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Research Article

Identification of proteins from membrane preparations by a combination of MALDI TOF-TOF and LC-coupled linear ion trap MS analysis of an Antarctic bacterium *Pseudomonas syringae* Lz4W, a strain with unsequenced genome

Multidimensional protein identification technology helps in identifying a large number of proteins with ESI by sequencing several peptides with MS/MS methods. When ionization and separation of different hydrophobic and hydrophilic peptides in a single process are difficult, a combination of LC-coupled linear ion trap MS and MALDI TOF/TOF can be used for identification of proteins as shown in the present study. We have used this combinational approach to identify membrane proteins of the Antarctic bacterium *Pseudomonas syringae* Lz4W, which are separated by SDS gel electrophoresis. Although the genome of *P. syringae* Lz4W has not been sequenced, the known genome sequences of mesophilic *Pseudomonas* species have been used for the identification of the proteins. Broadly, many membrane proteins, proteins with a wide range of molecular weight and *pI* including some integral membrane proteins could be identified using this procedure. Some of the identified proteins are involved in low temperature adaptation.

Keywords:Antarctic bacteria / Membrane proteins / MS / *Pseudomonas sp* / Psychrophiles
DOI 10.1002/elps.200700750**1 Introduction**

About 20–30% of all the genes in an organism encode membrane proteins. However, membrane proteomes characterized so far are few [1, 2]. This problem is mainly because of the physicochemical nature of these proteins. The membrane-spanning regions are extremely hydrophobic and the extra membrane regions are hydrophilic. These properties lead to difficulties in formulating a general strategy for the analysis of these proteins. In general, multidimensional protein identification technology helps in the identification of a large number of proteins using ESI, and sequencing the peptides adopting MS/MS methodologies [3, 4]. Using this approach, several proteins in complexes, sub-cellular components, cells and tissues are identified [5–7].

Using a combination of SDS-PAGE and LC-MS/MS, the membrane proteome of *Halobacterium salinarium* was identified [8]. Recent studies, aimed at identifying the complete membrane proteome of a *Corynebacterium glutamicum*, revealed that only 50% of the proteins could be identified [2].

The problems associated with the solubility of proteins and losses of hydrophobic peptides make it difficult to generalize the procedure. The current methodologies and approaches, aimed at identifying the complete membrane proteome, are far from being satisfactory. Hence, there is a great demand for developing new strategies/methodologies for identifying the membrane proteins. Currently, several methodologies are being developed for the ionization and detection of these molecules by various mass spectrometric methods using detergents [9–12]. Unfortunately, the presence of detergents and salts in the sample hinders the analysis of proteins and peptides in MS methods [13,14]. The methods of ionization such as MALDI and ESI also play an important role in detecting the peptides, depending on the chemical nature of the peptide. In a recent study, it has been shown that the signal intensity of the precursor ion in the LC-ESI experiment increases, with increase in hydrophobic nature of the peptide due to greater efficiency in the ionization of these peptides [15].

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Abbreviations: GRAVY, grand average of hydropathy; Xcorr, cross correlation

Several investigations have been carried out in our laboratory and elsewhere to elucidate the mechanism of cold adaptation of bacteria using some Antarctic bacterial isolates as a model system [16–20]. Several lines of evidence are also available, indicating that membranes and membrane components play an important role in cold adaptation of bacteria [21–25].

The possible role of a membrane protein of the Antarctic bacterium *Pseudomonas syringae* in sensing the environmental temperature was studied [26, 27]. These studies revealed the importance of identifying the membrane proteins in understanding their role in cold adaptation.

In the present study, the membrane proteins of the Antarctic bacterium *P. syringae* Lz4W were separated on SDS-PAGE and the bands were excised and digested with trypsin. Subsequently, LC-ESI MS/MS and MALDI TOF/TOF were used simultaneously to analyze the resultant peptides for identification of proteins. This helped in increasing the sequence coverage of a large number of proteins. Although the genome of *P. syringae* Lz4W had not been sequenced, the genome sequence of several *Pseudomonas* species, which were available, had been used for the identification of the proteins of *P. syringae* Lz4W.

2 Materials and methods

2.1 Chemicals and reagents

All the chemicals required for culturing bacteria such as yeast extract, peptone and agar were purchased from Himedia (Mumbai, India). All the solvents used in this study were procured from Merck (Darmstadt, Germany). The chemicals for gel electrophoresis, acrylamide, bis acrylamide, TEMED, ammonium persulfate, SDS, Tris, glycine, urea, CHAPS, molecular weight markers, etc. were all obtained from Bio-Rad (Hercules, CA, USA). α -Cyano-4-hydroxy-cinnamic acid and trypsin of spectroscopic grade were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Standard protein tryptic digest of β -galactosidase from *E. coli* and peptide calibration standards were obtained from Applied Biosystems (Foster City, CA, USA).

2.2 Bacterial growth and culture conditions

The Antarctic psychotropic bacterium *P. syringae* Lz4W was routinely grown in Antarctic bacterial medium, consisting of bacto peptone 0.5% w/v and yeast extract 0.2% w/v at 22°C as described earlier [28]. This bacterium had the ability to grow between 0 and 30°C with an optimum temperature of ~22°C. The strain was grown to optical density 1.0 at 600 nm and harvested. It contains 1×10^8 cells per mL of culture. About 500 mg of cells were taken for the membrane preparations.

