

UNIVERSITA' DEGLI STUDI DI VERONA

**Dottorato di ricerca in
biotecnologie applicate**

**Ciclo XXII
S.S.D.: AGR/07**

**Study of *Bacillus thuringiensis* behaviour
in food environment by genome – wide
transcriptome analysis**



**Tesi di dottorato di: Francesca Colla
Anno accademico 2009/2010**



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DIPARTIMENTO DI
BIOTECNOLOGIE

DOTTORATO DI RICERCA IN
BIOTECNOLOGIE APPLICATE

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Study of Bacillus thuringiensis behaviour in food environment by
genome – wide transcriptome analysis

S.S.D. AGR/07

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* Per l'elenco dei Settori Scientifico-Disciplinari (SSD) si veda il D.M. del 4 Ottobre 2000, Allegato A "Elenco dei Settori Scientifico –Disciplinari" reperibile sul sito del Ministero dell'Università e della Ricerca al seguente indirizzo: http://www.miur.it/atti/2000/alladm001004_01.htm

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CHAPTER 1

INTRODUCTION: THE GENUS *BACILLUS*



1. The genus *Bacillus*

In 1872, Ferdinand Cohn, characterized the bacterium *Bacillus subtilis*. This Gram-positive organism, capable of growth in the presence of oxygen, and able to form a unique type of resistant cell called endospore, represented the first member of what was to become a large and diverse genus of bacteria named *Bacillus*, in the Family *Bacillaceae*. The ubiquity and diversity of these bacteria, the resistance of their endospores to chemical and physical agents, the developmental cycle of endospore formation, the ability to produce antibiotics, the toxicity of their spores and protein crystals for many insects, have attracted ongoing interest since their discoveries in the 1870s (Kennet Todar, 2009).

1.1 Classification and Phylogeny

The heterogeneity in ecology, physiology, and genetics of *Bacillus* species made difficult to categorize the genus *Bacillus*. The modern concept of the genus *Bacillus* can be ascribed largely to the work of Nathan R. Smith, Francis E. Clark, and Ruth E. Gordon; in the 1930's these group of scientists, developed a definition of the genus *Bacillus* as comprising “rod-shaped bacteria capable of aerobically forming refractile endospores that are more resistant than vegetative cells to heat, drying, and other destructive agencies”. First attempts to classify *Bacillus* species were based on two main characteristics: aerobic growth and endospore formation. This resulted in grouping of many bacteria possessing different physiology and occupying a variety of habitats. In Bergey's Manual of Systematic Bacteriology (1st ed. 1986), the G+C content of known species of *Bacillus* is reported to range from 32 to 69% (Holt, 1986), illustrating the genomic heterogeneity of the genus. There are variation from species to species, but sometimes it can be observed profound differences in G+C content within strains of the same species. Phylogenetic classification reported in the Bergey's Manual of Systematic Bacteriology (2nd ed. 2004) groups the two most prominent types of endospore-forming bacteria, clostridia and bacilli, in the two different Classes of *Firmicutes*: *Clostridia* and *Bacilli*. The Phylogenetic evidence, mainly based upon RNA analysis of the small subunit of ribosomes (16S rDNA) indicated that *Bacillus* species showed a kinship with several non spore-forming bacteria like *Enterococcus*, *Lactobacillus*, *Listeria* and *Staphylococcus*. With the advent of ssRNA analysis, *Bacillus* genus, was divided into several families of endospore-forming currently assigned to four genera in the family *Bacillaceae*. Within this family, the genus *Bacillus*, is distinguish from the strictly

anaerobic *Clostridium* spp. for its ability to grow in the presence of air. Many *Bacillus* species can be allocated to one of six taxa that have distinguishable physiologies. This is generally consistent with the division of the genus based on spore morphologies. The six groups are: *B. polymyxa* group (I), *B. subtilis* group (II), *B. brevis* group (III), *B. sphaericus* group (IV), and thermophiles (V and VI). Group I includes species that are facultative anaerobes and can grow strongly in the absence of oxygen. A variety of sugars are fermented to produce acid, and endospores are ellipsoidal. Species belonging to the *B. subtilis* group, are phylogenetically and phenotypically consistent. All these bacteria produce acids from a wide range of sugars and some strains, like *B. cereus* and *B. licheniformis*, are facultative anaerobes. *B. licheniformis* can use glucose only under anaerobic conditions but grows poorly anaerobically. Although *B. subtilis* is generally considered an aerobe, it can grow and sporulate slowly also in anaerobic conditions. When glucose, with nitrite is the terminal electron acceptor, it grows strongly anaerobically. These bacteria are therefore an intermediate stage between the true facultative anaerobes of the group I strains and the strict aerobes in groups III and IV. This is reflected in their production of acid from several sugars (Leuschner, *Bacillus* - Central Science Laboratory, York, UK. 2008). The oval endospores produced by these bacteria do not swell the mother cell and are generally located centrally or subterminally. Group III represents strict aerobes that generally do not produce acid from sugars. They produce ellipsoidal spores that swell the mother cell. In group IV all species produce spherical spores that may swell the mother cell and contain l-lysine or ornithine in the cell wall. All species are strictly aerobic, but some have a limited ability to produce acids from sugars. Thermophilic species of the Group V are heterogeneous physiologically and morphologically, and grow optimally at $> 50^{\circ}$ C. Most produce oval spores that swell the mother cell. In group VI are thermophilic and acidophilic species which membranes are characterized by the presence of omega-alicyclic fatty acid.

2. General properties of bacilli

2.1 Structure surface of *Bacillus*

Like many gram-positive bacteria, the properties of adhesion, resistance and tactical responses, making the surface of *Bacillus* species rather complex.

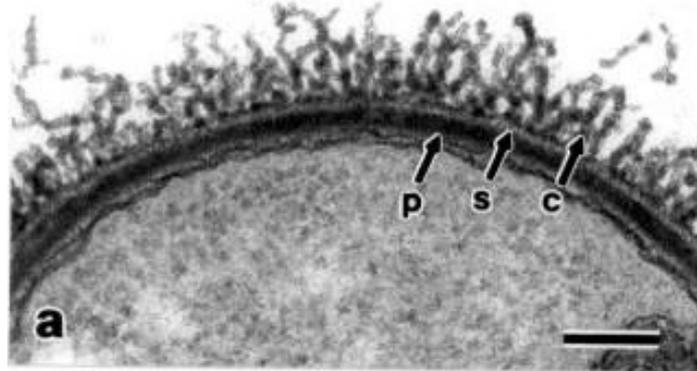


Fig.1 *Bacillus* surface. C=capsule, S=S-layer, P=peptidoglycan (from Kenneth Todar PhD.2009 University of Wisconsin-Madison).

The surface of vegetative cells is a laminated structure consisting of a capsule, a proteinaceous S-layer, several multi-layers peptidoglycan, and proteins located on the outer surface of the plasma membrane.

2.1.1 S-layers

S-layers consist in crystalline surface layers of protein or glycoprotein subunits. They can be found in *Bacillus*, like in other bacteria but their function is not completely understood. Since it covers the entire cell surface, it seems likely that it can act as a molecular sieve, preventing large molecules from entering or leaving the cell. Other roles which have been ascribed to bacterial S-layers include protection of the cell from predation and provision of attachment sites for exoenzymes. It has recently been shown that in some Gram-positive bacteria S-layers can mask the negative charge of the peptidoglycan sheets and prevent agglutination processes.

2.1.2 Capsules

Bacillus species can produce different types of capsules: those of *B. anthracis*, *B. subtilis*, *B. megaterium*, and *B. licheniformis*, contain poly-D- or L-glutamic acid. *B. circulans*, *B. megaterium*, *B. mycoides* and *B. pumilus*, produce carbohydrate capsules, or with more complex polysaccharides. Some polysaccharides produced by *Bacillus* may react with antisera of other genera of bacteria, including human pathogens: is the case of *B. mycoides* with *Streptococcus pneumoniae* or *B. pumilus* with *Neisseria meningitides*. The capsule is very important for determining virulence in *B. anthrax*: since it is not produced

by the closest *B. cereus* and *B. Thuringiensis*, can be used as a criterion for distinguishing between these species.

2.1.3 Cell wall

Bacillus genus does not present much variability in the structure of the cell wall as occur in many Gram-positive bacteria. The wall of all *Bacillus* species consists of peptidoglycan of the mesodiaminopimelic acid (DAP) (Weiss et al.1981). This type of polymer is the same type as the one universally found in Gram negative bacteria. DAP can be directly cross-linked to D-alanine, as in the *Enterobacteriaceae*; in other cases, like in most Gram-positive bacteria, two tetrapeptide side chains of peptidoglycan connect DAP and D-alanine, by an interpeptide bridge. The presence of teichoic acids bounded to muramic acid residues has been reported in large amount for all species. However, the type of teichoic acids varies widely between *Bacillus* species. As in many Gram-positive bacteria *Bacillus* species present lipoteichoic acids associated with the cell membrane, which seem to be involved in the synthesis of teichoic acids in the cell wall.

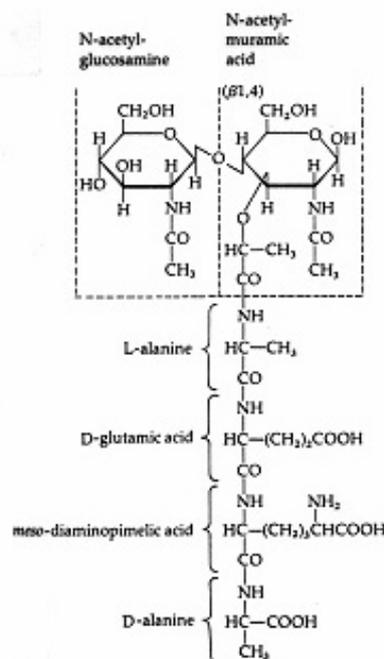


Fig.2 Schematic representation of muropeptide subunit of *Bacillus* peptidoglycan, without intrapeptide bridge for DPA and D-alanyne connection

2.1.4 Flagella

Most of the spore-forming aerobic bacteria, are mobile and possess peritrichous flagella that cell use to move in the environment in response to external stimuli through the chemotaxis mechanism; the composition of alkaliphile *Bacillus* species like *B. firmus*, present a low content in basic amino acids, thought to render cell more stable at pH value up to 11. Flagellar system and chemotaxis has been extensively studied in *B. subtilis*.

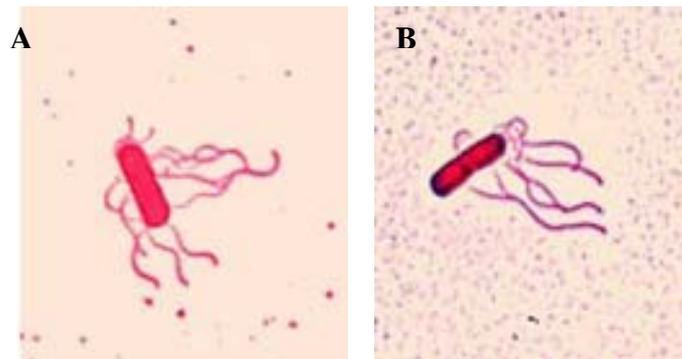


Fig.3 Flagellar strains: *B. cereus* (A) *B. brevis* (B) (from Kenneth Todar PhD.2009 University of Wisconsin-Madison).

2.2 Growth conditions and nutritional requirements

Spore-forming bacteria are generally chemoheterotrophs able to implement the process of respiration using a variety of simple organic compounds like sugars amino-acids and organic acids. In some cases they can ferment carbohydrates with reactions that produce glycerol and butanediol. Species such as *B. megaterium* need no organic factors for growth, while others require amino acids or vitamins. Most are mesophilic with an optimal of temperature growth between 30 and 45 degrees. Some species are thermophilic with optimal growth around 65 ° C. Psychrophiles species are few but are able to develop and sporify even at 0 ° C. *Bacillus* species can grow in a wide range of pH between 2 and 11. In laboratory environment, and optimal growing conditions, they present a regeneration time of about 25 minutes.

2.3 *Bacillus* endospore

2.3.1 Sporulation

Gram positive bacteria belonging to the *Bacillus* genus can undergo a complex developmental cell differentiation process what allows them to adapt to changing

environmental conditions and lack of nutrients by producing highly resistant spores. This process, called sporulation, involves progression through different stages including initiation, chromosome segregation, sporulation-specific cell division (asymmetric in rod-shaped bacteria), differential gene expression and specific signal transduction mechanisms. The return pathway, leading to vegetative cell growth, involves spore germination followed by outgrowth of the germinated spore. All of these aspects of sporulation have been studied for many years in great detail and have had both a substantial impact on our understanding of many other basic cell processes and have started to fuel applied spore research with new ideas.

In general, factors that may affect sporulation the ability to sporulate are pH, oxygen, and temperature. Sporulation appears to be favoured by conditions which result in a decreased growth rate in the presence of adequate energy and carbon source reserves. When sufficient nutrients are present, the vegetative cell divides rapidly by cell division, but environmental triggers like nutrient depletion and/or population density do initiate the sporulation process, ultimately resulting in the bacterial spore (Barak and Wilkinson, 2005; Eichenberger et al, 2004; Errington 2003; Piggot en Hilbert, 2004; Wang et al, 2006). Spore development involves an unequal cell division, the smaller cell (forespore) being engulfed by the larger one so that the endospore develops inside the mother cell. In many species, the cell is distended by the spore. Spore formation, which takes several hours, is accompanied by morphological, physiological, and biochemical changes, and the resulting refractile spore is structurally very different from a vegetative cell. The formation of a spore is an expensive and complex process for the bacterial cell. Spores are only made under conditions where cell survival is threatened such as starvation for certain nutrients or accumulation of toxic wastes. Regulation of sporulation is tight and the first few steps are reversible. This helps the cell conserve energy and only sporulate when necessary. Initiation of spore formation is controlled by Spo0A, a transcriptional factor which modulates gene expression during the transition from the exponential to the stationary phase. *Spo0A* is the response regulator of a two-component, signal transduction regulatory system, and in growing cells exists predominantly in the dephosphorylated state. Under conditions in which sporulation is initiated, it is phosphorylated by a phosphorelay involving a number of kinases and is thereby able to activate or repress gene expression by binding specific DNA targets (“0A boxes”) found upstream of regulated genes. Subsequently, changes in gene expression are controlled by the synthesis

and activation of alternative σ factors which associate with RNA polymerase and alter the promoter specificity of the enzyme. Five σ factors are known to be produced at various stages.

Sporulation is a seven step process during which cell will be dramatically reorganized. The first stages of sporulation are involved in forming a separate compartment for the spore in the mother cell. Activation of Spo0A and σ^H in the predivisional cell leads to asymmetric division. Once this occurs, sporulation is irreversible. The next stages involve laying down the various layers of the spore. Both the spore and the mother cell plays a role in this process. Within the two compartments, spore development is orchestrated by RNA polymerase σ^E regulated gene expression in the mother cell and σ^F regulated gene expression in the forespore. In the final stages, regulated by σ^K in the mother cell, and σ^G in the forespore (Wang et al, 2006), the spore dehydrates its cytoplasm while at this stage the mother cells lysis as a consequence of programmed cell death (Lewis, 2000), releasing the spore in the environment. The sporulation pathway is depicted in Figure 4.

