PlcRa, a New Quorum-Sensing Regulator from *Bacillus cereus*, Plays a Role in Oxidative Stress Responses and Cysteine Metabolism in Stationary Phase

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**Abstract**

We characterized a new quorum-sensing regulator, PlcRa, which is present in various members of the *B. cereus* group and identified a signaling heptapeptide for PlcRa activity: PapRa². We demonstrated that PlcRa is a 3D structural paralog of PlcR using sequence analysis and homology modeling. A comparison of the transcriptomes at the onset of stationary phase of a *ΔplcRa* mutant and the wild-type *B. cereus* ATCC 14579 strain showed that 68 genes were upregulated and 49 genes were downregulated in the *ΔplcRa* mutant strain (>3-fold change). Genes involved in the cysteine metabolism (putative CymR regulon) were downregulated in the *ΔplcRa* mutant strain. We focused on the gene with the largest difference in expression level between the two conditions, which encoded -AbrB2- a new regulator of the AbrB family. We demonstrated that purified PlcRa bound specifically to the *abrB2* promoter in the presence of synthetic PapRa², in an electrophoretic mobility shift assay. We further showed that the AbrB2 regulator controlled the expression of the *yrrT* operon involved in methionine to cysteine conversion. We found that the *ΔplcRa* mutant strain was more sensitive to hydrogen peroxide- and disulfide-induced stresses than the wild type. When cystine was added to the culture of the *ΔplcRa* mutant, challenged with hydrogen peroxide, growth inhibition was abolished. In conclusion, we identified a new RNPP transcriptional regulator in *B. cereus* that activated the oxidative stress response and cysteine metabolism in transition state cells.

**Introduction**

The *Bacillus cereus* group includes well known spore-forming pathogens of mammals (*B. anthracis* and *B. cereus*) and insects (*B. thuringiensis*). *B. cereus* is frequently associated with food-borne infections causing gastroenteritis [1]. The capacity of *B. cereus* to sporulate allows this bacterium to resist the usual cleaning procedures used in the food industry, resulting in the presence of *B. cereus* in many raw and processed foods, such as rice, spices, milk, vegetables, meats and various desserts [1].

At the end of the vegetative growth phase, bacterial cells face a number of challenges, including a decrease in the nutrient content of their environment. Under these conditions, spore-forming bacteria may initiate sporulation, producing spores that can survive in unfavorable environmental conditions [2]. Bacteria use various strategies to cope with environmental changes during the transition between the vegetative and sporulation phases [3]. The production of degradative enzymes and antimicrobial compounds responsible for the lysis of targeted cells provides *Bacillus subtilis* with new nutrients [3]. In parallel, a general stress response may be activated during the transition phase, due to the accumulation of oxidative products and changes in the pH of the medium [4]. Cellular responses may be controlled by a range of sensors and activators, including two-component systems, quorum-sensing systems and other transcriptional regulators [3,5].

Quorum sensing regulation appears to be a consequence of interbacterial communication by which bacteria of one or even different species sense about their current population density and react in a defined way to that information. These communication systems are based on the secretion and recognition of cell-cell signalling molecules, termed autoinducers [6]. The PlcR/PapR quorum sensing system is activated during the transition phase in most members of the *B. cereus* group [7]. This system controls the expression of genes encoding exported virulence factors, including degradative enzymes, enterotoxins and hemolysins [8]. PlcR is activated by binding to PapR, a signaling peptide produced as a propeptide under the control of PlcR. PapR undergoes extracellular processing, to generate an active heptapeptide [9], which is then re-imported into the bacterial cell via the oligopeptide permease system, OppABCDF [10]. Within the cell,
PapR interacts with PlcR and the resulting complex binds PlcR target sites on DNA [11], resulting in the activation of the PlcR regulon, which contains 40 genes [7].

The structure of PlcR has been resolved. This molecule has a unique folding pattern, due to the presence of an HTH DNA-binding domain and a peptide-binding regulatory domain composed of five tetratricopeptide repeats (TPR) [12]. A TPR is a structural 34-amino acid repeat motif present in various eukaryotic and prokaryotic proteins. There may be from 3 to more than 16 tandem repeats [13]. Determination of the structure of PlcR resulted in the identification of a new family of central regulatory quorum sensors (the RNPP family) found exclusively in Gram-positive bacteria with a low G+C content [12]. These quorum sensors include NprR from B. cereus [14], PrgX from Enterococcus faecalis [15] and RAP phosphatases from B. subtilis [16,17]. All these RNPP regulators are activated through a secreted signaling peptide that interacts with the TPR activation domain.

In this work, we characterized PlcRa, a novel quorum-sensing type regulator of the RNPP family and identified PapRa2, a new signaling heptapeptide. We constructed a ΔplcRa mutant strain from the B. cereus ATCC 14579 type strain [18] and identified PlcRa-controlled genes through a whole-genome microarray from the ATCC 14579 genome: BC0988 (PlcRa), BC1158 (PlcRb), previously named PcrR2 in B. anthracis [19] and BC2443 (PlcRc). These putative regulators display about 29% sequence identity and about 50% similarity to PlcR. The PlcRa, PlcRb and PlcRc proteins have high levels of overall sequence identity (85%). A small ORF, BC0989, encoding a putative peptide with a potential signal sequence is located upstream from the plcRa gene. We named this gene popRa. In contrast, the plcRb and plcRc genes are not associated with such genes. As all characterized RNPP regulators are activated by exported peptides with regulatory functions, we decided to focus on the plcRap/popRa locus. We describe here the analysis of plcRa in the B. cereus ATCC 14579 strain. Genome comparisons revealed genes for PlcRa (identity >94%) in B. thuringiensis BMB171, serovar chinesis CT-43, HD-789, HD-771 strains (4 out of 7 complete genomes) and B. cereus B4264 and G9842 strains (3, including ATCC 14579, out of 13 complete genomes) but no such gene was present in the genome of B. anthracis (data not shown). The 297-amino acid PlcRa protein displays 29% identity and 51% similarity to PlcR (Figure 1A). Given this high identity score over its entire sequence, PlcR is a relevant template for homology modeling [20]. We therefore constructed a homology model for PlcRa, based on the structure of the PlcR dimer solved at a resolution of 2.6 Å [12]. Each PlcR monomer displays a unique folding pattern, with an HTH domain at the N-terminus, followed by a linker helix connecting the HTH domain to the 5 TPR domains and serving as an anchoring platform for dimerization. The packing of the five TPR domains defines, for each monomer, a pocket that binds the PapR activator peptide [12]. The PlcRa homodimer model, composed of A and B chains, was constructed progressively, beginning with chain A, followed by the addition of chain B and ending with modeling of the dimer (Figure 1B). As expected, the homology model of the PlcRa homodimer was found to be highly helical. Each monomer has, at its N-terminus, an HTH domain followed by a linker helix of 27 residues (Figure 1A) that connects the HTH to the five TPRs and anchors the two monomers together (Figure 1B). Thus, the homology model of PlcRa constructed here is very similar to the X-ray structure of PlcR. By analogy with PlcR [12], we suggest that the five TPR motifs may be arranged similarly, to form a pocket that is responsible for peptide binding. The popRa gene encodes a 93-amino acid polypeptide, which is longer than PapR (45 amino acids). As described for PapR, a typical Gram-positive N-terminal signal peptide was identified for PlcRa with SignalP program (Figure 2A) [21]. Interestingly, alignment of PapR and PlcRa sequences showed similarity over a short segment corresponding to the PapR C-terminus including the heptapeptide (ADLPFF), which is the physiological activator of PlcR (Figure 2A) [9]. Based on this sequence alignment, the CSIPYEY fragment of PapRa was proposed as a consistent candidate for a signaling heptapeptide. We docked CSIPYEY into the dedicated pocket of PlcRa and minimized the energy of the complex with CHARMM [22]. This docking procedure showed that PapRa could fit into the PlcRa pocket formed by the five TPRs (Figure 2B). Overall, these homology modeling and docking analysis suggested that PlcRa is a 3D structural homolog of PlcR. For confirmation and characterization of PlcRa as a regulator, we first conducted a genetic analysis of the plcRa gene and searched for target genes using a comparative transcriptome analysis approach with the ΔplcRa strain.

