Adaptive responses in *Bacillus cereus* group bacteria – microarray comparisons and follow-up studies

by
Karoline Fægri

Thesis for the degree of *philosophiae doctor*
Department of Pharmaceutical Biosciences
School of Pharmacy
Faculty of Mathematics and Natural Sciences
University of Oslo
2010

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Series of dissertations submitted to the
Faculty of Mathematics and Natural Sciences, University of Oslo
No. 955

ISSN 1501-7710

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Cover: Inger Sandved Anfinsen.
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Acknowledgements

The present work was carried out in the period May 2002-September 2009 at the University of Oslo, first at The Biotechnology Center, and from 2005 at The School of Pharmacy, Faculty of Mathematics and Natural Sciences. The work was supported by a grant from The Norwegian Research council from 2002 until September 2007. Additional support was provided from The School of Pharmacy from September 2007 until September 2008, and from my parents from August 2006 and up until present day.

I would like to thank my supervisor, Anne-Brit for her enthusiasm and never-ending optimism, and for sharing her knowledge and experience with me. I am grateful to Ole Andreas Økstad for his contribution as co-supervisor, and to Solveig Ravnum, who was my co-supervisor in the beginning, for teaching me what I know about lab-work with RNA and microarrays. I would also like to thank Endre Anderssen, whose help and advice on microarray analysis has been invaluable to me.

I am grateful to my co-authors and collaborators for allowing me to be part of their projects, and for participating in mine. I would also like to thank all past and present members of our group for sharing their knowledge, and for all the good times and frustrations we have shared. Everyone else in the ZEB building also deserves thanks for creating an enjoyable and social working atmosphere.

Finally, I would like to thank my parents for their unfailing support and encouragement, and most of all Anders, Rasmus and Daniel, who lived through it all without complaint, and who always make my day better. I love you very much.

Blindern, March 2010

Abbreviations

ADP	Adenosine diphosphate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
cDNA	complementary DNA
CIRCE	controlling inverted repeat of chaperone expression
CFU	Colony forming unit
COG	Clusters of orthologous groups
DNA	Deoxyribonucleic acid
FDR	False discovery rate
INRA	Institute Nationale de la Recherche Agronomique
LB broth	Luria Bertani broth
LB agar	Luria Bertani agar
LIMMA	Linear methods for microarrays
ORF	Open reading frame
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNPP	Rap, NprR, PlcR, and PrgX
RT-qPCR	Reverse transcription followed by quantitative polymerase chain reaction
SASP	Small, acid soluble proteins.
TCA	Tricarboxylic acid
TPR	Tetratricopeptide repeat
wt	wildtype

List of papers included

I Michel Gohar, Karoline Fægri, Stéphane Perchat, Solveig Ravnum, Ole Andreas Økstad, Myriam Gominet, Anne-Brit Kolstø, Didier Lereclus:

The PlcR virulence regulon of *Bacillus cereus*.

PLoS ONE. 2008 Jul 30;3(7):e2793.

II Karoline Fægri, Stéphane Perchat, Christina Nielsen-Leroux, Nalini Ramarao, Didier Lereclus, Anne-Brit Kolstø:

Characterization of the NprR regulon in bacteria of the *Bacillus cereus* group.

Submitted.

III Karoline Fægri, Annette Fagerlund, Ewa Jaroszewicz, Ida Kristin Hegna, Wolfgang M. Egge-Jacobsen, Lillian Reiter, Per Einar Granum, Ole Andreas Økstad, Anne-Brit Kolstø:

A transiently filamentous phenotype associated with pleiotropic changes in *Bacillus cereus* ATCC 14579.

Manuscript.

IV Sara Salvetti, Karoline Fægri, Emilia Ghelardi, Anne-Brit Kolstø, Sonia Senesi:

Global gene expression profiles of *Bacillus cereus* during active swarming migration.

Submitted.

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Outline of thesis.

The purpose of this thesis is to summarize the results from four different studies where microarray technology has been used as an exploratory tool to gather new insights about transcriptional responses to various changes in bacteria from the *B. cereus* group. Two of the projects are very similar, as they both aimed to find genes regulated by a transition state regulator. The third project involved comparison of swarm-differentiated cells with non-swarming cells. The fourth project originated from an observation that certain variants of *B. cereus* ATCC 14579 grow as long filaments during the exponential phase, while other grow as single cells or short chains of visibly separated cells. Microarrays and other techniques were used to further characterize the variants and describe phenotypic differences and similarities.

What these projects have in common, apart from the use of microarray technology, is that they involve, in some way or another, adaptive responses of the bacteria. The regulators PlcR and NprR regulate adaptive responses during the transition phase, while swarming is an adaptive form of cellular differentiation which allows the bacteria to rapidly colonize a surface. Filamentous growth may also be an adaptive response, although this is most likely not the case in this study. However, phenotypes observed during study of the variants included increased transcription of genes involved in adaption to stress. Effects were also seen on the process of endospore formation, an adaptive response to extreme conditions.

On this background, I will start the thesis with an introduction to the *Bacillus cereus* group and some of the adaptive responses relevant to the various projects. I will then summarize the main results from the papers. As microarray analysis has been a cornerstone in the work presented in this thesis, I will discuss methodological choices in some detail before I proceed to discuss the results.

During discussion of the results, I will attempt to place our observations in a broader context and describe how they comply with what is already known. I will also use the opportunity to report some additional observations made while analyzing our results and to discuss some of the observations from another angle or in more detail than there is room for in an article. Finally, I will sum up our conclusions and outline future perspectives for some of the projects.

1. Introduction.

In the year 2009, which marks the 200th anniversary of Charles Darwin and the 150th anniversary of *The origin of species* (Darwin, 1950 reprint), there has been much talk about the survival of the fittest. In such a perspective, bacteria belonging to the genus *Bacillus* must be considered very fit, something their ancient ancestry indicates (Cano & Borucki, 1995; Vreeland *et al.*, 2000). Members of the *Bacillus cereus* group, which have been the targets of this work, are no exception. Their ability to exploit widely divergent habitats, and to endure extreme conditions through various pathways of differentiation, makes "adapt and survive" a catch phrase of this group. In this introduction, I will present the *B. cereus* group and examine closer some of the adaptive responses these bacteria display in different phases of life, with emphasis on those which have been relevant to this work. I will also give a brief introduction to the microarray technology, which has been central in the studies presented here.

1.1 The genus *Bacillus* and the *Bacillus cereus* group.

The genus *Bacillus* belongs to the family *Bacillaceae* (Priest, 1993). Bacteria belonging to this genus are gram-positive or gram-variable rod-shaped. They are aerobic or facultative anaerobes, and usually motile. They are also capable of forming heat resistant endospores, which are metabolically inert and can endure extreme conditions. When conditions turn favorable, the endospores can germinate to vegetative cells. The genus *Bacillus* is unusually large, with highly variable GC content, and a further subdivision into six subgroups has been proposed (Priest, 1993). The *Bacilllus subtilis* subgroup (group II) includes *Bacillus subtilis*, which is the type species of the genus, and 17 other species with a GC-content between 33 and 45 % (Priest, 1993). The *B. cereus* group, also referred to as *B. cereus sensu lato*, is a subdivision within the *B. subtilis* group and comprises the closely related species *B. cereus sensu stricto, Bacillus thuringiensis, Bacillus anthracis, Bacillus mycoides, Bacillus pseudomycoides,* and *Bacillus weihenstephanensis*. These bacteria all have characteristic traits, and, with the exception of the two last species, they were classified as different species at a time when classification was based on phenotypes. Below, I will briefly describe the characteristic properties of the various species:

Bacillus cereus sensu stricto (Frankland, 1887) includes members of the *B. cereus* group which do not fit any of the other species definitions. Many strains carry conjugative plasmids. Some strains are pathogenic, frequently causing emetic (Agata *et al.*, 1995) or diarrhoeal food poisoning (Hauge, 1950). The emetic toxin, a dodecadepsipeptide, is non-ribosomally synthesized, and the genes encoding the responsible enzymes are localized on a large plasmid (Ehling-Schulz *et al.*, 2006a; Hoton *et al.*, 2005), while gastroenteritis is caused by the secretion of chromosomal virulence factors such as the Hbl and Nhe enterotoxins (Arnesen *et al.*, 2008; Michelet *et al.*, 2006).

Bacillus thuringiensis is characterized by its ability to produce entomopathogenic toxins during sporulation, and is widely used as a biopesticide (Schnepf *et al.*, 1998). The toxin-encoding genes reside on plasmids.

Bacillus anthracis is a mammalian pathogen and the causative agent of anthrax (Mock & Fouet, 2001; Passalacqua & Bergman, 2006). Its potent virulence is due to the presence of two plasmids named pXO1 (182 kb), encoding the the anthrax toxin components, and pXO2 (95 kb), encoding an antiphagocytotic γ-D-glutamic acid capsule.

Bacillus weihenstephanensis is characterized by its psychrotolerant growth as well as characteristic sequence signatures in rDNA genes and in the gene encoding the cold shock protein *cspA* (Lechner *et al.*, 1998). However, psychrotolerant strains are also found among other members of the *B. cereus* group (Guinebretiere *et al.*, 2008). Pathogenicity of *B. weihenstephanensis* strains is generally regarded as low (Stenfors *et al.*, 2002), but strains producing the cereulide toxin characteristic of emetic *B. cereus* strains, have been described (Thorsen *et al.*, 2006)

Bacillus mycoides (Flugge, 1886; Lewis, 1932) and ***Bacillus pseudomycoides*** (Nakamura, 1998) are non-motile and characterized by their ability to grow as filaments (rhizoidal phenotype). They produce intricate colony patterns when grown on agar plates (Di Franco *et al.*, 2002).

Bacillus cytotoxicus has recently been suggested as a separate species due to large phenotypic and genetic differences from other *B. cereus* strains (Lapidus *et al.*, 2008). *Bacillus cytotoxicus* strains have smaller genomes than strains in other species of the *Bacillus cereus* group, and certain genes appear to be absent, which indicates that these strains may be distinguishable by simple metabolic tests (Lapidus *et al.*, 2008). The species is characterized by moderate thermotolerance (Guinebretiere *et al.*, 2008).

As can be seen from the list above, the characteristic phenotypic features can be encoded by a small number of genes, in some cases residing on plasmids that may be exchanged between cells. At the chromosomal level, these species are all very similar, and genetic variation within one species may be as large as the variation between species (Helgason *et al.*, 1998; Helgason *et al.*, 2000). The *B. anthracis* strains are genetically monomorphic, and in a phylogenetic tree of the *B. cereus* group they can all be found on the same branch in a cluster of pathogenic *B. cereus* and *B. thuringiensis* strains (Kolstø *et al.*, 2009). On the other hand, a *B. thuringiensis* which loses the plasmid encoding the Cry-toxin, becomes indistinguishable from a *B. cereus*, and strains from these two species cannot be separated in phylogenetic studies (Carlson *et al.*, 1994; Helgason *et al.*, 1998; Helgason *et al.*, 2000). An overweight of food and clinical samples are found in the large group which has become known as clade I, while soil samples tend to cluster within clade II, regardless of their species definition (Helgason *et al.*, 2004; Priest *et al.*, 2004). On this genetically interweaved background, it has been argued that *B. thuringiensis, B. cereus,* and *B. anthracis* may in fact be one species (Helgason *et al.*, 2000; Rasko *et al.*, 2005), but for practical reasons the distinctions are kept. *B. weihenstephanensis, B. mycoides* and *B. pseudomycoides* tend to form phylogenetic subgroups within the *B. cereus* group (Lechner *et al.*, 1998; Nakamura, 1998), and their species definition may be more appropriate, or they may not have been sufficiently investigated (Bartoszewicz *et al.*, 2009) For more phylogenetic information, see also http://mlstoslo.uio.no.

The two strains used in this study, the *B. cereus* type strain ATCC 14579 (Frankland, 1887; Ivanova *et al.*, 2003) and *B. thuringiensis* 407 Cry^{-}, are closely related. *B. thuringiensis* 407 was originally an insect isolate from Brasil, but has been cured of its Cry-encoding plasmids (Lereclus *et al.*, 1989). Therefore it has lately also been referred to as a *B. cereus* strain (Guillemet *et al.*, 2009).

It is hypothesized that bacteria in the *B. cereus* group primarily live as symbionts in vertebrate or invertebrate hosts, and, with the exception of *B. anthracis*, only occasionally act as invasive pathogens (Jensen *et al.*, 2003). The main argument is that all members of the *B. cereus* group can grow saprophytically in a nutrient-rich environment. This is rarely found in nature, but frequently encountered in the guts of insects and higher animals. This line of reasoning was supported by analysis of the metabolic potential of the strains *B. cereus* ATCC 14579 and *B. anthracis* A2012, finding only 14-15 coding sequences for polysaccharide degradation, but around fifty protease-encoding sequences (Ivanova *et al.*,

2003). However, it has later been shown that both *B. cereus* ATCC 14579 and *B. cereus* ATCC 10987 can metabolize a variety of carbohydrates (Mols *et al.*, 2007), and, as mentioned above, *B. cereus* ATCC 14579 has been shown to grow in soil extract (Vilain *et al.*, 2006). There are also reports that *B. cereus, B. thuringiensis,* and even *B. anthracis* can grow in the plant rhizosphere (Dunn & Handelsman, 1999; Emmert & Handelsman, 1999; Park *et al.*, 2008; Saile & Koehler, 2006). Taken together, these reports indicate that bacteria in the *B. cereus* group can flourish in a variety of habitats.

Due to the bioterrorism potential of *B. anthracis*, the *B. cereus* group has been in focus in recent years, and an impressive number of genomes have been sequenced. Genomes from more than fifteen strains have been closed, and partially assembled sequence exists for around sixty more strains (http://www.ncbi.nlm.nih.gov), taking the total number up to eighty-four (January 2010). Even though *B. anthracis* is somewhat oversampled in this collection, the vast amounts of genetic information available offer unique opportunities for phylogenetic studies and genetic comparisons across strains.

1.2. Communication and multicellular behavior.

Communication and the ability to act as a community is one of the keys to the successful adaptive responses of bacteria (Shapiro, 1998; Shapiro, 2007). In the early 1960's, it was reported that competence (i.e. the ability to take up DNA from the environment) could be induced by a chemical substance isolated from a bacterial culture that had already reached the competent phase (Pakula & Walczak, 1963; Tomasz & Hotchkiss, 1964; Tomasz, 1965). This is an early description of intercellular communication in bacteria, and in the following decades there were numerous discoveries of chemical signaling substances that induce differentiation or coordinated behavior in bacteria.

Quorum sensing has been used as a collective term to describe activation of a certain behavior as a result of density-dependent signaling mechanisms (Bassler & Losick, 2006; Dunny & Leonard, 1997). Chemical signal substances constitutively expressed at a low level are secreted from individual cells, and their concentration in the environment will thus depend on the cell density. Through evolution, the system has reached a balance where the signal substance reaches a threshold concentration which results in activation at the optimal cell density for the specific activity (Dunny & Leonard, 1997). This type of

mechanism enables the bacteria to coordinate growth, movement and biochemical secretion (Shapiro, 1998).

Multicellular behavior plays an important role in many adaptive responses (Shapiro, 1998). In addition to the competence development mentioned previously, biofilm formation, sporulation, bacterial swarming, and activation of virulence factors are some examples of behavior that is induced by accumulation of signal substances in the environment. The joint efforts of a bacterial population secreting virulence factors or exhibiting colonizing behavior is far more effective than the uncoordinated efforts of single bacterial cells.

The chemical structures of the signaling substances are diverse, but peptides or modified peptides are commonly used in gram-positive bacteria (Dunny & Leonard, 1997). The peptides are frequently synthesized as larger precursor molecules and processed to the active form posttranslationally (Dunny & Leonard, 1997). After secretion, the signaling molecule may be reimported into the cell (Declerck *et al.*, 2007) or interact with surface receptors (Dunny & Leonard, 1997). In the latter case, the receptor domain is often in the N-terminal end of a histidine kinase that is part of a two-component regulatory system (de Been *et al.*, 2006). In the cytosolic C-terminal end is a phosphotransferase domain. In a simple system, when the receptor binds the signal substance, the phosphotransferase will phosphorylate and thus activate the cytosolic response regulator component of the two-component system (de Been *et al.*, 2006). The activated response regulator will bind to DNA regions and activate or repress genes to elicit the adaptive response (de Been *et al.*, 2006). The regulon, i.e. the genes controlled by a given regulator, may vary from only a few to several hundred genes.

Complex processes with great consequences for the cell, such as competence development or sporulation, have a far more complex system for signal transduction. In these cases, the activation of the receptor by a signal substance will start a phosphorelay between different signal transduction proteins (de Been *et al.*, 2006; Dunny & Leonard, 1997). More than one signal substance may be involved, and each step in the chain of phospohorylation events may be influenced by intracellular events, as in the process of sporulation (Fujita & Losick, 2005). In this way, the information from the environment will be integrated into a complex regulatory network where the sum of many factors decides the outcome for the cell.

1.2.1. The RNPP family of quorum sensors.

Structural and phylogenetic analysis indicates that quorum sensors which bind their signaling peptide directly, belong to a protein superfamiliy, named RNPP after its current members: Rap, NprR, PlcR, and PrgX (Declerck *et al.*, 2007). The signaling peptides of these proteins are encoded right downstream of the sequence encoding the regulator itself. They are synthesized as precursor peptides and cleaved during or after export out of the cell. The active peptide is then reimported, and activates or inhibits its sensor by binding to a tetratricopeptide repeat (TPR) domain on the molecule (Declerck *et al.*, 2007). Thus, in these cases, the signal is not mediated by a two-component system. The regulators NprR and PlcR, which are central in this work, are both quorum sensing activated members of the RNPP protein superfamily, Their signaling mechanisms are, as far as they are known, described in more detail below.

1.2.1.1. Activation of the PlcR regulator.

The transcriptional regulator PlcR (phospholipase C regulator) controls a regulon dominated by extracellular and cell wall-associated virulence factors (Agaisse *et al.*, 1999; Gohar *et al.*, 2002; Lereclus *et al.*, 1996). The regulator is activated at the transition into stationary phase, and reaches maximum activity two hours later (Gohar *et al.*, 2002). Its expression is dependent on the signaling peptide PapR (peptide activated by PlcR) (Slamti & Lereclus, 2002). PapR is synthesized as a 48 amino acid peptide and exported out of the cell. In the extracellular environment it is cleaved, and the active heptapeptide is reimported via an oligopeptide permease (opp) system (Bouillaut *et al.*, 2008; Gominet *et al.*, 2001). When PapR binds to the PlcR TPR-domain, the PlcR regulator is activated and able to bind to the PlcR binding sequence (Agaisse *et al.*, 1999). Through binding sites upstream of both *papR* and *plcR* itself, PlcR autoregulates its own transcription and activation in a positive feedback loop (Lereclus *et al.*, 1996; Okstad *et al.*, 1999). Fig. 1 shows the process of PlcR-activation, and the positive feedback loop.

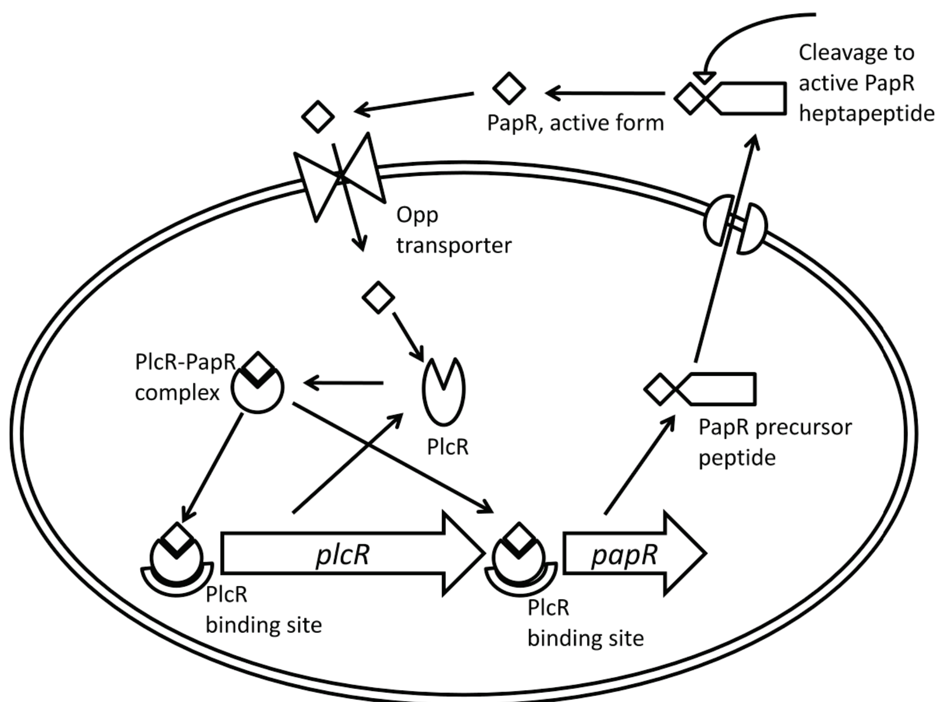

Fig.1. The activation and positive feedback loop of PlcR.

1.2.1.2. Activation of the NprR regulator.

Downstream of the gene encoding NprR (neutral protease regulator) is a gene sequence specifying NprX, a 43 amino acid peptide with a putative export signal (Perchat *et al.*, 2007). The *nprR* and *nprX* genes are located right upstream of the gene *nprA*, encoding a metalloprotease. Studies of deletion mutants lacking *nprA*, *nprR*, and/or *nprX* in a *B. thuringiensis* strain carrying a chromosomal transcriptional fusion between *nprA* and *lacZ,* showed that NprR and NprX are required for *nprA* transcription (Perchat *et al.*, 2007). Lack of *nprX* can be complemented by adding to the growth medium a synthetic peptide corresponding to the C-terminal part of NprR (Perchat *et al.*, 2007), suggesting that this part of NprX acts as a signaling peptide. NprR does not appear to possess autoregulatory activity (Stéphane Perchat, personal communication). Fig. 2 shows the process of NprR-activation as far as it is known.

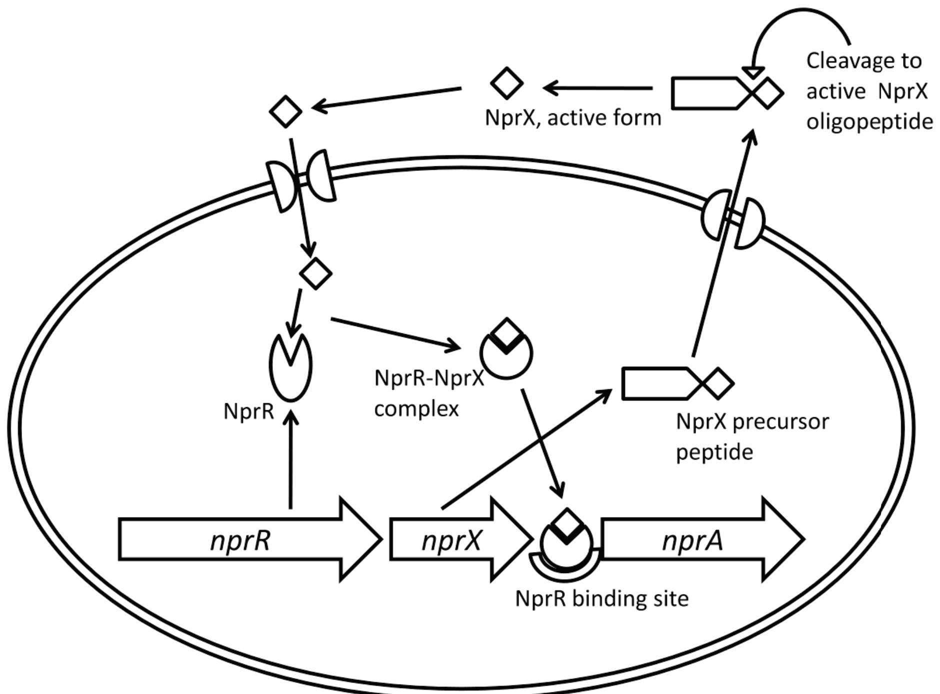

Fig. 2. Possible method of NprR-activation

1.3. Bistability

The previous chapter may leave the impression that quorum sensing results in identical behavior throughout the bacterial community. In reality, the response will always be heterogenous. Even during well established multicellular behavior such as biofilm formation, competence and sporulation, only part of the cell population will be induced (Chung *et al.*, 1994; Hadden & Nester, 1968; Veening *et al.*, 2005b). Furthermore, no matter how good the growth environment, a tiny fraction of the cells will still enter pathways such as competence and sporulation (Veening *et al.*, 2005b).

Rather than constituting imperfections of the quorum sensing system, this intrinsic instability can be regarded as an adaptive mechanisms which increases the long term probability of survival for the community (Veening *et al.*, 2008a). Pathways such as competence and sporulation have great costs to the individual cell in terms of growth arrest and energy-intensive production (Smits *et al.*, 2006). If only a portion of the cells respond to the quorum sensing signal, the remaining cells can continue as before, or perhaps enter other pathways of differentiation. Such differentiation results in a robust and flexible

population with increased chance of survival, and allows division of tasks between cells in a community, e.g. matrix production in biofilm, which is only carried out by a fraction of the cells (Kearns, 2008; Veening *et al.*, 2008b)

Differentiation occurs as a consequence of stochastic processes (noise) that influence the biochemical reactions in a cell (Elowitz *et al.*, 2002). The effects will be integrated into the regulatory networks, and may be augmented or counteracted by positive and negative feedback loops (Gore & van Oudenaarden, 2009). Differentiation can occur as a continuum of phenotypes, as observed in individual bacteria swimming (Spudich & Koshland, 1976), but many quorum sensing phenomena are bistable, meaning that the cell does or does not enter a certain pathway (Veening *et al.*, 2008b).

A bistable switch is traditionally viewed as a regulatory network which creates polarized gene expression. If a transcriptional regulator reaches a threshold activity, an auto-regulatory positive feedback loop (or a pairwise number of negative feedback loops) is initiated, turning the system on and committing the cell to the regulatory pathway (Smits *et al.*, 2006). Below this threshold, the system is not auto-induced, and the cell does not enter the pathway. However, it now appears that some of the most important switches are not simply turned on or off, but will instead initiate different pathways in a stepwise fashion, dependent on their degree of activation and the affinity of the binding sites for their regulator. This is the case for DegU and Spo0A, two central regulators that have been shown to coordinate several types of multicellular behavior in *B. subtilis* (Fujita *et al.*, 2005; Murray *et al.*, 2009; Verhamme *et al.*, 2009).

As the scientific community has become aware of the concept of bistability, it has been recognized as a property of an increasing number of cellular pathways. Sporulation (Chung *et al.*, 1994), competence (Cahn & Fox, 1968; Hadden & Nester, 1968), biofilm formation(Chai *et al.*, 2008), persistence (Balaban *et al.*, 2004), motility (Mauder *et al.*, 2008), swarming (Calvio *et al.*, 2005; Calvio *et al.*, 2008; Senesi *et al.*, 2004), filament formation (Kearns & Losick, 2005), and protein secretion (Veening *et al.*, 2008a) are among the differentiation processes which have been reported to be under control of bistable or multistable regulators (Murray *et al.*, 2009).

1.4. Motility

In a world of limited resources, motility is an advantage, as it allows the organism to migrate towards nutrients and other favorable conditions, and away from unfavorable ones. Bacterial translocation can occur in a variety of ways: Swimming, swarming, gliding, twitching, sliding and darting, depending on the organism and the properties of the surface or surrounding medium (Henrichsen, 1972; Jarrell & McBride, 2008). Many bacteria have flagella which enable them to move by swimming in liquid media or swarming on a solid surface. However, the production and maintenance of flagella is structurally complicated, and requires energy and amino acid resources (McCarter, 2006). Therefore, flagellar activity is under strict regulatory control (Smith & Hoover, 2009b). In this section I will briefly describe the flagellar structure and regulation, and also the process of bacterial swarming, which is relevant to this work. Finally I will attempt to account for some of what is known about flagellar motility in the *B. cereus* group.

1.4.1. The flagellar organelle

1.4.1.1. Flagellar structure and function.

The flagellum consists of three main proteinaceous structures (Fig. 3.), reviewed by Terashima *et al* (2008) and Smith et al (2009b): The **basal body** contains the motor that powers flagellar rotation by conversion of ATP to ADP. This is the most complex structure of the flagellum, and it is anchored in the cell envelope. The **flagellar hook** is the link between the motor and the flagellar filament. It is a curved rod and converts the rotary motion provided by the basal body motor into wavelike movements by the flagellar filament. The **flagellar filament** is helical and, when moved by the hook, it pushes against a surface or the surrounding medium and makes the cell move. Inside, the flagellar filament is hollow. During growth, flagellins, the proteins that constitute the building blocks of the filament structure, are transported through the hollow fiber and added to its distal end.

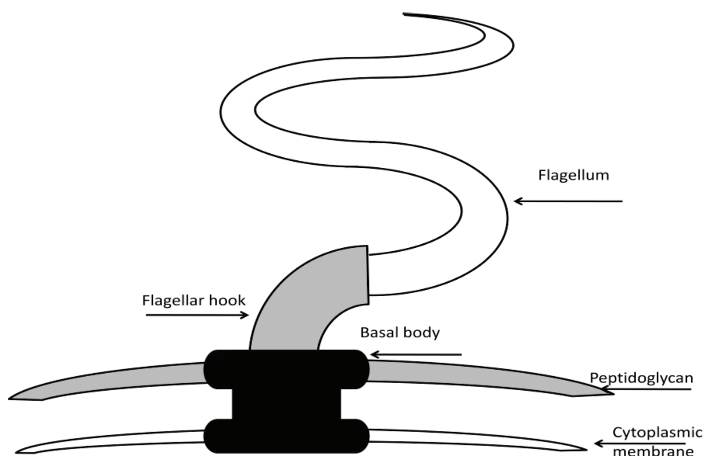

Fig. 3. A simplified model of the flagellar structure. Each of the three main structures are composed of a variety of subunits.

The rotational direction of the flagellum governs the directional movement of the bacterium and is controlled by a phosphorelay signaling cascade receiving input from several two-component systems sensing environmental factors such as pH, temperature, the presence of nutrients, and other chemical signals.

1.4.1.2. Regulation of flagellar biosynthesis.

As previously mentioned, flagellar biosynthesis consumes energy and amino acid resources, and is therefore tightly controlled . The composition of the regulatory networks varies between species, as reviewed by Smith and Hoover (2009b) and McCarter (2006). Tables 1 and 2 show important regulatory proteins in two low GC gram-positive bacteria: The model organism *B. subtilis* and *Listeria monocytogenes*.

