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Identification of a chloroform-soluble membrane mini-protein in *Escherichia coli* and its homolog in *Salmonella typhimurium*

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Abstract

Two homologous 29 amino acid-long highly hydrophobic membrane mini-proteins were identified in the Bligh-Dyer lipid extracts of *Escherichia coli* and *Salmonella typhimurium* using liquid chromatography/tandem mass spectrometry (LC/MS/MS). The amino acid sequences of the proteins were determined by collision-induced dissociation tandem mass spectrometry, in conjunction with a translating BLAST (tBLASTn) search, i.e. comparing the MS/MS-determined protein query sequence against the six-frame translations of the nucleotide sequences of the *E. coli* and *S. typhimurium* genomes. Further MS characterization revealed that both proteins retain the N-terminal initiating formyl-methionines. The methodologies described here may be amendable for detecting and characterizing small hydrophobic proteins in other organisms that are difficult to annotate or analyze by conventional methods.

INTRODUCTION

Only a handful of proteins and peptides that have been reported to be soluble in chloroform, including subunit *c* of the F_1F_0 ATP synthase [1], *Escherichia coli* multidrug resistance protein E (EmrE) [2], myelin proteolipid protein (PLP) [3], lung surfactants [4], and several small peptides [5]. These unusually hydrophobic proteins and peptides play diverse and critical cellular functions. Here, we report the unexpected identification of two small chloroform–soluble proteins (3 kDa) in the Bligh-Dyer lipid extracts [6] of the Gram-negative bacteria *E. coli* and *Salmonella typhimurium*. The full sequences of these homologous proteins were determined by using the partial amino acid sequences determined by collision-induced dissociation tandem mass spectrometry in database searches. Further characterization by accurate mass measurement and MS/MS revealed that both proteins retain their N-terminal initiating formyl-methionines.

EXPRIMENTAL PROCEDURES

Materials

All solvents were of HPLC grade and were obtained from VWR (West Chester, PA).

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Bacterial Growth Conditions

Wild type strains *E. coli* W3110 and *S. typhimurium* χ3761 were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract and 1% NaCl). Typically, a 100 ml cell culture, inoculated from an overnight culture grown to $A_{600} = 0.02$, was grown to $A_{600} = 2$. The cells were collected by centrifugation and washed with phosphate-buffered saline (PBS, pH 7.4). The cell pellets were resuspended in 152 ml of a single-phase Bligh-Dyer mixture consisting of chloroform/methanol/water (1:2:0.8, v/v/v), incubated at room temperature for 60 min, and centrifuged at 3000 × *g* to remove insoluble debris. The supernatant was transferred to a new glass tube and converted to a two-phase Bligh-Dyer system by adding chloroform and water to generate a mixture consisting of chloroform/methanol/water (2:2:1.8, v/v/v). The lower phase was dried under a stream of nitrogen and stored at −20 °C until analysis.

Electrospray Ionization/Mass Spectrometry

For negative ion ESI/MS analysis [7], each of the dried Bligh-Dyer lipid extracts of *E. coli* and *S. typhimurium* cells was first re-dissolved in 200 μl of chloroform. Typically, 5 μl of the solution was diluted into 200 μl of chloroform/methanol (2:1, v/v), followed by the addition of 1 μl of piperidine (Sigma-Aldrich). The solutions were infused at flow rates of 5–10 μl/min into the ESI source of the high-resolution QSTAR XL quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). The negative electrospray voltage was −4200 V. Other MS settings were as follows: Curtain Gas = 20 psi (pressure), Ion Gas Source = 20 psi, Declustering Potential = −55 V, and Focusing Potential = −265 V. Data acquisition and analyses were performed using Analyst QS software (Applied Biosystems/MDS Sciex).