The Expression of plcRa Is Activated at the Onset of the Stationary Growth Phase

We investigated the temporal regulation of plcRa gene expression, by constructing a P_{plcRa}$\text{-}\text{lacZ}^+$ transcriptional fusion in the low-copy-number plasmid pH304–18Z [23]. The P_{plcRa}$\text{-}\text{lacZ}^+$ fusion was introduced into the B. cereus wild-type strain and β-galactosidase activity was measured from $t_{-2}$ to $t_0$ (time zero, $t_0$, corresponds to the onset of the stationary growth phase, and $t_0$ is the number of hours before (−) or after time zero) during growth in LB medium. Expression of the P_{plcRa}$\text{-}\text{lacZ}^+$ fusion began at $t_{-1}$ and increased rapidly from $t_{0.5}$ to $t_{1.5}$ (Figure 3A). These findings suggest that plcRa expression is transiently activated early in the stationary phase. We then constructed a plcRa mutant (Table 1) as described in Material and Methods. Expression of the P_{plcRa}$\text{-}\text{lacZ}^+$ fusion was similar in the wild-type strain and in the ΔplcRa mutant strain during growth (data not shown), indicating that the plcRa gene is not autoregulated. The transcriptional start site identified by 5’RACE was located 46 bp upstream from the predicted start codon of plcRa (Figure 3B). The plcRa promoter region contains −10 and −35 DNA binding regions resembling those recognized by SigA [24].

Identification of PlcRa-controlled Genes

We characterized the regulatory role of PlcRa, by comparing the transcriptomes of the ΔplcRa and wild-type B. cereus ATCC 14579 strains during early stationary phase in LB medium. Assessments were carried out one hour after the onset of stationary phase ($t_1$), when plcRa expression increases, and two hours after the onset of stationary phase ($t_2$), when plcRa expression has reached a plateau.

In total, 117 genes were differentially expressed with a more than three-fold difference between the wild-type strain and the ΔplcRa mutant strain (Tables 2, 3). Forty-nine genes were more strongly expressed in the wild-type strain and 68 genes were less strongly expressed in the wild-type strain than in the mutant. In total, 12 genes were differentially expressed at $t_1$, 88 were
PicRa, a New RNPP Regulator from *Bacillus cereus*

**A**

**HTH domain**

| 1 | 10 | 20 | 30 | 40 | 50 | 60 | 70 |
|---|----|----|----|----|----|----|----|
| PlcR |  |  |  |  |  |  |  |
| PlcRa |  |  |  |  |  |  |  |

**Linker helix**

| 90 | 100 | 110 | 120 |
|----|-----|-----|-----|
| PlcR |  |  |  |  |
| PlcRa |  |  |  |  |

**TPR 1**

| 130 |
|-----|
| PlcR |  |
| PlcRa |  |

**TPR 2**

| 140 | 150 | 160 | 170 | 180 | 190 | 200 |
|-----|-----|-----|-----|-----|-----|-----|
| PlcR |  |  |  |  |  |  |
| PlcRa |  |  |  |  |  |  |

**TPR 3**

**TPR 4**

| 210 | 220 | 230 | 240 | 250 |
|-----|-----|-----|-----|-----|
| PlcR |  |  |  |  |
| PlcRa |  |  |  |  |

**TPR 5**

| 280 |
|-----|
| PlcR |  |
| PlcRa |  |

**B**

**Monomer α**

**Monomer β**

**Distribution of the domains of PicRa monomer**

| HTH | Linker helix | TPR | TPR | TPR | TPR | TPR |
|-----|--------------|-----|-----|-----|-----|-----|
| HTH | Linker helix | TPR | TPR | TPR | TPR | TPR |
| 89-127 | 134-168 | 172-212 | 216-253 | 259-290 |
differentially expressed at \( t_2 \) and 17 were differentially expressed at both \( t_1 \) and \( t_2 \). These transcriptome data were validated by RT-qPCR analysis on a set of 15 genes with new cultures (Tables 2, 3 and Table S1).

Most of the proteins encoded by the genes upregulated by PlcRa (expressed more strongly in the wild-type strain) fell into four main categories: sulfur metabolism, oxidative stress responses, peptide transport and iron metabolism (Tables 2, 3). The largest category, sulfur metabolism, comprised 20 proteins, 18 of which were orthologs of proteins involved in cysteine transport and metabolism in \( B. subtilis \) (Table 2) (see below).

The second category, oxidative stress response proteins, contained five proteins, including PerR and OhR, two major regulators of the oxidative stress responses in \( B. subtilis \) [5] and presumably in \( B. cereus \) [25,26]. PerR regulates many genes, including those of the ahpCF operon encoding the alkyl hydroperoxide reductase (AhpR), a detoxification system composed of two enzymes – hydrogen peroxide-forming NADH oxidase (nox-1) and peroxidase (AhpC) – that catalyzes the breakdown of molecular oxygen to hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) which is reduced by the second enzyme to water. This operon was downregulated in a \( plcRa^{-} \)-deficient mutant strain. The final protein in this group was a Dps-like miniferritin protein, the Dsp2 protein (BC5044), which...
The third group comprises four components of an uncharacterized oligopeptide permease system, Opp, which is thought to be required for the import of small molecules into the bacterial cell. The fourth category consists of five proteins involved in iron transport and metabolism. In addition to these factors, PlcRa induces the expression of genes encoding proteins of various known or unknown functions. These genes include the gene displaying the strongest upregulation by PlcRa one hour after entry into stationary phase (Table 3). This gene (BC244) encodes a regulatory protein belonging to the AbrB family analyzed in greater detail below.

