Restricting Fermentative Potential by Proteome Remodeling

AN ADAPTIVE STRATEGY EVIDENCED IN BACILLUS CEREUS

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Pathogenesis hinges on successful colonization of the gastrointestinal (GI) tract by pathogenic facultative anaerobes. The GI tract is a carbohydrate-limited environment with varying oxygen availability and oxidoreduction potential (ORP). How pathogenic bacteria are able to adapt and grow in these varying conditions remains a key fundamental question. Here, we designed a system biology-inspired approach to pinpoint the key regulators allowing Bacillus cereus to survive and grow efficiently under low ORP anoxic conditions mimicking those encountered in the intestinal lumen. We assessed the proteome components using high throughput nanoLC-MS/MS techniques, reconstituted the main metabolic circuits, constructed ΔohrA and ΔohrR mutants, and analyzed the impacts of ohrA and ohrR disruptions by a novel round of shotgun proteomics. Our study revealed that OhrR and OhrA are crucial to the successful adaptation of B. cereus to the GI tract environment. Specifically, we showed that B. cereus restricts its fermentative growth under low ORP anaerobiosis and sustains efficient aerobic respiratory metabolism, motility, and stress response via OhrRA-dependent proteome remodeling. Finally, our results introduced a new adaptive strategy where facultative anaerobes prefer to restrict their fermentative potential for a long term benefit. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.013102, 1–13, 2012.

Facultative anaerobes encompass all the major pathogens of the human gastrointestinal (GI) tract. The GI tract poses several challenges for pathogens because it is sliced into distinct niches with different oxygen concentrations and different oxidoreduction potentials (ORP) (1–3). Although much is known about gene expression and metabolism under fully aerobic and high ORP anaerobic condition (4, 5), our knowledge about the physiological impact of low ORP anoxic conditions and the underlying molecular mechanisms is scarce (6).

Bacillus cereus is a notorious food-borne pathogenic bacterium. Like the closely related Bacillus anthracis (7, 8), it is a recognized agent of GI tract infections (9–11). The critical step of infection takes place in the small intestine, where B. cereus has to grow and produce virulence factors to induce diarrheal disease (11, 12). Thus, how B. cereus adapts its catabolism and regulates its proteome across the range of physiologically relevant ORP and oxygen availabilities is important for its survival and growth. In B. cereus, anaerobic and aerobic catabolism work through different pathways. In the presence of oxygen, reducing equivalents generated by glycolysis and the TCA cycle (NADH and FADH) are reoxidized by the respiratory chain, resulting in the buildup of a proton motive force and the subsequent synthesis of ATP. Acetate excretion can occur aerobically when carbon flux into the cells exceeds TCA cycle capacity. In the absence of oxygen or other external electron acceptors (such as nitrate), NADH is reoxidized in terminal step fermentative reactions from pyruvate. When grown in pH-controlled anaerobic batch cultures (pH ~7), the fermentative by-products of B. cereus are lactate, succinate, acetate, and ethanol. The relative rate of formation of these products is influenced by the ORP of the growth medium, which directly impacts the intracellular redox state (6, 13–18). The intracellular redox state is dependent on the degree of oxidation or reduction of various redox-active species. Among these species, NAD(P)H/NAD(P) and low molecular weight thiol/disulfide (SH/S-S) compounds are of special significance because they mediate redox regulation through direct effects on proteins. The activities of many metabolic enzymes depend on the steady-state NAD(P)H/NAD(P) ratio, whereas proteins with essential SH/S-S groups can be regulated by post-translational modification involving cellular thiols and disulfides. The main low molecular weight thiols in B. cereus ATCC 14579 cells are bacillithiol and cysteine (19). On the other hand, the NAD(P)H/NAD(P) and SH/S-S ratios are closely related to cellular levels of reactive oxygen species (ROS). NAD(P)H and thiols contribute to ROS formation via...

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by strong temporal and/or spatial fluctuations in ORP and to maximize survival in stressful environments characterized with varying oxygen and ORP conditions such as that for redox homeostasis. Their high reactivity allows fast local oxidation of protein (i.e. protein disulfide bond formation) in the reducing cytoplasm. However, this high reactivity also leads to cell damage if cellular capacity to scavenge ROS is compromised (20–23).

In B. cereus, the expression of most of the genes encoding metabolic enzymes is modulated by the two redox systems, ResDE (a two-component system) (6, 24) and Fnr (a one-component system) (18, 25), and the catabolite control protein A (CcpA) (26). Despite the knowledge currently gathered throughout these studies, we still lack a global quantitative understanding of the contribution of the multilevel regulatory events that govern metabolic modes (anaerobic fermentation versus aerobic respiration). One potential strategy for tackling this issue is to use a systems biology approach (27), i.e. assessing the proteome components with high throughput techniques, rebuiding the main metabolic circuits, inactivating key regulators, and verifying their impacts. Here, we compare B. cereus ATCC 14579 proteomes established for cells grown under low ORP conditions (considered to mimic those encountered in the intestinal lumen (3)), oxic conditions (considered to mimic those encountered in zones adjacent to the mucosal surface (2)), and intermediary high ORP anaerobic conditions. This comparative analysis identified OhrA, a thiol-dependent peroxidase-like protein, as a putative low ORP sensor. OhrA is encoded by ohrA, which is the second cistron of the ohrR-ohrA operon in B. cereus ATCC 14579. The ohrR cistron is predicted to encode a MarR-like repressor of ohrA. The ΔohrR and ΔohrA mutants were constructed and analyzed for their effect on growth, glucose catabolism, and proteome composition under low and high ORP anaerobiosis and aerobicosis. Our results indicate that OhrA and OhrR are major factors controlling glucose catabolism and global proteome in B. cereus under both anaerobic fermentative and aerobic respiratory conditions.

