



# Methionine Residues in Exoproteins and Their Recycling by Methionine Sulfoxide Reductase AB Serve as an Antioxidant Strategy in *Bacillus cereus*

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During aerobic respiratory growth, *Bacillus cereus* is exposed to continuously reactive oxidant, produced by partially reduced forms of molecular oxygen, known as reactive oxygen species (ROS). The sulfur-containing amino acid, methionine (Met), is particularly susceptible to ROS. The major oxidation products, methionine sulfoxides, can be readily repaired by methionine sulfoxide reductases, which reduce methionine sulfoxides [Met(O)] back to methionine. Here, we show that methionine sulfoxide reductase AB (MsrAB) regulates the Met(O) content of both the cellular proteome and exoproteome of *B. cereus* in a growth phase-dependent manner. Disruption of *msrAB* leads to metabolism changes resulting in enhanced export of Met(O) proteins at the late exponential growth phase and enhanced degradation of exoproteins. This suggests that *B. cereus* can modulate its capacity and specificity for protein export/secretion through the growth phase-dependent expression of *msrAB*. Our results also show that cytoplasmic MsrAB recycles Met residues in enterotoxins, which are major virulence factors in *B. cereus*.

**Keywords:** methionine sulfoxide reductase, exoproteome, antioxidants, *Bacillus cereus*, metabolism

## INTRODUCTION

Reactive oxygen species (ROS) are by-products of aerobic metabolism, and respiration is considered to be the major intracellular source of ROS production in bacteria (Brynildsen et al., 2013; Imlay, 2013). Methionine residues in proteins are particularly susceptible to oxidation by ROS (Vogt, 1995; Stadtman et al., 2005), resulting in methionine-S-sulfoxides [Met-S-(O)] and methionine-R-sulfoxides [Met-R-(O); (Luo and Levine, 2009; Kim et al., 2014)]. Oxidized methionine can be repaired by the antioxidant enzymes, Met-S-(O) reductase (MsrA) and Met-R-(O) reductase (MsrB). Both Msr share a common catalytic mechanism to reduce Met(O) back to Met. This catalytic mechanism leads to the formation of an intramolecular disulfide bond in the Msr and involves thioredoxin (Trx), thioredoxin

reductase, and NADPH (Weissbach et al., 2002). It has been shown that MsrA reduces both oxidized proteins and low molecular weight Met(O)-containing compounds with a similar catalytic efficiency, whereas MsrB is specialized for the reduction of Met(O) in proteins. Interestingly, both Msr types preferentially repair unfolded proteins (Tarrago et al., 2012). The genes encoding MsrA and MsrB have been identified in most living organisms. Four different types of organization have been reported for *msrA* and *msrB*: (i) *msrA* and *msrB* genes are two separate transcription units, (ii) *msrA* and *msrB* cistrons are organized as an operon, (iii) *msrA* and *msrB* cistrons form a single open reading frame (ORF) to produce a two domain protein, and (iv) *trx*, *msrA*, and *msrB* cistrons form a single ORF to produce a three domain protein (Drazic and Winter, 2014).

Several studies have revealed the importance of Met oxidation and Msrs, especially regarding oxidative stress resistance and metabolism under stress conditions. In addition, Msrs have also been reported to be important virulence factors in pathogens by modulating a range of properties such as adherence (Wizemann et al., 1996; Giomarelli et al., 2006), motility (Hassouni et al., 1999), biofilm formation (Beloin et al., 2004), and *in vivo* survival (Alamuri and Maier, 2004). However, the importance of Met oxidation and Msr in the secretion of virulence factors under normal physiological conditions is largely unknown in pathogens, and in particular in *Bacillus cereus*.

*B. cereus* is a Gram-positive, motile human pathogen that is well-equipped to survive in various environments such as those encountered in soil, food and the human gastrointestinal tract (Stenfors Arnesen et al., 2008). These bacteria can grow in the presence or absence of oxygen (Rosenfeld et al., 2005; Duport et al., 2006). In the human intestine, *B. cereus* encounters oxic conditions in zones adjacent to the mucosal surface (Marteyn et al., 2010) and anoxic conditions in the intestinal lumen (Moriarty-Craige and Jones, 2004). In the presence of oxygen, *B. cereus* grows by means of aerobic respiration and secretes a large number of proteins into the extracellular compartment. These secreted proteins, and all the released proteins found in the pathogen's surrounding environment, constitute the *B. cereus* exoproteome (Clair et al., 2010, 2013; Laouami et al., 2014). We previously reported that the *B. cereus* exoproteome contained protein-bound Met(O) and that the accumulation of protein-bound Met(O) decreased significantly during aerobic respiratory growth, to reach its minimal value at the stationary phase (Madeira et al., 2015). Insofar as there is no ROS source and no Msr to reduce Met(O) back to Met in the extracellular milieu, we assumed that the time dynamic of protein-bound Met(O) in the *B. cereus* exoproteome could reflect the growth phase-dependent activity of an intracellular Msr. Here, we show that *B. cereus* encodes a functional MsrAB methionine sulfoxide reductase that is responsible for the decrease of the Met(O) content of the *B. cereus* exoproteome during aerobic respiratory growth. In addition, our results provide evidence that Met residues in exoproteins, especially enterotoxins, and their recycling by MsrAB, can serve as an antioxidant system that could trap ROS and maintain redox homeostasis in cells.

## MATERIALS AND METHODS

### Construction of a $\Delta$ *msrAB* Mutant and Its Complementation

Mutant construction was performed according to the procedure developed by Arnaud et al. (2004). The *msrAB* ORF was interrupted by insertion of a non-polar spectinomycin resistance expression cassette, *spc* (Murphy, 1985) as follows. A DNA fragment of 1,413 bp encompassing the *msrAB* ORF was amplified from *B. cereus* genomic DNA by PCR with primers 5'-gaattcTCATGCCTTGAAAGTTACGG-3' and 5'-agatctTTG GCGTAACGGTAATTGGT-3', which contained *EcoRI* and *BglII* restriction sites, respectively. The amplified DNA fragment was cloned into pCRXL-TOPO (Invitrogen). The resulting pCRXL*msrAB* plasmid was digested with *StuI*. A 1.5 kb *SmaI* fragment containing *spc* was purified from pDIA (Laouami et al., 2011) and ligated into *StuI*-digested pCRXL*msrAB*. The resulting plasmid, pCRXL*msrAB* $\Delta$ *spc*, was digested with *EcoRI* plus *BglII*. The *msrAB* $\Delta$ *spc* fragment was then subcloned into *EcoRI/BglII* sites of pMAD (Arnaud et al., 2004). This construct was used for *B. cereus* transformation (Omer et al., 2015). For complementation of the  $\Delta$ *msrAB* mutant with wild-type *msrAB* gene, the 1,413 bp *EcoRI-BglII* fragment was cloned into pHT304 (Arantes and Lereclus, 1991). *MsrAB* is under the control of its own promoter into pHT304-*msrAB*.

### *B. cereus* Strains and Growth Conditions

Wild-type *B. cereus* ATCC 14579 without its pBclin15 plasmid (Madeira et al., 2016a,b), its  $\Delta$ *msrAB* mutant and  $\Delta$ *msrAB*/pHT304*msrAB* complemented strains were grown in MOD medium supplemented with 30 mM glucose as the carbon source, as previously described (Madeira et al., 2016b). The inoculum was a sample of exponential subculture harvested by centrifugation, washed and diluted in fresh medium to obtain an initial optical density at 600 nm of 0.02. Three independent batch cultures (biological replicates) were carried out at 37°C for each strain.

### Analytical Procedures and Growth Parameters

*B. cereus* growth was monitored spectrophotometrically at 600 nm. The specific growth rate ( $\mu$ ) was determined using the modified Gompertz equation (Zwietering et al., 1990). Cells and filtered culture supernatants were harvested at the indicated growth stage as previously described (Madeira et al., 2015, 2016b). Exoproteins were immediately precipitated from the culture supernatant using trichloroacetic acid (TCA), as previously described, and stored at 4°C until analysis. The concentrations of substrate, and by-products in the filtered culture supernatants were determined with Enzytec Fluid kits purchased from R-Biofarm, as described by the manufacturer. Exoprotein concentration was determined by the Bradford protein assay (Pierce).

### Protein Sample Preparation, Trypsin In-gel Proteolysis, and Nano-LC-MS/MS Analysis

Protein extraction and subsequent digestion were performed as previously described (Madeira et al., 2015). Extracellular and

intracellular proteins from the 27 samples (biological triplicates from the three time conditions for the wild-type,  $\Delta msrAB$  and  $\Delta msrAB/pHT304-msrAB$  strains) were resolved on NuPAGE® 4–12% Bis-Tris gels (Invitrogen) that were run for a short (about 3 mm) electrophoretic migration using NuPAGE MES supplemented with NPAGE antioxidant as the running buffer (Hartmann and Armengaud, 2014). This avoids artefactual protein oxidation. For each of the 54 protein samples, the whole protein content was extracted as a single polyacrylamide band. The bands were subjected to proteolysis with sequencing grade trypsin (Roche) following the ProteaseMAX protocol (Promega), as previously described (De Groot et al., 2009; Clair et al., 2010). NanoLC-MS/MS experiments were performed using an LTQ-Orbitrap XL hybrid mass spectrometer (ThermoFisher) coupled to an Ultimate 3000 nRSLC system (Dionex, ThermoFisher; Dedieu et al., 2011; Madeira et al., 2015).

## Peptide and Protein Identification from MS/MS Datasets

MS/MS spectra were searched against an in-house polypeptide sequence database corresponding to an improved annotation of the *B. cereus* ATCC 14,579 genome (Madeira et al., 2016a). The MASCOT Daemon search engine (version 2.3.02; Matrix Science) was used to search tryptic peptides as previously described (Dupierris et al., 2009; Madeira et al., 2016a). The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository (<http://www.ebi.ac.uk/pride>) with the dataset identifiers, PXD006169 and 10.6019/PXD006169 (exoproteome) and, PXD006205 and 10.6019/PXD006205 (cellular proteome).

## Label-Free Comparative Proteomics

Analyses of changes of peptides and proteins in terms of abundance were achieved by comparing the spectral counts of proteins after voom transformation of abundance values using the R package LIMMA (Ritchie et al., 2015), as previously described (Madeira et al., 2016b). Data were normalized using the trimmed mean of *M*-values (TMM), implemented in the R package edgeR (Robinson et al., 2010). For quantitative comparisons, data were filtered to have two valid values in at least two biological replicates. Since we were specifically interested in the comparison between wild-type,  $\Delta msrAB$  mutant and the complemented strain  $\Delta msrAB/pHT-msrAB$ , we conducted differential analysis between WT and  $\Delta msrAB$ , as well as  $\Delta msrAB$  and  $\Delta msrAB/pHT-msrAB$ , and WT and  $\Delta msrAB/pHT-msrAB$ , individually. Differential protein and peptide abundances between WT and  $\Delta msrAB$ , between  $\Delta msrAB$  and  $\Delta msrAB/pHT-msrAB$ , and between WT and  $\Delta msrAB/pHT-msrAB$  were considered significant at stringent *p*-values ( $\leq 0.01$ ). The results are presented as log<sub>2</sub> fold-changes.

