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Quantitative analysis of cell surface membrane proteins using membrane-impermeable chemical probe coupled with ^{18}O labeling

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Abstract

We report a mass spectrometry-based strategy for quantitative analysis of cell surface membrane proteome changes. The strategy includes enrichment of surface membrane proteins using a membrane-impermeable chemical probe followed by stable isotope ^{18}O labeling and LC-MS analysis. We applied this strategy for enriching membrane proteins expressed by *Shewanella oneidensis* MR-1, a gram-negative bacterium with known metal-reduction capability via extracellular electron transfer between outer membrane proteins and extracellular electron receptors. LC/MS/MS analysis resulted in the identification of about 400 proteins with 79% of them being predicted to be membrane localized. Quantitative aspects of the membrane enrichment were shown by peptide level ^{16}O and ^{18}O labeling of proteins from wild-type and mutant cells (generated from deletion of a type II secretion protein, GspD) prior to LC-MS analysis. Using a chemical probe labeled pure protein as an internal standard for normalization, the quantitative data revealed reduced abundances in ΔgspD mutant cells of many outer membrane proteins including the outer membrane *c*-type cytochromes OmcA and MtrC, in agreement with previously investigation demonstrating that these proteins are substrates of the type II secretion system.

Keywords

cell surface proteins; membrane proteome; ^{18}O labeling; membrane-impermeable chemical probe; LC-MS

Introduction

Cell surface membrane proteins are essential for maintaining normal biological functions in both prokaryotic and eukaryotic cells, and often initiate the first responses to environmental stimuli. In spite of the biological significance of these surface membrane proteins, they present an analytical challenge for mass spectrometry (MS)-based proteomics because of their naturally low abundances and insolubility in aqueous solutions. Sub-cellular fractionation performed by either density gradient centrifugation^{1, 2} or differential centrifugation^{3, 4} has been widely used to separate proteins associated with various cellular compartments. While membrane proteins have been efficiently enriched using sub-cellular fractionation with the aid of detergents or organic solvents to extract hydrophobic membrane

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proteins,⁵ major drawbacks include cross-contamination and time-consuming sample preparation. More recently, 1D/2D SDS-PAGE,⁶⁻⁹ capillary electrophoresis,¹⁰⁻¹² and ion chromatography¹³⁻¹⁶ have been used to separate and detect membrane proteins from the whole cell proteome. Efficient separation can be achieved on the basis of one or two dimensional properties of size, mobility, isoelectric focusing point, and pKa values of cellular proteins; however, protein precipitation and poor reproducibility were encountered when these techniques were applied for membrane protein enrichment.¹⁷⁻²⁰

Aiming to characterize a certain subset of the proteome by specifically labeling and enriching the target proteins using a chemical probe, so called chemical proteomics,^{21, 22} has been developed during the studies of drug discovery^{23, 24}, post-translational modifications^{25, 26} and enzyme activities.^{27, 28} Specific enrichment of a sub-proteome not only decreases the complexity, but also enhances the detection of low- abundance proteins. The design of chemical probes usually consists of three components, the reactive group, the linker and the affinity tag. Enrichment of target proteins is achieved by specific reactions between chemical probes and target proteins followed by affinity enrichment using the affinity tag in the chemical probe. For example, biotinylation of extracellular lysine residues coupled with MS-based proteomics has proved effective for enriching and identifying cell membrane proteins.²⁹⁻³¹ More recently, this strategy has been successfully applied to identify and quantify cell surface glycoproteins.^{32, 33} Although a relatively high specificity of membrane protein enrichment was demonstrated using this biotinylation chemical probe strategy, accurate quantification of enriched membrane proteins is still challenged by low protein recovery and large experimental variations during affinity enrichment and MS-based analysis.

