysoplasmalogenase activity was measured by the coupled enzyme assay as described in the Experimental Procedures section. Protein concentrations were determined by Bradford assay. N.D. = not determined.

PAGE analysis showed a prominent band at 36 kDa (Fig. 3B, lanes 4 and 5). Although this M.W. is considerably smaller than that predicted from the amino acid sequence of LpYhhN-GFP (46 kDa), this behavior is not uncommon for integral membrane proteins [34]. Moreover, as shown in Fig. 4b, lane 4, the 36 kDa band is strongly fluorescent under UV light, indicating that it indeed contains GFP. At later stages of the purification, mass spectrometry analyses of a trypsin digest of material from the 36 kDa band confirmed that the protein contains LpYhhN (Supplementary Fig. 1). Importantly, the proteomics analysis identified a peptide from the N-terminus of LpYhhN, indicating that the protein in the 36 kDa band contains the full-length LpYhhN, and not an N-terminal truncation. As will be shown below (Fig. 4), TEV cleavage separates this band into GFP, which runs on the SDS–PAGE at its predicted mass of 20 kDa and un-tagged LpYhhN, which runs at 18 kDa, smaller than its predicted mass of 24.5 kDa. Rat TMEM86B also runs on an SDS–PAGE gel at a smaller MW than predicted, at 18–19 kDa. [28]. Based on all of these considerations, we conclude that the 36 kDa band is the LpYhhN-GFP fusion protein.

We also note that after mixing the protein with the SDS–PAGE loading buffer, heating of the samples to the usual 95 °C resulted in complete disappearance of the 36 kDa band and of the18 kDa band, presumably due to the formation of aggregates (Fig. 5). Thus, all gel samples were heated to only 40 °C for 30 min. This behavior is also seen for TMEM86B [28], as well as for certain other transmembrane proteins [34].

3.3. Cleavage of the LpYhhN-GFP with TEV protease and further purification of LpYhhN

The addition of TEV protease to the protein from the nickel column produced the expected cleavage pattern (Fig. 4A, lane 5). The band at 36 kDa corresponding to LpYhhN-GFP was depleted (compare lanes 4 and 5), and new bands appeared for GFP (20 kDa), LpYhhN (18 kDa), and TEV protease (26 kDa). Imaging of the gel under UV light confirmed that the bands corresponding to GFP and LpYhhN-GFP were fluorescent while bands corresponding to TEV and LpYhhN were not (Fig. 4B). A western blot probing with antibodies to GFP further confirmed the presence or absence of GFP for each band (Fig. 4C). Since both the GFP and TEV contained histidine tags, a "reverse" nickel column was performed to separate the un-tagged LpYhhN protein (Fig. 4A, lane 7) from the GFP and the TEV protease (Fig. 4A, lane 8). The final purified LpYhhN protein is also shown in Fig. 5, lane 4 (18 kDa). The final yield of the un-tagged LpYhhN protein from 3 L of *E. coli* was 270 μg and 1,862 units (Table 1, Purification 2). This was 2.3% of the total from the starting cell lysate.

3.4. Enzyme analysis

As was seen previously for TMEM86B, both un-tagged LpYhhN and the LpYhhN-GFP fusion required the continuous presence of 0.1% DDM or 2–4 mM diradylglycerophospholipid for maintenance of activity at 4 °C. For storage at −20 °C, the presence of 50% glycerol or 2–4 mM diradylglycerophospholipid was also required.

Typical of enzyme catalyzed reactions, the reaction velocity depended on the amount of enzyme added (data not shown), and heating the enzyme to 95 °C for 3 min resulted in complete loss of activity (Table 2). At constant enzyme concentration, the amount of product formed increased linearly with time for more than 10 min (Table 3). These observations indicate that the observed activity is due to the purified protein. The reaction velocity as a function of substrate

