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## Two-dimensional electrophoresis analysis of the extracellular proteome of *Bacillus cereus* reveals the importance of the PlcR regulon

Many virulence factors are secreted by the gram-positive, spore forming bacterium *Bacillus cereus*. Most of them are regulated by the transcriptional activator, PlcR, which is maximally expressed at the beginning of the stationary phase. We used a proteomic approach to study the impact of the PlcR regulon on the secreted proteins of *B. cereus*, by comparing the extracellular proteomes of strains ATCC 14579 and ATCC 14579  $\Delta plcR$ , in which *plcR* has been disrupted. Our study indicated that, quantitatively, most of the proteins secreted at the onset of the stationary phase are putative virulence factors, all of which are regulated, directly or indirectly, by PlcR. The inactivation of *plcR* abolished the secretion of some of these virulence factors, and strongly decreased that of others. The genes encoding proteins that are not secreted in the  $\Delta plcR$  mutant possessed a regulatory sequence, the PlcR box, upstream from their coding sequence. These proteins include collagenase, phospholipases, haemolysins, proteases and enterotoxins. Proteins for which the secretion was strongly decreased, but not abolished, in the  $\Delta plcR$  mutant did not display the PlcR box upstream from their genes. These proteins include flagellins and InhA2. InhA2 is a homologue of InhA, a *Bacillus thuringiensis* metalloprotease that specifically degrades antibacterial peptides. The mechanism by which PlcR affects the production of flagellins and InhA2 is not known.

**Keywords:** *Bacillus* sp / PlcR / Secretome / Virulence

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### 1 Introduction

*Bacillus cereus* causes diseases such as ophthalmitis, dermonecrosis, periodontitis and food-borne gastroenteritis. Yet, the factors that make strains of this gram-positive, spore forming bacterium pathogenic to humans are not precisely known, although several potential virulence factors secreted by *B. cereus* have been well characterized. All of them but one, the depsipeptide cereulide [1], are proteins encoded by genes dispersed on the bacterial chromosome [2]. Many of these proteinaceous virulence factors are degradative enzymes, such as proteases or phospholipases. Others are nonspecific toxins, such as enterotoxins or haemolysins. The transcription of the genes encoding these potential virulence factors is controlled by the pleiotropic regulator, PlcR [2, 3]. In mice, the lethal effect that follows nasal instillation of the *B. cereus* type strain ATCC 14579 is partly abolished when the *plcR*

gene is inactivated [4]. This suggests that the PlcR regulon is involved, at least in part, in the pathogenicity of *B. cereus* to humans.

PlcR is a 34 kDa transcriptional activator that is both auto-regulated [5] and under the control of the sporulation factor, Spo0A [6]. It was first identified in *Bacillus thuringiensis*, an entomopathogenic species that is closely related to *B. cereus* [3]. In *B. thuringiensis* PlcR acts as a positive regulator of the *plcA* gene, which encodes phosphatidyl inositol-specific phospholipase C (PI-PLC). A conserved palindromic sequence (called the PlcR box) is found upstream of the transcriptional start site of all PlcR regulated genes [3]. In *B. thuringiensis*, *plcR* expression starts at the end of the vegetative phase and is maximal two hours after the onset of the stationary phase [5].

The presence of a PlcR box upstream of the coding sequences suggests that PlcR regulates the transcription of at least 15 genes in *B. cereus* [2]. Most of these putative PlcR regulated genes encode potential virulence factors that may act synergistically during infection [7], confirming the physiological consistency of the PlcR regulon. Also, most of these proteins have a Sec-type signal peptide [2], suggesting that they are exported. Recent studies on the extracellular proteome led to the discovery of new potential virulence determinants belonging to the Rgg regulon in *Streptococcus pyogenes* [8] or to the Sar regu-

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**Abbreviations:** **PC-PLC**, phosphatidylcholine-specific phospholipase; **PI-PLC**, phosphatidylinositol-specific phospholipase; **PMF**, peptide mass fingerprint; **Smase**, sphingomyelinase

lon in *Staphylococcus aureus* [9]. In this study, we used 2-DE to investigate the importance and extent of the PlcR regulated extracellular proteins of *B. cereus* at the beginning of the stationary phase, when *plcR* expression is maximal.