2.3 Preparation of bacterial membranes (subcellular fractionation of proteins)

The bacterial membrane was prepared as described earlier [26]. Briefly, the cells were treated with lysozyme 60 μ g/mL in the membrane buffer (10 mM Tris, pH 8.0, 0.75 M sucrose, 2 mM EDTA and 1 μ M PMSF) and sonicated for 2 min in a Branson sonifier. The unbroken cells and cell debris were removed by centrifugation at 8000 rpm for 10 min in a Sorvall centrifuge. The entire membrane fraction was pelleted by ultracentrifugation at 30000 rpm for 2 h at 4°C in a tabletop ultracentrifuge TL-100 (Beckman). The supernatant was used as cytoplasmic fraction of proteins. The pellet was solubilized with 2% Triton X-100 in the membrane buffer for 30 min at room temperature (25°C) and centrifuged at 30 000 rpm for 2 h at 4°C to isolate the inner membrane proteins. The outer membrane proteins remained in the pellet. The fraction was dissolved in the lysis buffer containing 8.5 M urea, 1.98 M thiourea and 2% CHAPS. The soluble proteins were separated by SDS-PAGE. Protein concentrations were estimated by performing protein dye binding assay using Bio-Rad protein assay reagents and bovine serum albumin as standard.

2.4 SDS-PAGE and in-gel digestion with trypsin

Membrane proteins of *P. syringae* Lz4W were separated on a 10–12% SDS-gel according to the Laemmli protocol [29]. Typically, about 100–150 μ g of protein reduced and alkylated, dissolved in SDS-PAGE loading buffer and boiled for 5 min was loaded on different wells in the 1-mm gel. The gel was stained with Coomassie Blue after electrophoresis. Following destaining the gel, the prominent bands were cut out and subjected to in-gel trypsin digestion, using the protocol described earlier with minor modifications [30]. Briefly, the gel pieces were further cut to the smallest possible size and the bands were treated with 50% of 25 mM ammonium bicarbonate and ACN. Subsequently, the gel pieces were treated with ACN and the solvent was dried up with the help of a SpeedVac concentrator. Depending on the size of the gel band, 10–20 μ L of trypsin (10 μ g/mL in ammonium bicarbonate) was added to the gel pieces and incubated at 37°C for 16–18 h. The resultant peptides were extracted with 5% TFA in 50% ACN. The peptides were dried on a SpeedVac concentrator and re-dissolved in 8 μ L 0.1% TFA in 50% ACN before spotting them on the MALDI plate. For LC-ESI experiments the solvent was evaporated and the peptides were dissolved in a suitable volume of 5% ACN containing 0.2% formic acid and loaded manually through a loop. The sample was mixed with solvent A used in LC for loading on the column. During loading, the concentration was ~3% ACN.

2.5 MALDI-TOF/TOF MS

The mass spectra of the trypsin-digested proteins were obtained with the help of 4800 MALDI TOF/TOF mass spectrometer from Applied Biosystems. Mass spectra were recorded in reflector mode. The matrix used was α -cyano-4-hydroxycinnamic acid (CHCA). The MS/MS spectra of individual peptides were recorded, with air as CID gas by using 1 keV energy. The peptides were selected based on their relative abundance. Proteins were identified using the GPS explorer software supplied by the manufacturer. This program uses MASCOT search tools for the identification of proteins using the National Center for Biotechnology Information (NCBI) database. MS/MS spectra were submitted for database searching of proteins from *Pseudomonas* sp. Using MS/MS data, several proteins could be identified. The sequences of the top-scoring peptides were selected. The protein hits with these sequences were identified. The peptide summary report essentially groups the peptide matches into protein hits and the protein score was derived by combining the ions scores for ranking the proteins. The highest-scoring proteins that contained one or more peptides were selected for identification. The MASCOT peptide score 33 was considered as significant ($p < 0.05$). The precursor ion mass tolerance and the MS/MS fragment ion tolerances were set to 0.2 and 0.25 Da, respectively.

2.6 LC-MS/MS of trypsin-digested proteins

All the MS and MS/MS experiments for peptide identification were performed using a Finnigan LTQ ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA). The instrument was operated with the X_calibur file to acquire a full MS scan in the mass range m/z 200–2000. Briefly, the tryptic peptides (10 μ L) obtained from each band of the SDS-gel were loaded manually onto a C-18 pre-column (from Applied Biosystems) connected to an RP column Biobasic C18 (100 mm \times 0.18 mm) with a pore size of 300 \AA and a particle size of 5 μ m. The flow rate was set at 3 μ L/min. The mobile phases A and B were 0.2% formic acid in water and 0.2% formic acid in 95% ACN, respectively. The gradient was started at 10 min and ramped to 60% B till 40 min and 100% B at 55 min and retained at 100% B till 65 min. The column was equilibrated with solvent A for 10 min before the next sample was injected. MS and MS/MS spectra were obtained at a heated capillary temperature of 200°C, and ESI voltage of 4 kV. The normalized collision energy was set at 35, which was known to be optimal for fragmenting peptides. MS/MS of the top seven peptides with a signal threshold above 500 counts was acquired with 30 m/s activation time, with a repeat duration of 30 s.