Most of the proteins encoded by genes downregulated by PlcRa (lower expression in the wild-type strain versus plcRa-deficient mutant strain) belonged to two major categories (Table S1). The first one consisted of general stress Sigma factor SigB, its associated regulatory proteins RbsV, RbsW and RbsP and six SigB-controlled proteins [28]. The second consisted of 31 prophage proteins encoded by the genes of two prophages, phBC6A52 and phBC6A51, harbored by the chromosome of strain ATCC 14579 [10]. Our transcriptome analysis indicated that PlcRa downregulated the expression of about 50% of phBC6A52 genes (49, total ORFs number) and 13% of phBC6A51 genes (75, total ORFs number) [29]. BC1852 and BC1857 encode phBC6A51 prophage proteins thought to be involved in DNA repair: an SbcC-like chromosomal ATPase and an SbcD-like protein, both related to bacterial SMC-like (structural maintenance of chromosome) proteins [30]. This is the first report of a bacterial regulator controlling the expression of numerous phage genes in this strain, or even in B. cereus [29,31,32]. PlcRa downregulated several other genes unrelated to these two main categories, including a three-gene operon encoding the components of the Hbl enterotoxin, a virulence determinant thought to be involved in diarrheal disease. Expression of the hbl operon is activated by PlcR at the onset of the stationary phase [1]. PlcRa is a pleiotropic regulator activated at the onset of stationary phase. Since PlcRa controls regulators, some PlcRa-controlled genes may be indirect PlcRa targets.

Figure 3. Analysis of plcRa expression. A. Kinetics of plcRa gene expression. Specific β-galactosidase activity (U/mg protein) of strain B. cereus ATCC 14579 harboring the transcriptional P_{plcRa}=lacZ fusion. Time zero corresponds to the onset of the stationary growth phase, and t_i is the number of hours before (–) or after time zero. The cells were grown at 37°C in LB medium. Error bars are shown. B. Determination of the transcriptional start site of plcRa. The 5′ RACE-PCR method was used to identify the transcriptional start site of plcRa. The start site (+1, in bold typeface) and the −10 and −35 putative promoter elements from the vegetative sigma factor are shown in bold typeface and underlined. The putative ribosome-binding site sequence and putative start codon of plcRa are shown in bold typeface and underlined.

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Table 1. Strains used.

| Strain      | Relevant genotype | Source or reference |
|-------------|-------------------|---------------------|
| BCATCC 14579| Reference wild-type B. cereus strain | [18]                |
| ΔplcRa      | ΔplcRa::tet       | This study          |
| ΔabrB2      | ΔabrB2::tet       | This study          |
| ΔplcRa-ΔabrB2 | ΔplcRa::tet, pHT304ΔplcRa ery | This study |
| plcRa/Z     | ppxyn::lacZ ery | This study          |
| abrB2/Z     | ppxyn::lacZ ery | This study          |
| yrrT/Z      | ppxyn::lacZ ery | This study          |
| plcRa/Z-ΔplcRa | ppxyn::lacZ ery ΔplcRa::tet | This study          |
| abrB2/Z-ΔplcRa | ppxyn::lacZ ery ΔplcRa::tet | This study |
| abrB2/Z-ΔplcRa ΔpapRa | P_{pxyl}::lacZ ery pHT1618D_papRa kana ΔplcRa::tet | This study |
| yrrT/Z-xyfΔabrB2 | P_{pxyl}::lacZ ery pHT1618D_pxyf-ΔabrB2 kana | This study |
| yrrT/Z-xyfΔabrB2ΔplcRa | P_{pxyl}::lacZ ery pHT1618D_pxyf-ΔabrB2ΔplcRa::tet | This study |
| abrB2/Z-xyfΔpapRa | P_{pxyl}::lacZ ery pHT1618D_pxyf-ΔpapRa kana | This study |
| abrB2/Z-xyfΔpapRaΔplcRa | P_{pxyl}::lacZ ery pHT1618D_pxyf-ΔpapRaΔplcRa::tet | This study |

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is homologous to the Dsp2 protein of B. anthracis (BA5290) that has recently been shown to play a major role in oxidative stress resistance [27].
**Table 2.** Sulphur metabolism genes expressed more strongly in the wild-type strain than in the *B. cereus* plcRa mutant straina.

| Locus tagb synonymf | Function/similarity | Wild type/ΔplcRα expression ratio | microarray analysis | qRT-PCR analysis |
|---------------------|----------------------|-----------------------------------|-------------------|-----------------|
|                     |                      | t1     | t2     | t2       |
| BC1421 cysH         | Phosphoadenosine phosphosulfate reductase | 1      | 2      |          |
| BC1422 sat          | Sulfate adenylytransferase | 1      | 6      | 21      |
| BC1423 cysC         | AdenylylSulfate kinase | 1      | 8      |          |
| BC1424              | Putative ferredoxin-nitrite reductase/sulfite reductase | 1      | 5      | 32      |
| BC1425              | Hypothetical protein | 1      | 5      |          |
| BC1426 sumT         | Uroporphyrin-III C-methyltransferase | 1      | 2      |          |
| BC1427 sirB         | Sirohydrochlorin ferrochelatase | 1      | 4      |          |
| BC1428 sirC         | Precorrin-2 dehydrogenase | 1      | 2      |          |
| BC4369 ynrT         | AdoMet-dependent methyltransferase | 1      | 12     | 8       |
| BC4368 mtnN         | methylthioadenosine/5-adenosylhomocysteine nucleosidase | 1      | 4      |          |
| BC4367 mccA         | Cystathionine β-synthase | 1      | 9      |          |
| BC4366 mccB         | Cystathionine γ-lyase | 1      | 16     | 15      |
| BC4392 ynrO         | Cysteine desulphhydrase | 1      | 3      |          |
| BC4393 cymR         | Cysteine metabolism repressor protein (RRF2 family) | 1      | 3      |          |
| BC0075 cysK         | OAS-thiol-lyase | 1      | 3      |          |
| BC2617              | Cysteine dioxygenase | 1      | 2      |          |
| BC4003 metE         | Cobalamin-independent methionine synthase | 1      | 3      |          |
| BC4242 tcyP         | Sodium-cysteine symporter | 1      | 7      | 3       |
| BC4751              | Putative sulfite reductase (flavoprotein alpha-subunit) | 1      | 3      |          |
| BC4789 luxS         | S-ribosylhomocysteine lyase/AI2 production | 1      | 5      | 3       |

**PlcRa Upregulates Cysteine Metabolism Genes**

Twenty of the 49 genes upregulated by PlcRa encode proteins involved in sulfur metabolism (Table 2). These genes were found to be differentially expressed only at t2. The sulfur metabolism genes of *B. cereus* remain poorly annotated, despite the availability of several *B. cereus* genomes. We therefore reconstructed the sulfur metabolism pathway, by searching for orthologs of *B. subtilis* genes in the *B. cereus* ATCC 14579 genome. The transport of sulfur sources [33] and the two major cysteine biosynthetic pathways in *B. subtilis* – the thiolation pathway, which requires sulfide, and the reverse transsulfuration pathway, which converts homocysteine to cysteine, with cystathionine formed as an intermediate [34,35] – are conserved in *B. cereus* ATCC 14579. We were able to identify all the enzymes and transporters required for these pathways other than those for the reduction of sulfite to sulfide (Table 2, Figure S1).

