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NEWS & VIEWS

Is Proteomics a Reliable Tool to Probe the Oxidative Folding of Bacterial Membrane Proteins?

Vivianne J. Goosens,¹ Ruben A.T. Mars,¹ Michiel Akeroyd,² Andre Vente,² Annette Dreisbach,¹ Emma L. Denham,¹ Thijs R.H.M. Kouwen,² Tjeerd van Rij,² Maurien Olsthoorn,² and Jan Maarten van Dijk¹

Abstract

The oxidative folding of proteins involves disulfide bond formation, which is usually catalyzed by thiol-disulfide oxidoreductases (TDORs). In bacteria, this process takes place in the cytoplasmic membrane and other extra-cytoplasmic compartments. While it is relatively easy to study oxidative folding of water-soluble proteins on a proteome-wide scale, this has remained a major challenge for membrane proteins due to their high hydrophobicity. Here, we have assessed whether proteomic techniques can be applied to probe the oxidative folding of membrane proteins using the Gram-positive bacterium *Bacillus subtilis* as a model organism. Specifically, we investigated the membrane proteome of a *B. subtilis* *bdbCD* mutant strain, which lacks the primary TDOR pair BdbC and BdbD, by gel-free mass spectrometry. In total, 18 membrane-associated proteins showed differing behavior in the *bdbCD* mutant and the parental strain. These included the ProA protein involved in osmoprotection. Consistent with the absence of ProA, the *bdbCD* mutant was found to be sensitive to osmotic shock. We hypothesize that membrane proteomics is a potentially effective approach to profile oxidative folding of bacterial membrane proteins. *Antioxid. Redox Signal.* 18, 1159–1164.

Introduction

THE OXIDATIVE REACTION necessary for disulfide bond formation can occur spontaneously. However, efficient disulfide bond formation between the correct cysteine residues *in vivo* is catalyzed by specific enzymes known as thiol-disulfide oxidoreductases (TDORs) (4). In the Gram-positive bacterium *Bacillus subtilis*, thiol oxidases have been identified that are referred to as *Bacillus* disulfide bond proteins (Bdb) (4). These TDORs are of critical importance in the application of *B. subtilis* as a cellular factory for secreted proteins with disulfide bonds.

Four Bdb proteins have been identified in *B. subtilis*, namely, BdbA, B, C, and D. The genes for these proteins are grouped in pairs on the genome: *bdbA* and *bdbB* are found in the *Sp* β prophage region, while *bdbC* and *bdbD* form an operon on the core genome (4). BdbC and BdbD form a redox pair important for oxidative folding of the competence proteins ComEC (2) and ComGC (4), while BdbB and BdbC are connected to the correct folding of the *Sp* β prophage-encoded bacteriocin sublancin 168 (4). Apart from these proteins

no further native TDOR substrates have been identified in *B. subtilis*. However, both BdbC and BdbD are needed for the heterologous secretion of the alkaline phosphatase PhoA of *E. coli* in an active and protease-resistant state (4).

The available data imply that BdbC and BdbD make up the primary oxidative TDOR unit in *B. subtilis*, and, consistent with this view, the *bdbC* and *bdbD* genes are expressed throughout the cell cycle under a wide range of physiologically and industrially relevant conditions (8). That the expression of *bdbC* and *bdbD* is not specific for cells that are competent for genetic transformation suggests the possible existence of BdbC and BdbD substrates that are not associated with competence. However, despite extensive molecular biological and proteomics analyses, no such substrates were identified in the cell wall or spent culture media of *B. subtilis* (unpublished observations). This suggested that particular membrane proteins might be substrates for oxidative folding by BdbC and BdbD.

The primary objective of the present studies was to investigate whether membrane proteomics approaches can be applied to identify membrane proteins of *B. subtilis* that are produced in a BdbCD-dependent manner. Specifically, the

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Innovation

Approximately 30% of all genomes are predicted to encode membrane proteins. However, compared to water-soluble proteins, membrane proteins are substantially less studied due to their high overall hydrophobicity. This intrinsic property of membrane proteins makes them notoriously difficult to analyze at a proteome-wide level, and it has especially hampered the identification of specific post-translational modifications. Accordingly, relatively few membrane-associated oxidatively folded proteins have been identified. Here, we have investigated whether recent advances in membrane protein extraction techniques, and gel-free mass spectrometry can be applied to identify TDOR-dependent membrane proteins in *B. subtilis*. Importantly, *B. subtilis* produces many different proteases and incorrectly folded proteins are therefore rapidly degraded. Hence, the absence of certain proteins from a mutant lacking the main membrane-associated TDORs BdbC and BdbD was regarded as indicative of BdbCD-dependence or association with BdbCD-dependent proteins. The changes observed in the membrane proteome of *bdbCD* mutant cells reveal novel and unanticipated links between TDOR activity and membrane-associated proteins.