2.7 Identification of proteins of *P. syringae* Lz4W

LC-MS/MS profiles of the peptides from *P. syringae* Lz4W were used for searching the sequence similarities to data

from *Pseudomonas* sp. available in the NCBI database. For this purpose, the database of *P.seudomonas* sp. is downloaded from NCBI (<http://www.ncbi.nlm.nih.gov/>). The database includes 99 128 protein sequences (Updated up to July 2007). Most of the genes of *P. syringae* Lz4W show maximum identity with that of *P. syringae* pv. *tomato* DC 3000 and *P. fluorescence* Pf01 (Acc. No. NC_004578 and NC_007492) (M. K. Ray *et al.*, unpublished observations). The organisms mainly included in the database search, in addition to these are *P. putida* F1, *P. syringae* pv. *phaseolicola* 1448A, *P. fluorescence* pf- 5, *P. putida* KT 2440, *P. entomophila* L48, *P. syringae* pv. *syringae* B728a, *P. aeruginosa* 2192, *P. aeruginosa* PA01, *P. fragi*, *P. stutzeri*, *P. aeruginosa* UCB 1P-PA14, *P. putida* GB-1, *P. putida* W 619, *P. aeruginosa* PACS2, *P. aeruginosa* PA7 and *P. aeruginosa* C 3719. The database search was carried out using SEQUEST program Turbo-SEQUEST (Thermo Finnigan). The database was searched after setting the precursor ion and fragment ion mass tolerances at 1.4 and 1 Da, respectively. The MS/MS spectra of +1, +2 and +3 (multiply charged) peptides were searched against the database of *P.seudomonas* sp. The database search parameters included two missing cleavages by trypsin, oxidation of methionine and carbamidomethyl cysteine. The cross correlation (Xcorr) scores of singly, doubly and triply charged peptides greater than 1.8, 2.5 and 3.5, respectively, were fixed for identifying the proteins. A list of peptide sequences that had the highest Xcorr values was identified [31, 32]. Other parameters of $\Delta C_n > 0.1$ are selected for anticipated results in addition to Xcorr score. The values of ΔC_n obtained for a large number of peptides are very much higher than the selected criteria, suggesting that the sequences obtained are exact matches. In addition, a filter for the estimation of false positives was adapted. The manufacturer supplied the algorithm used for calculating the probability scores. A minimum value of P (pep) was set at 0.001. This setting allows a cut-off of less than 0.1% false positives. P (pro) is an extrapolation of protein probability. The procedure adapted in this method uses the highest P (pep) within each protein to be equivalent to P (pro). The peptides obtained from *P. syringae* Lz4W have picked up maximum homology hits with the proteins from different mesophilic *P.seudomonas* sp. The peptide identification results are integrated by DTA select to display the list of proteins identified over the whole fractions and the peptide lists belonging to each protein [33]. The final list of proteins is prepared after manual verification, removing the peptides that have not contributed to the sequence coverage.

Both the methods (MALDI and LC MS/MS) identified the same proteins. Sequences of some peptides were obtained from MALDI TOF/TOF MS and others were from LC-MS/MS for the same protein. Both the results were combined for final identification. Normally, the proteins identified exhibited maximum sequence similarity with the proteins of a particular *Pseudomonas* sp. This was shown along with the accession number and the name of the *P.seudomonas* sp. in the results.

2.8 GRAVY scores of proteins and peptides, subcellular localization of proteins

The grand average of hydrophathy (GRAVY) scores of proteins and peptides, a measure of hydrophobic and hydrophilic character of these molecules are computed, using the website (<http://www.expasy.org/tools/protparam.html>) and the values are calculated according to the method of Kyte Doolittle scale [34]. The sub-cellular localization of the proteins was carried out using a program PSORT b ver 2.0 (<http://www.psort.org>) by choosing Gram negative strain and normal output format that displays the results with final predictions and associated scores, as reported earlier [35]. Cytosolic proteins were identified clearly with good scores (8–9.5 on a scale of 10). The program used does not detect lipoprotein motifs. In general, this method helped in compartmentalizing the identified proteins.

3 Results

3.1 Isolation and separation of membrane proteins from the Antarctic *P. syringae* Lz4W by 1-D SDS-PAGE

The samples of inner and outer membrane proteins of the Antarctic bacterium *P. syringae* Lz4W were prepared as described in Section 2. The membrane proteins were pre-fractionated by Triton X-100 solubilization method and separated by SDS-PAGE. A representative gel picture is shown in Fig. 1. Proteins were resolved from high molecular weight to low molecular weight separating regions of the gel, which was good enough for subsequent analysis. The protein bands were cut out from the gel as shown in Fig. 1. The gel pieces containing bands were further digested with trypsin. The resultant peptides were then analyzed with the help of LC-MS/MS and MALDI TOF/TOF.

3.2 MS of the trypsin-digested membrane protein fractions using LC-coupled ESI MS/MS

In the present study, a combination of MALDI and ESI methods was used to enlarge the number of peptides obtained from trypsin digestion of various proteins. This enabled identification of the membrane proteins and also increased the sequence coverage.

LC-MS/MS, in combination with database searching, was an excellent strategy for identifying a large number of proteins in the mixture. Pursuing this strategy, the tryptic peptides of the proteins obtained from each gel band were separated using LC followed by peptide fragmentation accomplished by a mass spectrometer. Peptides belonging to different proteins were separated by reverse phase chromatography and the mass spectra were obtained simultaneously.