Regulation in *B. subtilis* (table 1) resembles the hierarchical and temporally spaced control observed in *Escherichia coli* and *Salmonella typhimurium*. Here a class 1 gene encoding a master regulator induces transcription of class 2 genes coding for the proteins needed for synthesis of the basal body and the flagellar hook, as well as transcription factors inducing transcription of class 3 genes encoding the flagellin genes needed for the flagellar filament. This cascade ensures that proteins are synthesized in the right order (Smith & Hoover, 2009b).

Table 1. Central regulatory proteins involved in motility in *B. subtilis*.

Protein	**Function**	**Found in *B. cereus***	**References**
DegU	**Transition state regulator:** Activates *swrAA* transcription at low concentrations. Inhibits transcription of the *fla/che* operon at high concentrations	no*	(Amati *et al.*, 2004; Calvio *et al.*, 2008; Tokunaga *et al.*, 1994)
SwrAA (formerly SwrA or Ifm),	**Master regulator (class 1 gene):** Enhances transcription of the *fla/che* operon containing class 2 genes, class 3 activators, most chemotaxis genes and *sigD* (encoding σ^D)	no	(Calvio *et al.*, 2005; Kearns *et al.*, 2004; Senesi *et al.*, 2004; Smith & Hoover, 2009b)
σ^D	**Transcription factor:** Positively regulates transcription of flagellins (class 3 genes) and induces *swrAA* transcription	no	(Calvio *et al.*, 2008; Smith & Hoover, 2009b)
FlgM	**Anti-sigma factor:** Prevents σ^D from binding to its promoter region until the flagellar basal body has been synthesized	no	(Bertero *et al.*, 1999; Ghelardi *et al.*, 2002; Mirel *et al.*, 1994)

*http://img.jgi.doe.gov, http://www.kegg.com, http://blast.ncbi.nlm.nih.gov/Blast.cgi

The *fla/che* operon of *B. subtilis* is preceded by a σ^A promoter, and is transcribed at a low rate also in absence of the master regulator SwrAA, so that a *swrAA* deletion mutant will still display swimming motility (Kearns *et al.*, 2004). To induce swarming or full swimming motility of *B. subtilis* in the exponential phase, both DegU and SwrAA are required (Calvio *et al.*, 2008 and references therein).

When in need of a regulatory model for the *B. cereus* group, it is common to look to *B. subtilis*. However, as will be evident from column three in Table 1, *B. subtilis* is not likely to provide a useful model for all aspects of motility in the *B. cereus* group. Therefore, I will also briefly explain the regulation of flagellar biosynthesis in another relative, *L. monocytogenes*. Regulation of flagellar biosynthesis in this organism is different from what is found in *B. subtilis* and most other bacteria. Here, a central repressor controls all genes involved in motility (Grundling *et al.*, 2004; Shen & Higgins, 2006). When repression is lifted, flagellar genes are activated in a non-hierarchical manner (Shen *et al.*, 2006). Central regulatory proteins are listed in table 2.

Table 2. Central regulatory proteins involved in motility in *L. monocytogenes*.

Protein	Function	Found in *B.cereus*	References
DegU	**Transition state regulator:** Activates GmaR transcription at low temperatures	no	(Shen *et al.*, 2006)
MogR	**Repressor:** Controls genes involved in motility	yes	(Grundling *et al.*, 2004; Shen & Higgins, 2006) (Smith & Hoover, 2009a)
GmaR	**Anti-repressor:** lifts MogR-repression of genes involved in motility	Complete *gmaR* only found in strain NVH391-98*	(Shen *et al.*, 2006)

*http://img.jgi.doe.gov, http://www.kegg.com, http://blast.ncbi.nlm.nih.gov/Blast.cgi

Listeria may perhaps serve as a better model for regulation of flagellar biosynthesis in the *B. cereus* group, since these species do have *mogR* (Smith & Hoover, 2009a), but a complete *gmaR* has only been found in NVH391-98 (http://Blast.ncbi.nlm.nih.gov).

1.4.2. Bacterial swarming.

Bacterial swarming, as reviewed in (Fraser & Hughes, 1999), is a type of multicellular behavior displayed by bacteria growing on a solid surface. Swarming bacteria develop elongated multinucleate, nonseptate, hyperflagellated cells which align and move coordinately across the surface in large rafts. Cells do not divide in the swarming state, but will dedifferentiate in order to divide. Swarming functions as an effective way to colonize a surface, and a primary requirement for swarm-cell differentiation is a solid surface of appropriate viscosity (Fraser & Hughes, 1999). Sufficient cell density is also important, and the medium has to be rich enough to support flagellar biosynthesis and the energy required for movement (Eberl *et al.*, 1996).

Exactly how the bacteria sense the solid surface, is not certain, but in one case the polar flagellum is reported to be involved (McCarter *et al.*, 1988), and there appears to be a link between chemotaxis and swarming, as mutations in genes involved in chemotaxis frequently reduce or abolish swarming (Harshey, 1994; Harshey & Matsuyama, 1994). On this background it has been speculated that the chemotaxis phosphorelay may be involved in the integration of signals leading to swarm-cell differentiation (Fraser & Hughes, 1999).

1.4.3. Flagellar motility in the *B. cereus* group.

Both *B. cereus, B. thuringiensis,* and *B. weihenstephanensis* are known to exhibit flagellar motility, but the details of its regulation are not well known. As shown previously, regulation is likely to be different from what is observed in *B. subtilis*, but may be partly similar to that of *L. monocytogenes*, despite the absence of DegU. Genes involved in chemotaxis are also reported to be organized differently in *B. subtilis* and *B. cereus* (Celandroni *et al.*, 2000). Repeated attempts to knock out MogR in *B. cereus* ATCC 14579 have not been successful, possibly indicating that MogR is essential in this organism (Annette Fagerlund, personal communication).

In addition to swimming motility, members of the *B. cereus* group are reported to swarm on LB agar with agar concentrations between 0.4-2.5 %, with an optimal range between 0.7 % and 1.2/1.5 % (Ghelardi *et al.*, 2002; Senesi *et al.*, 2002). Under normal conditions, swarm-differentiated cells are only found in the outermost rim of the colony (Ghelardi *et al.*, 2002; Salvetti *et al.*, 2009; Senesi *et al.*, 2002). This way of swarming has also been demonstrated in other bacteria (Calvio *et al.*, 2005; Eberl *et al.*, 1999; Harshey & Matsuyama, 1994).

As could be expected, genes involved in chemotaxis and flagellar expression affect motility, but different genes affect motility, chemotaxis and flagellar expression to variable extent, and other genes may also affect swarming. Table 3 shows the effects of some mutations which have been studied in the *B. cereus* group. However, some of these genes have unknown function, and for several of them, it is not known how they inhibit the swarming process. The conclusion must be that, in fact, we know very little about swarming in bacteria from the *Bacillus cereus* group, and perhaps even less about the regulation of motility in general.

Table 3. Effects of mutations on cellular aspects related to motility.

Nonfunctional gene	flagella	motile	Swarming	chemotaxis	reference
fliY	yes	yes	no	no	(Senesi *et al.*, 2002)
flhA	no	no	no	no	(Ghelardi *et al.*, 2002)
flhF	yes	yes	no	yes	(Salvetti *et al.*, 2007)
*B.t.*407 homologues of BT9727_0602 (hyp. protein) BT9727_2595 (sarcosine oxidase, beta subunit) BT9727_3195 (acetyltransferase) BT9727_2764 (*katX* catalase) BT9727_3344 (*oppA*, ABC transporter, substrate-binding protein) BT9727_5059 (dGTP triphosphohydrolase) *	yes	yes	no	yes	(Salvetti *et al.*, 2009)

* These genes were found by searching a *B. thuringiensis* 407 mini-Tn10 insertion library for isolates deficient in swarming, but with intact motility and chemotaxis.

1.5. Stress responses

Bacteria are continuously exposed to different types of stress from the environment, and they are equipped to handle temperature fluctuations, various types of chemical stress (pH, high salt concentrations, ethanol etc.), and desiccation, as well as attacks from hostile microorganisms and host immune systems (Abee & Wouters, 1999; Hecker *et al.*, 2007). The genes involved in different stress responses vary. Some genes are specific to one particular stress response, while other genes are activated by several types of stress (Periago *et al.*, 2002). Stress responses have been studied extensively in *B. subtilis* (Hecker & Volker, 2001; Hecker *et al.*, 2007), and also in members of the *B. cereus* group, particularly *B. cereus* ATCC 14579 (Browne & Dowds, 2001; den Besten *et al.*, 2009; den Besten *et al.*, 2006; Mols *et al.*, 2007; Periago *et al.*, 2002; van Schaik *et al.*, 2004; van Schaik *et al.*, 2007), revealing extensive adaption to severe stress by pre-exposure to mild stress, and also that adaption led to a large degree of cross-protection between various types of stress.

Transcription of many stress response genes is induced by heat. Such genes are considered part of the heat shock response, though their functions may be diverse. In *B. subtilis,* the heat-inducible genes are classified according to their regulatory mechanism (Derre *et al.*, 1999; Hecker *et al.*, 1996). This classification has been adopted also by related species. Below, I briefly present the five classes presently known, and account in more detail for the classes relevant to this work.

Class I genes are regulated by the HrcA (heat regulation at CIRCE) repressor, which binds to the CIRCE (controlling inverted repeat of chaperone expression) operator sequence. Class I genes have a σ^A-dependent promotor, and include the classical chaperone genes *groEL*, *groES*, *dnaK*, and *dnaJ* (Schulz & Schumann, 1996), and will be presented in more detail below.

Class II genes are positively regulated by σ^B, an alternative sigma factor involved in general stress response (Hecker *et al.*, 2007). These genes are activated both by heat and other stresses, e.g. starvation or chemical stresses such as salt and ethanol. This is the largest group of stress response genes.Van Schaik *et al.* have investigated the σ^B regulon of *B. cereus* ATCC 14579, finding that it is smaller than what is observed in many other bacteria (van Schaik & Abee, 2005; van Schaik *et al.*, 2007).

Class III genes are negatively regulated by CtsR (class three stress gene repressor) (Derre *et al.*, 1999). This class includes genes encoding the protease ClpP and the Clp ATPases ClpC and ClpE in *B. subtilis*. (Derre *et al.*, 1999). In *L. monocytogenes* the ClpB ATPase is also part of this regulon (Chastanet *et al.*, 2004). The class III heat shock proteins are presented in more detail below

Class IV genes encode other heat-inducible proteins whose regulatory mechanisms are so far unknown. ClpX and FtsH are members of this class (Derre *et al.*, 1999).

Class V genes are regulated by the CssSR two-component system (control of cellular response to secretion stress sensor and -regulator, respectively), and are important for the response to secretion and heat stress (Darmon *et al.*, 2002; Hyyrylainen *et al.*, 2001). This class includes HtrA and HtrB, and the CssSR system also autoregulates its own transcription.

1.5.1. Chaperones.

The class I Heat Shock proteins are considered the classical chaperones. Chaperones are protein complexes which assist folding and translocation of other proteins, and prevent protein aggregation in the cell, reviewed in (Lund, 2001). The classical chaperones are the GroE complex and the DnaK chaperone systems. The GroE complex consists of two large rings, each made up of GroEL subunits, which form a cavity with a protected environment where proteins can fold. A smaller protein, GroES, caps the cavity reversibly, and is involved in admission and release of folded proteins (Lund, 2001).

The DnaK chaperone system works by different principles. DnaK binds hydrophobic areas of unfolded or partially folded proteins, thus protecting them from aggregation until they can fold properly (Lund, 2001). DnaJ and GrpE promote binding and release of DnaK to the protein chains, respectively (Lund, 2001). Under physiological conditions, the activity of DnaK is particularly important for nascent proteins, while under stressful conditions such as elevated temperatures, the action of DnaK can prevent aggregation of proteins which have become completely or partially unfolded (Lund, 2001).

In *Bacillus* and *Listeria* species, transcription of the *groESL* and *hrcA-grpE-dnaKJ* operons are both regulated by the HrcA repressor, which is the first gene of the operon. HrcA thus represses its own transcription (Schulz & Schumann, 1996; Yuan & Wong, 1995). GroE modulates HrcA activity (Mogk *et al.*, 1997), possibly through a titration mechanism where GroE is necessary for correct folding of HrcA, and high levels of other non-native proteins will occupy GroE and reduce the folding of active HrcA (Lund, 2001; Mogk *et al.*, 1998).

1.5.2. Clp proteases and the CtsR regulator.

Many of the class III heat shock proteins are subunits of Clp proteases. Clp proteases are ATP-dependent proteases which degrade aggregated or improperly folded proteins. They are also involved in posttranscriptional regulation through specific degradation of particular proteins (Frees *et al.*, 2007; Striebel *et al.*, 2009; Zolkiewski, 2006). The proteolytic complex consists of an ATPase and a proteolytic subunit, ClpP (Frees *et al.*, 2007). The ATP-binding subunit confers substrate specificity and unfolding activity (Zolkiewski, 2006). The ATPase-binding subunits also have independent unfolding and chaperone activity (Frees *et al.*, 2007; Striebel *et al.*, 2009; Zolkiewski,

2006). In fact, the ClpB-ATPase does not associate with ClpP at all, but is still an important stress protein due to its unfolding of aggregated proteins (Zolkiewski, 2006).

The Clp ATPases ClpC and ClpX are found in most low GC gram-positive bacteria (Frees *et al.*, 2007). Other Clp ATPases have a more variable distribution. Table 4 shows which Clp proteins are found in the *B. cereus* group and the related Gram positive species *B. subtilis* and *L. monocytogenes*. Table 4 also shows which Clp proteins are under control of CtsR, the negative regulator of class III stress proteins. In *B. subtilis,* CtsR is reported to be active as a dimer, and specifically degraded at 37°C in a process involving Clp (Derre *et al.*, 2000). CtsR controls its own transcription as well as two proteins involved in its regulation, McsA and McsB (Frees *et al.*, 2007). In *B. subtilis,* CtsR binding to its repressor sites is controlled by McsA, McsB, and ClpC (Frees *et al.*, 2007; Kirstein & Turgay, 2005; Kirstein *et al.*, 2005; Kirstein *et al.*, 2007).

Table 4. The presence of Clp proteins in the *B. cereus sensu lato, L. monocytogenes and B. subtilis.*

Species	protein	CtsR-regulated	References
Bacillus cereus sensu lato	ClpP1	yes	(Fedhila *et al.*, 2002a)
	ClpP2	no	(Fedhila *et al.*, 2002a)
	ClpC	yes*	(Fedhila *et al.*, 2002a), this work
	ClpX	no	(Frees *et al.*, 2007)
	ClpB	yes*	This work
Listeria monocytogenes	ClpP1	yes	(Chastanet *et al.*, 2004; Nair *et al.*, 2000)
	ClpP2	no	(Chastanet *et al.*, 2004)
	ClpC	yes	(Nair *et al.*, 2000)
	ClpX	no	(Frees *et al.*, 2007)
	ClpB	yes	(Chastanet *et al.*, 2004)
	ClpE	yes	(Nair *et al.*, 2000)
Bacillus subtilis	ClpP	yes	(Derre *et al.*, 1999; Msadek *et al.*, 1998)
	ClpC	yes	(Derre *et al.*, 1999; Msadek *et al.*, 1994)
	ClpX	no	(Frees *et al.*, 2007)
	ClpE	yes	(Derre *et al.*, 1999)

*Presence confirmed by sequencing, CtsR binding site found with Genome2D (Baerends *et al.*, 2004), paper III.

1.6. Sporulation

Bacterial endospores are differentiated cells which are metabolically inactive. They can survive without nutrients for long periods of time and germinate when the conditions are once again favorable. Endospores are resistant to heat, UV radiation, organic solvents, and a variety of other harmful environmental influences (Setlow, 2006; Setlow, 2007). Within the endospore core, DNA is protected by small acid soluble proteins (SASPs) and dehydrating dipicolinic acid (Driks, 2002). The core is surrounded by a peptidoglycan cortex, a protein spore coat, and sometimes also an exosporium (Driks, 2002; Madigan *et al.*, 1997) Sporulation is an important part of the success of the genus *Bacillus* and their notorious reputation as industrial contaminants (Barak *et al.*, 2005). Still, the differentiation into an endospore is energy-intensive, and also means the cell will be unable to divide and multiply (Hoch, 1993). Massive sporulation will therefore only be seen under conditions which do not support other survival strategies.

1.6.1. Spo0A and initiation of sporulation.

The master regulator of sporulation is Spo0A (sporulation factor 0A), which needs to be phosphorylated (Spo0A~P) in order to become active. In *B. subtilis*, phosphorylation processes start around the transition to stationary phase and proceeds through a phosphorelay integrating environmental and cellular signals in a variety of phosphorylation and dephosphorylation reactions as indicated in Fig. 4, including a positive feedback loop (Burbulys *et al.*, 1991; Fujita & Losick, 2005; Garti-Levi *et al.*, 2008; Ohlsen *et al.*, 1994; Perego *et al.*, 1994; Perego, 1998; Perego, 2001; Strauch *et al.*, 1990; Strauch *et al.*, 1992). As a result, the concentration of Spo0A and its degree of phosphorylation increases gradually.

As members of the Spo0A regulon (Molle *et al.*, 2003) have different binding constants for the regulator in the upstream region of each respective gene, the regulon is gradually activated (Fujita *et al.*, 2005). This allows Spo0A, together with the regulator DegU, to sequentially activate different transition state adaptive responses and genes needed for sporulation. There are indications that this gradual increase, mediated through the phosphorelay, is necessary for efficient sporulation (Fujita & Losick, 2005). Perhaps even more important to the cell, various survival strategies are activated which may make sporulation unnecessary (Doi, 1989; Phillips & Strauch, 2002): Motility increases, and new metabolic pathways are derepressed, enabling the cells to metabolize new substances.

Degradative enzymes are excreted into the environment (Veening *et al.*, 2008a) in an attempt to make available nutrients from the surroundings, or even cannibalize other bacteria (Gonzalez-Pastor *et al.*, 2003), and some genes may develop competence (Veening *et al.*, 2005b). At higher concentrations, Spo0A~P may activate SinI, and the cells may form a biofilm where the bacteria are protected by an extracellular matrix (Chai *et al.*, 2008).

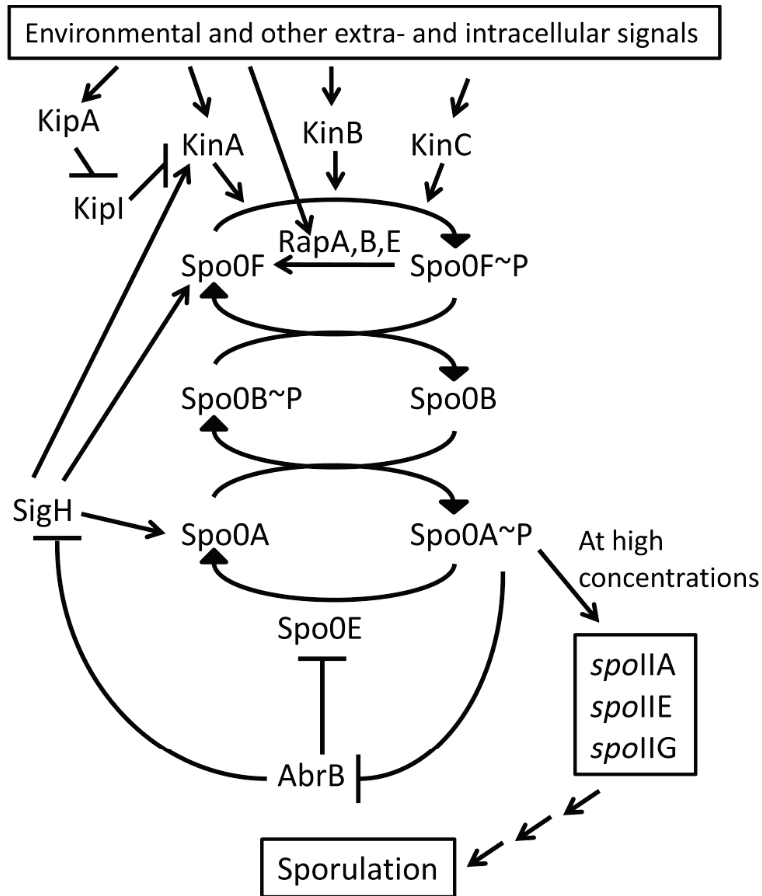

Fig. 4. Some central proteins in the process of SpoA-phosphorylation in *B. subtilis* (Phillips & Strauch, 2002; Veening *et al.*, 2005a)

If the level of phosphorylated Spo0A reaches a (high) threshold concentration, transcription of Spo0A-regulated genes directly involved in sporulation is activated, and

the cell will be committed to sporulation (Chung *et al.*, 1994; Fujita *et al.*, 2005). The cell will then proceed to stage II of the sporulation process and divide asymetrically (Yudkin & Clarkson, 2005). Interestingly, there are indications that the Spo0A~P level continues to increase and maintain a regulatory function in the mother cell (Fujita & Losick, 2003). Sporulation is a bistable differentiation process, and for a planktonic liquid culture in an efficient sporulation medium, about half the population will reach this activation threshold and sporulate (Chung *et al.*, 1994; Fujita *et al.*, 2005; Veening *et al.*, 2005a).

1.6.2. The stages of sporulation.

The timing of the sporulation process depends on both the medium and the strain, but under laboratory conditions *B. subtilis* normally uses at least 7-8 hours, starting from the end of the logarithmic growth phase. The process of endospore formation is divided into different stages based on cytological changes (Barak *et al.*, 2005; Doi, 1989) (stage I is no longer recognized as a separate stage):

Stage 0: When the cell reaches a threshold concentration of phosphorylated Spo0A (Spo0A~P), it is committed to sporulation, even though it is still vegetative. This threshold concentration is reached at the end of the logarithmic growth phase or later. Enzymes involved in Spo0A activation and regulation are shown in figure 4.

Stage II: The forespore septum appears and divides the cell assymetrically into a small forespore compartment and a larger mother cell compartment.

Stage III: The forespore is engulfed into the cytoplasm of the mother cell. Both cell membranes are intact, resulting in a double membrane around the forespore. Formation of calcium dipicolinate starts to dehydrate the forespore.

Stage IV: The spore cortex (peptidoglycan) starts to develop between the inner and outer forespore membrane. At this stage the forespore starts to refract light.

Stage V: Inner spore coat proteins are deposited on the outer forespore membrane. The complex inner spore coat has a multilayered, laminar structure.

Stage VI: The proteinaceous outer spore coat is deposited on the surface of the inner spore coat. The spore matures during this stage, and becomes increasingly resistant to heat and chemicals as the spore coats develop.

Stage VII: The mature spore is released from the mother cell.

Cellular regulation of sporulation after asymetric septum-formation has been studied most closely in *B. subtilis* (Eichenberger *et al.*, 2004; Piggot & Losick, 2002; Wang *et al.*, 2006).

1.6.3. Sporulation in members of the *B. cereus* group.

The process of sporulation in members of the *B. cereus* group appears in many respects to follow that of *B. subtilis* (de Vries *et al.*, 2004), but differences in regulation would be expected. There are observed differences compared to *B. subtilis,* for instance in sporulation histidine kinases and the number of phosphatases (Anderson *et al.*, 2005; Brunsing *et al.*, 2005). Furthermore, Spo0B of *B. anthracis* Sterne has been found to exhibit autophosphorylation and ATPase activity, in contrast to *B. subtilis* Spo0B which is only a phosphotransferase (Mattoo *et al.*, 2008). The phosphatase Spo0E, on the other hand, has the same role in the two organisms, and *B. subtilis* Spo0E is active in *B. anthracis* (Dubey *et al.*, 2009).

1.7. Virulence

With the exception of *B. anthracis*, most members of the *B. cereus* group are not very invasive, but are considered opportunistic pathogens (Kotiranta *et al.*, 2000). Virulence varies greatly both between and within species, from strains accepted for use as probiotics (Hong *et al.*, 2005) to *B. anthracis,* the causative agent of anthrax (Mock & Fouet, 2001; Passalacqua & Bergman, 2006). The genes encoding the virulence factors leading to anthrax reside on two large plasmids, pXO1 and pXO2, and differ from those displayed by most other members of the group (Kolstø *et al.*, 2009; Mock & Fouet, 2001). In fact, PlcR, a pleiotropic regulator of virulence in the *B. cereus* group, is non-functional in *B. anthracis* (Agaisse *et al.*, 1999; Mignot *et al.*, 2001). However, there are reports of *B. cereus* strains which have caused disease with anthrax-like symptoms and harbor plasmids similar to pXO1 and pXO2 (Hoffmaster *et al.*, 2004; Hoffmaster *et al.*, 2006; Klee *et al.*, 2006). *B. mycoides* and *B. pseudomycoides* both have functional PlcR, but are rarely implied in infection, while *B. cereus, B. thuringiensis,* and *B. weihenstephanensis* all include strains with potential to cause disease (Ehling-Schulz *et al.*, 2004; Kramer &

Gilbert, 1989; Lapidus *et al.*, 2008; Stenfors *et al.*, 2002; Thorsen *et al.*, 2006). Thus, the discussion of virulence below will be relevant to strains within all these three species.

1.7.1. The virulence regulator PlcR

PlcR (phospholipase C regulator) is a pleiotropic regulator of virulence factors in members of the *B. cereus* group (Agaisse *et al.*, 1999). *plcR* transcription is activated at the transition to stationary phase and reaches its maximum two hours later (Lereclus *et al.*, 1996) (for activation mechanism, see section 1.2.1.1). PlcR positively regulates transcription of a large regulon, dominated by secreted and cell wall-associated virulence factors, including cytotoxins (e.g. cytotoxin K), enterotoxin complexes (hemolysin BL and nonhemolytic enterotoxin), immune inhibitors (InhA2), phospholipases, proteases, and a variety of other degradative enzymes (Agaisse *et al.*, 1999; Gohar *et al.*, 2002). Proteins controlled by PlcR make up more than eighty percent of the secretome during the transition to stationary phase in a rich medium (Gohar *et al.*, 2002). Disruption of PlcR reduced, but did not abolish virulence in insect larvae, mice and an ophthalmic rabbit model system (Callegan *et al.*, 2003; Salamitou *et al.*, 2000). Motility was also reduced in PlcR-deficient strains (Callegan *et al.*, 2003)

PlcR is a quorum sensor, responding to the cell population density, and positively regulates its own transcription (Gominet *et al.*, 2001; Lereclus *et al.*, 1996). However, it is also influenced by other regulators in the cell; *plcR* transcription is repressed, possibly indirectly, by Spo0A in its phosphorylated state (Spo0A~P), and will therefore not be activated under conditions favoring sporulation (Lereclus *et al.*, 2000). Deletion of the two-component system YvfTU resulted in 50 % lower expression of PlcR, but this only affected a smaller number of PlcR-controlled genes, and transcription of the genes encoding haemolysin BL-components were actually upregulated (Brillard *et al.*, 2008).

1.7.2. Members of the *B. cereus* group as human pathogens.

As noted above, most members of the *B. cereus* group rarely cause serious disease in humans. However, there are examples of systemic infections, particularly in immunocompromised individuals (Drobniewski, 1993). When introduced through traumatized skin such as burns or wounds, *B. cereus* can cause severe local infections (Drobniewski, 1993). Likewise, if *B. cereus* or *B. thuringiensis* is introduced into the eye,

serious eye infection may occur, which may result in loss of sight within 24 hours (David *et al.*, 1994; Miller *et al.*, 2008).

Still, the diseases most commonly associated with members of the *B. cereus* group in humans are gastrointestinal or emetic food poisoning of short, but rather unpleasant duration (Drobniewski, 1993; Hauge, 1950; Kramer & Gilbert, 1989). The emetic disease is an intoxication caused by preformed cereulide, a non-ribosomally synthesized peptide, while the diarrhoeal disease is usually considered a toxicoinfection caused by vegetative cells secreting toxins and degradative enzymes, the majority of which are controlled by PlcR. Cytotoxin K (CytK) (Lund *et al.*, 2000) as well as the three-component enterotoxins hemolysin BL (Hbl) (Beecher & Macmillan, 1991) and non-hemolytic enterotoxin (Nhe) (Granum *et al.*, 1999; Lund & Granum, 1999) are thought to play a central role in gastrointestinal infection (Arnesen *et al.*, 2008; Kotiranta *et al.*, 2000), but most likely act together with other virulence factors to create the enterotoxic effect (Arnesen *et al.*, 2008).

1.7.3. Members of the *B. cereus* group as insect pathogens.

B. thuringiensis is regarded as the primary insect pathogen of the *B. cereus* group, due to its ability to produce large amounts of insecticidal crystal toxins (Schnepf *et al.*, 1998). These crystal toxins are encoded by *cry* genes residing on plasmids, Different *cry* genes have specificity towards different insect larvae, and combinations of toxins yield synergistic effects (Schnepf *et al.*, 1998). The Cry toxins are produced in large amounts during sporulation, e.g. in soil, and several *cry* genes are preceded by a Spo0A binding site, but transcription may also be induced by sporulation-independent mechanisms (Lereclus *et al.*, 1995; Schnepf *et al.*, 1998).

When ingested by susceptible insect larvae, the crystal toxins attack the peritrophic membrane lining the insect midgut (Soberon *et al.*, 2009). There are indications that the toxins may act synergistically with virulence factors encoded in the chromosome, such as chitinase and the PlcR-regulated InhA2 (Fedhila *et al.*, 2002b; Liu *et al.*, 2002). Without the toxin-encoding plasmids, *B. thuringiensis* is indistinguishable from *B. cereus* (Helgason *et al.*, 2000; Rasko *et al.*, 2005). However, even though some *B. cereus* strains are reported to live symbiotically in the insect gut (Margulis *et al.*, 1998), *B. cereus* strains as well as *B. thuringiensis* strains cured of the *cry* plasmid still exhibit pathogenicity in oral and intrahemocoelic insect infection models (Bouillaut *et al.*, 2005; Fedhila *et al.*, 2002a;

Salamitou *et al.*, 2000). Thus, it is also possible to discover chromosomally encoded genes which lead to attenuated virulence when rendered non-functional. Examples of such genes are *ilsA*, *inhA2*, *fur*, *flhA*, *plcR*, *papR, clpP1* (although the effect of *clpP1* deletion is temperature-dependent) (Bouillaut *et al.*, 2005; Fedhila *et al.*, 2002a; Fedhila *et al.*, 2002b; Fedhila *et al.*, 2003; Fedhila *et al.*, 2006; Salamitou *et al.*, 2000; Slamti & Lereclus, 2002), and genes of unknown function, such as *yqgB/ yqfZ* (Fedhila *et al.*, 2004). Virulence was strongly attenuated in an insect model by simultaneous deletion of all three *inhA*-encoding genes in *B. thuringiensis* 407 (Guillemet *et al.*, 2009). Thus it is apparent that chromosomally encoded virulence factors also play a role in insect pathogenicity.