membrane proteome of a *B. subtilis* *bdbCD* mutant strain was analyzed by mass spectrometry (MS) and compared to the membrane proteome of the parental *B. subtilis* 168 strain. Notably, the extracytoplasmic compartments and the growth medium of *B. subtilis* are highly proteolytic due to the production of a large number of cell wall-associated and extracellular proteases (9). A potential BdbC-BdbD substrate would incorrectly fold in the absence of these TDORs and therefore becomes a readily degradable target for these proteases (3, 4). This degradation could potentially also extend to the interacting partners of BdbC and BdbD substrates. Therefore, we considered the absence of particular proteins upon mutation of *bdbCD* as an indicator for potentially direct or indirect Bdb relationships.

Results and Discussion

Mass spectrometric identification of changes in the membrane proteome of *bdbCD* mutant cells

In the present studies, the membrane proteomes of two strains, a *B. subtilis* double mutant (*bdbCD*) devoid of BdbC and BdbD and its parental strain 168, were analyzed by gel-free liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and the identified proteins were subsequently compared. Quality of the fractionation was assessed on the basis of different protein banding patterns upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1A). The absence of BdbD from the membrane of the *bdbCD* mutant strain was confirmed by Western blotting (Fig. 1B). Membrane proteins from *B. subtilis* 168 and the *bdbCD* strain were extracted twice, generating two biological replicate experiments. Each sample was injected three times, thereby generating three technical replicates per biological replicate. To confirm the presence of a single protein, a minimum of two unique peptides of this protein were needed. Taking these constraints into account, a total number of 681 membrane-associated proteins were identified in our MS runs, of which 43% were predicted to contain transmembrane domains (Supplementary Table S1; Supplementary Data are available online at www.liebertonline.com/ars). To consider a particular protein as a lead for BdbCD-dependence, it had to be identified in both biological replicates.

The localization of identified proteins was predicted by comparing the results from six different membrane protein prediction algorithms. The number of algorithms predicting whether a protein is localized to the membrane is given in Table 1. No reliable programs predicting protein folds and disulfide bond formation are available as yet. Therefore, for the purpose of our studies, we only determined the number of cysteines and the presence of at least one cysteine was considered suggestive of a potential for disulfide bond formation.

Comparisons between the *B. subtilis* 168 and the *bdbCD* mutant membrane proteomes showed that the majority of the proteins observed were identified in both strains; however, a subset of 18 proteins listed in Table 1 showed reproducible variation. Specifically, 15 proteins present in at least two of the

FIG. 1. Subcellular fractionation of *Bacillus subtilis*. Cells were fractionated and the quality of the fractionation was subsequently assessed on the basis of different protein banding patterns upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). (A) Cytoplasmic (Cyto), membrane (Mem) and cell wall (Wall) fractions were collected from cells of a *B. subtilis* *bdbCD* mutant strain (*bdbCD*) or the parental strain 168 (168) as described in the Materials and Methods. Next, the proteins in these fractions were separated by SDS-PAGE. (B) The absence of BdbD from membrane fractions that were used for proteomics analyses was verified by Western blotting with BdbD-specific antibodies. Molecular weight (Mw) markers are indicated (in kDa).

TABLE 1. DIFFERENCES BETWEEN THE MEMBRANE PROTEOMES OF A *BACILLUS SUBTILIS* 168 *BdbCD* MUTANT STRAIN AND THE PARENTAL STRAIN 168

	# Cys ^a	# prog predict membrane ^b	<i>B. subtilis</i> 168 A			<i>B. subtilis</i> 168 B			<i>bdbCD</i> A			<i>bdbCD</i> B		
			WT A1	WT A2	WT A3	WT B1	WT B2	WT B3	CD A1	CD A2	CD A3	CD B1	CD B2	CD B3
BdbD	2	5	8	11	13	10	11	12						
BglP	1	6	2	2	2	3	2	2						
DegS	1	1		3	3	3	3	3						
TcyP	1	4	2	2	3	3	4	2						
ProA	4	1		2	3	3	2	3						
PyrAB	8	0	8	10	12	16	14	14						
PyrAA	6	0	4	5	5	2		2						
PyrE	4	0		3	3	3	4	4						
PyrH	1	0			2		2	2						
PyrF	2	1			2	2	2	4						
YbxA	0	0	2					2						
LytA	1	4		2	2			2						
SipU	0	4			3			2						
YxaI	1	5	2					2						
GlxK	1	0			2			2						
ResD	1	1							3	3	2	4		2
CypD	8	0							2					2
MsmR	4	0							3					2