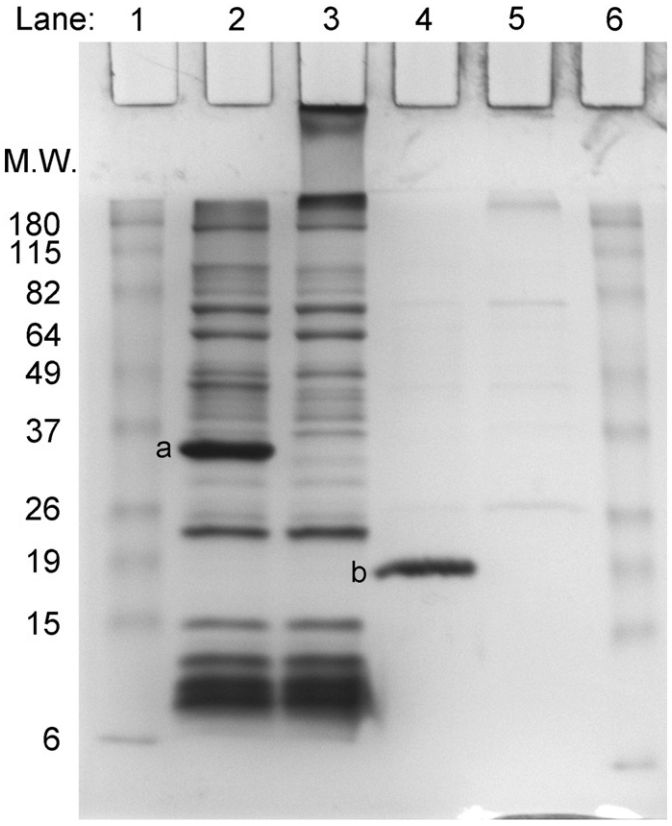

Fig. 5. LpYhhN-GFP and LpYhhN protein form aggregates in SDS gel samples heated to 95 °C. Each lane of the 11% SDS–PAGE gel was loaded with approximately 15 μg of total protein. Lanes 1 and 6 contain MW standards. Lanes 2 and 3 contain LpYhhN-GFP protein from the first nickel column, and lanes 4 and 5 contain the un-tagged LpYhhN protein from the second nickel column. The samples in lanes 2 and 4 were heated to 40 °C after adding the SDS loading buffer, while the samples in lanes 3 and 5 were heated to 95 °C. Band **a** in lane 2 is the LpYhhN-fusion protein. Band ***b*** in lane 4 is the un-tagged LpYhhN protein. Notice that heating to 95 °C (lanes 3 and 5) leads to disappearance of distinct bands for LpYhhN-GFP and LpYhhN, presumably due to formation of aggregates. This does not happen for most of the *E. coli* membrane proteins that are seen in the background (compare lanes 2 and 3). However, a similar phenomenon with membrane proteins has been noted previously [48].

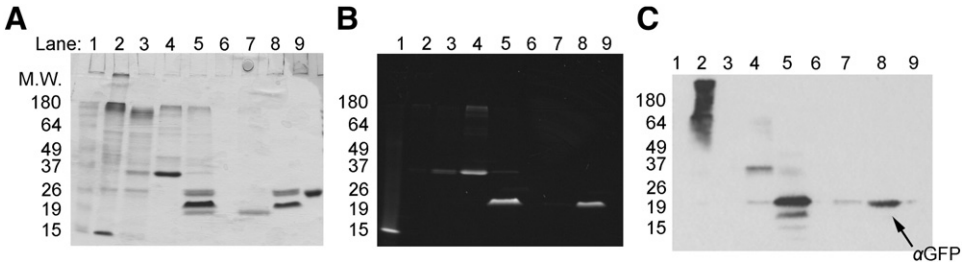

Fig. 4. SDS–PAGE of the LpYhhN-GFP TEV cleavage. (A) Coomassie-stained 11% SDS–PAGE gel. Lane 1, MW standard. Lane 2, DDM treated *Escherichia coli* membranes. Lane 3, solubilized membranes (130,000 ×*g* supernatant). Lane 4, pooled fractions from the nickel column. Lane 5, sample from lane 4 treated with TEV protease. Lane 6, empty. Lane 7, flow-through from the nickel column after TEV treatment. The band at 18 kDa is the LpYhhN protein. Lane 8, imidazole-eluted fraction showing bands for TEV (26 kDa) and GFP (20 kDa). Lane 9, TEV protease (Invitrogen, 30 U). (B) The same gel as panel A, but visualized under UV-light (prior to staining with Coomassie) to show GFP-containing bands. (C) A similar gel as panels A and B transferred to PVDF membrane and probed with antibodies against GFP. Each gel lane contained approximately 10 μg of total protein.

Table 2
Effects of heat and various compounds on LpYhhN-GFP lysoplasmalogenase activity.

Treatment	% of control activity
None	100
Enzyme heated at 95 °C for 5 min	0
Addition of $MgCl_2$ 10 mM	75 (7)
Addition of EDTA 2 mM	100 (7.1)
Addition of lysophosphatidic acid 250 μM	100 (6.3)
Addition of PMSF 500 μM	100 (9.2)

Reaction velocities were determined by the coupled enzyme assay. The substrate was 450 μM ethanolamine lysoplasmalogen. A control reaction without enzyme yielded 8 nmol of aldehyde per minute per ml. One microgram of LpYhhN-GFP protein from fractions 8–14 of the nickel column was added to a 0.52 ml reaction. The standard deviation in parentheses was calculated from three independent measurements.

concentration, for both ethanolamine and choline lysoplasmalogen substrates, is shown in Fig. 6 for the un-tagged LpYhhN protein. The curves followed rectangular hyperbolas characteristic of the binding isotherm. At the lower levels of substrate examined, 3, 6, and 10 μM , the rates of activity fell along the curves shown in Fig. 6 (upper 2 curves). (Only the 10 μM velocity point is shown on the graph.) These lower concentrations of substrate are probably below the CMC of lysoplasmalogen under these conditions. The apparent V_{max} values were 11 and 12 μmol/min/mg for ethanolamine and choline lysoplasmalogen substrates, respectively. The apparent K_m value for both substrates was 45 μM. The kinetic behavior of the LpYhhN-GFP fusion protein was very similar (data not shown).

In the kinetic experiments described in the previous paragraph, the lipid substrate was presented to the enzyme as a sonicated aqueous suspension. To test if LpYhhN can also hydrolyze lysoplasmalogens embedded within a lipid bilayer, substrates were presented to the enzyme as components of liposomes. In these experiments, the fraction of lysoplasmalogen relative to total phospholipid was varied from 5% to 20%. As can be seen in Fig. 6, LpYhhN was indeed able to hydrolyze lysoplasmalogens when presented in liposomes, and at constant lysoplasmalogen concentration, the velocity tended to increase as the mole % of lysoplasmalogen increased.