2. Aims of the research and summary of papers.

In the work presented in this thesis, microarray technology was applied to address various questions of an exploratory nature, questions which would be difficult to address effectively using traditional methods. Comparative transcriptomic analysis may provide insights into the global effects of deletion mutations or cellular differentiation. The method has the potential to reveal the transcriptional rationale behind an observed phenotype, and also transcriptional changes which do not necessarily result in a visible phenotype under the applied conditions. Below I will briefly present the major aim(s) of each study and give a summary of the resulting paper.

Paper I:

Aim: To establish a regulon for the transition state transcriptional activator PlcR, which is important to virulence in the *B. cereus* group. We wanted to verify differential expression of genes found by other methods to belong to the PlcR regulon, as well as to identify new members of this regulon (paper I)

Summary of results: a PlcR regulon was determined, which consisted of forty-five genes controlled by twenty-eight PlcR binding sites. Twenty-two PlcR-controlled proteins were secreted, and eighteen were bound to the cell wall or the outside of the cytoplasmic membrane. Many of the exported proteins were involved in virulence and degradation, supporting the view that PlcR plays a role during host invasion, by activating defensive as well as invasive mechanisms. Several exported proteins also have potential regulatory or environmental sensing functions, possibly indicating that the PlcR regulon integrates many signals from the environment. Of the cytoplasmic proteins, four had confirmed (PlcR) or putative regulatory functions.

Based on the PlcR binding sites found to be active, a new consensus sequence was proposed. Areas surrounding the active sites were found to be significantly more AT-rich than areas surrounding inactive binding sites.

Paper II

Aim: To establish a regulon for the transition state transcriptional represssor NprR and its signalling molecule NprX. As little was previously known about NprR, an important aim was also to gain a better understanding of the role of this regulator during the transition phase.

Summary of results: Global comparison of gene transcription between *B. thuringiensis* 407 *nprA'-lacZ* Δ*nprR-nprX* and its isogenic strain using microarrays, showed that NprR-NprX affected one hundred and eight genes, encoding proteins with a broad range of functions. Genes more than four times up- or downregulated, as well as two genes with high functional similarity to some of these genes, were considered most likely to be members of an NprR regulon. Differential expression of these genes in the two strains was confirmed by RT-qPCR and the genes were all found to be at least four times up- or downregulated. With the exception of one operon, all genes were downregulated in the mutant relative to the reference strain, indicating that NprR primarily functions as a transcriptional activator. Some of the genes found to be differentially expressed, were also compared by introducing lacZ fusions of their upstream regions into *B. thuringiensis* 407 *nprA'-lacZ* and the Δ*nprR-nprX* mutant. These experiments confirmed our observations. On this background, the forty-two genes were proposed to make up an NprR regulon. Of these, one fourth are degradative enzymes, indicating that the NprR regulon may have a role in nutrient acquisition during the transition to stationary phase.

A BLAST search revealed that complete or partial hits to *nprR* was found in all but one of the currently sequenced members of the *B. cereus* group (*B. cereus* H3081.97). No hits were found outside the *B. cereus* group. A search for orthologs to genes belonging to the NprR regulon in fifteen completed genomes, identified a subset of eleven genes to be practically absent from genomes belonging phylogenetically to clade I, which harbors the *B. anthracis* cluster and many clinical isolates.

Paper III.

Aim: To describe phenotypic differences and similarities between three variants of *B. cereus* ATCC 14579, two which displayed filament formation during exponential growth (14579-L1 and 14579-L2), and one which grew as single cells or short chains during all stages of growth (14579-S1). The aim was also to establish a genetic or regulatory cause for the observed differences.

Summary of results: Microarray comparisons of the variants 14579-S1 and 14579-L1, isolated from the same liquid culture, revealed increased transcription of genes encoding class I and III stress proteins in the filamentous variant, 14579-L1. This was confirmed by RT-qPCR, and mass spectrometry showed that differences in expression could also be found for some of the corresponding proteins. Sporulation experiments showed the sporulation efficiency of 14579-L1 to be only one tenth of that observed for 14579-S1 and 14579-L2. Non-sporulated cells, though non-viable, did not lyse.

14579-L2 shared most of the morphological phenotypes of 14579-L1, including a rugged colony edge when grown on agar plates, and the failure to lyse, both in contrast to 14579-S1. However, as already mentioned, 14579-L2 was sporulation proficient, and RT-qPCR showed no upregulation of genes encoding stress proteins. All three variants were stable over time, indicating that the observed differences had a genetic basis, but a cause for the phenotypic differences and similarities could not be determined.

Paper IV.

Aim: To study global effects of swarming on gene expression and investigate the regulatory mechanisms behind the swarm-differentiated cells.

Summary of results: Microarray comparison of swarm-differentiated versus non-swarming cells of *B. cereus* ATCC 14579 found 290 genes to be significantly affected by swarming. Half the genes were found to be upregulated, while the other half was downregulated. Genes involved in motility, signal transduction mechanisms, and intracellular trafficking and secretion were among the COG categories dominated by upregulated genes during swarming, in accordance with the hyperflagellated, multicellular

nature of the swarming state. Among the most highly upregulated genes, were also genes encoding proteins with a potential role in antimicrobial resistance, and further studies demonstrated increased resistance towards daptomycin and also towards sodium tellurate compared to non-swarming colonies.

Transport and metabolism of nucleotides, carbohydrates, and lipids (three different COG categories) were all dominated by downregulated genes during swarming. In addition, a substantial number of genes involved in energy production were found to be downregulated, but this was most likely due, at least in part, to differences in oxygen availability between swarming and non-swarming cells. Upregulation of genes, however, did not seem to be dependent on oxygen conditions. The virulence regulator PlcR and many genes controlled by this regulator, showed reduced transcription during swarming, while the *hbl* toxins showed increased transcription, presumably by a PlcR-independent mechanism.

3. Methodological considerations.

In this chapter, I will briefly introduce the principles of microarray technology, and I will discuss in some detail the choices made during analysis of the microarrays. Microarray experiments have been central in the projects presented in this thesis. Even though the microarray results may constitute a small part of the final papers, they have provided much of the foundation for further studies. Microarray results are not only affected by the experimental procedure. Image processing and data analysis also have a considerable impact on the final results. These are details which there is not room for in article, but which still influence the outcome, and hopefully they may be of interest to others who work with this technology.

3.1. Microarray technology, principles and a short history.

Microarray technology is based on the principle of hybridization (Knudsen, 2004a). Two complementary single strands of DNA/RNA will hybridize to form a double strand. Adenine binds to thymine or uracil, while cytosine binds to guanine. A microarray is an arrangement of one-stranded nucleotide probes attached to a matrix. Depending on the type of array, one or two samples are fluorescently labeled, denatured, and applied to the matrix, where nucleotide sequences in the samples will bind to matching probes by hybridization. RNA is converted to cDNA in the labeling process.

After hybridization, the slide is scanned. The intensity of the fluorescent signal for a given probe is used as a measure of the amount of the matching nucleotide in the sample (Knudsen, 2004a). For two-color arrays, the two applied samples bind by competitive hybridization, and the scanning is done at two different wavelengths, returning two values for each probe. For these arrays, the result is given as a ratio of the signal intensities of the two samples to be compared, or a ratio of the sample relative to a reference. Fig. 5 shows an overview of the workflow for the laboratory part of a microarray experiment.

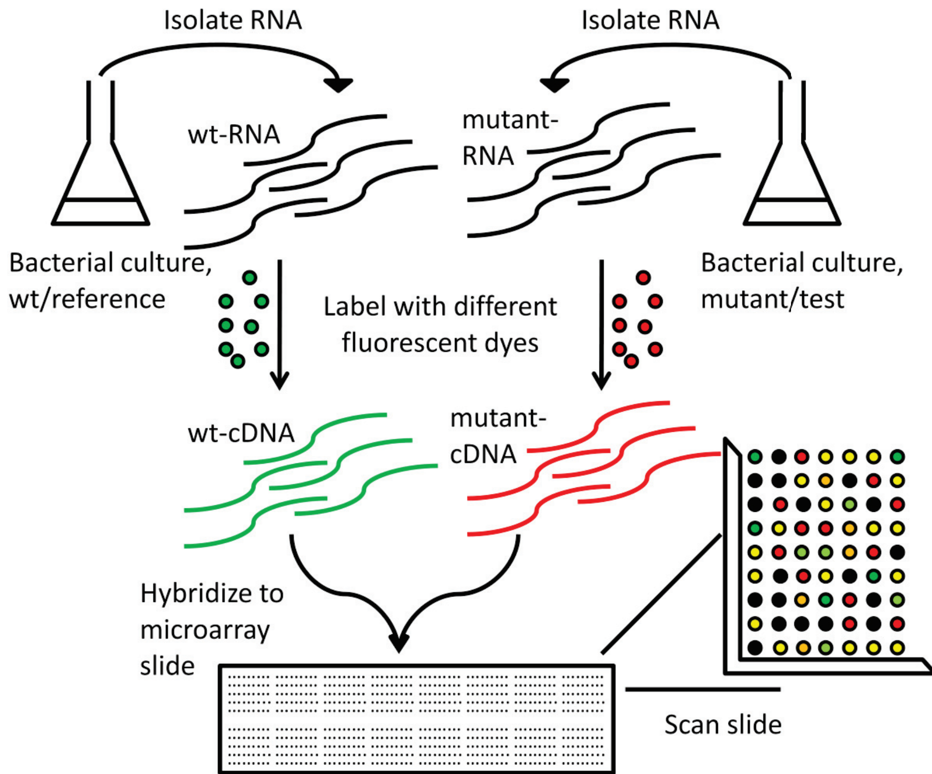

Fig. 5. The steps of a two-color spotted array gene expression experiment

Hybridization is also the basis of classical techniques such as southern and northern blotting, and was, as such, well established. Immobilized probes were also used previously, in membrane based dot blots and macroarrays. The novelty of microarrays was in the massive parallel capacity for multiple testing. This was among the first true high throughput techniques, allowing simultaneous hybridization of a sample to thousands of probes printed on a surface. The expression of all genes in a genome could be investigated simultaneously. This was particularly feasible for microbial genomes, due to their relatively small size.

In the beginning, two types of microarrays dominated the field: Affymetrix chips and two-color arrays spotted on microscope glass slides. Affymetrix synthesizes short nucleotide sequences directly on to the chip with high density of probes per area (Ragoussis & Elvidge, 2006). This allows the creation of so-called tiling arrays, which cover the entire genome sequence with overlapping probes, and can be used to discover

small variations and mutations in the genome as well as gene expression studies (Ragoussis & Elvidge, 2006). Affymetrix chips are one-color arrays, where one sample is hybridized to each chip. However, affymetrix chips are expensive and require highly specialized scanners (Dufva, 2005).

Spotted arrays were a cheaper alternative. Here the array is made by small amounts of nucleotide probes printed on the surface of a glass slide (Dufva, 2005). In the beginning, probes were based on cDNA, but as an increasing number of sequenced genomes became available, the use of synthetic oligonucleotides became more common (Barrett & Kawasaki, 2003; Kawasaki, 2006). Due to their lower resolution, spotted arrays were primarily used for gene expression studies.

After a couple of years, other high density array platforms appeared, such as two-color arrays from Agilent (Wolber *et al.*, 2006), a variety of array types from NimbleGen (Roche) (Kirmizis *et al.*, 2004; Nuwaysir *et al.*, 2002; Okou *et al.*, 2007; Selzer *et al.*, 2005; Wong *et al.*, 2004), and also high density bead arrays from Illumina (Fan *et al.*, 2006), to mention a few of the most successful platforms. This allowed the creation of tiling arrays for more platforms than Affymetrix, and expanded the possible applications of microarrays (Kapranov *et al.*, 2003).

Lately, however, arrays have met competition from the new high-throughput sequencing methods such as SOLiD (ABI) (Cloonan *et al.*, 2008) and Solexa (Illumina) (Mortazavi *et al.*, 2008), leading some scientists to predict the imminent obsolescence of microarrays (Shendure, 2008). Direct sequencing of RNA avoids several of the weaknesses inherent in the microarray technology. Cross hybridization and artifacts introduced through reverse transcription and fluorescent labeling of your sample are not a problem, and better reproducibility between different platforms should be expected. Furthermore, sequencing may uncover unknown active transcripts and alternative splicings, while a probe-based technique will be limited by the design of the probes. With sequencing, *a priori* knowledge of the genome sequence is not absolutely required, but will still be a great advantage to those attempting to make sense of the vast amount of data that is generated. However, at a similar cost, sequencing is at the moment not outcompeting high-density arrays for gene expression studies (Bloom *et al.*, 2009). Arrays are by now a well established technique, and will, at least for some time still, be a more economical alternative than sequencing, particularly for large numbers of samples. The technique is likely to be valuable for quite a

while yet, particularly within diagnostics, though its use may shift in the direction of preliminary analysis and preparative purposes (Blow, 2009).

3.2. The microarray slides.

The microarrays used in this study are spotted arrays, custom printed on aminosilyl-covered glass slides. The probes are 70-mer oligos designed for *B. anthracis* Ames, with supplementary probes for *B. anthracis* A2012 and *B. cereus* ATCC 14579 where the sequence in these strains is less than 93 % identical to the corresponding *B. anthracis* Ames sequence (ungapped alignment), or for unique ORFs in these two strains. One probe has been designed for each ORF. Probes matching two hundred ORFs found in *B. cereus* ATCC 10987, but not in the three other strains, are also included. Each probe is printed twice, in a pair, on the array, and the entire array is printed twice on each slide, resulting in a total number of four replicates for each probe on a slide. Negative and positive controls (housekeeping genes) are included.

The presence of probes for similar genes from multiple strains poses a risk of crosshybridization, but the main weakness of the slides stems from the sequencing of the *B. cereus* ATCC 14579 (Ivanova *et al.*, 2003), where many genes were found to carry mutations which render them non-functional. For such pseudo-genes, no probes were designed. However, resequencing the gene frequently shows that the alleged mutations are sequencing errors (Salvetti *et al.*, 2007, paper III), and that the gene is in fact functional and may be expressed. Fortunately, such genes may in many cases be detected by the *anthracis* probes. Also, as annotation differs between strains, transcribed areas not designated as ORFs in *B. cereus* ATCC 14579 may be registered by probes for other strains. Significant hits to *anthracis* probes were therefore investigated to uncover whether they were a result of cross hybridization or contained new information.

3.3. Experimental design.

A suitable experimental design is important to get the desired information from the experiments. When comparing a wild type and a mutant, or two different conditions, direct comparison by hybridisation of the two sample types to the same slide was chosen as an efficient and easily interpretable method, which was also easy to analyze properly. The

disadvantage of such a design is that it can not easily be expanded to accommodate new sample types, unlike a design with a common reference.

For the PlcR-experiments, a hybridization loop (fig. 6) was set up to maximize technical and biological variation in as few experiments as possible. However, though theoretically possible, it was in reality difficult to distinguish between technical and biological replicates during analysis. When the biological material is a bacterial culture, biological replicates are easy to obtain, and give more valuable information than technical replicates. Thus, for the other analyses in this work, all slides represent independent biological replicates, and four slides were normally used for each experiment. Dye swap (i.e. alternating the assignment of the fluorescent dyes between the two conditions tested) was performed between biological replicates to avoid dye bias.

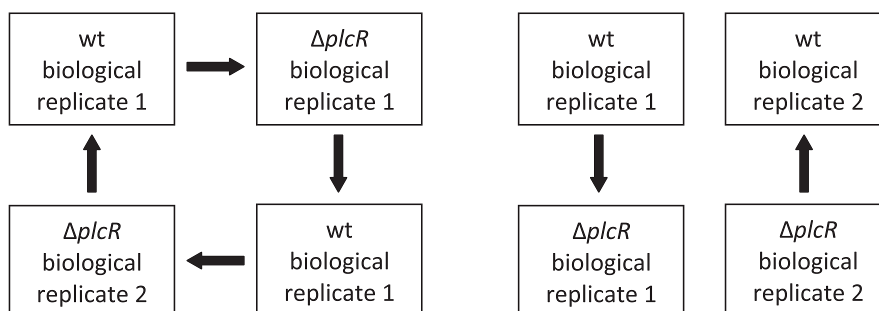

Fig. 6. To the left, the design used for most of the PlcR experiments. To the right, the design used for the other experiments, which only uses two slides to compare two biological samples. The slide at the bottom of the arrow was labeled with Cy3 and the slide at the tip of the arrow with Cy5.

3.4. Scanning

The scanner used for the microarray slides was a GenePix 4000b scanner (Molecular devices). During scanning, intensity of the scanning beams was adjusted to achieve a ratio close to 1 between the sums of the fluorescent signals in the raw image. A common piece of advice for microarray scans is to avoid saturated spots. However, as the samples are prepared using total RNA, probes corresponding to ribosmal RNA can be expected to reach saturation. It has been reported that low-intensity signals (intensities < 200) lead to erroneous expression ratios which may also affect the total intensity normalization factor, while a small number of saturated spots appeared to have few global

effects (Lyng *et al.*, 2004). Most of the slides were therefore scanned at intensities where a small number of spots were saturated. A drawback of this is that bacground intensities around the spots may increase. However, the probes will normally appear as black holes in the background and do not seem to be affected to a large extent by the background.

3.5. Microarray analysis

Numerous programs and scripts exist to normalize microarray data and make a statistical model based on the results. Though some methods are undisputably better than others, a more common situation is that each choice has its advantages and disadvantages. In the following, I will discuss the microarray analysis, and choices made along the way. Generally, it can be said about the analyses in this work that they are conservative, and that robustness has been given priority over statistical power. The filtering scripts used before and after normalization were made by Endre Anderssen, and have only been slightly modified to suit our use.

3.5.1. Analysis of the raw images.

Initial analysis of the raw image consists of three steps (Yang *et al.*, 2002):

Gridding: A grid of probe identifiers is fitted to the raw image, localizing and identifying each spot

Segmentation: The boundaries of each spot are determined, and pixels are classified as belonging to the spot (foreground) or to the surroundings (background)

Intensity extraction: Signal intensities of the foreground and background are calculated for each wavelength. In addition, various quality measures may be calculated.

The Genepix Pro 6.1. software (Molecular Devices, 2005) was used for the raw image analysis. The program performs the steps above automatically, but manual intervention is necessary to secure proper gridding and segmentation, something which is not unusual for spotted arrays (Knudsen, 2004c). Adaptive circle segmentation was used (Knudsen, 2004c).

During the raw image analysis, spots of dubious quality or unduly influenced by background were also marked (flagged bad) to make it possible to weigh these down or

remove them during downstream analysis. In addition, many spots were automatically flagged by the software as “not found”.

3.5.2. Filtering.

After raw image analysis, signal intensities and additional quality information about each spot was exported from the GenePix result file into the statistical computing platform R, version 2.7.1. (http://www.r-project.org), and further data analysis was performed with the package LIMMA (Linear models for microarray data) (Smyth, 2005). The main focus of our analyses was to find genes which could reliably be assumed to be affected by the mutation or the conditions under investigation. As internal replicates on the slides were averaged during downstream analysis, it was considered important to remove replicate spots of dubious quality. Spots which were flagged “bad” or “not found”, as well as very small spots, were excluded from the analysis, as their values were considered unreliable. Spots with weak or saturated signal intensities at both wavelengths were removed, while spots with one good signal and one weak or saturated signal were kept. Due to the nature of saturation, and the tendency of the GenePix scanner to overestimate weak signals (Lyng *et al.*, 2004), these spots would most likely have underestimated expression ratios and return some false negatives, but some of these genes could be both significant and interesting. Likewise, spots with one weak and one saturated signal were kept, as their results would be very significant even if their expression ratios were grossly underestimated.

3.5.3. Weighting

Control spots were weighted down so as not to influence the normalization. For technical reasons, they were removed before the statistical models were made. Normal spots were given a quality weight based on the spread in signal intensity within each spot (Bruland *et al.*, 2007). The quality weights were used during normalization and when internal replicate spots were averaged. The averaged quality weights were also used when the statistical model was made, but in this case the weights were also dependent on the number of replicates on which the averaged values were based, and this normally had a far higher impact than the quality weights.

3.5.4. Background correction

Background correction is common, and based on the assumption that the fluorescence registered from the background will also cover the spot and increase the signal intensity. As described above, however, the two signals were seldom additive in our experiments, but usually appeared as dark circles against the surrounding background. This was particularly striking in arrays with high background, where subtraction of the background values would substantially affect signal intensities. In such cases, subtracting the background would only increase noise in the data (Knudsen, 2004c). There are also studies reporting that background correction may increase the variability of low-intensity spot values and reduce the number of differentially expressed spots found (Bruland *et al.*, 2007; Yang *et al.*, 2002). On these grounds, background intensities were not subtracted during microarray analyses performed in this work. Instead, spots which appeared to be affected by the background signal were flagged bad during the raw image analysis, and subsequently excluded from downstream analysis. However, to stabilize spots with very low signal intensities, a small offset (usually in the size range 10-50) was added to all signal intensities. This slightly dampened the expression ratios, but usually had only a small or negligible effect on those spots which were not removed during filtering.

3.5.5. Normalization

The purpose of normalization is to remove systematic bias from the data. There are several ways to normalize, but, unless external controls are used, they all make assumptions about the distribution of the data (Knudsen, 2004b). In this work, data were normalized using global loess (Smyth & Speed, 2003), which stipulates that the data are centered around zero, i.e. that the sum of signal intensities at each wavelength is equal, but takes into account that the bias in signal-ratios often depends on the signal intensities (Knudsen, 2004b; Smyth & Speed, 2003). Equal sums of signal intensities were also assumed during scanning, when the power of the multiplier tubes was adjusted to achieve a total signal ratio close to one, and is based on the fact that we started with equal amounts of RNA in the two samples to be compared. Printtip-loess was not used, as intensive filtering of some areas of a slide could leave few spots on which to base the normalization. In addition, dye bias was corrected by dye swap between biological replicates (4.3.1.).

3.5.6. Merging internal replicates

After normalization, technical replicates within each slide were averaged, and the averaged value given a weight based on the quality weights of the original spots and the number of replicates which had been averaged. Averaging the spots decreases the granularity/resolution of the data, which may cause problems if analyzing data from low-intensity scans, where the spread in signal intensity is smaller. However, the methods which include technical replicates in the statistical model assume the same correlation between all spots (Smyth *et al.*, 2005), something that does not apply to our array design where the four replicates are located in two pairs far apart. Therefore we preferred to average the replicates, as this method was robust and applicable to all genes regardless of how many and which replicates were left after filtering. For slides scanned at higher intensities, the ranking of genes was very similar between analyses with merged and unmerged replicates, and there was a tendency that genes predicted to be cotranscribed would be treated more similarly in genelists based on merged replicates.

3.5.7. Transformation

The averaged values were compared across slides to calculate $\log_2$ expression ratios and significance estimates using linear models and moderated t- statistics as described by Smyth (Smyth, 2004). FDR-correction ($p = 0.05$) was used to correct for the problem of multiple testing (Benjamini & Hochberg, 1995; Knudsen, 2004b).

3.5.8. Separate normalization of positive controls

Probes for twelve typical housekeeping genes were used as positive controls on the slide. Probes from this set were frequently found to be differentially expressed, which posed a problem, as the controls could not be separated from the ordinary probes, and could not be analyzed together with the normal data. To obtain expression ratios for the probes used as positive controls, a separate analysis was made where the probe IDs matching those of the positive controls were not removed, but analyzed exactly as the the other data, but this time influencing normalization. From this analysis, only results for the probes corresponding to the positive controls were extracted and added to the remaining data.

3.6. Performance of the analysis method.

During this work, various approaches to experimental design, scanning and analysis have been tried and reconsidered, and some of this experience has been accounted for above. The resulting analysis method was applied to a variety of scientific questions, finding from zero to several hundred significant genes. Due to the strict filtering criteria, the analysis is conservative and will most likely not find all true positives, particularly among weakly expressed genes. However, a major aim has been to avoid false positives. In this respect, it is reassuring that strong results can generally be reproduced by RT-qPCR or other methods. For the genes picked out as likely candidates to be under direct control of NprR (paper II), fourfold or higher differential expression could be confirmed for all the suggested genes by quantitative RT-PCR. The observed increase in differentially expressed genes from T1 to T3 in the NprR experiments is also reasonable, as NprR activity was expected to increase during this period.

For the genes found to be significantly upregulated in the microarray comparison between filamentous and planktonic variants (paper III), most of the fold changes were lower than fourfold. Still, upregulation of eleven out of thirteen genes could be confirmed by quantitative RT-PCR, even though, in this case, there was no overlap between the samples used for microarrays and those used for RT-qPCR. The microarray results were also compared with results from the same experiment carried out in LB broth without added glucose (data not shown). Nine of the thirteen genes were found in both genelists, and all operons except BC_3705-BC_3706, were represented. BC_3705-BC_3706 were also the genes which could not be confirmed by RT-qPCR. As both genes in the operon were found to be differentially regulated in one study, the divergent results may be caused by biological variability as well as analytical artifacts. Microarray results for paper IV have not been confirmed by other methods. A validation would strengthen the data, and be particularly valuable for the hypothetical proteins.

The examples above indicate that the analysis method yields fairly reproducible results. Still, the power of microarrays is in its use as an exploratory, hypothesis-generating tool, and fold changes are often more reproducible than ranking genes based on p-values alone (Shi *et al.*, 2008), which was also evident in paper I, where the results were used not only to find new candidate genes, but also to validate genes already known to be controlled by PlcR. Of course, an appropriate p-value cut-off should be set to balance the sensitivity and specificity of the results, but it is the differential expression of genes and the possible

interactions between different genes in the gene list which may provide answers to the questions. How large a transcriptional difference has to be in order to be considered relevant, depends on both the gene and the study. Therefore, a variety of cut-offs have been used in this work. In all cases, knowledge of the organism and its cellular processes is necessary to properly interpret the biological significance of the observations, and to design suitable follow-up studies.

4. Discussion.

In this section, I will attempt to place some of the results from individual subprojects in a larger context. I will also use the opportunity to discuss some aspects of the various parts of the project in more detail and perhaps from a different angle than what the format of a regular article permits. I will start by discussing the impact of adaptive responses on parts of the regulatory and sensory machinery of the cell, and then I will look at NprR and PlcR and their roles in the transition state regulatory network. I will also briefly look at the phylogenetic distribution of the PlcR regulon.

The impact of oxygen availability on the study of transcriptional differences will be discussed, as this background is also relevant for the following discussion of the connection between motility and virulence, seen in light of the results from the PlcR- and swarming projects. Finally, I will discuss in more detail some of the unsettled issues of paper III, regarding the transient filamentation and occasional sporulation deficiency observed in variants of *B. cereus* ATCC 14579.

4.1. Adaptive responses affect the sensory and regulatory networks of the cell.

Adaptive responses are activated by one or more environmental or internal stimuli, and the responses themselves may in their turn modulate the sensory as well as the regulatory apparatus the cell. One example of this is the number of genes encoding proteins with potentially sensory or regulatory functions in the PlcR regulon (paper I). Also during swarming and NprR-deletion, effects are seen on genes with regulatory and sensory functions, though these appear to be of a more indirect nature (paper II, table S2, and paper IV, table S1). On this background, we wanted to investigate effects of adaptive responses on two-component systems, which make up an important part of the cell's system to sense and respond to environmental stimuli (de Been *et al.*, 2008). For each microarray experiment, the 100 two-component genes found in *B. cereus* ATCC 14579 by de Been et al (2006) were extracted, and genes with a p-value < 0.1 were considered potentially interesting candidates. The results for each study are listed in table 5. Microarray comparisons from paper IV and the study of *nprR-nprX* deletion at T1 found no affected two-component genes.

Table 5. Two-component system genes whose transcription is affected by adaptive responses in the *B. cereus* group.

locus tag	gene name	annotation/predicted function* (HK = histidine kinase, RR = response regulator)	log2FC	p-value
PlcR after 3 hours.				
BC_0577	yufL	HK C4-dicarboxylate (citrate) uptake/metabolism	-0,8	0,005
BC_0882	comA	RR natural competence	-0,6	0,086
BC_1477	resD	RR aerobic/anaerobic respiration/virulence	-0,5	0,021
BC_1478	resE	HK aerobic/anaerobic respiration/virulence	-0,5	0,011
BC_1654	cheV	RR chemotaxis protein	-0,7	0,004
BC_4470		HK Sporulation kinase (uroporphyrinogen-III synthase)	-0,6	0,026
BC_4836	yxdJ	RR cell wall stress response, antimicrobial resistance	0,5	0,007
BC_5353	yocF	HK membrane fatty acid saturation/desaturation	0,6	0,026
BC_5462	yycG	HK fatty acid biosynthesis, virulence	0,4	0,021
PlcR after 5 hours.				
BC_1627	cheY	RR chemotaxis protein	-0,4	0,090
BC_3100		HK sporulation initiation	-1,7	0,070
BC_4470		HK Sporulation kinase (uroporphyrinogen-III synthase)	-0,8	0,005
NprR after 3 hours				
BC_4170	spo0A	RR stage 0 sporulation protein A	-0,6	0,008
BC_5336	spo0F	HK Sporulation initiation phosphotransferase F	-0,5	0,042
swarming				
BC_1627	cheY	RR chemotaxis protein	0,5	0,028
BC_1628	cheA	HK chemotaxis protein	0,8	0,024
BC_4589	phoP	RR phosphate uptake/metabolism	0,9	0,085

As activation of two-component systems often leads to regulatory changes (de Been *et al.*, 2008), small changes in transcription of genes encoding two-component sensors or regulators may potentially have a considerable downstream impact. However, looking at small changes in differential expression requires caution, and the results must be seen in context with the purpose of the regulator as well as observations. Still, it is notable that the number of affected two-component genes found in each study was small and could frequently be associated with each other or the adaptive response under study. Two of the three two-component genes found to have increased transcription during swarming are involved in chemotaxis, while the two genes found to be downregulated by the *nprR-nprX*

deletion both are stage 0 sporulation factors. This may indicate a positive effect of NprR-NprX on sporulation, but such an hypothesis would have to be further investigated. As can be seen from table 5, deletion of *plcR* also negatively affected some genes involved in sporulation initiation. This is somewhat more surprising, as high levels of Spo0A are known to inhibit PlcR activation, directly or indirectly (Lereclus *et al.*, 2000). However, initiation of sporulation is a complex process, and the exact effects of this downregulation remains to be elucidated, as *B. cereus* ATCC 14579 carries as many as fourteen histidine kinases with putative roles in the sporulation phosphorelay (de Been *et al.*, 2006).