Eighteen PlcRa-controlled genes were identified as putative homologs of genes involved in cysteine metabolism in *B. subtilis* (Table 2, Figure S1): a cystine (the oxidized form of cysteine) transporter (*tcpP*) [33] and proteins involved in the biosynthesis of cysteine from sulfate (the *cysH* operon and *cysK* gene) or methionine (the *yrtT*-mtnN-mccAB operon and *luxS* gene) [35]. In *B. subtilis*, the expression of these genes is repressed by the transcriptional regulator CymR, in response to cysteine availability [35,36]. BC4393, which is 76% identical to CymR from *B. subtilis*, is probably the global negative regulator of cysteine metabolism in *B. cereus*. By screening for the *B. subtilis* CymR box sequence, we identified putative CymR-binding motifs upstream from *cysK*, *cysH*, * tcpP*, *ynrT* and *luxS* (Figure S2). Thus, PlcRa upregulates 14 probable members of the CymR regulon in *B. cereus*. The cymR gene itself was downregulated in a *plcR* mutant strain (ratio 3, Table 2). A lacZ transcriptional fusion was constructed with the promoter region of the cymR gene and introduced into the wild-type strain and the *plcR* mutant. We found that the cymR gene expression was constitutive (Figure S3) as described in *B. subtilis* (I. Martin-Verstraete, unpublished results, [37]). A significant but small difference (ratio 2) in β-galactosidase activity was transiently observed between the wild-type strain and the *plcR* mutant over a short period, one hour after the onset of stationary phase (Figure S3). We hypothesized that PlcRa activated CymR-controlled genes independently of CymR or that PlcRa upregulated CymR-controlled genes through the modulation of CymR activity.

**Transcriptional Control of the BC2444 (abrB2) Gene by PlcRa**

For characterization of PlcRa as a transcriptional regulator, we searched for a direct target gene candidate. Microarray analysis indicated that the PlcRa-regulated gene BC2444 was differentially expressed at both t1 and t2 and that this gene presented the highest differential expression ratio at t2 (6-fold in the microarray analysis and 50-fold in RT-qPCR analysis) (Table 3). This gene encodes a putative regulator 50% identical to the AbrB regulator of *B. subtilis* [38] and 85% identical to that of *B. cereus* [BC0042] [39].
We therefore renamed BC2444, *abrB2*. Given the high levels of early activation of this regulator gene observed here, we decided to investigate its expression kinetics. We analyzed the expression of a plasmid-borne P*abrB2*-lacZ transcriptional fusion in the wild-type and *DplcRa* strains (Figure 4). We found that *abrB2* expression increased sharply from \( t_0 \) to \( t_2 \). In the *plcRa* mutant, *abrB2* expression was strongly reduced while the introduction of *plcRa* in trans restored its expression. Thus, *abrB2* expression is activated by the presence of PlcRa at the onset of stationary phase.

### Purified PlcRa Binds Specifically to the *abrB2* Promoter in the Presence of PapRa

To further understand the role of PlcRa on the *abrB2* expression and distinguish between direct or indirect effects, electrophoretic mobility shift assays (EMSA) were performed with the same fragment present in plasmid P*abrB2*-lacZ (Figure 4) using purified PlcRa. A biotin end-labeled DNA fragment containing the *abrB2* promoter region was incubated in the presence of increasing PlcRa concentrations (Figure 5A). We did not observe gel-retardation under these conditions.

### Table 3. Genes expressed more strongly in the wild-type strain than in the *B. cereus plcRa* mutant strain*

| Locus tag* synonym | Function/similarity | Wild type/\(\Delta plcRa\) expression ratio | microarray analysis | qRT-PCR analysis |
|-------------------|---------------------|-------------------------------------------|-------------------|-----------------|
| *Oxidative stress response* | | | | |
| BC0518 *perR* | Peroxide stress response/metal-dependent repressor protein (Fur family) | 3 | 1 |
| BC0377 *ahpC* | Alkyl hydroperoxide reductase small subunit | 1 | 3 | 2 |
| BC0376 *ahpF* | Alkyl hydroperoxide reductase large subunit | 1 | 2 |
| BC5044 *dps2* | Dps-like miniferritin/antioxidant protein | 3 | 2 | 3 |
| BC4474 *ohnR* | Organic hydroperoxide resistance regulatory protein | 1 | 3 |
| *Peptide/nickel transport* | | | | |
| BC0242 | Oligopeptide transport system permease protein oppB-like | 1 | 4 | 3 |
| BC0243 | Oligopeptide transport system permease protein oppC-like | 1 | 4 |
| BC0244 | Oligopeptide transport ATP-binding protein oppD-like | 1 | 5 | 3 |
| BC0245 | Oligopeptide transport ATP-binding protein oppF-like | 1 | 3 |
| *Iron transport/metabolism* | | | | |
| BC5380 | Ferrichrome-binding protein | 3 | 1 |
| BC5381 | Ferrichrome transport ATP-binding protein fhuC | 2 | 1 |
| BC5382 | Ferrichrome transport system permease protein fhuG | 3 | 1 |
| BC5383 | Ferrichrome transport system permease protein fhuB | 2 | 1 |
| BC1154 | Ferrochelatase | 4 | 1 |
| *Miscellaneous* | | | | |
| BC2444 | Putative transition state regulatory protein (AbrB family) | 6 | 3 | 50 (\(t_0\)) |
| BC3727 *yhgG* | Formate/nitrite transporter | 3 | 1 |
| BC3225 | Macrolide efflux protein | 3 | 1 |
| BC4925 | NADH dehydrogenase | 3 | 1 |
| BC1224 | Acetyltransferase | 1 | 3 |
| BC3662 *ccdA* | Ribosomal-protein-alanine acetyltransferase | 1 | 4 |
| BC3338 | Hydrolyase | 1 | 3 |
| BC4660 *acuA* | Acetoin utilization protein AcuA | 1 | 3 |
| BC1225 | 2'-5' RNA ligase | 1 | 3 |
| *Hypothetical protein* | | | | |
| BC2445 | Hypothetical protein | 3 | 2 |
| BC2446 | Hypothetical membrane-spanning protein | 2 | 2 |
| BC1074 | Hypothetical protein | 1 | 4 |
| BC3506 | Hypothetical protein | 1 | 4 |
| BC4208 | Hypothetical protein | 4 | 1 |
| BC5260 | Hypothetical protein | 1 | 3 |
| Total 29 | | | | |

*See Table 2 for legends.

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We observed no increase in \( \beta \)-galactosidase activity when PapRa7 was added to the culture after \( t_{2/3} \). Moreover, when the peptide was added to the culture during the exponential growth phase, the increase in \( \beta \)-galactosidase activity coincided strictly with entry into stationary phase (data not shown). The addition of synthetic PapRa7 in the culture positively affected \( abrB2 \) expression at the onset of stationary phase in a PlcRa-dependent manner. A plasmid harboring the \( papRa \) gene under the control of a xylose-inducible promoter was constructed and subsequently introduced into a wild-type strain containing the P\( _{yrrT} \)\(-\lacZ\) and into the \( plcRa\)-deficient mutant previously described. When xylose was added at \( t_{2/3} \), the \( \beta \)-galactosidase activity strictly increased at the onset of stationary phase compared to the level observed in the culture without xylose (Figure 6B) whereas in the \( plcRa \) mutant we did not observe any change of the weak \( \beta \)-galactosidase activities (data not shown). We demonstrated that the level of PapRa production influenced \textit{in vivo} the activity of PlcRa.