We discovered that the OhrRA system restricts B. cereus fermentative capacity under low ORP anoxic growth conditions while boosting motility, respiratory metabolism and resistance against external ROS. We conclude that the OhrRA system may function in B. cereus as a major redox signal transduction system to co-regulate catabolism, motility, and oxidoreductive stress resistance in an environment with varying oxygen and ORP conditions such as that encountered in the human intestine. Finally, our findings provide detailed insights into the metabolic events required to maximize survival in stressful environments characterized by strong temporal and/or spatial fluctuations in ORP and oxygen levels.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Wild type, ΔohrA, and ΔohrR B. cereus ATCC 14579 (28) strains were grown at 37 °C in synthetic MOD medium supplemented with 30 μmol glucose as carbon source, as previously described (12). The pH of these cultures was maintained at 7.2 by automatic addition of 2 N KOH. The bioreactor was equipped with a Mettler Toledo polarographic oxygen electrode coupled with feedback regulation to maintain the set point dissolved oxygen tension (PO2) via air sparging and agitation speed. The bioreactor was sparged with air alone to set a PO2 value of 100%. A PO2 of 0% was obtained by continuously flushing the medium at 20 ml/h with either pure N2 or pure H2 gas previously filtered through a Hungate column. ORP was measured using a redox-combined electrode (AgCl, Mettler Toledo), and the values were corrected to the reference electrode value (Eref = +200 mV at 37 °C). Each bioreactor was inoculated with a culture grown overnight under aerobicosis or anaerobiosis in MOD medium supplemented with carbon source and the required electron acceptors. A sample of the overnight culture was diluted in fresh medium to obtain an initial optical density at 560 nm of 0.02. The cells were harvested by centrifugation at the early exponential growth phase, i.e. 1.5 h after reaching the maximal growth rate (when μ = 80 ± 10% of μmax) and immediately frozen at −80 °C until analysis. Supernatants were kept for metabolite and glucose assays. For motility and disk diffusion assays, B. cereus cells were grown in LB medium under aerobic or anaerobic conditions. For anaerobic growth, the cells were incubated in an Oxoid anaerobic jar.

Growth was performed at room temperature at 15 °C.

**RNA Isolation, Operon Mapping, and Expression Analysis**—To investigate the transcriptional organization and expression of the ohrR-ohrA locus, RT-PCR was performed with total RNA isolated from B. cereus ATCC 14579 as previously described (12). Fragments corresponding to ohrA, ohrR, and the ohrA-ohrR intergenic regions were amplified by RT-PCR using the Titan one Tube RT-PCR system following the manufacturer’s protocol (Roche Applied Science). The primer pairs used for ohrA, ohrR, and the ohrA-ohrR intergenic region were 5′-CAGGTGGAAAGAAATGGGAAA-3′ plus 5′-GCCGAAACCAC-CATCTGTAT-3′, 5′-TGTTTTTCTATTTACGCTGC-3′ plus 5′-TGTAAGCATCTGTGGGTA-3′, and 5′-CGAAGAAGCTCATA-ACCA-3′ plus 5′-TACCCGAAACGATGCGTACA-3′, respectively. To check whether contaminant genomic DNA was present, each sample was tested in a control reaction without reverse transcriptase. The 5′ end of ohrA-ohrR mRNA was mapped from a 5′-RACE PCR product obtained with the 3′/5′-RACE kit (Roche Applied Science). Briefly, the first strand cDNA was synthesized from total RNA with ohrA-ohrR-specific SP1 primer (5′-TGTAAGCATTGCTGCGT-3′), avian myeloblastosis virus reverse transcriptase, and the deoxynucleotide mixture of the 3′/5′-RACE kit following the manufacturer’s instructions. After purifying and dA-tailing the cDNA, PCR with the (dT)-anchor oligonucleotide primer and the ohrA-ohrR-specific SP2 primer (5′-TTCGCTTCGTTTCTGTTTG-3′) followed by a nested PCR with SP3 primer (5′-CATCTGTGGTCCCCATAGC-3′) led to a PCR product of ~140 bp, as revealed by 2% agarose gel electrophoresis. This PCR product was purified and sequenced. Real-time RT-PCR was performed using SYBR Green technology on a LightCycler instrument (Roche Applied Science), as described previously (6).

Construction of ΔohrA and ΔohrA Mutant Cells and Complementation—ΔohrA and ΔohrR cell mutants were constructed as follows. A BglII-Sall DNA fragment of 1,489 bp encompassing the ohrA and ohrR ORFs was amplified by PCR using chromosomal DNA as template and the primer pair 5′-AGATCTTCTTGAATCTGA-CAATCGGG-3′ plus 5′-GGCCAGACCAGGAAACGATGCGTACA-3′. The amplified DNA fragment was cloned into pCRXL-TOPO (Invitrogen). The resulting pCRXLohr plasmid was then digested with Hpal and BsgI, respectively. A 1.5-kb Smal fragment containing the entire...
spectinomycin resistance gene spc (29) was purified from pDia(14). This purified DNA fragment was ligated into HpaI-digested pCRXLohr and BsgI-digested pCRXLohr, respectively. The resulting plasmids pCRXL-ohrA-spcc/H9004 and pCRXL-ohrR-ohrA-spcc/H9004 were both digested with EcoRI plus BglII. The resulting ohrA-spcc/H9004 and ohrR-ohrA-spcc/H9004 fragments were subsequently inserted between the corresponding pMD4 sites. The resulting plasmids were introduced into B. cereus strains by electroporation. The ohrA and ohrR genes were deleted by a double-crossover event (30). Chromosomal allele exchanges were confirmed by PCR with oligonucleotide primers located upstream and downstream of the DNA regions used for allelic exchange. To confirm the nonpolar effect of the spectinomycin resistance cassette insertion in ohrR (which precedes ohrA in the ohrR-ohrA operon), fragments encompassing the ohrA and ohrR-ohrR intergenic region mRNA were amplified from total RNA isolated from ΔohrR cells as described above. A low copy number plasmid, pHT304 (31), was used to complement the ohrR and ohrA genes in trans. The pCRXL-ohrA-spcc/H9004 and pCRXL-ohrR-ohrR-spcc/H9004 plasmids were digested with EcoRI plus BamHI, and the ohrA-spcc/H9004 and ohrR-ohrR-spcc/H9004 fragments were cloned into pH3T04. The integrity of the inserts in the recombinant vectors was verified by sequencing, after which the vectors were used to transform B. cereus mutant strains.