PlcR was found to directly activate transcription of the gene encoding the response regulator *yufM* (BC_0578, paper I), but effects were also seen on other two-component genes, such as that encoding the corresponding sensor kinase *yufL* (BC_0577) as well as the two component system genes *resE* (BC_1478) and *resD* (BC_1477), which have been shown to activate transcription of PlcR-activated toxins under low oxidoreductive conditions in a manner that is at least partially PlcR-independent (Duport *et al.*, 2006). ResD is also shown to bind to the *plcR* upstream region (Esbelin *et al.*, 2009). In this context, it is interesting to note that *plcR*-deletion leads to reduced transcription of these two genes, possibly indicating that PlcR can activate enterotoxin production also by indirect regulation and the possibility of mutual influence of PlcR and the ResDE two-component system on each other.

The PlcR data should be regarded with extra caution, as the three hour sampling point is at the break in the logarithmic growth curve, meaning that even small changes in growth between samples may potentially lead to large variations in transcription, which may wrongly be attributed to the differences under study, as observed for genes involved in arginine biosynthesis in *B. subtilis* (Comella & Grossman, 2005). At the five hour sampling point, the bacterial density is much higher in the wild type than in the plcR deletion mutant, and this may also affect gene regulation.

4.2. The roles of NprR and PlcR in the transition state regulatory network.

Cells in a culture reach the transition state when the culture medium can no longer support exponential growth, e.g. due to shortage of nutrients, accumulation of waste products or other stress. In response, the cell will activate alternative metabolic pathways

as well as new transport systems, and secrete degradative enzymes and virulence factors into the environment to acquire more nutrients and out-compete other bacteria (Bron *et al.*, 1998; Gilois *et al.*, 2007; Harwood, 1992). Depending on the environment and the severity of the food shortage, various adaptive responses may be activated, including the PlcR- and NprR regulons.

The transition state regulator PlcR is widely recognized as a major regulator of extracellular virulence in the *B. cereus* group (see section 1.7.1.), and this role is further emphasized by the PlcR regulon identified in paper I. *plcR* deletion mutants show attenutated virulence in various infection models (Callegan *et al.*, 2003; Salamitou *et al.*, 2000). However, virulence was not abolished in these model systems, indicating that other factors also contribute to virulence in the *B. cereus* group. AbrB, another important transition state regulator, has been shown to control production of the emetic toxin cereulide and anthrax toxin genes (Lucking *et al.*, 2009; Saile & Koehler, 2002).

Though not as dominated by secreted and cell wall proteins as the PlcR regulon, the NprR regulon (paper II) also includes a large number of degradative enzymes. This indicates that an important role of this regulon is to make available new nutrients through various degradative processes targeted at compounds in the environment. The various enzymes involved in chitin degradation could imply a role of NprR in entomopathogenicity, since chitin is an important component of the peritrophic membrane lining the insect gut (Terra, 2000). However, chitin is also one of the most abundant biopolymers on earth, found in fungi and present in large amounts in the soil (Shahidi & Abuzaytoun, 2005; Vaaje-Kolstad *et al.*, 2009). Virulence effects of the chitinases may therefore perhaps be more likely to be of a protective or opportunistic nature. In *B. anthracis*, NprA was found to readily digest proteins belonging to the human extracellular matrix and proteins involved in the blood coagulation cascade (Chung *et al.*, 2006; Chung *et al.*, 2008), but NprA is found to be a dominant component of the extracellular proteome under conditions favoring sporulation, i.e. in minimal media with access to oxygen (Chitlaru *et al.*, 2006; Chung *et al.*, 2006; Donovan *et al.*, 1997). These conditions are quite different from what would be expected during an infection, and makes invasive virulence less likely to be a major function of the NprR regulon. However, it is interesting to note that there appears to be a certain redundancy between the regulons of PlcR and NprR, and also the regulon of the transition state regulator AbrB, e.g. they all control a variety of immune inhibitors with similar functions (Guillemet *et al.*, 2009). Deletion of all three

immune inhibitors strongly attenuates virulence in an insect model (Guillemet *et al.*, 2009). Thus, it seems that the NprR regulon may contribute to protection of the cell as well as more general nutrient acquisition. Hopefully, functional studies will give us a better understanding of the role of NprR and its regulon in the near future.

4.3. Phylogenetic variations in the composition of the PlcR- and NprR regulons.

Both PlcR and NprR have been shown to be specific to, and widely distributed in the *B. cereus* group (Agaisse *et al.*, 1999). In paper II, a preliminary investigation of the phylogenetic distribution of the forty-two genes belonging to the suggested NprR regulon was also performed by searching for genes with orthologous neighborhoods (http://img.jgi.doe.gov) in fifteen completed genomes (paper II, Table S4), three of which were *B. anthracis* genomes. This revealed that a subset of eleven genes were practically absent from the genomes which belonged phylogenetically to clade I (Didelot *et al.*, 2009; Kolstø *et al.*, 2009; Tourasse & Kolstø, 2008, http://mlstoslo.uio.no). Thus, the NprR regulon varies between different phylogenetic groups. This, of course, also allows for the possibility that the NprR regulon in clade I strains may include other genes not detected by our experiments, and this may also be the case for the PlcR regulon.

Later, a similar search was made for the forty annotated members of the PlcR regulon (paper I) in the same fifteen genomes (Table 6) (http://img.jgi.doe.gov). These results showed that most genes in the PlcR-regulon are widely distributed in members of the *B. cereus* group. Only three genes showed a similar distribution to the subset of eleven genes found in the NprR regulon: BC_0362, BC_3527, and BC_5351 are, with one exception, absent from the investigated clade I genomes and present in nearly all clade II and III genomes (Table 6) (Kolstø *et al.*, 2009; Tourasse & Kolstø, 2008, http://mlstoslo.uio.no). In addition, *hbl* genes were found in all investigated clade II genomes, but only in a minority of the investigated clade I genomes. These results correspond well with other reports about the distribution of toxin genes in the *B. cereus* group (Ehling-Schulz *et al.*, 2005; Ehling-Schulz *et al.*, 2006b; Moravek *et al.*, 2006). Recent phylogenetic studies by Didelot *et al* partly confirmed these observations (Didelot *et al.*, 2009). They found an overweight of diarrhoeal isolates in clade II, something which was attributed to the more abundant presence of *hbl* genes in *B. thuringiensis* (Rivera *et al.*,

2000) which dominate clade II (Didelot *et al.*, 2009), while emetic strains were partly clonal, and mainly found in clade I. However, isolates from lung, wounds, or bloodstream were found to be evenly distributed between clades I and II, which emphasizes the opportunistic nature of such infections.

In summary, neither the presence or absence of specific virulence genes, nor the phylogenetic background can explain the large strain to strain variations in virulence. Part of the reason for this may lie in the opportunistic nature of many infections, but studies of supernatant effects on cell cultures clearly show variations in the pathogenic potential between strains. The effect is largely due to differences in toxin concentration, and toxin production efficiency may be more important to explain strain virulence than genotype or exact composition of the toxin cocktail (Fagerlund *et al.*, 2007; Moravek *et al.*, 2006; Rivera *et al.*, 2000). This emphasizes the importance of understanding the regulatory mechanisms which control production of virulence factors.

Table 6. Distribution of PlcR-regulated genes in fifteen *B. cereus* group genomes, as found by searching for orthologous gene neighborhoods (http://img.jgi.doe.gov.)

gene	Name/function	*Bacillus anthracis* Ames	*Bacillus Anthracis* Ames Ancestor	*Bacillus Anthracis* Sterne	*Bacillus cereus* AH187	*Bacillus cereus* AH820	*Bacillus cereus* ATCC 10987	*Bacillus cereus* ATCC 14579	*Bacillus cereus cytotoxis NVH391-398*	*Bacillus cereus* B4264	*Bacillus cereus E33L*	*Bacillus cereus* G9842	*Bacillus cereus* Q1	*Bacillus thuringiensis* Al Hakam	*Bacillus thuringiensis* konkukian 97-27	*Bacillus weihenstephanensis* KBAB4
BC_0362	unknown							x		x		x			x	x
BC_0556	*colC*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
BC_0576	*mcpA*	x	x	x		x		x	x	x	x	x	x	x	x	x
BC_0577	*yufL*	x	x	x		x	x	x	x	x	x	x	x	x	x	x
BC_0578	*yufM*	x	x	x		x	x	x	x	x	x	x	x	x	x	x
BC_0666	*inhA2*	x	x	x	x	x	x	x		x	x	x	x	x	x	x
BC_0670	*plcB*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
BC_0671	*smase*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
BC_0991	*slpA*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
BC_1081	*prp2*	x	x	x				x	x	x		x				x
BC_1082	regulator?	x	x	x		x		x		x	x	x				x
BC_1110	*cytK*					x	x	x	x	x	x				x	
BC_1713	unknown	x	x	x	x	x	x	x	x	x	x	x	x		x	x
BC_1809	*nheA*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
BC_1810	*nheB*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
BC_1811	*nheC*			x	x	x	x	x	x	x	x	x	x	x	x	x
BC_2410	*tetR*	x	x	x	x	x	x	x		x	x		x	x	x	x
BC_2411	Drug efflux	x	x	x	x	x	x	x		x	x		x	x	x	x
BC_2552	unknown	x	x	x	x	x		x		x	x	x	x		x	x
BC_2735	*nprP2*	x	x	x	x	x		x		x	x	x	x	x	x	x
BC_3102	*hblB*					x		x		x		x		x	x	x
BC_3103	*hblL1*					x		x		x		x		x	x	x
BC_3104	*hblL2*					x		x		x		x		x	x	x
BC_3161	*colA*	x	x	x				x			x					
BC_3383	*nprC*	x	x	x		x		x		x	x				x	
BC_3384	*mpbE*	x	x	x		x		x		x	x				x	
BC_3385	*tlpA*	x	x	x		x		x	x	x	x				x	
BC_3527	unknown							x		x		x				
BC_3746	lipoprotein	x	x	x	x*	x	x	x	x	x	x	x		x	x	x
BC_3747	sensory box	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
BC_3761	*plcA*	x	x	x	x	x	x	x		x	x	x	x	x	x	x
BC_3762	*sfp*	x	x	x		x	x	x	x	x	x	x	x*	x	x	x
BC_4509	permease	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
BC_4510	ATP-binding	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
BC_4511	*lppC*	x	x	x	x	x	x	x		x	x	x	x	x	x	x
BC_4999	protease	x	x	x	x	x	x	x		x	x		x	x	x	x
BC_5101	*clo*	x	x	x	x	x	x	x		x	x	x	x	x	x	x
BC_5349	*papR*	x	x	x	x	x	x	x	x	x	x	x	x		x	x
BC_5350	*plcR*				x	x	x	x	x	x	x	x	x	x	x	x
BC_5351	*nprB*							x		x		x				x

* The gene is located on a plasmid.

4.4. Differences in oxygen availability during swarming affect gene transcription.

The effects of swarming on the transcriptional activity in *B. cereus* ATCC 14579 was studied in paper IV. In the *B. cereus* group, swarm-differentiated cells are normally found only in the outer rim of a swarming colony (Ghelardi *et al.*, 2002; Salvetti *et al.*, 2009; Senesi *et al.*, 2002). Thus, when we harvest a swarming colony, a greater proportion of the cells are not in a swarm-differentiated state, which will reduce the transcriptional differences observed. However, strong trends should still give a consistent, albeit somewhat muted, response. Given that synthesis and operation of flagella is quite energy-intensive (McCarter, 2006), and that swarming is also hypothesized to require much energy (Eberl *et al.*, 1996; Eberl *et al.*, 1999), it was therefore surprising that many of the genes found to be most strongly downregulated during swarming, were involved in energy production and conversion (COG category C). A closer look revealed, however, that the downregulated genes are involved in fermentation and oxidative respiration, while there appears to be a weak upregulation of some genes associated with the TCA-cycle. Most likely, these changes are not directly brought about by swarming, but result from a difference in oxygen availability under swarming and non-swarming conditions. As the swarming colony grows in a thin layer covering a large area, it is reasonable to assume that oxygen availability will be much better than in a regular colony, where the conditions may turn anaerobic for the lower layers. Thus, when we extract RNA from the entire population, differential expression due to differences in oxygen availability can be expected. In fact, of the twenty-one genes more than threefold downregulated during swarming in our study (Table 7), thirteen genes were also found to be significantly affected in a study comparing anaerobic to aerobic growth in *B. cereus* ATCC 14579 (van der Voort & Abee, 2009). Of these thirteen, eleven genes were upregulated during anaerobic growth (Table 7, column 5), and it is therefore likely that the observed downregulation during swarming is, at least partly, due to lower oxygen availability for the non-swarming colony, resulting in an upregulation of these genes in the non-swarming control RNA. Among the thirteen genes were the arginine deiminase operon *arcABDC* (BC_0406-BC_0409), which is reported to be activated by anaerobic growth conditions (Maghnouj *et al.*, 2000; van der Voort & Abee, 2009), and its regulator (BC_0410) as well as genes involved in various forms of fermentation (BC_0491(-BC_0492), BC_2220), oxidative phosphorylation (BC_4792), and anaerobic respiration (BC_2134, BC_2128).

Under anaerobic conditions, the L-lactate dehydrogenase BC_4996, which is involved in fermentation, was found to be upregulated. This gene was not significantly affected during swarming, but another L-lactate dehydrogenase, BC_4870, was strongly downregulated. Relaxing the search criteria to include genes with smaller downregulation and higher p-values than those in the Table 7, revealed even more genes which showed the same pattern of upregulation under anaerobic growth conditions (van der Voort & Abee, 2009) and downregulation during swarming (data not included in Table 7). Differences in oxygen availability did not seem to have a similar impact on the genes found to be strongly upregulated. Among the twenty-five genes which were threefold or more upregulated during swarming (data not shown), only one gene, BC_5123, encoding a hypothetical protein, was affected by anaerobic conditions in the study by van der Voort and Abee (2009).

Of the ten genes in table 7 not found to be upregulated under anaerobic conditions (van der Voort & Abee, 2009), eight code for hypothetical proteins. The genes BC_5026-BC_5027 are particularly interesting as they are more than fivefold downregulated during swarming. These genes are also negatively affected by anaerobic growth according to van der Voort and Abee (2009), possibly indicating that their true downregulation during swarming could be even more extreme. Further studies of their effects on swarming could be potentially interesting.

Table 7. Genes more than threefold downregulated during swarming (p < 0.05).

Locus tag	Name	Fold change non-swarming / swarming	p-value	Significant effect of anaerobic conditions*
BC_0248	hypothetical protein	4,4	0,02	
BC_0250	hypothetical protein	4,2	0,02	
BC_0406	arginine deiminase	3,9	0,02	up
BC_0407	ornithine carbamoyltransferase	10,4	0,02	up
BC_0408	Arginine/ornithine antiporter	7,1	0,02	up
BC_0409	carbamate kinase	3,4	0,03	up
BC_0410	Transcription regulator. Crp family	4,0	0,02	up
BC_0491	formate acetyltransferase	5,0	0,04	up
BC_0492	pyruvate formate-lyase-activating enzyme	6,5	0,03	up
BC_0564	lipoprotein. putative	4,6	0,03	
BC_1000	hypothetical protein	3,7	0,02	
BC_2128	nitrite extrusion protein	3,1	0,03	up
BC_2134	uroporphyrin-III C-methyltransferase	3,5	0,02	up
BC_2220	alcohol dehydrogenase	3,5	0,02	up
BC_3526	Collagen adhesion protein	3,8	0,04	
BC_3766	hypothetical protein	3,4	0,02	
BC_4751	hypothetical protein	5,6	0,03	
BC_4792	cytochrome d ubiquinol oxidase. subunit I	4,4	0,03	up
BC_4870	L-lactate dehydrogenase	3,0	0,04	
BC_5026	hypothetical protein	5,7	0,03	down
BC_5027	hypothetical protein	5,8	0,04	down

* According to the study by van der Voort and Abee (2009).

4.5. Motility and Virulence.

A coupling of virulence to motility in general and swarming in particular is reported for many bacteria (Ghelardi *et al.*, 2002; Ghelardi *et al.*, 2007; Grant *et al.*, 1993; Gueriri *et al.*, 2008; Overhage *et al.*, 2008; Verstraeten *et al.*, 2008). This link is stronger than just the invasive advantage which can be expected from a motile compared to a non-motile bacterium: In several examples, genes involved in motility and virulence are coregulated or influence each other's regulation (Bouillaut *et al.*, 2005; Clernmer & Rather, 2008; Overhage *et al.*, 2008). In the *B. cereus* group, motility was shown to be reduced in a *B. thuringiensis* 407 Cry$^-$ strain with an insertional deletion in the virulence regulator PlcR (Callegan *et al.*, 2003). This is consistent with observations made during microarray comparisons of the the Δ*plcR* mutant with wildtype *B. cereus* ATCC 14579

(paper I), where motility genes (BC_1634-BC_1671) were downregulated in the Δ*plcR* mutant compared to the wild type at T2. Effects of motility on enterotoxin production and secretion has also been reported: An insertional deletion of the *flhA* gene, encoding a protein in the flagellar export apparatus, which rendered the mutant deficient in flagella formation, also affected secretion of Hbl, production of Hbl, Nhe and CytK, and showed attenuated virulence in an insect model system and an endophthalmitis model (Arnesen *et al.*, 2008; Bouillaut *et al.*, 2005; Callegan *et al.*, 2005; Ghelardi *et al.*, 2002). Hbl secretion has been reported to increase during swarming, and secretion through the flagellar apparatus has been suggested (Ghelardi *et al.*, 2007), although this is controversial. Other studies conclude that coordination of the flagellar apparatus with several PlcR regulated genes is likely to be controlled at the transcriptional level (Bouillaut *et al.*, 2005; Fagerlund, 2008). On this background, we wanted to investigate the potential link between swarming and PlcR-regulated genes. Effects of swarming on antimicrobial resistance will also be discussed.

4.5.1. Swarming negatively affects expression of most PlcR-regulated genes.

If there is an influence of swarming on virulence or *vice versa*, one could perhaps expect some overlap between the genes affected by swarming (paper IV) and by the virulence regulator PlcR (paper I). Of the 290 genes which were found to be significantly affected during swarming, around half (160) were also affected by the PlcR deletion at T0 and/or T2, but there were no clear trends that genes were affected in the same way by swarming and by PlcR-activation. Rather, for somewhat more than half the genes (51 of the 79 genes affected at T0, and 70 of the 122 genes affected at T3), the two factors appeared to have opposite effects.

When the genes of the PlcR-regulon (paper I) were extracted from the swarming microarray data and analyzed, seventeen were found to be affected ($p < 0.1$) during swarming (Table 8). Twelve of these genes were downregulated during swarming, including the gene encoding the PlcR regulator itself. As there are several reports in the *B. cereus* group of upregulation of virulence genes under oxygen-limited conditions (Duport *et al.*, 2004; Duport *et al.*, 2006; Klichko *et al.*, 2003; Rosenfeld *et al.*, 2005; Zigha *et al.*, 2007), the relative downregulation of *plcR* and PlcR-regulated genes during swarming may, at least partly, be related to differences in oxygen availability (see section 4.4). Therefore, it is interesting to note that three genes encoding Hbl components (BC_3102-

BC_3104), as well as a serine protease (BC_3762) and a methyl-accepting chemotaxis protein (BC_0576) are upregulated. This confirms earlier observations of increased Hbl production during swarming (Ghelardi *et al.*, 2002; Ghelardi *et al.*, 2007), and indicates that activation of these genes during swarming is PlcR-independent and must be caused by other regulatory mechanisms.

Upregulation of *hbl* genes simultaneously with downregulation of PlcR and several members of the PlcR regulon has also been reported in a deletion mutant of *yvfTU*, which encodes a two-component system (Brillard *et al.*, 2008). In fact, of the eleven PlcR-regulated genes found to be affected by the *yvfTU* mutant, ten are among the seventeen genes found to be affected during swarming, and the change in expression (up- or down) has the same direction as observed during swarming (Table 8). Thus swarming and *yvfTU* deletion seem to have similar effects on PlcR-regulated genes. The downregulation of PlcR and parts of its regulon, does not necessarily signify a reduction in virulence during swarming, but strongly indicates that virulence during swarming is caused by PlcR-independent mechanisms (paper IV).

Table 8. PlcR-regulated genes affected by swarming (p < 0.1).

gene	name	fold change Δ*plcR*/wt	p-value	Δ*yvfTU*-effect
BC_0556	microbial collagenase.putative	0,6	0,05	
BC_0576	methyl-accepting chemotaxis protein	1,5	0,06	
BC_0670	phospholipase C	0,7	0,07	down
BC_0671	sphingomyelinase C	0,6	0,08	down
BC_0991	S-layer protein. putative	0,7	0,08	
BC_1809	enterotoxin	0,6	0,05	down
BC_1810	enterotoxin	0,6	0,04	down
BC_1811	Non-expressed Enterotoxin C	0,6	0,02	
BC_2735	neutral protease	0,6	0,03	
BC_3102	Hemolysin BL binding component precursor	1,5	0,04	up
BC_3103	Hemolysin BL lytic component L1	1,4	0,07	up
BC_3104	Hemolysin BL lytic component L2	1,6	0,02	up
BC_3761	1-phosphatidylinositol phosphodiesterase	0,7	0,08	
BC_3762	serine protease. subtilase family	1,5	0,03	
BC_5101	thiol-activated cytolysin	0,6	0,04	down
BC_5350	Transcriptional activator plcR	0,7	0,05	down
BC_5351	Bacillolysin	0,6	0,02	down

4.5.2. Swarming may lead to increased antimicrobial resistance.

Antimicrobial resistance is another aspect of virulence, and swarming motility has been found to induce antimicrobial resistance in a variety of species (Lai *et al.*, 2009). In paper IV, the swarming colony showed an increased resistance towards daptomycin, and this was potentially attributed to the strong upregulation (six-fold) of BC_1435 and BC1436. These genes showed some similarity to the *liaI* and *liaH* genes (Jordan *et al.*, 2006). Deletion of *liaH* has been shown to increase daptomycin susceptibility (Hachmann *et al.*, 2009). However, LiaH belongs to the phage shock protein family, and could theoretically be activated by phage activity, consistent with the massive upregulation of transcription of genes belonging to the prophage phBC6A51. It would be interesting to investigate further the potential role of LiaH in the increase in antimicrobial resistance during swarming, and whether the response was specific to daptomycin, or the result of a more general increase in antimicrobial resistance caused by the swarm-cell differentiation. Due to their highly increased transcription, it would also be relevant to investigate if *liaH* and/or *liaI* could have a more direct role in the swarming process.

4.6. Filamentous growth – accident or adaptive response.

In paper III, we investigated similarities and differences between *B. cereus* ATCC 14579 variants which grow as filaments in the exponential phase (14579-L1 and 14579-L2) and a variant which does not display such growth (14579-S1). In spite of many interesting observations, a cause (or causes) for the filament formation could not be determined. It thus remains an open question whether the observed filament formation is part of the natural diversity and adaption of the cells, or a sign of suboptimal changes at the genetic level. In this chapter, I will review some known cases of filament formation as a natural trait or adaptive respsonse, and discuss whether this is applicable to our observations. I will also examine some genetic changes which may result in filament formation in *B. cereus* group species and closely related bacteria, and comment on their relevance to this case. Finally, I will discuss whether the phenotypes observed for the various variants may be causally related or are more likely to result from common, underlying mechanisms.

4.6.1. Filamentous growth as a natural trait or adaptive response.

Throughout the work with filamentous and planktonic morphotypes of *B. cereus* ATCC 14579, the planktonic form has been used as reference or wild type. However, it is interesting to note that the original Frankland and Frankland article (1887) describes *B. cereus* (the ancestor to what would become *B. cereus* ATCC 14579) as having a very variable colony morphology. Rough-edged colonies, similar to those made by the filamentous forms studied in paper III, appeared to be as common as the smooth, well-rounded colonies, indicating that filament formation may have been every bit as natural as planktonic growth. Their description of various colony types arising from a single CFU (colony forming unit) indicates that morphological revertants can be expected.

Studies also show that particular growth conditions can promote filamentous growth. *B. cereus* growing symbiotically in insect guts, was reported to grow filamentously with one end attached to the intestinal wall (Margulis *et al.*, 1998). In culture, sporulation and the degree of filament formation of the intestinal bacteria were influenced by environmental and chemical signals. Addition of insect intestinal content to the culture reduced sporulation and induced the formation of long filaments. It has also been shown that *B. cereus* ATCC 14579 and various soil isolates prefer the filamentous form when growing in liquid soil extract or in soil microcosms, and it is hypothesized that the alternative growth form may be induced by the presence of Ca^{2+} ions or other components of soil substances, while LB medium promotes planktonic growth (Vilain *et al.*, 2006). In our study, filamentous growth in the exponential phase of 14579-L1 and –L2 was observed in several different growth media, including LB, while 14579-S1 was consistently planktonic under the same conditions, and the variants could not be induced to switch phenotypes by changing the liquid growth medium.

Unlike swarm-differentiated cells, filamentous bacteria are often non-motile or have reduced flagellar motility (Hsueh *et al.*, 2007; Kearns & Losick, 2005; Monk *et al.*, 2004; Msadek *et al.*, 1998). It has therefore been hypothesized that filamentous growth represents an alternative form of motility by enabling the bacteria to move by the forces inherent to cell elongation (Vilain *et al.*, 2006). This would be a useful trait under saprophytic growth conditions, and in an environment which does not support swarming or swimming. This type of motility may be comparable to bacterial sliding, described by Henrichsen and observed in *B. anthracis* (Henrichsen, 1972). A similar type of motility was observed in the *plcR* deletion mutant under low-nutrient conditions (Hsueh *et al.*,

2007). The *plcR* deletion mutant exhibited increased production of biosurfactant compared to the wild type strain. However, it is unclear whether biosurfactant production and filament formation are closely linked, and whether PlcR has a more generally suppressive effect on sliding motility.

In conclusion, there is convincing evidence that environmental factors may induce filamentous growth in strains which usually display planktonic growth. In our study, however, filament formation during exponential growth was not affected by change of growth media, and did not appear to be an adaptive response, but a consistent phenotype of the affected variants.

4.6.2. Filamentous growth as a result of changes in genes associated with cell wall metabolism.

Any mutation which leads to a septated, but filamentous phenotype, must in some way affect the process of cell wall growth or cell separation. In *B. subtilis clpP* and *clpC* deletion mutants, reduced degradation of MurAA is believed to be a major cause for filamentous growth during the exponential phase (Kock *et al.*, 2004). MurAA catalyzes the first committed step in peptidoglycan biosynthesis and was found to be a substrate for the ClpCP complex (Kock *et al.*, 2004). Correspondingly, overproduction of MurAA in *B. subtilis* leads to a twisted, filamentous phenotype (Kock *et al.*, 2004). In our studies, however, transcriptional effects on the *murAA* ortholog BC5288 were not seen in microarray analysis, and *clpC* and *clpP1* did in fact show increased transcription as well as increased protein levels in 14579-L1 (Table 4). Posttranslational effects on protein stability or cell wall production have not been investigated, but no accumulation of cell wall material was observed in the AFM images.

There are also several examples of mutations in autolysins involved in cell separation which result in a filamentous phenotype (Fukushima *et al.*, 2006; Ohnishi *et al.*, 1999; Vollmer *et al.*, 2008). However, in our study, neither microarray results, nor RT-qPCR showed any transcriptional differences with respect to autolysins. All autolysins appeared to be transcribed, but mutations can not be ruled out. The investigation was complicated by the fact that it is not known which hydrolases control cell separation in the *B. cereus* group. LytE, LytF, and CwlS, the endopeptidases responsible for cell separation in *B. subtilis* (Fukushima *et al.*, 2006; Ishikawa *et al.*, 1998; Ohnishi *et al.*, 1999), do not appear to have close orthologs in the *B. cereus* group, and proteins involved in their

regulation in *B. subtilis*, such as σ^D and IseA (Ohnishi *et al.*, 1999; Yamamoto *et al.*, 2008), are not found in *B. cereus* group organisms. Thus, the process of cell separation is likely to be differentially regulated. Members of the *B. cereus* group also differ from *B. subtilis* in that their cell wall is covered with teichuronic acids instead of the teichoic acids coating *B. subtilis* and many other bacteria (Molnar & Pragai, 1971), and this further increases the likelihood of divergent mechanisms of cell separation.

The two-component system WalKR (formerly YycGF), has been found to control cell wall metabolism in *B. subtilis* and many other Gram-positive bacteria (Bisicchia *et al.*, 2007; Dubrac *et al.*, 2008), and may be likely to do so also in the *B. cereus* group. The walR and walK genes (BC_5462-BC5463) were not affected at the transcriptional level in our microarray data. Searching the *B. cereus* ATCC 14579 genome with the WalR consensus binding sequence used for *B. subtilis/Staphylococcus aureus* (Dubrac *et al.*, 2008) in Genome2D (Baerends *et al.*, 2004) revealed sixty-nine potential binding sites, but none were upstream of genes found to be differentially regulated between 14579-L1 and 14579-S1 (data not shown). It is therefore unlikely that the observed phenotype in 14579-L1 is due to differential transcriptional regulation by WalR.

Autolysin function may also be shut down by indirect effects mediated at the post-transcriptional level. Examples of such effects are non-functional export systems or changes which affect attachment of the autolysin to the cell wall. In *B. anthracis,* several autolysins showed reduced activity in a deletion mutant lacking the *csaB* gene responsible for pyruvylation of cell wall associated polysaccharides (Mesnage *et al.*, 2000). It was shown that the pyruvylation was necessary for attachment to the cell wall of proteins carrying an SLH binding domain. This indicates that some autolysins involved in cell separation in *B. anthracis* attach to the cell wall by SLH domains. *B. cereus* ATCC 14579 also has several autolysins with an SLH domain and contains the *csaB* gene, even though this was not found with the primers used by Mesnage *et al* (Annette Fagerlund, personal communication). However, unlike Mesnage *et al*, we found no differences between the filamentous and short variants when comparing 14579-S1, 14579-L1, and 14579-L2 by zymogram analysis (results not shown).

There are of course even more mutations which can lead to aberrant cell morphology with and without co-occurrence of other phenotypes. Though we have tried to investigate their relevance to our study, to examine them all is beyond the scope of this discussion. The aim of this section has rather been to draw the attention of the reader

towards various cellular systems where defects may lead to filament formation, and also to point out some of the differences between the *B. cereus* group and the closely related *B. subtilis*. This does not provide us with an answer to the cause of the filamentous phenotypes, but points out some areas which will be relevant for future studies.