High-performance MS coupled with stable isotope labeling has increasingly become a popular strategy for quantitative proteomics. Stable isotope labeling methods include metabolic labeling (SILAC),³⁴ chemical labeling on specific functional groups using reagents such as ICAT,³⁵ and enzymatic transfer of ¹⁸O from water to the C-terminus of peptide (¹⁸O labeling).^{36, 37} With ¹⁶O/¹⁸O labeling, paired peptide samples are labeled with either H₂¹⁶O or H₂¹⁸O via a trypsin-catalyzed oxygen exchange reaction. The oxygen atom (either ¹⁶O or ¹⁸O) from water is incorporated into the C-terminus in each tryptic peptide, thus providing an isotopic tag for relative quantification.³⁸

In this study, we demonstrated a quantitative proteomic strategy for measuring the relative abundance changes of membrane proteins. The strategy specifically enriches membrane proteins, using a membrane-impermeable chemical probe followed by ¹⁶O/¹⁸O labeling and then identifies and quantifies the enriched proteins using the accurate mass and time (AMT) tag approach^{39, 40}. To further improve LC-MS reproducibility and accuracy of quantification, a chemical probe-labeled pure protein is used as an internal standard for normalization. We applied the strategy to investigate membrane proteome changes in the gram-negative bacterium *Shewanella oneidensis* MR-1 in which membrane proteins play a critical role in mediating extracellular electron transfers.⁴¹⁻⁴³ Gaining insight into protein changes under different cellular conditions has important implications for biogeochemical cycling of metals, biotransformation of contaminants, and current generation in microbial fuel cells.

Our study involved both wild type *Shewanella* and a *gspD* deletion (Δ *gspD*) mutant. As a key component of the bacterial type II secretion system (T2SS), GspD is required for or implicated in translocating the outer membrane proteins MtrC (SO1778), OmcA (SO1779), DmsA (SO1429) and DmsB (SO1430) across the bacterial outer membrane.^{44, 45} Our results revealed that *gspD* deletion significantly altered abundances of a group of membrane proteins specifically within the cell membrane envelop, including the outer membrane

proteins MtrC, OmcA, DmsA, and DmsB, which is in agreement with previous observations^{44, 45}.

Experimental Procedures

Cell Culture

Generation of $\Delta gspD$ mutant cells has been described elsewhere.⁴⁶ Briefly, starter cultures of wild type and a $\Delta gspD$ mutant of *Shewanella oneidensis* MR-1 cells were generated by transferring a single colony to 5 ml Luria-Bertani (LB) broth and then incubating 8 h at 30 °C with rotary shaking (150 rpm). An aliquot of 1 ml of the starter culture was innoculated into 40 ml M1 minimal medium⁴⁷ supplemented with 20 mM lactate in a sealed serum bottle and incubated on a rotary shaker at 150 rpm at 30 °C overnight. The cells were harvested at mid-log phase ($OD_{600} = 0.6$) by centrifuging at $3000 \times g$ at 4 °C for 20 min.

Chemical probe labeling and cell lysis

Sulfo-NHS-SS-Biotin (Pierce, Rockford, IL) labeling was performed according to the manufacturer's instructions. Briefly, the cells were pelleted at $3000 \times g$ at 4 °C for 10 min, and then washed twice in 40 ml ice-chilled PBS buffer (150 mM sodium phosphate, 100 mM NaCl, pH 7.5) and resuspended in 20 ml PBS. A fresh aliquot of sulfo-NHS-SS-Biotin chemical probe dissolved in 1 ml PBS buffer was added to the cell suspension to a final concentration of 0.5 mM and then allowed to interact with the sample by gentle shaking for 30 min on ice, after which 1 ml 1 M Tris buffer (pH 7.5) was added to quench the reaction. The chemical probe labeled sample was washed with 1 ml 50 mM Tris buffer (pH 7.5) three times to remove excess chemical probe, as well as proteins not associated with cell membrane. The cell pellet was resuspended in 1.3 ml radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) that contained a 1:100 dilution of protease inhibitor cocktail (Pierce, Rockford, IL) and then lysed by conducting 10 cycles of pressurization in a NEP3229 Barocycler (Pressure Biosciences, West Bridgewater, MA). Each cycle consisted of 20 s exposure to 35,000 psi followed by 10 s exposure to ambient pressure. The cell debris was removed from the sample via centrifugation at $16,000 \times g$ at 4 °C for 60 min. The protein concentration of the supernatant was estimated by using the BCA protein assay (Pierce Rockford, IL). To characterize the nonspecific binding during the affinity enrichment, control experiments were performed in parallel, following the same procedure as detailed above, but without chemical probe labeling.