## Real-time RT-PCR and 5'RACE Assays

Total RNA was prepared as described previously (Omer et al., 2015). Real-time RT-PCR was performed using the iScript™ One-Step RT-PCR kit with SYBR® Green following the manufacturer's protocol (Biorad). The *msrAB*-specific primer

pair used in this study was: 5'-TTCTGGTACACAGGTGGTC-3' and 5'-AAAGCGTCCACTCTGCTCAA-3'. Gene expression was normalized by the  $\Delta\Delta CT$  analysis. The 16s rDNA was used as the reference gene in the calculations. The 16S rDNA-specific primer pair was 5'-TCCAAGTATGATGGCGGAC-3' and 5'-TCACGCCAGATTCTTTTTC-3'. Rapid amplification of 5' complementary cDNA ends (5'RACE) was performed using the 5'/3' RACE kit (Sigma). The *msrAB* specific primers SP1, SP2 and SP3 were: 5'-ATGTCCCGTCGTTTCTGAAC-3, 5'-TCAAATGGCGAAACCATACA-3' and 5'-CCATACACCAGAAGCACCCT-3', respectively.

## Protease Activity Assay

Sigma's non-specific protease activity assay was used to determine the protease activity of filtered culture supernatant. In this assay, casein acts as a substrate. Tyrosine, which is released on hydrolysis of casein by proteases, is able to react with Folin-Ciocalteu's reagent to produce a blue chromophore. The quantity of this chromophore was measured by means of its absorbance value by spectrophotometry. Absorbance values generated by the activity of the protease were compared to a standard curve, which was generated on the basis of known quantities of tyrosine. From the standard curve, the activity of protease samples was determined in units, corresponding to the amount in micromoles of tyrosine equivalents released from casein per minute. Experiments were performed twice for each of the 27 filtered culture supernatants. Statistical differences were evaluated by the Student's *t*-test.

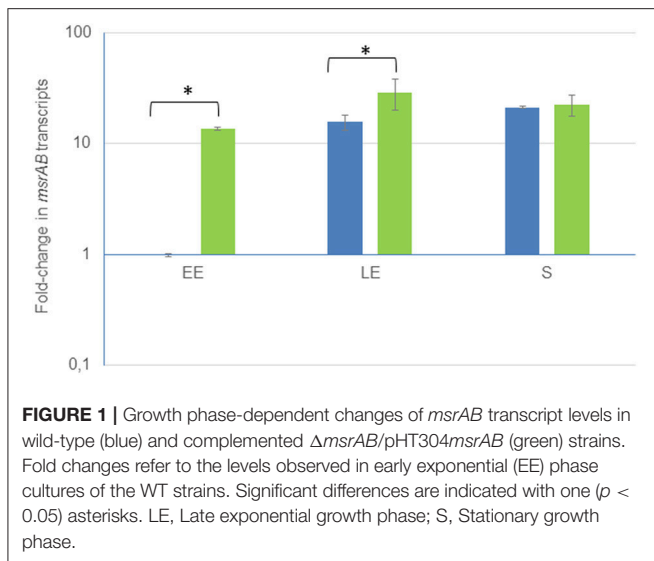
## Long-Term Survival

The survival of WT,  $\Delta msrAB$  mutant, and complemented  $\Delta msrAB$  mutant were determined as follows. After 24 h incubation at 37°C on glucose containing MOD medium, cultures were transferred to 4°C. An aliquot of each culture was collected before and after 1, 2, 3, 4, and 5 days of exposure to 4°C. Viable cells were determined by serial dilution of cultures in PBS, plating on LB agar, and incubation overnight (37°C). Experiments were performed in triplicate. Statistical differences were evaluated by the Student's *t*-test.

## RESULTS

### *msrAB* Expression Is Growth Phase Dependent

Genome analyses of *B. cereus* ATCC 14579 identified an ORF (BC\_5436) encoding a cytoplasmic protein annotated as MsrAB (NP\_835097). This predicted cytoplasmic protein is composed of 321 amino acids and has a molecular weight of 36,938 Da. MsrAB and its gene *msrAB* are strongly conserved in members of the *B. cereus* group (data not shown). We mapped the transcriptional start site of *msrAB* by 5'RACE. The transcriptional start site (G) was located 23 nt upstream of the translational start codon and was preceded by a region similar to  $\sigma E$  consensus-35 (TAATATG) and -10 (CATACTG) boxes separated by 13 nt. Furthermore, *msrAB* appeared to be followed by an inverted repeat ( $\Delta G^\circ = 23.6$  kcal/mol) that may be a transcriptional terminator (Figure S1). This indicates that *msrAB* may be transcribed as a single unit.



To determine whether there is any regulation of *msrAB*, mRNA levels were measured at early exponential (EE), late exponential (LE) and stationary (S) growth phases. **Figure 1** shows that there was about a 30-fold increase in *msrAB* expression for cells harvested at the S growth phase compared with the EE growth phase. *B. cereus* *msrAB* expression was thus maximal over the stationary phase. Similar stationary phase-induced expression of *msr* genes has been documented in several bacteria (Moskovitz et al., 1995; Vattanaviboon et al., 2005; Alamuri and Maier, 2006; Singh and Singh, 2012).

## MsrAB Contributes to *B. cereus* Respiratory Metabolism

To investigate the role of MsrAB in *B. cereus*, we constructed a non-polar  $\Delta msrAB$  mutant and a  $\Delta msrAB$ -complemented strain using a multicopy pHT304-based plasmid (Arantes and Lereclus, 1991). Expression of *msrAB* in the complemented strain was under the control of its own promoter. We did not detect *msrAB* mRNA by RT-PCR in the mutant, proving that the genomic disruption of the gene generated a *msrAB*-null mutant. **Figure 1** shows that *msrAB* was overexpressed in the strain  $\Delta msrAB/pHT304msrAB$  at the EE and LE growth phases. Therefore, *msrAB* expression level was not restored by complementation.

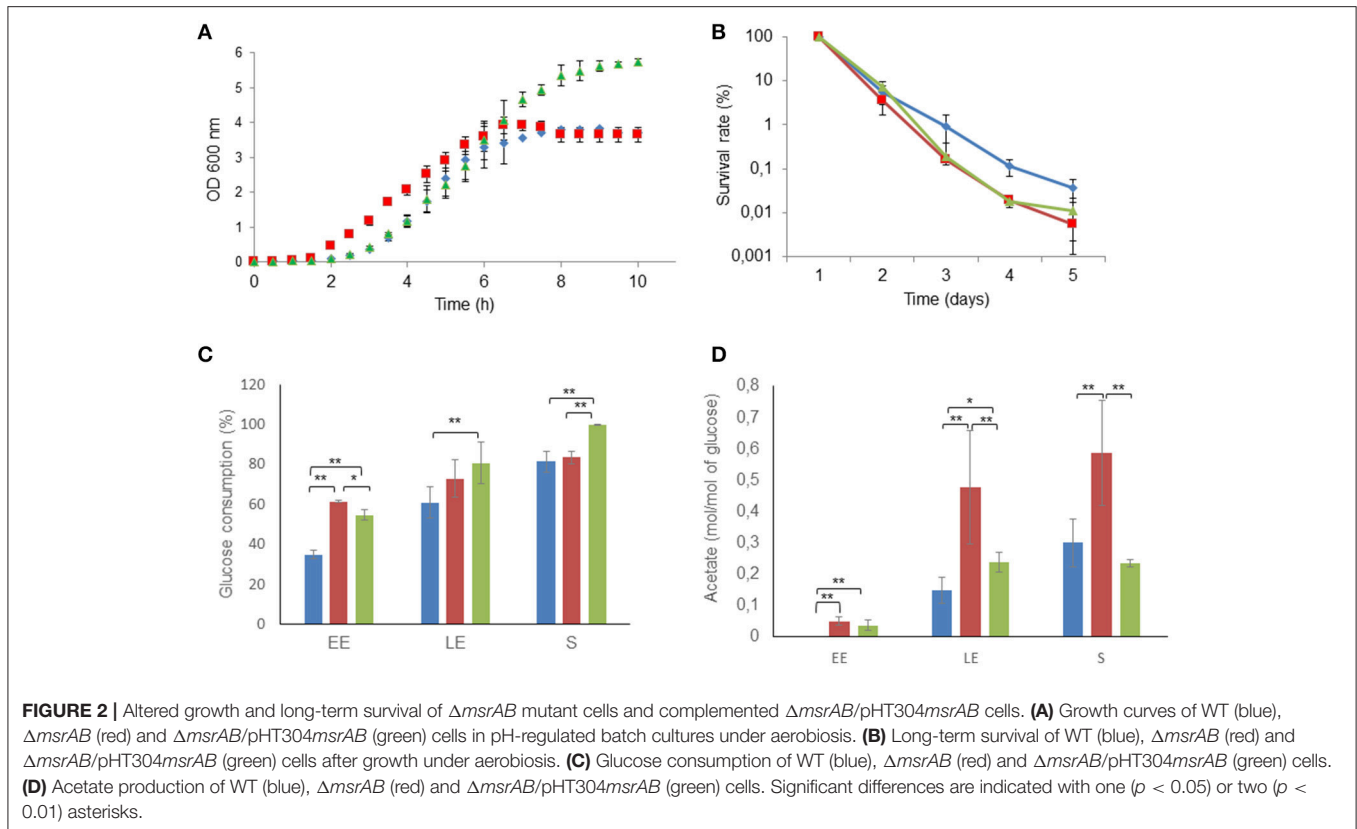
The growth characteristics of the three strains,  $\Delta msrAB$ ,  $\Delta msrAB/pHT304msrAB$ , and the parental wild-type strain (WT), were determined under pH-regulated aerobic respiratory conditions in synthetic MOD medium. **Figure 2A** shows that the lag phase was 2.5-fold lower in the  $\Delta msrAB$  strain ( $0.7 \pm 0.1 \text{ h}^{-1}$ ) than in the  $\Delta msrAB/pHT304msrAB$  ( $1.8 \pm 0.9 \text{ h}^{-1}$ ) and WT ( $1.9 \pm 0.2 \text{ h}^{-1}$ ) strains. Exponential growth kinetics were similar in the three strains for the first 6 h. After this initial growth time, WT and  $\Delta msrAB$  cultures entered stationary phase. In contrast,  $\Delta msrAB/pHT304msrAB$  continued to grow and reached the stationary growth phase at a higher final biomass ( $2.6 \pm 0.1 \text{ g.L}^{-1}$ ) than  $\Delta msrAB$  ( $1.9 \pm 0.1 \text{ g.L}^{-1}$ ) and WT ( $1.8 \pm 0.2 \text{ g.L}^{-1}$ ). The viabilities of  $\Delta msrAB$  and