4.6.3. Filament formation, sporulation, and stress – is there a connection?

The filamentous variants 14579-L1 and 14579-L2 shared several phenotypic traits related to cell growth and loss of lytic properties. At the same time, they also showed distinct differences, as 14579-L1 additionally showed increased transcription and expression of class I and III heat shock proteins and a sporulation efficiency which was only one tenth of that observed for 14579-L2 and 14579-S1. Table 9 shows similarities and differences between the three variants with respect to the investigated traits.

Table 9. Phenotypic similarities and differences between the variants 14579-S1, 14579-L1, and 14579-L2

phenotypic trait	14579-S1	14579-L1	14579-L2
filamentous growth in exponential phase	–	+	+
Elongation of individual cells	–	+	+
Rough colony morphology	–	+	+
Cell lysis after death or sporulation	+	–	–
Sporulation efficiency (%)	35 ± 13	5 ± 3	56 ± 25
increased transcription of class I and III stress genes relative to 14579-S1 (RT-qPCR)	–	+	–
increased expression of class I and III stress proteins relative to 14579-S1 (ICPL MS)	–	+	NA

As our studies indicate that genetic differences are a likely cause for the observed phenotypes, there are several possible reasons for the partially overlapping phenotypes of the two filamentous variants:

1. The two variants have the same mutation, but14579-L1 carries an extra mutation which results in additional phenotypes.
2. The two variants have the same mutation, but 14579-L2 has developed compensatory mutations which reverse some of the original phenotypes.

3. The two variants have different mutations which affect different parts of the same regulatory network.

The true cause remains unknown at this point, but some insights may be derived from a discussion of whether the additional phenotypes of 14579-L1 are likely to be related to filamentous growth. Filamentous growth is frequently induced by various types of stress both in *Bacillus* species and other bacteria, but cells will often return to planktonic growth when the stress is removed (den Besten *et al.*, 2009; Giotis *et al.*, 2007; Mattick *et al.*, 2000; Mattick *et al.*, 2003). In our study, filamentous growth occurred without external stress, but an upregulation of class I and III heat shock proteins was still observed at both the transcriptional and protein level in 14579-L1.

Class I and III heat shock proteins have chaperone and/or proteolytic activity and are involved in protein maintenance. Many proteins require the assistance of chaperones to fold properly even under physiological conditions, and the number of proteins found to be associated with chaperones rises under stressful conditions (Wickner *et al.*, 1999). Traditionally, refolding by chaperones and degradation by proteases have been regarded as two different fates which may befall an improperly folded protein. However, these two paths are more intertwined than originally recognized, and Clp ATPases not only act as proteolytic subunits, but also have protein unfolding and chaperone activity (Frees *et al.*, 2007; Striebel *et al.*, 2009; Zolkiewski, 2006). (Gottesman *et al.*, 1997; Wickner *et al.*, 1999). A model has been proposed where both chaperones and proteases are part of a posttranslational quality control system based on kinetic principles, the purpose of which is to prevent protein aggregation through assisted folding/refolding or degradation of nonnative proteins (Gottesman *et al.*, 1997; Wickner *et al.*, 1999).

All the genes which showed increased transcription and translation in 14579-L1 (paper III, Table 4), belong to this proposed system of quality control. It is therefore possible that their increase reflects an increased need for protein maintenance, perhaps as a result of stress or reduced function in other genes involved in protein quality control. Such stress could possibly also induce filament formation during exponential growth. However, no upregulation of other stress proteins, such as σ^B or genes belonging to the σ^B regulon (Class II heat shock proteins) (van Schaik *et al.*, 2007) was observed, even though σ^B is reported to be activated by various types of stress in *B. cereus* ATCC 14579 (van Schaik *et al.*, 2004). Still, if there is a connection, it seems more likely that the filamentous

phenotype and the upregulation of class I and III heat shock proteins have a common, underlying cause, than that one is caused by the other

It is not known whether increased expression of class I and III heat shock proteins by itself could reduce sporulation efficiency. Studies in *B. subtilis* indicate that upregulation of the *hrcA* and *ctsR* regulons by deletion of their respective repressors does not have negative effects on wet heat resistance of spores, and no sporulation deficiency is mentioned (Melly & Setlow, 2001). There are also other studies which suggest that neither overproduction, nor deletion of members of the class I heat shock proteins affect sporulation in various bacteria (Grandvalet *et al.*, 1998; Homuth *et al.*, 1997). For the Clp proteins which belong to the CtsR regulon, deletion is associated with sporulation deficiency in both *B. subtilis* and *B. thuringiensis* (Fedhila *et al.*, 2002a; Msadek *et al.*, 1994; Msadek *et al.*, 1998). In the *B. subtilis clpP* deletion mutant, the effect on sporulation efficiency is mediated through Spo0E, which dephosphorylates Spo0A~P (Nanamiya *et al.*, 2000). If this is also the case in the *B. cereus* group, up-regulation of ClpP would seem more likely to accelerate sporulation than decrease it. However, the sporulation process is complex, and the proper timing of activation events has been shown to be important (Fujita & Losick, 2005), therefore the exact effect of such up-regulation is difficult to predict. ClpC is suggested to play a role in degradation of σ^H in *B. subtilis*. σ^H is important for the activation of several genes involved in sporulation, and it is posible that elevated levels of ClpC could inactivate σ^H and in this way prevent the inititiation of sporulation. In any case, further studies will be needed to determine the cause or causes behind the partially overlapping phenotypes of 14579-L1 and 14579-L2.

5. Conclusions and future perspectives.

The establishment of the PlcR regulon for *B. cereus* ATCC 14579 in paper I in many ways concluded several years of research on the effects of this transition state regulator by various methods (Agaisse *et al.*, 1999; Fedhila *et al.*, 2003; Gohar *et al.*, 2002; Salamitou *et al.*, 2000). The results corresponded well with was previously known, and confirmed the role of PlcR as a major regulator of extracellular virulence factors. At the same time, they also provided new information, particularly about intracellular proteins whose transcription is activated by PlcR, and the ability of PlcR to integrate a variety of environmental inputs and modulate its response through the control of other proteins with sensory or regulatory potential

In paper II, we showed that the transition state regulator NprR controls a large regulon of genes encoding extra- and intracellular proteins. This uncovered a new level of post-exponential regulation in the *Bacillus cereus* group. Further studies of NprR and its regulon will provide a better understanding of the functional role of NprR in the transition state regulatory network. Also, determination of the NprR binding site will make it easier to distinguish between direct and indirect effects of NprR activity.

The transiently filamentous variants of *B. cereus* ATCC 14579 studied in paper III, had partially overlapping phenotypes, but the 14579-L1 variant was found to carry additional phenotypic traits setting it apart from the other two variants studied. The stability of the phenotypes in both variants indicated that their cause is genetic, but we were not able to determine any affected loci. Furthermore, the phenotypic differences between the filamentous variants may indicate that they are affected in different loci, or that one variant carries additional mutations. In order to find out more about the nature of the genetic differences, sequencing of the different variants is probably a necessary and natural next step.

Paper IV demonstrated the effects of swarming on the transcriptome of *B. cereus* ATCC 14579. Many of the observed changes resulted from differences in oxygen availability between swarming and non-swarming colonies, but the study also uncovered genes strongly affected by swarming independently of oxygen availability. These genes may have a potentially important role in swarming, and are candidate targets for knock-out mutations or overexpression in follow-up studies.

Over several years, large amounts of microarray data have accumulated in our lab, and most of it has been poorly exploited. It would be highly interesting to reanalyze the data in a way that would make them suitable for downstream analysis (this would require that weak and uncertain genes are weighted down rather than excluded where possible, as most types of downstream analysis can't handle missing data). The best slides could then be used for meta-analysis with pattern-finding techniques and clustering methods such as K-means clustering and PCA (principal component analysis) clustering. Hopefully, this would make it possible to find genes which show co-varying or opposite expression profiles under various conditions. Such information could increase our understanding of the regulatory networks of *B. cereus* and might lead to new and interesting research projects.

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I

The PlcR Virulence Regulon of *Bacillus cereus*

Michel Gohar[1,2]*, Karoline Faegri[3], Stéphane Perchat[1], Solveig Ravnum[3], Ole Andreas Økstad[3], Myriam Gominet[4], Anne-Brit Kolstø[3], Didier Lereclus[1]

1 INRA, Génétique microbienne et Environnement, Guyancourt, France, **2** INRA, Microbiologie et Génétique Moléculaire, Thiverval-Grignon, France, **3** Department of Pharmaceutical Biosciences, University of Oslo, Oslo, Norway, **4** Institut Pasteur, Paris, France

Abstract

PlcR is a *Bacillus cereus* transcriptional regulator, which activates gene expression by binding to a nucleotidic sequence called the 'PlcR box'. To build a list of all genes included in the PlcR regulon, a consensus sequence was identified by directed mutagenesis. The reference strain ATCC14579 sequenced genome was searched for occurrences of this consensus sequence to produce a virtual regulon. PlcR control of these genes was confirmed by comparing gene expression in the reference strain and its isogenic Δ-*plcR* strain using DNA microarrays, *lacZ* fusions and proteomics methods. The resulting list included 45 genes controlled by 28 PlcR boxes. Forty of the PlcR controlled proteins were exported, of which 22 were secreted in the extracellular medium and 18 were bound or attached to cell wall structures (membrane or peptidoglycan layer). The functions of these proteins were related to food supply (phospholipases, proteases, toxins), cell protection (bacteriocins, toxins, transporters, cell wall biogenesis) and environment-sensing (two-component sensors, chemotaxis proteins, GGDEF family regulators). Four genes coded for cytoplasmic regulators. The PlcR regulon appears to integrate a large range of environmental signals, including food deprivation and self cell-density, and regulate the transcription of genes designed to overcome obstacles that hinder *B. cereus* growth within the host: food supply, host barriers, host immune defenses, and competition with other bacterial species. PlcR appears to be a key component in the efficient adaptation of *B. cereus* to its host environment.

Citation: Gohar M, Faegri K, Perchat S, Ravnum S, Økstad OA, et al. (2008) The PlcR Virulence Regulon of *Bacillus cereus*. PLoS ONE 3(7): e2793. doi:10.1371/journal.pone.0002793

Editor: Ching-Hong Yang, University of Wisconsin-Milwaukee, United States of America

Received April 9, 2008; **Accepted** June 23, 2008; **Published** July 30, 2008

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Funding: This work was carried out with the financial support of the « ANR- Agence Nationale de la Recherche - The French National Research Agency » under the « Programme National de Recherche en Alimentation et nutrition humaine », project « ANR-05-PNRA-013, B.CEREUS ».

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: michel.gohar@ jouy.inra.fr

Introduction

In pathogenic bacteria, the production of virulence factors is often coordinately regulated in response to changes in the bacterial cell environment, with various types of regulatory processes being employed. In Gram-positive bacteria, these processes may involve two-component systems [1,2], alternative sigma factors [3] or stand-alone transcription regulators [4]. In some cases, the three regulatory mechanisms act together, each controlling a part in the production of virulence factors. This situation is found for instance in the nosocomial infection agent *Staphylococcus aureus*, in which more than 40 cell-surface or secreted proteins involved in bacterial virulence are controlled by a complex network involving the transcriptional regulator SarA, the two component regulator Agr and the general stress response regulator SigB [5]. In some species, a master regulator controls most of the virulence factors, which are therefore members of the same regulon. Virulence regulons may include a large number of genes: for example, the PrfA regulon of the food-borne pathogen *Listeria monocytogenes* includes 73 genes located on the chromosome [6]. Functional analysis of genes included in virulence regulons and a precise understanding of their regulation provide means to determine how environmental signals are integrated by virulence regulators and which strategies are used by bacterial cells to survive and develop within their host environment.

In *Bacillus cereus*, the transcriptional regulator PlcR (Phospholipase C Regulator) controls most known virulence factors [7]. *B. cereus* is a sporulating low-GC Gram-positive bacterium widely distributed in the environment and genetically close to two other pathogens: the human pathogen *B. anthracis*, which is the cause of anthrax and was implicated in the killing of five people in the US in the fall of 2001, and the insect pathogen *B. thuringiensis*. *B. cereus* is a food-poisoning pathogen frequently diagnosed as the causative agent of gastroenteritis [8] but it may also cause more severe diseases such as endophthalmitis [9] or meningitis [10]. PlcR controls the expression of several enterotoxins, haemolysins, phospholipases and proteases [7,11]. PlcR has been shown to bind to DNA on a specific sequence called the 'PlcR box', located upstream from controlled genes, and at various distances ahead of the −35 box of the sigma A promoter [11,12]. The transcription of *plcR* starts shortly before the onset of the stationary phase t_0 and reaches a plateau two hours later (t_2) [13]. *plcR* transcription is autoinduced [13], and is repressed by the sporulation factor Spo0A [14]. PlcR needs PapR to be active: this peptide is expressed as a propeptide under the control of PlcR, is exported out of the cell, is processed to form the active peptide either during export or in the extracellular medium, and is captured back by the cell through the oligopeptide permease system OppABCDF [12,15,16]. Thus, the three partners PlcR, OppABCDF and PapR function as a quorum-sensing system. Therefore, PlcR integrates at least two classes of signals: cell growth state through Spo0A and self cell density through PapR [12,14].

Although several *B. cereus* genes have been demonstrated to be controlled by PlcR, no detailed study of the whole PlcR regulon has been undertaken until now. Moreover, several *B. cereus* group

genomes have now been sequenced, presenting the possibility of building a virtual PlcR regulon by searching for matches with the PlcR box consensus sequence. Using this method, a virtual regulon was in fact proposed after the sequences of *B. cereus* ATCC14579 and other strains were published [17–19]. However the presence of a PlcR box is not sufficient to classify a gene as PlcR-regulated: experimental evidence is also required. In addition, PlcR may recognize sequences diverging from the previously defined consensus sequence, as has been reported in a study of metalloprotease gene *inhA2* regulation [20]. In order to define all factors involved in the coordinated PlcR-based virulence response in *B. cereus*, we have undertaken an extensive study to map the complete PlcR regulon in the ATCC 14579 reference strain, utilizing mutagenesis experiments and *in silico* predictions in combination with proteomics and transcriptomics analyses.

Results

Directed mutagenesis defines a new PlcR box

A consensus sequence was previously determined by alignment of the promoter regions of 13 PlcR-controlled genes [11]. However, the PlcR box located upstream from *inhA2*, a gene known to be under PlcR control, diverged from this original consensus sequence by one base. Therefore, we investigated which substitution could be introduced in the consensus sequence while still maintaining significant PlcR-dependent activity. Thus, we mutated half of the palindromic nucleotide sequence of the PlcR box located upstream from *plcA*, a PlcR-regulated gene coding for a phosphatidyl-inositol specific phospholipase C (PI-PLC) and used to report PlcR activity [12]. The recognition of the mutated PlcR boxes by PlcR was investigated in the ATCC14579 strain by a transcriptional fusion between the modified promoter region of *plcA* and *lacZ*, carried on the pHT304-18Z plasmid [21]. The results, displayed in Table 1, showed that the first and the last nucleotide of the 16 bp consensus sequence can be replaced by any base without a greater than five-fold drop in expression of *plcA-lacZ*. However, deletion of A or T or their replacement by C or G in position 7–8 in the middle of the sequence, or a replacement of C by G in position 5 of the sequence, leads to a dramatic loss of activity. Similarly A_2, T_3 or G_4 could not be replaced by another base. Therefore, the PlcR target sequence identified in the mutagenesis experiment was 'ATGhAwwwwTdCAT', were h, w and d stand for, respectively: C, A or T; A or T; G, A or T. In addition to the previous consensus sequence 'TATGnAnnnnTn-CATA', this sequence was retained for the subsequent *in silico* analysis step.

Searching for PlcR boxes in the sequenced *B. cereus* genome

We searched for the two PlcR target sequences TATG-nAnnnnTnCATA and ATGhAwwwwTdCAT in the *B. cereus* ATCC14579 genome sequence. We identified a total of 69 boxes located at least 35 bp, but not more than 700 bp, upstream from a putative coding sequence. These boxes may control as many as 138 genes, as the same box could act on several genes putatively organized into an operon and/or that were divergently transcribed (see supplementary material, Table S1). Included in this list are genes that have not been annotated in the published genome sequence of the ATCC14579 strain (Ivanova *et al.*, 2003; http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi?view=1), but for which expression was confirmed by proteomic or genetic means (Bc0361a, this study; BC3763, [7]; Bc2463a, Bc3185a, Bc5101a, [22]).

Microarray analysis

We used microarrays to determine the ratio of expression between the wild type ATCC14579 strain and the isogenic Δ*plcR* strain for the 138 genes identified in the *in silico* procedure. For this determination, we chose two time points in the growth curve: the onset of the stationary phase (t_0), because PlcR expression increases sharply at this point, and two hours later (t_2), after PlcR expression reaches a plateau. Most of the genes, which on the basis of genome annotation were expected to be transcribed as part of an operon structure, displayed similar expression ratio values. Only genes with a relative expression ratio greater than 2.5 at t_0 or at t_2, and a significance value (p) smaller than 0.2, were considered for subsequent analysis. Consequently, 75 genes (Figure 1, blue box) were considered as not controlled by PlcR under standard culture conditions and were discarded. No microarray data were available for 25 genes. For most of the 38 remaining genes, transcription was enhanced by PlcR both at t_0 and at t_2 (Figure 1). Noticeably, the *clO* hemolysin, the *cytK* cytotoxin and the enterotoxins *hblC*, *hbLD*, *hblA* and *nheA*, *nheB*, *nheC*, were the genes most strongly induced by PlcR with relative expression ratios of 10 to 50 in the Δ*plcR* mutant versus wildtype cells. The expression of genes coding for other secreted proteins, including proteases (*sfp*, *nprB*, *nprC*, *mpbE*, *colA*, and *colC*) and phospholipases (*plcA*, *plcB* and *smase*) was also induced by PlcR, with a ratio of induction ranging from 3 to 30 (see supplementary Table S1). The expression of a high number of genes coding for cell-surface proteins appeared also to be induced by PlcR, although at a lower level than for secreted proteins. By contrast, only a few genes coding for cytosolic proteins, including four transcriptional regulators, had their expression significantly induced by PlcR. Data relating to *plcR*

Table 1. Effect of base mutation on the *plcA* PlcR box activity.

	T_1	A_2	T_3	G_4	C_5	A_6	A_7	T_8	A_9	T_{10}	T_{11}	T_{12}	C_{13}	A_{14}	T_{15}	A_{16}
A	**20**	**100**	**100**	**1**	*ND*	**100**	**100**	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	**100**
T	**100**	*ND*	*ND*	*ND*	*ND*	**4**	**10**	**100**	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	**90**
G	**70**	**1**	*ND*	**100**	**6**	**3**	**3**	**2**	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	**40**
C	**20**	*ND*	**0**	*ND*	**100**	**2**	**2**	**7**	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	**45**
	T	A	T	G	N	A	N	N	N	N	T	N	C	A	T	A

Each column corresponds to each position of the PlcR box located in the region upstream from *plcA* (BC3761). The unmodified sequence is given in the first line, and the subsequent next lines give the effect of a base exchange by A, T, G or C. Last line gives the original consensus sequence. The *plcA* promoter regions including the modified PlcR boxes are transcriptionally fused with *lacZ*, and each modified PlcR box activity is expressed as the percentage of beta-galactosidase activity relative to the unmodified PlcR box. *ND* means 'not determined'.
doi:10.1371/journal.pone.0002793.t001

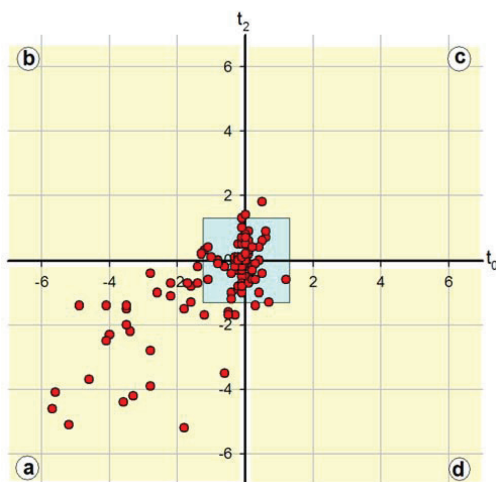

Figure 1. ΔplcR-wt expression ratios as determined by microarray experiments. Ratios of expression between the wildtype strain and the delta *plcR* strain as determined by microarrays. The log2 of these ratios were plotted at t_2 *vs* t_0. Each red circle represents the values obtained for one gene. Inside the blue square at the center of the figure are the genes for which the expression ratios were equal or less than 2. Genes for which the transcription was induced by PlcR both at t_0 and at t_2 are in the yellow square 'a', whereas genes induced only at t_2 or only at t_0 are in the yellow squares 'b' or 'd', respectively. Genes repressed by PlcR both at t_0 and at t_2 are in the yellow square 'c'.
doi:10.1371/journal.pone.0002793.g001

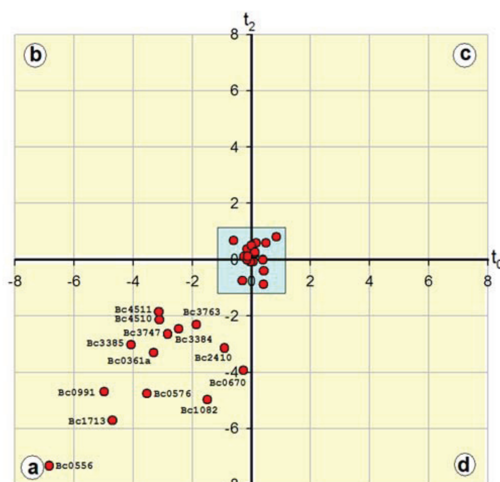

Figure 2. ΔplcR-wt expression ratios as determined by *lacZ* fusions. Ratios of expression between the wildtype strain and the delta *plcR* strain as determined by lacZ fusions. The log2 of these ratios were plotted at t_2 *vs* t_0. Each red circle represents the values obtained for one gene. Inside the blue square at the center of the figure are the genes for which the expression ratios were equal or less than 2. The transcription of all the other genes was induced by PlcR both at t_0 and at t_2 (yellow square 'a').
doi:10.1371/journal.pone.0002793.g002

and *papR* were discarded, because insertion of the Km^R cassette into the *plcR* gene introduced a promoter upstream from the regions recognized by the microarray 70-mer oligos. These genes have however previously been shown to be PlcR-regulated [11,13]. Finally, 6 genes appeared to be repressed by PlcR at a ratio of 2 to 6 in this microarray analysis, either at t_0 (BC4986) or at t_2 (BC0069, BC1736, BC3520, BC4982, BC4983).

Transcriptional fusions

Results from the microarray analysis were then crossed with data from previous proteomic or genetic analysis [7,11–13,22–24]. All genes encoding secreted proteins and identified by DNA microarray analysis were confirmed as belonging to the PlcR regulon, including BC2463a, BC3185a and BC5101a for which microarray results could not be produced, except for *colC*, for which no data from previous reports was available. Some cell surface and cytosolic proteins (*inhA2*, *prp2* and *plcR*) were also confirmed as part of the PlcR regulon. However, for 39 genes, some of which were in operon, no previous results were available regarding their control by PlcR. Therefore, we constructed 30 transcriptional fusions between the genes promoter region including the PlcR box and a *lacZ* gene, to determine if they were truly controlled by PlcR. Two genes that were not previously predicted to be in the ATCC14579 strain genome sequence, but which were located downstream from PlcR boxes, were included in the analysis: *cwh* (BC3763) and a small open reading frame located downstream from BC0361, which we named BC0361a. The ratios of β-galactosidase (encoded by *lacZ*) activity between the wild type strain and the *ΔplcR* strain was plotted at t_0 *vs* t_2 (Figure 2; the kinetics of expression obtained between t_{-1} and t_4 are shown in supplementary, Figure S1). Among the 30 promoter regions assayed by *lacZ* fusions, 14 were controlled by PlcR whereas the remaining 16 were not. Microarray results were missing or gave low ratio values for these 16 PlcR-independent genes.

Proteomic study

We have observed in a previous two-dimensional protein gel electrophoresis analysis of the *B. cereus* ATCC14579 secretome at t_2 that most of the extracellular proteins disappeared upon inactivation of *plcR* [7]. However, in the same time a large number of spots appeared on the gel obtained from the mutant strain. This suggests that PlcR is possibly a repressor for some extracellular proteins. We identified the protein content of 103 of these spots by peptide mass fingerprints and by N-terminal sequencing (see supplementary material, Figure S2 and Table S2), and only one of the proteins was encoded by a gene preceded by a PlcR box. This protein, the fructose bisphosphate aldolase FbaA, was shown by *lacZ* fusion not to be controlled by PlcR (Figure S1). Therefore no repressor role for PlcR acting on genes coding for secreted proteins was identified. The appearance of cytosolic proteins in the culture supernatant if *plcR* is inactivated was due to a greater cell lysis in the mutant strain than in wildtype: this lysis, determined by measuring isocitrate dehydrogenase activity in the bacterial cells and culture supernatant, was 1% in the wildtype strain and 15% in the mutant strain at t_2.

Final list of the PlcR-controlled genes

We built a list of PlcR-controlled genes using the data generated. We added three genes coding for antibacterial peptides to this list, which were previously shown to be controlled by PlcR: *sppc1*, *sppc2* and *sppc3* [22]. The final list included 45 genes controlled by 28 PlcR boxes, as the same PlcR box may control several genes (Table 2 and Figure S4). Genes coding for secreted

Table 2. List of the PlcR-controlled genes in the ATCC14579 strain.

Gene n°	Gene ID	Name	Function	Localisation
Bc1809	30019951	*nheA*	Enterotoxin	Extracellular
Bc1810	30019952	*nheB*	Enterotoxin	Extracellular
Bc1811	30019953	*nheC*	Enterotoxin	Extracellular
Bc3102	30021214	*hblB*	Enterotoxin	Extracellular
Bc3103	30021215	*hblL1*	Enterotoxin	Extracellular
Bc3104	30021216	*hblL2*	Enterotoxin	Extracellular
Bc5101	30023138	*clo*	Hemolysin I, cereolysin	Extracellular
Bc1110	30019265	*cytK*	Hemolysin, cytotoxin	Extracellular
Bc3761	30021854	*plcA*	Phospholipase (phosphatidyl inositol)	Extracellular
Bc0670	30018852	*plcB*	Phospholipase (phosphatidyl choline)	Extracellular
Bc0671	30018853	*smase*	Phospholipase (sphingomyelin)	Extracellular
Bc2735	30020906	*nprP2*	Neutral protease	Extracellular
Bc3383	30021487	*nprC*	Neutral protease	Extracellular
Bc5351	30023381	*nprB*	Neutral protease	Extracellular
Bc0556	30018742	*colC*	Protease, collagenase	Extracellular
Bc3161	30021271	*colA*	Protease, collagenase	Extracellular
Bc3384	30021488	*mpbE*	Protease, Enhancin	Extracellular
Bc3762	30021855	*sfp*	Protease, subtilase family protease	Extracellular
Bc5101a	NA	*sppc1*	Peptide with anti-bacterial activity	Extracellular
Bc2463a	NA	*sppc2*	Peptide with anti-bacterial activity	Extracellular
Bc3185a	NA	*sppc3*	Peptide with anti-bacterial activity	Extracellular
Bc5349	30023379	*papR*	Peptide, signaling molecule	Extracellular
Bc0576	30018762	*mcpA*	Methyl-accepting chemotaxis transducer protein	Cell wall
Bc3385	30021489	*tlpA*	Methyl-accepting chemotaxis transducer protein	Cell wall
Bc0577	30018763	*yufL*	Two-component system sensor	Cell wall
Bc3747	30021841		sensory box / GGDEF family protein	Cell wall
Bc4509	30022587		ABC transporter, permease subunit	Cell wall
Bc4510	30022588		ABC transporter, ATP-binding protein	Cell wall
Bc2411	30020542		Drug efflux protein	Cell wall
Bc3763	NA	*cwh*	Cell wall hydrolase	Cell wall
Bc0991	30019146	*slpA*	S-layer protein A, autolysin	Cell wall
Bc3746	30021840		Predicted hydrolase or acyl transferase. Lipoprotein?	Cell wall
Bc0666	30018848	*inhA2*	Metalloprotease – lipoprotein	Cell wall
Bc4999	30023039		CAAX amino terminal protease family, 6 TM domains	Cell wall
Bc4511	30022589	*lppC*	Acid phosphatase, lipoprotein	Cell wall
Bc2552	30020679		Unknown, 2 transmembrane domains	Cell wall
Bc1713	30019857		Unknown, membrane spanning protein	Cell wall
Bc3527	30021629		Unknown, membrane spanning protein	Cell wall
Bc0361a	NA		Unknown, 1 TM domain	Cell wall
Bc0362	30018570		Unknown, lipoprotein	Cell wall
Bc0578	30018764	*yufM*	Two-component system regulator	Cytoplasm
Bc2410	30020541	*tetR*	Regulator, TetR family	Cytoplasm
Bc1082	30019237		Ribosomal protein alanine acetyl transferase; regulator ?	Cytoplasm
Bc5350	30023380	*plcR*	Transcriptional regulator	Cytoplasm
Bc1081	30019236	*prp2*	Unknown	Cytoplasm

Sppc stands for 'small peptide regulated by PlcR in *B. cereus*'. Sppc genes are wrongly annotated in the ATCC14579 genome. Bc2463a, BC3185a and Bc5101a are located between the PlcR box and, respectively, Bc2463, Bc3185 and Bc5101. Bc0361a is located between the PlcR box and Bc0361. Overall, 22 PlcR-controlled proteins are secreted, 18 are located in the cell wall and 5 are located in the cytosol. Determination of protein subcellular localisation was based on signal peptides, hydrophobic domains and cell-wall/membrane anchoring motifs presence.
doi:10.1371/journal.pone.0002793.t002

proteins made up 49% of the regulon, whereas genes coding for proteins associated to the membrane or to the peptidoglycan (cell wall proteins) represented 40%. The 22 secreted proteins were toxins, phospholipases, proteases, peptides with antibacterial activity and included one cell-cell communication peptide (PapR); however, the 18 cell wall proteins were annotated as being involved in cell immunity, drug efflux transport, cell wall biogenesis, and environment-sensing (in connection with regulation systems). Environmental sensors included two chemotaxis proteins, McpA and TlpA, the two-component system sensor YufM and one GGDEF family protein. The GGDEF family protein displays three conserved domains: a dinucleotide cyclase and a phosphodiesterase domain, involved in regulating the intracellular level of cyclic dinucleotide diguanylate, a second messenger, in response to ligands detected by the PAS domain [25]. One protein, InhA2, was possibly involved in cell immunity. InhA2 is a member of the Immune Inhibitor A metalloprotease family, previously shown to specifically degrade antibacterial peptides [26] and involved in bacterial virulence [20]. The cytosolic proteins controlled by PlcR were PlcR itself, a TetR family regulator, a two-component response regulator, a protein of unknown function and a protein homologous to the RimL ribosomal alanine acetyl transferase. Therefore, all cytoplasmic PlcR-controlled proteins of known function are likely to be regulators. The TetR family regulators are transcriptional repressors involved in the biosynthesis of antibiotic efflux pumps and the response to osmotic stress [27]. The two-component response regulator YufM is in an operon with its sensor component YufL and a chemotaxis transducer protein McpA, all under PlcR transcriptional control. RimL belongs to the GNAT superfamily of acetyltransferases [28] and acetylates a ribosomal protein interacting with elongation factors EF-Tu and EF-G [29,30].

