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

Michel Gohar, Ole Andreas Økstad, Nathalie Gilois, Vincent Sanchis, Anne-Brit Kolsto, Didier Lereclus

Michel Gohar[1,2]
Ole Andreas Økstad[3]
Nathalie Gilois[1]
Vincent Sanchis[1,4]
Anne-Brit Kolstø[3]
Didier Lereclus[1,4]

[1]INRA, Unité de Recherches de Lutte biologique, Guyancourt, France
[2]Aventis CropScience Gent, Belgium
[3]Biotechnology Centre of Oslo and School of Pharmacy, University of Oslo, Oslo, Norway
[4]Institut Pasteur, Unité de Biochimie Microbienne, Paris, France

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 Δ *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 Δ*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 Δ*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 PRO 0232

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 autoregulated [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-

Correspondence: Dr. Michel Gohar, INRA, Unité de Recherches de Lutte biologique, La Minière, 78285 Guyancourt Cedex, France
E-mail: gohar@diamant.jouy.inra.fr
Fax: +33-130438097

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

 1615-9853/02/0606–784 $17.50+.50/0

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 Δ*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_{600} 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 μm). 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 μL of rehydration buffer. We loaded 20 μg protein *per* IPG strip for spot quantification and 100 μg 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 × 20 × 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 × 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_4HCO_3 pH 8 (1:1). They were then dried and rehydrated in 50 mM NH_4HCO_3 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 Δ*plcR* strains grew differently in culture (Fig. 1). During vegetative growth, *B. cereus* ATCC 14579 Δ*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 Δ*plcR* grew faster than the wild-type strain (Fig. 1), which resulted in a higher bacterial density in the ATCC 14579 Δ*plcR* strain at t_2 (Table 1) when the samples were taken for subsequent analysis. Although the bacterial density of the ATCC 14579 Δ*plcR*

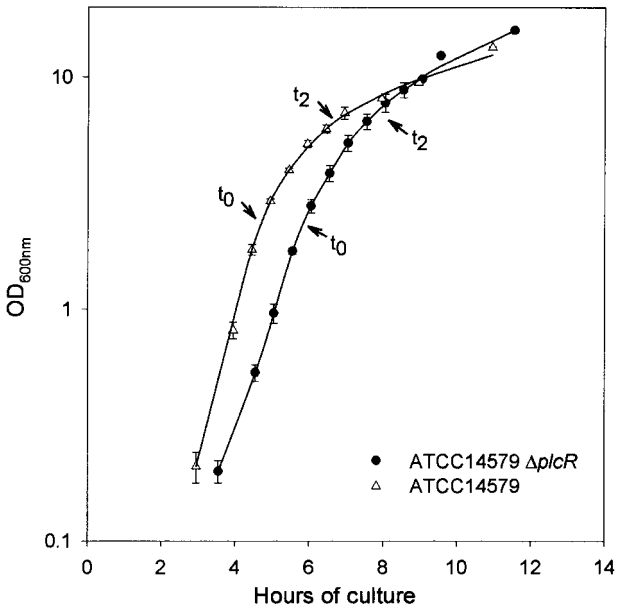

Figure 1. Growth curves of *B. cereus* ATCC 14579 and *B. cereus* ATCC 14579 Δ*plcR* in LB medium. Each point is the mean of four to six independent experiments. Vertical bars: standard error of the mean.

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 Δ*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 Δ*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 Δ*plcR* strains for growth parameters, protein content of the culture supernatant and the number of spots on the 2-DE gels.

	wild-type[a)]	mutant[b)]	*p* value[c)]
Doubling time (h) in vegetative phase	0.36 ± 0.01 (6)	0.46 ± 0.03 (6)	0.006
OD_{600} at t_0	3.5 ± 0.7 (6)	3.6 ± 0.7 (6)	0.890
OD_{600} 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

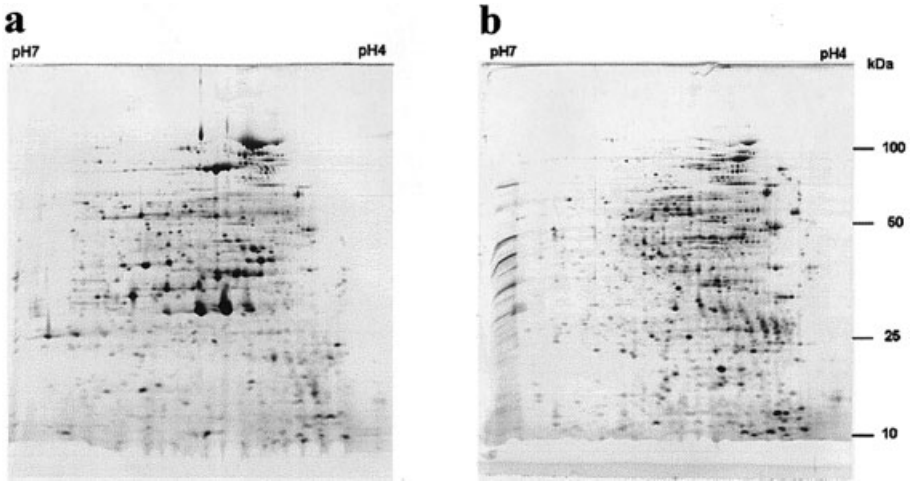

Figure 2. Comparison of 2-D gels from the wild-type (a) and Δ*plcR* (b) strains. For each strain, the culture supernatant was harvested in early stationary phase at t_2 and 20 μg proteins from the supernatant were separated by 2-DE and silver stained.