Analytical Procedures and Physiological Parameters—B. cereus growth was monitored spectrophotometrically at 560 nm and calibrated against cell dry weight measurements, as previously described (12). Specific growth rate (μ) was determined using the modified Gompertz equation (32, 33). Glucose, lactate, ethanol, formate, acetate, and succinate concentrations were determined using Difffchamp, R-Biopharm, and Roche Applied Science kits. Specific glucose consumption rate, defined as the differential change in glucose concentration with time, was calculated from the equation

\[ \frac{\Delta [\text{glucose}]}{\Delta t} = \mu Y_g, \]

where \( \mu \) is specific growth rate (h\(^{-1}\)), and \( Y_g \) is biomass yield (g-mol carbon substrate\(^{-1}\)).

Protein Sample Preparation—After thawing on ice, cell pellets were suspended in 1 ml of a lysis buffer consisting in 7.8M urea (Sigma), 2.2 M thiourea (Sigma), 4.5% w/ w CHAPS (Sigma), 44.5 mM DTT (Sigma), 2.2 mM Trizma base (Sigma)/HCl (Sigma) pH 7.5, and one tablet of antiprotease mini-mixture (Roche Applied Science). The suspension was transferred into a 2-ml sterilized tube containing 400 mg of 0.1-mm-diameter zirconium/silica beads (VWR). Each tube was placed in a Precellys 24 disruptor (Bertin Technologies) and shaken in 95 °C, and loaded on 4–12% gradient NuPAGE gels (Invitrogen). The resulting peptide mixtures were diluted 1:20 in 0.1% lithium dodecyl sulfate 1 l of lysis buffer and redisrupted. These bands were destained, and their protein contents were estimated as 10 polyacrylamide bands of equal volume from top to bottom. After incubation for 45 min at room temperature, the samples were centrifuged at 9,000 × g for 4 min, and 600 μl of supernatant was recovered. The pellets were washed twice with 600 μl of lysis buffer and redisrupted. The supernatants were then pooled. Protein concentration of each lysate was determined using the Bio-Rad protein assay (Bio-Rad). A volume of 30 μl of lithium dodecyl sulfate 1× sample buffer (Invitrogen) was added to 40 μg of proteins. The samples were then sonicated for 10 min using a transonic 780H sonicator, boiled for 5 min at 95 °C, and loaded on 4–12% gradient NuPAGE gels (Invitrogen). The gels were operated with MES buffer, run at 150 V (Invitrogen), and then stained with Coomassie Blue Safe stain (Invitrogen). After overnight destaining, the whole protein content from each well was excised as 10 polycrylamide bands of equal volume from top to bottom. These bands were destained, and their protein contents were treated with iodoacetamide and then proteolyzed with proteasMAX (Promega). The resulting peptide mixtures were diluted 1:20 in 0.1% trifluoroacetic acid.

NanoLC-MS/MS Analysis—NanoLC-MS/MS analyses were performed on an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo-Fisher) coupled to an UltiMate 3000 LC system ( Dionex-LC Packings) operated as previously described (12). Peak lists were generated on Matrix Science Mascot Daemon software (version 2.2.2) using the extract_msn.exe data import filter (ThermoFisher) from the Thermo-Fisher Xcalibur FT software package (version 2.0.7). Data import filter options were set at: 400 (minimum mass), 5,000 (maximum mass), 0 (grouping tolerance), 0 (intermediate scans), and 1,000 (threshold). MS/MS spectra were searched using MASCOT 2.2.04 software (Matrix Science) against an in-house database containing all the annotated protein sequences of B. cereus ATCC 14579. This database comprises 5,255 polypeptide sequences specified by B. cereus ATCC 14579 chromosome (NC_004722) and plasmid pBClin15 (NC_004721), totaling 1,455,982 amino acids. Search parameters for trypptic peptides were as previously described (34): full trypsin specificity, a mass tolerance of 10 ppm on the parent ion, and 0.6 Da on the MS/MS spectra, static modifications of carboxyamidomethylated Cys (+57.0215), and dynamic modifications of oxidized Met (+15.9949). Maximum number of missed cleavages was set at 2. MASCOT results were parsed using IPRMa 1.2.24 software (35) filtering with a p value less than 0.05. A protein was considered valid when at least two different peptides were detected in the same experiment. The false-positive rate for protein identification was estimated using the appropriate decoy database as below 0.1% at these parameters. Mass spectrometry data were deposited in the PRIDE PRoteomics IDENTifications database (36) under accession numbers 17312–17338. Peptide characteristics are reported in supplemental Table S4. The database comprises 14,679 unique peptide sequences. Supplemental Table S2 shows the 461,191 assigned spectra and the peptide coverage for the 1,344 proteins identified in this study. Significant variations were assessed using Patternlab software (37), as previously described (12). Supplemental Table S3 reports log₂ (fold change) and p values obtained with the T-fold statistical method adapted for high throughput hypothesis tests (37). A BH-FDR statistical test was calculated to evaluate the global false discovery rate for each comparison.

