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OhrRA functions as a redox-responsive system controlling toxinogenesis in *Bacillus cereus*

Gérémy Clair[a,b,c], *Alain Lorphelin*[c], *Jean Armengaud*[c], *Catherine Duport*[a,b,*]

[a]*Université d'Avignon et des Pays de Vaucluse, UMR408, Sécurité et Qualité des Produits d'Origine Végétale, F-84000 Avignon, France*
[b]*INRA, UMR408, Sécurité et Qualité des Produits d'Origine Végétale, F-84914 Avignon, France*
[c]*Laboratoire de Biochimie des Systèmes Perturbés, CEA Marcoule, DSV-iBEB-SBTN-LBSP, F-30207 Bagnols-sur-Cèze cedex, France*

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ABSTRACT

Bacillus cereus OhrR is a member of the subgroup of the MarR (multiple antibiotic resistance) family of transcriptional regulators that use a cysteine-based redox sensing mechanism. OhrA is a thiol-dependent, peroxidase-like protein. The dual OhrRA system triggers *B. cereus* adaptation in response to redox changes, such as those encountered in the environment of the gastrointestinal tract. Here, we investigated the role of OhrRA in toxinogenesis. Comparative shotgun analysis of exoproteomes from Δ*ohrA*, Δ*ohrR* and wild-type cells revealed significant changes in the abundance levels of toxin-related proteins depending on the extracellular redox potential. We complemented these data by measuring the DNA binding activity of reduced and oxidized recombinant OhrR on toxin and putative toxin promoter regions. Furthermore, transcriptomic data and OhrRA-dependent, antiproliferative activity of the *B. cereus* exoproteome on Caco-2 human epithelial cells were recorded. The results indicate that OhrR controlled toxin gene expression directly or indirectly in a redox- and toxin-dependent manner, and may function as a repressor or an activator. Moreover, we found that OhrR restricts toxin-dependent antiproliferative activity of the *B. cereus* exoproteome whatever the growth conditions, while the restrictive impact of OhrA occurs only under low ORP anoxic conditions.

Biological significance
B. cereus is a notorious foodborne pathogen which causes gastroenteritis. Fatal and severe cases have been reported. The pathogenicity of *B. cereus* is intimately associated with the production of epithelial cell-destructive toxins in the small intestine. The small intestine poses several challenges for a pathogen because it is sliced into various niches with different oxygen concentrations and different redox potentials. We recently showed that the organic hydroperoxide resistance OhrRA system was crucial to the successful adaptation of *B. cereus* to extreme redox environments such as those encountered in the lumen (high reducing anoxic environment) and on the intestinal epithelium (transient oxic environment). Here we provide evidence that this bacterial system is a major virulence determinant in *B. cereus* in that it coordinates toxinogenesis in a redox dependent manner. Specifically, our comparative exoproteomic analyses reveal that OhrR strongly restricts *B. cereus* toxinogenesis under high reducing anoxic conditions while OhrA boosts toxinogenesis. Based on exoproteomic analyses, we further examined the role of OhrR and found that it functions as a

* *Corresponding author*. Tel.: +33 432 722 507; fax: +33 432 722 492.
E-mail address: catherine.duport@univ-avignon.fr (C. Duport).

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redox-dependent transcriptional regulator of toxin and putative toxin genes. These findings provide novel insights into the weapons used by *B. cereus* to control its toxinogenic potential and, as a result its toxicity against human epithelial cells.

1. Introduction

Bacillus cereus is a ubiquitous, globally distributed, foodborne pathogen. This human pathogen [1,2] is mainly associated with two types of gastrointestinal (GI) infection, both causing diarrhea and vomiting after the ingestion of contaminated food. The diarrheal syndrome is mainly attributed to toxins secreted by vegetative cells colonizing the human small intestine [3–5]. A total of 14 toxin-related proteins were identified in the culture supernatant of *B. cereus* cells grown in low oxidoreduction potential (ORP) anoxic conditions, which are considered to mimic those encountered in the human intestine [6]. These 14 toxin-related proteins include the four components (HblL1, HblL2, HblB and HblB′) of hemolysin BL (Hbl), the three components (NheA, NheB, NheC) of the nonhemolytic enterotoxin, Nhe, and the single-component protein, CytK [5,7–9]. Hbl, Nhe and CytK are secreted, pore-forming cytotoxins, which are currently considered to be the main causative agents of diarrheal syndrome [9–11]. However, several other toxin-related proteins may contribute to this syndrome, including hemolysin I (cereolysin O, HlyI), which is a thiol-activated, cholesterol-binding cytolysin, hemolysin II (HlyII) and enterotoxin FM (EntFM) [12–17]. Enterotoxin-like EntA, EntB and EntC, which are not regulated by the pleiotropic virulence gene regulator PlcR, cannot be excluded as causative agents of diarrheal syndrome [6]. Although toxin-related proteins most probably play a prevalent role in *B. cereus* pathogenicity, other proteins present in the extracellular milieu may be required, such as components of flagella [18], cell-wall peptidases [13,19,20], metalloproteases [21], antioxidant enzymes [22], siderophores for iron acquisition from the host [23] and moonlighting proteins [24–26]. All of these exoproteins are classified as virulence factors and constitute the major part of the *B. cereus* exoproteome as observed by shotgun, high-throughput methodology [6,27].