## 2 Methods

### 2.1 Bacterial strains and culture conditions

We used the *B. cereus* reference (type) strain ATCC 14579 and the *B. cereus* mutant strain ATCC 14579  $\Delta plcR$  [4]. These strains were grown in 100 mL Luria Bertani (LB) broth at 30°C in 1 litre flasks on a rotary shaker at 175 rpm. Cultures were seeded at OD<sub>600</sub> 0.01 with cells in vegetative phase in LB broth, and culture supernatants were harvested 2 h ( $t_2$ ) after the onset of the stationary phase. The onset of the stationary phase ( $t_0$ ) was defined as being the breakpoint in the vegetative phase slope. Four cultures were grown for each strain for spot quantification and a single culture was used to identify the spots.

### 2.2 Sample preparation

Immediately after harvesting the bacterial cells, culture supernatants were centrifuged at 8000 rpm for 20 min at 4°C, and the supernatant of the centrifugation was rapidly filtered through a membrane (pore size = 0.2  $\mu$ m). Proteins were then precipitated twice using the deoxycholate-tetrachloroacetic acid method [10]. The pellet was washed twice with ethanol: ether (1:1), dried and stored at –80°C until use. The protein content of the pellet was determined with the Bradford method [11].

### 2.3 Two-dimensional electrophoresis

Protein pellets were resuspended in sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.6% Pharmalytes (Amersham Pharmacia Biotech, Uppsala, Sweden), 2 mM tricarboxyethylphosphine (TCEP) (Pierce, Chester, UK), 40 mM Tris), and subsequently diluted in IPG rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.6% Pharmalytes, 2 mM TCEP). IPG strips (17cm length) in the linear pH range 4–7 (Bio-Rad, Hercules, CA, USA) were chosen because preliminary experiments revealed no proteins below pH 4 and few proteins above pH 7. The IPG strips were rehydrated for 16 h with 400  $\mu$ L of rehydration buffer. We loaded 20  $\mu$ g protein *per* IPG strip for spot quantification and 100  $\mu$ g protein *per* IPG strip for protein identification. The proteins were separated by IEF on an Amersham Pharmacia Multiphor II horizontal electrophoresis system for 32 000 Vh. The IPG strips were then equilibrated for

15 min in 50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 1% SDS and 1% DTT and then for 20 min in 50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 1% SDS, 1.6% iodoacetamide. The second dimension was run on a 10% to 15% gradient SDS-PAGE, on a 16  $\times$  20  $\times$  0.1 cm Bio-Rad Protean II xi vertical slab gel. Gels were silver stained [12] for spot detection and quantification or stained with Coomassie Blue G250 [13] for protein identification.

### 2.4 Gel analysis and spot detection and quantification

Gels were scanned at 300 dpi and 8 bits depth on a SHARP JX-330 scanner equipped with a film scanning unit and analysed with the ImageMaster 2D programme from Amersham Pharmacia Biotech. The spots were quantified after normalisation and spot volumes (pixel intensity  $\times$  area) are expressed as a percentage of the total volume of the spots on the gel.