Motility and Disk Diffusion Assays—The medium used for swimming assays was LB containing 0.3% (w/v) Bacto-agar (Difco). The cells were grown to mid-log phase and adjusted to the same density as evaluated by A\(_{600}\) measurements. Then a 10-μl drop of exponential growth phase culture was spotted on a plate and incubated at 37 °C, and swimming distances were measured after 72 h. For disk diffusion assays, the cells were grown to mid-log phase. Then 100 μl of cells at an A\(_{600}\) of ~0.4 were mixed with 4 ml of LB medium containing 0.75% agar and poured onto plates containing 25 ml of LB agar. Next, 6-mm paper disks containing 10 μl of hydrogen peroxide (0.8 or 0.08 M) were placed on top. The clear zones were measured after incubating plates for 24 h at 37 °C.

RESULTS

Analysis of Central Carbon Metabolism in Early Grown B. cereus Cells—To elucidate how B. cereus adapts to low ORP anaerobic conditions considered as mimicking those encountered in the intestinal lumen (3), we used hydrogen gas as a nonselective and nontoxic reducing agent for the growth medium (initial ORP = −410 mV; pO\(_2\) = 0%). The GI tract can also contain substantial amounts of H\(_2\) (38). Bacteria were grown in pH- and temperature-regulated bioreactors using glucose as the sole and limiting carbon source (pH 7, 37 °C, 30 mM glucose). The reference condition was growth under high ORP anaerobiosis (initial ORP = −10 mV; pO\(_2\) = 0%). B. cereus was also grown under aerobiciosis (initial ORP = +140 mV; pO\(_2\) = 100%), which is considered as mimicking the conditions encountered in zones adjacent to the mucosal surface (2). Like all of the facultative anaerobes, B. cereus
ATCC 14579 (28) cells grow slower and less efficiently in anoxic fermentative conditions than in oxic respiratory conditions (see supplemental Table S1). The fermentative end products arising from the pivotal intermediates pyruvate and acetyl-CoA (A-CoA) are lactate, formate, ethanol, and acetate, which we systematically quantified. Producing these products in a given specific ratio allows *B. cereus* to grow efficiently in both low and high ORP anoxic conditions (Fig. 1A and supplemental Table S1) (17).

High throughput characterization of the proteome from *B. cereus* ATCC 14579 harvested at the early exponential phase was achieved for all three culture conditions via an extensive shotgun nanoLC-MS/MS analysis performed with triplicate biological samples. For this purpose, the proteins from

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High throughput characterization of the proteome from *B. cereus* ATCC 14579 harvested at the early exponential phase was achieved for all three culture conditions via an extensive shotgun nanoLC-MS/MS analysis performed with triplicate biological samples. For this purpose, the proteins from
the nine samples were resolved by their molecular weight by SDS-PAGE. After migration, the gel was sliced into 10 polyacrylamide bands and subjected to trypsin proteolysis, and the resulting peptide mixtures were analyzed by tandem mass spectrometry with a LTQ-Orbitrap XL mass spectrometer. A total of 216,443 MS/MS spectra were recorded through the analysis of these 90 nanoLC-MS/MS runs. A total of 107,480 different MS/MS spectra (see supplemental Table S4) were confidently assigned to 1,142 proteins (see supplemental Table S3). The detected proteins were catalogued into 13 functional groups. Fig. 1B and supplemental Table S2 show that the global distribution of proteins among these functional groups did not change significantly in the three different growth conditions studied. However, the percentages of MS/MS spectra assigned to central metabolism and translation-related proteins were significantly different in low ORP anoxic conditions (15.3 ± 0.3 and 28.01 ± 1.9%, respectively) compared with high ORP anoxic conditions (20.5 ± 0.5 and 24.7 ± 1.6%, respectively) and oxic conditions (20.1 ± 0.9 and 23.4 ± 2.1%, respectively). These results indicate that low ORP anaerobically grown cells require more translation-related proteins and fewer central metabolism-related proteins than high ORP anaerobically and aerobically grown cells. The central metabolism-related proteins were then catalogued into five groups according to function: 1) glycolysis, 2) fermentation, 3) TCA cycle, 4) ATP synthesis, and 5) others. Fig. 1C shows that the main differences observed in the three conditions arise from proteins involved in fermentation and TCA cycling, which represented 27 and 13% of intracellular proteins under anaerobiosis and 7 and 30% of intracellular proteins under aerobicism, respectively. This proteomic remodeling is representative of changes in mode of glucose catabolism, i.e., respiration under aerobicism and fermentation under anaerobiosis. The metabolic pathways used by B. cereus in anaerobic fermentative and aerobic respiratory conditions were meticulously analyzed, as presented in supplemental Fig. S1. This process included systematically performing blast analyses to annotate wrong gene functional assignments and retrieve missing functions. Supplemental Fig. S1 also reports the fold changes observed for each detected protein, taking into account spectral counts as input.

Although changes in extracellular ORP did not impact the ratios between fermentation and TCA cycle groups, phosphotransacetylase (Pta, NP_835048.1) was significantly downregulated (log$_2$ = −0.8; p value = 0.013 relative to high ORP anoxic conditions), and dihydroxyacetone acetyltransferase (SucB, NP_831035.1) was significantly up-regulated (log$_2$ = 1.5; p value = 0.017 relative to high ORP anoxic conditions) under low ORP anoxic conditions (Fig. 2 and supplemental Table S3). SucB is the lipoylated E2 subunit of the 2-oxo acid dehydrogenase multienzyme complex (OGDHC) that catalyzes the oxidative decarboxylation of 2-oxoglutarate to succinyl-CoA (see supplemental Fig. S1). In addition to SucB, OGDHC also contains SucA (E1 component, NP_831036.1) and LpdV (E3 component, NP_833875.1), which can catalyze the redox processes between lipoylated E2 enzyme and NADH/NAD$^+$ in both reductive and oxidative directions. OGDHC also catalyzes ROS production (39, 40). Our proteomic data indicate that 1) SucB is the only 2-oxo-acid dehydrogenase subunit that preferentially accumulates under low ORP anaerobiosis and 2) abundance of SucB may be fairly similar in low ORP anoxic and fully oxic conditions (Fig. 2 and supplemental Table S3). It is thus possible that SucB may contribute to redox signaling under both low ORP anaerobiosis and aerobicism (39).