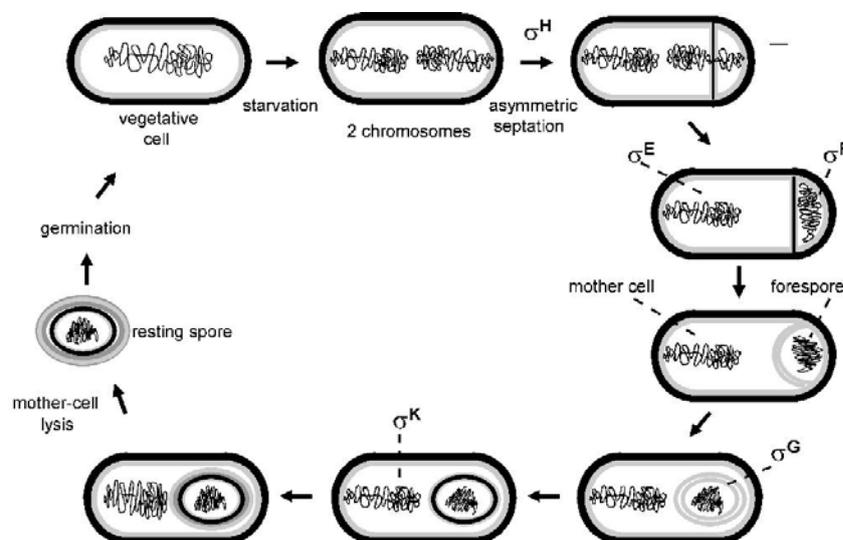


Fig. 4 The morphological stages of sporulation. Patrick Stragier. Annual Review of Genetics, 1996

2.3.2 The structure of bacterial spores

The complex structure of the spore protects the cellular compartment from environmental challenge providing a formidable resistance against harsh conditions (Table 1). Another typical spore property, crucial for its longevity is the spore dormancy. The cross section

of the spore in figure 5, reveals all the spore compartments including the core, the inner membrane, the cortex, the outer membrane and the coat layers followed by the exosporium.

Table 1Resistance capacity of growing cells and dormant spores of *B. subtilis*

Treatment	Treatments required to kill 90% of the population	
	Growing cells	Dormant spores
UV radiation (254 nm)(KJ m ⁻²)	36	330
Wet heat (90°C) (min)	<0.1	18
Dry heat (120°C) (min)	<0.01	18
H ₂ O ₂ (15% at 23°C) (min)	<0.2	50
Formaldehyde (25 g/l) (min)	<0.1	22
Nitrous acid (100 mmol/l) (min)	<0.2	100
Freeze dryings (number of cycles)	< 1	>20

Setlow, 2005

The outermost layer is the exosporium, which is a thin covering made of proteins. The exosporium is the primary site of contact with the environment, including host defences; it is not present on *B. subtilis* spores, but seems to be conserved among pathogenic bacilli on members of the *B. cereus* group. It is a loose-fitting, balloon-like structure composed of a paracrystalline basal layer and an external hair-like nap (Gerhardt, P., 1967). The filaments of the hair-like nap are apparently formed by a single collagen-like glycoprotein, whereas the basal layer is composed of a number of different proteins in tight and loose associations. The exosporium is the least understood part of the spore structure, but its presence on pathogenic Bacilli and, suggests a possible role in interactions with host organisms. Below there is the spore coat which is made up of highly cross-linked keratin and layers of spore-specific proteins. The role of many individual coat proteins remains unclear. The outer membrane is located between the cortex and inner coat layers. Its function is still not well elucidated, It is however an essential structure during formation of the spore (Piggot, 2004). The cortex consists of loosely cross-linked peptidoglycan. It is of important to maintain spore dormancy and

heat resistance, and is thought to contribute to the dehydrated state of the core (Nicholson et al, 2000 and references therein). The cortex composition is similar between many spore-forming bacteria, including clostridia (Atrih and Foster, 2001). During spore germination, the cortex must be degraded quickly to allow the expanding spore core. During spore dormancy, spore cortex lytic enzymes are present in the dormant spore although in an inactive state. The two crucial cortex lytic enzymes in *B. subtilis* spore germination are CwlJ and SleB. CwlJ is synthesized in the mother cell during sporulation, and located in the outer layers of the spore, whereas SleB, synthesized in the forespore, is targeted to both the spore inner membrane and the outer spore layers (Bagyan and Setlow, 2002; Chirakkal et al., 2002). We know that the SleB protein of *B. subtilis* is a muramidase but we do not yet know how it is activated during germination. Not all sporeformers have the SleB, CwlJ pair of germination-specific cortex lytic enzymes. The inner membrane, surrounds the spore core as selective permeability barrier. The germination receptors and gene products of the *SpoVA* operon, essential parts of the germination apparatus, (Vepachedu and Setlow, 2005) seems to be partly incorporated in this membrane. This suggested that the inner membrane plays an important role in the first stages of germination. Moreover, after the activation of the germination receptors, the inner membrane contributes in signal transduction directly to other parts of the spore as germination signal. Core contains the components of the vegetative bacterial cell (the cell wall, cytoplasmic membrane, cytoplasm, nucleoid, DNA, ribosomes, etc.) as well as significant quantities of dipicolinic acid and Ca^{2+} ions. The water content of endospores is only about 10-30% of the water content of vegetative cells; therefore, endospores are capable of surviving at levels of dehydration that would kill vegetative cells. The low water content also provides the endospore with chemical resistance (to chemicals such as hydrogen peroxide) and it causes the remaining enzymes of the spore cell to become inactive.

One chemical produced by endospores that is thought to lend to their high resistance is dipicolinic acid. This chemical has been found in the spore cell of all endospores examined. Dipicolinic acid interacts with calcium ions to form calcium dipicolinate (DPA), which is the main substance believed to lend endospores their resistance and represents about 10% of the dry weight of an endospore. The spore also contains small acid-soluble spore proteins (SASPs). These function to protect DNA from UV radiation,

desiccation and dry heat, and they also serve as a carbon and energy source during the germination process (conversion back to a vegetative cell).

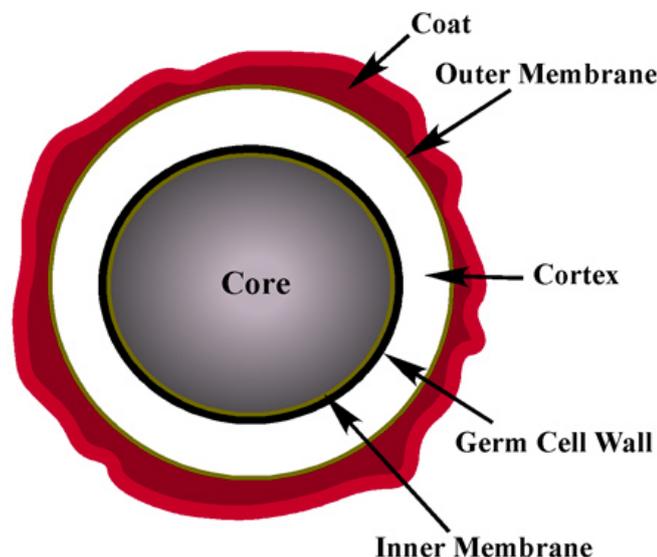


Fig.5 Schematically representation of the spore structure,(from Department of Microbiology, Cornell University. 2007)

2.3.3 Germination process

In addition to its intrinsic interest, spore germination has attracted applied interest, because it is through germination that spores ultimately cause food spoilage and poisoning (Setlow et al., 2003). In order to initiate germination and restore vegetative growth when conditions become favourable, bacterial spores must be able to monitor their external environment. Spore germination, as defined as those events that result in the loss of the spore-specific properties, is an essentially biophysical and degradative process (Moir and Smith, 1990). The spore's inner membrane increases in fluidity (Stewart et al., 1979) and ion fluxes resume; monovalent cations, potassium and sodium, move across the spore membrane, and calcium ions and dipicolinate are excreted. The peptidoglycan of the spore cortex is degraded, and the coat layers are partially degraded (Atrih et al., 1998; Atrih et al., 1999). ATP synthesis and oxidative metabolism resume (Otani et al., 1986), DNA damage is repaired (Nicholson et al., 1997) and the DNA-complexing small acid-soluble proteins (SASPs) are degraded by a specific protease (Nessi et al., 1998), providing a source of amino acids for outgrowth. It occurs without any need for new

macromolecular synthesis, so the apparatus required is already present in the mature dormant spore. Germination in response to specific chemical nutrients requires specific receptor proteins, located at the inner membrane of the spore. After penetrating the outer layers of spore coat and cortex, germinant interacts with its receptor: one early consequence of this binding is the movement of monovalent cations from the spore core, followed by Ca^{2+} and dipicolinic acid (DPA). Germinant molecules are able to activate these receptors, probably by allosteric interaction (Wolgamott and Durham, 1971). This initiates a cascade of processes that gradually degrade the protective structures of the spore and resume cellular processes and its metabolism, ultimately leading to the vegetative cell (Hornstra, 2007). In some species, an ion transport protein is also required for these early stages. Early events including loss of heat resistance, ion movements and partial rehydration of the spore core, can occur without cortex hydrolysis, although the latter is required for complete core rehydration and colony formation from a spore. In *B. subtilis* two crucial cortex lytic enzymes have been identified: one is CwlJ, which is DPA-responsive and is located at the cortex-coat junction. The second, SleB, is present both in outer layers and at the inner spore membrane, and is more resistant to wet heat than is CwlJ. Cortex hydrolysis leads to the complete rehydration of the spore core, and then enzyme activity within the spore protoplast resumes. We do not yet know what activates SleB activity in the spore, and neither do we have any information at all on how the spore coat is degraded (Moir, 2005).

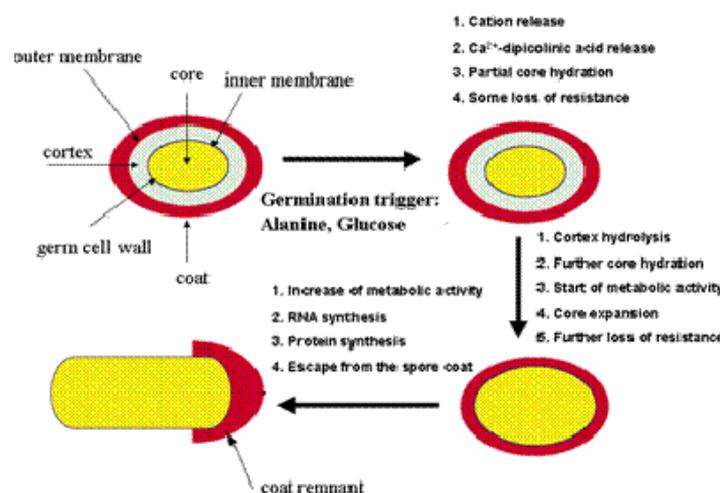


Fig. 6 *Bacillus* spore germination. Kort et al. 2005

2.3.4 Bacilli receptors

Bacillus spores are equipped with a specific set of germination receptors that monitor the environment for proper outgrowth conditions. As signalling molecules herein function germinants, often amino acids or ribosides, which are able to initiate germination when present in appropriate concentration and mixture in close proximity of the spore (Foerster and Foster, 1966; Gould, 1969). The process of germination involves interaction of chemical germinants with presumed specific receptors in the spore, and the transduction of this signal in some way. There is no evidence of bulk transport or metabolism of germinant (Scott and Ellar, 1978). The full molecular details of the signal transduction process in spore germination are not yet clear, but reasonable hypotheses can be constructed with the available information, most of which is derived from studies with *B. subtilis*. One hypothesis to explain the germination-associated changes is that the earliest events in germination would involve membrane changes that alter permeability properties, leading to a redistribution of ions and water in the spore, and thereby activate lytic enzymes (Keynan, 1978); evidence of inhibition of spore germination by ion channel blockers supports this (Mitchell, 1986), as does the likely membrane association of *gerA* gene products (Moir and Smith, 1990). The germinant has to first permeate the outer coat and cortex layers of the spore before coming in contact with the germinant receptors.

The *gerA* operon in the genome of *B. subtilis*, encoding for the germination (Ger) receptor GerA, was the first germination operon described (Zuberi et al, 1987), and was shown to be involved in L-alanine initiated germination. Later, *gerB* and *gerK* were described, both involved in a germination response on a mixture of L-asparagine, D-glucose, D-fructose and K⁺ (AGFK response) (Corfe et al, 1994).

Genomes of almost all sporeformers contain at least one, and usually several of these receptor operons, leading to the conclusion that sporeformers respond to different types of germinant via multiple receptors, encoded in gene clusters that have diverged from some common ancestor(s). Sometimes more than one receptor is involved in the response to single or multiple germinants (McCann et al., 1996; Barlass et al., 2002; Ireland and Hanna, 2002). The *gerA* operon, like most of its homologues, encodes three proteins, GerAA, GerAB and GerAC. These all have a potential association with the membrane – GerAA and AB are integral membrane proteins – GerAA has a predicted membrane-bound domain that would span the membrane at least five times, whereas GerAB is predicted to have 10 membrane spans, and is classified in evolutionary terms as a

subfamily of single component membrane transporters (Jack et al., 2000)(it is the only one of the three proteins that has homology to any other known protein outside the spore-forming bacteria). The GerAC protein is a predicted lipoprotein. All are expressed in the developing spore compartment, and would therefore be targeted to the inner membrane of the spore. Experiments using antibodies against GerAA and GerAC proteins showed that they were present in the inner membrane, rather than the outer layers of the spore (Hudson et al., 2001) and experiments on GerBA showed that this too was present in the inner membrane (Paidhungat and Setlow, 2001). Evidence that receptor proteins directly bind germinant comes only, so far, from analysis of mutant phenotypes. A germinant may bind without mediating transport, but causing allosteric changes within the membrane protein(s). Therefore, we need a better understanding of these receptor proteins, which has been hindered by the failure of attempts so far to overexpress and characterize the membrane-associated components.

3. Occurrence of *Bacillus* spp. in the environment

Members of the genus *Bacillus* have a ubiquitous environmental distribution. The endospore production is basic for the dispersion of *Bacillus* spp..The reservoir of these bacteria is the soil. Strains have been isolated from the extremes of deserts and Antarctic samples.. The extreme spore resistance capacities have amazed many scientists and have been studied, trying to reveal the mechanisms behind spore resistance (Nicholson et al, 2000 and references therein; Setlow, 2005). They have been proved to be the most durable type of cells found in nature: thanks to their dormant state, they can survive for extremely long periods, even millions of years. Because of this incredible resistance, the presence of spores may causes several problems wherever hygienic and sterile conditions are a prerequisite, such as in the food industry and in medical environments. Spores are able to resist most of the preservation techniques currently applied and as a consequence are responsible for infections, serious food-borne illnesses and significant amount of food spoilage (Brul et al, 2006). Industry has developed preservation methods to reduce the microbial contamination on food products. As a result of these efforts our food can be regarded as safe. Unfortunately, currently used methods are not fully effective against spores as a consequence of their incredible resistance capacities (Oomes and Brul, 2004). The omnipresence of *Bacillus* spores in the environment inevitably results in the presence of spores in agricultural and dairy products. In recent years, consumers have shifted their

preferences on “fresh-like” foods, since having better taste and texture characteristics, these products are expected to be also healthier. However, the use of milder food processing conditions, basal to accommodate these preferences, facilitate the presence of spores in food products, for frequently not completed inactivation of spore. Furthermore, the lack of microbial competition after the treatments, facilitates the rapid facilitates the rapid release of vegetative cells from germinating spores.

3.1 The *Bacillus cereus* group

The *Bacillus cereus* group comprises a highly homogeneous subdivision of the genus *Bacillus* that exhibit highly divergent pathogenic properties.