Enrichment of labeled protein and enzymatic digestion

The cell lysate was gently shaken with NeutrAvidin Agrose (Pierce, Rockford, IL) at room temperature for 1 h, after which the avidin beads were washed three times using RIPA buffer to remove unbound proteins. The labeled proteins were eluted from the beads by incubating with 200 μ l elution buffer (10 mM DTT, 6 M Urea) for 1 h. The eluted proteins were denatured at 60 °C for 30 min and then diluted 10 fold using 100 mM ammonium bicarbonate solution. 10 μ g sequencing grade modified porcine trypsin (Promega, Madison, WI) was added to digest labeled proteins overnight at 37 °C. The digested samples were loaded onto a 1-ml SPE C18 column (Supelco, Bellefonte, PA) and washed with 4 ml of 0.1% trifluoroacetic acid (TFA)/5% acetonitrile (ACN). Peptides were eluted from the SPE column with 1 ml of 0.1% TFA/80% ACN and then lyophilized. The resulting peptide samples were reconstituted in 25 mM ammonium bicarbonate, and residual trypsin activity was quenched by boiling the samples for 10 min and immediately placing the samples on ice for 30 min. Equal amounts (as assessed by BCA protein assay) of wild type and $\Delta gspD$ mutant peptide samples were analyzed using LC-MS, using the same LC column for each sample type.

Internal standard preparation

Bovine serum albumin (BSA) was biotinylated with 1 mM Sulfo-NHS-SS-Biotin in PBS buffer at room temperature for 10 min. The labeled BSA was purified using a 3-kDa mass filter spin column (Millipore, Billerica, MA), and the unreacted chemical probe was removed by washing three times with PBS buffer. Equal amounts of biotinylated BSA were spiked into cell lysates of both wild type and $\Delta gspD$ mutant MR-1 samples to serve as an internal standard.

Trypsin-catalyzed ^{16}O and ^{18}O labeling

Trypsin-catalyzed ^{16}O and ^{18}O -labeling was performed for wild-type and mutant cells, respectively, as previously described.³⁷ After residual trypsin activity was quenched, the digested peptide sample was lyophilized to dryness and initially reconstituted in 100 μL of 50 mM NH_4HCO_3 and 10 mM CaCl_2 in either ^{18}O -enriched water (95%, ISOTEC, Miamisburg, OH) or ^{16}O water. Sequencing grade modified porcine trypsin (Promega, Madison, WI) was added in a 1:50 trypsin:peptide ratio to the digests and allowed to mix continuously for 5 h at 37 °C. After labeling, the sample was acidified by adding 5 μL of formic acid. The labeled sample was lyophilized and reconstituted in 25 mM ammonium bicarbonate, and the peptide concentration was measured using a BCA assay. Equal amount of peptides from wild type and $\Delta gspD$ mutant samples were mixed and subjected to LC/MS/MS analysis.

In addition to quantifying ^{18}O labeled enriched membrane protein samples, the whole cell lysate proteomes for wild-type and mutant cells were also quantified using the same ^{18}O labeling strategy.

LC-MS/(MS) analysis

Peptide samples were analyzed using a fully automated, custom-built, four-column capillary LC system coupled online using an in-house manufactured ESI interface⁴⁸ to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The capillary columns were made by slurry packing 3 μm Jupiter C18 bonded particles (Phenomenex, Torrance, CA) at 8000 psi into a 70-cm long, 75- μm i.d. fused-silica capillary (Polymicro Technologies, Phoenix, AZ). Mobile phase A consisted of 0.2% acetic acid and 0.05% formic acid in water and mobile phase B consisted of 0.1% formic acid in 90% acetonitrile/10% water. 5 μL aliquots of each peptide sample were injected onto the reversed-phase column for LC-MS analysis. Mobile phase A was maintained at 100% for 20 min after which a nonlinear exponential gradient elution was generated using a 10 ml stainless steel mixing chamber to increase the composition of the mobile phase to 80% B over 100 min. High mass accuracy spectra were collected via an orbit-trap analyzer, and the six most intensive peaks in the previous MS spectrum were selected for MS/MS in the linear ion trap. Three technical replicates were analyzed for each experimental sample to improve the coverage of peptide and protein identifications.