Liquid Chromatography/Tandem Mass Spectrometry

LC/MS/MS analysis was performed in the positive ion mode using a Shimadzu LC system (comprising a solvent degasser, two LC-10A pumps and a SCL-10A system controller) coupled to a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (as above). LC was operated at a flow rate of 200 μL/min with a linear gradient as follows: 100% A was held isocratically for 2 min and then linearly increased to 100% B over 14 min and held at 100% B for 4 min. Mobile phase A consisted of methanol/acetonitrile/aqueous 1 mM ammonium acetate (60/20/20, v/v/v). Mobile phase B consisted of 100% ethanol containing 1 mM ammonium acetate. A Zorbax SB-C8 reversed-phase column (5 μm, 2.1 × 50 mm) was purchased from Agilent (Palo Alto, CA). The post-column split diverted ~10% of the LC flow to the ESI source. Positive ion mass spectra were acquired with an electrospray voltage of +5500 V. The collision-induced dissociation tandem mass spectra were obtained with collision energy of +50 V (laboratory frame of reference) and with nitrogen as the collision gas.

RESULTS

Two unknown species, each with a molecular weight of 3 kDa, were observed upon ESI/MS analysis of the total lipid extracts of *E. coli* and *S. typhimurium* cells. Figures 1A and 1B show the negative ion ESI mass spectra of the species present in the Bligh-Dyer extracts of the *E. coli* and *S. typhimurium* cells, respectively. The major ion species, with mass-to-charge (*m/z*) ratios ranging from 600 to 800, were identified by MS/MS to be the $[M-H]^-$ ions of the major bacterial phospholipids, including phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) [8]. In both spectra, there exist unknown 3 kDa species, whose triply charged ions appeared near *m/z* 1000. As shown in the magnified ion peaks in the insets, the charge state (3-) was determined as the reciprocal of the spacing between two adjacent isotopic peaks (differing by 1 atomic mass unit) [9]. The monoisotopic masses of

these species, determined by accurate mass measurements of the $[M-3H]^{3-}$ ions, were 3023.740 Da and 2981.686 Da for the *E. coli* and *S. typhimurium* proteins, respectively. These unknown species were also detected by LC/MS operated in the positive ion mode. Figure 2 shows the zoomed-in spectra of the triply charged (protonated) $[M+3H]^{3+}$ ions at *m/z* 1008.9 and *m/z* 994.9 for the unknown protein species from *E. coli* and *S. typhimurium*, respectively.

To identify these unknown 3 kDa species, collision-induced dissociation MS/MS was performed on the triply charged ions (in both positive and negative ion modes). While the negative ion MS/MS spectra of the $[M-3H]^{3-}$ ions were not structurally informative (data not shown), the positive ion MS/MS spectra of the $[M+3H]^{3+}$ ions (acquired through targeted LC/MS/MS) showed fragmentation patterns characteristic of a peptide. As illustrated in Figure 3, the partial amino acid sequences deduced from the product ion spectra of $[M+3H]^{3+}$ ions at *m/z* 1008.9 (*E. coli*) and *m/z* 994.9 (*S. typhimurium*) are: GL(I)L(I)VAVGLTAALH (*E. coli*) and GVL(I)VAGGL(I)TAALH (*S. typhimurium*). The isobaric I and L residues could not be discerned by the low-energy CID MS/MS used in this study. Our initial attempt at searching these partial sequences against the protein sequence database (prior to annotation) yielded no matches. We then performed a translating BLAST (tBLASTn) search, *i.e.* comparing the partial amino acid sequences against the six-frame translations of the nucleotide sequences of the bacterial genomes. This yielded matches for both proteins. The full sequences of the two proteins were identified as: MSTDLKFSLVTTIIVLGLIVAVGLTAALH (*E. coli*) and MSTDLKFSLITTLIVLGVIVAGGLTAALH (*S. typhimurium*). Differing by only three amino acid residues, these proteins are apparently derived from orthologous genes. The *E. coli* protein was later annotated as YnhF in the EcoGene database (http://www.ecogene.org), containing updated information on the *E. coli* K-12 genome and proteome sequences.