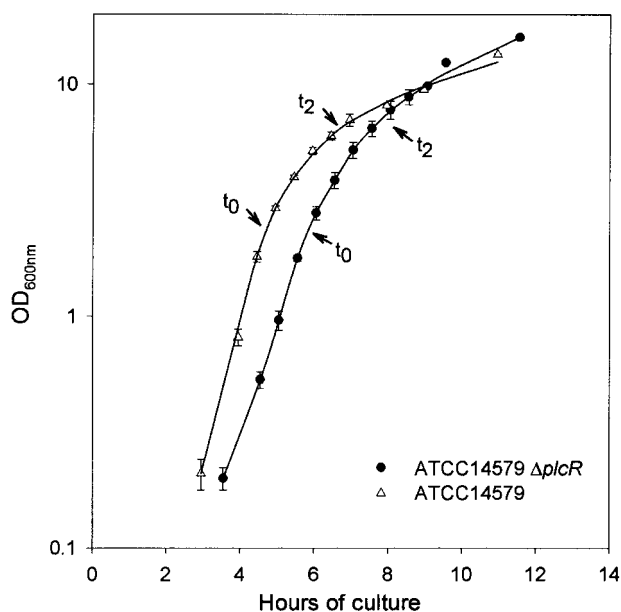
### 2.5 Protein identification

Gels stained with Coomassie Blue were fixed with 5% acetic acid and scanned. The spots were immediately excised, washed three times in acetonitrile: 50 mM NH<sub>4</sub>HCO<sub>3</sub> pH 8 (1:1). They were then dried and rehydrated in 50 mM NH<sub>4</sub>HCO<sub>3</sub> pH 8, 3.2% trypsin (Promega, Madison, WI, USA) and incubated for 16 to 18 h at 37°C. The digested proteins were analysed by MALDI-TOF mass spectrometry by the “Unité de Recherches en Biochimie et Structure des Protéines” from INRA (Jouy-en-Josas, France). The peptide mass fingerprints (PMF) were used to search the *B. cereus* strain ATCC 14579 partially sequenced genome database from Integrated Genomics (<http://wit.integratedgenomics.com>) by use of the MS-Fit tool of ProteinProspector.

## 3 Results

### 3.1 Growth curves

The *B. cereus* ATCC 14579 wild-type and  $\Delta plcR$  strains grew differently in culture (Fig. 1). During vegetative growth, *B. cereus* ATCC 14579  $\Delta plcR$  grew more slowly than the wild-type strain (as illustrated by the doubling time, Table 1). The two strains reached  $t_0$  at the same bacterial density (Table 1). However, after the onset of the stationary phase, ATCC 14579  $\Delta plcR$  grew faster than the wild-type strain (Fig. 1), which resulted in a higher bacterial density in the ATCC 14579  $\Delta plcR$  strain at  $t_2$  (Table 1) when the samples were taken for subsequent analysis. Although the bacterial density of the ATCC 14579  $\Delta plcR$



**Figure 1.** Growth curves of *B. cereus* ATCC 14579 and *B. cereus* ATCC 14579  $\Delta plcR$  in LB medium. Each point is the mean of four to six independent experiments. Vertical bars: standard error of the mean.

strain was higher at  $t_2$ , the protein concentration in the culture supernatant was higher for the wild-type strain (Table 1). This means that the total amount of protein secreted *per cell* by the wild-type strain is almost double (1.9 times) that secreted by the  $\Delta plcR$  mutant.

### 3.2 Comparison of 2-D gels

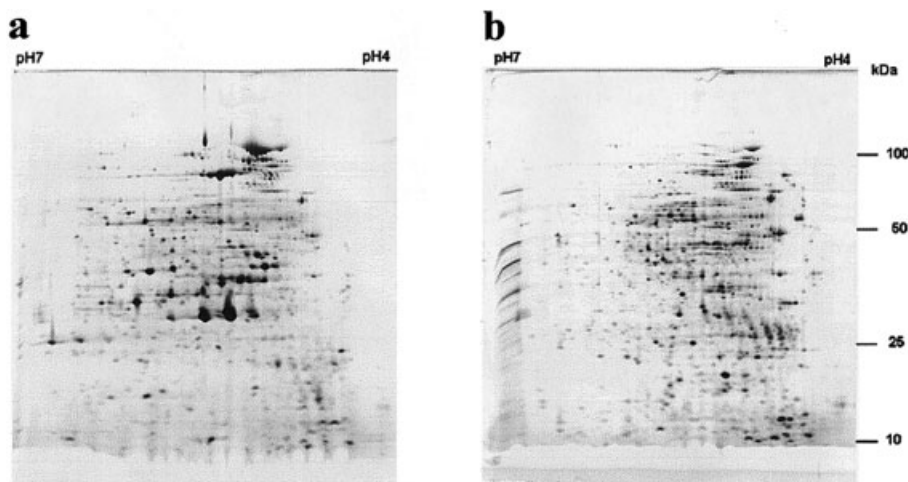
The comparison of the extracellular proteomes revealed major differences between the two strains. The 2-D gel for the wild-type strain (Fig. 2a) was highly heterogeneous, with large, dense protein spots. In contrast, the