\textbf{AbrB2 Controls \( yrrT \) Expression}

To investigate the possible role of AbrB2 in the regulation of PlcRa-controlled genes, we constructed a deletion mutant strain (Table 1). We tested the effect of \( abrB2 \) deletion on the transcription of the \( yrrT \) operon, which encodes proteins involved in methionine-to-cysteine conversion \[34\] (Figure S1). We constructed a transcriptional fusion between \( lacZ \) and the \( yrrT \) promoter region and investigated the kinetics of P\( _{yrrT} \)\(-\lacZ\) expression in the wild-type and \( \Delta plcRa \) and \( \Delta abrB2 \) mutant strains during growth. To understand the role of AbrB2 on \( yrrT \) expression, we expressed \( abrB2 \) under the control of a xylose-inducible promoter (P\( _{xylA} \)) in \( \text{pHT1618} \). We introduced this plasmid into the wild type, \( \Delta plcRa \) and \( \Delta abrB2 \) strains containing the P\( _{yrrT} \)\(-\lacZ\) fusion (Figure 7). In the absence of xylose, an increase in \( \beta \)-galactosidase activity was detected at the onset of stationary phase in the wild-type strain, but not in either of the mutant strains (Figure 7). In the presence of xylose, \( \beta \)-galactosidase activity increased at the onset of stationary phase in both the \( plcRa \) and \( abrB2 \) mutant strains, reaching levels similar to those for the wild type. Thus, the down-regulation of \( yrrT \) expression due to \( plcRa \) inactivation was complemented by \( abrB2 \) in trans. In addition, the expression of \( abrB2 \) under the control of the \( xylA \) promoter in the presence of xylose also restored the expression of the \( yrrT \) fusion in a \( \Delta plcRa \) background. These findings demonstrated that the PlcRa-dependent control of \( yrrT \) is mediated by AbrB2.

\textbf{High Sensitivity of the \textit{pIcRa} Mutant Strain to Peroxide and Disulfide Stresses}

PlcRa controls the expression of genes encoding proteins involved in the oxidative stress response and cysteine biosynthesis. Previous studies in \( B. \ subtilis \) and \( S. aureus \) have established strong links between cysteine metabolism and oxidative stress [5,35,37,40]. It has been shown in \( B. \ subtilis \) [5] and \( B. \ anthracis \) [41,42,43] that cysteine itself and cysteine-containing molecules such as bacillilhiol or CoenzymA play a key role in protection against oxidative stress. We therefore evaluated the sensitivity of the wild-type and \( plcRa \) mutant strains to \( H_2O_2 \) and diamide, a compound that causes thiol oxidation and disulfide stresses. We first demonstrated that the addition of \( H_2O_2 \) (1 mM) or diamide (10 mM) to LB medium at the start of stationary phase had no dramatic effect on \( plcRa \) expression (Figure S4). The viability of the \( \Delta plcRa \) and wild-type strains was then assessed after the addition of \( H_2O_2 \) (1 mM) or diamide (10 mM) to the LB medium (Figure 8AB). Survival rates for the \( \Delta plcRa \) strain were lower than those for the wild-type strain, by a factor of six in the presence of \( H_2O_2 \) and...
10 in the presence of diamide. The introduction of plcRa, in trans, into the ΔplcRa strain restored the wild-type phenotype. Thus, plcRa inactivation led to an increase in sensitivity to H2O2 and disulfide-induced stresses, suggesting a role for PlcRa in the regulation of the peroxide and disulfide stress defense system of *B. cereus*.

**The Addition of Cystine Improves the Stress Resistance of the plcRa Mutant**

In *B. subtilis*, cysteine depletion induces the expression of cysteine synthesis genes, such as those of the *cysH* operon, which is involved in cysteine production from sulfate, or the *yrrT* operon, which is involved in methionine-to-cysteine conversion [34,44]. These operons were downregulated in the plcRa mutant (Table 2, Figure S1). We hypothesized that this would result in lower intracellular levels of cysteine in the plcRa mutant. Moreover, we found that the expression of the *P* *yrrT*-lacZ fusion was induced at the onset of stationary phase in the wild-type strain, suggesting cysteine depletion in the growth medium leading to the induction of the methionine-to-cysteine conversion pathway. No such induction was observed in the plcRa mutant strain (Figure 7). We thus investigated the effects of cystine addition during peroxide stress. Cystine (1 mM) was added to the culture in mid-exponential growth phase. We first demonstrated that the addition of cystine to LB medium in mid-exponential growth phase did not modify plcRa expression (data not shown). No growth difference was observed for the wild-type and ΔplcRa mutant strains with or without cystine (Figure 8C). Two hours later, at the end of exponential phase, H2O2 (0.4 mM) was added to the medium. Both strains presented a growth arrest one hour after the H2O2 addition which was characterized by a OD600 measurements drop. In addition, in the presence of H2O2, OD600 measures of the ΔplcRa mutant strain were lower than the wild type strain. When cystine was added, the growth arrest for both strains was abolished (Figure 8C). Thus, cystine significantly reduced the sensitivity of these cells to H2O2 stress in our growth conditions. Moreover, these results strongly suggest that cystine transport is efficient in the ΔplcRa mutant, as in the wild type and it might be due at least partly to the TcyABC system (BC0872–BC0873–BC0874) that is not controlled by PlcRa (Figure S1). These results demonstrated a role for PlcRa in the regulation of the oxidative stress defense system of *B. cereus* in relation with cysteine biosynthesis.

**Discussion**

We characterized PlcRa, a new member of the RNPP family of transcriptional regulators in the *B. cereus* group. All RNPP regulators are activated through a secreted signaling peptide that interacts with the TPR activation domain [12]. Our comparative modeling of the PlcRa protein indicates a folding similar to PlcR, with a DNA-binding domain and five TPR motifs putatively involved in the peptide binding. A small gene, *papRa*, encoding a putative exported peptide is present upstream from *plcRa*. Based
on a sequence alignment with the PapR peptide, the CSIPYEY fragment -PapRα2- was proposed as a good candidate for a signaling heptapeptide. PapRα2 corresponds to an internal region of the carboxy-terminal part of PapRα. This is dissimilar to findings for PapR whose mature form corresponds to the C-terminal end [12]. We first demonstrated that in the plcRa mutant, abrB2 expression was strongly reduced while the introduction of plcRa in trans restored its expression. Then, we demonstrated in vitro that PlcRa binds specifically to the abrB2 promoter, and that its binding requires the presence of PapRα2. Moreover, the addition of this heptapeptide in the culture as well as the overexpression of the papRa gene enhanced abrB2 expression significantly, in a PlcRa-dependent manner, indicating that PapRa modulates PlcRa activity. Taken together, our data suggest that PapRa, in the form of PapRα2, can function as an extracellular signal. In addition, the expression of plcRa and abrB2 genes was strongly activated at the onset of stationary phase suggesting a transcriptional regulation in relation with cell density. Together, our data indicate that PlcRa/PapRa is probably a new quorum sensing system in B. cereus. The production and the maturation of PapRa and the binding of PapRα2 with the TPR activation domain of PlcRa remain to be established.