B. cereus belongs, together with *B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides* and *B. pseudomycoides*, to this group of closely related microorganism.

The reservoir of these bacteria is the soil, but they are widely distributed in the environment, or commensal inhabitants of the intestines of insects. Occasionally these species can cause food poisoning and soft tissue infections, particularly of the eye. *B. cereus* is an opportunistic human pathogen most commonly associated with food poisoning (Drobniowski et al. 1993). Other members of this group, currently classified as *B. thuringiensis*, are primarily insect pathogens widely used as a biopesticide (Schnepf et al 1998). A third pathogenic phenotype is exhibited by *B. anthracis*: it is the causal agent of anthrax, a zoonotic disease that can be lethal to humans. *B. mycoides*, *B. pseudomycoides* and psychrotolerant *B. weihenstephanensis* are less well characterized. Although the latter one, capable of growing efficiently at temperatures of 4°C, may form a hazard in food products stored at low temperatures (Hornstra 2007). The genome analysis of *B. weihenstephanensis*, revealed the presence of toxin genes (Stenfors et al, 2002), but has not yet been demonstrated its responsibility in food-borne disease.

Despite first studies on *B. cereus* group started in 19th century, the relationships between some of these organisms have yet to be completely resolved. The very high genetic relationship between *B. cereus*, *B. anthracis* and *B. thuringiensis*, makes genome based differentiation complicated or even impossible (Helgason et al, 2000; Ivanova et al, 2003). Conventional markers of chromosomal diversity, such as 16S and 23S rRNA genes, are essentially identical (Ash et al. 1991, 1992) . Several studies using different techniques like pulsed-field gel electrophoresis of chromosomal DNA (Carlson et al. 1994), genomic mapping (Carlson et al.1996), multilocus enzyme electrophoresis

(Helgason et al. 1998, 2000), BOX-PCR fingerprinting (Kim et al. 2001), multilocus sequence typing (MLST) (Helgason et al. 2004) and amplified fragment length polymorphism (AFLP) analysis (Ticknor et al. 2001), have also been suggested that these species are so closely related that they should be considered as one species.

On the other hand, some differences in terms of phenotype within these species, allow easy identification using classical methods; *B. cereus* and *B. thuringiensis* present hemolytic activity, are mobile, resistant to penicillin, and are able to degrade tyrosine and produce phosphatase, while *B. anthracis* does not show any of these characteristics. *B. thuringiensis* produces parasporal toxic crystals, known as δ -endotoxins, which allows to discriminate it from *B. cereus* (Schoeni and Wong, 2005 and references therein). Moreover, pathogenicity patterns of these species are very different. Most of the genes responsible for virulence of these bacteria are plasmid located. In some case, loss of the plasmid corresponds to loss of virulence, making impossible to distinguish between bacteria belonging to this group. The evolutionary relationships between all members of the group should be important, not only for understanding the evolution of virulence in the *B. cereus* group, but also for rapidly increasing of scientific and political importance that these organisms have acquired in recent years from.

3.2 *B. cereus* as pathogenic organism

B. cereus is an opportunistic human pathogen that can cause two types of food-borne infections. The emetic syndrome is caused by toxin production in the food product before consumption, while the diarrhoeal syndrome is the result of ingested *B. cereus* spores that germinate in the human intestine and produce enterotoxins in the intestinal tract (Granum, 2001; Schoeni and Wong, 2005).

3.2.1 Emetic syndrome

Emetic syndrome is a typical example of food intoxication caused by a toxin called cereulide, that lead to nausea and vomiting 1-6 hours after ingestion of contaminated food (Kramer en Gilbert, 1989; Ehling-Schulz et al, 2004). Similar symptoms are caused by *Staphylococcus aureus* enterotoxin (Granum and Lund, 1997). Cereulide is a heat and pH stable circular dodecadepsipeptide (Fig. 7) consisting of three repeating units of four amino acids, each consisting of D-O-leucine, D-alanine, L-O-valine and L-valine (Agata et al. 1994; Agata et al. 1995b). The structure resembles that of the known potassium

ionophore valinomycin (see Figure 7). Indeed cereulide has been shown to be toxic to mitochondria by acting as a potassium ionophore. Symptoms are generally mild, and patients recover within 24 h, but occasionally fatal cases resulting from emetic syndrome have been reported (Mahler et al, 1997).

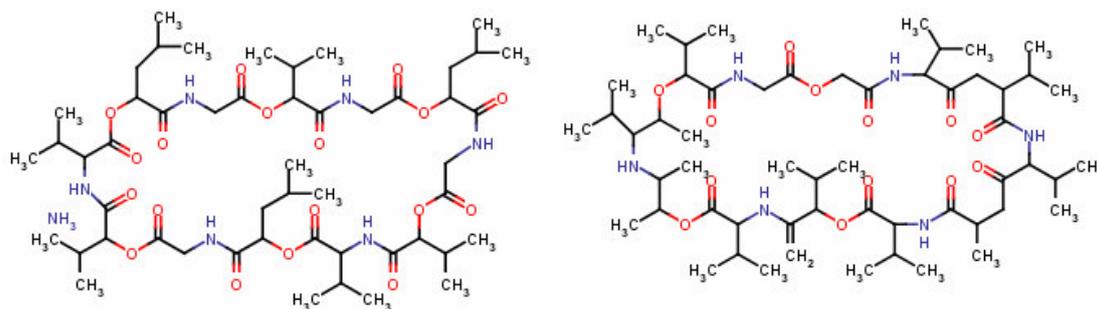


Fig.7 Comparison of the amino acid compositions of cereulide [D-Ala – D-O-Leu – L-Val – L-O-Val]₃ (left) and of valinomycin [D-Val – L-O-Ala – L-Val – D-O-Val]₃ (right) (Teplova et al. 2006)

In 2004 two research groups have shown that the production of cereulide in *B.cereus* is the result of a complicated mechanism operated by a non ribosomal peptide synthetase (NRPS) complex (Toh et al. 2004; Horwood et al. 2004).

3.2.2 Diarrhoeal syndrome

The diarrhoeal syndrome is caused by the production of enterotoxins in the small intestine after ingestion of food contaminated by *B.cereus* vegetative cells. This typical toxicoinfection is characterized by abdominal pain, cramps and diarrhoea, occurring 8 to 16h after ingestion (Granum and Lund, 1997). The enterotoxins cause disturbance of the water (solute transport) affecting the epithelial lining of the small intestine. Symptoms of the disease are very similar to those caused by the food-borne disease from *Clostridium perfringens*, but the pathogenic mechanism appears to be different: the *C. perfringens* enterotoxin is released during sporulation in the small intestine, whereas the enterotoxins of *B. cereus* are produced during growth in the small intestine (McClane, 1997; Granum, 2007).

During the 1980's and 1990's, with the discovery and identification of enterotoxins, many advances in the study of diarrheal syndrome have been possible. New molecular biology techniques allowed to acquire knowledges on the production and regulation of

enterotoxins. Based on epidemiological data it was estimated that $10^3 - 10^8$ cells per gram of food are sufficient for the manifestation of disease symptoms (Granum and Lund, 1997). Several virulence factors produced by *B. cereus* have been described, of which three-component enterotoxins hemolysin BL and non-hemolytic NHE are well characterized (Beecher and Wong, 1997; Lindback et al, 2004). Another single component toxin, enterotoxin T has been described (Agata et al, 1995) encoded by the *bceT* gene, but the role of this enterotoxin in *B. cereus* initiated food poisoning remains to be elucidated. Generally the symptoms associated with diarrheal syndrome are rather mild, but a strain of *B. cereus* producing CytK toxin, responsible for necrotic enteritis, caused the deaths of three people in France (Lund et al, 2000).

Haemolysin BL (HBL)

The first described enterotoxin in *B. cereus* is the hemolytic toxin BL (HBL). Because of its observed effects *in vivo* and *in vitro* (Kramer and Gilbert, 1989), it was initially defined diarrhoeagenic factor, fluid accumulation factor and vascular permeability factor (Shinagawa et al. 1991a, Shinagawa et al. 1991b, Sutherland and Limond, 1993). HBL is a three component protein toxin, encoded by 3 genes organized in 1 operon *hblA*, *hblC* and *hblD* genes encode the B, L1, and L2 components, respectively. A fourth gene has been found in this operon, *hblB*, but its function has not yet been defined. The molecular weight of B-component is 38 kDa, 40 kDa for the L1-component, and 45 kDa the L2-component.

Non haemolytic enterotoxin (NHE)

In 1996 investigation of over 300 strains from various sources, including strains from a number of outbreaks, revealed that another unknown enterotoxigenic complex, with cytotoxic effects, could have been the causative agent in some of the *B.cereus*-associated food-borne disease (Granum et al. 1996). This three component enterotoxin, named NHE, was discovered after an outbreak in Norway (Lund and Granum, 1996). Even though NHE contain several structural resemblances to HBL complex, and lead to symptoms similar to those caused by HBL, it lacked the haemolytic activity and the cytotoxic potential of the two three component is different (Lund and Granum, 1997).

Cytotoxin K

Cytotoxin K (CytK) may also be involved in *B. cereus* food poisoning. This toxin causes more severe diarrhoea including necrotic enteritis. It consists of a single protein toxin with a molecular weight of approximately 34 kDa. CytK belongs to a family of β -barrel channel-forming toxins (including *Staphylococcus aureus* leucocidins and *Clostridium perfringens* b-toxin). It is necrotic and haemolytic (Lund et al., 2000), and also cytotoxic for intestinal epithelia (Hardy et al., 2001). It was first characterized in *B. cereus* strain 91-98: a strain isolated from cases of food-borne disease that in France was responsible for the death of three people (Lund et al. 2000). None of the other, enterotoxin genes, previously described (*hbl* and *nhe*) was detected in this strain, further implicating CytK as a major virulence factor. However the cytotoxin K discovered in this outbreak, appeared to be the strongest form discovered so far. Later research detected a less potent cytotoxin K variant, also named cytotoxin K like or cytK-2, with approximately 89% amino acid homology to the original cytotoxin K (cytK-1) but 20% less toxic on human intestinal Caco-2 cells and Vero-cells. However, several *B. cereus* isolates possess the cytK gene, (Guinebretiere et al., 2002); although the mere presence of a gene possibly involved in virulence is not sufficient to confer pathogenicity, the transcription level of the gene could be important for virulence (Brillard and Lereclus, 2004).

enterotoxin T (Bc-D-ENT)

Another single component toxin, enterotoxin T (bc-D-ENT), has been described in *B. cereus* (Agata et al, 1995). The bc-D-ENT enterotoxin, encoded by the *bceT* gene, is capable of causing fluid accumulation in ligated rabbit ileal loops (Punyashthiti & Finkelstein, 1971), showing cytotoxicity towards Vero cells (Konowalchuk *et al.*, 1977). Unlike for the three first mentioned enterotoxins (Hbl, Nhe and CytK), has not yet been shown bc-D-ENT was involved in food poisoning. The role of this enterotoxin in *B. cereus* food poisoning remains to be elucidated.

3.2.3 Mode of action of the enterotoxins

The two three-component enterotoxins HBL and NHE present a similar mode of action (Lund and Granum, 1997). According to cytotoxicity experiments with VERO-cells all three components of the HBL-complex are necessary for maximal enterotoxic activity, (Powell, 1987; Rousset and Dubreuil, 2000; Belaiche, 2000; Black et al. 2005). The

optimal ratio of each component for HBL activity is 1:1:1 (Beecher et al. 1995). The latest model for the action of HBL studied by Beecher and Lee Wong (1997), suggested that all three components bind to the target cells leading to their lysis. Even for the activity of NHE toxin all three component complex are request, although in this case the optimal ratio is 10:10:1 (NHE-A : NHE-B : NHE-C) (Lindback et al. 2004).

The mode of action of cytotoxin K is different from that described for both HBL and NHE. The amino-acid sequence of cytotoxin K suggests that cytK belong to the β -barrel channel-forming protein family such as the β -toxin from *Clostridium perfringens* and α – and γ - haemolysin of *Staphylococcus aureus* (Hardy et al. 2001). Its symptoms include severe epithelial lesions and bloody diarrhoea.

3.2.4 Regulation of enterotoxin expression

More than one regulator system have been identified to be important in the *B. cereus* virulence regulation.

The transcriptional regulator PlcR (Phospholipase C Regulator) takes part in the control of most known virulence factors in *B. cereus*: enterotoxin, haemolysins, phospholipases and proteases (Michel et al 2008). It also regulates phospholipase C expression, then is called the phospholipase C regulator (PlcR). Transcription of PlcR is autoinduced (Lereclus et al 1996) and is repressed by the sporulation factor Spo0A (Lereclus et al 2000). To be active PlcR needs the PapR peptide. PapR 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 (Slamti et al. 2002, Gominet et al 2001, Declerck et al 2007). Thus, the three partners PlcR, OppABCDF and PapR function as a quorum-sensing system. PlcR integrates at least two classes of signals: cell growth state through Spo0A and self cell density through PapR (Michel et al 2008).

However, it seems that other systems may interact with PlcR, assuming a role in regulating the pattern expression of *B. cereus* virulence factors. Variability and adaptability, are crucial characteristics of all the organisms that possess the ability to survive and prosper in a wide variety of environmental conditions; often virulence factors allow them to conquer many different niches throughout the course of infection. Recognition of specific signals and conversion of this information into specific transcriptional responses, are basal to cope with a variety of environmental situations. In

many cases, signalling through a single two-component system results in a coordinated change in expression of multiple genes whose products play a role in adaptation to a particular environment. Several study focused on the importance of two-component signal transduction systems in controlling both metabolism and virulence factors in *B. cereus* (Duport et al. 2006). One of the major controlling factors of gene expression in *B. subtilis* during fermentative (Nakano et al 1997, Cruz Ramos et al 2000) microaerobic and aerobic growth (Hartig et al 2004) is ResDE two-component system; moreover it regulates virulence in *Staphylococcus aureus* under low-oxygen conditions (Yarwood et al 2001). Homologs of the *B. subtilis* ResDE was found in *B. cereus*. It was demonstrated that ResDE, play an important role in the regulation of enterotoxin expression in *B. cereus*. This two-component regulatory system consists of a histidine sensor kinase (*ResE*), bound to the cell membrane, and a cytoplasmic response regulator (*ResD*), (Fig.2). Signals related to oxygen limitation are perceived by ResE that undergoes autophosphorylation at a conserved histidine residue. ResD phosphorylation level is determined by the balance between both activities of ResE as phosphate donor for ResD, and phosphatase of phosphorylated ResD. In *B. cereus* *resE* mutant strain, abolition of enterotoxin production was observed in all the conditions examined (Duport et al 2006).

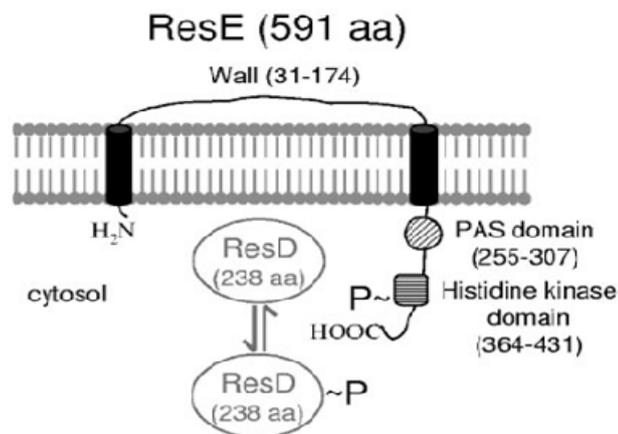


Fig. 8 Gene organization of the *B. cereus* chromosome region containing *resDE*. (Duport et al 2006.)