Prior to purification of un-tagged LpYhhN, several properties of the enzyme in its GFP-tagged form were characterized (Table 2). Addition of EDTA, sodium fluoride, PMSF, phosphatidic acid, lysophosphatidic acid, monoacylglycerophospho-choline, and monoacylglycerophospho-ethanolamine to the reaction were tested and found to have no effect. Addition of 10 mM Mg^{2+} lowered the activity by 25%. The pH optimum of the LpYhhN-GFP enzymes is between 6.5 and 7.0, which is similar to TMEM86B.

The experiments described to this point used an assay for lysoplasmalogenase activity that detects the release of fatty aldehyde, the reduction of which is coupled to the oxidation of NADH. To further verify that the reaction being catalyzed is indeed that of a lysoplasmalogenase, the LpYhhN-GFP enzyme-substrate mixture was subjected to lipid extraction at various time points [38] followed by TLC of the lipid fraction containing lysoplasmalogen, and the aqueous fraction containing glycerophosphoethanolamine (Table 3). The spots corresponding to lysoplasmalogen and glycerophosphoethanolamine were scraped and quantified by phosphorus assay [39]. A 1:1:1 stoichiometry was observed between the amount of lysoplasmalogen that disappeared, and the amounts of glycerophosphoethanolamine and of aldehyde that were formed. These results are consistent with the conclusion that the enzyme is cleaving the vinyl-ether bond at the *sn-1* carbon of lysoplasmalogen to release the expected products. TLC plates for reactions with LpYhhN-GFP were essentially identical to those performed with TMEM86B protein purified previously from rat liver, suggesting that the two enzymes are catalyzing the same reaction.

To characterize the substrate specificity of the LpYhhN enzyme, reactions were performed using other types of lipids as potential substrates, including 1-alkenyl-glycerol, two plasmalogens (alkenyl-2-acyl-*sn*-glycero-3-phospho-choline and -ethanolamine), the monoacyl glycerophospholipids 1-acyl-*sn*-glycero-3-phospho-choline and ethanolamine, and lysophosphatidic acid (1-acyl-*sn*-glycerol-3-phosphatidic acid) (Table 4). None of these substrates yielded detectable levels of product using either assay, suggesting that the hydrolytic activity of the LpYhhN-GFP protein is highly specific for the vinyl-ether bond at the *sn-1* and for a hydroxyl group at *sn-2* positions, as is the case for the TMEM86B enzyme.

4. Discussion

4.1. The YhhN protein of L. pneumophila is a lysoplasmalogenase

Lysoplasmalogenase catalyzes the hydrolytic cleavage of the vinyl ether bond of lysoplasmalogens to form a fatty aldehyde and glycerophospho-ethanolamine (or -choline). In a previous study, we purified the lysoplasmalogenase enzyme from rat liver microsomes, and identified the protein as TMEM86B [28]. TMEM86B is a member of the larger YhhN family of proteins, which are present in 138 species of eukaryotes and 1205 species of bacteria, many of which are known human pathogens, including *M. tuberculosis*, *L. pneumophila*, *Versinia pestis*, and *B. multivorans*.

In this work, we asked if the YhhN proteins of bacteria, which are about 30% identical in sequence to TMEM86B, also function as lysoplasmalogenase enzymes, despite the fact that many of the bacteria that encode them do not contain endogenous plasmalogens. To answer this question, we cloned and expressed the YhhN gene of *L. pneumophila* as a GFP fusion protein in *E. coli*. The membranes were isolated and solubilized and the LpYhhN-GFP protein was purified by nickel chromatography, cleaved with TEV protease, and the mixture was run over a second nickel column to isolate the un-tagged LpYhhN protein. The results of the enzyme assays are clear: both the LpYhhN-GFP fusion and the un-tagged LpYhhN protein exhibit abundant levels of lysoplasmalogenase activity. This is to our knowledge the first demonstration of a biochemical activity for a

Table 3
Quantitative correlation between substrate depletion and product formation in the LpYhhN lysoplasmalogenase reaction.

Time (min)	Amount of product formed or substrate depleted (nmol/2 ml)		
	1-Alkenylglycero-phospho-ethanolamine depleted [b]	Aldehyde produced [a]	Glycerophosphoethanolamine produced [b]
0	0	0	0
5	118 (13)	120 (6)	120 (11)
10	230 (21)	250 (17)	235 (19)

Concentration of ethanolamine lysoplasmalogen substrate was 400 μM. The enzyme source was 5 μg of LpYhhN-GFP from fractions 8–14 of the nickel column (Fig. 3, lanes 4–5). The reactions were run in duplicates, and the standard errors are in parentheses.

[a] Enzyme assay 1 (coupled enzyme assay) was used to determine rates.

[b] Enzyme assay 2 (determined by TLC assay described in the Experimental Procedures section.)