Leads were generated by comparing the detected membrane proteins of *B. subtilis* 168 and a *bdbCD* mutant. The two biological replicate experiments are indicated by A and B, each with three technical replicate injections (1–3). Low numbers of unique peptide detection are common when working with hydrophobic membrane proteins. Nevertheless, the higher the number of unique peptides identified per protein per technical replicate as indicated in the columns, the greater we considered the probability of the protein in that sample.

^aNumber of cysteine residues within particular proteins.

^bNumber of algorithms predicting membrane protein localization.

B. subtilis 168 biological replicates were not identified in the samples of the *bdbCD* mutant strain. As expected, BdbD was found to be absent from the *bdbCD* mutant, thereby serving as an unambiguous internal standard. BdbC was not identified in the 168 strain, but this can be explained by the fact that BdbC has four transmembrane domains and relatively small cytoplasmic/extracytoplasmic domains. Of the 15 membrane-associated proteins missing from the *bdbCD* strain, the following were predicted to be membrane-associated: the β -glucoside permease BglP, the cysteine transporter TcyP, the minor signal peptidase SipU, the lipoprotein LytA, and the protein of unknown function YxaI. Other proteins included the glutamate-5-semialdehyde dehydrogenase ProA, the putative glycerate kinase GlxK, 5 pyrimidine metabolism-related proteins (PyrAA, PyrAB, PyrH, PyrE, and PyrF), the sensor kinase DegS, and the protein YbxA, which is linked to an ABC transporter of an unknown function. Three proteins were detected in the *bdbCD* strain, but not in the parental 168 strain, and were suggestive of BdbCD compensatory mechanisms. They included ResD, the NADPH-cytochrome P450 reductase CypD, and the transcription regulator MsmR.

Phenotypic assessment of *BdbCD* associations

One of the limits of MS analyses is that the failure to detect a particular protein does not unambiguously demonstrate its absence. Therefore, although MS is a powerful tool to identify novel leads, these leads should be confirmed at least in those cases where a suitable detection assay is available. Such assays are often indirect, and for many proteins no suitable assays are as yet available. This is particularly true for the membrane proteome. Nevertheless, we performed functional

analyses to follow-up on three potential leads for BdbCD-dependent membrane protein folding, of which at least one (*i.e.*, ProA) was shown to be meaningful.

A number of the proteins identified here are related to cytoplasmic functions (*e.g.*, DegS, ProA, and the 5 pyrimidine-related proteins). This may imply that these proteins were possibly cytoplasmic contaminants. However, in other extensive *B. subtilis* proteomic studies, these proteins have consistently been identified in the membrane fraction (9). These studies used various different extraction and MS-techniques each with their own pros and cons when considering membrane proteomics. Hence, a consistency covering not only these three studies but also the present studies performed here suggests potential membrane-related roles for these proteins, and direct or indirect associations with BdbCD (9). Further, regarding the proteins involved in pyrimidine metabolism, most of the corresponding genes form part of a pyrimidine operon. This *pyr* operon includes the gene for the membrane-associated protein PyrP. It is noticeable that the Pyr proteins all contain a large number of cysteine residues, and they thus have the capacity to form disulfide bonds as well as a Pyr protein complex at the membrane interface. Moreover, the Pyr proteins have been associated with thiol formation under oxidative stress conditions (6). The consistent membrane association and potential disulfide bond formation is thus suggestive of a membrane-associated complex. Therefore, although no functional analysis of the localization and oxidative folding of proteins involved in the pyrimidine metabolism was proven, a Bdb-Pyr relationship does deserve further in-depth investigations.

Both DegS and ResD form part of two-component regulatory systems, DegS-DegU and ResE-ResD, respectively.

Contrasting results regarding these two-component systems were obtained in our studies. While DegS was observed in the membrane fraction of the *B. subtilis* 168 strain, but not in the membrane fraction of the *bdbCD* mutant, the opposite was observed for ResD. The available assays for DegS and ResD are all related to their known roles in the cytoplasm. Specifically, DegS is an important regulator of motility and protease activity (7), and ResD controls expression of the ResA and ResE proteins (5). As evidenced by the absence of the respective phenotypes from the *bdbCD* mutant, DegS and ResD are presumably still present and active in the cytoplasm of cells lacking BdbC and BdbD, performing their known roles relating to motility, protease activity, and transcriptional activation. However, as yet undefined roles of DegS or ResD at the membrane may be affected by the absence of BdbCD. Hence, the lack of detectable DegS- or ResD-related phenotypes could relate to specific roles that these proteins may be performing at the membrane interface, and this warrants further research.