Subsequent studies described another redox regulator that may act in synergy with ResDE to control the expression of fermentation and enterotoxin genes, demonstrating that,

although important, ResDE is not essential for both fermentative metabolism and enterotoxin expression. This transcriptional regulator, known as CRP-Fnr (fumarate and nitrate reduction regulator), is member of the cyclic AMP receptor protein, and play an important role in modulating the expression of many metabolic genes in several facultative or strictly anaerobic bacteria (Korner et al 2003). Their functions also include the control of virulence factors (Baltes et al 2005, Bartolini et al 2006, Schmiel et al 2000). Furthermore, the one-component CRP-Fnr regulators are known to act coordinately with two-component regulators homologous to ResDE in response to two environmental signals: oxygen availability and the presence of alternative electron acceptors. CRP-Fnr family proteins, are characterized by a nucleotide-binding domain that extends from the N terminus over 170 residues to a C-terminally located helix-turn-helix structural motif. A short C-terminal sequence with four cysteine residues follow this DNA-binding domain. Three Cys residues from this C terminus together with one Cys residue from the central part of the protein bind a $[4\text{Fe-4S}]^{2+}$ center that serves as a redox sensor (Reents et al 2006). Transcription of *hbl* and *nhe* was dramatically (90%) down-regulated after CRP-Fnr mutation experiment in *B. cereus* strains (Duport et al. 2007).

The production of major virulence factors hemolysin BL (Hbl) and nonhemolytic enterotoxin (Nhe) in the food-borne pathogen *B. cereus*, seems to be regulated through complex mechanisms. A recent study led by Esbelin and colleagues (2009) clarified some aspects of the *B. cereus* virulence regulation, suggesting a strict interaction between the three system previously described. The response regulator ResD was shown to interact directly with promoter regions of the enterotoxin regulator genes *resDE*, *fnr* and *plcR* and the enterotoxin structural genes *nhe* and *hbl*, but with different affinities. Moreover, phosphorylation state of ResD results in a different target expression pattern. This finding led to the conclusion that enterotoxin expression and fermentative metabolism may be controlled coordinately at the transcription level. It was also clearly defined the role of ResDE two component system, as a sentinel capable of sensing redox changes, and coordinating responses that modulates *B. cereus* virulence.

3.3 Presence of toxin in other *Bacillus* spp.

In some instances, enterotoxin production from non-*B. cereus* species has been reported. Isolates of *B. circulans*, *B. lentus*, *B. licheniformis*, *B. mycoides*, *B. subtilis*, and *B.*

thuringiensis demonstrated positive results using a commercial RPLA assay (*Bacillus cereus* enterotoxin reverse passive latex agglutination) to detect the L2 component of the HBL-complex (Beattie & Williams 1999). Toxin production by two environmental strains of *B. pumilus* were reported by Hoult & Tuxford (1991).

Toxin production by other *Bacillus* spp. has largely been limited to that of *B. thuringiensis*, a member of the *B. cereus* group.

The species *B. thuringiensis*, *B. anthracis*, *B. cereus*, differ in 16S rRNA sequence by only nine nucleotides, leading many to the conclusion that these could be considered a single species (Ash et al. 1991). Phenotypic differences within this group are very few, but the pathogenicity patterns differ significantly. *B. thuringiensis*, is characterized by the presence of large crystalline endotoxin molecules which form during sporulation. The toxin is insect-specific, and several classes of these toxin molecule, that target particular order of insects, were isolated (Schnepf et al. 1998). However conjugative transfers of many plasmids among *Bacillus cereus* and *B. thuringiensis* are demonstrated (Yuan et al. 2007, Van der Auwera et al. 2007). *B. cereus* and *B. thuringiensis* are not able to be differentiated strictly on the basis of biochemical characteristics (Carlson et al. 1994, Damgaard et al. 1996, Yamada et al. 1999). Standard methods for detection of *B. cereus*, have been failed to distinguish these two organisms. Damgaard et al. (1996) isolated several enterotoxin-producing strains of *B. thuringiensis* from pasta, bread, and milk. Perani et al. (1998) found that 29% of *B. thuringiensis* strains isolated from the environment produced *B. cereus*-like enterotoxins. From the reports mentioned above emerged that an exhaustive investigation into the ubiquity of enterotoxin genes in various *Bacillus* spp. has not been done. Little attention has been paid, either in model systems or in food environment, to assess the conditions that could support toxin expression in non-*B. cereus* isolates.

4. *Bacillus thuringiensis*

4.1 General characteristics

Bacillus thuringiensis, like the food-borne and opportunistic pathogen *Bacillus cereus*, belong to the *Bacillus cereus sensu lato* family. In 1901, a Japanese biologist, Ishiwata Shigetane, discovered a previously not described bacterium as the causative agent of a disease in silkworms. *B. thuringiensis* was originally considered a risk for silkworm rearing but it has become the heart of microbial insect control. It can form a parasporal

crystal during the stationary phase of its growth cycle and was initially characterized as an insect pathogen. In 1956, T. Angus demonstrated that the insecticidal activity was attributed largely or completely (depending on the insect) to crystalline protein inclusions formed in the course of sporulation. This observation led to the development of bioinsecticides based on *B. thuringiensis* to control certain insect species, especially among the orders Lepidoptera, Diptera, and Coleoptera. The earliest commercial production began in France in 1938, under the name Sporeine. In 1982, Gonzalez et al. revealed that the genes coding for crystal proteins were harboured on transmissible plasmids. Schnepf and Whiteley (1981) first cloned and characterized the genes coding for crystal proteins (*cry*) from plasmid DNA of *B. thuringiensis* subsp. *kurstaki* HD-1, toxic to larvae of tobacco. This bacterium has quickly become of commercial interest as useful alternative or supplement to synthetic chemical pesticides in forestry and agriculture, and it is now the most widely used biologically produced pest control agent. In 1995, *B. thuringiensis*-based bioinsecticides represented about 2% of the total global insecticide market.

4.2 Ecology and serotyping

B. thuringiensis seems to be indigenous to many environments (Chaufaux et al. 1997 Martin et al. 1989). Strains have been isolated worldwide from many habitats, including soil (Hastowo et al. 1992, Martin et al. 1989), insects (Carozzi et al 1991), stored-product dust (Burgess et al. 1977, Meadows et al. 1992). Isolation typically involves heat treatment for spores selection. Studies on *B. thuringiensis* spores persistence in the laboratory, field or forest environment revealed that, although rapid declines in population and toxicity have been noted, *B. thuringiensis* spores can survive for many years after spray applications (Addison et al. 1993).

For the identification and classification of *B. thuringiensis* strains H serotyping, based on the immunological reaction to the bacterial flagellar antigen, flagellin, has been established as a typing method (de Barjac et al. 1962). Today, the widely diverse *B. thuringiensis* strains are classified into more than 69 different H serotypes (Lecadet et al. 1999) and 13 sub-antigenic groups, giving 82 serovars, have been defined as subspecies. Although serotyping is the most common classification method used throughout the world, it has limitations since only reflects one characteristic of the species and prove unreliable as a predictor of insecticidal activity. The production of the parasporal crystal,

which defines the quality of *B. thuringiensis*, is rather too narrow a criterion for taxonomic classification (Lysenko et al. 1983). The frequent isolation from the same serotype strain, of several new strains, having innate *cry* genes that were not known previously, also demonstrated that H-serotyping might not be enough to represent the molecular characteristics of *B. thuringiensis* species.

4.3 *B. thuringiensis* Cry proteins

Individual Cry toxin has a defined spectrum of insecticidal activity, usually restricted to a few species in one particular order of Lepidoptera (butterflies and moths), Diptera (flies and mosquitoes), Coleoptera (beetles and weevils) and nematodes.

Natural isolates of *B. thuringiensis* can produce several different crystal proteins; certain combinations of Cry proteins have been shown to exhibit synergistic effects. On the other hand different target specificity could be perhaps even undesirable, (Hofte et al. 1989, Lambert et al. 1992).

The toxins were originally classified into four classes according to their amino acid sequence homology and insecticidal specificities (Hofte et al. 1989). CryI toxins are toxic to lepidopterans; CryIIs are toxic to lepidopterans and dipterans; CryIIIs are toxic to coleopterans; CryIVs are toxic to dipterans. CryV and CryVI classes, were added for the toxins active against nematode (Feitelson et al. 1992). Each new protoxin discovered, acquires a name consisting of the mnemonic Cry and four hierarchical ranks (consisting of numbers) (e.g., Cry25Aa1), depending on its place in a phylogenetic tree (Crickmore et al. 1998).

The ongoing discovery of new *B. thuringiensis* toxin genes and rapid accumulation of information on their insecticidal activities has prompted the construction of a database on "*Bt toxin specificity*": "The *Bacillus thuringiensis* toxin specificity database" <http://www.glf.cfs.nrcan.gc.ca/Bacillus>. A 500 delta-endotoxin list with corresponding access name for NCBI database sequences, is available in this site; biological specificity is also a component of the original nomenclature.

4.4 Mode of action of *B. thuringiensis* Cry proteins

During the sporulation process, *B. thuringiensis* cells produce parasporal crystalline inclusions containing polypeptides (δ -endotoxins). These protoxins, plasmid encoded by *cry* genes, present molecular masses ranging from 50 to 140 kDa and are toxic to a variety

insect species (Angsuthanasombat et al. 1993). Upon ingestion by the susceptible insect larvae, these inclusions are solubilised in the alkaline environment of the midgut and proteolytically digested to release the toxic fragments (Brown et al. 1990). During this proteolytic activation, δ -endotoxins undergo extensive proteolysis at both their *C* and *N* termini to produce a mature toxic moiety that has a molecular mass of approximately 60 kDa. A multistage process is generally accepted to describe the mode of action of Cry toxin. First, the activated toxins, then pass through the peritrophic matrix, binds to highly specific receptors located on the apical microvillus membrane of epithelial midgut cells (Bravo et al. 1992, Hofmann et al. 1988). After toxin binding to the receptor, a change in the toxin's conformation, allow toxin insertion into the membrane. Perhaps, following an oligomerization, the toxin oligomer induces the formation of a lytic pore in the midgut epithelial membrane that that leads to osmotic cell lysis , cessation of feeding, and death of the larva (Sacchi et al. 1986, Lorence et al. 1995). Receptor binding is a key factor in specificity of activated Cry toxins The activated toxin readily binds to specific receptors on the apical brush border of the midgut microvilli. Two different insect proteins have been identified as receptors for Cry toxins: a 120-kDa aminopeptidase N (APN), of also called Cry1Ac toxin-binding protein, and the 210-kDa cadherin-like glycoprotein, called Cry1Ab toxin-binding protein, each purified from brush border vesicles of susceptible. Insect glycolipids were additionally suggested as a receptor in nematodes (Griffitts et al. 2005). Recent data suggest that toxicity is correlates with irreversible binding that could reflect a tighter interaction of the toxin with the receptor or might be related to insertion of the toxin into the membrane (Liang et al. 1995).

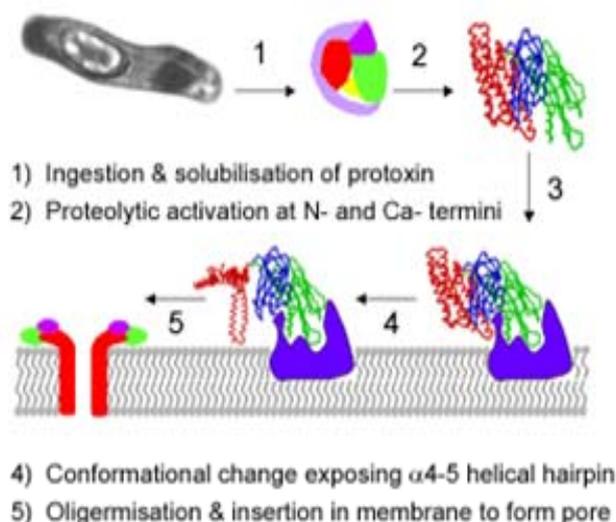


Fig.9 Proposed mode of action for cry toxin

4.5 Transcriptional mechanisms of *cry* gene

The expression of *cry* genes is considered to be largely sporulation dependent. The development of sporulation, is controlled at the transcriptional level by the successive activation of σ -factors, which bind the core RNA polymerase to allow the transcription of sporulation-specific promoters. σ A are the primary sigma factor of vegetative cells; five factors called σ H, σ F, σ E, σ G, and σ K, appear in that order in a temporally regulated fashion during development of *B. thuringiensis* cell cycle. Several *cry* gene promoters have been identified, and their sequences have been previously determined (Yoshisue et al. 1993, Dervyn et al. 1995, Brizzard et al. 1991, Brown et al. 1993). Consensus sequences recognized by *B. thuringiensis* RNA polymerase containing σ E or σ K, were found after alignment of promoter regions of these genes (Agaisse et al. 1995, Baum et al. 1995). The results are that is likely to be σ E or σ K -dependent. Low-level of *cry* genes transcripts has been also detected during the transition phase of *B. thuringiensis* biological cycle, lasting until the onset of sporulation (Poncet et al. 1997, Yoshisue et al. 1995). It is thought that this expression may be due to the σ H RNA polymerase, and it is suggested that Spo0A represses this weak transition phase expression, when the cells enter the sporulation phase (Poncet et al. 1997). One case of *cry* gene expressed during vegetative growth was described (Malvar et al. 1994, Sekar et al. 1988). The *cry3Aa* gene expression, isolated from the coleopteran-active *B. thuringiensis* var. *tenebrionis* seems to

be activated by a non-sporulation-dependent mechanism. The *cry3Aa* gene promoter, resembles promoters recognized by the primary sigma factor of vegetative cells, σ_A .

4.6 Development of *B. thuringiensis* biopesticides

First Insecticidal *B. thuringiensis* products were commercialized in France in the late 1930s (Lambert et al. 1992). By 1995, the U.S. Environmental Protection Agency (EPA), registered 182 Bt-based products, but in 1999 constituted less than two percent of the total sales of all insecticides (Carpenter et al. 2001, EPA et al 2001). As insect pests have become resistant to chemical insecticides. The use of Bt has strongly increased. As reported by Beegle and Yamamoto (Beegle et al. 1992), the early Bt formulations presented several problems. Standardization was based on spore count rather than potency, the products often contained subsp. *thuringiensis* of low potency.

After serotyping of Kurstaki HD-1 by Barjac and Lemille, this *B. thuringiensis* subsp. became the basis for products competitive with chemical insecticides for performance and cost. For many years, all of the *B. thuringiensis* companies produced only subsp. *kurstaki*. However, other varieties, such as the Coleoptera-active Bt subsp. *Tenebrionis* (Krieg et al. 1983) and the Diptera-active subsp. *israelensis* (Goldberg et al. 1977), have come to be used worldwide for the control of larvae of pest. Today *B. thuringiensis* subsp. *israelensis* applications comprise up to 50% of all insecticide applications. The relevant works of screening and isolation of new *B. thuringiensis* strains performed during years, finally resulted in the production of insect specific commercial products. Some of the most frequently used are listed in table 2.