$\Delta msrAB/pHT304msrAB$  cells, harvested at S growth phase, were similar to the viability of WT after 2 days but declined by more than 100-fold after 5 days of storage at  $4^\circ\text{C}$  (**Figure 2B**). This suggests that *msrAB* expression impacts the metabolic activity of *B. cereus* cells at the end of growth (Chubukov and Sauer, 2014). **Figure 2C** shows that the  $\Delta msrAB$  and  $\Delta msrAB/pHT304msrAB$  strains consumed higher amounts of glucose than WT at the beginning of exponential growth. The  $\Delta msrAB/pHT304msrAB$  culture could be distinguished from the  $\Delta msrAB$  culture by continued glucose consumption between the LE and S growth phases (**Figure 2C**). At the end of growth,  $\Delta msrAB/pHT304msrAB$  consumed a higher level of glucose than  $\Delta msrAB$  and WT. During aerobic respiratory growth, glucose is catabolized into  $\text{CO}_2$  through the TCA cycle, and acetate is excreted as a by-product of overflow metabolism (Madeira et al., 2015; Duport et al., 2016). **Figure 2D** shows that  $\Delta msrAB$  cells, and to a lesser extent  $\Delta msrAB/pHT304msrAB$  cells, excreted higher amounts of acetate than WT cells during exponential growth. Acetate accumulation stopped at the LE growth phase in the  $\Delta msrAB$  and  $\Delta msrAB/pHT304msrAB$  cultures while it continued to accumulate between the LE and S growth phases in the WT culture. Taken together, these results suggest that *msrAB* expression impacts on the metabolic activity of *B. cereus* under aerobiosis.

To determine whether the alteration of glucose catabolism was associated with changes in extracellular protein production, extracellular proteins were extracted from culture supernatants of the three *B. cereus* strains, harvested during the EE, LE, and S growth phases (Madeira et al., 2015). **Figure 3A** shows that the  $\Delta msrAB$  culture supernatant accumulated a higher amount of exoproteins than that of WT at the LE phase. However,  $\Delta msrAB$  supernatant had 50 and 90% fewer exoproteins in the EE and S growth phases, respectively, compared with WT. This decreased exoprotein concentration could have resulted from a higher protease activity in the  $\Delta msrAB$  culture supernatant. To test this hypothesis, we quantified the protease activity of the  $\Delta msrAB$ ,  $\Delta msrAB/pHT304msrAB$  and WT culture supernatants against casein. **Figure 3B** shows that the  $\Delta msrAB$  culture supernatant sustained a higher protease activity than WT, markedly in the EE and S growth phases. These changes in protease activity were only partially rescued in  $\Delta msrAB/pHT304msrAB$ . However, unlike  $\Delta msrAB/pHT304msrAB$ , there was no correlation between the protease activity and the amount of exoproteins in  $\Delta msrAB$  at LE phase (**Figure 3A**). This indicates that changes in *msrAB* expression could be selective for certain extracellular proteases.

## MsrAB Modulates the Proteome Profile of *B. cereus*

To determine if altered metabolism in  $\Delta msrAB$  and  $\Delta msrAB/pHT304msrAB$  was associated with cellular and exoproteome profile changes, we quantified protein abundance level differences between  $\Delta msrAB$ ,  $\Delta msrAB/pHT304msrAB$ , and WT cells in the EE, LE and S growth phases. Exoproteome and cellular proteome samples were prepared from supernatant cultures and whole-cell lysates, respectively. A total of 200,746 and 71,676 MS/MS spectra were recorded from cellular proteome and exoproteome samples, respectively. A total of 922 proteins were identified in the cellular proteome (Table S1) and 371

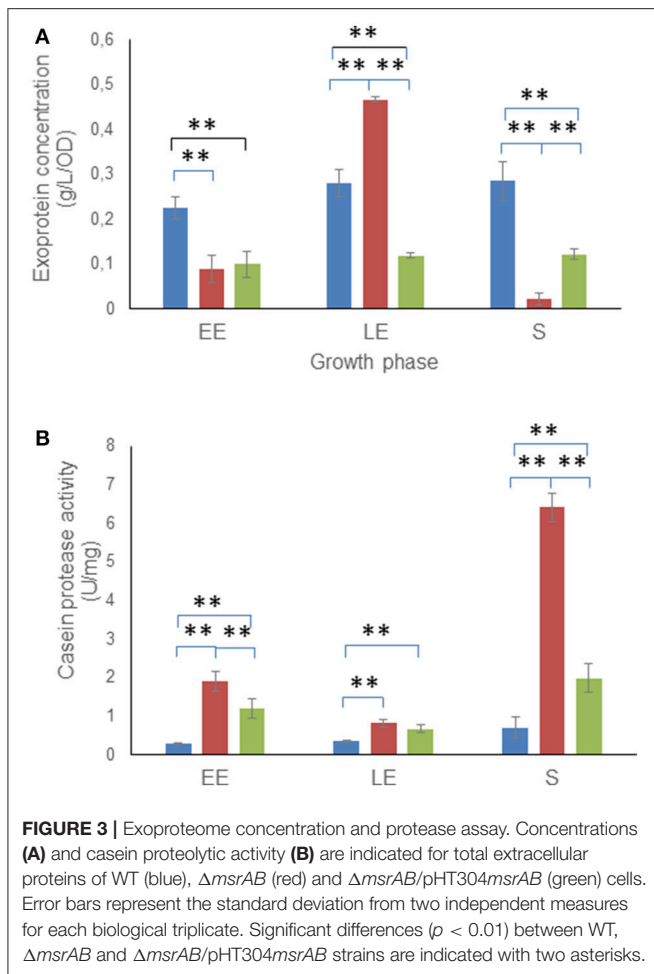


proteins were identified in the exoproteome (Table S2), based on the confident detection of at least two different peptides. A two-sample *t*-test was then conducted separately between WT and  $\Delta msrAB$ , and between  $\Delta msrAB$  and  $\Delta msrAB/pHT304msrAB$ . All proteins with a  $p \leq 0.01$  and at least a 2-fold change ( $\log_2$  fold-change  $\geq 1$ ) were considered to be differentially modulated in terms of abundance. A total of 64 and 78 proteins were found to vary in abundance in  $\Delta msrAB$  compared with WT in the cellular proteome and exoproteome fractions, respectively. The majority (80%) of these proteins were not rescued in  $\Delta msrAB/pHT304msrAB$  (data not shown). The Venn diagrams presented in **Figure 4** show the growth phase distribution of the identified proteins. Less than 2% of proteins showed abundance level changes in all three growth stages, indicating that *msrAB* modulates *B. cereus* cellular and exoproteome mainly in a growth phase-dependent manner. The impact of *msrAB* disruption appeared to be more important at the LE and S than the EE growth phase in the cellular proteome (**Figure 4A**), according to its expression (**Figure 1**).

### Cellular Proteins

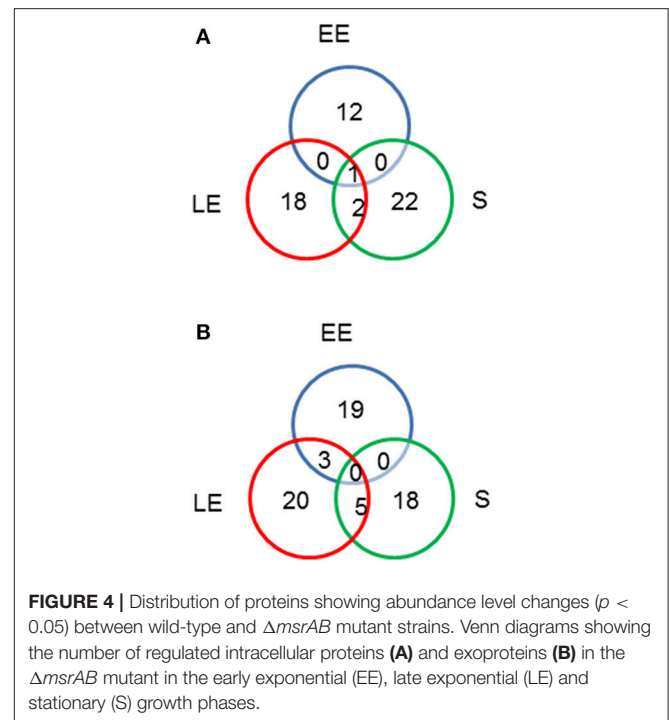
**Table 1** lists the identities and putative functions of the cellular proteins differentially produced in  $\Delta msrAB$  compared with WT. In the EE phase, three proteins impacted by *msrAB* disruption were classified as carbohydrate metabolism enzymes. The glycolytic enzyme, Tpi (triose phosphate isomerase), and the two TCA enzymes, Mqo (malate:quinone oxidoreductase) and FumB (fumarate hydratase), were less abundant in  $\Delta msrAB$

than WT. Tpi catalyzes the interconversion of dihydroacetone phosphate (DHAP) and glyceraldehyde-3-phosphate to prevent DHAP accumulation. It has been shown that a reduction of Tpi activity redirected the carbon flux from glycolysis to the pentose phosphate pathway (PPP), which provides the redox power for antioxidant systems (Ralser et al., 2007). FumB catalyzes the reversible hydration of fumarate to malate, and Mqo oxidizes malate to oxaloacetate and reduces quinone via a one-transfer electron reaction (Kabashima et al., 2013). By decreasing FumB and Mqo levels,  $\Delta msrAB$  cells can thus decrease TCA activity, and consequently respiratory chain activity and ROS production. The reduction of respiratory chain capacity could be compensated by increasing glycolytic flux and increasing overflow metabolism (acetate excretion), as observed in **Figure 2**. Only one stress response-related protein (USP) was shown to be differentially produced in  $\Delta msrAB$  at EE phase. As recently reported, USP may function as a protein regulator of downstream effectors of nucleotide-binding protein cycling. This activity depends on the energy status (ATP level) of the cells (Banerjee et al., 2015). A decrease in the UPS abundance level in  $\Delta msrAB$  could thus reflect a change in ATP availability and/or demand at EE phase.  $\Delta msrAB$  also sustained a higher level of RibD whatever the growth phase. The gene encoding RibD belongs to the putative operon *ribDEAH*, which encodes RibD, a pyrimidine deaminase/reductase, RibE, the  $\alpha$ -subunit of riboflavin synthase, RibA, the GTP cyclohydrolase/3,4-dihydroxy 2-butanone 4-phosphate (3,4-DHBP) synthase, and RibH, the  $\beta$ -subunit of riboflavin (RibH). These enzymes form a pathway that produces



one riboflavin molecule from GTP and ribulose-5-phosphate (Vitreschak et al., 2002). RibA and RibH were more highly produced in  $\Delta msrAB$  than in WT at LE growth phase and RibE was more highly produced at S growth phase. Together these results suggest the increased production in  $\Delta msrAB$  of riboflavin, which is known to be an element of antioxidant defense (Abbas and Sibirny, 2011). One stress-related protein, named AcpD (annotated as an azoreductase), which was not detected in WT cells (Table S1), was significantly induced at both LE and S growth phases in  $\Delta msrAB$  cells. AcpD is a putative FMN-NAD(P)H-dependent quinone oxidoreductase that catalyzes the two-electron reduction of quinones to quinols. This protein could play an important role in managing oxidative stress in the absence of *msrAB* by maintaining the reduced antioxidant form of quinone (Ross et al., 2000; Ryan et al., 2014). Several proteins related to the biosynthesis of amino acids were upregulated at the LE and S phases. This suggests that an increase in the intracellular content of these amino acids may be part of the adaptive response to the lack of MsrAB.