The ProA protein, which was identified in membranes of the *B. subtilis* 168 strain but not in the membranes of the *bdbCD* mutant, contains four cysteine residues. ProA is involved in the synthesis of proline, an important constituent of peptides and proteins. The *bdbCD* mutant strain was therefore tested for a possible proline auxotrophy in chemically defined media. However, the mutant was able to grow normally under proline-limited conditions (data not shown). Notably, proline

serves a second important role as a major osmoprotectant (1). The ability of the *bdbCD* strain to withstand osmotic shock was therefore also investigated. Osmotic shock was induced by the addition of 1.1 M NaCl to exponentially growing cells and the cell viability was measured using a live-dead stain. As was to be expected for cells with significantly reduced ProA levels, the *bdbCD* strain showed a strong sensitivity to osmotic shock, and this phenotype was fully reversed when the *bdbCD* mutant was complemented through the ectopic expression of *bdbCD* from a plasmid (Fig. 2).

In conclusion, our present proteomics analyses show that membrane proteomics can be applied to identify potentially TDOR-dependent membrane proteins and processes. Specifically, our studies have led to the identification of a new phenotype of *bdbCD* mutant *B. subtilis* cells, namely, sensitivity to osmotic stress. This is a biologically highly relevant finding, because *B. subtilis* is regularly exposed to major osmotic insults in its natural habitat, the soil.

Notes

Bacterial strains and growth

Bacterial growth was performed at 37°C and cultures were shaken at 250 rpm, and growth was measured by optical density readings at 600 nm. Media used in this study included the Luria Bertani (LB) broth, the phosphate-limited medium

FIG. 2. Increased sensitivity of *bdbCD* mutant cells to osmotic shock. The survival of cells challenged by osmotic shock with NaCl was assessed by live/dead staining and subsequent flow cytometry. (A) Percentages of dead cells detected with the live/dead stain after salt shock. The *bdbCD* strain was complemented with plasmid expressing BdbC and BdbD (*BdbCD* + CD). Values represent the results of three independent experiments. The standard deviation between experiments is indicated. (B) Representative flow cytometry data indicating shifts in color spectrum upon live/dead staining. A shift toward the left implies an increase in dead cells, where green fluorescence is measured on the x-axis and the number of cell counts on the y-axis.

TABLE 2. BACTERIAL STRAINS USED IN THIS STUDY

Plasmid	Properties	Reference
pHB- <i>bdbCD</i>	pHB201 vector carrying the <i>bdbCD</i> genes; Em ^R ; Cm ^R	(3)
Strains	Properties	Reference
<i>B. subtilis</i> 168	<i>trpC2</i>	(9)
<i>B. subtilis</i> 168 <i>bdbCD</i>	<i>trpC2</i> ; <i>bdbCD</i> ::Sp ^R	(3)

LPDM (0.25% glucose, 0.21 mM KH₂PO₄ [pH 7.0], 0.025% casamino acids, 5 mM L-arginine, 1 mg Tryptophan, and 50% Hulets salts [50 mM Tris pH 7, 3.03 mM (NH₄)₂SO₄, 6.8 mM trisodium citrate, 3.04 mM FeCl₃, 1 mM MnCl₂, 3.5 mM MgSO₄, and 0.01 mM ZnCl₂], and the chemically defined minimal M9 medium (8) supplemented with tryptophan. When appropriate, the growth media were supplemented with 100 µg/ml spectinomycin, 2 µg/ml erythromycin, or 5 µg/ml chloramphenicol. The bacterial strains used in this study are detailed in Table 2.

Membrane protein enrichment and extraction

Cultures were grown to an OD₆₀₀ of 2. Membrane fractions were prepared as described previously (9) with minor adaptations. Protoplast disruption was performed by sonication (Soniprep 150; Beun de Ronde BV) in a high salt buffer (20 mM Tris, 10 mM EDTA, and 1 M NaCl). All buffers used included freshly added protease inhibitors (Complete Protease Inhibitor cocktail; Roche) except for the solubilization buffer. The membrane protein fraction was TCA-precipitated overnight at 4°C.