Table 2 Bt-based biopesticide active ingredients and products (ROH et al. 2007)

Bt subspecies	Strain	Product name	Company	Target insect ^b
<i>kurstaki</i>	– ^c	Foray	Valent Bioscience Co.	Lepidopteran
	–	Biobit	Valent Bioscience Co.	Lepidopteran
	BMP123	BMP123	Becker Microbial Products, Inc.	Lepidopteran
	EG2348	Condor	Ecogen Inc.	Lepidopteran
	EG2371	Cutlass	Ecogen Inc.	Lepidopteran
	ABTS-351	Dipel	Valent Bioscience Co.	Lepidopteran
	EG7481	Crymax	Ecogen Inc.	Lepidopteran
	EG7826	Lepinox	Ecogen Inc.	Lepidopteran
	SA-11	Javelin	Certis USA	Lepidopteran
	SA-12	Thuricide	Certis USA	Lepidopteran
	<i>aizawai</i>	GC-91	Agree	Certis USA
NB200		Florbac	Valent Bioscience Co.	DBM, AW
ABTS		XenTari	Valent Bioscience Co.	DBM, AW
NT0423		Tobaggi ^d	Dongbu Hannong Chemicals	DBM, AW
GB413		Solbichae ^d	Green Biotech Co.	DBM, AW
<i>israelensis</i>	–	Gnatrol	Valent Bioscience Co.	Mushroom fly
	–	Bactimos	Valent Bioscience Co.	Mosquito & BF
	AM-65-52	VectoBac	Valent Bioscience Co.	Mosquito & BF
	SA3A	Teknar	Valent Bioscience Co.	Mosquito & BF
	BMP 144	BMP	Becker Microbial Products, Inc.	Mosquito & BF
<i>tenebrionis</i>	NB-176	Novodor	Valent Bioscience Co.	CPB, ELB

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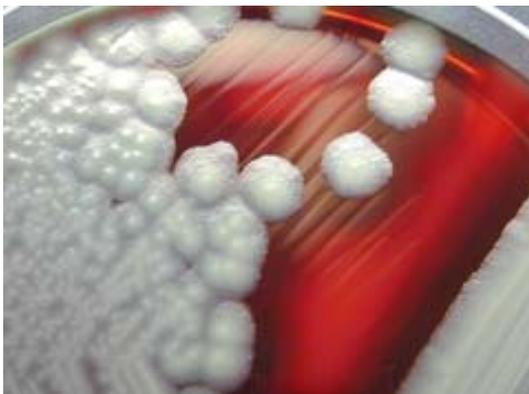
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DISTRIBUTION AND EXPRESSION PROFILES OF
GENES CODING FOR
BACILLUS CEREUS-LIKE ENTEROTOXINS IN
BACILLUS THURINGIENSIS STRAINS OF
COMMERCIAL INTEREST



1. Introduction

Bacillus thuringiensis, is a ubiquitous gram-positive, spore-forming bacterium known to exhibit specific insecticidal activities against certain insects, especially within the orders Lepidoptera, Diptera, and Coleoptera; it forms a parasporal crystal during the stationary phase of its growth cycle: these toxins, the so-called crystal proteins (Cry protein) or δ -endotoxins, are plasmid encoded by different *cry*-type genes. Many different genes encoding the *B. thuringiensis* endotoxins have been isolated and characterized. The high specificity of the Cry proteins against insects is mainly due to specific receptors in the insect gut, which are not present in the mammalian gut: these toxins are therefore considered harmless to humans. Due to its specific activity against insects and minimal environmental impact, *Bacillus thuringiensis* is used extensively around the world as a pesticide in forestry and agriculture as useful alternative or supplement to synthetic chemical pesticides (Schnepf et al. 1998): it is therefore of great commercial interest.

Bacillus thuringiensis, like the foodborne and human opportunistic pathogen *B.cereus*, belongs to the *Bacillus cereus* sensu latu family.

It is well known that *B. cereus* is associated with two forms of human food poisoning, the emetic and the diarrhoeal syndrome; the pathogenic mechanism of the emetic syndrome is a typical example of food intoxication: *B. cereus* can grow in food prior to consumption producing the emetic toxin (cereulide), a heat and pH stable circular dodecadepsipeptide that disrupt the energy production by mitochondria, passing the mitochondrial membrane and inducing vacuolization of cells (Mikkola et al. 1999). The main symptoms are nausea and vomiting, similar to the symptoms caused by *Staphylococcus aureus* enterotoxin (Granum and Lund, 1997). The diarrhoeal syndrome is a typical example of a toxicoinfection, caused by enterotoxins after the ingestion of food contaminated with *Bacillus cereus*; the enterotoxins affect the epithelial lining of the small intestine causing disturbance of the water solute transport: this may lead to diarrhoea and abdominal cramps within 8-16 hours after consumption of contaminated food. At least four different enterotoxins have been characterized: the three-component haemolysin BL (HBL), the non haemolytic enterotoxin E (NHE), the one-component hemolytic toxins CytK and the bc-D- ENT enterotoxin T (Granum and Lund 1997, Hansen et al., 2003).

The tripartite hemolytic heat-labile enterotoxin HBL, is the product of an operon that includes *hblA*, *hblD*, and *hblC* genes, which respectively encode the binding subunit (B) and the L₁ and L₂ lytic components (Phelps et al 2002). The subunits of the *B. cereus*

NHE also include two lytic components, NH₁ and NH₂, and a third gene product that remains uncharacterized. A third enterotoxin, the so called bc-D-ENT has been described; it is a single component protein enterotoxin with activity towards vascular permeability, composed of a single 41-kDa subunit: the exact role of this toxin is still unclear compared to what is known about HBL and NHE enterotoxins. A fourth enterotoxin, cytotoxin K (CytK), is a single-component toxin once reported to be involved in a severe food poisoning case that caused the deaths of three individuals (Lund et al. 2000).

Recent studies showed that most of these genetic determinant have been found frequently not only in *B. cereus* strains, but also in *B. thuringiensis* strains (Gaviria Rivera et al. 2000, Hansen and Hendriksen, 2001), while the emetic toxin, whose synthetase gene cluster is located on a pXO1-like virulence plasmid, has been found in both *B. cereus* and *B. weihenstephanensis* strains (Thorsen et al. 2006), but not to date in *B. thuringiensis* (Ehling-Schulz et al., 2006). Classical biochemical and morphological methods of classifying bacteria have failed to distinguish *B. thuringiensis* from *B. cereus* which are closely related and indistinguishable phenotypically and genetically except that the former harbours insecticidal plasmid (Schnepf et al 1997, Logan et al. 1984, Priest et al. 1988). However, conjugative transfers of many plasmids among *Bacillus cereus* sensu latu family are demonstrated (Yuan et al. 2007, Van der Auwera et al. 2007) especially between *B. cereus* and *B. thuringiensis*, in soil, insect larvae, culture media and foodstuffs (Battisti et al.1985, Yuan et al. 2007, Van der Auwera et al. 2007). Despite these knowledges, because of its strong insecticidal activities, plant protection products based on selected strains of *B. thuringiensis* are used worldwide in the production of fruits and vegetables in greenhouses and in the field.

B. thuringiensis strains has been isolated from pasta, pitta bread and milk (Damgaard et al. 1996), several ready-to-eat foods (Rosenquist et al. 2005), fresh fruits and vegetables (Frederiksen et al. 2006), as well as from cabbage for human consumption (Hendriksen and Hansen 2006); since some of these isolated strains were indistinguishable from commercial *B. thuringiensis* subsp. *kurstaki* HD-1, this suggested these strains might be residuals of biopesticides applied in the field (Frederiksen et al. 2006, Hendriksen and Hansen, 2006). The capacity of these bacteria to form highly resistant dormant particles, called spores, enables them to survive in extreme conditions, and throughout the manufacturing line. The last decade, when consumer preferences have shifted to mildly processed food, new opportunities arose for spore forming spoilage and pathogenic

organisms. Current food preservations are only moderately successful against spores; application of milder treatments in food processing may not be sufficient to eliminate the spores that under favourable conditions, can germinate, growing out to vegetative cells and produce virulence factors. Taking into account these finding and previous consideration, it stands to reason that the presence of *B. thuringiensis* in foodstuff and its impact on food safety still warrant further investigations.

The purpose of the first part of this study was the isolation of *B.thuringiensis* strains, from commercial bioinsecticide products, to investigate the distribution of gene coding for *B. cereus*-like enterotoxins, to evaluate the enterotoxin expression profiles and to assess the presence of corresponding proteins. Then a food model, vegetable based, was developed to evaluate the behavior of *B. thuringiensis* spores artificially added to the food matrix, after the simulation of an industrial processing treatment. SEM and SEM-X ray technique were used to follow the trend of *B. thuringiensis* biological cycle, from dormant spore to vegetative cell. Morphological changes in the structure of the spore were observed and described in detail, at different time during the germination process. In order to achieve more information on the internal spore structure, we tried a SEM carbon coating technique; SEM X-ray analysis was used to examine the release of calcium DPA from *B. thuringiensis* spore which is one of the initial events of the germination process.

2. Materials and methods

2.1 Bacterial strains isolation

The *Bacillus thuringiensis* strains examined in this study were isolated from the following commercial bio-isecticidal products: Delfin, BAC, Vectobac DT, Lepinox, Rapax, Jack pot, B 40, Biolarkim 14, Thuricide HPC, DiPel PRO.

After resuspending in sterile water, decimal dilutions of commercial powdered insecticides, were plated on BCA selective medium plates (Bacillus Cereus Agar Base), and incubated at 30°C for up to 72h, for bacterial strains isolations.

Crystalline inclusion were observed by phase contrast microscopy in all the products tested. For all subsequent analysis, the isolated *B.thuringiensis* strains were cultured on Brain Heart Infusion (BHI) broth at 37°C, on continuous shaking, or in BHI medium agar plates at 37° C.

2.2 DNA extraction for PCR and REP

For DNA preparation, isolated bacteria were cultured on Brain Heart Infusion (BHI, OXOID) broth and incubated overnight at 37°C, on continuous shaking. Genomic DNAs for fingerprinting and toxin profile analysis, were extracted from 1.0-ml aliquots of the cultures by the use of FTA Starter Pack (WHATMAN), in accordance with the manufacturer's instructions.

2.3 Detection of crystal proteins

The *B. thuringiensis* isolates were plated on BHI and then inspected for the presence of intracellular crystals by phase-contrast microscopy, after growth for 2–3 days at 30°C. PCR analyses were carried out to detect the insecticidal toxin genes from all the isolates: the X62821 pair of primers reported by Fuping Song et al. 2003, 5'GCTGTCTACCATGATTCGCTTG3', 5'CAGTGCAGTAACCTTCTCTTGCA3' were used to amplify the conserved regions of *cryII*-type genes; the DiplA 5'CAAGCCGCAAATCTTGTGGA3' and DiplB 5'ATGGCTTGTTTCGCTACATC3' primer set were used for *cryIV* gene detection (Carozzi et al. 1991). Amplifications were carried out in a Mastercycler Ep Gradient S Eppendorf PCR for 32 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 5 min. PCR reaction mix was electrophoresed on a 0.8% agarose gel in TAE1X buffer with SYBR® Safe DNA gel stain (Invitrogen).

2.4 Repetitive extragenic palindromic DNA sequence (REP)

Repetitive Extragenic Palindromic (REP) PCR using (GTG)₅ primer was used to identify *Bacillus thuringiensis* isolates at the strain level.

REP-PCR reaction was carried out in a total volume of 25 µL with 10ng DNA, 0.5µM primer and MasterMix PCR (Promega). Amplification was performed in a Mastercycler Ep Gradient S (Eppendorf) as follows: initial denaturation at 95°C for 7 min, followed by 30 cycles at 90°C for 30 s, 40 °C for 1 min and 65°C for 8 min, and a final extension at 65°C for 16 min. Profiles obtained were analysed on a 2% agarose gel in TAE1X with SYBR® Safe DNA gel stain (Invitrogen) and a 100bp ladder (Promega corporation) was loaded for molecular weight standard.

2.5 Detection of genes coding for enterotoxins

PCR assay was used to characterize toxin profiles of *Bacillus thuringiensis* strains isolated from bio-insecticides. The primer sequences earlier described in a study done in 2001 by Hansen and Hendriksen, were used to detect genes encoding the production of each of the enterotoxins HBL, NHE and bc-D-ENT. Primers and condition described by Swiecicka e Mahillon were employed for *cytK* gene detection; primer set is listed in Table 2. PCR analysis were carried out in a total volume of 25µL that contained 5ng of DNA, 0.5µM of each primer and the GoTaq Green Master Mix (Promega). Reaction was performed in a Mastercycler Ep Gradient S (Eppendorf) with an initial denaturation of 5min at 95°C, followed by 30 cycles PCR each comprising 15 s at 94°C, 45s at annealing temperatures (indicated in Table 2 for each primer pair) and 1min at 72°C, final extension was 7 min at 72°C. PCR reaction mix was electrophoresed on a 1% agarose gel in TAE1X buffer with SYBR® Safe DNA gel stain (Invitrogen), and photographed.

Table 1. Primer set for detection of enterotoxigenic genes in *B.thuringiensis* isolated strains. For HBL and NHE complex, PCR analysis were performed to amplified at least two genes in both the three component operons : *hblC* and *hblD* genes for the hemolytic toxin, *nheB* and *nheC* genes for the non hemolytic toxin.

Genes	Primer name	SEQ (5'-3')	Annealing temperatures	Amplification product
hblC	L2A Fw	5'-AATGGTCATCGGAACTCTAT-3'	52	749pb
	L2B Rv	5'-CTCGCTGTCTGCTGTTAAT-3'		
hblD	HBLD-N	5'-AATCAAGAGCTGGTCACGAAT-3'	51	429pb
	HBLD-C	5'-CACCAATTGACCATGCTAAT-3'		
nheB	nheB 1500 S Fw	5'-CTATCAGCACTTATGGCAG-3'	50	769pb
	nheB 2269 A Rv	5'-ACTCCTAGCGGTGTTCC-3'		
nheC	nheC 2820 S Fw	5'-CGGTAGTGATTGCTGGG-3'	50	581pb
	nheC 3401 A Rv	5'-CAGCATTTCGTACTIONGCGCAA-3'		
bceT	BCET-N	5'-TTACATTACCAGGACGTGCTT-3'	53	428pb
	BCET-C	5'-TGTTTGTGATTGTAATTCAGG-3'		
cytK	cytK _f	5'-GATAATATGACAATGTCTTTAAA-3'	52	617pb
	cytK _r	5'-GGAGAGAAACCGCTATTTGT-3'		

2.6 RNA preparation

For RNA extraction, isolated strains were grown overnight at 37°C in Brain Heart Infusion (BHI) broth, in anaerobic condition, until cells reached stationary phase of growth. After centrifugation of 1.0-ml aliquots of the cultures, pellets were treated with 2.0 ml of RNA Protect Bacteria Reagent (Qiagen) solution and stored at -20°C. Total RNA was extracted using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Quantification of total RNA was performed with Ultraspec 2100 pro

Amersham Biosciences spectrophotometer after enzymatic treatment with DNase (Ambion) for 30 min at 37°C.

2.7 Analysis of toxins expression by RT-PCR

Reverse transcription (RT) was performed using 1 ng of total RNA from commercial *Bacillus thuringiensis* strains cells. To test the presence of mRNA for genes coding toxins previously detected by PCR (hblB – hblC, nheB – nheC, cytK, bceT), Verso™ 1-Step RT-PCR Reddy Mix™ Kit (Thermo Scientific) protocol was used.