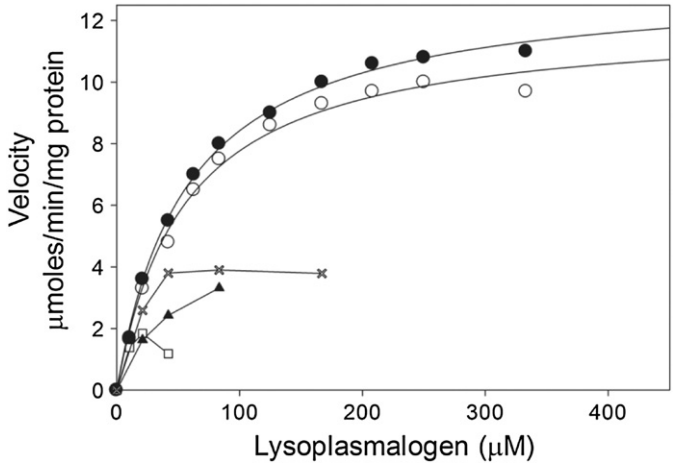

Fig. 6. Lysoplasmalogenase reaction velocity as a function of substrate concentration. The upper 2 curves show reactions with lysoplasmalogen added as sonicated aqueous suspensions. Substrates were choline-lysoplasmalogen (filled circles) or ethanolamine (open circles). The three lower curves, show reactions in which choline lysoplasmalogen was added as a component of liposomes, to mimic the conditions present in cell membranes; higher levels of liposome substrates could not be used as the initial absorbance at 340 nm was too high (greater than 2.3) for accurate reading in the coupled enzyme assay. The molar % ratios of choline lysoplasmalogen to phosphatidyl choline and phosphatidyl ethanolamine were varied. The relative amounts of these lipids were 5%, 47.5%, 47.5% (open squares), 10%, 45%, 45% (filled triangles), and 20%, 40%, 40% (crosses), respectively. The 0.52 ml reactions contained 1.2 μg of the purified LpYhhN protein shown in Fig. 5, lane 4. Reaction velocities were determined by the coupled enzyme assay, as described in the Experimental Procedures section. The plotted values are the mean of two independent measurements that differed by less than 10%. The curves are representative of two separate experiments performed on different days.

bacterial YhhN-like protein, and the first bacterial enzyme found that cleaves the vinyl ether bond of a lysoplasmalogen substrate.

4.2. The physical and chemical properties of the LpYhhN protein are similar to TMEM86B

LpYhhN is an integral membrane protein of a similar size as TMEM86B (216 vs. 226 amino acids). Sequence analysis predicts it to have eight transmembrane helices. The hydrophobic properties that require 1% DDM for solubilization and the continued presence of DDM or diradylglycerophospholipid for maintenance of activity are the same for both proteins.

The pH optimum, apparent kinetic parameters, and substrate specificities of LpYhhN are also very similar to those of TMEM86B. The V_{max} values of 11 and 12 μmol/min/mg for LpYhhN with ethanolamine and choline lysoplasmalogen, respectively, are only slightly lower than the corresponding values of 17 and 24 for TMEM86B. Similarly, the K_m value of 45 μM for LpYhhN with both substrates is very close to K_m value of 50 μM for TMEM86B. Like TMEM86B, LpYhhN is highly specific for the *sn-2*-deacylated (lyso) form of plasmalogen, and has no activity on the diradyl plasmalogens. The requirement for an *sn-2* hydroxyl in the substrate may also be important mechanistically, as it has been suggested that it could participate directly in the hydrolysis reaction [25].

One notable difference between LpYhhN and TMEM86 was found. Lysophosphatidic acid inhibits TMEM86B with a K_i of about 50 μM [28] but does not inhibit LpYhhN, even at concentrations as high as 500 μM (Table 4). We do not at present understand the biological significance of this observation.

Table 4
Substrate specificity of LpYhhN lysoplasmalogenase.

	Velocity (nmol·min^{-1}·ml^{-1})
Ethanolamine lysoplasmalogen[a]	8.3 (0.8)
Choline lysoplasmalogen[a]	8.1 (0.75)
Alkenyl glycerol[a]	0
Ethanolamine plasmalogen[a]	0
Choline plasmalogen[a]	0
Monoacylglycerophospho-ethanolamine[b]	0
Monoacylglycerophospho-choline[b]	0
Lysophosphatidic acid[b]	0

Substrate concentrations were 400 μM; 0.7 μg of LpYhhN fusion protein from fraction 8–14 of the nickel column was added to 0.52 ml reaction cuvette. The reactions were run in duplicates or triplicates, and the standard errors are shown in parenthesis.

[a] Enzyme assay 1 (coupled enzyme assay) was used to determine rates.

[b] Enzyme assay 2 (determined by TLC assay described in the Experimental Procedures section).

4.3. Could bacteria that do not contain endogenous plasmalogens or lysoplasmalogens be exposed to them during an infection?

Anaerobic bacteria synthesize and contain plasmalogens in their membranes, but aerobic and facultative anaerobic bacteria, such as *L. pneumophila* and *M. tuberculosis*, do not [1]. The absence of plasmalogens and lysoplasmalogens in many of the bacteria that encode a YhhN gene is interesting and at first appears paradoxical. However, the host cells in which the bacteria reside, such as amoeba (*L. pneumophila*) and lung macrophages (*L. pneumophila* and *M. tuberculosis*), are known to contain abundant levels of plasmalogens. Moreover, pneumocytes and surfactant have 12% and 4%, respectively, of their phospholipids in the plasmalogen subclass [49–51].