2-D gel for the ATCC 14579  $\Delta plcR$  strain was much more homogeneous (Fig. 2b). On average, 533 spots were detected in the wild-type strain. This was not significantly different from the 773 spots found in the mutant strain on average (Table 1). Coomassie Blue staining revealed about 120 spots in both strains. However, comparative gel analysis showed that most of the large extracellular protein spots in the wild-type strain were affected by the mutation in *plcR*: some of them completely disappeared, whereas others were strongly decreased in volume in the mutant strain. If spot filtering was used to discard the spots which, when cumulated, gave a volume of less than 1%, 96 spots appeared to be totally controlled by *PlcR* (*i.e.* they disappeared in the mutant strain) and 37 spots were strongly affected by *PlcR* (*i.e.* they were decreased by a factor equal to or greater than 5). We can rule out the possibility that these proteins will be produced later by the mutant strain, as this strain grew faster than the wild-type strain in stationary phase.

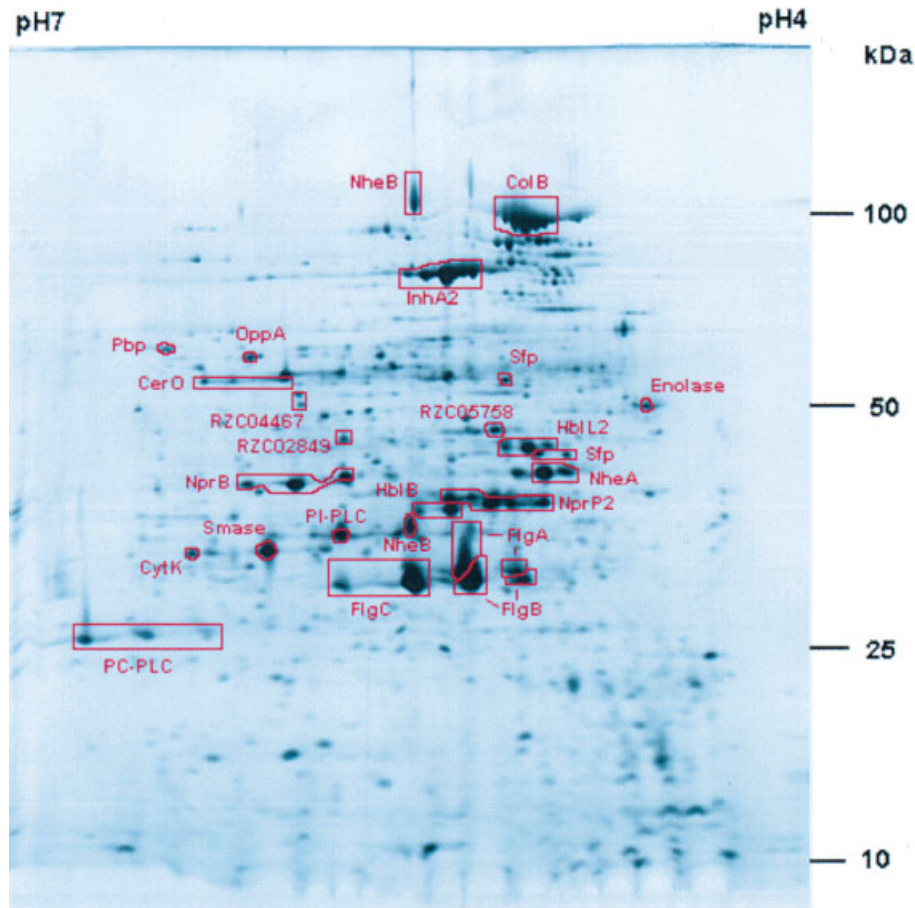
**Table 1.** Comparison of the wild-type and the  $\Delta plcR$  strains for growth parameters, protein content of the culture supernatant and the number of spots on the 2-DE gels.