The promoter region of PlcR-regulated genes contains a highly conserved palindromic sequence (TATGNN4TNCATA), constituting the PlcR binding site [7]. Despite the structural similarities between PlcR and PlcRa, no palindromic sequence was found in the promoter regions of PlcRa-regulated genes, including abrB2, a direct PlcRa target. We were unable to identify a conserved motif upstream from abrB2 and other PlcRa-controlled genes, using various bioinformatic tools.

The timing of plcRa expression (t0.5 to t1.5) suggests an additional regulatory mechanism that prevents constitutive expression by SigA and determines stationary phase expression. We have shown that the plcRa gene is not autoregulated. In light of knowledge of the regulatory network controlling transition state in B. subtilis model [3] we can speculate for a switch from vegetative sigma to transition sigma factor or for the activation of an activator or for the inactivation of a repressor. Overall these results suggest a tightly controlled plcRa expression at the onset of stationary phase, and this regulatory mechanism, different from the plcR expression activation [7,8], remains to be elucidated.

PlcRa principally positively regulates the transcription of genes involved in regulation, cysteine synthesis and oxidative stress resistance. It also downregulates the expression of numerous phage genes and this regulation may be indirect or direct. We investigated the expression and the role of a major PlcRa directed gene, abrB2. This gene encodes an AbrB-like regulator, and displays the highest level of upregulation at t1 whereas most of the genes (35/49) positively controlled by PlcRa displays upregulation only at t2. This could suggest that PlcRa may regulate gene expression indirectly, via AbrB2 at least for the genes induced at t2. This is the case for the ywgT operon encoding proteins involved in methionine-to-cysteine conversion: the expression of abrB2 under the control of a xylose-inducible promoter bypasses the requirement for PlcRa for ywgT expression.

Our results indicate the existence of links between PlcRa and the response(s) to stress stimuli. Indeed, we showed that the ΔplcRa mutant is more sensitive to H2O2 and diamide stresses than the isogenic wild-type strain. B. cereus group species respond to oxidative stress by the activation of different cellular defence

Figure 6. Addition of synthetic PapRa7, or overexpression of papRa enhanced abrB2 gene expression in a PlcRa-dependent manner. A. Expression of the P_uveg/lacZ fusion in the wild-type and in the ΔplcRa mutant strains in the presence of synthetic PapRa7. The cells were grown at 37 °C in LB medium and PapRa7 was added at t0.5 (onset of stationary phase) at different concentrations: 2 μM or 4 μM or 20 μM. Dashed lines correspond to LB cultures with PapRa7, and thick line corresponds to LB culture without PapRa7. B. Expression of the P_uveg/lacZ transcriptional fusion in the wild-type strain carrying pHT1618Pxyl’-PapRa, in the form of PapRa7, can function as an extracellular signal. In addition, the expression of the papRa gene enhanced abrB2 expression in a PlcRa-dependent manner, indicating that PapRa modulates PlcRa activity. Taken together, our data suggest that PapRa, in the form of PapRa7, can function as an extracellular signal. In addition, the expression of plcRa and abrB2 genes was strongly activated at the onset of stationary phase suggesting a transcriptional regulation in relation with cell density. Together, our data indicate that PlcRa/PapRa is probably a new quorum sensing system in B. cereus. The production and the maturation of PapRa and the binding of PapRa7 with the TPR activation domain of PlcRa remain to be established.

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mechanisms. These are composed of scavenging enzymes, as well as protection and repair systems presumably organized in highly sophisticated networks [5,25,26,45]. Our transcriptome analysis showed the downregulation of one iron uptake system in the plcRa-deficient mutant (Table 3). An induction of iron and manganese uptake systems in response to H2O2 stress has been demonstrated in a B. cereus ATCC 14579 transcriptome study [25], and in B. anthracis combined proteomic and transcriptomic analysis [45]. H2O2 stress induces the synthesis of many proteins and enzymes, such as catalases, thioredoxin reductase, ferroxidase and peroxidases, responsible for eliminating H2O2 from the cells [25,45,46]. We therefore suggest that the lower level of resistance to H2O2 stress in the plcRa deficient mutant may be at least partially due to the lower expression level of AhpCF, a major two-enzyme detoxification system, and/or Dps2, a Dps-like miniferritin (Table 3) [27]. One of our results was apparently contradictory: perR (ratio 3, Table 3) which encodes a repressor, was found to be weakly expressed in a plcRa mutant strain, together with PerR-presumably controlled genes, ahpCF operon and dps2 gene. It was also observed in B. cereus [25] and in B. anthracis [45] that H2O2 treatment modified the expression of both PerR-controlled genes and the perR gene itself in the same manner, rather than in the opposite manner as expected. However, this increased RNA level was not correlated with an increased protein level [45] and it is well established in B. subtilis that PerR is activated through conformational change [5].

In the ΔplcRa mutant, the expression of genes encoding proteins involved in cysteine synthesis from sulfate or methionine is downregulated (Table 2, Figure S1), and cysteine addition to a ΔplcRa culture improved H2O2 stress resistance (Figure 8C). It has previously been shown that modifications in the intracellular concentration of cysteine lead to increased sensitivity to oxidative stresses [5,37,40,47]. For example, in B. subtilis and Staphylococcus aureus, cymR-deficient strains, which accumulate cysteine due to the derepression of genes involved in cysteine synthesis, are highly sensitive to H2O2, disulfide, paraquat, copper- and tellurite-induced stresses [40,47]. Indeed, the range of acceptable intracellular cysteine concentrations is narrow, as this concentration must be kept below the toxicity threshold but above the minimum requirement for protein synthesis and the production of essential molecules, including compounds required for thiol homeostasis, which plays an important role in protection against oxidative stress [5,41]. We suggest that the plcRa mutant had a lower intracellular cysteine concentration, resulting in a higher susceptibility to both H2O2 and disulfide stresses generated by the thiol oxidant diamide. Indeed, cysteine is the direct precursor of low-molecular weight (LMW) thiol molecules such as bacillithiol [41] and Coenzyme A [42,43]. These molecules are the key actors in the maintenance of cytosolic redox balance and in adaptation to the

Figure 7. AbrB2 controls the expression of yrrT operon, involved in methionine to cysteine conversion. β-galactosidase specific activity (U/mg protein) of wild-type (black circles), ΔplcRa (black triangle) and ΔabrB2 (black diamonds) strains harboring both pH7304_yrrT'-lacZ and pH1618K3P_yrr-abrB2 plasmids, in HCT. See legends figure 5 for growth conditions. White symbols indicate cultures in the presence of xylose.
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Figure 8. Sensitivity to peroxide and disulfide stress of a B. cereus plcRa mutant. We assessed the viability of wild-type (WT), ΔplcRa (plcRa) and complemented ΔplcRa (plcRa+) strains in early stationary phase. Early stationary-phase cells grown in LB medium (OD 0.4) were treated for 10 minutes with 1 mM H2O2 (A) or for 40 minutes with 10 mM diamide (B) in LB and plated on LB. The results shown are the mean values for survival, expressed as a %, with standard deviations, and are representative of three independent experiments. **: P<0.01. C. The addition of cystine.
PlcRa, a New RNPP Regulator from Bacillus cereus

The chromosome of B. cereus plcRa and abrB2 genes were disrupted by homologous recombination with the pRN5101 heat-sensitive vector [51]. For the B. cereus plcRa and abrB2 mutant constructs, a tetracycline cassette carrying the tet gene was cloned for cloning [52] (Table 1). Each molecular construct, containing the 5′- and 3′-end flanking regions of the target gene and the positive selection cassette, was inserted between the HindIII and BamHI sites of pRN5101.