Data Analysis

Peptides were identified using the SEQUEST algorithm to search the whole genome database of *Shewanella oneidensis* MR-1 in combination with the peptide sequence of BSA. Because the chemical probe specifically reacts with primary amines, dynamic modification on lysine with the remainder of the chemical probe after DTT cleavage was used as a search parameter during SEQUEST analysis. Identified peptides were filtered⁴⁹ and a minimum of two unique peptides were required for confident identification of proteins. The false discovery rate (FDR) was determined by searching against a decoy database containing both forward and reversed peptide sequences, and was estimated to be <1%. Peptides identified

from all datasets of both wild-type and mutant cells were used to generate an AMT tag database that served as a reference database for identifying and quantifying peptides in subsequent LC-MS analyses.

Data analysis procedures are described in detail elsewhere.^{50, 51} Briefly, each MS spectrum was deisotoped using Decon2LS software to generate a peak mass and intensity for each detected species. VIPER software was then employed to search for isotopic paired peaks (4.0085 Da mass difference between light and heavy members of the pair), and match the light members (¹⁶O members) of the pairs to information in the database within a 2.5 ppm mass error and 1% NET tolerance to identify the peptides and protein of origins. At the same time, VIPER calculated the ¹⁶O/¹⁸O ratio to report as the peptide abundance ratio.³⁷ The average ¹⁶O/¹⁸O ratio of all identified peptide pairs for a particular protein was reported as the protein abundance ratio for that protein. The average protein abundance ratio calculated from technical replicates was further normalized based on the BSA abundance ratio in wild-type and mutant samples. The average of normalized protein abundance ratio values from three experimental replicates was reported as the final protein abundance ratios of wild-type to *ΔgspD* mutant.

Results

Identification of enriched membrane proteins

Gram-negative bacteria consist of outer-membrane, periplasm and inner-membrane, which form a sealed envelope around the cytoplasm. In this study, the steps of cell membrane envelope protein enrichment and identification included i) *in situ* labeling of membrane proteins using aqueous soluble/membrane impermeable chemical probe, Sulfo-NHS-SS-Biotin, ii) cell lysis and affinity enrichment of biotinylated proteins, iii) protein elution and trypsin digestion, and iv) protein identification by LC/MS/MS (Figure 1).

In this study, a total of 3,789 unique peptides were identified by LC/MS/MS representative of 470 proteins (Figure. 2a and Supplementary Table 1). Protein subcellular localizations were predicted by PSORTb v 2.0⁵² and CELLO v.2.5.⁵³ The predictions were further compared with previous experimental reports. 62 identified cytoplasmic proteins were also identified in the control (Supplementary Table 2), suggesting they were resulted from non-specific binding to the avidin column. Subtracting these non-specific proteins from the list, 408 proteins were specifically enriched using the membrane impermeable chemical probe and 79% of them were predicted to be membrane proteins (Figure 2a). In addition, enriched membrane proteins generated 2,780 unique peptides, which corresponded to 87% of the total identified peptides (Figure 2b). This high percentage of membrane proteins among the enriched sub-proteome demonstrates the relatively high efficiency and specificity of the method for membrane protein enrichment and identification. To compare the identifications of chemical probe enriched proteins, *S. oneidensis* MR-1 whole cell lysate were analyzed by LC/MS/MS, which resulted in 434 protein identifications with only 18% of them were predicted to be membrane proteins (Supplementary Figure 1 and Supplementary Table 6). The sharp contrast of membrane protein percentages demonstrated the membrane proteins enrichment specificity by the chemical probe.

Because affinity enrichment was carried out on the protein rather than peptide level, only a small number of peptides were identified with a thiol tag modification on the internal lysine cleaved from the disulfide bond of the chemical probe (Supplementary Table 1). While both modified and unmodified peptides were used to identify chemical-probe-labeled proteins, unmodified peptides were more likely to be subject to LC/MS/MS due to their higher abundance than that of modified peptides. However, the identification of modified peptide sequences has provided a direct evidence of protein labeling by the chemical probe. One

example of modified peptide was originated from an outer membrane protein, OmcA (SO1779), which was found previously to be cell surface-exposed⁵⁴ (Figure 3). The identification of modified peptide from OmcA demonstrates that this residue is accessible to the ionic chemical probe under the cell labeling condition.