A protein was considered validated when at least two different peptides were found in the same sample. We found only one peptide assigned to MsrAB and did not validate its presence in the cellular proteome. To determine whether MsrAB is a



true cellular protein, we carried out further analyses using a Q-exactive HF mass spectrometer. Five and 19 peptides assigned to MsrAB were detected in the cellular proteome of WT and  $\Delta msrAB/pHT304msrAB$ , respectively, at LE and S growth phases (Figure S1). No peptide was detected in the exoproteome, proving that MsrAB is cytoplasmic.

## Exoproteome

Table 2 lists the exoproteins that were considered as differentially produced in  $\Delta msrAB$  supernatant. The majority of the metabolism and stress/detoxification-related proteins were less abundant in  $\Delta msrAB$  compared with WT, regardless of growth phase. These proteins were predicted to be cytosolic and, accordingly, we found that they were more abundant in the cellular proteome compared with the exoproteome (Table S3). In contrast, the majority of the cell wall/surface-associated proteins, transporters and degradative/adhesin proteins, which were predicted to be secreted proteins, were increased in  $\Delta msrAB$  compared with WT, especially at the EE and LE growth phases. This suggests that *msrAB* deletion could favor the accumulation of some secreted exoproteins at the expense of cytosolic proteins. Interestingly, two predicted secreted foldases, PrsA1 and PrsA2, showed significant increases in their abundance levels in  $\Delta msrAB$ , especially at LE growth phase. PrsA1 and PrsA2 have been predicted to function as peptidyl-prolyl isomerases at the bacterial membrane-cell wall interface, to assist in the folding and stability of exported proteins (Vitikainen et al., 2004). In addition, we noted increased abundance levels of a bacterial type I signal peptidase protein (SPase) in  $\Delta msrAB$  compared with WT at LE phase. SPases function at the terminal step of the general secretory pathway by releasing translocated proteins from the

**TABLE 1** | Cellular proteins with significant abundance level changes ( $|\log_2|\text{fold-change} > 1, p < 0.01$ ) in  $\Delta\text{msrAB}$  compared with WT.

| Functional class                      | NP no.                 | Gene no.         | Protein name  | Protein description  | Log <sub>2</sub> fold-change        |       |       |
|---------------------------------------|------------------------|------------------|---------------|--|-------------------------------------|-------|-------|
|                                       |                        |                  |               |  | EE                                  | LE    | S     |
| Carbohydrate metabolism               | <b>NA</b>              | <b>BC5137</b>    | <b>Tpi</b>    | <b>Triosephosphate isomerase</b>                           | -1.50                               |       |       |
|                                       | NP_834982              | BC5320           | Ccr           | PTS system, glucose-specific IIA component                 |                                     | 1.82  |       |
|                                       | NP_834343              | BC4637           | Ack           | acetate kinase   |                                     |       | 1.09  |
|                                       | NP_832706              | BC2959           | Mqo           | Malate:quinone oxidoreductase                              | -2.82                               |       |       |
|                                       | NP_831487              | BC1712           | FumB          | Fumarate hydratase   | -2.57                               |       |       |
|                                       | NP_833692              | BC3973           | PdhA          | Pyruvate dehydrogenase E1 component alpha subunit          |                                     |       | 1.02  |
|                                       | NP_833555              | BC3834           | SucC          | Succinyl-CoA synthetase subunit beta                       |                                     |       | 1.05  |
|                                       | Enterotoxin            | NP_834610        | BC5239        | EntA   | Enterotoxin A                       |       |       |
| Lipid metabolism                      | <b>NP_830401</b>       | <b>BC0584</b>    |               | <b>Acetyltransferase</b>                                   |                                     |       | -2.04 |
| Cell wall and cell surface metabolism | NP_830495              | BC0682           | SrtA          | Sortase  |                                     | -3.01 |       |
|                                       | NP_834255              | BC4548           | IsdA1         | Cell surface protein                                       |                                     |       | -4.19 |
| Purine metabolism                     | NP_832069              | BC2306           | BacF          | Glycine-AMP ligase   |                                     | -3.94 |       |
|                                       | <b>NP_831124</b>       | <b>BC1343</b>    | <b>QueE</b>   | <b>Organic radical activating protein</b>                  |                                     |       | 2.63  |
|                                       | <b>NP_831122</b>       | <b>BC1341</b>    | <b>QueC</b>   | <b>Aluminum resistance protein</b>                         |                                     |       | 3.28  |
| Pyrimidine metabolism                 | NP_833606              | BC3886           | CarB          | Carbamoyl phosphate synthase large subunit                 |                                     | -4.35 |       |
|                                       | NP_833803              | BC4085           | Pdp           | Pyrimidine-nucleoside phosphorylase                        |                                     | 2.14  |       |
| DNA binding and repair                | NP_831634              | BC1861           |               | Helicase   | -3.15                               |       |       |
|                                       | NP_831628              | BC1855           |               | Chromosome segregation ATPase                              | -2.46                               |       |       |
|                                       | NP_834171              | BC4459           | HsdM          | Type I restriction-modification system methylation subunit |                                     | -2.51 |       |
|                                       | NP_831628              | BC3769           | MutS          | DNA mismatch repair protein                                |                                     | -2.58 |       |
| Aminoacid metabolism                  | NP_833492              | BC1546           | Aat           | Aspartate aminotransferase                                 |                                     |       | 1.74  |
|                                       | NP_831735              | BC1965           | ThrC          | Threonine synthase   |                                     | 3.41  | 3.13  |
|                                       | NP_831736              | BC1966           | ThrB          | Homoserine kinase  |                                     | 2.64  | 3.30  |
|                                       | NP_832070              | BC2307           |               | Glycine-AMP ligase   |                                     | -3.31 |       |
|                                       | NP_831552              | BC1779           | IlvC2         | Ketol-acid reductoisomerase                                |                                     |       | 1.58  |
|                                       | <b>NP_831190</b>       | <b>BC1410</b>    | <b>HisF</b>   | <b>Imidazole glycerol phosphate synthase subunit HisF</b>  |                                     | 2.05  |       |
|                                       | <b>NP_831186</b>       | <b>BC1406</b>    | <b>HisD</b>   | <b>Histidinol dehydrogenase</b>                            |                                     |       | 3.41  |
|                                       | NP_831734              | BC1964           | Hom1          | Homoserine dehydrogenase                                   |                                     | 2.86  | 2.35  |
|                                       | <b>NP_830438</b>       | <b>BC4331</b>    | <b>AroE</b>   | <b>Shikimate 5-dehydrogenase</b>                           | 2.06                                |       |       |
|                                       | Amino sugar metabolism | NP_834865        | BC5201        | MnaA   | UDP-N-acetylglucosamine 2-epimerase |       |       |
| Translation                           | <b>NP_831277</b>       | <b>BC1498</b>    | <b>RrpsA</b>  | <b>30S ribosomal protein S1</b>                            | -3.21                               |       |       |
|                                       | <b>NP_830015</b>       | <b>BC0135</b>    | <b>RpsS</b>   | <b>SSU ribosomal protein</b>                               | 2.06                                |       |       |
| Motility                              | NP_831407              | BC1629           | CheC          | Flagellar motor switch protein                             |                                     | -2.38 |       |
|                                       | NP_831428              | BC1651           | FglE          | Flagellar hook protein                                     |                                     |       | -3.26 |
|                                       | NP_831435              | BC1658           | FlaB          | Flagellin  |                                     | -2.30 |       |
|                                       | NP_831415              | BC1637           | FlgL          | Flagellar hook-associated protein                          |                                     |       | -3.28 |
|                                       | NP_834158              | BC4446           | MreB          | Rod shape-determining protein                              |                                     |       | 1.05  |
| Rod shape-determining proteins        | NP_834531              | BC4831           |               | ABC transporter ATP-binding protein                        |                                     | 3.67  |       |
| Transporters                          | NP_834524              | BC4824           |               | ABC transporter ATP-binding protein                        |                                     | -2.11 |       |
|                                       | NP_833512              | BC3790           |               | Nucleoside transport ATP-binding protein                   |                                     | -3.36 |       |
|                                       | NP_830967              | BC1182           | OppD          | Oligopeptide transport ATP-binding protein                 |                                     | -1.78 |       |
|                                       | NP_832817              | BC3071           | CutC          | copper homeostasis protein cutC                            |                                     |       | 3.00  |
|                                       | <b>NP_834331</b>       | <b>BC4625</b>    | <b>UspA</b>   | <b>Universal stress protein</b>                            | -3.19                               |       |       |
|                                       | Stress response        | <b>NP_835071</b> | <b>BC5410</b> | <b>AcpD</b>  | <b>Azoreductase</b>                 |       | 4.87  |
| Chaperones                            | NP_830954              | BC1168           | ClpB          | ATP-dependent chaperone                                    | -2.99                               |       |       |
|                                       | NP_830829              | BC1043           | PrsA1         | Peptidylprolyl isomerase                                   |                                     |       | 1.08  |
|                                       | NP_833827              | BC4109           | RibD          | Diaminohydroxyphosphoribosylaminopyrimidine deaminase      | 2.78                                | 2.92  | 4.42  |

(Continued)