**Analysis of Chaperones and Stress Response Protein Abundance Pattern**—Among the 60 proteins classified as stress response and the 9 proteins classified as chaperonin-related proteins, GTP-binding protein TypA, iron-binding ferritin-like antioxidant protein 2 (Dps2), alkyl hydroperoxide reductase F (AphF), catalase A (KatA), organic hydroperoxide resistance protein A (OhrA), and DnaK chaperone Hsp70 were significantly more abundant in low ORP than in high ORP anaerobically grown cells (Fig. 2 and supplemental Table S3). TypA, AphF, KatA, and DnaK were also significantly more abundant in aerobically grown cells than in high ORP anaerobically grown cells, as reported in other microorganisms (41). As a result, the abundances of these proteins were found to be statistically unchanged between low ORP anoxic and oxic conditions (see supplemental Table S3). However, the low p values associated to the log$_2$ (fold change) of Dps2 (log$_2$ = −1.4, p value = 0.017) and OhrA (log$_2$ = −0.7, p value = 0.077) in oxic conditions versus low ORP anoxic conditions indicated that it is reasonably likely that both Dps2 and OhrA are more abundant in low ORP anaerobically grown cells than in aerobically grown cells (see supplemental Table S3). OhrA is a putative Cys-based thiol-dependent peroxidase (22, 42–44). It was recently reported that lipoylated proteins, such as SucB, are the physiological reducing agents for OhrA-like *Xylella fastidiosa* (45). The presence of higher levels of both OhrA and SucB and to a lesser extent BC4157 (a lipoamide acyltransferase component of branched chain α-keto acid dehydrogenase complex; Fig. 2) under low ORP anoxic conditions raises thus the question of the role of OhrA in redox homeostasis.

**Genetic Organization of the B. cereus ohrRA Locus**—Genome analysis of B. cereus ATCC 14579 indicated that the gene encoding OhrA is the second cistron of the *ohrR-ohrA* operon (Fig. 3A). The first cistron is predicted to encode OhrR, a MarR-like repressor of OhrA that could function as a redox sensor via its cysteine residues (Fig. 3B) (46, 47). RT-PCR experiments demonstrated that *ohrR* and *ohrA* were actually co-transcribed under both aerobicism and anaerobiosis, whatever the redox conditions (Fig. 3A). A transcriptional start site (T) located 46 bp from the *ohrR* start codon was identified by 5′-RACE PCR. Upstream of this start site, we identified a potential αA-type −10 sequence, TATATT, that is preceded

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by a typical −35 sequence TTGACA (48). Inspection of the 5′-untranscribed region revealed the presence of a palindromic sequence spanning positions +8 to +31. Each symmetrical arm contains a highly conserved binding site for OhrR (49). Furthermore, the ohrRA locus is followed by an inverted repeat (change in Gibbs free energy (ΔG), −19 kcal/mol) that

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**Fig. 2.** Changes in protein abundances under low ORP anaerobiosis compared with high ORP anaerobiosis and under aerobiosis compared with high ORP anaerobiosis. Only changes satisfying statistical criteria (p value < 0.05) at least under low ORP compared with high ORP anoxic conditions are shown. **Solid colors** indicate fold change values that satisfied Student t test statistical criteria (p value < 0.05), and **hatching** indicates that the statistical criteria are not satisfied.
may be a transcriptional terminator. This suggests that ohrR and ohrA form a bicistronic transcriptional unit. This genetic organization differs from that observed in *B. subtilis* (49), where ohrA and ohrR are transcribed in the opposite direction, but was similar to that observed in other sequenced members of the *B. cereus* group (see supplemental Table S5). Sequence analysis also showed that the stop codon of ohrR is located close to the start codon of ohrA (2-bp upstream) and overlaps with the Shine-Dalgarno sequence of ohrA, suggesting a putative translational coupling between ohrA and ohrR (50). ohrA encodes a protein of 138 amino acid residues with 65% sequence identity and 74% sequence similarity to *B. subtilis* OhrA. The two active cysteines of *B. cereus* OhrR are in conserved positions in relation to those of *B. subtilis* OhrA (51). OhrR encodes a protein of 150 amino acid residues with 59% sequence identity and 77% sequence similarity to *B. subtilis* OhrR. The amino-terminal cysteine residue (Cys) that corresponds to C15 of *B. subtilis* OhrR was conserved in *B. cereus*. This cysteine residue has been shown to be important for response to organic hydroperoxide in *in vivo* and *in vitro* (49, 51). In addition to the C13 cysteine, *B. cereus* also contains three other cysteine residues, which are absent in *B. subtilis* OhrR. However, two of them (Cys and Cys) are often found in other bacteria (51), whereas Cys was atypical. This might be a signature of *B. cereus* OhrR, because it has been found in other sequenced members of the *B. cereus* group. This also raises the possibility that *B. cereus* OhrR could function differently from OhrR from other bacteria.