Assessment for the accuracy of membrane proteome quantification

Accurate quantification requires high reproducibility in both sample processing and LC-MS analysis. Sample preparation in this study consisted of multiple steps of affinity enrichment (that includes binding, washing, and elution), tryptic digestion, SPE desalting, and trypsin catalyzed ¹⁸O labeling, which could all affect the reproducibility and quantification accuracy. To address this issue, we introduced an internal standard of biotinylated BSA by spiking equal amounts of internal standard in both wild-type and mutant samples before the affinity enrichment of the chemical probe labeled proteins. The final abundance ratio of BSA in wild-type and mutant samples was then used to normalize the abundance ratios of identified membrane proteins. To achieve reproducible and accurate quantification using LC/MS, peptides from mutant and wild-type cells were ¹⁸O and ¹⁶O labeled, respectively, and equally mixed prior to LC/MS/MS analysis. The relative abundances are measured by the relative ratios of co-eluted isotopic peptide pairs. Finally, the protein abundance ratios were calculated by averaging values of three experimental replicates to mitigate possible experimental variations.

Peptides were identified by applying the AMT tag strategy in which LC/MS detected features were matched to a previously established AMT tag database using the in-house software tool, VIPER. VIPER first searches for isotopic paired peaks (4.0085 Da mass difference between light and heavy members of the pair), and then matches the light members (¹⁶O members) of the pairs to the AMT tags in the database to identify the peptides and protein of origins. At the same time VIPER calculates the ¹⁶O/¹⁸O ratio to report as the peptide abundance ratio. Figure 4a and 4b show the distribution of average mass errors and average NET errors for all initially matched ¹⁶O-peptides, illustrating that a majority of peptides were identified within a mass error of 2 ppm and a NET error of 1% against customized AMT database. The FDR was estimated to be less than 1% by performing peak matching to an 11 Da-shifted AMT tag database.⁵⁵ Reproducibility of technical and experimental replicates was evaluated by pair-wise Pearson correlation coefficients for detected peptide peak intensities. High reproducibility was observed for three technical and experimental replicates with an average correlation coefficient of 0.98±0.01 and 0.93±0.01, respectively (Figure 4C and 4D).

Membrane proteome differences between wild-type and mutant cells

To identify specific membrane proteome differences between wild-type and mutant cells generated from the deletion of the type II secretion protein GspD, we quantitatively analyzed membrane proteins enriched by a membrane-impermeable chemical probe from both strains. After filtering with 2 ppm and 1% NET error, 1169 unique peptides were identified with isotopic pairs that corresponded to 357 proteins (Supplementary Table 3). Only those proteins with at least two observations of isotopic peptide pairs out of three experimental replicates were used for protein quantification. The protein abundance ratio was calculated by averaging all identified peptide abundance ratios, and then the protein values were normalized using the ratio of the BSA standard. The final protein abundance ratios of wild type to mutant cells, i.e., the average values from three experimental replicates, are provided in Table 1 and Supplementary Table 4. Note that abundance ratios were removed by filtering if the relative standard deviation of three experimental replicates was > 1 or if it had been previously identified in the control experiment as a non-specific binding protein.

Protein abundance ratios displayed in a heat map along with their subcellular localizations reveal the overall abundance of both outer membrane and periplasmic proteins were reduced with the deletion of *gspD* (Figure 5a). No major differences were observed in the abundance of inner membrane proteins between wild type and the mutant cells. The cytoplasmic proteins most likely enriched from non-specific labeling did not show any consistent pattern in expression ratio. To confirm that the observed decreased abundances of membrane proteins in the $\Delta gspD$ mutant was due to membrane protein translocation and not to decreased expression, ^{18}O labeling was used to quantify the relative protein abundances of whole cell lysates of wild type and $\Delta gspD$ mutant cells (Figure 5b and Supplementary Table 5). No significant protein abundance changes were observed for any subcellular proteins from the deletion of *gspD* on the whole cell level, which suggests that deletion of *gspD* had minimal effects on global protein expression, but significant effects on protein localization.