Analysis of the nucleotidic sequences of the active PlcR boxes

The 28 active PlcR boxes that we determined were scattered all along the chromosome (Figure S3, supplementary material). Consequently, no pathogenicity island could be found in the *B. cereus* chromosome. Alignment of the active PlcR boxes led us to a new consensus sequence, shown as a logo in Figure 3. To investigate whether nucleotide sequences surrounding active PlcR

Figure 3. PlcR consensus sequence. The height of the letter representing a base is proportional to its frequency at each position in the alignment. For each position, the most frequent base is drawn in blue, followed by green and pink for less frequent bases.
doi:10.1371/journal.pone.0002793.g003

boxes could exhibit additional properties required for the box to be recognized by PlcR, a comparison of sequences upstream or downstream from the active and inactive PlcR boxes was performed. We found that in the vicinity of the active PlcR boxes, the AT-content was much higher than in the vicinity of inactive boxes (Figure 4). Downstream from all the active PlcR boxes, we identified a putative -10 σ^A binding sequence (Figure S5), suggesting that the PlcR-regulated genes may be transcribed by a σ^A-associated RNA polymerase.

Discussion

Building a list of PlcR-controlled genes

In 1999, Agaisse and colleagues used a genetic screen to identify PlcR-regulated genes. They reported 13 genes encoding exported proteins, mostly toxins and degradative enzymes [11]. As a consequence, PlcR appeared to be a pleiotropic virulence regulator controlling extracellular factors. That study also led to the definition of a PlcR target sequence, to which the active complex PlcR/PapR binds [12]. Later, the sequencing of *B. cereus* genomes in combination with proteomic and genetic studies revealed that PlcR may control a much higher number of genes, all of which were not, at least not directly, involved in virulence [7,17,24,31]. Indeed, the role of PlcR in virulence has been extensively documented [31–34]. Various studies have also suggested that PlcR could acts on other functions, including sporulation [35] and biofilm formation [36]. We therefore systematically investigated the PlcR regulon to understand better the role of PlcR during bacterial infection, and have provided the first comprehensive, genome-wide characterization of the complete PlcR virulence regulon based on functional experiments. A virtual PlcR regulon was constructed *in silico* using the PlcR DNA target sequence defined through mutagenesis experiments, and was investigated by transcriptional studies using DNA microarrays and *lacZ* fusions. The resulting data were cross-analyzed with data from proteomic studies, to build a list of 45 genes positively controlled by PlcR under standard culture conditions. The genes were scattered along the chromosome, and did not form a pathogenicity island. Aligning the sequences of the PlcR boxes located upstream from these genes led to the identification of the PlcR consensus binding sequence wTATGnAwwwwTnCATAw.

Inactive PlcR boxes

A high number of PlcR boxes turned out to be inactive under our culture conditions. Alignment of the sequences located upstream and downstream from the PlcR boxes revealed that, for active boxes, these sequences are significantly more AT-rich. Therefore, in addition to the consensus sequence, the genetic environment of the PlcR box could be critical for the binding of PlcR to its box, and/or could be important for the transcription activity of the promoter. Also, we found cases in which a PlcR box is placed between two divergently transcribed genes, and PlcR controls the transcription of only one of these genes (for example, Bc0555/Bc0556). Thus, the binding of PlcR to its box is required but not sufficient to activate the transcription of genes located

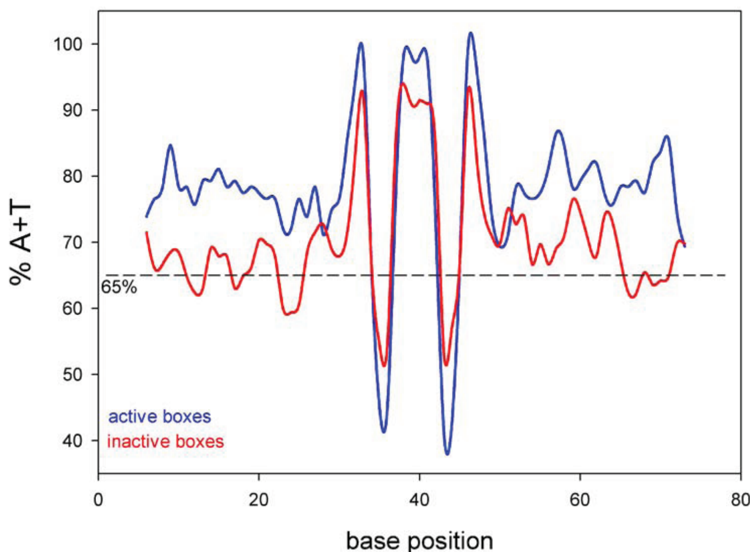

Figure 4. Percentage of A+T in the vicinity of PlcR boxes. Active boxes are plotted in blue whereas inactive boxes are plotted in red. The dashed line represents the average A+T percentage for the ATCC14579 chromosome. The difference between active and inactive boxes for the A+T percentage is highly significant (Qui-square test, $p<0.001$).
doi:10.1371/journal.pone.0002793.g004

downstream. A putative σ^A -10 region consensus sequence was found downstream from all the active PlcR boxes. In various conditions, including the host, the transcription of genes not controlled by PlcR despite the binding of the regulator to the PlcR box may require alternative sigma factors, such as σ^B, σ^H, or ECF factors. In *B. anthracis* [37], *L. monocytogenes* [38,39] and *S. aureus* [5], σ^B has been shown to be involved in virulence under some culture conditions. Similarly, σ^H is required for toxin gene expression in *B. anthracis* [40]. These two sigma factors are likely to be expressed in early stationary phase under standard culture conditions [37,40]. Finally, it seems unlikely that we may have missed any PlcR-regulated genes in this study due to no expression in LB medium because no additional genes were identified in a screen for genes specifically induced during growth *in vivo* [31].

Role of the PlcR regulon

Ninety percent of the genes included in the final list of PlcR-controlled genes encode proteins either secreted or located at the cell wall, i.e. at the interface between the bacterial cell and its environment – including the eukaryotic host. Proteases and phospholipases, in addition to enterotoxins and hemolysins, have been found located at this interface. These enzymes are likely to be involved in host tissue degradation. Phosphatidylcholine-specific phospholipase C (PC-PLC) and sphingomyelinase were previously shown to induce hemolysis [41,42] and the InhA2 metalloprotease is involved in protecting the bacterial cell from host immune defenses [26]. Proteins involved in peptidoglycan synthesis and modification (four genes) are also likely to be involved in bacterial cell protection by strengthening the cell wall, as suggested by the significantly greater tendency of cell lysis observed for the *plcR* mutant strain than the wildtype strain (this study). Furthermore, three secreted antibacterial peptides and four drug efflux transporters shown to be controlled by PlcR for the first time here may protect the cell from competition with other bacterial species and their bacteriocins.

Thus, these functions may work together to provide nutrition and bacterial cell protection in a hostile host environment (Figure 5). The bacterium may feed on host tissues by producing toxins, phospholipases and proteases. Proteins, peptides and amino acids have been suggested as the preferred nutrient sources for *B. cereus* [17], possibly linked to the growth of the bacterium as a human and animal pathogen. Meanwhile, other functions of the regulon may inhibit the growth of other bacterial species in the same niche, inactivate host antibacterial peptides, and increase cell wall resistance to lysis.

Sensing the host environment

Sensing the surrounding environment is necessary for a bacterium to react appropriately to changes. Bacterial pathogens often use two-component systems to sense their host environment, and promote or repress the transcription of genes in response to changes in this environment [1,2]. Interestingly, as shown here for the first time, four sensors are included in the *B. cereus* PlcR virulence regulon, only one of which (YufL) is part of a two-component system. The other sensors are chemotaxis proteins (McpA and TlpA) or a GGDEF-family regulator producing a second messenger. This variety in sensor types is likely to reflect a variety in the types of signals providing input to the cell. Furthermore, genes controlled by PlcR-dependent transcriptional regulators could add to the list of genes controlled by PlcR, and extend the regulon size. However, these regulators could also recruit genes already controlled by PlcR. If so, PlcR-controlled sensors and their regulators could modulate the transcription of

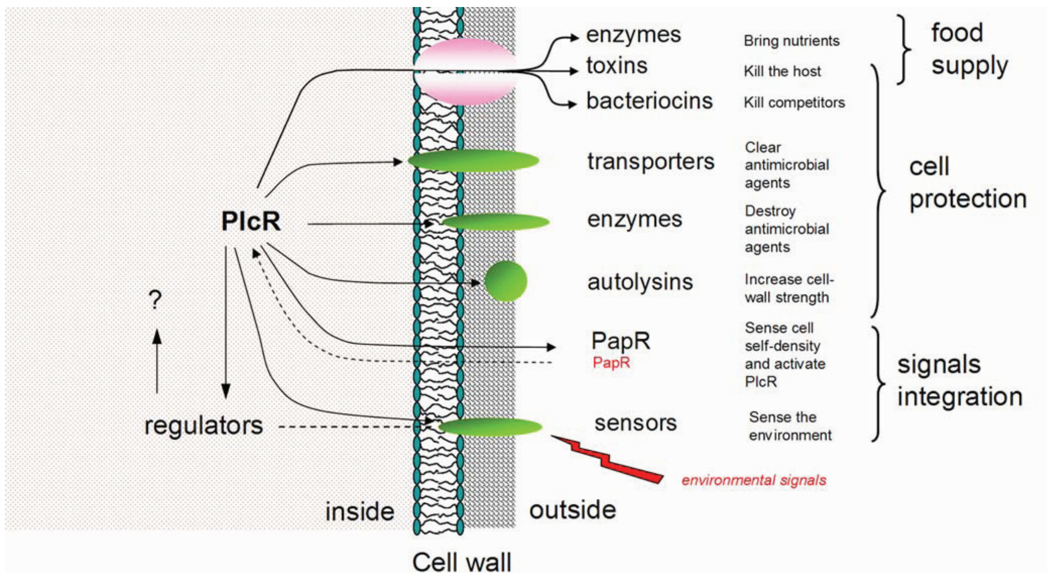

Figure 5. Overview of the PlcR regulon organization. PlcR positively controls (dark line) the transcription of a vast array of genes coding for proteins located in the cell wall or in the extracellular space. Cell wall proteins are designed in green. Secreted proteins are exported through the SecA machinery designed in pink. Environmental signals are sensed by cell-wall sensors and act *via* (dashed line) regulators on undetermined genes or proteins. PlcR requires PapR to be active (dashed line). Signals integrated by PlcR and PlcR-controlled regulators are designed in red.
doi:10.1371/journal.pone.0002793.g005

subgroups of PlcR-controlled genes. It was recently suggested that regulators other than PlcR could act on the expression of PlcR-controlled genes [43,44]. Accordingly, *L. monocytogenes* internalins, which constitute a subgroup of the PrfA virulence regulon, are simultaneously controlled by σ^B, itself acting on gene transcription in response to stress signals [39]. These observations have led us to propose the following hypothesis for the integration of PlcR/PapR-related environmental signals in *B. cereus*: PlcR triggers the transcription of its regulon, including the sensor proteins, in response to food deprivation sensed *via* transition state regulators and in response to its own cell density sensed *via* PapR. In turn, the PlcR-controlled sensors repress (or promote) transcription of subgroups of genes in response to host signals. The PlcR/PapR quorum-sensing system thus provides an efficient way to integrate several environmental signals and produce a gene expression profile continuously adapted to a changing host environment, such as that experienced by the bacterium during infection.

Materials and Methods

Strains and culture conditions

The *B. cereus* strains used in this study were the type strain, ATCC14579, and the isogenic Δ*plcR* strain [32], obtained by insertion of a Km^R cassette in *plcR*. The two strains were grown in Luria Bertani broth (LB) at 30°C. Cultures were harvested at the onset of the stationary phase (t_0) or two hours later (t_2). The onset of the stationary phase (t_0) was defined as the breakpoint in the vegetative phase slope.

Directed mutagenesis of the PlcR box

Point mutations were introduced into the PlcR box of the *plcA* promoter region by PCR amplification with primer Bc-plc matching the 5′ end of the *plcA* gene, and primers pRX1 to pRX18 carrying a modified PlcR box (supplementary Table S3). Each PCR product (a 390-bp DNA fragment) was digested with *Xba*I and *Hin*dIII enzymes and cloned between the *Xba*I and *Hin*dIII sites in pHT304-18Z [21]. The nucleotide sequence of each DNA fragment was determined and analyzed by Genome express (France) by using oligonucleotides UP and OVG flanking the DNA fragments cloned into pHT304-18Z. Plasmids carrying the *plcA'-lacZ* transcriptional fusions were introduced into *B. cereus* ATCC14579 by electroporation, and ß-galactosidase activity produced by the recombinant clones was measured two hours after entry in the stationary phase in LB medium.

Microarray analysis

Harvested bacterial cells were incubated in an equal volume of ice-cold methanol for 5 minutes before centrifugation at 4°C and 4000 rpm. RNA isolation was performed with the RNeasy Midi Kit (Qiagen, Germany) together with the RNase-Free DNase Set (Qiagen, Germany). For microarray slide preparation, 70-mer oligos from the whole genomic *B. cereus* ATCC14579 ORFs (released at NCBI in 2003) were designed and synthesized by Qiagen-Operon (Germany). The oligos were printed in 50% DMSO on UltraGAPS™ gamma amino silane-coated slides from Corning (USA), at the Norwegian Radiumhospital (DNR). The microarray slides were prehybridized before use for 30–60 minutes in a 5×SSC/0.1 % SDS/0.1 % BSA solution at 42°C, according to the UltraGAPS™ Coated Slides instruction manual from Corning. The slides were then washed three times in MQ H_2O, once in isopropanol and finally spun dry.

cDNA synthesis, labeling and purification was carried out with the FairPlay™ microarray labeling kit (Stratagene, CA, USA), using 500 ng random hexamers (Applied Biosystems, CA, USA) on 20 µg of RNA, and with amino-allyl coupling of Cy3 and Cy5 dyes from Amersham Biosciences (GE Healthcare Bio-Sciences AB, Sweden). After purification, the samples were concentrated with a Microcon column (Millipore, MA, USA) and hybridization solution was added to a final concentration of 30 % formamide, 5×SSC, 0.1 % SDS and 0.1 mg/mL sperm DNA, based on the UltraGAPS™ Coated Slides instruction manual from Corning (USA). Labeled DNA were denatured at 95°C for 2 minutes, and incubated at 42°C before hybridization. The samples were hybridized in a hybridization chamber (Monterey Industries, CA, USA), humidified with 5×SSC for 16 hours in a 42°C water bath. After hybridization, the slides were washed at 42°C in 0.5×SSC/0.01 % SDS and in 0.06×SSC, and finally at room temperature in isopropanol before they were spun dry.

The slides were scanned with an Axon 4000B scanner. Gridding, spot annotation and calculation was carried out using GenePix Pro 6.0 software. The R platform [45] and LIMMA [46,47] were used for filtering, normalization and further analysis (for details, see supplementary material). P-values were computed using false discovery rate correction of 0.05.

Transcriptional fusions

Transcriptional fusions were constructed in the pHT304-18Z plasmid, between the X*ba*I and P*st*I or H*ind*III and B*am*HI cloning sites of the plasmid [48]. Primers used for PCR-amplification of the promoter regions cloned are listed in supplementary Table S4. The resulting plasmids were then transferred into *B. cereus* strains ATCC14579 or ATCC14579 Δ*plcR* by electroporation. For β-galactosidase activity measurement, bacterial cells were lysed using the FastPrep 120 system (Savant), and β-galactosidase-specific activities were measured as described previously [49]. The specific activities are expressed in units of β-galactosidase $milligram^{-1}$ of protein (Miller units). Two to four assays were performed for each transcriptional fusion.

Two-dimensional electrophoresis

Protein extracts were prepared from the culture supernatants and subjected to two-dimensional electrophoresis as described earlier [7]. The spots were immediately excised and stored at −70°C until use. Proteins were identified by peptide mass fingerprint and by N-terminal sequencing. Peptide mass fingerprints were generated after trypsin-digestion and MALDI-TOF analysis (Biobac, INRA, Jouy-en-Josas, France), and proteins were identified using ProteinProspector or Mascot programs. N-terminal sequencing was performed by Prof. K. Sletten at the Biotechnology Center, University of Oslo, Norway.

Sequence analysis

PlcR boxes were searched in the sequenced genome of the ATCC14579 strain using the 'find sequence' tool of Vector NTI (Invitrogen). The same method was used to find σ^A −10 boxes in the promoter regions of PlcR-controlled genes. The consensus sequence for PlcR binding ('PlcR box') was drawn as a logo where, at each nucleotide position, the letter height is proportional to the frequency of the base and to the weight of the position in the sequence [50]. The content in A+T bases in sequences upstream and downstream from the inactive and active PlcR boxes were compared using a chi-square test.

Supporting Information

Table S1 Microarray results for genes with a PlcR box in their promoter region
Found at: doi:10.1371/journal.pone.0002793.s001 (0.26 MB PDF)

Table S2 Proteins identified in the culture supernatant of the delta-PlcR ATCC14579 strain harvested at t2
Found at: doi:10.1371/journal.pone.0002793.s002 (0.19 MB PDF)

Table S3 Primers used for the directed mutagenesis of the PlcR box
Found at: doi:10.1371/journal.pone.0002793.s003 (0.04 MB PDF)

Table S4 Primers for transcriptional fusions
Found at: doi:10.1371/journal.pone.0002793.s004 (0.05 MB PDF)

Figure S1 Results from lacZ fusions
Found at: doi:10.1371/journal.pone.0002793.s005 (0.13 MB PDF)

Figure S2 Two-dimensional gel electrophoresis of the Δ-plcR ATCC14579 supernatant
Found at: doi:10.1371/journal.pone.0002793.s006 (0.97 MB PDF)

Figure S3 Location of PlcR boxes on the ATCC14579 chromosome
Found at: doi:10.1371/journal.pone.0002793.s007 (0.02 MB PDF)

Figure S4 Genetic environment of the 45 PlcR-regulated genes
Found at: doi:10.1371/journal.pone.0002793.s008 (0.26 MB PDF)

Figure S5 Putative -10 σ^A boxes located downstream of PlcR boxes for PlcR-controlled genes
Found at: doi:10.1371/journal.pone.0002793.s009 (0.60 MB PDF)

Acknowledgments

We wish to thank Professor Knut Sletten (Biotechnology Centre, University of Oslo, Norway) for N-terminal sequencing, Dr. Nicolas J. Tourasse (Department of Pharmaceutical Biosciences, University of Oslo, Norway) for microarray bioinformatics support, and Jean-Claude Huet and Céline Henry (BioBac, INRA, France) for mass spectrometry.

Author Contributions

Conceived and designed the experiments: MG ABK DL. Performed the experiments: MG KF SP MG. Analyzed the data: MG KF SP SR OA MG ABK DL. Contributed reagents/materials/analysis tools: KF. Wrote the paper: MG.

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gene	name	Function	PlcR box	Location[a]	Dist[b]	r0[c]	p0[c]	r2[c]	p2[c]
BC0069	*spoIIE*	stage II sporulation protein E	catgcaaatttgcatg	64359	257	NA	NA	1.3	0.175
BC0313		Methyltransferase	aatgaaattttgcatt	283956	152	-0.3	0.978	0.0	0.985
BC0360	*ampS*	aminopeptidase AmpS	tatgcaattttgcata	341478	427	0.0	0.998	-0.5	0.005
BC0361		polysaccharide deacetylase-like protein	tatgcaattttgcata	341478	427	-0.6	0.006	-0.2	0.700
BC0361a		unannotated 57-amino acids peptide	tatgcaattttgcata	341478	427	NA	NA	NA	NA
BC0362		hypothetical protein	tatgcaattttgcata	341478	98	-2.8	0.002	-0.4	0.290
BC0442		tellurium resistance protein. putative	tatgaatgttttcata	430642	112	0.6	0.015	0.7	0.000
BC0443		tellurium resistance protein	tatgaatgttttcata	430642	112	0.6	0.032	0.9	0.003
BC0444		tellurium resistance protein	tatgaatgttttcata	430642	112	0.5	0.049	0.6	0.014
BC0445		tellurium resistance protein. putative	tatgaatgttttcata	430642	112	0.1	0.739	0.6	0.104
BC0446		conserved hypothetical protein	tatgaatgttttcata	430642	112	0.4	0.077	0.0	0.970
BC0447		tellurite resistance protein. putative	tatgaatgttttcata	430642	112	-0.1	0.720	0.7	0.043
BC0555		glycine betaine transporter	tatgaaatattgcatt	535372	165	0.1	0.541	-0.2	0.659
BC0556	*colC*	microbial collagenase.putative	tatgaaatattgcatt	535372	94	-3.4	0.000	-2.2	0.002
BC0576	*mcpA*	methyl-accepting chemotaxis protein	tatgcaaaaatgcata	559643	98	-1.6	0.000	-0.8	0.119
BC0577	*yufL*	sensory box histidine kinase	tatgcaaaaatgcata	559643	98	-0.8	0.009	0.0	0.982
BC0578	*yufM*	response regulator	tatgcaaaaatgcata	559643	98	-0.5	0.059	-1.6	0.160
BC0597		nicotinate phosphoribosyltransferase. putative	aatgaaattatgcata	589180	94	-0.2	0.442	-0.3	0.116
BC0598	*nprR*	Transcriptional activator NprR	aatgaaattatgcata	589180	91	NA	NA	0.1	0.934
BC0599		Transposase	aatgaaattatgcata	589180	91	0.5	0.000	-0.4	0.000
BC0600		Transposase	aatgaaattatgcata	589180	91	0.5	0.000	-0.4	0.000
BC0666	*inhA2*	immune inhibitor A metalloprotease	catgcaattttgcata	662055	307	-1.8	0.020	-5.2	0.000
BC0670	*plcB*	phospholipase C	tatgaacatttgcata	668446	124	-4.9	0.001	-1.4	0.007
BC0671	*Smase*	sphingomyelinase C	tatgaacatttgcata	668446	124	-4.1	0.002	-1.4	0.087
BC0672		hypothetical protein	tatgaacatttgcata	668446	124	-2.2	0.025	-0.7	0.156
BC0673		Flavin-dependent dehydrogenase	tatgaacatttgcata	668446	124	-0.3	0.514	-1.7	0.157
BC0757		major facilitator family transporter	catgaaaaattgcata	746136	480	-0.2	0.779	-0.1	0.579
BC0759		phospholipase. putative	catgaaaaattgcata	746136	235	-0.1	0.402	-0.5	0.206
BC0860		Multidrug resistance protein B	tatgaaaatatacatc	834200	682	0.2	0.438	-0.2	0.925
BC0917		hypothetical Cytosolic Protein	tatgtaaaaatgcata	905398	343	-0.2	0.001	0.0	0.895
BC0990		hypothetical protein	tatgaattttttgcata	976350	347	0.1	0.438	-0.1	0.701
BC0991	*slpA*	S-layer protein. putative	tatgaattttttgcata	976350	116	-2.8	0.000	-2.8	0.000
BC0992		hypothetical protein	tatgaattttttgcata	976350	116	-0.2	0.990	0.0	0.994
BC1081	*prp2*	PlcR-regulated protein PRP2	tatgtaatattgcata	1069156	61	-1.1	0.022	-0.6	0.131

gene	name	Function	PlcR box	Location[a]	Dist[b]	r0[c]	p0[c]	r2[c]	p2[c]
BC1082		acetyltransferase. GNAT family	tatgtaatattgcata	1069156	58	NA	NA	NA	NA
BC1110	*cytK*	Cytotoxin K,	tatgcataattgcata	1092541	82	-4.0	0.000	-2.3	0.009
BC1111		phosphoesterase	tatgcataattgcata	1092541	195	0.2	0.387	-0.6	0.055
BC1112		HD domain protein	tatgcataattgcata	1092541	195	-0.4	0.018	-0.4	0.626
BC1349		acetyltransferase. GNAT family	tatggaactttgcata	1323471	42	-0.1	0.561	-0.3	0.119
BC1350		hypothetical protein	tatggaactttgcata	1323471	278	0.1	0.095	0.2	0.373
BC1429		hypothetical protein	tatggaaatttccata	1389495	231	-0,1	0.740	0.1	0.897
BC1641	*flgB*	flagellar basal-body rod protein FlgB	tatgtaagggtacata	1596806	97	-0.4	0.065	-1.2	0.000
BC1642	*flgC*	flagellar basal-body rod protein FlgC	tatgtaagggtacata	1596806	97	-0.4	0.049	-1.0	0.000
BC1643	*fliE*	flagellar hook-basal body complex protein FliE. putative	tatgtaagggtacata	1596806	97	-0.2	0.333	-0.9	0.042
BC1644	*fliF*	Flagellar M-ring protein FliF	tatgtaagggtacata	1596806	97	-0.2	0.133	-0.8	0.027
BC1645	*fliG*	flagellar motor switch protein FliG	tatgtaagggtacata	1596806	97	-0.1	0.508	-0.2	0.646
BC1646		conserved hypothetical protein	tatgtaagggtacata	1596806	97	-0.1	0.742	-0.8	0.168
BC1647		flagellum-specific ATP synthase. putative	tatgtaagggtacata	1596806	97	-0.1	0.615	-0.7	0.146
BC1655		conserved hypothetical protein	tatgcaattttacata	1608869	162	0.4	0.065	0.4	0.346
BC1713		hypothetical Membrane Spanning Protein	tatgcagaattgcata	1662411	68	-2.6	0.000	-1.0	0.000
BC1734		ABC transporter. ATP-binding protein	tatgaaattattcata	1678356	472	0.3	0.409	-0.1	0.864
BC1735		Export ABC transporter permease protein	tatgaaattattcata	1678356	472	0.1	0.679	0.9	0.000
BC1736		Export ABC transporter permease protein	tatgaaattattcata	1678356	472	-0.1	0.854	1.3	0.000
BC1809	*nheA*	Enterotoxin,	tatgcataattgcata	1764706	527	-3.6	0.003	-4.4	0.000
BC1810	*nheB*	Enterotoxin,	tatgcataattgcata	1764706	527	-3.3	0.001	-4.2	0.000
BC1811	*nheC*	Non-expressed Enterotoxin C,	tatgcataattgcata	1764706	527	-4.6	0.000	-3.7	0.000
BC1956		DNA-binding response regulator	gatgcatttattcatg	1903735	457	-0.4	0.073	NA	NA
BC1957		sensor histidine kinase	gatgcatttattcatg	1903735	457	0.7	0.958	-1.3	0.826
BC1975		hypothetical Exported Protein	tatgtaggcatgcata	1922975	458	-0.4	0.079	-1.2	0.000
BC2138		hypothetical protein	aatgaaataatacata	2083572	270	0.1	0.531	0.3	0.378
BC2356		acetyltransferase. GNAT family	gatgaatatatacata	2302720	308	0.1	0.068	-0.7	0.004
BC2409		hypothetical protein	tatgcaatattacata	2353229	74	-1.0	0.141	0.1	0.660
BC2410	*tetR*	Transcriptional regulator. TetR family	tatgcaatattacata	2353229	253	-0.3	0.998	NA	NA
BC2411		ABC transporter. permease protein. putative	tatgcaatattacata	2353229	253	NA	NA	-1.3	0.830
BC2463a	*sppc2*	small peptide PlcR-controlled	tatgcaatattgcata	2427276	90	NA	NA	NA	NA
BC2466	*colB*	Microbial collagenase	tatgtaattttgcata	2431934	525	0.2	0.481	NA	NA
BC2552		hypothetical protein	tatgcaaatatgcata	2526975	82	-4.1	0.000	-2.5	0.000
BC2706		Acetyltransferase	tatgtagctattcata	2687769	299	0.1	0.998	NA	NA