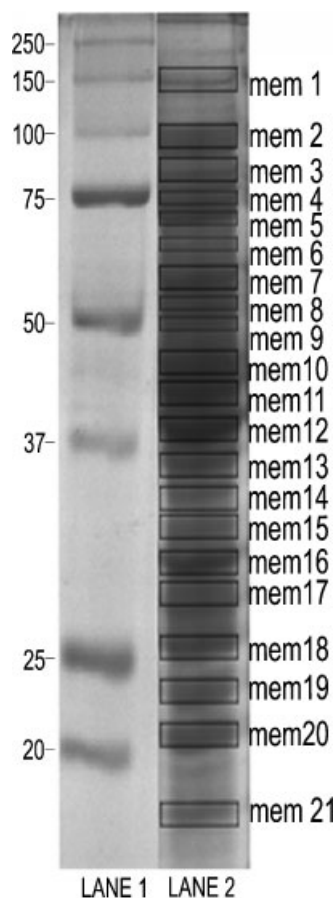


Figure 1. A representative gel picture of SDS-PAGE analysis of membrane proteins of an Antarctic bacterium *P. syringae* Lz4W. Membrane proteins are separated by SDS-PAGE and stained with CBB. Gel pieces are cut for trypsin digestion as shown in the Figure. Lane 1 shows the molecular weight markers 20–250 kDa and Lane 2 shows proteins from membrane preparations.

Supporting Information Fig. 1 shows the MS/MS profiles of tryptic peptides of a gel band, marker mem 16, from Fig. 1. The MS/MS spectra of the peptides obtained from the membrane proteins present in the band are shown. Supporting Information Fig. 1A–C shows the MS/MS spectra of different peptides derived from competence lipoprotein, the outer membrane protein OprF and cell division protein. Similarly, Supporting Information Fig. 2 shows the mass spectra of the tryptic peptides from another band (mem 1) from the gel. The MS/MS spectra of different peptides from the organic solvent tolerance protein are shown in Supporting Information Fig. 2A–D. Thus, from a single band of the gel several proteins are identified.

3.3 MS of trypsin-digested membrane protein fractions using MALDI-CID MS

In addition to LC-ESI MS/MS analysis, MALDI TOF mass spectra were also recorded for all the digests of the gel bands. Supporting Information Fig. 3A shows the peptide

mass fingerprint of gel band mem 4 that consisted of ABC transporter-like protein. The resultant peptides of the protein were fragmented by CID to obtain the sequence of these peptides. The sequences of the peptides were determined using mainly the y-type, b-type ion fragments by submitting the mass spectrum to the databases (Supporting Information Fig. 3). The immonium ions formed during fragmentation with MALDI TOF/TOF were also used to identify the amino acids potentially present in the sequences in these peptides (Supporting Information Fig. 3B and C).

3.4 Identification of membrane proteins: a case study with *P. syringae* Lz4W

The proteins of *P. syringae* Lz4w are identified with the help of LC-ESI MS/MS. Even though the genome sequence of

this species is not known, the proteins of *P. syringae* Lz4W are identified using the known genome sequence database of different *Pseudomonas* sp. at NCBI (the same used for Sequest). Detecting the sequences of many peptides is very useful in identifying the proteins from the bacteria of unknown genomes, such as those of the Antarctic *P. syringae* strain. Thus, a combination of MALDI CID MS and LC ESI MS/MS has helped in enlarging the sequences of a number of peptides. This, in turn, has helped in increasing the number of the proteins identified from the bacterium. Supporting Information Fig. 4 shows, as an example, the sequence of the ABC transporter protein along with the sequence coverage of the protein using a combination of MALDI and ESI methods.

Table 1 presents the membrane proteins and membrane-associated proteins that were identified from *P. syringae* Lz4W. Altogether 294 proteins were identified in this study using this method. Proteins of the Antarctic

Table 1. Membrane proteins of *P. syringae* Lz4W identified by a combination of MALDI and ESI MS