While it is thus clear that plasmalogens would be present in the cells infected by *L. pneumophila*, the extent to which *lyso*plasmalogens could accumulate is unknown. In mammals, lysoplasmalogens are formed from plasmalogens by the action of calcium-independent PLA_2 ($iPLA_2$) enzymes that remove the acyl chain at *sn-2*. Some of the mammalian $iPLA_2$ enzymes (group VI) are highly selective for plasmalogens [52,53]. The $iPLA_2$ enzyme present in macrophages [13] and type II pneumocytes [12] is activated during certain physiological and pathophysiological conditions [11]. Thus, during an infection with *L. pneumophila,* lysoplasmalogens could potentially be released from plasmalogens of host macrophages, pneumocytes, and surfactant.

It is also possible that plasmalogens could serve as substrates for one or more of the many bacterial phospholipase A. Although we have found no studies in which plasmalogens or lysoplasmalogens were tested as substrates for a bacterial phospholipase A, significant sequence homologies exist between VipD, a patatin-like phospholipase (PLP) of *L. pneumophila,* strain Philadelphia (AAU28879.1), and the mammalian $iPLA_2$ gamma enzymes (group VI) (NP_001242940.1), which hydrolyze plasmalogens [52,53]. The homologous regions include the conserved catalytic residues of $iPLA_2$, including the GDSRG sequence, in which the arginine is replaced by a lysine in VipD. VipD has phospholipase A activity with diacyl glycerophospholipid substrates [34]; plasmalogen was not tested as a substrate. If the VipD enzyme (and or other phospholipases A) of *L. pneumophila* does have activity on plasmalogens, it could conceivably provide a source of lysoplasmalogen for the bacterium to encounter.

4.4. What is the biological role for a bacterial lysoplasmalogenase?

Although our data have not yet answered this question, LpYhhN could conceivably serve to protect the bacteria from lysis by lysoplasmalogen. Such a role would be analogous to lysophospholipases of *L. pneumophila* that protect it from monoacyl glycerophospholipids (monoacyl GPL) [54], and to mammalian lysophospholipases that protect their cells from monoacyl GPLs [55]. The high V_{max} and the low K_m of LpYhhN are compatible with a role for the enzyme in maintaining lysoplasmalogens at low levels to prevent lysis of the bacterial cytoplasmic membrane. The CMC for lysoplasmalogens ranges from approximately 1 to 25 μM, depending on the length of the alkyl chain and whether or not albumen is present [20]. If present at concentrations

above their CMC values, lysophospholipids can lyse cell membranes (hence their name).

L. pneumophila produces both secreted and cell associated lysophospholipase activities with monoacyl GPL [54]. *L. pneumophila* showed reduced growth when exposed to 50–200 μM lysophosphatidyl choline (LPC), indicating that LPC is cytotoxic. Mutation of the gene for the secreted lysophospholipase increased the sensitivity of the bacteria to LPC. Conversely, overexpression of the secreted lysophospholipase decreased the sensitivity of the bacteria to LPC. It was concluded that the lysophospholipase served to protect the bacteria from toxic effects of the lysolipid [54]. It must be emphasized, however, that the lysophospholipase of this study was secreted outside the bacterial cell, whereas LpYhhN would presumably remain membrane bound. How LpYhhN would come into contact with lysoplasmalogens remains to be determined.

There are no studies on the effects of 1-alkenyl GPL (lysoplasmalogen) on bacteria. However, the structures and physical properties of monoacyl GPL and lysoplasmalogen are similar; they are both amphiphilic molecules [14,20]. At levels below their CMC, monoacyl GPLs are able to move across lipid bilayers and cell membranes [20]; these studies have not been done with lysoplasmalogens yet. Both monoacyl GPL and monoalkenyl GPL increase membrane fluidity and disrupt lipid bilayers. Lysoplasmalogens, with their vinyl ether linkage at *sn*-1, cause an even greater disturbance of bilayer structure than monoacyl GPL [14]. Both of these mono-radyl GPLs have been shown to lyse cell membranes at levels near their CMCs [19,20].

4.5. Hydrolytic cleavage of a vinyl ether bond is a rare reaction in biology.

There is only one other enzyme-catalyzed reaction known in nature that involves cleavage of a vinyl-ether bond. The reaction is catalyzed by the bacterial enzyme isochorismatase, which cleaves isochorismate to form pyruvate and 2,3-dihydroxybenzoate [56]. Isochorismatase is a globular, water-soluble enzyme, with an α/β-type fold [57]. This contrasts markedly with lysoplasmalogenase, an α-helical transmembrane protein. Thus, it is not likely that YhhN family proteins will bear any structural resemblance to isochorismatase, despite the common chemistry of the reactions. Sequence comparisons reveal only a very short, 22 amino acid region with 23% identity between the two proteins, which is unlikely to be statistically significant, given the short length. The fact that this region does not correspond to any of the conserved regions containing putative catalytic residues of YhhN family proteins (Fig. 2), or to the known catalytic residues of isochorismatase [57], further suggests that the two proteins do not share a common fold or arrangement of catalytic residues.