**Physiological Characteristics of B. cereus ΔohrA and ΔohrR Mutants**—We created ΔohrA and ΔohrR mutants by introducing a spectinomycin resistance cassette into the corresponding genes (30). The ΔohrR mutant showed a significantly lower growth rate than the wild type under aerobicosis (−29 ± 3%) and to a lesser extent under anaerobiosis (−22 ± 2%; Fig. 4A), probably because of lower glycolytic fluxes (or glucose uptake rates) under aerobicosis and anaerobiosis (Fig. 4B). Fermentative ΔohrA cells secreted lower amounts of lactate compared with wild type cells (Fig. 4C), indicating that the rate-limiting step in glucose uptake is the NAD⁺-generating lactate pathway. Unexpectedly, ΔohrR mutant showed a significantly higher growth rate than the wild type under low ORP anaerobiosis (1.17 ± 0.01 h⁻¹ versus 0.75 ± 0.01 h⁻¹ in the wild type), whereas there were no significant differences under high ORP anaerobiosis or aerobicosis (Fig. 4A and supplemental Table S1). Fig. 4B shows that the high growth rate of low ORP anaerobic ΔohrR cells is associated with high glycolytic flow. Fig. 4C shows that 1) fermentative ΔohrR cells secreted similar amounts of lactate under low and high ORP anaerobiosis (0.66 ± 0.15 and 0.63 ± 0.15, respectively), whereas wild type cells secreted higher amounts of lactate under low ORP anaerobiosis (0.68 ± 0.05 versus 0.48 ± 0.12 under high ORP anaerobiosis); 2) fermentative ΔohrR cells secreted higher amounts of acetate, formate, and ethanol when grown under low ORP anaerobiosis (0.20 ± 0.04, 0.13 ± 0.07, and 0.10 ± 0.02 versus 0.12 ± 0.02, 0.05 ± 0.01, and 0.070 ± 0.005, respectively, in the wild type); and 3) respiratory ΔohrR cells secreted higher amounts of acetate than the wild type cells (0.20 ± 0.04 versus 0.14 ± 0.02) under aerobicosis. Taken together, these results indicate that disruption of ohrR1 abolished the redox-dependent regulation of the NAD⁺-generating lactate pathway under anaerobiosis, 2) allows PfI-dependent pathways to reach their peak capacity under low ORP anaerobiosis and consequently allows *B. cereus* to grow faster, and 3) restricts TCA cycle capacity under aerobicosis (16, 52). We concluded that both ohrA and ohrR are major factors controlling glucose catabolism.
Impact of ohrA and ohrR Mutations on Proteome Remodeling—We used the same robust high throughput proteomics free label method as previously carried out for the wild type characterization to analyze the quantitative proteome changes caused by either ohrR or ohrA disruptions in B. cereus cells grown at low density under low ORP anaerobiosis, high ORP anaerobiosis, and full aerobiosis. Once again, three biological replicates were obtained for each of the three culture conditions. For each mutant, nine samples were analyzed proteomically by cumulating 10 nanoLC-MS/MS runs per sample. A total of 503,810 and 290,481 MS/MS spectra were recorded for /H9004 ohrR and /H9004 ohrA mutants, respectively, when analyzing the 2 × 90 nanoLC-MS/MS runs. A total of 1,344 proteins were identified after appending all the mass spectrometry results recorded in this study. These proteins were quantified by applying the spectral count method in the 27 proteomes. Like the wild type strain, ΔohrA and ΔohrR mutants showed crystal clear proteomic remodeling between high ORP anoxic and oxic growth conditions and, to a lesser extent, between low ORP and high ORP anoxic conditions. A, maximal specific growth rates (μmax, h⁻¹) of wild type (black), ΔohrA mutant (blue), and ΔohrR mutant (red) under aerobiosis, high ORP anaerobiosis, and low ORP anaerobiosis. B, maximal specific glucose uptake rates of wild type (black), ΔohrA mutant (blue), and ΔohrR mutant (red) under aerobiosis, high ORP anaerobiosis, and low ORP anaerobiosis. Yields of fermentative by-product from wild type B. cereus, ΔohrA mutant, and ΔohrR mutant under aerobic growth, high ORP anaerobiosis, and low ORP anaerobiosis. C, yields of fermentative by-product from wild type B. cereus, ΔohrA mutant, and ΔohrR mutant under aerobic, high ORP anaerobiosis, and low ORP anaerobiosis. Yields of lactate (orange), acetate (brown), formate (green), and ethanol (purple) are expressed as mol of product/mol of consumed glucose. Glucose and glucose by-product concentrations are mean values obtained from triplicate cultures. See supplemental Table S1 for details on the mean values.

Impact of ohrA and ohrR Mutations on Proteome Remodeling—We used the same robust high throughput proteomics free label method as previously carried out for the wild type characterization to analyze the quantitative proteome changes caused by either ohrR or ohrA disruptions in B. cereus cells grown at low density under low ORP anaerobiosis, high ORP anaerobiosis, and full aerobiosis. Once again, three biological replicates were obtained for each of the three culture conditions. For each mutant, nine samples were analyzed proteomically by cumulating 10 nanoLC-MS/MS runs per sample. A total of 503,810 and 290,481 MS/MS spectra were recorded for ΔohrR and ΔohrA mutants, respectively, when analyzing the 2 × 90 nanoLC-MS/MS runs. A total of 1,344 proteins were identified after appending all the mass spectrometry results recorded in
higher abundances.

Dehydrogenase (Bdh) in low ORP anaerobically grown cells. Fumarate dehydrogenase A (FumA), isocitrate dehydrogenase isoenzyme B (MdhB) and isocitrate dehydrogenase (Icd) are the two proteins that showed a similar decrease in abundance in wild type relative to mutants under aerobiosis compared with high ORP anaerobiosis (Fig. 6B, solid line). Our data also revealed that the abundance pattern of some antioxidant proteins, OhrA showed the highest abundance in wild type cells whatever the conditions. In addition to TCA cycle enzymes, whose abundance strongly decreases in aerobically grown ΔohrR cells, the largest measurable decreases in protein abundance were observed for flagellar and chemotaxis-related proteins under the three growth conditions tested (log₂ < −2.5, p < 0.05, relative to wild type; Fig. 6B). As a result, ΔohrR cells showed decreasing swimming ability compared with wild type cells (supplemental Fig. S2).

Our data also revealed that the abundance pattern of some chaperones and stress-related proteins is differently modulated in ΔohrR and ΔohrA cells (Fig. 6B and supplemental Table S3). Among them, DnaJ chaperone was significantly down-regulated in ΔohrR cells but remained unchanged in ΔohrA cells whatever the conditions. In addition, the GroES chaperone and the cold shock protein CspB were strongly up-regulated in ΔohrR cells (log₂ > 2.5, p < 0.05, relative to wild type), which could explain the experimentally evidenced quicker adaptation of these mutant cells to a temperature drop (supplemental Fig. S4).