TABLE 1 | Continued

| Functional class                | NP no.           | Gene no.      | Protein name | Protein description  | Log <sub>2</sub> fold-change |      |       |
|---------------------------------|------------------|---------------|--------------|--|------------------------------|------|-------|
|                                 |                  |               |              |  | EE                           | LE   | S     |
| Riboflavin biosynthesis         | NP_833829        | BC4111        | RibA         | Bifunctional 3,4-dihydroxy-2-butanone 4-phosphate synthase |                              | 2.87 |       |
|                                 | <b>NP_833828</b> | <b>BC4110</b> | <b>RibE</b>  | <b>Riboflavin synthase subunit alpha</b>                   |                              |      | 2.20  |
|                                 | NP_833830        | BC4112        | RibH         | Riboflavin synthase subunit beta                           |                              | 1.68 |       |
|                                 | NP_833832        | BC4114        | BioB         | Biotin synthase  |                              |      | 3.40  |
| Biotin biosynthesis             | <b>NP_831123</b> | <b>BC1342</b> |              | <b>6-pyruvoyl tetrahydrobiopterin synthase</b>             | 2.16                         |      |       |
| Folate biosynthesis             | NP_833540        | BC3819        | Dxr2         | 1-deoxy-D-xylulose 5-phosphate reductoisomerase            | 2.75                         |      |       |
| Terpenoid backbone biosynthesis | NP_831099        | BC1317        | PhaB         | Acetoacetyl-CoA reductase                                  | -2.53                        |      |       |
| Uncategorized                   | NP_829927        | BC0025        |              | Unknown  |                              | 2.67 |       |
|                                 | NP_832675        | BC2927        |              | Prolyl endopeptidase                                       |                              |      | 4.18  |
|                                 | NP_831667        | BC1894        |              | Phage protein  | -2.84                        |      |       |
|                                 | NP_831673        | BC1901        |              | phage protein  |                              |      | -2.43 |
|                                 | NP_834610        | BC4938        |              | NADH dehydrogenase   |                              | 2.03 |       |
|                                 | NP_834043        | BC0622        |              | L-threonine 3-dehydrogenase                                |                              |      | 2.14  |
|                                 | NP_830802        | BC1016        |              | Unknown  |                              | 3.04 |       |
|                                 | NP_834559        | BC4860        |              | Unknown  |                              |      | -1.97 |
|                                 | NP_829986        | BC0105        |              | Unknown  |                              |      | 1.87  |
|                                 | NP_834083        | BC4371        |              | Unknown  |                              |      | 1.57  |

Proteins showing abundance level restored in  $\Delta msrAB/pHT304msrAB$  are indicated in bold. EE, early exponential growth phase; LE, late exponential growth phase; S, stationary growth phase. NA, Not Annotated. Green and red highlights indicate increased and decreased protein levels, respectively.

cytoplasmic membrane at a defined cleavage site (Craney et al., 2015). This Spase could thus function in conjunction with PrsA proteins to sustain a higher secretion level of some proteins (Alonzo et al., 2011).

Several proteins classified as degradative enzymes showed higher abundance levels in  $\Delta msrAB$  compared with WT (Table 2) and could contribute to the high protease activity of the  $\Delta msrAB$  extracellular milieu (Figure 3B). Interestingly, we showed that the abundance level of Npr600, a predicted bacillolysin, was restored in  $\Delta msrAB/pHT304msrAB$  at LE phase. Npr600 could thus be a major contributor to the protease activity of  $\Delta msrAB$  at LE phase (Altincicek et al., 2007).

### ***msrAB* Regulates the Dynamic of the Met(O) Content of the *B. cereus* Proteome**

We identified peptides with oxidized Met in  $\Delta msrAB$ ,  $\Delta msrAB/pHT304msrAB$ , and WT, in both the cellular proteome and the exoproteome at EE, LE, and S growth phases, as previously described (Madeira et al., 2015). The Met(O) content of both the cellular proteome and the exoproteome was estimated by comparing the number of Met(O) to the total number of Met residues identified in each of the three biological samples obtained for each growth phase in each of the three strains (Tables S4, S5). Figure 5 shows that the Met(O) content of WT and  $\Delta msrAB/pHT304msrAB$  decreased similarly in the cellular proteome (Figure 5A) and exoproteome (Figure 5B) during growth. The Met(O) content of the  $\Delta msrAB$  intracellular proteome also decreased during exponential growth and was lower than the Met(O) content of WT at LE phase and higher

at S growth phase. More importantly, the Met(O) content of the  $\Delta msrAB$  exoproteome remained constant during growth and accounted for  $38 \pm 3\%$  of total Met residues. Taken together, these results indicate that MsrAB regulates the dynamic of the Met(O) content of the proteome, especially at the exoproteome level.

To identify peptides exhibiting significant differences in Met(O) content in  $\Delta msrAB$  compared with WT, we conducted a *t*-statistical analysis. For a robust analysis, we considered a peptide as containing an oxidized Met residue when it was identified in at least two biological replicates. The lists of intra- and extracellular peptides showing significant Met(O) level changes ( $\log_2$  fold-change > 1 and  $p \leq 0.01$ ) according to growth phase are presented in Tables 3, 4.

### **Cellular Proteome**

The number of peptides with Met(O) content changes was lower at the EE (6) than the LE (13) and S (19) growth phases. Only one peptide, a RibH-related peptide, showed similar changes in the two growth phases (Table 3). At the EE growth phase, we noted that the subunit E2 of the pyruvate dehydrogenase complex (PdhC), which interconnects glycolysis with acetate metabolism, had one peptide with a decreased Met(O) level in EE phase. This could impact the activity of this enzyme and contribute to the metabolic perturbation observed in  $\Delta msrAB$  at EE phase (Figure 2; Martin et al., 2005). At the LE growth phase, the majority of the identified peptides showed a lower Met(O) content in  $\Delta msrAB$  compared with WT at LE phase. This is consistent with the results presented in Figure 4. At



**TABLE 2** | Exoproteins with significant abundance level changes ( $|\log_2(\text{fold-change})| > 1, p < 0.01$ ) in  $\Delta\text{msrAB}$  compared with WT.

| Functional class                               | NP no.           | Gene no.         | Protein name  | Protein description                                | Log <sub>2</sub> fold-change             |       |       |  |
|--|------------------|------------------|---------------|--|--|-------|-------|--|
|  |                  |                  |               |  | EE                                       | LE    | S     |  |
| Metabolism                                     | NP_833767        | BC4049           | HPr           | Phosphocarrier protein HPr                         |  |       | -2.24 |  |
| Carbohydrate                                   | NP_834306        | BC4600           | Pfk           | 6-phosphofructokinase                              | -3.09                                    |       |       |  |
|  | NP_833689        | BC3970           | PdhD          | Dihydrolipoamide dehydrogenase                     |  | -2.43 |       |  |
|  | <b>NA</b>        | <b>BC5138</b>    | <b>Pgk</b>    | <b>Phosphoglycerate kinase</b>                     |  | -1.79 |       |  |
|  | NP_834571        | BC4898           | Pgi           | Glucose-6-phosphate isomerase                      |  | -2.30 |       |  |
|  | NP_833346        | BC3616           | Acn           | Aconitate hydratase                                | -3.30                                    |       |       |  |
| Fatty acid and phospholipid                    | NP_833485        | BC3761           | PlcA          | 1-phosphatidylinositol phosphodiesterase precursor |  | 1.15  |       |  |
| Amino acids                                    | NP_830183        | BC0344           | RocA          | 1-pyrroline-5-carboxylate dehydrogenase            | -3.18                                    |       |       |  |
|  | <b>NA</b>        | <b>BC3705</b>    | <b>GlnA1</b>  | <b>Glutamine synthetase, type I</b>                |  | -3.45 |       |  |
|  | NP_834978        | BC5316           | GlyA          | Serine hydroxymethyltransferase                    | -2.58                                    |       |       |  |
|  | NP_831022        | BC1238           | TrpA          | Tryptophan synthase subunit alpha                  | -2.21                                    | -2.67 |       |  |
|  | <b>NP_833521</b> | <b>BC3799</b>    | <b>Asd</b>    | <b>Aspartate-semialdehyde dehydrogenase</b>        |  | -1.53 | 2.30  |  |
|  | NP_834652        | BC4981           | DcyD          | Cysteine desulfhydrase                             | -1.87                                    |       |       |  |
|  | <b>NP_830053</b> | <b>BC0185</b>    | <b>RocF</b>   | <b>Arginase</b>                                    |  | -1.57 |       |  |
|  | NP_830056        | BC0188           | GlmM          | Phosphoglucosamine mutase                          |  |       | 3.81  |  |
| Amino sugar and nucleotide sugar               |                  |                  |               |  |  |       |       |  |
| Nucleotide                                     | NP_835123        | BC5468           | AdSS          | Adenylosuccinate synthetase                        | -2.53                                    |       |       |  |
| Butanoate                                      | NP_831099        | BC1317           | PhaB          | Acetoacetyl-CoA reductase                          | -2.56                                    |       |       |  |
| Gamma Hexachlorocyclohexane degradation        | NP_834220        | BC4511           | LppC          | Acid phosphatase                                   |  |       | -2.34 |  |
| Ubiquinone and other terpenoid-quinone         | NP_832068        | BC2305           | DhbB          | Isochorismatase                                    | 2.45                                     |       |       |  |
| Toxins   | NP_832699        | BC2952           | EntB          | Enterotoxin/cell-wall binding protein              | 2.25                                     |       |       |  |
|  | NP_832844        | BC3101           | HblB          | Hemolysin BL binding component precursor           |  | 1.69  |       |  |
|  | NP_833256        | BC3523           | HlyII         | Hemolysin II                                       |  | -1.78 |       |  |
| Degradative enzymes & adhesins                 | NP_83404223      | BC4514           | VanY4         | D-alanyl-D-alanine carboxypeptidase                | 1.77                                     |       |       |  |
|  | NP_833486        | BC3762           | Sfp           | subtilisine like serine protease                   | 3.14                                     |       |       |  |
|  | <b>NP_830673</b> | <b>BC0887</b>    | <b>CnaA</b>   | <b>Collagen adhesion protein</b>                   | 2.96                                     |       | 3.19  |  |
|  | NP_831437        | BC1660           | MltB          | Soluble lytic murein transglycosylase              | 1.65                                     |       |       |  |
|  | NP_835018        | BC5357           | CnaC          | Collagen adhesion protein                          | 1.77                                     |       |       |  |
|  | NP_835020        | BC5359           | YwaD          | Aminopeptidase Y                                   |  | 2.13  |       |  |
|  | <b>NP_830419</b> | <b>BC0602</b>    | <b>Npr600</b> | <b>Bacillolysin</b>                                |  | 2.70  |       |  |
|  | NP_832233        | BC2473           | Blm           | Beta-lactamase                                     |  | 2.05  |       |  |
|  | NP_831066        | BC1284           | InhA2         | Immune inhibitor A precursor                       |  |       | -4.17 |  |
|  | NP_831063        | BC1281           | CalY          | Cell envelope-bound metalloprotease (camelysin)    |  |       | -2.85 |  |
|  | NP_830483        | BC0670           | PlcB          | Phospholipase C                                    |  |       | -2.42 |  |
|  | Motility         | NP_831428        | BC1651        | FglE   | Flagellar hook protein                   | 2.63  |       |  |
|  |                  | <b>NP_831414</b> | <b>BC1636</b> | <b>FlgK</b>  | <b>Flagellar hook-associated protein</b> | 2.66  | -3.63 |  |
| NP_831435                                      |                  | BC1658           | FlaB          | Flagellin  |  | -3.70 |       |  |
| NP_831415                                      |                  | BC1637           | FlgL          | Flagellar hook-associated protein                  |  |       | -4.14 |  |
| NP_831421                                      |                  | BC1643           | FliE          | Flagellar hook-basal body protein                  |  |       | -2.72 |  |
| Cell wall and cell surface associated proteins | NA               | BC3763           |               | Cell wall hydrolase                                | 3.31                                     |       |       |  |
|  | NP_831197        | BC1417           | YvgJ3         | phosphoglycerol transferase                        | 2.61                                     |       |       |  |
|  | NP_831682        | BC1911           | Ami           | N-acetylmuramoyl-L-alanine amidase                 | -2.73                                    |       |       |  |