2.8 Enterotoxin assay

Bacillus thuringiensis UC10070 var. *kurstaki*, was tested for the ability to product the diarrheal enterotoxins HBL (haemolytic fraction L2) with the reverse passive latex agglutination test using BCET-RPLA toxin detection kit (Oxoid). After 1% inoculum of the *B. thuringiensis* strain in BHI broth, and incubation at 37°C for 18 hours on shaking (250 cycles/min), the culture was centrifuged; supernatants was sterile filtered and stored at -20°C until the assay were performed following the manufacturers' instructions.

2.9 Spores production

Bacillus thuringiensis UC10070 var. *kurstaki*, was selected to generate spores for the following experiments in this study. Growth density and sporulation frequencies were initially compared by using the following three agar media: BHI medium (OXOID, UK), Peptonised Milk (1%peptonized milk, 1% dextrose, 0.2% yeast extract, 1.216 mM MgSO₄, 0.072 Mm FeSO₄, 0,139 mM ZnSO₄, 0.118mM MnSO₄). BP medium (Bacillus Genetic Stock Center, Ohio State University) 0,7% Bactopeptone (Difco), 0.68% KH₂PO₄, 0.012% MGSO₄, 1.7% Agar, 1.216 mM MgSO₄, 0.072 Mm FeSO₄, 0,139 mM ZnSO₄, 0.118mM MnSO₄, Glucose 0.3%.

The pH of Peptonised Milk and BP medium, were adjusted to 7.0 with KOH: salts were added at 55°C after sterilization by autoclaving.

Plates with each of the three medium considered, were inoculated with 500µl of *B. thuringiensis* UC10070 overnight cultures, and incubated for 4 days at 37°C. Spores were harvested and purified by extensive washing with MilliQ water at 4°C .

The spore crops were inspected by phase-contrast microscopy for the presence of vegetative cells, germinating spores, and debris. To estimate the number of spores

formed, 1-ml volume of spore suspension was then placed in a 1.5-ml tube, heated at 60°C for 20 min, diluted and plated on BHI agar. Colonies were counted after 12 h of growth at 37°C.

2.10 Food model preparation

Three commercial UHT vegetable creams based on pepper, artichoke and spinach, and one pasteurized purée, compounded from courgettes, potatoes and milk, were tested with the aim of identify the best to support the growth of the microorganism object of this study. The commercial UHT vegetable creams (20 ml) were dispensed in sterile tube and stored at – 20°C.

Fresh courgettes and potatoes, were washed, trimmed, peeled, and added to UHT milk with a ratio of 3:1:1; the mixture obtained, named CPM model, was heated at 100°C for 15 min, and dispensed in sterile tubes with rates of 20ml before freezing at – 20°C.

Before proceeding with the experiments, values of pH and a_w on the four vegetable mix was recorded. After inoculum in the four vegetable matrices with 10^7 ufc/ml of *B. thuringiensis* cells, growth ability of *B. thuringiensis* will be monitored by plate counting in Brain Heart Infusion up to a week of storage at 4°C and room temperature.

2.11 *B. thuringiensis* spores germination assay in food model

In this step of the work, the CPM model was tested for the *B. thuringiensis* spores germination assay. Recipe of the CPM model was modified by adding bacteriological agar to a concentration of 15g/l : mixture was mixed, heated at 100°C for 15 min, and then dispensed in 20 ml plates. After spreading of 100 µl of 10^7 ufc/ml of *B. thuringiensis* spores, CPM plates were anaerobically stored with Anaerocult® A mini foil bags, and heat treated for 15 minutes at 70 ° C, to activate spores. Germination process was monitored by phase contrast microscope: observations were carried out on spores, outgrowing spores, vegetative and sporulating cells: specifically at 10', 40' , 2 h, 12 h and 24h after heat activation.

2.12 Sample preparation for scanning electron microscopy (SEM)

Samples for scanning electron microscopy (SEM) were prepared as follows: a square section of about 1 cm each side of the food model after inoculum with spores suspension, was taken from each plate in the times previously listed during germination process. They were then dehydrated stepwise in ethanol 50%, 75%, 95%, and finally 100%, for overnight each at room temperature. Critical point drying was performed in a Baltec CPD030 dryer. When carbon coating was used, the specimens were mounted on SEM discs and coated with carbon for electrical conductivity. Finally, they were observed with a Philips XL30 ESEM scanning electron microscope. Gold coated samples were prepared as above described and then specimens were coated with gold for electrical conductivity as described by Palumbo et al. (2004). The specimens were analyzed under both low and high vacuum SEM conditions: high vacuum 3×10^{-4} Pa, 7000 count rate by dead time 33%, dwell time 60 milliseconds and low vacuum 1.199×10^2 Pa (Bassi et al. 2008).

2.13 X-ray microanalysis

Germination was studied in spore samples by means of X-ray microanalysis using the carbon coating technique for sample preparation: the specimens were mounted on SEM discs and coated with carbon for electrical conductivity. SEM images of single specimen fields (ranging from 1-5 μ m) were taken. X-ray microanalysis was then performed using a Philips XL30 ESEM. The elements calcium, phosphorus and sulphur were each detected using energy-dispersive X-ray microanalysis (Edax model Genesis 2000 XMS 60 SEM, Mahwah, NJ 07430, USA) and the Detector (CDU-UTW Shappire, software SEM genesis). The following instrumental conditions were maintained constant throughout the analysis: high vacuum 3×10^{-4} Pa, 7000 count rate by dead time 33%, dwell time 60 msec and low vacuum $1,199 \times 10^2$ Pa. Element microanalysis was used to detect variation in the calcium content of spores.

3. Results

3.1. Characterisation of the *B. thuringiensis* isolated strains

After culturing of commercial bio-pesticide products considered in this study, a total of 10 *Bacillus thuringiensis* strains were isolated (Table 3). *Bacillus thuringiensis* var.

kurstaki H3a 3b was present in BAC, Delfin, Lepinox, Rapax, Jack Pot and Thuricide HPC; *B. thuringiensis* var *israelensis* H:14 were isolated from Biolarkim 14 and Vectobac DT, *Bacillus thuringiensis* var. *kurstaki* ABTS-351 from DiPel PRO DF, *Bacillus thuringiensis* var. *aizawai* was present in B40. All the isolates were confirmed to be *B. thuringiensis*, belonging to the *B. cereus* group, because of their content of either crystal proteins, visualized by phase-contrast microscopy or *cry* genes, as detected by PCR. As expected, from the 10 isolates, 8 strains were positive for *cry-IIA* gene, which is present in the commercially used strains *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *aizawai*. In addition, 2 strains out of the 10 *B. thuringiensis* isolates, belonging to serovar *israelensis*, were positive for *cry-4* gene (Table 3).

Profiles obtained after amplification of repetitive extragenic palindromic DNA sequences (REP-PCR) (Fig 1), confirmed the isolation of three different *B. thuringiensis* serovars from the products analyzed: *B. thuringiensis* var. *kurstaki*, *B. thuringiensis* var. *israelensis* and *B. thuringiensis* var. *aizawai*.

Table 2. Classification of *B. thuringiensis* strains isolated from bio-insecticidal products

Bio-insecticidal commercial names	Strains	Name	Serotype	Cry gene
Delfin	<i>B.Thuringiensis</i> var. <i>kurstaki</i>	UC10070	H3a 3b	<i>cry-IIA</i>
BAC	<i>B. thuringiensis</i> var. <i>kurstaki</i>	UC10071	H3a 3b	<i>cry-IIA</i>
Vectobac DT	<i>B. thuringiensis</i> var. <i>israelensis</i>	UC10072	H14	<i>cryIV</i>
Lepinox	<i>B. thuringiensis</i> var. <i>kurstaki</i>	UC10073	H3a 3b	<i>cry-IIA</i>
Rapax	<i>B. thuringiensis</i> var. <i>kurstaki</i>	UC10074	H3a 3b	<i>cry-IIA</i>
Jack pot	<i>B. thuringiensis</i> var. <i>kurstaki</i>	UC10075	H3a 3b	<i>cry-IIA</i>
B 40	<i>B. thuringiensis</i> var. <i>aizawai</i>	UC10076	-	<i>cry-IIA</i>
Biolarkim 14	<i>B. thuringiensis</i> var. <i>israelensis</i>	UC10077	H14	<i>cryIV</i>
Thuricide HPC	<i>B. thuringiensis</i> var. <i>kurstaki</i>	UC10078	-	<i>cry-IIA</i>
DiPel PRO DF	<i>B. thuringiensis</i> var. <i>kurstaki</i>	UC10079	ABTS-351	<i>cry-IIA</i>

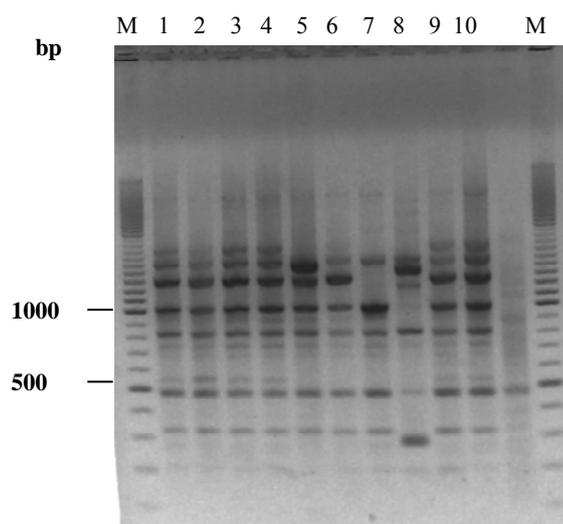


Fig. 1. Agarose gel electrophoresis of REP-PCR. Shown are *B.thuringiensis* subsp. *kurstaki* H3a 3b from Delfin, Bac, Lepinox, Rapax, Jack pot, (lanes 1, 2, 3, 4, 9), *B. thuringiensis* subsp. *aizawai* from B40 (lane 8), isolates indistinguishable from the Dipel strain (lane 10), and *B. thuringiensis* var *israelensis* from Vectobac DT and Biolarkim 14 and (lanes 5, 6)

3.2. Detection and expression of enterotoxigenic genes in *B. thuringiensis* isolated strains

The PCR analysis revealed that all the *B. thuringiensis* strains isolated from commercial products tested (Delfin, BAC, Vectobac DT, Lepinox, Rapax, Jack pot, B 40, Biolarkim 14, Thuricide HPC, DiPelPRO) harbored genes for HBL and NHE enterotoxins, as well as the genes coding for CytK and bc-D-ENT toxins (Table 4).

To clarify whether the enterotoxigenic genes are not only present but also expressed in laboratory medium, RT-PCR was performed on three *B. thuringiensis* strains each belonging to a different *serovar* from those isolated. All the four toxic genes examined resulted expressed at the conditions tested (Table 5).

Table 3. Detection of enterotoxigenic genes by PCR analysis on *B. thuringiensis* isolates.

n°	Strains tested	Serotype	hblC	hblD	nheB	nheC	cytK	bceT
5	<i>B. t</i> var. <i>kurstaki</i>	H3a 3b	+	+	+	+	+	+
2	<i>B. t</i> var. <i>israelensis</i>	H14	+	+	+	+	+	+
1	<i>B. t</i> var. <i>aizawai</i>	-	+	+	+	+	+	+
1	<i>B. t</i> var. <i>kurstaki</i>	ABTS-351	+	+	+	+	+	+
1	<i>B. t</i> var. <i>kurstaki</i>	-	+	+	+	+	+	+

Table 4. Enterotoxigenic genes expression of *B. thuringiensis* isolates assessed by RT-PCR

Strains tested	serotype	Genes					
		hblC	hblD	nheB	nheC	cytK	bceT
<i>B. t. var. kurstaki</i>	H3a 3b	+	+	+	+	+	+
<i>B. t. var. israelensis</i>	H14	+	+	+	+	+	+
<i>B. t. var. aizauwai</i>	-	+	+	+	+	+	+

3.3 Enterotoxin production

The production of the L2 component of HBL enterotoxin, involved in the diarrhoeal syndrome, was assessed in the supernatant of *B. thuringiensis* UC10070 broth culture. The enterotoxigenic activity observed, are reported in figure 2. Detection of L2 component from hemolysin BL, gave positive result in the strain object of the study for the growth condition tested.

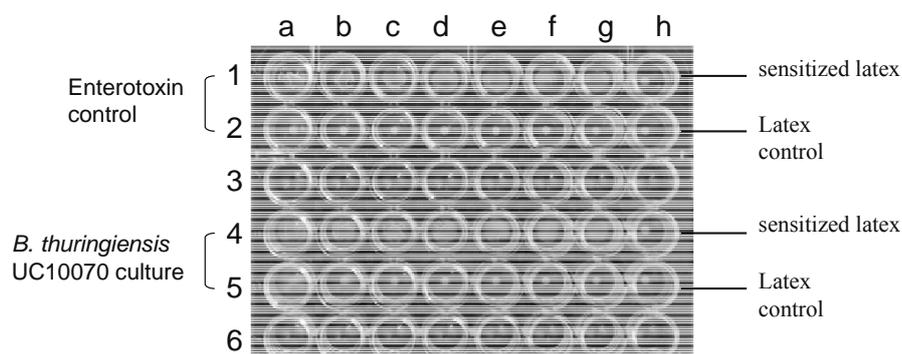


Fig.2 Enterotoxin-Reversed Passive Latex Agglutination reaction. Wells from “a” to “g” contain serial dilutions of the samples; well “h” is the negative control. The first row, shows the positive agglutination reaction generated by the control enterotoxin, provided by the kit (the respective negative control is in row 2). The positive response of sensitized latex added to the *B. thuringiensis* culture is clearly shown in the fourth row.

3.4 Selection of sporification medium and spores production

As it is worldwide used for large-scale production of bio-insecticides, *Bacillus thuringiensis* UC10070 var. *kurstaki*, was selected to generate spores for the following analysis. Experiments were performed to identify the best medium to induced sporification. All the three media tested, yielded high spores counts (Table 5), however intact vegetative cells were still visible in BHI and Peptonised milk media. On the contrary although lower counts were achieved on BP agar, only very few vegetative cells and germinating spores (less than 1%) were observed in this medium, when the spore

crops were inspected by phase-contrast. Hence, spore of *B. thuringiensis* UC10070 were produced from cells cultured in BP by incubation for 4 days at 37°C, allowing to obtain a spore suspension of 1.5×10^8 ufc/ml.

Table 5. Comparison of the growth efficiency of *B. thuringiensis* strains in three media. Standard deviations were as follows: BHI/ 7,08E+08, Peptonized milk/1,41E+08, BP/5,63E+07.

Strain	N° of CFU/ml in:		
	BHI	Peptonized milk	BP
<i>B.Thuringiensis</i> UC10070	1,22E+09	2,57E+08	1,51E+08

3.5 Food model development

In this study four types of different vegetables matrix were tested for the ability to support the development of commercial *B. thuringiensis* spores and the following growth of vegetative cells. Chemical analysis of the primary factors affecting microbial growth, pH and free water availability (a_w), allowed a first selection of the best model to use for analysis. Values observed are listed in Table 6.

Table 6. pH and a_w parameters assessed in the four vegetables matrix considered.