Among the antioxidant proteins, OhrA showed the highest abundance decrease in both low ORP anaerobically and aerobically grown ΔohrR cells (Fig. 6B). Lack of OhrA could therefore provide one explanation for the lower resistance against external H₂O₂ of both ΔohrR and ΔohrA cells under both anaerobiosis and aerobiosis (see supplemental Fig. S3). Fig. 6B also shows that the abundance pattern of antioxidant proteins was differently modulated by ohrR disruption in the three growth conditions tested. As a result, redox-dependent regulation of the abundance pattern of antioxidant proteins did not occur in ΔohrR cells (see supplemental Table S3). OhrR

Analysis of Protein Abundance Patterns in ΔohrR and ΔohrA Cells—A large fraction of fermentative and TCA cycle-related enzymes showed significant abundance changes in anaerobically and aerobically grown ΔohrR and ΔohrA cells (see supplemental Table S3). The largest decreases in TCA cycle enzyme abundance were observed in aerobically grown ΔohrR cells: 12 and 3 proteins showed significant abundance changes (log₂ < −1.5, p < 0.05 relative to wild type) in ΔohrR and ΔohrA cells, respectively. Among them, lactate dehydrogenase A (LdhA) and isocitrate dehydrogenase (Icd) are the two proteins that showed a similar decrease in the two mutant cells. Fumarate dehydrogenase A (FumA), succinate dehydrogenase A (SdhA), succinyl-CoA subunit α and β (SucC and SucD), and the E1 and E2 components of 2-oxo acid dehydrogenase (SucA and SucB) showed higher decreases in ΔohrR cells than in ΔohrA cells. Fermentative enzyme abundance was globally decreased in both anaerobically grown ΔohrA cells and ΔohrR cells (Fig. 6A). The largest significant decreases were observed for lactate dehydrogenase A (LdhA) in anaerobically grown ΔohrA cells (log₂ < −1.5, p < 0.05, relative to wild type), butanediol dehydrogenase (Bdh) in low ORP anaerobically grown ΔohrA and ΔohrR cells (log₂ < −1.8, p < 0.05, relative to wild type) and pyruvate formate lyase (PflA) in anaerobically grown ΔohrR cells (log₂ < −1.48, p < 0.05, relative to wild type). These data suggest that the redox-dependent regulation pattern of central metabolic enzymes is modulated by OhrA and OhrR. Interestingly, our data also suggest that OhrA and OhrR could differentially control the abundance patterns of functionally redundant isoenzymes (53). Lactate dehydrogenase isoenzymes LdhA, LdhB, and LdhC (14) showed particularly remarkable patterns, i.e. 1/1/0.3–1/0.8/0.3–1/0.5/1 in ΔohrA cells and 2.5/1/1–1/0.6/0.4–1/0.4/1 in ΔohrR cells relative to wild type cells under low or high ORP anaerobiosis and aerobiosis, respectively. In addition to TCA cycle enzymes, whose abundance strongly decreases in aerobically grown ΔohrR cells, the largest measurable decreases in protein abundance were observed for flagellar and chemotaxis-related proteins under the three growth conditions tested (log₂ < −2.5, p < 0.05, relative to wild type; Fig. 6B). As a result, ΔohrR cells showed decreasing swimming ability compared with wild type cells (supplemental Fig. S2).

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Fig. 5. Impact of ohrA or ohrR disruptions on B. cereus global proteome. A, Venn diagrams representing numbers of proteins showing significant abundance changes in wild type (black), ΔohrA (blue), and ΔohrR (red) mutants under aerobiosis compared with high ORP anaerobiosis (solid line) and under high ORP anaerobiosis compared with low ORP anaerobiosis (dashed line). B, cumulative distribution of proteins showing significant abundance changes (p value < 0.05) under aerobiosis compared with high ORP anaerobiosis (solid line) and under low ORP anaerobiosis compared with high ORP anaerobiosis (dashed line) in wild type (black, 171 and 59 proteins, respectively), ΔohrA mutants (blue, 137 and 64 proteins, respectively), and ΔohrR mutants (red, 159 and 28 proteins, respectively). Log₂ (fold change) < 0 indicates lower abundance under aerobiosis or low ORP anaerobiosis compared with high ORP anaerobiosis, whereas log₂ (fold change) > 0 indicates higher abundances.

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thus governs the redox-dependent regulation of the abundance pattern of antioxidant proteins, including OhrA.

**Transcriptional Control of ohrRA**—Transcription of *ohrRA* was investigated using real-time RT-PCR with total RNA obtained from wild type, ∆ohrA and ∆ohrR cells cultivated under low and high ORP anaerobiosis and aerobic conditions. We found that *ohrRA* mRNA was more abundant in low ORP anaerobically grown and aerobically grown wild-type cells than in high ORP anaerobically grown wild-type cells (log₂ratio 1.2 and 1.0, respectively; *p* < 0.05). This is consistent with the proteomic data and indicates that the redox and oxygen-dependent regulation of *ohrRA* may mainly occur at the transcriptional level. Fig. 7 also shows that 1) *ohrR* disruption significantly up-regulated *ohrRA* transcription under both low ORP anaerobiosis and aerobic conditions (log₂ratio 1.3 and 1.9, respectively; *p* < 0.05) but did not significantly change *ohrRA* transcription under high ORP anaerobiosis; 2) *ohrA* disruption significantly down-regulated *ohrRA* transcription under low ORP and high ORP anaerobiosis (log₂ratio ~2.3 and ~3.2, respectively, *p* < 0.05). Taken together, these results suggest that 1) under low ORP anaerobiosis, *ohrRA* transcription could be oppositely controlled by OhrR (down-regulation) and OhrA (up-regulation), 2) under high ORP anaerobiosis, *ohrRA* transcription could be controlled mainly by OhrA (up-regulation), and 3) under aerobic conditions, *ohrRA* transcription could be controlled mainly by OhrR (down-regulation). We concluded that the OhrRA-dependent transcription of *ohrRA* is redox-dependent. However, these transcriptomic results are not fully concordant with the proteomic data, which show that OhrA was significantly less abundant in ∆ohrR cells, whatever the growth conditions (Fig. 5B). This suggests that OhrA abundance may be mainly modulated at the post-transcriptional level when OhrR is lacking.