(Continued)

TABLE 2 | Continued

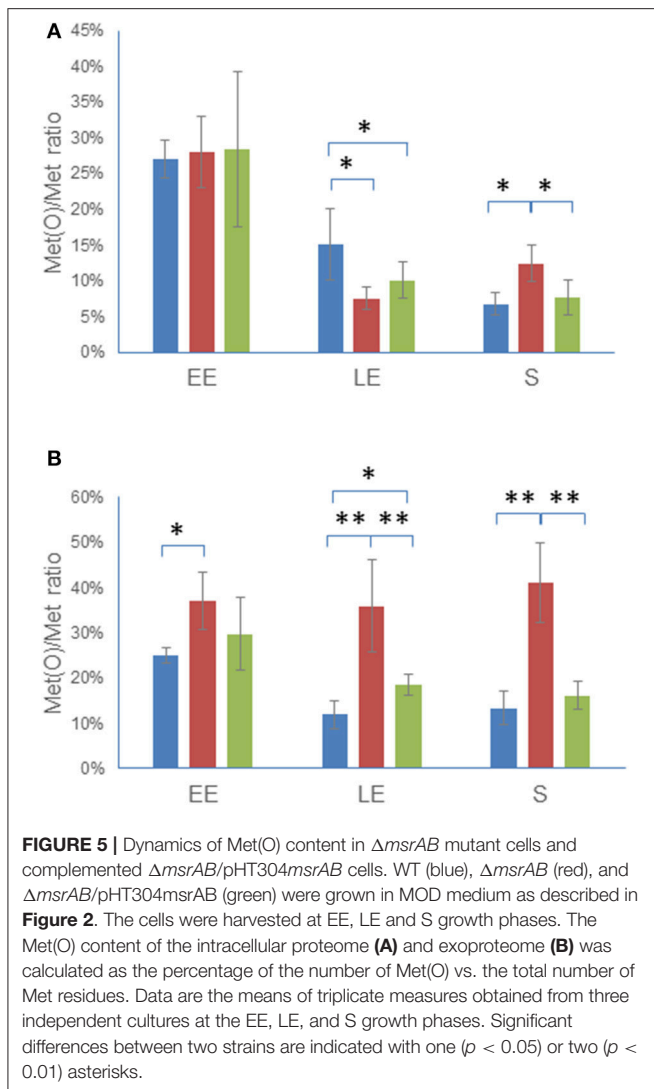
| Functional class           | NP no.           | Gene no.      | Protein name | Protein description                            | Log <sub>2</sub> fold-change |       |       |
|----------------------------|------------------|---------------|--------------|--|------------------------------|-------|-------|
|                            |                  |               |              |  | EE                           | LE    | S     |
|                            | NP_830492        | BC0679        | Smc          | Cell wall-binding protein                      | 1.12                         |       |       |
|                            | NP_832677        | BC2929        | PgdA         | Peptidoglycan N-acetylglucosamine deacetylase  |                              | 2.67  |       |
|                            | NP_831846        | BC2078        |              | Hypothetical Membrane Spanning Protein         |                              |       | -3.07 |
|                            | NP_832595        | BC2846        | DltD         | Protein dltD precursor                         |                              |       | -2.25 |
|                            | NP_833426        | BC3698        | CwpC         | Cell wall endopeptidase                        |                              |       | 2.34  |
|                            | NP_833984        | BC4270        |              | Penicillin-binding protein                     | 3.48                         | 1.94  |       |
| Protein export             | NP_833266        | BC3533        | VanW         | Vancomycin B-type resistance protein vanW      | 1.67                         |       |       |
| Transport                  | NP_832816        | BC3070        | SipA         | Signal peptidase I                             |                              | 2.04  |       |
|                            | NP_831789        | BC2021        | ZnuA         | High-affinity zinc uptake system protein       | 1.48                         |       |       |
|                            | NP_834656        | BC4985        |              | ABC transporter substrate-binding protein      |                              |       | -3.93 |
|                            | NP_830083        | BC0215        | OppA2        | Oligopeptide-binding protein oppA              |                              |       | -2.53 |
|                            | NP_830606        | BC0816        |              | Periplasmic component of efflux system         |                              | 3.09  | 2.50  |
|                            | NA               | BC1596        |              | Permease                                       | 3.60                         | 2.54  |       |
| Stress/detoxification      | NP_831779        | BC2011        | Dps1         | Non-specific DNA-binding protein               | -2.31                        | -2.32 |       |
|                            | <b>NP_834714</b> | <b>BC5044</b> | <b>Dps2</b>  | <b>Non-specific DNA-binding protein</b>        |                              |       | -3.47 |
|                            | <b>NP_833272</b> | <b>BC3539</b> | <b>CspB</b>  | <b>Cold shock protein</b>                      |                              |       | -5.13 |
|                            | <b>NP_830215</b> | <b>BC0376</b> | <b>AhpF</b>  | <b>Alkyl hydroperoxide reductase subunit F</b> |                              |       | -1.99 |
| Protein folding            | NP_830947        | BC1161        | PrsA2        | Peptidylprolyl isomerase                       |                              | 2.80  |       |
|                            | NP_830829        | BC1043        | PrsA1        | Peptidylprolyl isomerase                       |                              | 2.57  |       |
|                            | <b>NP_834192</b> | <b>BC4480</b> | <b>Tig</b>   | <b>Trigger factor</b>                          |                              |       | -2.14 |
| Translation                | NP_830029        | BC0149        | RpmD         | 50S ribosomal protein L30                      | 1.30                         |       |       |
|                            | NP_833528        | BC3806        | RpsO         | 30S ribosomal protein S15                      |                              |       | -2.38 |
|                            | NP_830008        | BC0128        | FusA         | Elongation factor G                            |                              | -2.59 |       |
|                            | NP_830009        | BC0129        | Tuf          | Elongation factor Tu                           |                              | -2.79 | 2.95  |
| Transcriptional regulators | NP_830591        | BC0801        | LytR2        | LytR family transcriptional regulator          | 1.98                         |       |       |
|                            | NP_834928        | BC5265        | LytR1        | LytR family transcriptional regulator          | 2.88                         |       |       |
|                            | NP_831739        | BC1669        | LytR3        | LytR family transcriptional regulator          |                              | 3.42  | 2.73  |
| Cell division              | NP_829962        | BC0065        |              | Cell division protein DIVC                     |                              | 3.39  |       |
| Uncategorized              | NP_831643        | BC1870        |              | Phage protein                                  | 1.30                         |       |       |
|                            | NP_831665        | BC1892        |              | Phage protein                                  |                              | -1.58 |       |
|                            | NP_831667        | BC1894        |              | Phage protein                                  |                              | -1.89 | -1.70 |
|                            | NP_831675        | BC1903        |              | Phage protein                                  |                              |       | -2.16 |
|                            | NP_832991        | BC3251        |              | Unknown  |                              | -2.71 |       |
|                            | NP_835021        | BC5360        |              | Unknown  |                              | 2.05  |       |
|                            | NP_830068        | BC0200        |              | Unknown  |                              | 1.64  |       |
|                            | NP_832874        | BC3133        |              | Unknown  |                              |       | -1.86 |
|                            | NP_833260        | BC3527        |              | Unknown  |                              | 1.75  |       |

Exoproteins with abundance level restored in  $\Delta$ msrAB/pHT304msrAB are indicated in bold. EE, early exponential growth phase; LE, late exponential growth phase; S, stationary growth phase. NA, Not Annotated. Green and red highlights indicate increased and decreased protein levels, respectively.

the S growth phase, the majority of the identified peptides (12/18) showed a higher Met(O) level in  $\Delta$ msrAB compared with WT. Among these 12 peptides, 6 are RibH-related peptides. Two of these six peptides had their Met(O) level restored in  $\Delta$ msrAB/pHT304msrAB (Table 3). RibH contains four Met residues: all of these were more highly oxidized in  $\Delta$ msrAB than in WT at the S growth phase and two were more highly oxidized in  $\Delta$ msrAB than in  $\Delta$ msrAB/pHT304msrAB. RibH is thus a target of MsrAB activity and the major contributor to the difference observed between  $\Delta$ msrAB and WT on the one hand, and  $\Delta$ msrAB and  $\Delta$ msrAB/pHT304msrAB on the other, at the S growth phase (Figure 5).

## Exoproteome

Table 4 shows that peptides with differential Met(O) contents belong to 21 proteins, including eight toxin-related proteins. The LE growth phase sustained the highest number of peptides with increased Met(O) levels (10); the majority of these peptides (9/10) had their Met(O) level restored in  $\Delta$ msrAB/pHT304msrAB, indicating a direct impact of MsrAB. Among the proteins with increased oxidation of Met residues were the degradative enzyme, PlcA, the flagellin, FlaA, and the four toxin-related proteins, NheA, HblB, EntC and EntD. Only PlcA and HblB showed increased abundance levels at LE growth phase (Table 2). FlaB was the protein for which we detected the largest number of



Met(O) peptides and Met residues with differential oxidation (7 Met residues). All of these residues were less oxidized in  $\Delta msrAB$  compared with WT at LE phase. In addition, we observed that FlaB was less abundant in  $\Delta msrAB$  at LE phase (**Table 2**). The loss of Met-oxidized peptides could thus be due to degradation of protein copies. This is possibly also the case for HlyII (**Tables 2, 4**). In S growth phase, the peptides with increased Met(O) content belong to the putative N-acetylmuramoyl-L-alanine amidase CwlC and the toxin-related EntA, HblB, and HblL2. All HblL2-bound Met were not equally susceptible to *msrAB* disruption, as one Met residue was more oxidized at the S growth phase, one was more oxidized at the LE growth phase and two were less oxidized, especially at the EE growth phase. Taken together, the results indicate that MsrAB regulates the dynamic of the Met(O) level of the exoproteome by controlling the Met(O) level of target peptides in a growth phase- and protein-dependent manner. Importantly, our results indicate that virulence factors such enterotoxins, degradative enzymes, and flagella components are MsrAB targets.