S No.	Name of the protein identified ^{a)}	Accession number	Molecular weight/pI	Number of residues (sequence span)/ sequence coverage %	Species name ^{b)}
1	Ribonuclease E and G	gi 77384390	118 889.9/4.25	1082 (9–681)/21.07	<i>P. fluorescens</i> pf0-1
2	RNA helicase rhIE	gi63254432	67 992.6/10.53	629 (140–236)/5.56	<i>P. syringae</i> pv. <i>syringae</i> B728a
3	Molecular chaperone DnaK	gi 70734322	68 434.3/4.68	638 (35–635)/22.88	<i>P. fluorescences</i> Pf-5
4	Ribonuclease R	gi 70734079	97 416.5/8.77	875 (298–5887)/6.63	<i>P. fluorescences</i> Pf-5
5	ABC transporter-like protein	gi 77460103	70 388.0/5.12	640 (211–616)/11.25	<i>P. fluorescens</i> pf0-1
6	Aminopeptidase N	gi 70730645	98 688.2/4.97	885 (273–43)/5.19	<i>P. fluorescences</i> Pf-5
7	Cell division protein FtsZ	gi 70732379	41 642.5/4.76	397 (176–259)/5.79	<i>P. fluorescences</i> Pf-5
8	Outer membrane porin	gi 77459840	48 290.6/5.02	441 (315–59)/10.20	<i>P. fluorescens</i> pf0-1
9	ABC transporter, ATP-binding protein	gi 71735836	59 007.8/4.93	529 (46–523)/10.39	<i>P. syringae</i> pv. <i>phaseolica</i> 1448A
10	Peptidase M41, FtsH	gi 82737622	69 319.5/5.44	634 (213–599)/7.1	<i>P. putida</i> F1
11	Succinate dehydrogenase	gi 71737800	63 314.7/5.81	590 (127–493)/13.55	<i>P. syringae</i> pv. <i>phaseolica</i> 1448A
12	PpiC-type peptidyl-prolyl <i>cis-trans</i> isomerase	gi 70731749	25 113/5.25	237 (35–192)/22.36	<i>P. fluorescences</i> Pf-5
13	TonB-dependent copper receptor	gi 70734150	76 754.1/6.05	710 (295–618)/13.8	<i>P. fluorescences</i> Pf-5
14	Ttgc	gi 5091481	52 816/5.29	484 (377–392)/3	<i>P. putida</i>
15	Peptidoglycan associated lipoprotein OprL precursor	gi 70732082	17 730.9/5.70	165 (60–142)/40.6	<i>P. fluorescences</i> Pf-5
16	Outer membrane protein OprF	gi 70729258	34 546.3/5.68	325 (88–227)/20.92	<i>P. fluorescences</i> Pf-5
17	Outer membrane ferric enterobactin receptor	gi 26988966	81 029.1/5.03	744 (171–511)/4.17	<i>P. putida</i> KT2440
18	Outer membrane protein	gi 84325654	86 582.7/4.85	781 (55–195)/2.56	<i>P. aeruginosa</i> 2192
19	Organic solvent tolerance protein	gi 77461353	105 537.9/5.44	937 (277–873)/6.51	<i>P. fluorescens</i> pf0-1
20	SecA protein	gi 82739998	105 945/5.45	939 (230–764)/5.22	<i>P. putida</i> F1
21	Bacterial surface antigen family protein	gi 26990088	86 461.4/4.97	787 (115–297)/4.32	<i>P. putida</i> KT2440
22	Aconitate hydratase	gi 28870907	93 643.6/4.97	866 (36–842)/28.18	<i>P. syringae</i> pv. <i>tomato</i> str. DC3000
23	Protein export protein SecD	gi 77460840	67 330.1/9.61	623 (97–255)/5.46	<i>P. fluorescens</i> pf0-1
24	Ubiquinol-cytochrome c reductase, cytochrome b	gi 70732402	45 886.1/7.91	403 (172–301)/7.69	<i>P. fluorescences</i> Pf-5
25	YjK	gi 32492877	34 758.9/5.07	311 (35–251)/19.94	<i>P. putida</i>
26	Outer membrane porin, OprD family	gi 70733819	46 157.0/6.16	427 (57–106)/10.07	<i>P. fluorescences</i> Pf-5
27	Acetolactate synthase III large subunit	gi 70732564	62 792.8/6.50	574 (1–559)/18.29	<i>P. fluorescences</i> Pf-5
28	Outer membrane porin	gi 77461638	48 167.9/5.72	447 (142–361)/3.80	<i>P. fluorescens</i> pf0-1
29	DNA gyrase subunit B	gi 26986758	89 975.9/5.57	806 (180–538)/6.82	<i>P. putida</i> KT2440
30	Outer membrane receptor proteins, mostly Fe transport	gi 84325518	78 737.7/5.90	719 (143–182)/5.56	<i>P. aeruginosa</i> 2192

Table 1. Continued.