**DISCUSSION**

Coping with reductive stress originating from low ORP conditions (3) is one of the prerequisites enabling pathogens to establish a successful infection in the human intestine. However, the molecular events necessary for bacteria to cope with
this stress are poorly understood. Obtaining low ORP conditions in in vitro cultures is a technical challenge, which could explain why the overwhelming majority of the relevant literature only reports results from experiments on high ORP anaerobic growth conditions, such as those generated by nitrogen gas in our study. Here, using H₂-generated low ORP conditions and B. cereus as a model bacterium, we provide the first report at both proteomic and metabolic levels to describe how facultative anaerobes overcome reductive stress to grow efficiently under pH-regulated and carbohydrate-limited conditions.

Compared with high ORP conditions (initial ORP = –10 mV), low ORP conditions (initial ORP = –410 mV) did not lead to marked changes in growth rate and metabolic activity, illustrating the capacity of B. cereus to efficiently maintain its intracellular redox state in response to reductive stress (13). Current knowledge suggests that reductase homeostasis is governed by the antioxidant system that buffers reductants and oxidants (23, 54, 55). The results presented here indicate that OhrA could be a major component of the antioxidant system allowing B. cereus to cope with reductive stress and maintain efficient growth under low ORP anaerobiosis. Although B. cereus seems to require similarly high levels of OhrA to grow efficiently under both low ORP anaerobiosis and aerobicosis, the results obtained with ΔohrA mutant strain indicated that the OhrA-dependent control of growth was stronger in aerobic respiratory conditions than in high reductive fermentative conditions. In X. fastidiosa, peroxidase activity of OhrA is supported by lipoylated proteins such as SucB, i.e. the reduced form of lipoylated SucB donates electrons to OhrA to cleave ROS (45). Our study showed that B. cereus synthesizes higher levels of both OhrA and SucB under low ORP anaerobiosis. Therefore, we cannot exclude the possibility that high levels of OhrA could be necessary to prevent excessive accumulation of the reduced form of lipoylated SucB (by regenerating the oxidative form) and excessive OGCD-dependent ROS production (by detoxifying ROS) under high reductive conditions. When OhrR is lacking, redox-dependent modulation of the antioxidant protein abundance pattern did not occur in B. cereus. This suggests that OhrR is a major redox regulator of the B. cereus antioxidant system, which includes OhrA. In addition, OhrR modulates the abundance of key glycolytic enzymes and anabolism-related proteins (supplemental Fig. S3). By controlling the abundance of key enzymes from these two metabolic blocks in a redox-dependent manner, OhrR may 1) modulate glycolytic flux and restrict growth under low ORP conditions (56) and 2) sustain high TCA capacity and limit energy spilling through an overflow metabolism under aerobicosis when cells are grown in glucose-limited conditions (16). A key question is how OhrR controls its own abundance, the abundance pattern of antioxidant proteins, and, more generally, proteome remodeling. These controls processes could involve bacillithiol- and lipoyl-dependent systems (21, 45), interplay of direct or indirect transcriptional regulation, and post-transcriptional regulation via OhrA. Evidently, further intensive work is now required to unravel the complexity of the regulatory network involving OhrRA. Such work hinges on first purifying and biochemically characterizing the atypical B. cereus OhrR. Finally, our strategy to use a wide reaching shotgun proteomic study to pinpoint the key proteins involved in B. cereus adaptation to low ORP anoxic conditions has proven successful, because we demonstrated that the two-component OhrRA system, which is only described to function as a protection system against organic hydroperoxide toxicity in several bacteria (49, 57, 58), is actually a key two-component system of redox signaling in B. cereus.

Surprisingly, we found that OhrRA-dependent proteome regulation sustains high flagellar protein synthesis and high resistance against exogenous ROS, such as produced by H₂O₂, whatever the redox conditions. In the natural evolutionary context, this may help bacteria to rapidly navigate toward more energy-favorable niches to create a hostile environment for competing microorganisms (59, 60) and better resist the ROS produced by intestinal microflora and inflammatory conditions (61, 62). A recent report (58), together with our closer examination of the B. cereus OhrRA homologs (as illustrated by the phylogenetic trees reported in supplemental Table S5), suggests that the ohrRA genes have been sprayed among bacteria by lateral transfer. We thus propose that the evolution in B. cereus enabling growth in human host environments could have arisen via a somehow recent acquisition of the ohrRA genes, with the synthesis of their products acting as master regulators of the proteome pattern. In addition, the fact that ohrRA genes that encode proteins with markedly varying sequences (see supplemental Table S5) occur in the B. cereus group and in other bacteria indicates that OhrR and OhrA could contribute to the acquisition of strain-specific features for successful colonization of the GI tract. More generally, our findings highlight how the disruption of just one gene can dramatically transform metabolic capabilities and expand the range of environments in which microorganisms can compete. Specifically, our findings with our ΔohrR mutant show that a facultative anaerobe can grow under high reductive fermentative conditions as fast as under aerobic respiratory conditions in a carbohydrate-limited environment. Finally, and challenging current dogma in microbiology, our data reveal that global proteome remodeling in response to oxygen availability and ORP changes probably does not occur to optimize catabolic pathways per se, as is usually considered in facultative anaerobes, but rather to maximize survival under changing environmental conditions.

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