## DISCUSSION

Methionine (Met) residues in proteins and their recycling by methionine sulfoxide reductases (Msrs) are part of the antioxidant system produced by aerobic microorganisms. The antioxidant system keeps a steady-state control over ROS production-detoxification (Levine et al., 1996; Kim, 2013). The tight regulation of ROS production and detoxification represents the basis for the maintenance of an appropriate redox homeostasis, which is central for growth.

While Met residues in cellular proteins are well-recognized as antioxidants, the relative importance of Met residues in extracellular proteins has hitherto not been established. In this study, we used next-generation proteomics on wild-type *B. cereus* and an MsrAB mutant to demonstrate that Met residues in exoproteins could be reversibly oxidized to Met(O), probably before their exportation. In addition, we provide the first evidence that *B. cereus* can modulate its capacity and specificity for protein export (secretion) through the growth phase-dependent expression of the methionine sulfoxide reductase-encoding gene, *msrAB*.

As reported for other *msr* genes in several bacteria, *msrAB* expression is lower in exponentially grown *B. cereus* cells than in growth-arrested cells. The low level of *msrAB* expression is probably sufficient to maintain a proper activity of the antioxidant system during exponential growth phase. The increased expression of *msrAB* at the end of growth would serve to minimize the accumulation of oxidative damage on ROS-affected molecules (Dukan and Nystrom, 1999). However, the expression level of *msrAB* in *B. cereus* cells is not by itself sufficient to prevent premature growth arrest under full aerobic conditions as growth can be prolonged by overproducing *msrAB*. In WT cells, premature growth arrest allows the cells to survive for extended time periods, suggesting that MsrAB could be a regulator of normal lifespan of *B. cereus* (Koc et al., 2004).

Considering the primary antioxidant function of MsrAB, variation of other antioxidant proteins was expected in MsrAB-deficient cells as a part of putative compensatory mechanisms or due to altered interactions with MsrAB (Alamuri and Maier, 2006). We observed abundance level changes in antioxidant proteins, mainly at LE phase, due to the lack of protection normally conferred by the high expression of *msrAB*. Neutralizing ROS without quelling its production may prove to be onerous to *B. cereus*. Our results indicate that *B. cereus* reprograms its proteome to both counteract and inhibit the formation of ROS in *msrAB*-deficient cells. This proteome modification leads to novel metabolic networks that allow the alleviation of TCA cycle activity, the main metabolic network that supplies NADH for oxidative phosphorylation. When the machinery involved in oxidative phosphorylation is severely impeded by the ROS challenge, glucose uptake is enhanced to satisfy the ATP need by substrate level phosphorylation. Increased carbon flow also maintains constant levels of glycolytic intermediates as macromolecular precursors and boosts carbon flow through the PPP, which produces large amount of NADPH, a key molecule that is used to

**TABLE 3** | Cellular peptides with significant Met(O) level changes ( $\log_2[\text{fold-change}] > 1, p < 0.01$ ) in  $\Delta\text{msrAB}$  compared with WT.

|   | Gene no | NP no     | Protein name | Description                                       | Peptide name  | Met(O) peptide detected by GC-MS/MS  |    |   | $\log_2$ fold-change |    |       |
|---|---------|-----------|--------------|---|---------------|--|----|---|----------------------|----|-------|
|   |         |           |              |   |               | EE   | LE | S | EE                   | LE | S     |
| Glycolysis                                  | BC3971  | NP_833690 | PdhC         | Alpha-keto acid dehydrogenase subunit E2          | PdhC_2        | HTAPHVTL <b>M</b> DEVDVTELVAHR   |    |   |                      |    | -2.32 |
| Amino sugar and nucleotide sugar metabolism | BC5288  | NP_834951 | MurA         | UDP-N-acetylglucosamine 1-carboxyvinyltransferase | MurA_1        | ASVQ <b>V</b> MGPLLAR  |    |   |                      |    | 2.38  |
| Amino acid metabolism                       | BC1238  | NP_831022 | TrpA         | Tryptophan synthase, subunit $\alpha$             | TrpA_4        | EVQMPFV <b>L</b> MTYLNPLVAFGK  |    |   |                      |    | -1.69 |
|   | BC1237  | NP_831021 | TrpB         | Tryptophan synthase, subunit $\beta$              | TrpB_1        | ETPLYA <b>E</b> MTK  |    |   |                      |    | -1.57 |
|   | BC1232  | NP_831016 | TrpE         | Anthranilate synthase component I                 | TrpE_2        | A <b>M</b> EINLENEKR   |    |   |                      |    | -1.02 |
| Transcriptional regulators                  | BC0102  | NP_829983 | CipC         | Negative regulator of genetic competence          | CipC_6        | VIEL <b>S</b> MDEAR  |    |   |                      |    | -3.29 |
|   |         |           |              |   | CipC_8        | VM <b>T</b> LD <b>M</b> GTWAGTK  |    |   |                      |    | -1.99 |
|   |         |           |              |   | CipC_9        | VM <b>T</b> LD <b>M</b> GTWAGTK  |    |   |                      |    | -2.40 |
| Stress response                             | BC0613  | NP_830430 | ArsR1        | ArsR family transcriptional regulator             | ArsR1_1       | I <b>S</b> EEDVQ <b>M</b> LR   |    |   |                      |    | 1.51  |
|   | BC5044  | NP_834714 | Dp2          | Non-specific DNA-binding protein                  | Dps2_12       | K <b>G</b> M <b>E</b> IAQSDDEMTSDLLGGIYTELEKHAWMLR   |    |   |                      |    | -1.86 |
|   | BC0377  | NP_830216 | AhpC         | Alkyl hydroperoxide reductase C22                 | Dps2_9        | G <b>M</b> EIAQSDDEMTSDLLGGIYTELEKHAWMLR   |    |   |                      |    | -2.20 |
|   | BC0376  | NP_830215 | AhpF         | Alkyl hydroperoxide reductase subunit F           | AhpC_3        | IEY <b>I</b> MIGDPTR   |    |   |                      |    | -2.34 |
|   |         |           |              |   | AhpF_18       | VSAGDDN <b>V</b> SK <b>D</b> MLALVDELAT <b>M</b> SSK   |    |   |                      |    | 1.14  |
| Chaperone                                   | BC1155  | NP_830941 | KatE         | Catalase  | KatE_5        | <b>M</b> NP <b>N</b> RL <b>T</b> NOGAPVGD <b>N</b> Q <b>S</b> RTAGR  |    |   |                      |    | 2.00  |
|   | BC0295  | NP_830146 | GroEL        | Chaperone   | GroEL_23      | SALQ <b>N</b> AA <b>S</b> VA <b>M</b> FL <b>T</b> TEAVADK <b>P</b> EP <b>N</b> AP <b>M</b> PD <b>M</b> G <b>G</b> M <b>G</b> MG <b>M</b> MG <b>M</b>   |    |   |                      |    | -1.67 |
|   |         |           |              |   | GroEL_30      | SALQ <b>N</b> AA <b>S</b> VA <b>M</b> FL <b>T</b> TEAVADK <b>P</b> EP <b>N</b> AP <b>M</b> PD <b>M</b> G <b>G</b> M <b>G</b> MG <b>M</b> MG <b>M</b>   |    |   |                      |    | -1.51 |
| Riboflavin metabolism                       | BC1043  | NP_830829 | PraA1        | Peptidyl/prolyl isomerase                         | PraA1_6       | Q <b>V</b> L <b>N</b> N <b>M</b> W <b>M</b> E <b>K</b>   |    |   |                      |    | -1.59 |
|   | BC4112  | NP_833830 | RibH         | Riboflavin synthase, subunit $\beta$              | <b>RibH_1</b> | <b>A</b> GN <b>K</b> G <b>Y</b> E <b>S</b> A <b>V</b> A <b>I</b> E <b>M</b> A <b>H</b> L <b>S</b> K  |    |   |                      |    | 2.88  |
|   |         |           |              |   | RibH_3        | AG <b>T</b> K <b>A</b> GN <b>K</b> G <b>Y</b> E <b>S</b> A <b>V</b> A <b>I</b> E <b>M</b> A <b>H</b> L <b>S</b> K  |    |   |                      |    | 1.98  |
|   |         |           |              |   | <b>RibH_4</b> | <b>G</b> V <b>A</b> S <b>L</b> <b>K</b> <b>D</b> <b>I</b> <b>P</b> <b>I</b> <b>F</b> <b>V</b> <b>I</b> <b>F</b> <b>G</b> <b>V</b> <b>L</b> <b>T</b> <b>T</b> <b>E</b> <b>I</b> <b>E</b> <b>Q</b> <b>A</b> <b>I</b> <b>E</b> <b>R</b> |    |   |                      |    | 2.75  |
|   |         |           |              |   | RibH_5        | G <b>Y</b> E <b>S</b> A <b>V</b> A <b>I</b> E <b>M</b> A <b>H</b> L <b>S</b> K   |    |   |                      |    | 2.21  |
|   |         |           |              |   | RibH_8        | <b>M</b> A <b>S</b> S <b>G</b> K <b>Y</b> D <b>A</b> V <b>T</b> L <b>G</b> T <b>V</b> <b>I</b> R   |    |   |                      |    | 1.93  |
|   |         |           |              |   | RibH_9        | <b>M</b> V <b>F</b> E <b>G</b> H <b>L</b> V <b>G</b> T <b>G</b> L <b>K</b>   |    |   |                      |    | 1.92  |
|   |         |           |              |   | RibE_2        | V <b>G</b> S <b>M</b> T <b>E</b> S <b>F</b> L <b>O</b> E <b>N</b> G <b>F</b> L   |    |   |                      |    | 1.32  |
| Ribosome components                         | BC0146  | NP_830026 | RplF         | 50S ribosomal protein L6                          | RplF_1        | A <b>L</b> I <b>G</b> N <b>M</b> V <b>E</b> G <b>T</b> E <b>G</b> F <b>A</b> R   |    |   |                      |    | -1.81 |
|   | BC3825  | NP_833546 | RpsB         | 30S ribosomal protein S2                          | RpsB_1        | A <b>G</b> M <b>V</b> F <b>V</b> N <b>O</b> R  |    |   |                      |    | -2.93 |
|   | BC0135  | NP_830015 | RpsS         | SSU ribosomal protein S19p                        | RpsS_4        | K <b>H</b> P <b>V</b> Y <b>T</b> E <b>D</b> <b>M</b> V <b>G</b> H <b>K</b>   |    |   |                      |    | 2.20  |
| Translation apparatus                       | BC0129  | NP_830009 | Tuf          | Elongation factor                                 | Tuf_1         | E <b>T</b> D <b>K</b> P <b>L</b> <b>M</b> P <b>V</b> E <b>D</b> V <b>F</b> S <b>I</b> T <b>G</b> R   |    |   |                      |    | -1.90 |
|   |         |           |              |   | Tuf_21        | T <b>D</b> V <b>T</b> G <b>I</b> Q <b>L</b> P <b>E</b> G <b>T</b> E <b>M</b> M <b>P</b> G <b>D</b> N <b>I</b> E <b>M</b> T <b>I</b> E <b>L</b> I <b>A</b> P <b>I</b> A <b>I</b> E <b>E</b> G <b>T</b> K                              |    |   |                      |    | -1.76 |
|   |         |           |              |   | Tuf_30        | V <b>G</b> D <b>V</b> E <b>I</b> E <b>I</b> G <b>L</b> A <b>E</b> E <b>N</b> A <b>S</b> T <b>V</b> T <b>G</b> V <b>E</b> M <b>F</b> R <b>K</b>   |    |   |                      |    | 1.57  |
| Degradative enzyme                          | BC1191  | NP_831760 | TgC          | Murein endopeptidase                              | Tuf_5         | I <b>E</b> L <b>M</b> A <b>E</b> V <b>D</b> A <b>I</b> P <b>T</b> P <b>E</b> R <b>E</b> T <b>D</b> K <b>P</b> L <b>M</b> P <b>V</b> E <b>D</b> V <b>F</b> S <b>I</b> T <b>G</b> R  |    |   |                      |    | -2.95 |
| Motility                                    | BC1654  | NP_831431 | CheV         | Chemotaxis protein                                | TgC_2         | <b>N</b> I <b>M</b> D <b>Q</b> L <b>Y</b> G <b>E</b> F <b>N</b> K  |    |   |                      |    | -1.94 |
| Uncategorized                               | BC1225  | NP_831009 | -            | Unknown   | CheV_3        | V <b>N</b> I <b>A</b> E <b>D</b> S <b>A</b> M <b>L</b> R   |    |   |                      |    | 1.75  |
|   | BC4045  | NP_833763 | -            | NAD(P)H nitroreductase                            | BC1225_1      | <b>M</b> K <b>L</b> G <b>I</b> V <b>I</b> F <b>P</b> S <b>K</b>  |    |   |                      |    | 1.70  |
|   | BC4182  | NP_833896 | Gls24        | Unknown   | BC4045        | <b>M</b> S <b>V</b> E <b>Q</b> V <b>S</b> E <b>W</b> A <b>K</b>  |    |   |                      |    | 1.14  |
|   |         |           |              |   | Gls24_3       | A <b>E</b> H <b>M</b> L <b>D</b> M <b>G</b> D <b>T</b> L <b>G</b> K <b>V</b> E <b>I</b> A <b>P</b> E <b>V</b> I <b>E</b> V <b>I</b> A <b>G</b> I <b>A</b> A <b>E</b> V <b>E</b> G <b>V</b> A <b>A</b> M <b>R</b>                     |    |   |                      |    | -1.02 |