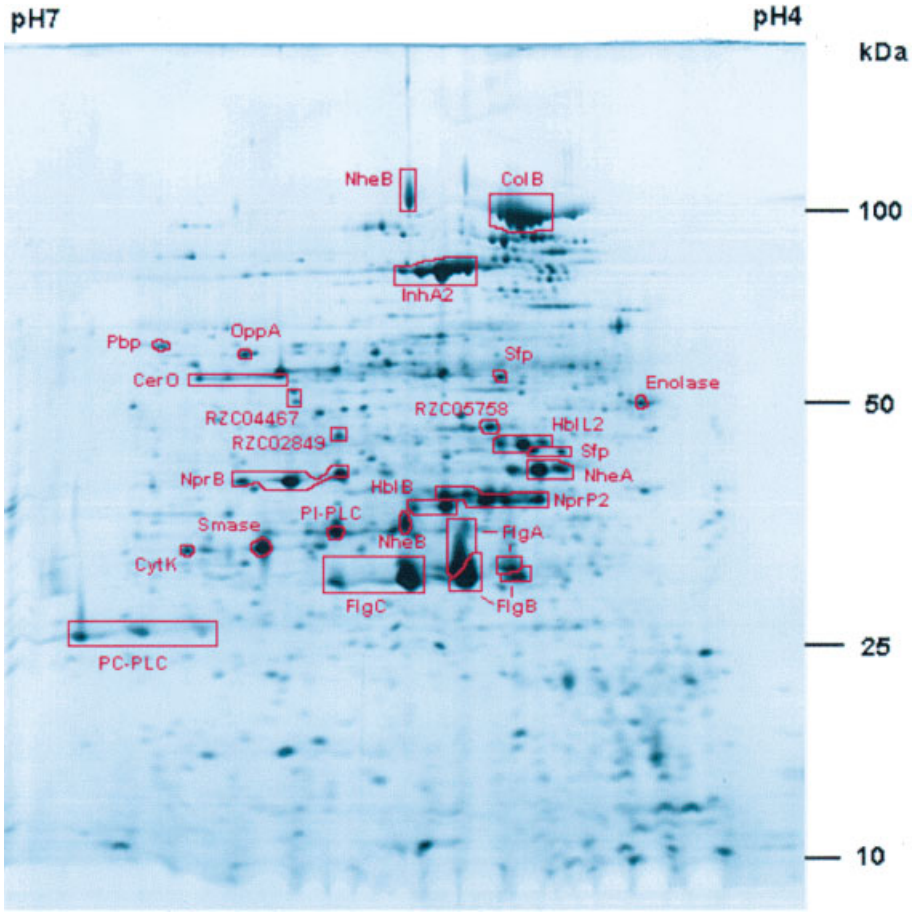

Figure 3. Identification of spots from the *B. cereus* ATCC 14579 wild-type strain. Proteins were extracted from a culture supernatant harvested at t_2, and 100 μg of these proteins were separated by preparative 2-DE. The proteins were identified by PMF. Protein spots that were identified are outlined 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 p*I* 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 enterotoxic 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 p*I*, depending on its oxidation state [17], which gave rise to several spots. The second haemolysin was the recently described cytolysin 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 Δ*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[a)]	Function	IG[b)] gene no.	M_r	p*I*	Δ*plcR*[c)]	localisation[d)]
Proteases						
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
Phospholipases						
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
Enterotoxins						
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
Non specific toxins						
CerO	Haemolysin, cereolysin O	RZC04911	57	6.2–6.3	0	extracellular
CytK	Haemolysin, cytolysin K	RZC04383	34	6.5	0	extracellular
Flagellum components						
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
Metabolism						
Enase**	Enolase	RZC02971	50	4.8	↑	cytoplasm
Transporter						
OppA*	Oligopeptide permease A	RZC04745	63	6.3	↑	membrane
Cell wall machinery						
Pbp-2B**	Penicillin-binding protein B	RZC04540	64	6.6	↑	membrane
Unknown						
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 similar M_r and p*I* 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 Δ*plcR* mutant strain (Fig. 4b). Amino acid