Terminology

Diradyl glycerophospholipids: phosphatidylcholine, 1,2-diacyl-*sn*-glycero-3-phosphocholine, diacyl GPC; phosphatidylethanolamine, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine, diacyl GPE;

Plasmalogens include: plasmenylethanolamine, 1-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine; plasmenylcholine, 1-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphocholine.

Monoradylglycerophospholipids: lysophosphatidyl-choline or ethanolamine are monoacylphosphatidyl-choline or ethanolamine, monoacyl GPC, monoacyl GPE, or monoacylGPL (L, for lipid); Lysophosphatidyl choline is also called LPC.

Lysoplasmalogens include: lysoplasmenylethanolamine, 1-alk-1′-enyl-*sn*-glycero-3-phosphoethanolamine; lysoplasmenylcholine, 1-alk-1′-enyl-*sn*-glycero-3-phosphocholine.

Names of the gene and protein; LpYhhN protein is the name we use in this paper for the protein product of the Lpg1991 gene of *L. pneumophila*, strain Philadelphia; LpYhhN fusion protein is the name we use in this manuscript for the LpYhhN-GFP-8His fusion protein.

The gene product of Lpg1991 is also named Q5ZU17_LEGPH.

Acknowledgments

This work was supported by the National Science Foundation Grant MCB-1021966 to CEB; the Ohio State University Development Fund (Physiological Chemistry) to MSJ; and the OSU Comprehensive Cancer Center Support (CORE) Grant NCI/NIH P30 CA16058 to L-C. W.

A special thank you to Adam Rauckhorst for many helpful discussions about lipid metabolism and mitochondrial biology, and for much help with the figures. We would like to thank Kathrin Wilczak and Dr. TuKiet Lam at the Yale MS & Proteomics Resource for the mass spectral data. We thank Angela Stamatikas, Warren Erdahl, Xinlei Pan, Christopher Smith, and Ron Louters for their valuable assistance in many ways. We appreciate the generosity of Dr. Daniel Schoenberg in sharing his FPLC apparatus with us, and of Dr. Jeff Kuret in sharing his French press and expertise with us.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.11.011.

References

[1] H. Goldfine, The appearance, disappearance and reappearance of plasmalogens in evolution, Prog. Lipid Res. 49 (2010) 493–498.
[2] L.A. Horrocks, M. Sharma, in: A. Neuberger, L.L.M. van Deenan (Eds.), New Comprehensive Biochemistry, Elsevier Biomedical Press, Amsterdam, 1982, pp. 51–95.
[3] X.L. Han, R.W. Gross, Plasmenylcholine and phosphatidylcholine membrane bilayers possess distinct conformational motifs, Biochemistry 29 (20) (1990) 4992–4996.
[4] X. Chen, R.W. Gross, Phospholipid subclass-specific alterations in the kinetics of ion transport across biologic membranes, Biochemistry 33 (46) (1994) 13769–13774.
[5] P.E. Glaser, R.W. Gross, Plasmenylethanolamine facilitates rapid membrane fusion: a stopped-flow kinetic investigation correlating the propensity of a major plasma membrane constituent to adopt an HII phase with its ability to promote membrane fusion, Biochemistry 33 (1994) 5805–5812.
[6] E. Yavin, S. Gatt, Oxygen-dependent cleavage of the vinyl-ether linkage of plasmalogens. 1. Cleavage by rat brain supernatant, Eur. J. Biochem. 25 (3) (1972) 431–436.
[7] E. Yavin, S. Gatt, Oxygen-dependent cleavage of the vinyl-ether linkage of plasmalogens. 2. Identification of the low molecular weight active component and the reaction mechanism, Eur. J. Biochem. 25 (3) (1972) 437–446.
[8] R.A. Zoeller, C.R. Raetz, Isolation of animal cell mutants deficient in plasmalogen biosynthesis and peroxisome assembly, Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 5170–5174.
[9] D.A. Ford, R.W. Gross, Plasmenylethanolamine is the major storage depot for arachidonic acid in rabbit vascular smooth muscle and is rapidly hydrolyzed after angiotensin II stimulation, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 3479–3483.
[10] L.J. Pike, X. Han, K.-N. Chung, R.W. Gross, Lipid rafts are enriched in arachidonic acid and plasmenyl ethanolamine and their composition is independent of caveolin-1 expression: a quantitative electrospray ionization/mass spectrometric analysis, Biochemistry 41 (2002) 2075–2088.
[11] P. Brites, H.R. Waterham, R.J.A. Wanders, Functions and biosynthesis of plasmalogens in health and disease, Biochim. Biophys. Acta 1636 (2004) 219–231.
[12] M.J. Angle, F. Paltauf, J.M. Johnston, Selective hydrolysis of ether-containing glycerophospholipids by phospholipase A_2 in rabbit lung, Biochim. Biophys. Acta 962 (1988) 234–240.
[13] D.P. Gaposchkin, H.W. Farber, R.A. Zoeller, On the importance of plasmalogen status in stimulated arachidonic acid release in the macrophage cell line RAW 264.7, Biochim. Biophys. Acta 1781 (4) (2008) 213–219.
[14] X.L. Han, R.W. Gross, Alterations in membrane dynamics elicited by amphiphilic compounds are augmented in plasmenylcholine bilayers, Biochim. Biophys. Acta 1069 (1) (1991) 37–45.
[15] R. Perichon, A.B. Moser, W.C. Wallace, S.C. Cunningham, G.S. Roth, H.W. Moser, Peroxisomal disease cell lines with cellular plasmalogen deficiency have impaired muscarinic cholinergic signal transduction activity and amyloid precursor protein secretion, Biochem. Biophys. Res. Commun. 248 (1998) 57–61.
[16] M. Schonefeld, S. Noble, A.M. Bertorello, L.J. Mandel, M.H. Creer, D. Portilla, Hypoxia-induced amphiphiles inhibit renal Na^+, K^+-ATPase, Kidney Int. 49 (1996) 1289–1296.
[17] S.D. Williams, D.A. Ford, Activation of myocardial cAMP-dependent protein kinase by lysoplasmenylcholine, FEBS Lett. 420 (1) (1997) 33–38.
[18] F. Facciotti, G.S. Ramanjaneyulu, M. Lepore, S. Sansano, M. Cavallari, M. Kistowska, S. Forss-petter, G. Ni, A. Colone, A. Singhal, J. Berger, C. Xia, L. Mori, G. DeLibero, Peroxisome-derived lipids are self antigens that stimulate invariant natural killer T cells in the thymus, Nat. Immunol. 13 (5) (2012) 474–480.
[19] E.L. Gottfried, G. Rapport, The biochemistry of plasmalogens: II. Hemolytic activity of some plasmalogen derivatives, J. Lipid Res. 4 (1) (1963) 57–62.
[20] H. Weltzien, Cytolytic and membrane-perturbing properties of lysophosphatidylcholine, Biochim. Biophys. Acta 559 (1979) 259–287.