S No.	Name of the protein identified ^{a)}	Accession number	Molecular weight/pI	Number of residues (sequence span)/ sequence coverage %	Species name ^{b)}
31	Cell division protein ZipA	gi 77458027	31 809.1/5.39	288 (158–273)/11.11	<i>P. fluorescens</i> pf0-1
32	Flagellin	gi9843801	51 055.7/5.09	503 (13–485)/27.44	<i>P. fluorescens</i> pf0-1
33	Outer membrane protein, OMP85 family	gi 70728566	87 392.4/5.07	796 (68–385)/11.68	<i>P. fluorescences</i> Pf-5
34	RND efflux system, outer membrane lipoprotein, NodT	gi 82738521	52 817.0/5.15	484 (377–435)/6.20	<i>P. putida</i> F1
35	Putative copper transport outer membrane porin OprC precursor	gi 15598985	79 254.9/6.00	723 (104–669)/8.85	<i>P. aeruginosa</i> PAO1
36	Type I efflux secretion system outer membrane protein, TolC	gi 104783903	52 853.0/5.62	478 (175–431)/17.15	<i>P. entomophila</i> L48
37	Outer membrane protein	gi 37519362	52 975.2/5.61	486 (310–334)/5.14	<i>P. fluorescens</i>
38	Porin B	gi 70731955	49 878.4/5.60	449 (50–350)/13.81	<i>P. fluorescences</i> Pf-5
39	CztO	gi 12484569	47 975.5/5.91	438 (312–333)/5.02	<i>P. fluorescens</i>
40	Putative OprD protein	gi 76556470	38 076.4/4.95	353 (119–257)/15.29	<i>P. fluorescens</i>
41	Translocation protein TolB precursor	gi 70732083	4703.3/9.52	433 (65–359)/9.70	<i>P. fluorescens</i> Pf-5
42	Amino acid ABC transporter, periplasmic amino acid-binding protein	gi 26987807	33 407/8.61	306 (36–217)/16.01	<i>P. putida</i> KT2440
43	OmpA family protein	gi 71734248	28 381.0/9.36	260 (241–256)/6.15	<i>P. syringae</i> pv. <i>phaseolicola</i> 1448A
44	Lipoprotein releasing system trans membrane protein LolE	gi 28869314	45 976.1/7.90	427 (247–278)/7.25	<i>P. syringae</i> pv. <i>tomato</i> str. DC3000
45	OmpW	gi 77461250	24 723.6/6.22	232 (32–232)/21.55	<i>P. fluorescens</i> pf0-1
46	Outer membrane protein, OprE1	gi 56291847	48 846.2/8.65	454 (439–454)/3.52	<i>P. fluorescens</i>
47	Peptidase aspartic, active site	gi 66044282	72 552.9/9.11	654 (349–372)/3.67	<i>P. syringae</i> pv. <i>syringae</i> B728a
48	Phosphotransferase system, fructose-specific IIBC component	gi 71737234	59 985.2/6.68	580 (149–175)/2.93	<i>P. syringae</i> pv. <i>phaseolicola</i> 1448A
49	Long-chain fatty acid transporter, putative	gi 26988421	45 692.1/5.44	421 (44–62)/4.51	<i>P. putida</i> KT2440
50	Porin D	gi 70732098	48 459.6/5.48	446 (146–411)/8.52	<i>P. fluorescens</i> Pf-5
51	Porin-like protein	gi 5712720	26 717.9/7.89	249 (96–105)/4.02	<i>Pseudomonas</i> sp. sp. BG33R
52	D-Alanyl-D-alanine carboxypeptidase	gi 82738224	44 367.6/6.57	403 (292–301)/2.48	<i>P. putida</i> F1
53	Binding-protein-dependent transport systems inner membrane component	gi 77460589	30 503.2/9.49	281 (268–281)/4.62	<i>P. fluorescens</i> pf0-1
54	Putrescine ABC transporter, periplasmic putrescine-binding protein	gi 28872419	39 617.4/5.64	365 (53–279)/12.05	<i>P. syringae</i> pv. <i>tomato</i> str. DC3000
55	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase	gi 70729131	36 518.3/6.71	341 (256–325)/9.09	<i>P. fluorescens</i> Pf-5
56	Extracellular solute-binding protein, family 1g	gi 77461626	40 375.9/8.03	370 (58–92)/8.11	<i>P. fluorescens</i> Pf0-1
57	Sulfate-binding protein	gi 28867539	37 104.2/7.62	338 (81–338)/9.47	<i>P. syringae</i> pv. <i>tomato</i> str. DC3000
58	Periplasmic thiosulfate-binding protein	gi 104780824	36 725.8/7.59	332 (108–306)/15.56	<i>P. entomophila</i> L48
59	PotF1	gi 13194612	40 054.5/8.05	370 (68–284)/14.86	<i>P. fluorescens</i>
60	Sulfate ABC transporter, periplasmic sulfate-binding protein	gi 71736716	36 708.7/5.71	332 (108–306)/15.66	<i>P. syringae</i> pv. <i>phaseolicola</i> 1448A
61	Antibiotic efflux pump outer membrane protein arpC precursor	gi 71152257	52 834.1/5.29	484 (377–435)/6.61	<i>P. putida</i>
62	Probable outer membrane protein precursor	gi 15600167	53 374.4/5.73	482 (365–409)/4.56	<i>P. aeruginosa</i> PAO1
63	Outer membrane porin, OprD family	gi 70732530	48 861.1/5.42	442 (316–360)/10.18	<i>P. fluorescens</i> Pf-5
64	Periplasmic or inner membrane associated protein	gi 929793	32 670.3/9.43	296 (142–158)/5.74	<i>P. fluorescens</i>
65	Thrombospondin type 3 repeat: OmpA/MotB:OmpF	gi 66045337	36 597.8/4.55	344 (232–246)/14.36	<i>P. syringae</i> B728a
66	NlpB/DapX lipoprotein	gi 70728837	40 475.5/4.86	371 (275–371)/6.74	<i>P. fluorescens</i> Pf-5
67	Lipoprotein	gi 104780119	20 685.7/9.30	194 (150–181)/22.68	<i>P. entomophila</i> L48

Table 1. Continued.