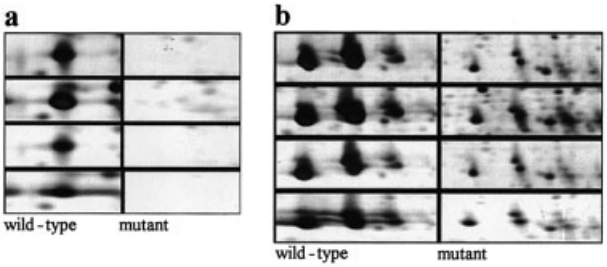

Figure 4. Comparison of gel areas containing PI-PLC (a) and flagellins (b) for the wild-type strain (left) and the Δ*plcR* mutant (right). The supernatant from four cultures were harvested at t_2 and the 20 μg proteins from these supernatants were separated by 2-DE and silver stained. The selected areas of the four silver stained 2-D gels are shown together for both strains.

sequence comparison of the three flagellins indicated that they were 75% to 89% identical. In contrast, several proteins, such as oligopeptide permease A, penicillin binding protein B and enolase, had their spots increased in the Δ*plcR* mutant 2-D gels.

3.4 Spot quantification

It could clearly be seen that the distribution of the spots in the wild-type gel was highly heterogeneous when the volumes of the spots were plotted in decreasing order (Fig. 5a). The experiments were fairly reproducible, as shown by the small standard errors. The 12 most abundant proteins accounted for 70% of the total protein volume. The three flagellins together appeared to account for 22% of the total volume, followed by collagenase B (10%) and by InhA2 (7.6%). Altogether 54% (in quantity) of the proteins secreted by *B. cereus* ATCC 14579 could not be detected in the Δ*plcR* mutant, indicating that they are totally controlled by PlcR (Fig. 5b). Proteases (mainly metalloproteases) constituted the main group of these proteins, followed by enterotoxins and phospholipases.

4 Discussion

Bacillus species secrete large amounts of proteins [21] that are important for their adaptation and survival in the environment. *B. subtilis* secretes about 200 proteins in the p*I* range 3–10 during the early stationary phase [22]. We detected about 500 secreted protein spots in *B. cereus* ATCC 14579 in the p*I* range 4–7. Despite this high number of spots, only a restricted number of proteins made a major contribution to the total amount of extracellular protein. Twelve proteins, representing 10% of the spots, contributed to more than 80% of the total spot volume. Most importantly, all of these major proteins can be considered to be potential virulence factors.

Flagellins and InhA2 were among the most abundant proteins found in the extracellular proteome of ATCC 14579. InhA (InhA1 and/or InhA2) is a major constituent of the *B. cereus* exosporium [23]. In *B. thuringiensis* flagellins are involved in the adherence of cells to host tissues [24], whereas InhA1 specifically degrades antibacterial peptides produced in response to bacterial invasion [19, 25]. InhA2 is involved in the synergistic effect of spores on the insecticidal activity of Cry insecticidal toxins in *B. thuringiensis* (Fedhila, Nel and Lereclus, submitted for publication). Consequently, flagellins and InhA2 can be considered to be virulence factors. The production of these two proteins is reduced in Δ*plcR* mutants. However, the PlcR box was not found upstream of the coding sequence of

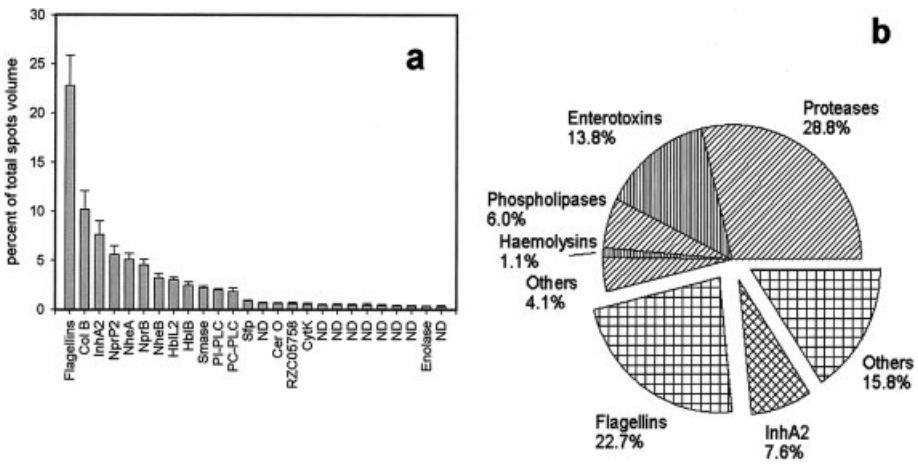

Figure 5. Spot quantification. (a) The relative volumes of spots, or of groups of spots representing highly homologous proteins, were pooled and plotted in decreasing order; vertical bars: standard error of the mean for four independent experiments; ND: spots not determined; (b) The relative volumes of spots in each functional protein family were pooled and plotted in a pie chart. Simple hatch: protein spots disappearing in the mutant strain. Cross hatch and exploded slices: protein spots not disappearing in the mutant strain.

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 cytolysin 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 substraction 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.

We wish to thank Céline Henry for mass spectrometry work on digested spots.

Received November 4, 2001

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., Ramisse, 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.