It was noted, for both the *E. coli* and *S. typhimurium* proteins, that there is a 28 Da difference between the sequence-predicated monoisotopic masses and those determined by MS. The sequence-predicated monoisotopic masses are 2995.740 Da (*E. coli*) and 2953.693 Da (*S. typhimurium*), while the MS-determined monoisotopic masses were 3023.740 Da (*E. coli*) and 2981.686 (*S. typhimurium*). This 28 Da difference implies that both proteins might be modified by a formyl (CO) group. Indeed, this possibility was confirmed by accurate mass measurements and MS/MS. The MS-determined monoisotopic masses (above) are in agreement with the predicated monoisotopic masses of 3023.735 Da (*E. coli*) and 2981.688 Da (*S. typhimurium*) for the formylated forms of both proteins. Furthermore, MS/MS showed that the formyl group is attached to the N-terminal methionine, as evidenced by the presence in the MS/MS spectra of the b_2 product ions at *m/z* 247.1 (the predicated b_2 ion mass for each of the N-formylated peptides is 247.1). Figure 4 depicts all inter-residual cleavage sites observed in the positive ion MS/MS spectra (Figure 3) of the $[M+3H]^{3+}$ ions. All sequence fragment ions (*b* and *y*) and their masses are listed in Table 1. The nomenclature used for naming the fragment ions is according to Roepstorff and Fohlman [10].

Although formyl-methione initiates all protein synthesis in Bacteria [11–13], in most cases the formyl group is subsequently removed by deformylase [14;15]. This is often followed by removal of the N-terminal methionine by methionine aminopeptidase [16]. Nonetheless, the retention of N-terminal formyl-methiones has been reported for several proteins and peptides, such as the aspartate chemoreceptor [17], the membrane-bound F_0 subunit *c* of ATP synthetase [18], and the chemotactic peptide, formyl-methinyl-leucyl-phenyanaline (f-MLF) [19].

DISCUSSION

Identification and structural characterization of hydrophobic proteins and peptides are challenging, as they are typically membrane-associated, and are difficult to extract or purify. The predominant MS-based proteomic techniques have, in fact, been developed for analyzing soluble proteins and peptides. As a result, hydrophobic proteins and peptides are under-represented in global proteomic analyses. However, encouraging technical advances have been made in recent years in the MS analysis of membrane-associated proteins [20–24]. In particular, the feasibility of high-resolution top-down MS/MS for determining post-translational modifications (PTM) of integral membrane proteins has been demonstrated [24].

The identification here of small membrane proteins from *E. coli* and *S. typhimurium* benefited unexpectedly from methods that were developed for lipid analysis. We envision that the methodologies described here could be equally amendable for the identification of unusually hydrophobic proteins in other organisms or cells. It is possible that by subjecting total Bligh-Dyer extracts (or other organic solvent extracts) to additional steps of fractionation, more hydrophobic proteins, especially those of very low abundance, could be uncovered.

A second distinctive feature of these small proteins is that they are encoded by genes defined by very small open reading frames. Small proteins and peptides play key roles in cellular processes including transport, intermediary metabolism, translation regulation, chromosome segregation, genome stability, and other biological and physiological functions [25]. However, correct annotation of genes encoded by small open reading frames (smORFs) is difficult. First, computational methods do not reliably predict small genes, as there are an enormous number of meaningless short ORFs. Second, their small size makes smORFs elusive targets for mutagenic screens [25;26]. As a result, knowledge of smORF function is rather limited, as compared to their longer counterparts. Detection by MS at the protein or peptide level, as demonstrated in this study, provides the most direct and convincing evidence for identifying the products of genes defined by small open reading frames. This is yet another example illustrating the important roles of proteogenomics, which typically utilizes MS-determined protein/peptide sequences to define novel open reading frames [27–29]. Currently, there are less than 60 proteins comprising fewer than 50 amino acids identified or predicted in the *E. coli* K-12 genome [30].

The fact that these membrane mini-proteins could be detected by direct infusion ESI/MS in the total lipid extracts of *E. coli* and *S. typhimurium* without any pre-fractionation is quite remarkable, implying that they may exist in significant quantities, playing crucial roles in these bacteria. The *E. coli* protein was annotated as YnhF. In the *E. coli* genome, *ynhF* is located upstream to *ydhP*, encoding a 389 amino acid-long protein predicted to be a transporter, containing 12 trans-membrane helices (Figure 5). It is possible that *ynhF*, predicted to have one trans-membrane segment, may function as a subunit of this transporter complex, similar to the role played by the membrane-bound subunit *c* (8 kDa) of the F_1F_0 ATP synthase complex, or the role of the KdpF subunit (3 kDa) in the K(+) translocating Kdp complex [31].