S No.	Name of the protein identified ^{a)}	Accession number	Molecular weight/pI	Number of residues (sequence span)/ sequence coverage %	Species name ^{b)}
68	ABC transporter, ATP-binding protein	gi 71734643	26 489.0/6.34	241 (78–241)/9.96	<i>P. syringae</i> pv. <i>phaseolicola</i> 1448A
69	Lipoprotein, putative	gi 71735571	20 372.7/9.95	193 (157–180)/12.44	<i>P. syringae</i> pv. <i>phaseolicola</i> 1448A
70	Outer membrane protein and related peptidoglycan-associated	gi 84322559	17 654.9/6.38	166 (61–143)/31.33	<i>P. aeruginosa</i> 2192
71	Major outer membrane lipoprotein I	gi 3201884	8823.5/6.79	83 (31–72)/49.78	<i>P. fluorescens</i>
72	OmpA/MotB	gi 77460622	17 708.8/5.01	165 (60–117)/29.09	<i>P. fluorescens</i> pf0-1
73	Outer membrane lipoprotein OprI	gi 77460232	8778.5/8.66	83 (26–72)/38.55	<i>P. fluorescens</i> pf0-1
74	Outer membrane protein, OmpW family	gi 70732833	25 012.4/6.28	232 (193–232)/22.41	<i>P. fluorescens</i> Pf-5
75	Sulfate ABC transporter, ATP-binding protein CysA	gi 70733701	37 009.2/5.92	329 (218–242)/9.12	<i>P. fluorescens</i> Pf-5
76	Glutamate/aspartate ABC transporter, periplasmic binding protein	gi 104780401	33 271.3/8.89	305 (198–214)/5.57	<i>P. entomophila</i> L48
77	Thioredoxin-disulfide reductase	gi 71736485	33 847.0/5.10	320 (119–222)/10	<i>P. syringae</i> pv. <i>phaseolicola</i> 1448A
78	Oligopeptide/dipeptide ABC transporter, ATP-binding protein-like	gi 77457036	35 953.4/8.88	326 (59–107)/9.20	<i>P. fluorescens</i> pf0-1
79	Putative permease of ABC sugar transporter	gi 116051177	30 720.5/9.24	281 (269–281)/4.62	<i>P. aeruginosa</i> UCBPP-PA14
80	Translocation protein TolB precursor	gi 70732083	47 503.3/9.52	433 (125–432)/9.70	<i>P. fluorescens</i> Pf-5
81	Translocation protein TolB	gi 77460623	45 481.3/9.15	414 (146–187)/7.72	<i>P. fluorescens</i> pf0-1
82	Periplasmic glucan biosynthesis protein Mdo G	gi 70733917	67 777/6.86	610 (60–343)/3.0	<i>P. fluorescens</i> Pf-5
83	Glutaminase-asparaginase	gi 632685	36 121/5.63	337 (156–169)/4.0	<i>Pseudomonas</i> , 7A
84	Imipenem outer membrane porin D precursor	gi 15596155	48 331/4.96	443 (48–53)/1.0	<i>P. aeruginosa</i> PA 1
85	Ubiquinol-cytochrome C reductase, iron-sulfur subunit	gi 77460913	21 005/7.68	197 (57–76)/10.0	<i>P. fluorescens</i> pf0-1
86	Dipeptide ABC transporter, periplasmic peptide-binding protein	gi 70728264	60 287/8.03	541 (228–251)/4.0	<i>P. fluorescens</i> Pf-5
87	Outer membrane efflux protein	gi 70734041	52 613/5.86	476 (274–429)/13.0	<i>P. fluorescens</i> Pf-5
88	Outer membrane protein Opr E3	gi 104779547	48 038/5.78	449 (29–41)/2.0	<i>P. entomophila</i> L48
89	Two-component regulatory protein lemA	gi 281611	101 694.5/5.32	929 (467–481)/1.61	<i>P. syringae</i>
90	Probable outer membrane protein presursor	gi 15598844	88 233/5.05	797 (68–126)/2.0	<i>P. aeruginosa</i> PA01
91	Malic enzyme, NAD-binding	gi 77456631	48 220.0/5.62	450 (91–437)/17.11	<i>P. fluorescens</i> pf0-1
92	Extracellular solute-binding protein, family 1	gi 66046612	37 706.3/5.65	341 (117–315)/15.25	<i>P. syringae</i> pv. <i>syringae</i> B728a
93	Signal recognition particle protein Ffh	gi 28868679	49 320.1/10.02	458 (36–342)/8.95	<i>P. syringae</i> pv. <i>tomato</i> str. DC3000
94	NAD-specific glutamate dehydrogenase	gi 71735459	181 999.6/5.45	1619 (118–1286)/5.49	<i>P. syringae</i> pv. <i>phaseolica</i> 1448A
95	OstA	gi 55833083	46 823/5.58	429 (104–297)/11.42	<i>P. fluorescens</i>
96	Putrescine ABC transporter, ATP-binding protein	gi 26991855	42 445.8/6.10	380 (173–201)/6.84	<i>P. putida</i> KT 2440
97	ATP synthase subunit C	gi 70733500	31 419.3/8.13	286 (213–286)/16.08	<i>P. fluorescens</i> Pf-5
98	ABC type amino acid transport protein	gi 23469844	28 419/6.07	251 (57–231)/15.93	<i>P. syringae</i> pv. <i>syringae</i> B728a

a) Membrane proteins, membrane-associated proteins, proteins consist of multiple localization sites and lipoproteins, which were included.

b) The proteins of *P. syringae* Lz4W identified have maximum sequence similarity with the other *Pseudomonas* sp which were shown.

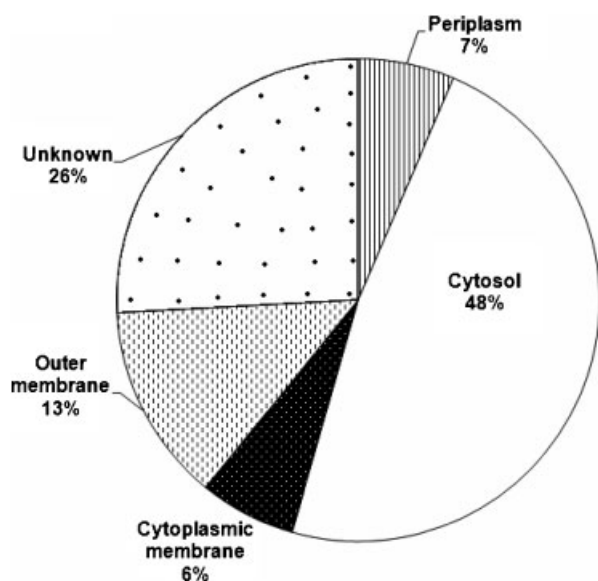


Figure 2. The predicted sub-cellular localization of different proteins identified using a program PSORT b Ver 2.0 from an Antarctic bacterium *P. syringae* Lz4W.