[21] P.B. Corr, R.W. Gross, B.E. Sobel, Amphipathic metabolites and membrane dysfunction in ischemic myocardium, Circ. Res. 55 (1984) 135–154.
[22] R.M. Kramer, D. Deykin, Arachidonoyl transacylase in human platelets. Coenzyme A-independent transfer of arachidonate from phosphatidylcholine to lysoplasmenylethanolamine, J. Biol. Chem. 258 (1983) 13806–13811.
[23] J. Gunawan, M. Vierbuchen, H. Debuch, Studies on the hydrolysis of 1-alk-1′-enyl-*sn*-glycero-3-phosphoethanolamine by microsomes from myelinating rat brain, Hoppe Seylers Z. Physiol. Chem. 360 (1979) 971–978.
[24] R.L. Wykle, J.M. Schremmer, A lysophospholipase D pathway in the metabolism of ether-linked lipids in brain microsomes, J. Biol. Chem. 249 (1974) 1742–1746.
[25] H.R. Warner, W.E. Lands, The metabolism of plasmalogen: enzymatic hydrolysis of the vinyl ether, J. Biol. Chem. 236 (1961) 2404–2409.
[26] J. Gunawan, H. Debuch, Liberation of free aldehyde from 1-(1-alkenyl)-*sn*-glycero-3-phosphoethanolamine (lysoplasmalogen) by rat liver microsomes, Hoppe Seylers Z. Physiol. Chem. 362 (1981) 445–452.
[27] M. Jurkowitz-Alexander, H. Ebata, J.S. Mills, E.J. Murphy, L.A. Horrocks, Solubilization, purification and characterization of lysoplasmalogen alkenylhydrolase (lysoplasmalogenase) from rat liver microsomes, Biochim. Biophys. Acta 1002 (1989) 203–212.
[28] L.C. Wu, D.R. Pfeiffer, E.A. Calhoon, F. Madiai, G. Marcucci, S. Liu, M.S. Jurkowitz, Purification, identification, and cloning of lysoplasmalogenase, the enzyme that catalyzes hydrolysis of the vinyl ether bond of lysoplasmalogen, J. Biol. Chem. 286 (2011) 24916–24930.
[29] D.W. Fraser, T.R. Tsai, W. Orenstein, W.E. Parkin, H.J. Beecham, R.G. Sharrar, J. Harris, G.F. Mallison, S.M. Martin, J.E. McDade, C.C. Shepard, P.S. Brachman, Legionnaire's disease: description of an epidemic of pneumonia, N. Engl. J. Med. 297 (1977) 1189–1197.
[30] B.S. Fields, R.F. Benson, R.E. Besser, Legionella and Legionnaires' disease: 25 years of investigation, Clin. Microbiol. Rev. 15 (2002) 506–526.
[31] L. Xu, Z.-Q. Luo, Cell biology of infection by *Legionella pneumophila*, Microbes Infect. 15 (2) (2013) 157–167.
[32] L.G. Tilney, O.S. Harb, P.S. Connelly, C.G. Robinson, C.R. Roy, How the parasitic bacterium *Legionella pneumophila* modifies its phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane, J. Cell Sci. 114 (2001) 4637–4650.
[33] S. Banerji, P. Aurass, A. Flieger, The manifold phospholipases A of *Legionella pneumophila*, identification, export, regulation, and their link to bacterial virulence, Int. J. Med. Microbiol. 298 (2008) 169–181.
[34] D. Drew, M. Lerch, E. Kunji, D.-J. Slotboom, J.-W. de Gier, Optimization of membrane protein overexpression and purification using GFP fusions, Nat. Methods 3 (4) (2006) 303–313.
[35] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[36] M.S. Jurkowitz-Alexander, Y. Hirashima, L.A. Horrocks, Coupled enzyme assays for phospholipase activities with plasmalogen substrates, in: E.A. Dennis (Ed.), Methods Enzymol, Phospholipases, 197, Academic Press, Orlando, Florida, 1991, pp. 79–89.
[37] M.S. Jurkowitz-Alexander, L.A. Horrocks, Lysoplasmalogenase: solubilization and partial purification from liver microsomes, in: E.A. Dennis (Ed.), Methods Enzymol. Phospholipases, 197, Academic Press, Orlando, Florida, 1991, pp. 483–490.
[38] E. Bligh, W. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[39] J. Gunawan, H. Debuch, Alkenylhydrolase: a microsomal enzyme activity in rat brain, J. Neurochem. 44 (1985) 370–375.