A recent study by Storz and co-workers [32] showed that YnhF is one of the small stress response proteins in *E. coli*. The expression level of *ynhF*, measured using a sequential peptide affinity (SPA) tag [30], is over 4-fold higher when cells were grown under aerobic versus low oxygen conditions, and is lower in minimal glycerol-grown cells, as compared to what is seen in minimal glucose-grown cells. These observations call for study on the

functions of these membrane mini-proteins, possibly to be approached initially through gene deletion.

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ABBREVIATIONS

ESI/MS	electrospray ionization/mass spectrometry
LC/MS	liquid chromatography/mass spectrometry
MS/MS	tandem mass spectrometry
ORF	open reading frame
PE	phosphatidylethanolamine
PG	phosphatidylglycerol

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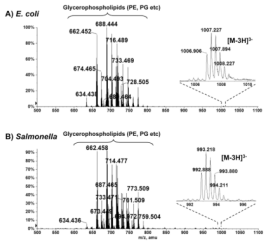

Figure 1.

Negative ion ESI mass spectra of the Bligh-Dyer extracts of *E. coli* (A) and *S. typhimurium* (B). The insets show the magnified ion peaks for the $[M\text{-}3H]^{3-}$ ions of the small 3 kDa proteins.

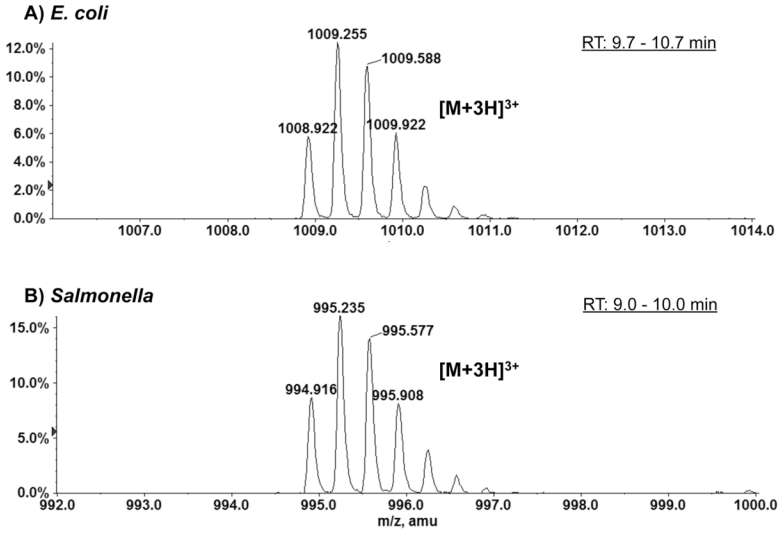

Figure 2.

Positive ion ESI mass spectra showing the $[M+3H]^{3+}$ ions of the small proteins in the Bligh-Dyer extracts of *E. coli* (A) and *S. typhimurium* (B). These positive ion mass spectra are averaged from the spectra acquired by LC/MS (during the LC elution times indicated in the figures).

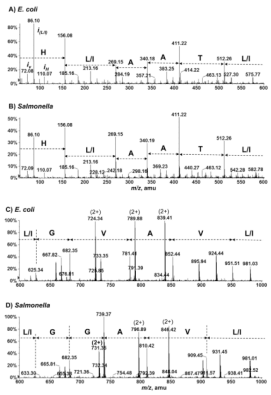

Figure 3.

Assignment of partial amino acid sequences based on the collision-induced dissociation MS/MS spectra of the small 3 kDa proteins from *E. coli* and *S. typhimurium*. LC/MS/MS spectra of $[M+3H]^{3+}$ ions at *m/z* 1008.9 (A and C) for *E. coli*, and *m/z* 994.9 (B and D) for *S. typhimurium*. Peaks labeled with i_V, $i_{L/I}$ and i_H correspond to the immonium ions of valine, leucine/isoleucine and histidine.

Figure 4.
Inter-residual cleavage sites observed in the MS/MS spectra of the $[M+3H]^{3+}$ ions at *m/z* 1008.9 and *m/z* 994.9 for the small proteins from *E. coli* and *S. typhimurium*, respectively.