	wild-type <sup>a)</sup>	mutant <sup>b)</sup>	<i>p</i> value <sup>c)</sup>
Doubling time (h) in vegetative phase	0.36 ± 0.01 (6)	0.46 ± 0.03 (6)	0.006
OD <sub>600</sub> at $t_0$	3.5 ± 0.7 (6)	3.6 ± 0.7 (6)	0.890
OD <sub>600</sub> at $t_2$	7.0 ± 0.4 (6)	8.8 ± 0.7 (5)	0.045
Proteins at $t_2$ (μg/mL)	46.8 ± 1.6 (4)	31.0 ± 3.1 (4)	0.004
Number of spots at $t_2$	534 ± 107 (4)	773 ± 134 (4)	0.214

Mean ± standard error of the mean, and (number of replications). (a) wild-type strain; (b), mutant strain; (c), Student *t*-test



**Figure 2.** Comparison of 2-D gels from the wild-type (a) and  $\Delta plcR$  (b) strains. For each strain, the culture supernatant was harvested in early stationary phase at  $t_2$  and 20 μg proteins from the supernatant were separated by 2-DE and silver stained.



**Figure 3.** Identification of spots from the *B. cereus* ATCC 14579 wild-type strain. Proteins were extracted from a culture supernatant harvested at  $t_2$ , and 100  $\mu\text{g}$  of these proteins were separated by preparative 2-DE. The proteins were identified by PMF. Protein spots that were identified are outlined in red and shown on an analytical gel matched with the preparative gel.

### 3.3 Protein identification

The proteins from the wild-type strain that were identified by MALDI-TOF are shown in Fig. 3 and listed in Table 2. Fourteen of the 23 proteins identified in the wild-type strain were not present in the mutant strain. Three of these proteins were phospholipases, four were from two enterotoxin complexes, one was a collagenase, two were neutral proteases, one was a serine protease and two were haemolysins.

The phospholipases were sphingomyelinase (Smase), phosphatidylinositol-specific phospholipase C (PI-PLC) and phosphatidylcholine-specific phospholipase C (PC-PLC). PC-PLC produced three spots with different  $pI$  values. This can be explained by the fact that this enzyme can complex up to nine zinc ions *per* molecule [14]. The two enterotoxin complexes were Hbl (haemolysin BL) and Nhe (nonhaemolytic enterotoxin). Hbl normally consists of three proteins: binding component B, which is encoded by the *hblA* gene and two lytic components (L1 and L2), which are encoded by *hblD* and *hblC*, respectively. The three genes are organised in an operon [15]. Similarly,

Nhe normally consists of three proteins (NheB, NheA and NheC) encoded by *nheB*, *nheA* and *nheC*, which form an operon [16]. However, only two components were visible on the 2-D gels for each of these enterotoxin complexes.

Three metalloproteases were identified, the neutral proteases NprP2 and NprB, and collagenase B, which forms a heavy pearl chain at the top of the gel. The serine protease, Sfp, was also observed. One of the haemolysins was cereolysin O (CerO). CerO has a variable  $pI$ , depending on its oxidation state [17], which gave rise to several spots. The second haemolysin was the recently described cytolyisin K [18]. All of these compounds are extracellular proteins that were strongly suspected to be directly controlled by the PlcR regulator because the genes encoding them were preceded by a PlcR box [2, 3].