Peptides with Met(O) levels restored in  $\Delta\text{msrAB/pHT304-msrAB}$  are indicated in bold. Met residues that are differentially oxidized are indicated in red. EE, early exponential growth phase; LE, late exponential growth phase; S, stationary growth phase. NA, Not annotated. Green and red highlights indicate increased and decreased protein levels, respectively.

**TABLE 4 |** Exopeptides with significant Met(O) level changes ( $\log_2[\text{fold-change} > 1, p < 0.01]$  in  $\Delta\text{msrAB}$  compared with WT).

| Functional class   | Gene no | NP no     | Protein name | Description                             | Peptide name       | Met(O) peptide detected by GC-MS/MS | log <sub>2</sub> fold-change |       |       |      |       |
|--|---------|-----------|--------------|---|--------------------|-------------------------------------|------------------------------|-------|-------|------|-------|
|  |         |           |              |   |                    |                                     | EE                           | LE    | S     | EE   | LE    |
| Carbohydrate metabolism<br>[O.8pt][-1pt] Amino acid metabolism | BC5135  | NP_834803 | Eno          | Enolase                                 | Eno_2              | LGANALGVSMVAIAHAAADFVGLPLYR         | -2.83                        | -2.67 | -2.73 |      |       |
|  | BC0344  | NP_830183 | RocA         | l-pyrroline-5-carboxylate dehydrogenase | RocA_4             | FMEVLEEAAGLPAGVWFVPGNGSEVGDYLDHPR   | -2.02                        |       |       |      |       |
| Translation<br>Cell wall and cell surface metabolism           | BC0119  | NP_830000 | RplJ         | Ribosomal protein L10                   | RplJ               | EGLLSMLLSVLQAPIR                    | -2.21                        |       |       |      | 2.44  |
|  | BC5234  | NP_834897 | CwlC         | N-acetylmuramoyl-L-alanine amidase      | CwlC_1             | SGPSHMGVYLGGSFIQAGDK                |                              |       |       |      |       |
| Enterotoxins   | BC0679  | NP_830492 | Smc          | Cell wall protein                       | Smc_3              | MNAVSTILEADKEILR                    | 2.35                         |       |       |      |       |
|  | BC5239  | NP_834902 | EntA         | Enterotoxin A                           | Smc_1              | GYNILTANPGMK                        | 1.80                         |       |       |      |       |
| Flagella   | BC0813  | NP_830603 | EntC         | Enterotoxin C                           | EntA_2             | VLTAAGHDLTANPNMK                    | -1.74                        |       |       |      |       |
|  | BC3716  | NA        | EntD         | Enterotoxin D                           | EntA_1             | VLTAAGHDLTANPNMK                    | -2.47                        |       |       |      | 1.87  |
|  | BC3102  | NP_832845 | HblB         | Hbl, component B                        | EntA_3             | VLTAAGHDLTANPNMK                    |                              |       |       |      |       |
|  | BC3104  | NP_832847 | HblL2        | Hbl, component L1                       | EntC_1             | GNIKIDVLPDPK                        | 2.32                         |       |       |      |       |
|  |         |           |              |   | EntC_3             | IDVLPDPK                            | 1.65                         |       |       |      |       |
|  |         |           |              |   | EntD_1             | VLTAAGHDLTANPNMK                    | 1.77                         |       |       |      |       |
|  |         |           |              |   | HblB_6             | SMNAYSMLIK                          | 2.39                         |       |       |      |       |
|  |         |           |              |   | HblB_2             | QLESDGFNVMK                         |                              |       |       |      | 2.21  |
|  |         |           |              |   | HblL2_7            | LIQTYIDQSLMSPNVQLEEVYALNTNQFLIK     | -3.10                        | -1.87 | -2.18 |      |       |
|  |         |           |              |   | HblL2_9            | SMLLLTQNDLHTFANQIDVELDLK            | -2.21                        |       |       |      |       |
|  |         |           |              |   | HblL2_10           | SMLLLTQNDLHTFANQIDVELDLKR           | -2.49                        |       |       |      | -1.93 |
|  |         |           |              |   | HblL2_12           | TQEYDLMKVIDTEK                      |                              |       |       |      | 1.96  |
|  |         |           |              | HblL2_8                                 | QDMKWSSELYPQLILNSK |                                     |                              |       |       | 2.09 |       |
|  | BC3523  | NP_833256 | HlyII        | Hemolysin II                            | HlyII_1            | ALEEQMNSINSVNDKLNK                  | -2.21                        |       |       |      |       |
|  | BC1809  | NP_831582 | NheA         | Nhe component A                         | NheA_2             | LIDLNQEMMR                          | 2.21                         |       |       |      |       |
|  | BC1810  | NP_831583 | NheB         | Nhe, component B                        | NheB_3             | TQTEYLNTIDTITALQNISQWYTMGSK         | -2.21                        | -2.27 | -1.65 |      |       |
|  |         |           |              |   | NheB_2             | TGSNALVMDLYLTIK                     |                              | -1.76 |       |      |       |
|  | BC1657  | NP_831434 | FliA         | Flagellin                               | FliA_9             | LDHNLNNVTSOATNMAAASQIEDADMAK        | -2.21                        | -1.67 |       |      |       |
|  |         |           |              |   | FliA_6             | ILNEAGISMLSQANQTPQMVSK              |                              | 2.92  |       |      |       |
|  |         |           |              |   | FliA_5             | ILNEAGISMLSQANQTPQMVSK              |                              | 2.19  |       |      |       |
|  |         |           |              |   | FliA_4             | ILNEAGISMLSQANQTPQMVSK              |                              | 1.78  |       |      | -1.78 |
|  |         |           |              |   | FliA_20            | MPRINTNINSMR                        |                              |       |       |      |       |
|  |         |           |              |   | FliA_7             | ILNEAGISMLSQANQTPQMVSK              |                              | -2.77 |       |      |       |
|  |         |           |              |   | FliA_8             | ILNEAGISMLSQANQTPQMVSK              |                              | -2.77 |       |      |       |
|  |         |           |              |   | FliA_9             | ILNEAGISMLSQANQTPQMVSK              |                              | -2.77 |       |      |       |
|  | BC1658  | NP_831435 | FliB         | Flagellin                               | FliB_14            | LDHNLNNVTSOATNMAAASQIEDADMAKEMSEMTK |                              | -2.29 |       |      |       |
|  |         |           |              |   | FliB_11            | LDHNLNNVTSOATNMAAASQIEDADMAK        |                              | -2.03 |       |      |       |
|  |         |           |              |   | FliB_15            | LDHNLNNVTSOATNMAAASQIEDADMAKEMSEMTK |                              | -1.67 |       |      |       |
|  |         |           |              |   | FliB_12            | LDHNLNNVTSOATNMAAASQIEDADMAK        |                              | -1.34 |       |      |       |
|  |         |           |              |   | FliB_26            | TNFNGNSFLDTTATPPGKDIEIQLSDASGDTMTLK |                              | -1.52 |       |      | -2.06 |

(Continued)



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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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