LC-MS/MS and data analysis

TCA-precipitated proteins were resuspended in 8 M urea with vortexing and sonication. About 100 mM NH₄HCO₃ was added to the samples, which were treated with 500 mM diethiothreitol for 30 min before being incubated in the dark for 30 min with 10 µl iodoacetamide (10 mM). Trypsin digestion was performed at 37 °C overnight with 20 µl of 250 µg/ml Trypsin, with a booster of 2–5 µl Trypsin for 1–3 h the next day before acidification with 5% formic acid (FA).

The complex peptide mix in the samples was separated by LC on a U-HPLC (Accela; Thermo Fisher Scientific) through a guard column (Poroshell 300 SB-C₃ 2.1 × 12.5 mm; Agilent) and C₁₈ column-reversed phase column (Zorbax SB-C₁₈ 2.1 × 50 mm; Agilent) at 50°C. Peptides were eluted at a constant flow rate of 0.4 ml/min for 275 min with a nonlinear gradient 5%–80% of buffers B (the BUFFER A 0.1% FA in water, and the buffer B 0.1% FA in acetonitrile, both UHPLC grade; Biosolve).

The peptides were identified with an LTQ-Velos (Thermo Fisher Scientific) coupled to an electrospray ion source. The survey scan was performed with an enhanced MS scan mass range of 300–2000. The 10 most intense doubly and triply charged precursor ions were chosen for MS/MS *via* CID with an exclusion time of 60 s. Each sample was injected individually three times resulting in three parallel MS/MS spectra per biological replicate. The *raw files generated were visu-

alized using Xcalibur (Thermo Fisher Scientific). This information was searched using the Sorcerer-Sequest (v.27, rev. 11; Thermo Fisher Scientific) against a *B. subtilis* 168 database, including a decoy reverse database (UniprotKB, release 2011_02–Feb, 2011). Parameters for database searches were the protease type (trypsin), variable modifications (deamination, oxidation, and carbamidomethyl), and a maximum of two missed cleavage sites. Charge-dependent Xcorr factors were applied for filtering the data (2+ /3+ at 2.5/2.8) and the deltaCn value had to be at least 0.09. In addition, ambiguous peptides were excluded from the analysis. A protein was regarded as identified, if at least two unique peptides were detected resulting in a false-positive rate of below 1%.

The proteins identified by MS were compared with the predicted integral membrane proteins and potentially membrane-associated proteins of *B. subtilis*. Proteins were considered potentially membrane-associated if they were identified as membrane associated in at least 2 of 6 membrane protein prediction algorithms used (TMHMM, TMMTOP, SOSIU, PHOBIUS, SCAMPI, and pSORT).

SDS-PAGE and Western blotting

Proteins were separated using SDS-PAGE (NuPAGE gels; Invitrogen). Gels were either stained with Simply blue™ Safe stain (Invitrogen) or semidry blotted onto nitrocellulose membranes (Protran; Schleicher&Schuell). Binding of polyclonal antibodies was monitored with fluorescent IgG secondary antibodies (IRDye 800 CW goat anti-rabbit from LiCor Biosciences) and the Odyssey Infrared Imaging System (LiCor Biosciences).

Osmostress assay

Overnight cultures in the LB broth were used to inoculate the fresh LB broth at a 1:200 dilution. These cultures were grown to mid-exponential phase (3 h). Samples were then diluted to an OD₆₀₀ of 0.05 and grown to an OD₆₀₀ of 0.4–0.5 before the addition of crystalline NaCl to a final concentration of 1.1 M. After 5-min incubation under vigorous shaking, cells were collected by centrifugation, and re-suspended in 0.85% NaCl before a 1:1 live/dead stain was added (SYTO 9:propidium iodide; LIVE/DEAD BacLight Bacterial Viability and Counting Kit; Invitrogen). The viability of salt-stressed cells was then measured by flow cytometry (Accuri C6 Flow Cytometer).

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Author Disclosure Statement

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R.H.M. Kouwen, Tjeerd van Rij, Maurien Olsthoorn, and Jan Maarten van Dijl declare no competing commercial interests or any other conflicts of interest. Michiel Akeroyd, Andre Vente, Thijs R.H.M. Kouwen, Tjeerd van Rij, and Maurien Olsthoorn are employees of DSM Biotechnology Center.

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Abbreviations Used

Bdb = *Bacillus* disulfide bond proteins
bdbCD = double *bdbC* and *bdbD* mutant strain
FA = formic acid
LC-MS/MS = liquid chromatography coupled to tandem mass spectrometry
MS = mass spectrometry
SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TDOR = thiol-disulfide oxidoreductase