gene	name	Function	PlcR box	Location[a]	Dist[b]	r0[c]	p0[c]	r2[c]	p2[c]
BC2707		Acetyltransferase	tatgtagctattcata	2687769	299	NA	NA	NA	NA
BC2735	*nprP2*	neutral protease	tatgcaaaattacata	2714407	98	-0.6	0.310	-3.5	0.002
BC2767		hypothetical Membrane Associated Protein	tatgtatttatacata	2745399	664	-0.1	0.998	0.0	0.855
BC2768		hypothetical protein	tatgtatttatacata	2745399	664	0.2	0.452	-0.6	0.104
BC2769		acetyltransferase. GNAT family	tatgtatttatacata	2745399	664	NA	NA	-0.8	0.009
BC2770		transcriptional regulator. DeoR family	tatgtatttatacata	2745399	664	NA	NA	NA	NA
BC2798		chitin binding protein. putative	aatgtataatttcatt	2766532	423	-0.1	0.914	-1.0	0.316
BC2922		hypothetical protein	aatgaattaattcatt	2879356	81	-1.2	0.002	0.3	0.836
BC2923		Acetyltransferase	aatgaattaattcatt	2879356	81	-0.3	0.998	NA	NA
BC2924		Acetyltransferase	aatgaattaattcatt	2879356	81	NA	NA	NA	NA
BC3019		hypothetical protein	catgtattaattcatt	2982086	430	-0.1	0.998	-0.8	0.214
BC3102	*hblA*	Hemolysin BL binding component precursor,	tatgtataattgcata	3067278	879	-3.5	0.000	-2.0	0.003
BC3103	*hblD*	Hemolysin BL lytic component L1,	tatgtataattgcata	3067278	879	-5.2	0.000	-5.1	0.000
BC3104	*hblB*	Hemolysin BL lytic component L2,	tatgtataattgcata	3067278	879	-5.6	0.000	-4.1	0.000
BC3161	*colA*	microbial collagenase. putative	tatgtattatttcata	3131867	66	-0.5	0.053	-1.7	0.078
BC3185a	*Sppc3*	small peptide PlcR-controlled	tatgcaatattgcata	3156120	92	NA	NA	NA	NA
BC3383	*nprC*	neutral protease	tatgtaattttgcata	3349558	174	-1.6	0.033	-1.3	0.533
BC3384	*mpbE*	enhancin family protein	tatgtaattttgcata	3349558	174	-1.4	0.075	-0.7	0.309
BC3385	*tlpA*	Methyl-accepting chemotaxis protein	tatgcaattttgcata	3351516	82	-1.2	0.195	-1.7	0.099
BC3461		conserved hypothetical protein	aatgaatatatacatc	3417962	291	-0.2	0.344	0.5	0.111
BC3463		arsenical pump family protein	aatgaatatatacatc	3417962	322	-0.3	0.147	0.1	0.068
BC3520		methyl-accepting chemotaxis protein	tatgcaatattacatg	3479263	616	0.5	0.416	1.8	0.754
BC3521		hypothetical protein	aatgtaatattacata	3481861	560	NA	NA	-0.4	0.297
BC3526		Collagen adhesion protein	tatgcaatattgcatt	3491783	481	0.2	0.419	-0.2	0.777
BC3527		hypothetical protein	tatgcaatattgcatt	3491783	76	-1.7	0.001	-0.7	0.078
BC3528		Sporulation kinase	tatgtaataattcata	3493194	554	0.3	0.409	-0.1	0.539
BC3688		conserved hypothetical protein	aatgcaataattcatc	3656825	615	0.0	0.968	NA	NA
BC3690		LexA repressor	aatgcaataattcatc	3656825	202	0.0	0.885	0.0	0.894
BC3739		exodeoxyribonuclease III	tatgaatacatacata	3704404	96	-0.3	0.010	-0.2	0.156
BC3740		Ada regulatory protein/6-O-methylguanine-DNA methyltransferase	tatgaatacatacata	3704404	43	0.1	0.998	NA	NA
BC3741		methylated-DNA--protein-cysteine S-methyltransferase	tatgaatacatacata	3704404	43	0.5	0.311	NA	NA
BC3742		DNA-3-methyladenine glycosidase	tatgaatacatacata	3704404	43	-1.1	0.029	0.4	0.278
BC3746		hydrolase. alpha/beta fold family	tatgcaattttgcatc	3710301	146	0.3	0.140	-1.4	0.172

gene	name	Function	PlcR box	Location[a]	Dist[b]	r0[c]	p0[c]	r2[c]	p2[c]
BC3747		sensory box/GGDEF family protein	tatgcaattttgcatc	3710301	237	-0.1	0.998	NA	NA
BC3761	*plcA*	1-phosphatidylinositol phosphodiesterase	tatgaaatattgcata	3725577	87	-2.2	0.000	-1.1	0.001
BC3762	*Sfp*	serine protease. subtilase family	tatgcaatatttcata	3727637	144	-3.5	0.004	-1.5	0.003
BC3763	*cwh*	unannotated cell-wall hydrolase	tatgcaatatttcata	3727637	151	NA	NA	NA	NA
BC4072	*sigF*	RNA polymerase sigma-F factor	catgaataattgcatt	4043452	291	NA	NA	-0.3	0.368
BC4142		DNA-damage-inducible protein P. putative	tatgaatttcttcata	4109378	522	0.4	0.184	-1.0	0.193
BC4346		conserved hypothetical protein	tatgcaattttgcata	4286809	504	-0.2	0.156	0.1	0.719
BC4496		glutamate racemase	tatgaaaattttcatt	4442741	82	-0.1	0.248	0.5	0.104
BC4508		rrf2 family protein	tatgcaaatgttcata	4456098	265	0.0	0.458	0.5	0.165
BC4509		Sodium export permease protein	tatgcaaatgttcata	4456098	265	-1.4	0.008	-0.2	0.336
BC4510		ABC transporter. ATP-binding protein	tatgcaaatgttcata	4456098	265	-1.8	0.000	-1.5	0.000
BC4511		acid phosphatase	tatgcaaatgttcata	4456098	62	-3.5	0.000	-1.4	0.023
BC4794		Spore germination protein PF	tatgcacaattgcata	4723091	468	NA	NA	NA	NA
BC4795		conserved domain protein	tatgcacaattgcata	4723091	198	-0.8	0.002	-0.1	0.966
BC4956		Ribosomal-protein-alanine acetyltransferase	tatgcagaattacata	4870034	74	NA	NA	NA	NA
BC4958		NAD(P)H dehydrogenase. quinone family	tatgcagaattacata	4870034	534	0.0	0.999	0.2	0.157
BC4979		conserved hypothetical protein	gatgaattaattcatc	4893912	634	-0.1	0.353	0.7	0.030
BC4980		nifU domain protein	gatgaattaattcatc	4893912	634	0.0	0.330	0.8	0.136
BC4981		aminotransferase. class V	gatgaattaattcatc	4893912	634	0.0	0.947	0.7	0.145
BC4982		conserved hypothetical protein	gatgaattaattcatc	4893912	634	-0.1	0.096	1.0	0.061
BC4983		ABC transporter. ATP-binding protein	gatgaattaattcatc	4893912	634	0.0	0.663	1.4	0.006
BC4984		ABC transporter. substrate-binding protein. putative	gatgaattaattcatc	4893912	634	0.5	0.313	-0.4	0.282
BC4985		ABC transporter. substrate-binding protein. putative	gatgaattaattcatc	4893912	634	0.3	0.385	-0.6	0.112
BC4986		ABC transporter. permease protein	gatgaattaattcatc	4893912	634	1.2	0.039	-0.6	0.172
BC4999		CAAX amino terminal protease family	tatgcaattttacata	4902529	89	-1.3	0.001	0.2	0.750
BC5093		Xanthine permease	tatgtaggatttcata	4998240	367	-0.2	0.720	0.0	0.989
BC5101	*clO*	thiol-activated cytolysin, *clO*	tatgcaatattacata	5004551	91	-5.7	0.000	-4.6	0.000
BC5101a	*sppc1*	small peptide PlcR-controlled	tatgcaatattacata	5004551	91	NA	NA	NA	NA
BC5286		Transcriptional regulator with ABC transporter ATP-binding domain and LytTR DNA-binding domain	tatggaagtttacata	5198202	285	NA	NA	NA	NA
BC5287		stage II sporulation protein D	tatggaagtttacata	5198202	285	0.4	0.998	NA	NA
BC5335	*fbaA*	fructose-bisphosphate aldolase. class II	tatgtatatctacata	5243458	77	0.2	0.382	0.4	0.070

gene	name	Function	PlcR box	Location[a]	Dist[b]	r0[c]	p0[c]	r2[c]	p2[c]
BC5349	*papR*	PapR protein	`tatgcaattatgcata`	5260284	55	1.0	0.000	2.0	0.000
BC5350	*plcR*	Transcriptional activator plcR	`tatgaaataatgcata`	5261256	81	3.6	0.000	3.8	0.000
BC5351	*nprB*	Bacillolysin	`tatgaaataatgcata`	5261256	71	-2.8	0.000	-3.9	0.000
BC5359		aminopeptidase. putative	`tatgcaaatatgcata`	5278003	119	-0.1	0.373	0.1	0.562
BC5445		superoxide dismutase. Mn	`gatgaattaatgcatt`	5369359	487	0.2	0.788	-0.3	0.414
BC5448		UDP-glucose 4-epimerase	`catgaaaaatttcatg`	5373858	243	0.0	0.998	0.2	0.001

Table S1: Microarray results for genes with a PlcR box in their promoter region.

Sequences retained as PlcR boxes were 'TATGNANNNNTNCATA' or ATGHAWWWWTWCAT'. PlcR boxes located more than 700bps and less than 35bps from the open reading frame were discarded. a: 'Location' means PlcR box location on the ATCC14579 chromosome. b: 'Dist.' is the distance between the PlcR box and the first ORF of the putative transcript. c: 'r0' and 'r2' are log2 ratio of gene expression between the PlcR mutant and the wild type strain; r0 was measured at the onset of stationary phase while r2 was measured 2 hours after the entry in stationary phase; p0 and p2 are fdr-corrected p-values, respectively for r0 and r2. Microarray results were missing for Bc0361a, BC2463a, BC3185a, BC3763 and BC5101a because these genes were not included in the microarray chips as they are not annotated in the ATCC14579 sequenced genome. NA means results not available.

Protein	Function	gene	Mw[a]	pI[a]	Wtype[b]	Localisation[c]	Seq cov[d]	N-terminal sequence	SP[e]
			Glycolysis						
G6pA	Glucose 6 phosphate isomerase	BC4898	48	5.0		cytoplasm	14%		No
FbaA	Fructose 6 phosphate aldolase	BC5335	28	5.2		cytoplasm	ND	XLVXMKXML	No
Eno	Enolase	BC5135	44-45	4.5-4.9 & 5.8	■	cytoplasm	31% - 10%		No
			Tricarboxylic acid cycle						
CitB	Aconitate hydratase	BC3616	106	4.9	■	cytoplasm	28%		No
FumA	Fumarate hydratase	BC0466	58	5.3		cytoplasm	10%		No
CitC	Isocitrate deshydrogenase	BC4593	39-40	4.9 & 5.2		cytoplasm	10%		No
MdH	Malate deshydrogenase	BC4592	32	5.1		cytoplasm	8%		1-30
PdhA	Pyruvate deshydrogenase alpha subunit	BC3973	36	5.6		membrane	21% - 4%		No
PdhB	Pyruvate deshydrogenase beta subunit	BC3972	33	4.5	■	membrane	20% - 4%		No
			Metabolism of other carbohydrates						
Tkt	Transketolase	BC3682	76	4.9		cytoplasm	7% - 3%		No
TreA	Trehalose 6 phosphate hydrolase	BC0632	73	5.0		cytoplasm	17%		No
YvyH	N-acetyl glucosamine epimerase	BC5201	35	5.7		cytoplasm	13% - 4%		No
			Amino acid metabolism						
GlyA	Serine hydroxymethyl transferase	BC5316	41	6.0		cytoplasm	9%		No
LeuDH	Leucine deshydrogenase	BC4162	40	5.4		cytoplasm	18% - 3%		No
AlaDH	Alanine deshydrogenase	BC0592	38	5.5 & 5.7		cytoplasm	24%		No
DapD	Tetrahydrodipicolinate succinylase	BC3981	23	5.5		cytoplasm	5%		No
RocA	Pyrroline carboxylate deshydrogenase	BC0344	48-52	5.3-5.7		cytoplasm	20% - 4%		No
			Nucleic acids metabolism						
Ndk	Nucleoside diphosphate kinase	BC1515	17	5.5	■	cytoplasm	7%		No
SmbA	Uridylate kinase	BC3823	23	5.7		cytoplasm	6%		No
Pdp	Pyrimidine nucleoside phosphorylase	BC4085	47	4.6		cytoplasm	11%		No
HprT	Hypoxanthine guanilate phosphorybosil transferase	BC0071	20	5.1		cytoplasm	3%		No
GTPase	GTPase	BC2005	18	5.0		membrane	ND	TEEQIIEAV	No
ATP_NADk	ATP-NAD kinase	BC4642	25	6.2		cytoplasm	10%		No
PnP-I	Purine-nucleotide phosphorylase I	BC4086	24	5.1		cytoplasm	9% - 2%		No
			Proteins and RNA synthesis						
Ef-G	Elongation factor G	BC0128	86-88	4.9-5.0		cytoplasm	5%	DAGKTTATERIL	No
EF-Ts	Elongation factor T	BC3824	31-32	5.2-5.4		cytoplasm	15% - 5%		1-56
GreA	Transcription elongation factor	BC4374	18	4.7		cytoplasm	8% - 6%		No
			Protein folding and general stress proteins						
Tig	Trigger factor (prolyl isomerase)	BC4480	52	4.3		cytoplasm	21% - 3%	KXXKLXXXV	No
GroEL	60 kDa chaperone	BC0295	61	4.8	■	cytoplasm	1%	KXIKFSXXA	No
TerD	Tellurium resistance protein terD	BC0443	21	4.4-4.6 & 5.3		cytoplasm	13%		No
GroES	10kDa chaperone	BC0294	15	4.4		cytoplasm	2%		No

CspD	Cold shock protein D	BC4859	15	4.5 & 4.7	■	cytoplasm	ND	MQXGKVKXFN	No
				Detoxification					
SodM	Superoxide dismutase manganese-dependent	BC5445	21	5.3	■	cytoplasm	5%		No
Tpx	Thiol peroxidase	BC4639	18	4.8		cytoplasm	10%		No
AhpF	Alkyl hydroperoxide reductase subunit F	BC0376	52	4.8		membrane	20%		No
AhpC	Alkyl hydroperoxide reductase (C22)	BC0377	20	4.7		cytoplasm	12% - 3%		No
				Ribosomal proteins					
RpsF	30S ribosomal protein S6	BC5476	16	5.6		cytoplasm	19% - 6%		No
				Mobility and chemotaxis					
FlgA	Flagellin A	BC1657	22-25	4.6-5.2	■	Flagellar	21% - 6%		No
FlgB	Flagellin B	BC1658	23-25	4.5-5.0	■	Flagellar	15% - 4%		No
FlgC	Flagellin C	BC1659	22-24	4.5-5.0	■	Flagellar	21% - 6%		No
FliD	Flagellar hook associated protein 2	BC1638	43	6.5		Flagellar	12% - 6%		No
				Membrane proteins (transport, binding and bioenergetics)					
DppE	Dipeptide binding protein	BC1179	57	6.4	■	membrane	29% - 7%		1-26
OppA	Oligopeptide-binding protein OppA	BC1185	55	6.1	■	membrane	16%		1-31
PtgA	PTS system, glucose-specific IIA component	BC5320	18	5.0 & 5.5		membrane	7%		No
				Cell wall proteins and endolysins					
LytE	N-acetylmuramoyl-L-alanine amidase	BC5196	48	5.9		membrane	10% - 5%		1-61
CwlA	N-acetylmuramoyl-L-alanine amidase	BC0888	20	6.2		membrane	13% - 2%		1-31
PlyBa	N-acetylmuramoyl-L-alanine amidase	BCp0021	20	6.5		cytoplasm	8%		No
MurA	N-acetyl glucosamine carboxyvinyl transferase	BC5288	40	5.9		membrane	16%		No
MdoB	Phosphoglycerol transferase	BC5232	40	5.2	■	membrane	19% - 13%		No
CwBP	Cell wall-binding protein	BC0679	51	5.9		extracellular	9% - 8%		1-26
CwlB	Cell wall endopeptidase, family M23/M37	BC3698	24	7.0	■	cytoplasm	14%		No
PbpA	Penicillin binding protein	BC4270	73	5.6		membrane	20%		1-35
				Unknown proteins					
Unknown	Phage protein	BC1894	34-35	5.5-5.7 & 6.6		membrane	7%		1-39
Unknown	Unknown (homologue pXO2 hypothetical protein)	BC3699	28	7.0		cytoplasm	14%		No
Unknown	Unknown (hypothetical cytosolic protein)	BCp0018	28	5.4	■	cytoplasm	7%		No
Unknown	Unknown	BC1862	27	4.4		cytoplasm	10%		No
Unknown	Unknown	BC2186	19	4.8		cytoplasm	10%		1-19
Unknown	Phage protein	BC1863	16	4.4		cytoplasm	4%		No
Unknown	Unknown (hypothetical cytosolic protein)	BCp0009	22	6.3		cytoplasm			No

Table S2: Proteins identified in the culture supernatant of the delta-PlcR ATCC14579 strain harvested at t2.

Proteins were identified by peptide mass fingerprint or by N-terminal sequencing on a Coomassie Blue-stained two-dimensional electrophoresis gel. (a): Mw and Ip are the molecular weight and the isoelectric point of the protein as determined on the gel. (b): the presence of the protein in the wild-type strain culture supernatant harvested in the

same conditions is indicated by a dark-filled square '■'. (c): subcellular localisation as predicted by PSORT. (d): sequence coverage of the protein in %. (e): presence of a signal peptide

Primer name	5' - 3' sequence [a]	Restriction site	Primer characteristics
OVG	CGTAATCTTACGTCAGTAACTTCCACAGTA		Complementary to *lacZ* 5' end
UP	CGCCAGGGTTTTCCCAGTCACGAC		Universal primer
Bc-plc	TGCTCTAGAGCTCCATGGTCCATTTG	*Xba*I	Complementary to *plcA* 5' end
pRX0	CCCAAGCTTCTATGCAATATTTCATATTG	*Hin*dIII	PlcR box upstream from *plcA*
pRX1	CCCAAGCTTC**A**ATGCAATATTTCATATTG	*Hin*dIII	PlcR box with mutation T_{1A}
pRX2	CCCAAGCTTC**C**ATGCAATATTTCATATTG	*Hin*dIII	PlcR box with mutation T_{1C}
pRX3	CCCAAGCTTC**G**ATGCAATATTTCATATTG	*Hin*dIII	PlcR box with mutation T_{1G}
pRX4	CCCAAGCTTCT**G**TGCAATATTTCATATTG	*Hin*dIII	PlcR box with mutation A_{2G}
pRX5	CCCAAGCTTCTA**C**GCAATATTTCATATTG	*Hin*dIII	PlcR box with mutation T_{3C}
pRX6	CCCAAGCTTCTAT**A**CAATATTTCATATTG	*Hin*dIII	PlcR box with mutation G_{4A}
pRX7	CCCAAGCTTCTATG**G**AATATTTCATATTG	*Hin*dIII	PlcR box with mutation C_{5G}
pRX8	CCCAAGCTTCTATGC**G**ATATTTCATATTG	*Hin*dIII	PlcR box with mutation A_{6G}
pRX9	CCCAAGCTTCTATGC**C**ATATTTCATATTG	*Hin*dIII	PlcR box with mutation A_{6C}
pRX10	CCCAAGCTTCTATGC**T**ATATTTCATATTG	*Hin*dIII	PlcR box with mutation A_{6T}
pRX11	CCCAAGCTTCTATGCA**G**TATTTCATATTG	*Hin*dIII	PlcR box with mutation A_{7G}
pRX12	CCCAAGCTTCTATGCA**C**TATTTCATATTG	*Hin*dIII	PlcR box with mutation A_{7C}
pRX13	CCCAAGCTTCTATGCA**T**TATTTCATATTG	*Hin*dIII	PlcR box with mutation A_{7T}
pRX14	CCCAAGCTTCTATGCAA**G**ATTTCATATTG	*Hin*dIII	PlcR box with mutation T_{8G}
pRX15	CCCAAGCTTCTATGCAA**C**ATTTCATATTG	*Hin*dIII	PlcR box with mutation T_{8C}
pRX16	CCCAAGCTTCTATGCAATATTTCAT**C**TTG	*Hin*dIII	PlcR box with mutation A_{16C}
pRX17	CCCAAGCTTCTATGCAATATTTCAT**G**TTG	*Hin*dIII	PlcR box with mutation A_{16G}
pRX18	CCCAAGCTTCTATGCAATATTTCAT**T**TTG	*Hin*dIII	PlcR box with mutation A_{16T}

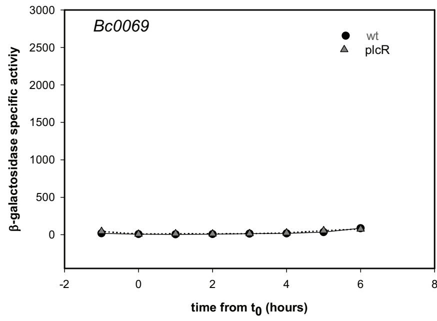
Table S3: Primers used for the directed mutagenesis of the PlcR box

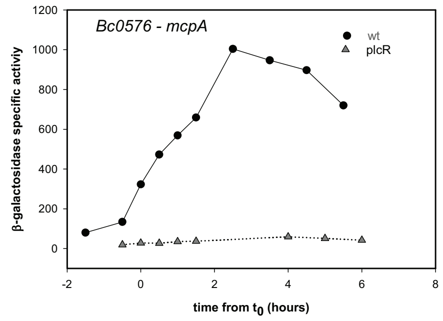
[a] The restriction sites are underlined, the wildtype PlcR box is underlined twice, and the modified nucleotides are in bold.

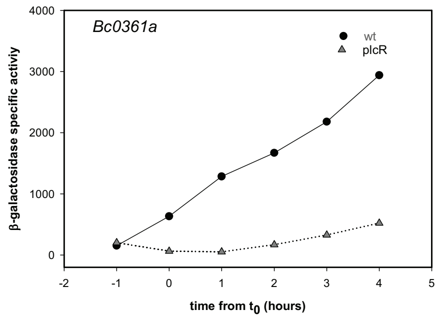
Gene	Forward primer	Reverse primer
Bc0069[a]	AACTGCAGCTGGCTAGAGCGTACGG	GCTCTAGAGCTAAAAATGCTAGCGGC
Bc0361a[a]	AAACTGCAGCACGCTATTCGCTTTAC	TGCTCTAGATCAGCGTTAAGACTAG
Bc0442[a]	AAACTGCAGGAGTTGTATGATAAAGGAGATCGG	GGTCTAGACAATTGGATCCCAGCCTAAG
Bc0556[a]	AACTGCAGCAGCCAAAATAATCATTGTAGTC	GCTCTAGATAAAGCCATTGTACTAATGCTAAGC
Bc0576[a]	AAACTGCAGGCGCCAAATAAATTAGGACG	GCTCTAGAATGTCTGCAAAGTACGTCGC
Bc0670[a]	AAACTGCAGTGGGATTGGACTAGTGTTTGG	GCTCTAGACTTCAGCAGACCAGCGC
Bc0991[a]	AAACTGCAGGTTTCAACGGACCAATCAAATGTC	GGTCTAGACCTTGTTTATTCGGTACTAACACTCGA
Bc1082[a]	AAACTGCAGCTATGGATAGAATTTTTTCATAATCAAA	GGTCTAGAGCATCAGTCTCTTTTACTAACCGT
Bc1111[a]	AAACTGCAGTATCTGTTACAACTTGTGACGTCG	GGTCTAGACCTATCGTTCGCAGAAATGG
Bc1641[a]	AAACTGCAGCGTTTACATAAAGAACTCCGC	GCTCTAGAGCAAGTGCATTTCATTCG
Bc1713[a]	AAACTGCAGCTTGTACAAATGTATGC	TGCTCTAGATGACGTGGAATGGAT
Bc2410[a]	AAACTGCAGACTTGCTCATATAAATCACCC	GCTCTAGATAAGAAACAAACGCTCAGC
Bc2466[a]	AACTGCAGCAAGCTAAGTTAGCATGTTTAATG	GCTCTAGATCCATATTTGGCGAACAAAG
Bc2707[c]	CCCAAGCTTCGCGCTTATAATAAGGAGGAC	CGGGATCCATCAGTTTCTGTCGCCTTCTC
Bc3384[a]	AAACTGCAGGAGACTTGGCTGAGCAATCTAAAG	GGTCTAGAGTTCGTTCATCAGCATGTAAGTGT
Bc3385[a]	AAACTGCAGTTATACATCAATGATGGGTTCCCC	GGTCTAGACCCCAAAACCCTATTAGGGATAAT
Bc3520[c]	CCCAAGCTTTGGGCAGAACATTAAAGC	CGGGATCCGCTTTATTTGCCCCAATCTTTC
Bc3521[c]	CCCAAGCTTTGTATAGCTACATGAGGATTTTGAC	CGGGATCCCCAAACCCTGTCCGAAAG
Bc3528[b]	CCCAAGCTTTACGAAAGAAGTTACAGCCTCACC	GGTCTAGAGCCAGAATACGTGATAGCTAAGG
Bc3740[a]	AAACTGCAGGCTCTTCAATGCCTAAACCATATG	GGTCTAGACGGTATTCTTGATTTGCATGATGG
Bc3747[a]	AAACTGCAGCGCAGCCATGTATGAT	TGCTCTAGACGAAGCGTACATCTGAAT
Bc3763[a]	AAACTGCAGCACTATTGATAGTGCCTCCTGTTC	GGTCTAGAGTGACATTGTAACGCTTTGCTATC
Bc4142[a]	AAACTGCAGCGCATTGACTCTGAAACGAC	GGTCTAGAACTCACTCCCTAATCAGAACGTT
Bc4510[a]	AAACTGCAGCTTCAGCCTCAGCTTTGTTAATCC	GGTCTAGACCTAGTACTTCACCTTTTGGTAACGAA
Bc4511[a]	AAACTGCAGCCCATGTAATCGAACCTTCATTAG	GGTCTAGAGCTGTTTGATACCATAAATCAGCC
Bc4794[a]	AACTGCAGCGTAGGACATCACTATCTGAGTTC	GCTCTAGATCCATTATAAGCCTTCGTCTTTTC
Bc4795[a]	AAACTGCAGCAATAATAACACCTTCCACAACTGCC	GGTCTAGAGTCGTCCTTCTCGCTTCATTAAAAT
Bc4958[a]	AAACTGCAGAACTATAAGGTTCCTCGTACGTCC	GGTCTAGACGTGACCTTTTCCTTCTAATTCAC
Bc4986[a]	AACTGCAGATTGTTTAATCAGTTAGAGAAACC	GCTCTAGATTGCCGTCATATATAACGTTTC
Bc5335[a]	AAACTGCAGGTCAAGCTGTGAGAAATGAGC	GCTCTAGAATTTTTCTTCTTCCGCAGC
Bc5359[a]	AAACTGCAGAGATGGAAAAATCATCCGAGAAGG	GGTCTAGACCCATTGATTTAAGCTGCATACCA

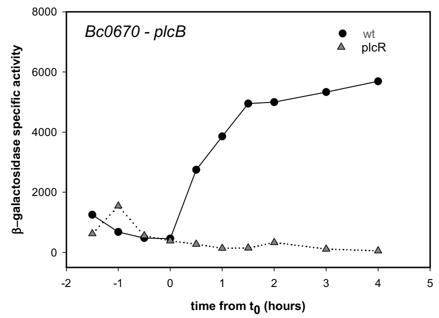
Table S4: Primers for transcriptional fusions.

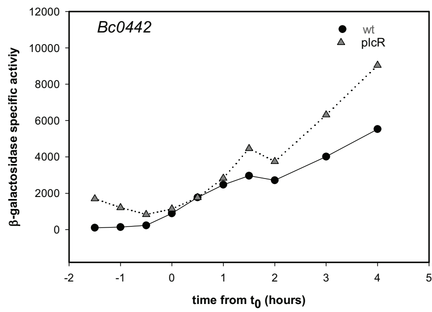
Primers used to amplify promoter regions of genes selected for lacZ fusions are given in the table. Restriction sites are underlined and were PstI and XbaI (a), HindIII and XbaI (b), or HindIII and BamHI (c).