Discussion

The MS-based membrane proteome quantification strategy couples a membrane-impermeable chemical probe as a membrane protein enrichment method with $^{16}\text{O}/^{18}\text{O}$ isotopic labeling and LC-MS analysis. Labeling with a biotinylated membrane-impermeable chemical probe followed by avidin enrichment enables enrichment of cell envelope membrane proteins from total cellular proteins. The negative charge on the reactive group of the chemical probe limits penetration of the probe into the cell membrane during labeling, which increases labeling specificity and efficiency for membrane proteins.^{56, 57} Compared to hydrophobic and membrane permeable chemical probes,⁵⁸ much higher specificity for membrane proteins was achieved using the negatively-charged chemical probe in this study.

The strategy was applied to *Shewanella oneidensis* MR-1, a microorganism that can transfer electrons, such as iron (hydr)oxides and dimethyl sulfoxide (DMSO) to substrates external to the cells.^{59, 60} Extracellular reduction of iron oxides or DMSO requires surface localization of MtrC (SO_1778) and OmcA (SO_1779) or DmsA (SO_1429) and DmsB (SO_1430), respectively. The type II secretion system is directly involved in translocating MtrC and OmcA across the bacterial outer membrane.⁴⁴ Inactivation of the type II secretion system diminished the bacterial ability to reduce DMSO, which suggests that DmsA and DmsB are translocated to the surface via bacterial type II secretion system.⁴⁵ Thus, deletion of *gspD*, a key component of the bacterial type II secretion system expectedly alters the surface localization of these proteins. Our results clearly showed that the amount of MtrC, OmcA, DmsA and DmsB found in the $\Delta gspD$ mutant was greatly reduced compared to wild type, which confirms previous results and demonstrates the quantitative capability of our method (Table 1).

In addition to MtrC, OmcA, DmsA, and DmsB, the abundances of three additional outer membrane lipoproteins (SO_0404, SOa_0110, and SOa_0112) and one β -barrel protein, MtrB (SO_1776) also appeared significantly reduced in $\Delta gspD$ mutant, suggesting that the type II secretion system has functional roles in regulating their localization to the outer membrane. All of the four known *S. oneidensis* MR-1 type II secretion substrates participate in electron transfer reactions and are distinct in that, with the exception of DmsB, they are predicted to be lipoproteins. Lipoproteins are proteolytically processed at the n-terminus by signal peptidase II and covalently modified with lipid extensions at the resulting N-terminal cysteine. In most gram negative bacteria the majority of lipoproteins are localized to the periplasmic side of the outer membrane via the Lol system instead of to the periplasmic side of the inner membrane.⁶¹

Our analyses also revealed that three putative lipoproteins (SO_0404, SOa_0110 and SOa_0112) were more abundant in surface protein enrichments of wild type versus the type

II secretion mutant, which suggests that they may be novel substrates of this system. The β -barrel protein MtrB also showed significantly lower abundance in $\Delta gspD$ mutant than wild-type, suggesting that the impact of this mutation also has secondary effects on abundances of non-type II secretion substrate. MtrB has been reported to form an interacting complex with Type II secretion substrate MtrC.⁶² Suppressed translocation of MtrC to the outer membrane due to the deletion of *gspD* may indirectly result in the decreased abundance or stability of MtrB.

In addition to outer membrane proteins, the abundances of 15 periplasmic proteins greatly decreased in $\Delta gspD$ mutant. The reason for this decrease is currently unknown, but may be due to the accumulation of outer membrane proteins such as MtrC and OmcA in the periplasm, which may down-regulate the expression or translocation of the periplasmic proteins. Consistent with this observation, our unpublished results showed that the abundance of periplasmic NifA decreased in $\Delta gspD$ mutant.⁴⁴ The specificity of membrane proteome changes was also confirmed by comparing the membrane proteome changes to the whole proteome in which no significant changes were observed. The data support that the observed changes in chemical probe enriched samples were specific to the membrane proteome and induced by protein translocation.