BamHI-Pol and XbaI-HindIII DNA fragments corresponding to the regions upstream and downstream from the plcRa gene were amplified from the B. cereus chromosome by PCR, with the primers R1–1 (5′-GGGAGTTTCAATTTATTTTCATG-3′) and R1–3 (5′-GCTGTAGTTATTTATTTTATGTAAC-3′), and the 5′- and 3′-end flanking regions of the target gene and the positive selection cassette, was inserted between the HindIII and BamHI sites of pRN5101.

RAPID AMPLIFICATION OF 5′-C’-DNA ENDS (5′RACE)

A 5′RACE experiment was conducted to map the transcriptional start sites of plcRa. B. cereus cultures were grown for four hours (t1). RNA (4 μg) was used for cDNA synthesis with the Superscript IITM reverse transcriptase (Invitrogen) and a gene-specific primer (BC0988GSP1: 5′-TCGAGAAGTTTTAAAAGTCTAGT-3′). The resulting cDNA was purified on S.N.A.P columns and a poly(dC) tail was added (Invitrogen 5′RACE system). The cDNA was amplified by PCR with an Abridged Anchor primer (Invitrogen) and a second gene-specific primer (BC0988GSP2: 5′-GAAATTTAGTTATGACACATGAC-3′), complementary to a region upstream from the binding site of the GSP1 primer. PCR products were isolated by gel extraction and inserted into the pGEM-T easy cloning vector (Promega). Three independent PCR products were sequenced.

CONSTRUCTION OF DELETION STRAINS

The chromosomal B. cereus plcRa and abrB2 genes were disrupted by homologous recombination with the pRN5101 heat-sensitive vector [51]. For the B. cereus plcRa and abrB2 mutant constructs, a tetracycline cassette carrying the tet gene was cloned for cloning [52] (Table 1). Each molecular construct, containing the 5′- and 3′-end flanking regions of the target gene and the positive selection cassette, was inserted between the HindIII and BamHI sites of pRN5101.

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GGTGAAGATCATTCCGGCGAGGGAGCCG-3') were used to check for correct chromosomal integration of the tet gene.

*HistID*-XhoI and EcoRI-BamHI DNA fragments corresponding to regions upstream and downstream from the *abrB2* gene were generated from the *B. cereus* chromosome by PCR, with the primers R3–1 (5'-CCCAAGCTTTGAGGGAGAAATG-GGA-3') R3–2 (5'-GGCTCTAGATGCTATCGCTTACG-GACTT-3') and R3–3 (5'-CGGAATTCCAGGAGAGT-GAACTG-3') and R3–4 (5'-GGCGATCCCAAATAGGATTATG-3'). External primers (R3–V1 5'-CCGCTATCTATGGTACAACG-3', R3–V2 5'-ATCTGTGGTCGTCGAGCAT-3') were used to check for correct chromosomal integration of the tet gene.

**Plasmid Construction**

pHT304†plcRa-lacZ, pHT304†abrB2-lacZ and pHT304†yrT-lacZ (Table 1) were obtained by inserting the DNA regions upstream (corresponding to the intergenic region) from the *B. cereus* ATCC 14579 plcRa, abrB2 and yrT genes between the XhoI and *Pst* cloning sites of pHT304–18Z [23]. The resulting plasmids were then transferred into *B. cereus* by electroporation.

**Microarray Hybridization and Data Analysis**

cDNA synthesis, Cy3/Cy5 labeling and cDNA purification were carried out as previously described by van Schaik et al. [28]. Microarray experiments comparing the transcription profiles of the wild-type strain and the *plcRa* deletion mutant were performed with two independent biological duplicates, with Cy3/Cy5 dye-swapping (GEO accession GSE30514). Custom-made Agilent *B. cereus* microarrays were hybridized with 200 ng of labeled cDNA for each sample. The DNA microarrays used in this study were of the 6×18K format [28]. Slides were scanned with an Agilent microarray scanner (G2565BA) and the data were extracted from the microarrays with Agilent Feature Extraction software (version 8.1.1.1). The data extraction procedure included LOWESS normalization of the raw data. The data were further processed as previously described [28], including the use of the web-based VAMPIRE platform [54] with a P-value threshold of 0.05. For gene annotation and metabolic routes, we used the PATRIC and KEGG databases.

**RT-quantitative PCR Analysis**

We generated cDNA from 1 μg of total RNA with the AffinityScript qPCR cDNA Synthesis kit (Stratagene, Agilent Technologies, France) and random hexamers. We checked cDNA quality with an Agilent 6000 Nano chip, on an Agilent 2100 bioanalyzer (Stratagene, Agilent Technologies, France). We carried out qPCR in triplicate, in a reaction volume of 20 μl containing 500 pg of cDNA, 15 μl of SYBR® Green PCR Master Mix (Applied Biosystems, Courtaboeuf, France) and 300 nM of each gene-specific primer. The primers were designed with Primer Express® (version 2.0), with the following parameters: mean product length of 70 base pairs (bp), mean primer Tm 59°C and mean primer size 20 bp. We generated standard curves for each set of primers, using serial dilutions (four dilutions) of cDNA obtained from total wild-type strain RNA collected at t2. We calculated the R² values for these dilution series and the efficiency of each primer set. Amplification was achieved with an ABI® PRISM 7900 (Applied Biosystems), with the following thermal profile: 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The specificity of each amplified PCR product was checked by melting curve analysis. Two endogenous controls, 16S and *infA* [28] from *B. cereus* ATCC 14579 were
tested, and \( uAgA \) was found to be the most reliable in our conditions. We therefore normalized the expression levels of the tested genes against those for the \( uAgA \) gene. The relative change in gene expression was recorded as the ratio of normalized target concentrations and was calculated by the comparative ΔΔCt method [55]. RQ Manager (Applied Biosystems) was used to generate expression ratios. The mean values for two independent experiments are presented. Standard deviations were less than 5% of the mean.