The regulation of *B. cereus* toxin gene expression mobilizes a complex machinery [28] that includes several pleiotropic regulators, such as the PlcR–PapR quorum sensing system [29,30], the CodY repressor [31], the ferric uptake regulator Fur [32], the catabolite control protein A (CcpA) [33], the two-component regulatory system ResDE, and the redox regulator Fnr. ResDE and Fnr are also involved in maintaining redox homeostasis by regulating catabolism [34–38]. Like ResDE and Fnr, the OhrRA system is involved in redox homeostasis [39]. Unlike ResDE and Fnr, the involvement of OhrRA in toxinogenesis has not been yet reported in the literature. The *B. cereus* OhrRA system comprises OhrA (organic hydroperoxide resistance protein A), an antioxidant protein, and OhrR, a potential pleiotropic regulator belonging to the MarR (multiple antibiotic resistance) family of winged, helix–turn–helix, DNA-binding proteins [40,41]. However, *B. cereus* OhrR appears to be an atypical member of this family because it contains four cysteine residues at its N-terminal domain while most members have two cysteines [39].

Here, we investigated the impact of *B. cereus* OhrR and OhrA on toxinogenesis. We compared the exoproteomes of Δ*ohrA* and Δ*ohrR* mutants with their parental strains from cells grown under three different ORP — one under aerobiosis and two under anaerobiosis. We found that OhrRA controls the toxinogenic profile of *B. cereus*. The effect of OhrRA on the production of toxin-related proteins is mediated at both the transcriptional (mainly by OhrR) and post-transcriptional (mainly by OhrA) levels. OhrR restricts toxin-dependent, antiproliferative activity of the *B. cereus* exoproteome on Caco-2 cells whatever the growth conditions, while OhrA restricts this activity only under low-ORP anaerobiosis.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli BL21 CodonPlus(DE3)-RIL (Stratagene) was used for the heterologous production of OhrR. *B. cereus* ATCC 14579 was used as the parental strain [42]. Strains lacking OhrA (Δ*ohrA*) and OhrR (Δ*ohrR*) were constructed previously [6]. Wild-type and mutant strains were grown in pH-regulated batch cultures on glucose-containing MOD medium [43,44] under low-ORP anaerobiosis (initial ORP = −410 mV, pO_2 = 0%), high-ORP anaerobiosis (iORP = −10 mV, pO_2 = 0%) and full aerobiosis (iORP = +140 mV, pO_2 = 100%), as previously described [39]. Supernatants were collected at the log/exponential growth phase, *i.e.* 1.5 h after reaching the maximal growth rate (when μ = 80 (±10) % of μ_{max}). After growth, the culture supernatants were first filtered through a 45-μm-pore-size, low-adsorption, cellulose-acetate membrane filter (Sartorius) and then through a 20-μm-pore-size filter. Filtered fractions (40 mL) were quickly frozen and kept at −20 °C until use.

2.2. Relative quantification of gene expression using real time RT-PCR

RT-PCR was performed using SYBR Green technology on a Lightcycler instrument (Roche applied Science) as described previously [34]. The primers used in this study are shown in Table S1.

2.3. Exoprotein sample preparation and nanoLC-MS/MS analysis

Three independent biological replicates were harvested for each of the three conditions (aerobiosis, low- and high-ORP anaerobiosis) and three strains (wild-type, and Δ*ohrA* and Δ*ohrR* mutant strains). Extracellular proteins were extracted from the 27 culture supernatants, subjected to SDS-PAGE, and then identified after trypsin proteolysis and nanoLC-MS/MS tandem mass spectrometry, as previously described [6]. The MASCOT search engine (version 2.2.04) was used to search all MS/MS spectra against an in-house polypeptide sequence database containing the sequence of all annotated proteins