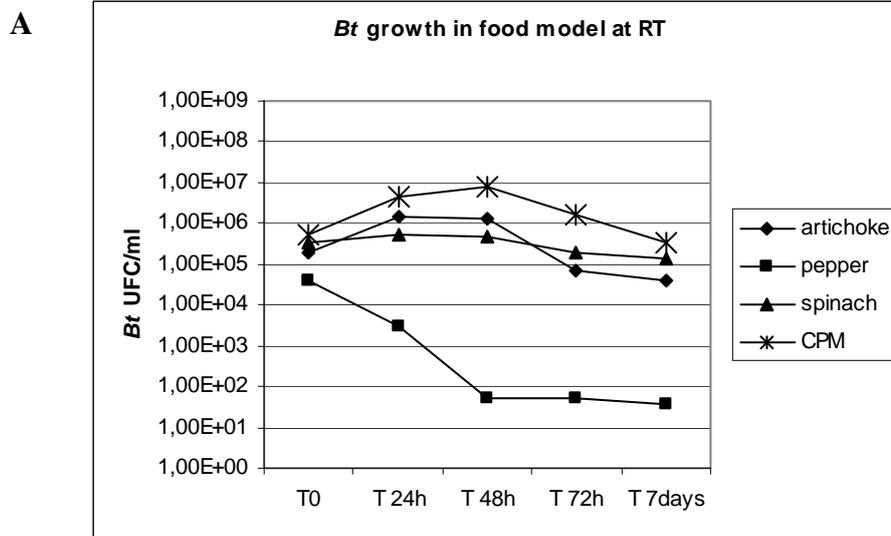
	pH	a_w
Pepper	4.63	0.97
Artichoke	5.63	0.94
Spinach	5.94	0.94
CPM	5.95	0.97

Pepper cream presented an optimum value of a_w , as the CMP model, but the pH seemed too restrictive to support the growth of *B. thuringiensis*. The cream of artichoke and spinach showed a pH value suitable for the development of the bacterium, but low water availability probably limited spores germination and growth of vegetative cells. CPM showed the optimum values for both parameters analyzed.

B. thuringiensis growth dynamics on the different vegetables considered, was monitored during a week; growth curves obtained are reported in figures 3.A and 3.B.. Some *B. thuringiensis* strains can grow up to 55°C while others can grow as low as 4- 5°C.

The experiment was performed in two different conditions: a permissive temperature (room temperature) and one more limiting (8°C), just to test the ability of *B. thuringiensis* UC10070, to grow in food model even at refrigerator temperature. Obviously, relevant differences were found in the kinetics of *B. thuringiensis* determined in each condition.

The results confirmed that room temperature could contribute to higher growth dynamics of *B. thuringiensis*; the temperature as low as 8°C delayed the onset of the bacterial growth but it doesn't arrest the *B. thuringiensis* biological cycle, as it can be observed by levelling of the kinetics curve. As expected CPM model resulted the best to support growth of *B. thuringiensis* cells; the low pH, seemed the most limiting parameter for microorganism development, as shown by the curve of cell death in pepper cream.



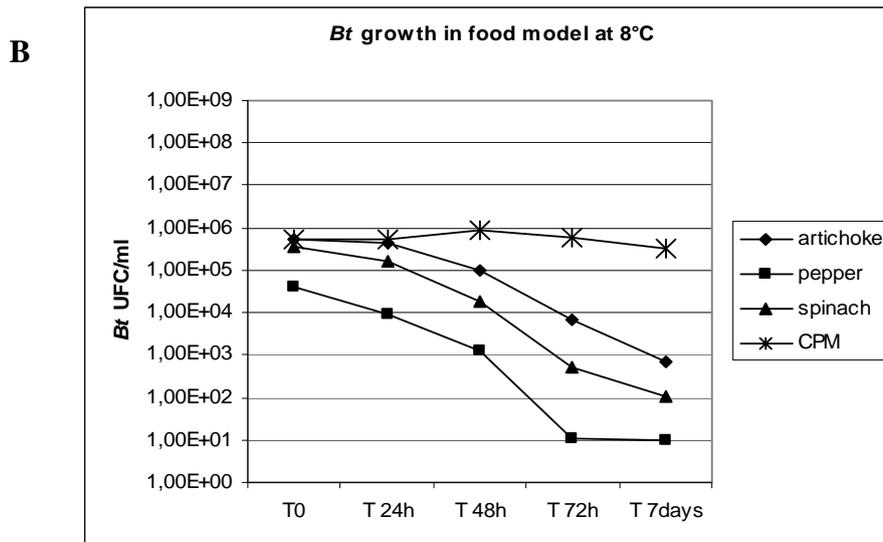


Fig.3 Kinetics of growth of *B. thuringiensis* observed in four different vegetables mix at room temperature (A) and refrigerator temperature (B).

Given the findings above, CPM model was chosen to test its ability to support, not only cell growth but also the germination process of *B. thuringiensis* spores for the next steps of the work. Recipe of the CPM model was modified by adding bacteriological agar in order to dispense the matrix on plates and to facilitate the spreading of *B. thuringiensis* spore suspension. The thermal treatment for 15' at 70°C, and incubation in anaerobic conditions, were used to reproduce, in food model, the industrial processing which foodstuffs are submitted, as for play the real trend of spore germination, outgrowth, and cell growth in processed foods.

3.6 SEM observations of the germination process

To identify the critical steps of *B. thuringiensis* germination, the process was monitored by scanning electron microscopy (SEM) and SEM-Xray techniques (Bassi et al. 2009) harvesting cells at various time points during growth in the CPM food model.

3.6.1 Gold coating results

First, observations were performed using the gold coating technique under high vacuum conditions. This technique allowed us to view morphological changes limited to the surface of *B. thuringiensis* cells and spores. Spores, obtained as described in section 2.9,

were transferred to CPM model plates and heated at 70°C for 15 minutes. This activation is usually followed by a cascade of reactions that results in rapid and significant morphological changes in the overall structure of spores.

In Fig.4 A-B-C-D, are shown dormant spores, and spores coated by cell wall residues (exosporium); the exosporium is not a universal part of the spore structure but seems to be conserved among pathogenic *Bacilli* and it is present on members of the *B. cereus* group. The spore coat, providing the spore with resistance against exogenous lytic enzymes, organic solvents and a range of oxidative chemicals (Driks, 1999; Nicholson et al. 2000, Setlow et al. 2000).

Parasporal body protein inclusions, containing Cry proteins, were observed (Fig. 5 A-B-C-D). The crystal protein is produced during sporulation and is accumulated both as an inclusion and as part of the spore coat. Parasporal inclusions produced by the present isolate morphologically fell into three groups: spherical (Fig. 5 C), rhomboid (Fig. 5 B-D), and irregular-shaped (Fig.5 D).

Ten minutes after heat shock in plates, the spores synchronously initiated germination (Fig. 6). Optical microscopic analysis of samples obtained at this time point, showed a nearly complete transition from phase-bright spores to phase-dark germinated cells, that coincides with the entrance of water to the core, resulting in a partly dehydrated core environment.

SEM microscopic analysis revealed that after 40 minutes, the outer layer of spores began to dissolve, spores began to break their external layer and assumed an elongated structure respect to the more round shape of dormant spores (Fig 7).

After two hours from heat activation, cells burst out definitely of the remaining protective spore structures (spore coat) and initiated chromosome segregation, as demonstrated by the septa formation and the first round of cell division. In figure 8, are shown round shaped cell forms. These are probably protoplasts which derive from lytic activity of new outgrowing cells, characterised by a weak cell wall.

Mature vegetative cells, could be observed 12 hours after spore activation, high cellular density together with a beginning of autolysis are represented in figure 9.