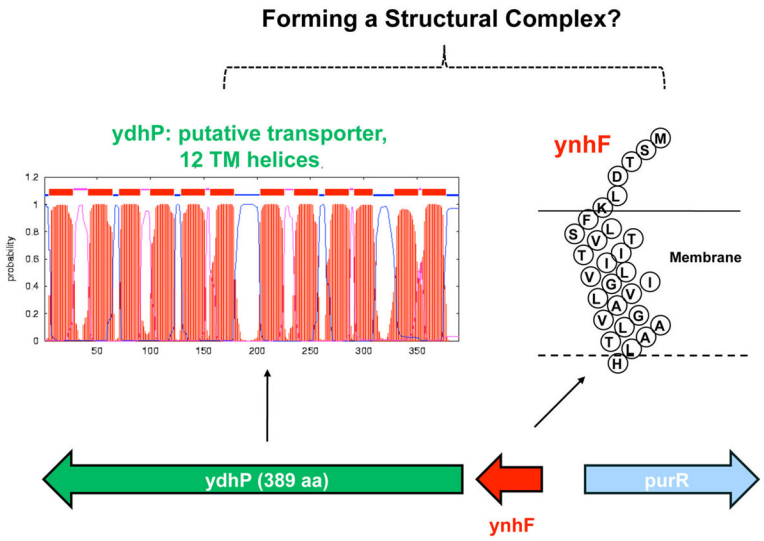

Figure 5.
Prediction of trans-membrane regions in YnhF and YdhP, encoded by adjacent genes. It is possible, given their genomic and membrane locations, that YnhF forms a complex with YdhP, a putative transporter.

Table 1

Sequence ions (*b* and *y*) observed in the collision-induced dissociation MS/MS spectra of the $[M+3H]^{3+}$ ions at *m/z* 1008.9 and *m/z* 994.9 of the small proteins from *E. coli* and *Salmonella*, respectively. The listed ion masses correspond to singly charged species. Abbreviation: f, formyl.

fMSTDLKFSLVTTIIVLGLIVAVGLTAALH (*E. coli*)				**fMSTDLKFSLITTLIVLGVIVAGGLTAALH (*S. typhimurium*)**			
	Observed		**Observed**		**Observed**		**Observed**
y_1	156.1			y_1	156.1		
y_2	269.2			y_2	269.2		
y_3	340.2			y_3	340.2		
y_4	411.2			y_4	411.2		
y_5	512.3			y_5	512.3		
y_6	625.3			y_6	625.3		
y_7	682.4			y_7	682.4		
y_8	781.4	b_{21}	2244.2	y_8	739.4	b_{21}	2244.2
y_9	852.4	b_{20}	2173.1	y_9	810.4	b_{20}	2173.1
y_{10}	951.5	b_{19}	2074.1	y_{10}	909.5	b_{19}	2074.1
y_{11}	1064.6	b_{18}	1961.1	y_{11}	1022.6	b_{18}	1961.1
y_{12}	1177.7	b_{17}	1847.8	y_{12}	1178.6	b_{17}	1861.8
y_{13}	1234.7	b_{16}	1790.8	y_{13}	1291.7	b_{16}	1804.9
y_{14}	1347.8	b_{15}	1677.8	y_{14}	1390.7	b_{15}	1691.8
y_{15}	1446.9	b_{14}	1578.8	y_{15}	1503.8	b_{14}	1592.8
y_{16}	1559.9	b_{13}	1465.5			b_{13}	1479.4
		b_{12}	1352.6			b_{12}	1366.6
		b_{11}	1251.6			b_{11}	1265.6
		b_{10}	1150.6			b_{10}	1164.6
		b_9	1051.5			b_9	1051.5
		b_8	938.4			b_8	938.4
		b_7	851.4			b_7	851.4
		b_6	704.3			b_6	704.3

fMSTDLKFSLVTTIIVLGLIVAVGLTAALH (*E. coli*)				fMSTDLKFSLITTLIVLGVIVAGGLTAALH (*S. typhimurium*)			
	Observed		**Observed**		**Observed**		**Observed**
		b_5	576.2			b_5	576.2
		b_4	463.1			b_4	463.1
		b_3	348.1			b_3	348.1
		b_2	247.1			b_2	247.1