bacterium *P. syringae* Lz 4W had maximum sequence similarity with proteins of the other *Pseudomonas* strains as shown in Table 1 and Supporting Information Table 1. MS/MS sequence analysis of various peptides indicated oxidation of methionine residues and missed cleavages numbering up to two in the digests. In some proteins, both methionine oxidized and unoxidized peptides were identified, suggesting that the oxidation was partial as it happened during boiling of the samples for loading on SDS-PAGE gels and also during trypsin digestion. The identification of methionine and oxidized methionine-containing peptide sequences afforded an additional aspect for the correct identification of these peptides. The peptides not contributing to the sequence coverage of the proteins are excluded from Table 1 and Supporting Information Table 1.

3.5 Assigning cellular localization of the proteins

An attempt was also made to assign the cellular localization of the proteins that were identified in this study (Fig. 2). Membrane and membrane-associated proteins, and cytosolic proteins constituted 26 and 48% of the total identified proteins, respectively. However, the location could not be assigned for the remaining 26% of proteins. These included proteins with multiple localization sites such as cytoplasmic membrane, outer membrane, etc. The occurrence of non-membrane proteins in the membrane fractions obtained by different methodologies was a very commonly encountered problem as was observed in other bacterial species [2, 11, 36]. Nonetheless, the bioinformatic tools helped in identifying the cellular localization based on the physicochemical properties of the proteins.

4 Discussion

4.1 Identification of proteins from the membrane fractions of *P. syringae* Lz4W

One of the objectives of the current investigation was to ionize and detect as many peptides as possible from the membrane protein fractions of the Antarctic bacterium *P. syringae* Lz4W. Previous studies had demonstrated that SDS and methanol-assisted protein solubilization and digestion methods greatly helped in the identification of a large number of membrane proteins [36]. Some other studies used detergents in the analysis to enlarge protein identification [37]. In the current study, a combination of MALDI and LC-coupled ESI ionization methods were used to increase the number of peptides in the identification of membrane proteins of an Antarctic bacterium *P. syringae* Lz4W. Using these procedures, 294 proteins were identified, including 98 membrane proteins and membrane-associated proteins (Supporting Information Table 1, Table 1).

The method used here has, in general, facilitated the identification of membrane proteins. The algorithm used for sub-cellular localization predicted 26% of them as unknown. These include proteins with multiple localization sites. Many proteins such as integral membrane proteins existing at multiple localization sites, with large periplasmic domains, trans-membrane domains, etc. are displayed with localization as unknown. These results were analyzed further by examining the distribution of localization scores displayed in the output. It is difficult to classify peripheral membrane proteins by bioinformatic methods. They help in general classification of proteins. Hence, the proteins identified may be regarded as either part of membrane proteome or proteins associated with membranes.

Several proteins reported here such as molecular chaperone Dna K, transcription factor Rho, aspartate ammonia-lyase and other proteins identified with a large number of peptides and spanning almost the complete sequence indicate that the methodologies adopted in this study are sufficient to identify proteins even from the bacterial species with unknown genome sequence. The typical sequence coverage of some proteins is 42% using the MS/MS sequences of peptides. The sequences of some peptides are obtained by both MALDI and ESI ionization methods and that helped in accurate identification of the proteins. The GRAVY scores obtained for various peptides identified in this study indicate that a large number of hydrophobic peptides, ~44%, are detected from the proteins reported in Table 1 using different ionization methods. After identifying the proteins by MS/MS sequencing some more peptides (PMF) corresponding to these proteins could be detected by manual verification by both MALDI-MS and ESI MS for several proteins (data not shown).

4.2 Significance of the identified proteins in cold adaptation *P. syringae* Lz4W

In the current study, some heat shock proteins and chaperones have been identified in the membrane fraction. This observation is consistent with the previous studies [38] that showed that chaperones played an important role in cold adaptation. Several chaperones and heat shock proteins are identified from the Antarctic strain of *P. syringae* Lz4W suggesting a high conservation of the protein function in psychrophilic bacteria.

Endoribonuclease (R Nase E), a key enzyme and a member of degradosome (a protein complex) involved in RNA decay and processing, was identified in the Antarctic *P. syringae* Lz4W [39]. R Nase E had been found in the membrane preparations in the present study, suggesting an association of degradosome with the membranes in the Antarctic bacterium. Interestingly, the other proteins associated with R Nase E in the degradosome complex, such as R Nase R and RhlE were also identified in the membrane preparation of *P. syringae*. These proteins were earlier identified with the help of N-terminal analysis by Edman degradation method [39]. This result was probably not surprising since, the R Nase E of mesophilic *E. coli* was earlier shown to be associated with membranes [40]. It is likely that degradosome protein works in membrane-associated compartments of the bacterial cell.

The present study has helped to identify the sequences of a large number of hydrophilic and hydrophobic peptides that formed part of membrane proteins of the Antarctic bacterium *P. syringae* Lz4W. Moreover, this study highlights the identification of proteins even in a bacterial species with unknown genome sequence. The strategies of using a combination of two different ionization methods adopted in this study are very useful in increasing the sequence coverage of several membrane proteins. These studies also helped in compartmentalization of the proteins inside the bacterial cell. This can contribute significantly to the basic understanding of the functional significance of the proteins.

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