We have provided the first experimental evidence that these extracellular proteins are positively regulated by PlcR in *B. cereus* and that they completely disappear when *plcR* is inactivated. For example, the protein spots corresponding to PI-PLC could not be detected in the  $\Delta plcR$  strain (Fig. 4a). One of the protein spots that was

**Table 2.** List of proteins identified by PMF from the wild-type strain 2-DE gel.

Protein <sup>a)</sup>	Function	IG <sup>b)</sup> gene no.	$M_r$	$pI$	$\Delta plcR^c)$	localisation <sup>d)</sup>
<b>Proteases</b>						
ColB	Collagenase	RZC05249	99–110	5.1–5.3	0	extracellular
InhA2*	Neutral protease	RZC05941	77–82	5.4–5.7	↓	extracellular
Sfp	Subtilase family protease	RZC02845	44–55	5.1–5.3	0	extracellular
NprB	Neutral protease	RZC04929	40–41	5.9–6.3	0	extracellular
NprP2	Neutral protease	RZC06042	37–38	5.3–5.5	0	extracellular
<b>Phospholipases</b>						
Smase	Sphingomyelinase	RZC01449	34	5.8	0	extracellular
PI-PLC	Phosphatidylinositol-specific phospholipase	RZC02846	34	6.2	0	extracellular
PC-PLC	Phosphatidylcholine-specific phospholipase	RZC01448	28	6.5–6.9	0	extracellular
<b>Enterotoxins</b>						
NheB	Enterotoxin Nhe, component B	RZC02615	35 & 105	5.3–5.7	0	extracellular
NheA	Enterotoxin Nhe, component A	RZC02616	41–42	5.1–5.2	0	extracellular
HblB	Enterotoxin Hbl, binding component B	RZC06097	36	5.5–5.6	0	extracellular
HblC	Enterotoxin Hbl, lytic component L2	RZC04946	44	5.1–5.3	0	extracellular
<b>Non specific toxins</b>						
CerO	Haemolysin, cereolysin O	RZC04911	57	6.2–6.3	0	extracellular
CytK	Haemolysin, cytolysin K	RZC04383	34	6.5	0	extracellular
<b>Flagellum components</b>						
FlgA*	Flagellin	RZC02639	32–35	5.3–5.5	↓	extracellular
FlgB*	Flagellin	RZC02640	31–32	5.2–5.4	↓	extracellular
FlgC	Flagellin	RZC06000	31–32	5.6–5.9	↓	extracellular
<b>Metabolism</b>						
Enase**	Enolase	RZC02971	50	4.8	↑	cytoplasm
<b>Transporter</b>						
OppA*	Oligopeptide permease A	RZC04745	63	6.3	↑	membrane
<b>Cell wall machinery</b>						
Pbp-2B**	Penicillin-binding protein B	RZC04540	64	6.6	↑	membrane
<b>Unknown</b>						
Unknown	unknown	RZC04467	51–54	6.1	↓	cytoplasm
Unknown	unknown	RZC02849	46	5.9	↑	cytoplasm
Unknown	unknown	RZC05758	47	5.3	=	cytoplasm

a) Protein names were determined by homology with the corresponding protein in *B. thuringiensis* (\*) or in *B. subtilis* (\*\*).

Unknown: no homology found in databases, function unknown;

b) Integrated Genomics numbering of genes from the *B. cereus* ATCC 14579 genome database;

c) Changes in protein quantities in the mutant compared to the wild-type strain: (↑), increased by a factor of 2 or more; (=), unchanged; (↓), decreased by a factor of 2 or more; (0): abolished;

d) Localisation as determined from PSORT or PEPSIG indications or from SWISS-PROT information.

strongly decreased (13 times) in the mutant strain was a homologue of the *B. thuringiensis* metalloproteases, InhA1 (formerly named InhA [19, 20]) and InhA2 [32]. InhA2 from *B. thuringiensis* is a 799 amino acid protein, whereas the *B. cereus* InhA2 protein stored in the Integrated Genomics database consists only of the first 690 amino acids. As the position of this protein has a simi-

lar  $M_r$  and  $pI$  to the full-length protein, it is likely that the C-terminal part of this protein has been omitted in the *B. cereus* database. The first 690 amino acids of the two orthologues were 97% identical. Three related flagellins, whose genes were found to be located on a same chromosomal cluster, were also strongly decreased (3 times) in the  $\Delta plcR$  mutant strain (Fig. 4b). Amino acid



the *InhA2* and flagellin genes. This suggests that these two groups of genes are not directly regulated by *PlcR*. The mechanism that negatively controls the production of flagellins and *InhA2* in a *plcR* deficient strain is not known.