In summary, we demonstrated the reproducibility, accuracy, and specificity of a quantitative analysis strategy for studying a cell membrane proteome. Quantitative results in terms of the relative membrane protein abundance differences between wild-type and mutant cells of *Shewanella oneidensis* MR-1 confirmed the role of the type II secretion system in outer membrane protein translocation and revealed many potential novel substrates for the T2SS, as well as for proteins that may have changed in abundance via association with T2SS substrates. This quantitative strategy of coupling a membrane-impermeable chemical probe with isotopic labeling can also be extended to studies of gram-positive bacteria and eukaryotic cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematic strategy of membrane protein enrichment and quantification in wild type and mutant cells.

Figure 2. Identification of chemical probe labeled proteins; a) number of identified proteins in different subcellular localizations; b) number of identified unique peptides generated by proteins in separate subcellular localizations.

Figure 3.

An example of MS/MS spectrum of chemical-probe labeled peptide originated from an outer membrane protein, OmcA (SO1779). The internal lysine was modified by the thiol tag which is the remaining part after chemical probe was cleaved by DTT.

Figure 4. Reproducibility analysis of ^{18}O labeling quantification; a) Mass error distribution and b) NET error distribution of all identified peptide from AMT database; The pairwise correlation of c) three experimental replicates and d) three technical replicates.

Figure 5.

Heat map of protein abundance ratio of wild-type over mutant cells in order of sub-cellular localization resulted from a) enriched membrane proteins, and b) whole cell lysate. ER means protein abundance ratio of wild type over ΔgspD mutant cells.

Table 1

Outer membrane protein abundance ratios of wild-type over *ΔgspD* mutant cells using cell-membrane-impermeable chemical probe enrichment and ¹⁸O labeling strategy. a: Bovine serum albumin. b: Average ratio of peptide abundance. c: Normalized protein abundance ratio using BSA standard. d: Average value of normalized protein abundance ratio. e: Relative standard deviation of average normalized expression ratio of three experimental replicates

Locus Tag	Product	Comment	Replica 1		Replica 2		Replica 3		Ave. Norm. ER ^d	R. Std of Norm.ER ^e
			Ave. ER ^b	Norm. ER ^c	Ave. ER	Norm. ER	Ave. ER	Norm. ER		
BSA ^a	Standard	Standard	0.77	1.00	0.81	1.00	0.56	1.00	1.00	0.00
SO_1429	outer membrane dimethyl sulfoxide reductase, molybdopterin-binding subunit, DmsA	surface lipoprotein	11.13	14.41	10.43	12.92	9.13	16.41	14.58	0.12
SO_1778	outer membrane decaheme cytochrome c lipoprotein, MtrC	surface lipoprotein	6.92	8.95	9.86	12.21	4.37	7.84	9.67	0.23
SO_1779	outer membrane decaheme cytochrome c, OmcA	surface lipoprotein	11.71	15.16	11.02	13.65	8.40	15.10	14.63	0.06
SO_1430	dimethyl sulfoxide reductase, FeS subunit, DmsB	Complexed with surface lipoprotein	15.22	19.71	23.09	28.59	6.50	11.68	19.99	0.42
SO_0404	zinc dependent metalloprotease domain lipoprotein	lipoprotein	4.77	6.18	5.22	6.46	4.04	7.26	6.63	0.08
SO_0918	acyl-homoserine lactone acylase, Aac	lipoprotein	0.61	0.79	0.63	0.78	0.27	0.48	0.69	0.25
SO_1044	ABC arginine transporter, periplasmic ligand-binding subunit, Artl	lipoprotein			2.08	2.57	1.95	3.51	3.04	0.22
SO_1065	peptidyl-prolyl cis-trans isomerase, FkIB_3	lipoprotein	0.51	0.67	1.40	1.74	1.14	2.04	1.48	0.49
SO_1210	globular tetratricopeptide repeat containing lipoprotein, Nlpl	lipoprotein	0.95	1.23	1.15	1.42			1.33	0.10
SO_1424	expressed lipoprotein	lipoprotein	0.86	1.11	0.99	1.22			1.17	0.07
SO_1831	expressed lipoprotein	lipoprotein	1.21	1.56	1.75	2.17	1.45	2.61	2.11	0.25
SO_2747	peptidoglycan-associated lipoprotein, Pal	lipoprotein	0.29	0.38	1.06	1.31			0.84	0.78
SO_2753	prolyl endopeptidase	lipoprotein	1.06	1.38	2.06	2.55			1.97	0.42
SO_3278	type I secretion system, membrane fusion protein, RND family	lipoprotein	0.32	0.42			0.56	1.00	0.71	0.58
SO_3343	expressed lipoprotein	lipoprotein	1.08	1.40			2.82	5.07	3.24	0.80
SO_3560	subfamily M16B unassigned peptidases	lipoprotein	0.41	0.53	1.44	1.78	0.57	1.02	1.11	0.57
SO_3564	peptidyl-dipeptidase Dcp_2	lipoprotein	1.11	1.43	0.93	1.15	2.01	3.61	2.07	0.65