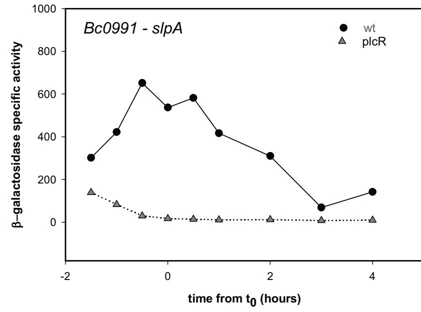

Bc0069
wt
plcR
β-galactosidase specific activity
time from t_0 (hours)

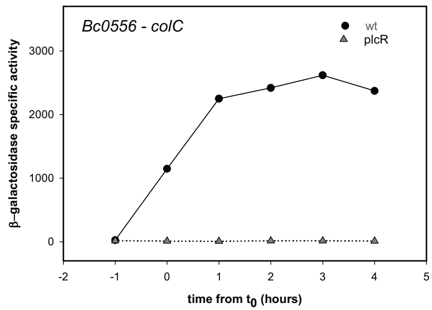

Bc0576 - mcpA
wt
plcR
β-galactosidase specific activity
time from t_0 (hours)

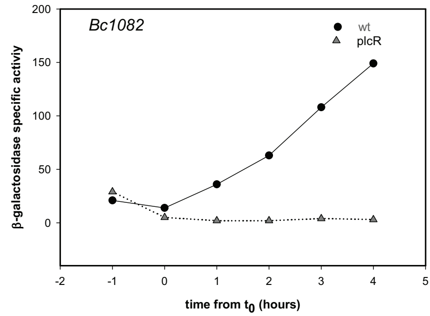

Bc0361a
wt
plcR
β-galactosidase specific activiy
time from t_0 (hours)

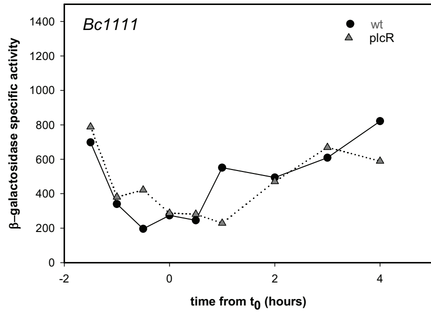

Bc0670 - plcB
wt
plcR
β-galactosidase specific activity
time from t_0 (hours)

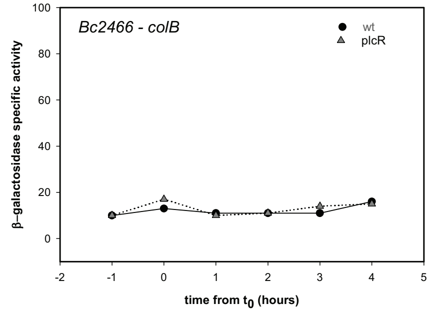

Bc0442
wt
plcR
β-galactosidase specific activiy
time from t_0 (hours)

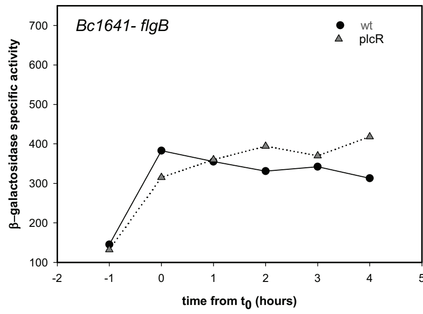

Bc0991 - slpA
wt
plcR
β-galactosidase specific activity
time from t_0 (hours)

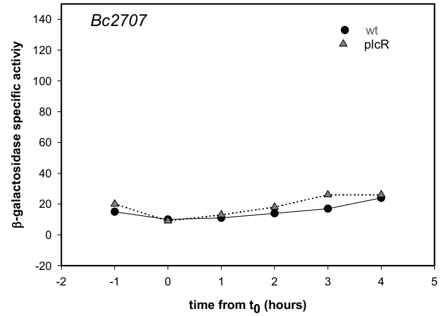

Bc0556 - colC
wt
plcR
β-galactosidase specific activity
time from t_0 (hours)

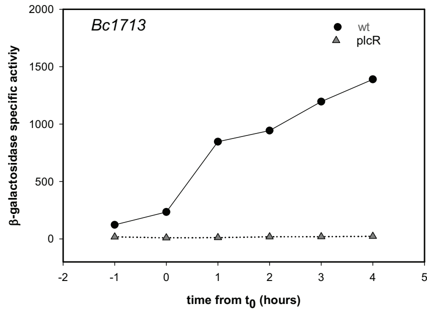

Bc1082
wt
plcR
β-galactosidase specific activity
time from t_0 (hours)

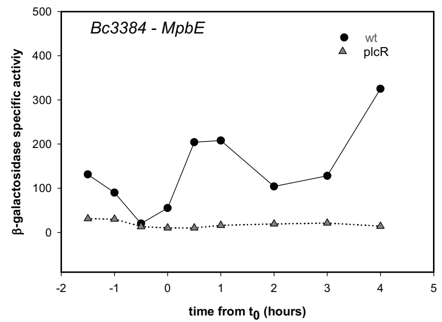

Bc1111
wt
plcR
β-galactosidase specific activity
time from t0 (hours)

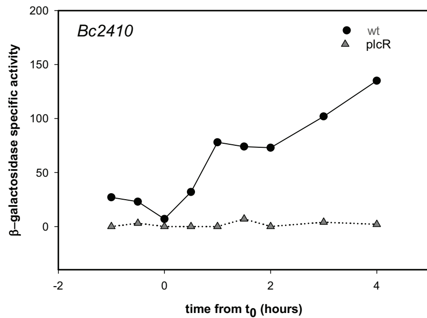

Bc2466 - colB
wt
plcR
β-galactosidase specific activity
time from t0 (hours)

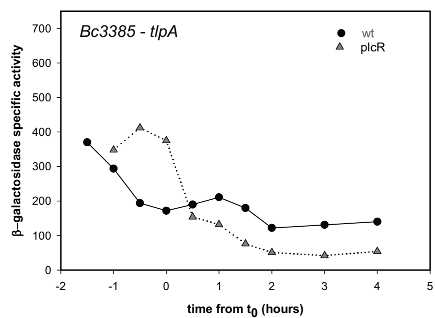

Bc1641- flgB
wt
plcR
β-galactosidase specific activity
time from t0 (hours)

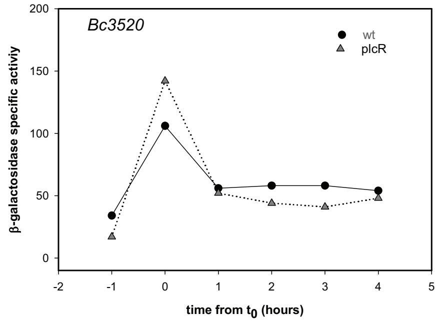

Bc2707
wt
plcR
β-galactosidase specific activiy
time from t0 (hours)

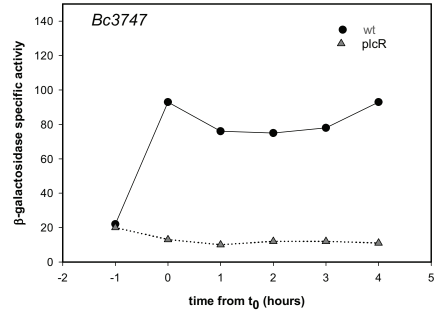

Bc1713
wt
plcR
β-galactosidase specific activiy
time from t0 (hours)

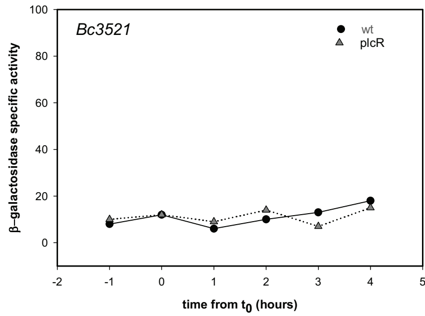

Bc3384 - MpbE
wt
plcR
β-galactosidase specific activiy
time from t0 (hours)

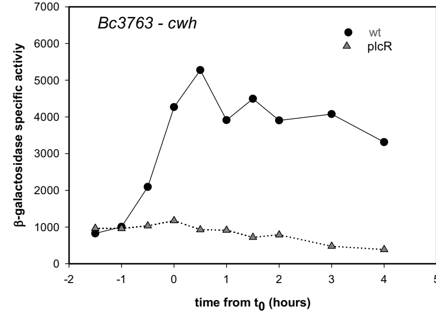

Bc2410
wt
plcR
β-galactosidase specific activity
time from t0 (hours)

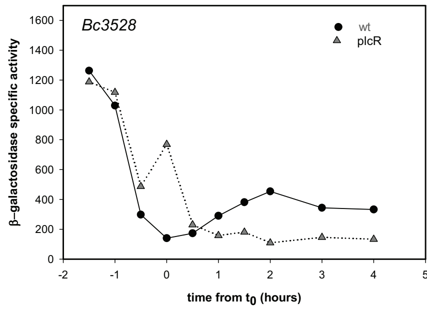

Bc3385 - tlpA
wt
plcR
β-galactosidase specific activity
time from t0 (hours)

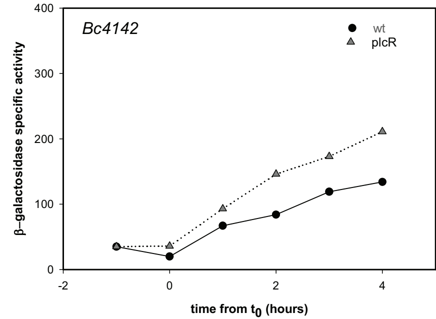

Bc3520
wt
plcR
β-galactosidase specific activiy
time from t_0 (hours)

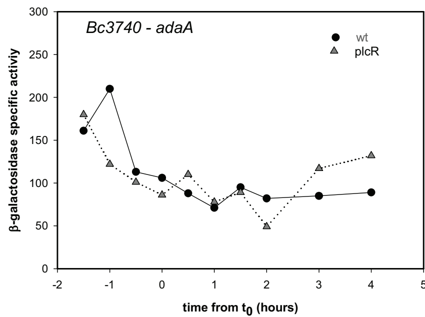

Bc3747
wt
plcR
β-galactosidase specific activiy
time from t_0 (hours)

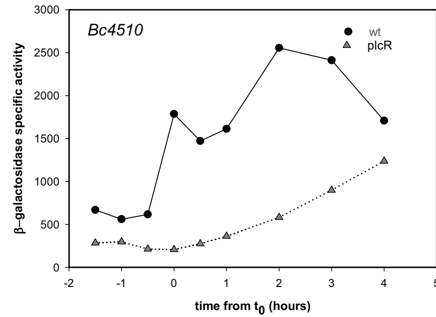

Bc3521
wt
plcR
β-galactosidase specific activiy
time from t_0 (hours)


Bc3763 - cwh
wt
plcR
β-galactosidase specific activiy
time from t_0 (hours)


Bc3528
wt
plcR
β-galactosidase specific activity
time from t_0 (hours)


Bc4142
wt
plcR
β-galactosidase specific activity
time from t_0 (hours)


Bc3740 - adaA
wt
plcR
β-galactosidase specific activiy
time from t_0 (hours)


Bc4510
wt
plcR
β-galactosidase specific activity
time from t_0 (hours)

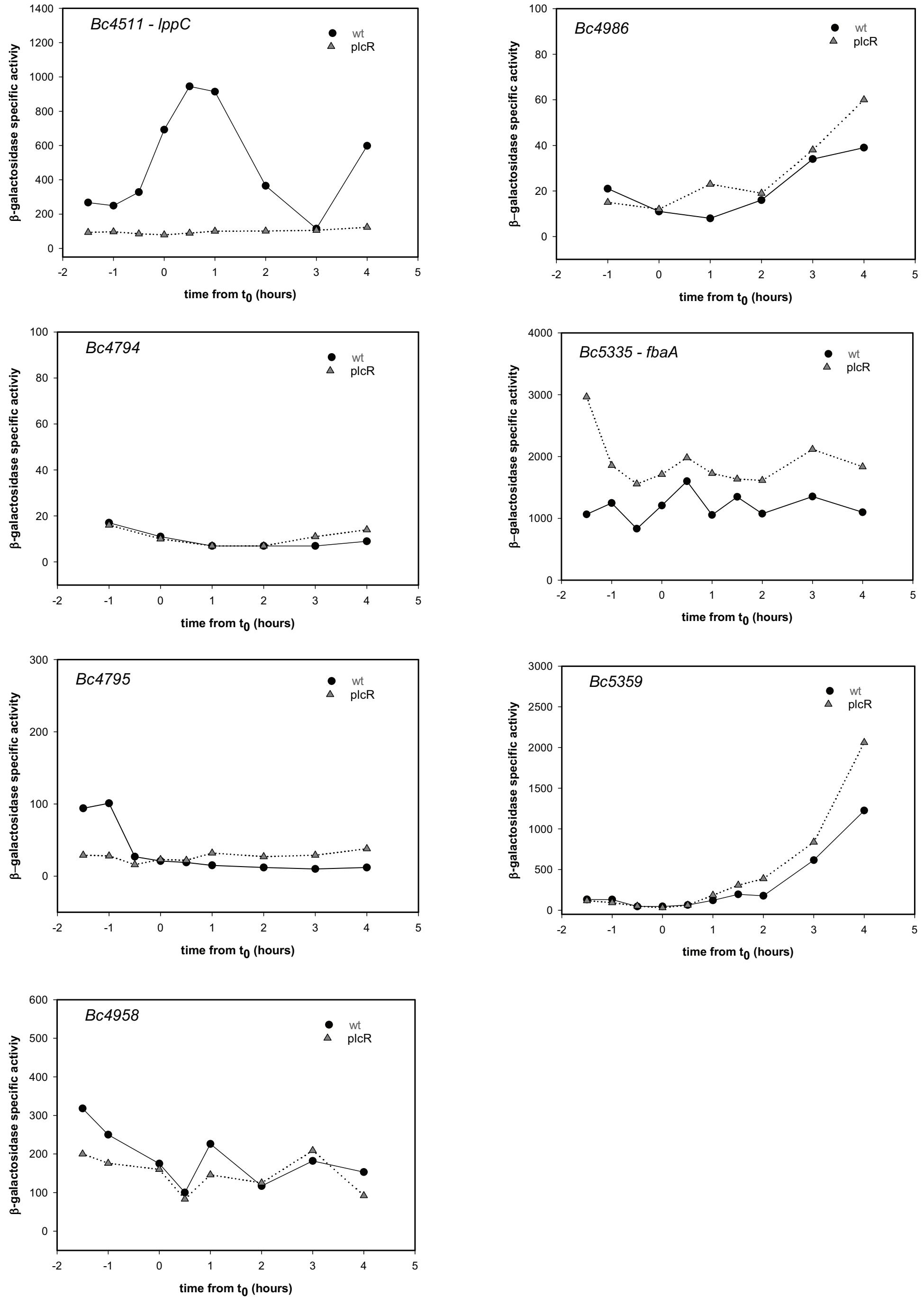


Figure S1: Results from *lacZ* fusions.
Transcriptional fusions were performed between genes promoter regions and *lacZ*. Beta-galactosidase activity is plotted as a function of time of for the ATCC14579 wild type and mutant strains.

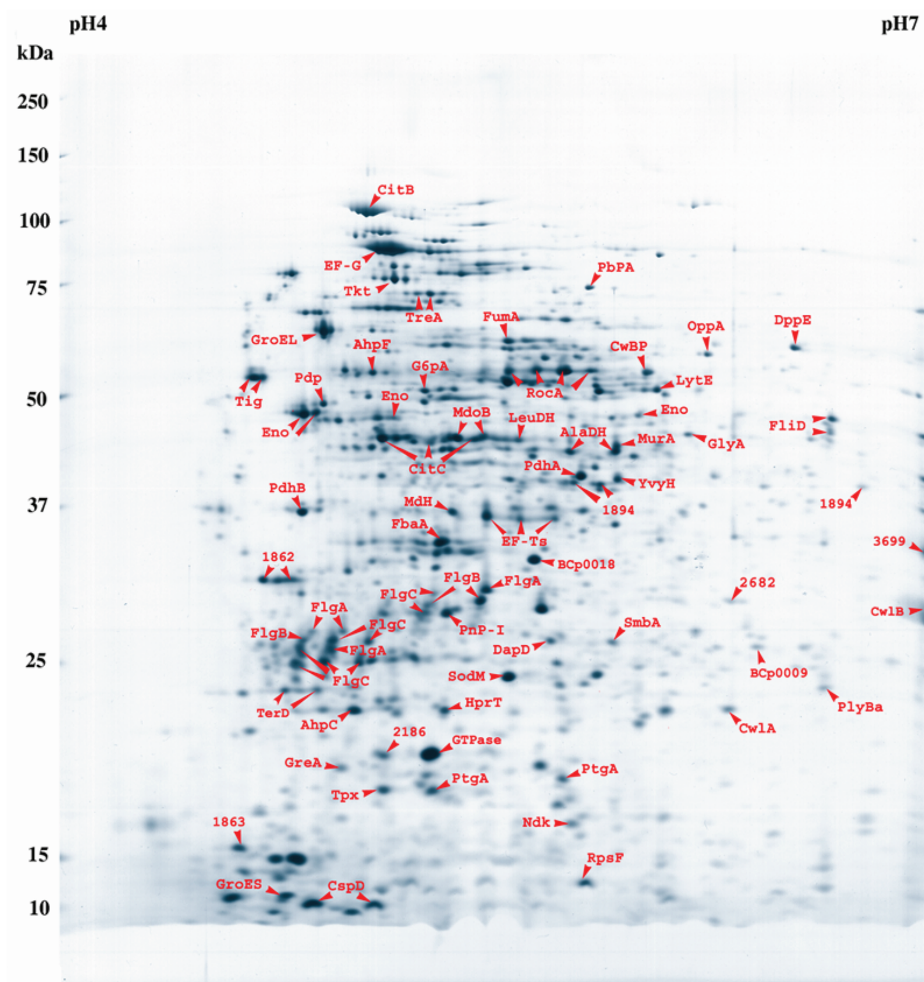


Figure S2: Two-dimensional gel electrophoresis of the Δ-plcR ATCC14579 supernatant.
The supernatant was harvested at t_2. The gel was silver-stained. In red, abbreviated name of the proteins present in the spots, and identified by mass spectrometry or by N-terminal sequencing (see table s3). Top scale: pH range; left scale: MW range

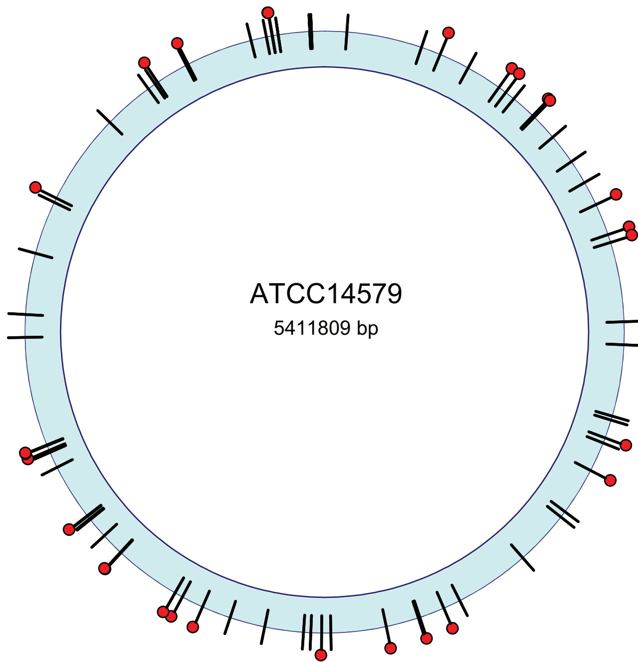
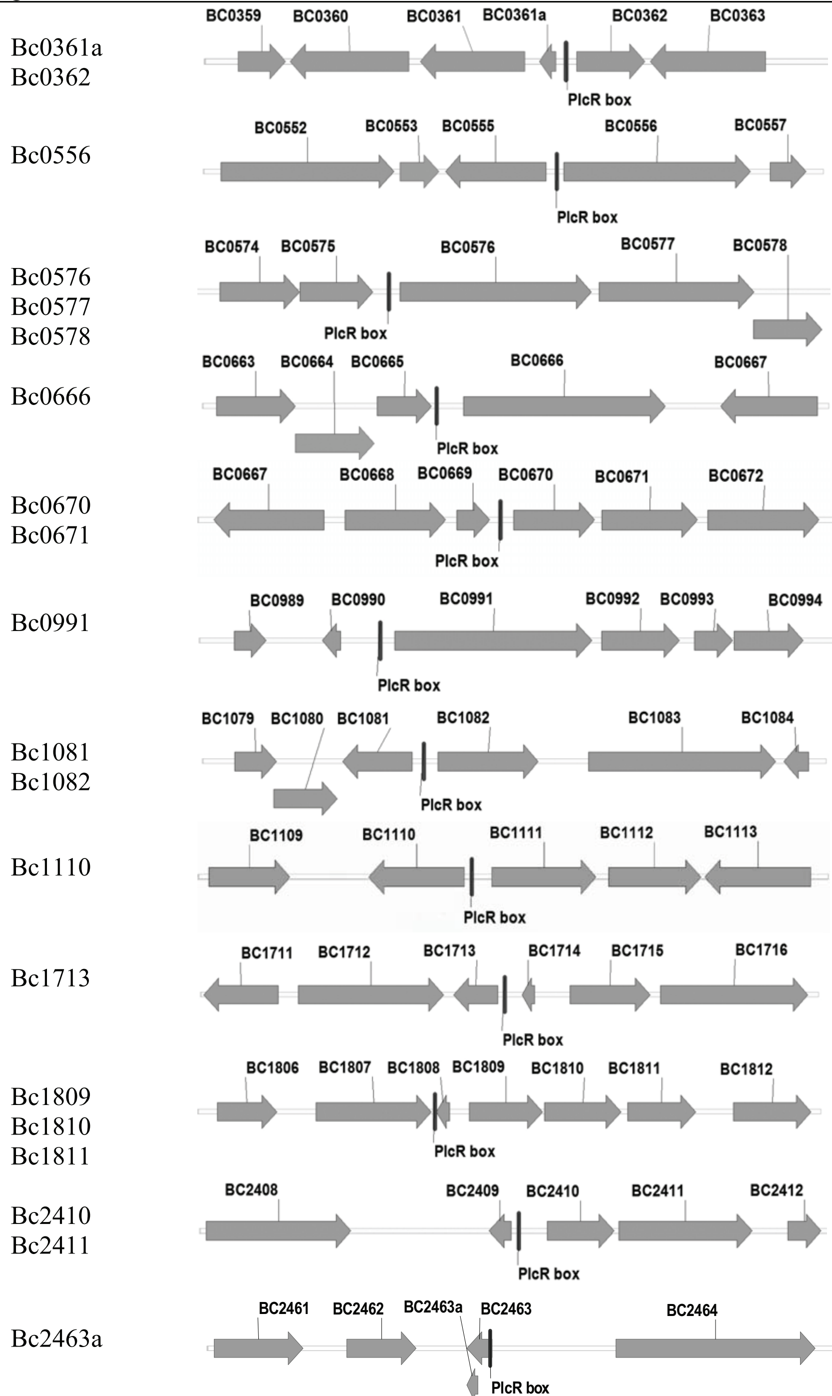
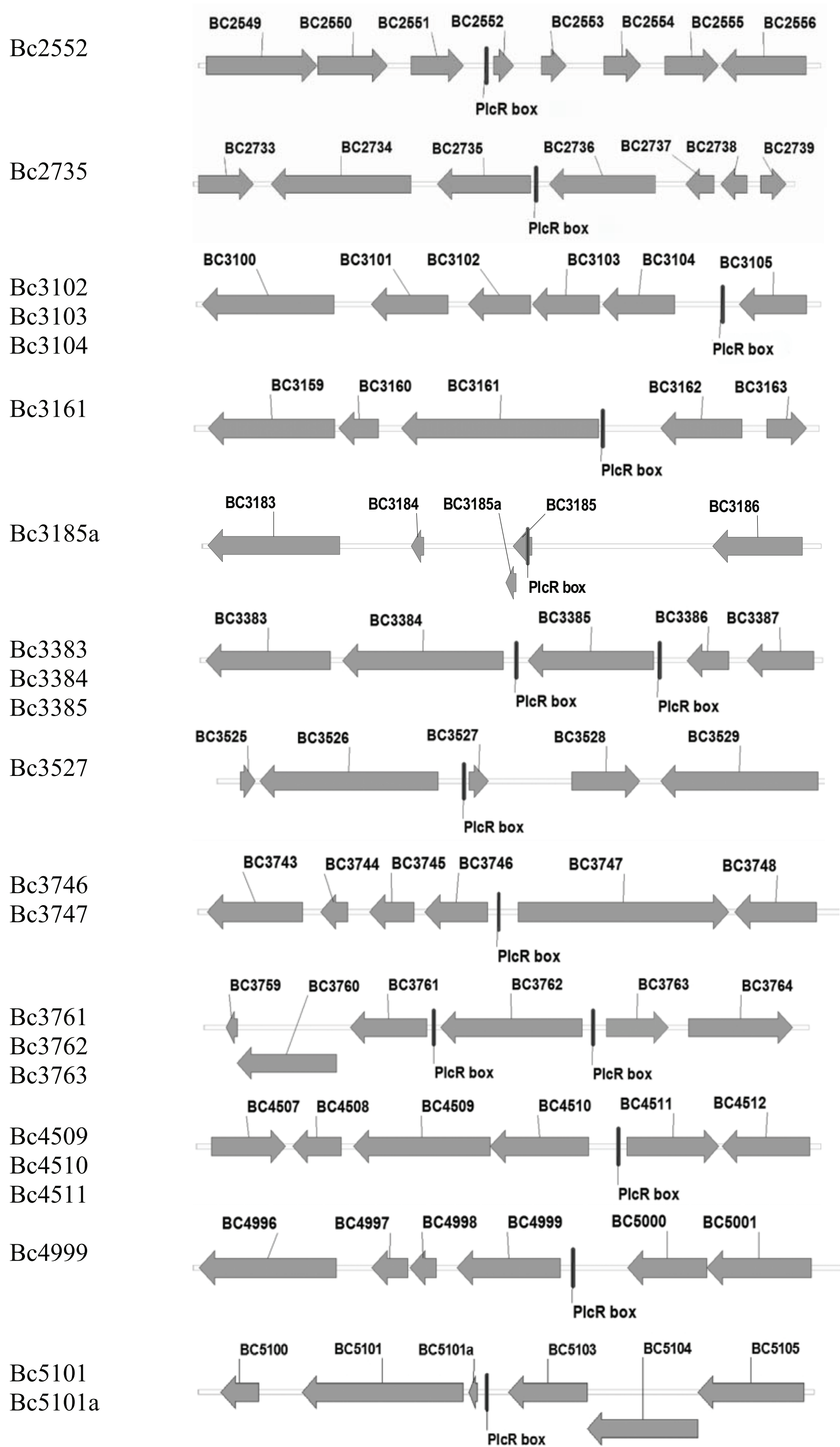


Figure S3: Location of PlcR boxes on the ATCC14579 chromosome.
Each dark line represents a PlcR box (active or inactive) and red circles show active PlcR boxes. The chromosome origin is placed on top of the blue circle.

PlcR-controlled genes	Genetic organization





Bc2552
BC2549
BC2550
BC2551
BC2552
BC2553
BC2554
BC2555
BC2556
PlcR box
Bc2735
BC2733
BC2734
BC2735
BC2736
BC2737
BC2738
BC2739
PlcR box
Bc3102
Bc3103
Bc3104
BC3100
BC3101
BC3102
BC3103
BC3104
BC3105
PlcR box
Bc3161
BC3159
BC3160
BC3161
BC3162
BC3163
PlcR box
Bc3185a
BC3183
BC3184
BC3185a
BC3185
BC3186
PlcR box
Bc3383
Bc3384
Bc3385
BC3383
BC3384
BC3385
BC3386
BC3387
PlcR box
PlcR box
Bc3527
BC3525
BC3526
BC3527
BC3528
BC3529
PlcR box
Bc3746
Bc3747
BC3743
BC3744
BC3745
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BC3747
BC3748
PlcR box
Bc3761
Bc3762
Bc3763
BC3759
BC3760
BC3761
BC3762
BC3763
BC3764
PlcR box
PlcR box
Bc4509
Bc4510
Bc4511
BC4507
BC4508
BC4509
BC4510
BC4511
BC4512
PlcR box
Bc4999
BC4996
BC4997
BC4998
BC4999
BC5000
BC5001
PlcR box
Bc5101
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BC5100
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BC5101a
BC5103
BC5104
BC5105
PlcR box

Bc5349
Bc5350
Bc5351

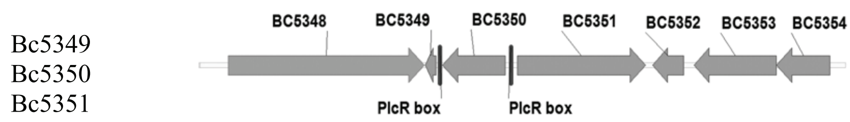

Figure S4: Genetic environment of the 45 PlcR-regulated genes.

Sequence	Gene
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TTGTGTATATGCTATGCATAATTGCATATGAGTTTAAAAATTATATCGATATAATTTTATCGGTTATTTGTAAGTAGTGTTTTCT	Bc1809
ATAAGGAGTGAATATGCAAAAATGCATAATTGACGAAAAAATTACATGTTATCATTATATATTGTTTTATATATGTTAATTGTAT	Bc0576
TACAAAAGACAATATGCAAATGTTCATATAAAAATATTATATTTTAATATATAATATAAAATGATTTTCTAACATCAAGGAGGAT	Bc4511
TAAAACGGCCAATATGCAATATTACATATTGAGAATCTTTTTACTCTTGATACAATATTCATACATCAAAATAAAAGGAATGTAT	Bc1081
CTAAAAAATGGGTATGCATAATTGCATATAATAGAGATAAATTTTCATGATATATTAAAATAAAAAATGTGGGTGATGGAATATG	Bc5349
TTGACCACAATTTATGTAATTTTGCATAATGTTACATAAATTGTGTATTTTCTCTTTTTTTAGACATTAATAGACAGTATACTTT	Bc2735
TTTTATATATATTATGCATTATTTCATATCAAAAATTGTCGAATTCACATTATTGTAGTGGTATGACAACTCAAAAAATTAGATT	Bc5350
AGCGGAATATATTATGCAATATTGCATTTGATAATCATTTTCACTGTAACTATAATGATTACAGGGTTACTATTAATCCAATATT	Bc3527
AATACATATCCTTATGCAAAATTGCATATTAATATGTATTCTTCTTACTTTAAAATTAGCTTAAATCACTTTCCAAATTATACAA	Bc0361a
AGGAAGAAATAATATGAAATATTGCATTTTATATTGTTACATCCAGATTTTATGTTAAATTCTTGAATGGAATATTAAAATAGGG	Bc0556
TCAAAACTCATCTATGCAATTATGCATAACCATCCATTCCGTTTTATTTTCATGTTACGATATAAATGTAATACGACATATATCG	Bc1110
AAAACGCGCGAATATGCAATTCTGCATATTCACACGTTTTATCACAATGTTATGATTTAATTTAGTTAAAGAACAAGTATACCGA	Bc1713
CTCATCATTTTATATGCAATTTTGCATCTCCACTTACAAATCACTAAAAAAACTTTACAATTCAAAGGATGACTAAATATACAAT	Bc3747
TTGTTAATACGATATGCAAATATGCATATCGTATTTTTTTGAGTTCTTTATGTGTTAAAATTAGATATAAAGATATGAAAATATA	Bc2552
TAAAAGAATTTTCATGCAATTTTGCATATTGTCTTTTAAATTTGTTATATAAGATATTTAATATATATCACATAACTCTTTATTT	Bc0666
GACAATTTTTGATATGAAATAATGCATAATATATATAAAATTCGGCAAATTTTTCATACAATTCATCTCACATACAATAATAGAT	Bc5351
CATGTAATTAAATATGAAATAATACATATTATAATAATTTATTAAGATAAAATGTTATATTTTTGGGTGGAATATATAAATGGAG	Bc3161
ATAATATTTTTATATGAACATTTGCATATTGTCTTTTGTATATATAGTAGTATGTCCTATATTAGAAAGATGAGCAAAATAATAA	Bc4510
AATGTATTTTCATATGCAATATTTCATATTCTGACTCTACACTAAACGAATATGAAACTTATAGTGATTATTTATTTTATATGTG	Bc3763
GTAGAGTCAGAATATGAAATATTGCATATGAAAATACATTGAAGTTCATATTTCTGATTACTATAATCTATTTAGTAGAAAGGGA	Bc3762
AAGTTTTCATAATATGTAAAATTGCATATTATTCTTTTTGAAAAAATTGTTATATTATCTATATATTTGTATTAAAGGATTTTTG	Bc4999
CTTGTTTTTTTATATGTAATATTGCATATCAGAGTGTATTGATAAAAGTGTAAGGGGTCACTATAATGAGGGCATAAAGATAAAT	Bc5101a
CTAATGTATAAATATGCAATATTGCATACGGAAATGTATTGTTATTATTGTAAGCGCTTACTATAATAAAGTTATAAAGATTTAT	Bc2463a
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TAACTTTACTTCTATGCAATATTTCATATTGATGAAATGTTATTTATTTTTTCTGAACTAGCTTATATTATATTCAATAAGTTTG	Bc3761
AGTAGTGGATGATATGCAAAATTGCATATTACAAAATAAACATCGTAATTTTTGGATTCTTGGTATAAAATACATTACTTAAACT	Bc3384
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AAGTTATAATGATATGAACATTTGCATATTTTAATTTATTGATAGAAATTTCATGAAAGGTGGGATATTCTAGTCATAGGTTAAC	Bc0670
TCCTTATAACAATATGAATTTTTGCATATTTTTAAATTCAATATATCCCTCAAAAACTTTTAATTGTATAATATATTTTGTCATA	Bc0991

Figure S5: Putative -10 σ^A boxes located downstream of PlcR boxes for PlcR-controlled genes. The presence of possible -35 boxes were considered for the determination of best -10 boxes locations. While there are 28 active PlcR boxes, 34 -10 σ^A boxes are shown because some of the PlcR boxes can work both in the forward and reverse directions.

II

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III

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IV

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