The other abundant proteins secreted by *B. cereus* ATCC 14579 were tightly controlled by *PlcR*. These include haemolysins (cereolysin O and cytolyisin K), phospholipases (PC-PLC, PI-PLC and, Smase), a collagenase, neutral proteases, a serine protease and two enterotoxin complexes (Hbl and Nhe). In the pathogenic bacterium *Clostridium perfringens*, collagenase, haemolysin, phospholipase C and sphingomyelinase are essential virulence factors [26]. The maximal toxicity of the *B. cereus* Hbl complex is retained when all three protein components are present [27]. 2-DE and PMF did not detect the HblL1 component of the Hbl enterotoxin complex, although this component has been detected in ATCC 14579 strain by immunoblotting of the culture supernatant [28]. This discrepancy may be due to the loss of the L1 component in our sample preparation or electrophoresis procedure, or to a blurred PMF signal for this component. However, the same study [28] reported that the *nhe* operon was poorly transcribed, whereas in the present study, NheA and NheB represented 5.1% and 4.5% of the total spots volume, respectively. This difference could be explained by a high stability of these two Nhe proteins. In contrast, the instability of NheC could explain why we were unable to detect this protein and why a previous attempt to isolate it failed [16]. Alternatively, *nheC*, the last gene of the operon, might be poorly or not transcribed. NheB was detected both at the expected location and at 105 kDa. This second spot could be due to the formation of a stable complex between NheB and another protein. Interestingly, the Nhe complex was initially thought to contain a 105 kDa protein [29] but this protein was found later to be a collagenase [30].

Some protein spots, such as enolase, penicillin binding protein or oligopeptide permease, showed an increased intensity in the mutant strain. These proteins do not have the *PlcR* box upstream from their genes. The apparent increase in intensity possibly results from the subtraction of 50% to 80% of the proteins total volume, which in turn increases the relative importance of the remaining spots. Indeed, the disruption of *plcR* resulted in a 50% decrease in amount of extracellular proteins in the mutant strain compared to the wild-type strain, at the onset of the stationary phase. Alternatively, the increase in the intensity of some spots may be the consequence of a lower degradation as a result of the absence of several *PlcR* dependent proteases. Interestingly, we found that five proteases almost completely disappeared in the mutant strain.

The extracellular proteome of *B. cereus* showed some common points with the recently published extracellular proteome of *B. subtilis* [22, 31]. In particular, oligopeptide permease A, enolase, possibly one flagellin and one penicillin binding protein migrate to roughly the same positions on 2-D gels. However, a striking difference between these two extracellular proteomes is the presence of virulence factors completely or partially controlled by *PlcR* on the *B. cereus* 2-DE gel. These proteins account for more than 80% of the total spots volume in *B. cereus* but are totally absent from the extracellular proteome of *B. subtilis*.

## 5 Concluding remarks

In conclusion, we have shown that at the onset of the stationary phase, *B. cereus* devotes a remarkably high percentage of its protein secretion capacity to the coordinated production of putative virulence factors, all of which are, directly or indirectly, positively regulated by *PlcR*.