Locus Tag	Product	Comment	Replica 1		Replica 2		Replica 3		Ave. Norm. ER ^d	R. Std of Norm.ER ^e
			Ave. ER ^b	Norm. ER ^c	Ave. ER	Norm. ER	Ave. ER	Norm. ER		
SO_3811	expressed lipoprotein of unknown function	lipoprotein	0.58	0.75	2.12	2.62			1.68	0.78
SO_3844	endothelin-converting enzyme, PepO	lipoprotein	1.28	1.66	3.56	4.40	2.36	4.24	3.43	0.45
SO_4693	type I secretion system, multidrug efflux pump, membrane fusion protein, AcrA	lipoprotein	1.65	2.14	1.61	2.00	1.56	2.80	2.31	0.18
SO_A0110	expressed lipoprotein	lipoprotein	4.53	5.86	10.81	13.39	1.81	3.25	7.50	0.70
SO_A0112	expressed lipoprotein	lipoprotein	7.05	9.12	10.07	12.47	1.96	3.52	8.37	0.54
SO_1637	beta barrel protein translocation component, BamA	β-barrel protein	2.16	2.80	2.70	3.34			3.07	0.12
SO_1776	outer membrane protein, MtrB	β-barrel protein	9.75	12.62	12.97	16.06	8.67	15.57	14.75	0.13
SO_2427	TonB-dependent receptor	β-barrel protein	2.14	2.78	3.20	3.96	0.59	1.05	2.60	0.56
SO_2469	TonB-dependent receptor	β-barrel protein	1.88	2.43	1.61	2.00	0.82	1.47	1.97	0.24
SO_2907	TonB-dependent receptor	β-barrel protein	3.61	4.67	4.00	4.96	1.67	3.00	4.21	0.25
SO_3099	outer membrane long-chain fatty acid transport protein, FadL-family	β-barrel protein	0.90	1.17	0.95	1.17	0.40	0.72	1.02	0.25
SO_3193	outer membrane polysaccharide export protein, OtnA	β-barrel protein	0.84	1.08	2.36	2.92	0.38	0.69	1.57	0.76
SO_3545	outer membrane porin	β-barrel protein	1.25	1.61	5.30	6.56	3.26	5.85	4.68	0.57
SO_3896	outer membrane porin, Omp35	β-barrel protein	2.09	2.70	3.73	4.62	1.22	2.18	3.17	0.41
SO_3904	type I secretion outer membrane protein, TolC	β-barrel protein	0.76	0.98	2.30	2.84	0.75	1.34	1.72	0.57
SO_4320	type I secretion system, outer membrane component, AggA	β-barrel protein	1.07	1.38	3.20	3.97	1.13	2.03	2.46	0.55
SO_4422	TonB-dependent ferric achromobactin receptor	β-barrel protein	2.24	2.91	1.08	1.34			2.12	0.52
SO_4694	outer membrane protein, TorF	β-barrel protein	1.17	1.51	0.67	0.82	0.57	1.03	1.12	0.32
SO_4743	TonB-dependent siderophore receptor	β-barrel protein	0.28	0.37			0.83	1.49	0.93	0.86
SO_A0114	outer membrane protein, OmpA	β-barrel protein	1.33	1.72	4.06	5.03	4.54	8.15	4.97	0.65