[40] G. Rouser, S. Fleischer, A. Yamamoto, Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots, Lipids 5 (1970) 494–496.
[41] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[42] M. Punta, P.C. Coggil, R.Y. Eberhardt, J. Mistry, J. Tate, C. Boursnell, N. Pang, K. Forslund, G. Ceric, J. Clements, A. Heger, L. Holm, E.L.L. Sonnhammer, S.R. Eddy, A. Bateman, R.D. Finn, The Pfam protein families database, Nucleic Acids Res. 40 (2012) D290–D301.
[43] A. Krogh, B. Larsson, G.I. von Heijne, F.L.L. Sonnhammer, Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes, J. Mol. Biol. 305 (2001) 567–580.
[44] G.E. Tusnády, I. Simon, Principles governing amino acid composition of integral membrane proteins: application to topology prediction, J. Mol. Biol. 283 (1998) 489–506.
[45] F. Sievers, A. Wilm, D. Dineen, T.J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Söding, J.D. Thompson, D.G. Higgins, Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega, Mol. Syst. Biol. 7 (2011) 539.
[46] S. Whelan, P.I. de Bakker, E. Quevillon, N. Rodriguez, N. Goldman, PANDIT: an evolution-centric database of protein and associated nucleotide domains with inferred trees, Nucleic Acids Res. 34 (2006) D327–D332.
[47] B. Miroux, J.E. Walker, Over-production of proteins in *Escherichia coli* mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels, J. Mol. Biol. 260 (1996) 289–298.
[48] H. Schagger, Chapter 3. Denaturing electrophoretic techniques, in: Gebhard Von Jagow, Hermann Schagger (Eds.), A Practical Guide to Membrane Protein Purification, Academic Press, New York, N.Y., 1994, pp. 59–79.
[49] A. Diagne, J. Fauval, M. Record, H. Chap, L. Douste-Blazy, Studies on ether phospholipids. II. Comparative composition of various tissues from human, rat and guinea pig, Biochim. Biophys. Acta 793 (2) (1984) 221–231.
[50] B. Rustow, I. Kolleck, F. Guthmann, R. Haupt, P. Stevens, Synthesis and secretion of plasmalogens by type-II pneumocytes, Biochem. J. 302 (1994) 665–668.
[51] M. Rudiger, I. Kolleck, G. Putz, R.R. Wauer, P. Stevens, B. Rustow, Plasmalogens effectively reduce the surface tension of surfactant-like phospholipid mixtures, Am. J. Physiol. 274 (1998) L143–L148.
[52] C.S. Beckett, P.J. Kell, M.H. Creer, J. McHowat, Phospholipase A_2-catalyzed hydrolysis of plasmalogen phospholipids in thrombin-stimulated human platelets, Thromb. Res. 120 (2) (2007) 259–268.
[53] D.J. Mancuso, C.M. Jenkins, R.W. Gross, The genomic organization, complete mRNA sequence, cloning, and expression of a novel human intracellular membrane-associated calcium-independent phospholipase A(2), J. Biol. Chem. 275 (14) (2000) 9937–9945.
[54] A. Fleiger, B. Neumeister, N.P. Cianciotto, Characterization of the gene encoding the major secreted lysophospholipase A of *Legionella pneumophila* and its role in detoxification of lysophosphatidylcholine, Infect. Immun. 70 (11) (2002) 6094–6106.
[55] A. Wang, E.A. Dennis, Mammalian lysophospholipases (Review), Biochim. Biophys. Acta 1439 (1999) 1–16.
[56] F. Rusnak, J. Liu, N. Quinn, G.A. Berchtold, C.T. Walsh, Subcloning of the enterobactin biosynthetic gene entB: expression, purification, characterization, and substrate specificity of isochorismatase, Biochemistry 29 (6) (1990) 1425–1435.
[57] J.F. Parsons, K. Calabrese, E. Eisenstein, J.E. Ladner, Structure and mechanism of *Pseudomonas aeruginosa* PhzD, an isochorismatase from the phenazine biosynthetic pathway, Biochemistry 42 (19) (2003) 5684–5693.