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## 6 References

- [1] Agata, N., Ohta, M., Mori, M., Isobe, M., *FEMS Microbiol. Lett.* 1995, **129**, 17–20.
- [2] Økstad, O. A., Gominet, M., Purnelle, B., Rose, M., Lereclus, D., Kolstø, A. B., *Microbiology*, 1999, **145**, 3129–3138.
- [3] Agaisse, H., Gominet, M., Økstad, O. A., Kolstø, A. B., Lereclus, D., *Mol. Microbiol.* 1999, **32**, 1043–1053.
- [4] Salamitou, S., Ramiisse, F., Brehelin, M., Bourguet, D. *et al.*, *Microbiology*, 2000, **146**, 2825–2832.
- [5] Lereclus, D., Agaisse, H., Gominet, M., Salamitou, S., Sanchis, V., *J. Bacteriol.* 1996, **178**, 2749–2756.
- [6] Lereclus, D., Agaisse, H., Grandvalet, C., Salamitou, S., Gominet, M., *Int. J. Med. Microbiol.* 2000, **290**, 295–299.
- [7] Beecher, D. J., Wong, A. C., *Microbiology*, 2000, **146**, 3033–3039.
- [8] Chaussee, M. S., Watson, R. O., Smoot, J. C., Musser, J. M., *Infect. Immun.* 2001, **69**, 822–831.
- [9] Ziebandt, A.-K., Weber, H., Rudolph, J., Schmid, R. *et al.*, *Proteomics*, 2001, **1**, 480–493.
- [10] Peterson, G. L., *Methods Enzymol.* 1983, **91**, 95–119.
- [11] Bradford, M. M., *Anal. Biochem.* 1976, **72**, 248–254.
- [12] Rabilloud, T., Charmont, S., in: Rabilloud, T. (Ed.), *Proteome Research: Two Dimensional Gel Electrophoresis and Identification Methods*, Springer, Heidelberg 2000, pp. 107–126.
- [13] Reisner, A. H., Nemes, P., Bucholtz, C., *Anal. Biochem.* 1975, **64**, 509–516.
- [14] Bjorklid, E., Little, C., *FEBS Lett.* 1980, **113**, 161–163.
- [15] Ryan, P. A., MacMillan, J. D., Zilinskas, B. A., *J. Bacteriol.* 1997, **179**, 2551–2556.
- [16] Granum, P. E., O'Sullivan, K., Lund, T., *FEMS Microbiol. Lett.* 1999, **177**, 225–229.



- [17] Cowell, J. L., Grushoff-Kosyk, P. S., Bernheimer, A. W., *Infect. Immun.* 1976, *14*, 144–154.
- [18] Lund, T., De Buyser, M. L., Granum, P. E., *Mol. Microbiol.* 2000, *38*, 254–261.
- [19] Lovgren, A., Zhang, M., Engstrom, A., Dalhammar, G., Landen, R., *Mol. Microbiol.* 1990, *4*, 2137–2146.
- [20] Grandvalet, C., Gominet, M., Lereclus, D., *Microbiology* 2001, *147*, 1805–1813.
- [21] Simonen, M., Palva, I., *Microbiol. Rev.* 1993, *57*, 109–137.
- [22] Antelmann, H., Tjalsma, H., Voigt, B., Ohlmeier, S. *et al.*, *Genome Res.* 2001, *11*, 1484–1502.
- [23] Charlton, S., Moir, A. J., Baillie, L., Moir, A., *J. Appl. Microbiol.* 1999, *87*, 241–245.
- [24] Zhang, M. Y., Lovgren, A., Landen, R., *J. Invertebr. Pathol.* 1995, *66*, 46–51.
- [25] Dalhammar, G., Steiner, H., *Eur. J. Biochem.* 1984, *139*, 247–252.
- [26] Rood, J. I., *Annu. Rev. Microbiol.* 1998, *52*, 333–360.
- [27] Dietrich, R., Fella, C., Strich, S., Martlbauer, E., *Appl. Environ. Microbiol.* 1999, *65*, 4470–4474.
- [28] Lindback, T., Økstad, O. A., Rishovd, A. L., Kolstø, A. B., *Microbiology* 1999, *145*, 3139–3146.
- [29] Lund, T., Granum, P. E., *FEMS Microbiol. Lett.* 1996, *141*, 151–156.
- [30] Lund, T., Granum, P. E., *FEMS Microbiol. Lett.* 1999, *178*, 355–361.
- [31] Hirose, I., Sano, K., Shioda, I., Kumano, M. *et al.*, *Microbiology* 2000, *146*, 65–75.
- [32] Fedhila, S. A., Nel, P., Lereclus, D., *J. Bacteriol.* 2002, *184*, in press.