**Overproduction and Batch Purification of 6His-tagged-PlcRa**

We used pET28a \( plcRa \) to transform *E.coli* strain BL21. The resulting strain was grown at 30°C in LB with kanamycine 20 \( \mu \g/ml-1 \) until mid-exponential growth phase (\( OD_{600} \) 0.7); IPTG was added (1 mM) and incubation continued for 4 h at 30°C. The cells were centrifuged at 3000 g for 10 min and resuspended in 1/50 of the culture volume of Lysis Buffer (50 mM NaH2PO4 pH 8, 300 mM NaCl, 10 mM imidazole). The cells were incubated during 30 minutes on ice with lysosome (1 mg/ml) and then disrupted by sonication, and cell debris was removed by centrifugation at 12 000 g for 20 min at 4°C. The resulting crude protein extracts were loaded onto a 0.5 ml Ni-NTA–agarose column (QIAGEN) during one hour at 4°C. After washing, 6His-tagged-PlcRa protein was eluted 4 times with 0.5 ml Elution Buffer (50 mM NaH2PO4 pH 8, 300 mM NaCl, 250 mM imidazole). Elution samples 2 and 3 were pooled and a Sephadex G-25 buffer exchange colonn was used (Pharmacia) for recovering 6His-tagged-PlcRa protein in Storage Buffer (10 mM Tris, pH7.5, 50 mM KCl, 1 mM DTT). Purified PlcRa aliquots were stored at -70°C. All purification steps were analyzed by SDS–PAGE in a 12% acrylamide gel. The molecular size reference marker was obtained from Bio-Rad. Protein concentrations were determined with the Bio-Rad protein assay.

**Electrophoretic Mobility Shift Assay (EMSA) Assays**

A 175-bp DNA probe of the \( abrB \) promoter region and a 180-bp DNA probe of the \( ilcA \) promoter region (negative control) were generated by PCR from BC14579 genomic DNA using 5’ end biotin oligonucleotide primers (Eurofins GENOMICS, LES Ulis, France). For competition assay, a 175-bp DNA probe of the \( abrB \) promoter region was generated by PCR from BC14579 genomic DNA using oligonucleotide primers (Eurofins GENOMICS, LES Ulis, France). All PCR fragments were extracted from gels with the QIAquick Gel Extraction Kit (Qiagen, France) and NanoDrop 2000 spectrophotometer (Thermo scientific) was used for DNA quantification. EMSA experiments were done according to the protocol of LightShift Chemiluminescent EMSA kit from Thermo Fisher Scientific (Brebieres, France) and was performed in a 20 \( \mu \)l reaction volume containing 10 mM Tris, pH7.5, 200 mM KCl, 1 mM DTT, 20 \( \mu \)M or 2 \( \mu \)M or 0.2 \( \mu \)M PapRa, and a non specific competitor, 250 ng final salmon sperm DNA. 5 fmol of DNA biotin probe and 200 nM or 1.2 \( \mu \)M or 2 \( \mu \)M of PlcRa were used for each reaction. Competition assay was done with \( abrB \) probe at 2.5 pmol. Electrophoresis was performed with non denaturing TBE-acrylamide gels (6%).

**Stress Assays**

Viability in the presence of \( H_2O_2 \) and diamide was assessed in cultures grown in LB medium until the onset of the stationary phase (\( \sim OD_600 \) 0.4). The final stock solutions of diamide (1M) or \( H_2O_2 \) (100 mM) was prepared in sterile demineralized water immediately before use. Cultures were then split in two, with one of the two halves exposed to 1 mM hydrogen peroxide (Sigma) for 10 minutes or 10 mM diamide (Sigma) for 40 minutes. Cells were serially diluted in 0.9% sodium chloride and viability was analyzed by assessing growth on LB agar. We determined the sensitivity of growth to hydrogen peroxide, by culturing cells either in LB medium alone or in LB supplemented with cystine (1 mM) until the end of exponential growth phase growth phase (\( \sim OD_600 \) 1). Cultures were split in two, and one half was exposed to 0.4 mM hydrogen peroxide (Sigma). Changes in OD600 were monitored until \( t_s \), to monitor growth arrest and estimate the effect of stress.

**Sequence Analysis**

Sequences were retrieved with Blast-tsp from the NCBI website, http://blast.ncbi.nlm.nih.gov/Blast.cgi, with PlcRa, NP_830774.1 used as the query sequence, the Blossum 62 matrix and all non redundant GenBank CDS translations, PDB, SwissProt, PIR &PRF databases.

**Homology Modeling**

Homology modeling of the PlcRa homodimer was performed with Modeler 8v0, using the crystal structure of the complex PlcR/ PapR as the template (group I, PDB entry 2QFC). We sequentially generated 30 models of PlcRa chain A and PlcRa chain B satisfying the spatial restraints imposed by the two-dimensional alignment with the target protein. The best model for each chain was selected on the basis of the score function in Modeler [20]. To build the homodimer, the homology model of each chain A and B was then superimposed to its corresponding A and B chains of the target structure using Dalilite from the EBI website (http://www.ebi.ac.uk/Dalilite/). The stereochemistry of the homodimer PlcRa was finally checked using MolProbity (http://molprobity.biochem.duke.edu/). Finally, minor repositionings of side chains were carried out using CHARMM forcefield implemented in Accelrys®. The binding of PapRa7 was optimized using CHARMM forcefield.

**Microarray Data Accession Number**

The microarray data presented in Tables 2, 3 and in supplementary data, have been deposited in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/projects/geo), under accession number GSE30514.

**Supporting Information**

**Figure S1** Reconstruction of the sulfur metabolism pathway in *B. cereus*: transport and biosynthesis of sulfur-containing amino acids. The putative proteins involved in the uptake and assimilation of inorganic (sulfate) and organic sulfur sources (sulfonates, cystine, methionine) are indicated by the corresponding genes. The BC numbers (ATCC 14579 strain) for *B. cereus* genes are shown, with gene names according to the orthologs in *B. subtilis*. "?" indicates genes probably involved in the pathway or a step for which a gene is lacking or remains to be identified. All the PlcRa-regulated genes involved in sulfur metabolism are indicated by a downward black arrow and the putative functions of all the corresponding proteins are presented in Table 2. Presumed direct targets of CymR are indicated in bold typeface. OAS, O-acetyl-serine; AdoMet, S-adenosyl-methionine.

**Figure S2** Identification of a motif common to the promoter regions of putative CymR targets in the *B.
**cereus ATCC 14579 strain.** An alignment of the promoter regions of the *cmaB, yerF, ytrP, hxxS, cyhl, cyhlB* and *BC1090* genes is presented. The consensus sequence for the CymR-binding site was determined with the WebLogo tool.

**Figure S3 Kinetics of cymR gene expression.** β-galactosidase specific activity (U/mg protein) of the wild-type (black circles), ΔpplRa (white triangles) strains harboring the transcriptional P<sub>cymR-lacZ</sub> fusion, in LB. Errors bars are shown. Time zero corresponds to the onset of the stationary growth phase, and *t*<sub>n</sub> is the number of hours before (−) or after time zero.

**Table S1** a. Genes with differences of 0.33 fold of less are presented. b. Locus tag in type strain ATCC 14579. c. The gene names indicated correspond to *B. subtilis* homologs, with the exception of *hhblL, hhblE, hhblF* which correspond to gene names in *B. cereus*. These genes were also analysed with qRT-PCR at t1 and the expression ratio was 0.1.

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**Author Contributions**

Conceived and designed the experiments: EH GAL MT TA IMV DL. Performed the experiments: EH GAL MT PW LB SM. Analyzed the data: EH GAL MT WP DL. Contributed reagents/materials/analysis tools: EH GAL MT TA IMV SM. Wrote the paper: EH GAL MT TA IMV DL.
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