Fig 4. Dormant spore of *B. thuringiensis* UC10070 in food model

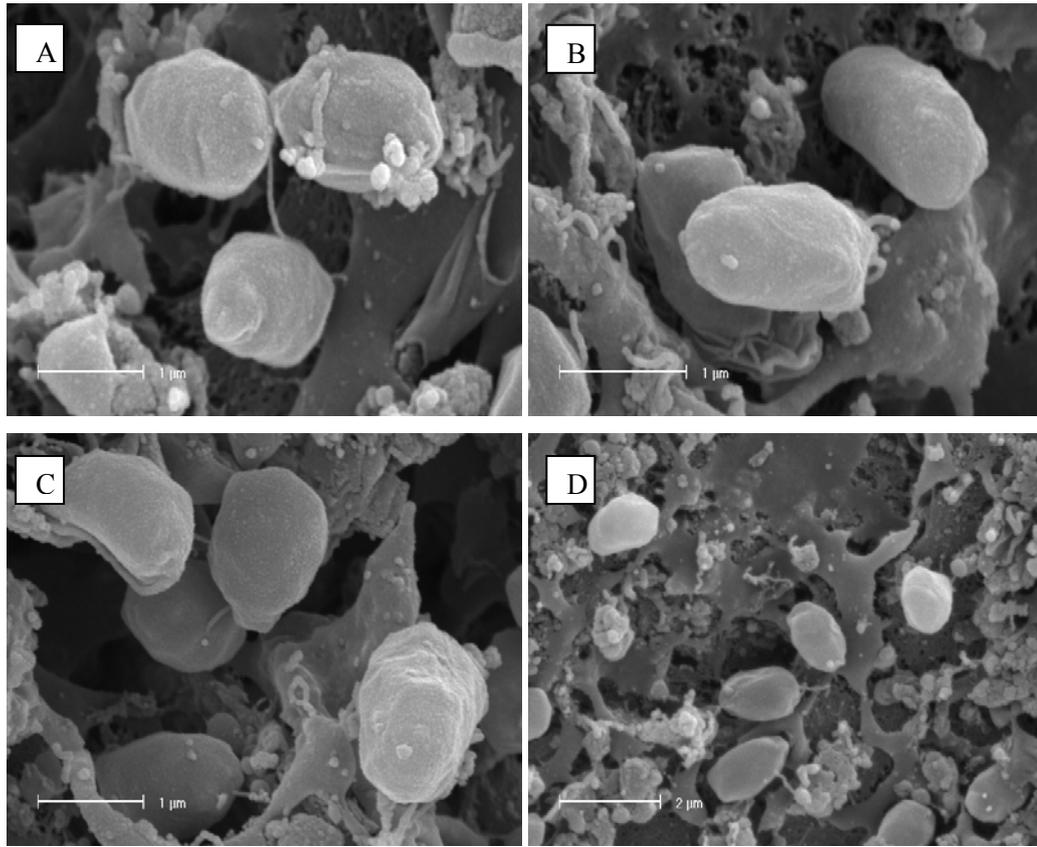


Fig.5 Crystal proteins in *B. thuringiensis* UC10070

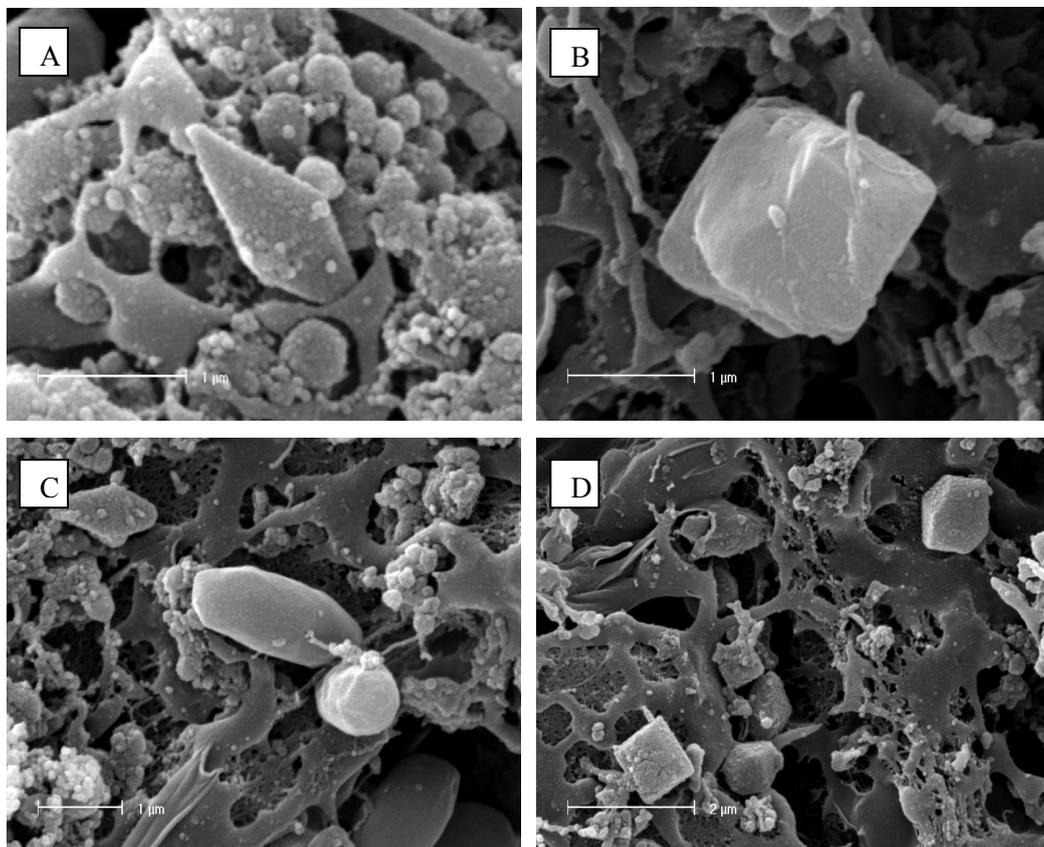


Fig.6 *B. thuringiensis* UC10070 spore 10 minutes after heat activation

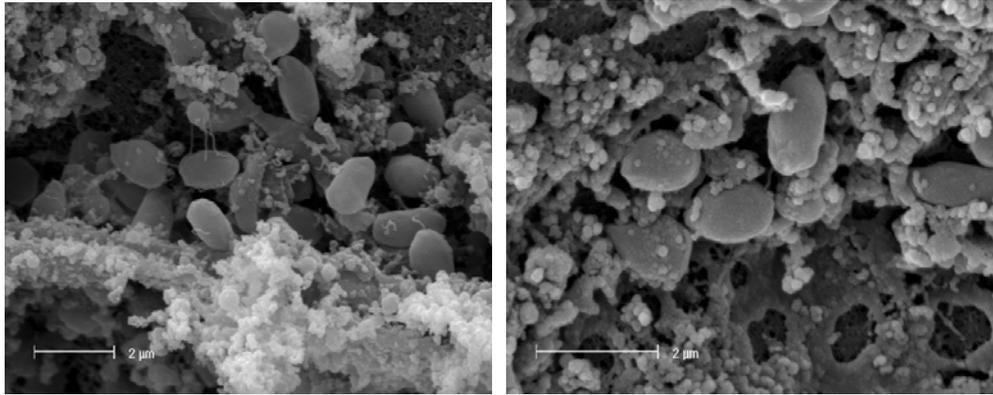


Fig.7 *B. thuringiensis* UC10070 spore 40 minutes after heat activation

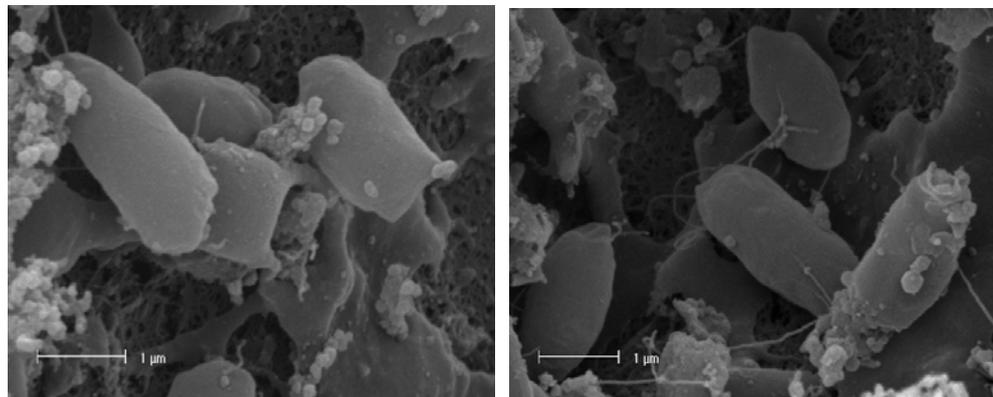


Fig.8 *B. thuringiensis* UC10070 cells 2 h after heat activation of spores

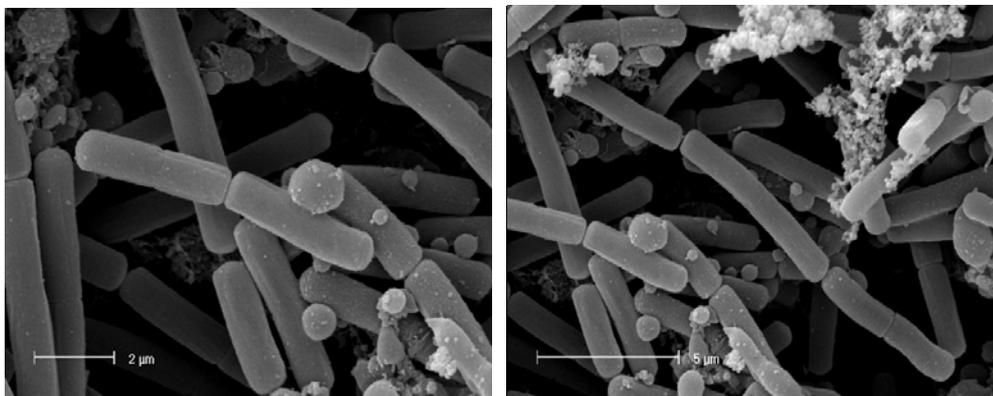
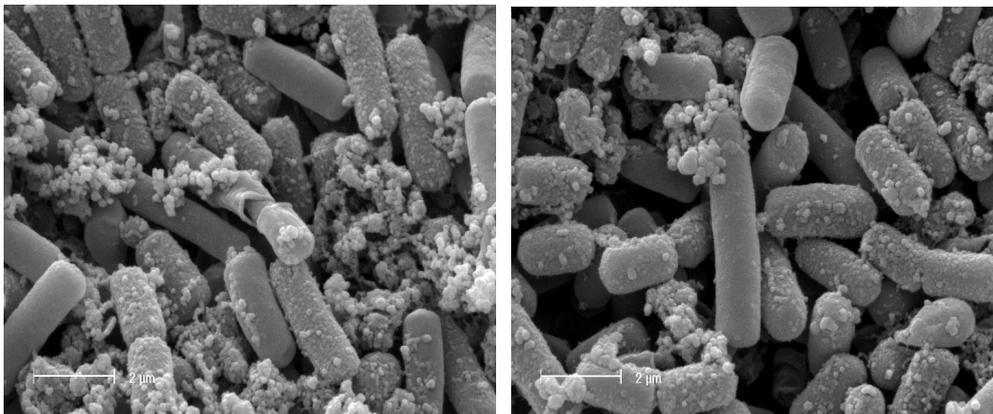


Fig.9 *B. thuringiensis* UC10070 cells 12 h after heat activation of spores



3.6.2. SEM X-ray analysis

To deeper investigate on the germination process, we used X-ray microanalysis (together with the high resolution carbon-coating SEM technique) to examine the release of calcium-DPA from *B. thuringiensis* spores. The release of this compound is one of the initial events of the germination process. Energy dispersive analysis of X-rays (EDAX) is a method to analyze the elemental composition at the ultrastructural level. When the electrons from external sources strike the atoms in the material, energy in the form of an X-ray photon is emitted, thus giving the characteristics X-ray of the element (Russ, 1978; Hall and Gupta, 1982; Murr, 1982; Hobbs et al., 1986; Hiom et al., 1995). A major advantage of EDAX is the ability to observe morphology and chemical composition of cells simultaneously (Hayat, 1980).

After heat treatment, the rapid release of the spore content of calcium dipicolinate, detected by SEM microanalysis gave a first evidence of immediate germination in food model. The release of calcium dipicolinate, started already after ten minutes from pasteurization as we can see in figure 12.

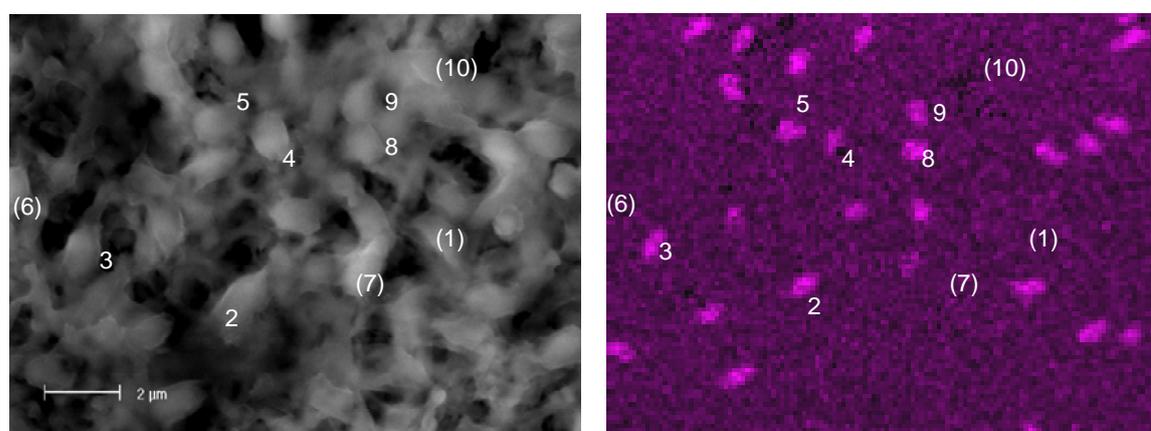


Fig.12 SEM (left) and X-ray microanalysis (right) map of the same picture. The spores (1),(6), (7) and (10) showed no calcium inside, as represented by the absence of a fluorescent spot. These spores have already triggered the germination process.

4. Discussion

In the first part of this study the isolation of *B. thuringiensis* strains from commercial bio-insecticide products was used to investigate the distribution of gene coding for *B. cereus*-like enterotoxins and to evaluate the enterotoxin expression profiles.

The pathogenic profile of 10 strains isolated from *B. thuringiensis*-based biopesticides was defined. PCR analysis revealed that all the 10 strains possess two genes of NHE and

HBL complexes, the two best-characterized three component toxins of *B. cereus*, as well as *cytK* and *bceT* single genes, coding for cytotoxin K and bc-D-ENT enterotoxins respectively; the importance of frequent occurrence of *bceT* in *B. cereus* organisms is not yet fully understood; the role of CytK in food borne disease needs further study, although it has recently been shown that *cytK* gene was strongly transcribed in a clinical strain responsible for the death of three persons (Lund et al 2000).

Reverse transcription assay, showed that toxic genes detected by PCR, are all expressed in the isolated strains; moreover agglutination tests gave positive results for the L₂ component of the toxic complex HBL in UC10070 *B. thuringiensis* strain analyzed.

As stated before the members of the *B. cereus* group, are closely related. Studies on *B. cereus*, *B. anthracis* and *B. thuringiensis* have shown that these organisms share relatively high levels of chromosomal base sequence identity.

No confirmed food borne illness cases caused by *B. thuringiensis* have been described yet. This, however, may also be due to the difficulties encountered in the discrimination between *B. cereus* and *B. thuringiensis*. There is considerable evidence that *B. thuringiensis* and *B. cereus* should be considered a single species: modern molecular methods including chromosomal DNA hybridization (Kaneko et al. 1978), phospholipid and fatty acid analysis (Black et al 1997, Kampfer et al. 1994), 16S rRNA sequence comparison (Ash C. et al 1991, Rossler D., et al. 1991), amplified fragment length polymorphism analysis (Keim P., et al. 1997), and genomic restriction digest analysis (Carlson C. et al. 1994, Carlson C. et al. 1996) proved to be far from providing effective methods to distinguish the two species supporting the single-species hypothesis. An attempt to distinguish *B. thuringiensis* isolates from *B. cereus* by analysis of a 16S rRNA variable region largely failed, yielding many false positives and negatives. The most reliable method for distinguishing the two species is still the detection of the parasporal inclusions in *B. thuringiensis*, though it was demonstrated that conjugative transfer of plasmids among *B. cereus* spp. are possible. Since commercial *B. thuringiensis* strains harbour and express genes for all of the four known *B. cereus* enterotoxins, HBL, NHE, bc-D-ENT and CytK, there is a risk that high levels of these organisms may cause human diseases.

Recent studies found high counts of *B. cereus*-like organisms in fresh cucumbers, tomatoes and other fresh vegetables; counts performed on fresh foods for sale in Danish retail shops, revealed that more than 10⁴ CFU/g of *B. thuringiensis* strains,

indistinguishable from the commercial strains from biopesticides, are present, more likely not due to growth of the organisms, but as natural contaminants or residues of *B. thuringiensis* insecticides (Rosenquist et al. 2005). Significant high counts of *B. cereus*-like organisms are also increasingly associated with heat-treated products, where improper cooling after cooking may induce growth of this organisms after spore germination. The CPM model proposed in this work was chosen for its ability to support growth of *B. thuringiensis* cells; moreover, the model was found to be an excellent substrate not only for cell growth but also for *B. thuringiensis* spores germination. By using CPM model it was possible to reproduce the real trend of spore germination, outgrowth, and cell growth, through the simulation of an industrial processing which foodstuffs are submitted; after pasteurisation treatment and anaerobic packaging, *B. thuringiensis* spores were activated from its dormant state, to recover the active metabolism that leads to the realisation of new vegetative cells and their duplication. This finding, together with the pathogenic profile described above, gave evidence that the increasing popularity of cooked chilled foods may lead to problems with spore-forming bacteria such as *B. cereus* and *B. thuringiensis*. To better investigate on the morphological changes during germination, we used SEM technique, combined with X-ray microanalysis. SEM methods could be used to observe the surface morphology of spores; X-ray microanalysis permitted us to distinguish which spores are germinating by determining their chemical compositions (i.e. release of calcium DPA). Moreover, morphology and chemistry could be studied simultaneously, enabling us to observe the structures of spores as they initiate the germination process. Our study provides a suitable method to acquire new knowledge on the biological cycle of opportunistic pathogens that can occur in processed foods.

B. thuringiensis exhibits a wide variety of insecticidal specificities against several insect species, which depends on the specificity of delta-endotoxin encoded by the *cry* genes; therefore, the Cry toxins seem the most prominent of a number of virulence factors allowing the development of the bacteria in insect larvae: more recent characterizations have shown that proteases and chitinases, may contribute to virulence. As these virulence factors are harmless to humans, an increasing use of this bacterium has been highlighted, to control pests in biological agriculture; however, our study suggests the importance of considering the ability of this bacterium to produce also a variety of other toxins and virulence factors that can affect humans.

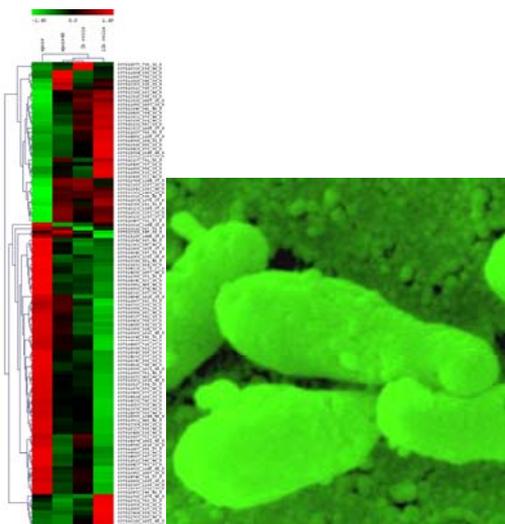
Additional investigations are needed to clarify whether the genes are expressed in food after contamination of the bacteria or spores, but taking this enterotoxigenic potential into account, as well as the fact that *B. thuringiensis* cannot be separated from *B. cereus* at the chromosomal level, vegetable producers and food authorities responsible for food safety, should consider the amount of *B. thuringiensis* insecticide residue left on products after harvest. Data obtained in this study, suggest that the authorization which permits the release of large amounts of *B. thuringiensis* in bioinsecticides should be revised, through adequate procedures to evaluate safety, and excluding the use of enterotoxigenic strains. The European Food Safety Authority has recommended that processors should ensure that levels of *B.cereus* bacteria between 10^3 and 10^5 /g are not reached at the day of consumption (Eur. Food Saf. Authority, 2005). It would be important to consider that this statement should apply also to residues of commercial enterotoxin-encoding *B. thuringiensis* strains.

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GENOME WIDE TRANSCRIPTOME ANALYSIS
OF *BACILLUS THURINGIENSIS*
SPORE GERMINATION OUTGROWTH AND TOXIN
PRODUCTION IN FOOD MODEL



1. Introduction

The *Bacillus cereus* sensu lato group, forms a highly homogeneous subdivision of the genus *Bacillus* and comprises six Gram-positive, spore forming species: *B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides* and *B. cereus*. Notorious is *B. anthracis*, the cause of the often-lethal disease anthrax; in 1877 it was the first bacterium shown to be responsible of a disease. The food pathogen *B. cereus*, is a normal inhabitant of the soil, but can be regularly isolated from foods such as grains and spices: it is responsible of two types of foodborne diseases leading to an emetic or diarrheal syndrome. The insect pathogen *B. thuringiensis*, is of great economical importance, being used worldwide as an insecticide.

The capacity of these bacteria to form highly resistant dormant particles, called spores, enables them to survive in extreme conditions and to occupy and complete a full life cycle within several different environmental niches (soil, decaying organic matter, plant surfaces, insect and mammalian guts). Under favourable conditions, spores may germinate, loosing their resistance capacities, and grow out to vegetative cells. The omnipresence of *Bacillus* spores in the environment inevitably results in the presence of spores in agricultural and dairy products. Since spores are able to resist most of the preservation techniques currently applied, they are responsible for infections, serious food-borne illnesses and significant amount of food spoilage (Brul et al, 2006). *Bacillus* spores are equipped with a specific set of germination receptors that continuously monitor the environment for proper outgrowth conditions: possibly the diversity in ecological background has resulted in a differently developed set of germination receptors, but little is still known about the life cycle of these bacteria. Availability of information by progress in bacterial genome sequencing, aids to more investigate *Bacillus* features.

Comparison of *B. anthracis*, *B. cereus*, and *B. thuringiensis* genomes (Ivanova et al. 2003, Han et al 2006), has allowed genome-scale comparison of sequences related to the physiology, sporulation and virulence of these bacteria; the very high genetic relationship found, made genome based differentiation complicated or even impossible and led to the conclusion that the three taxa should be considered a single bacterial species. Despite such biological arguments for unification, a separate species status for these bacteria has been maintained because of their distinctive pathogenic features; their pathogenicity patterns differ significantly: the principal virulence factors of *B. anthracis* are encoded by genes located on two plasmids pXO1 and pXO2 (Okinaka et al 1999). Similarly, the crystal

protein genes responsible for the major features of insect toxicity of *B. thuringiensis* isolates are almost invariably plasmid encoded (Schnepf et al 1998). The virulence genes of *B. cereus*, are chromosomal (Guttmann et al 2000, Ivanova et al 2003). The evolutionary relationships between all members of the group have yet to be definitively established; this is important, not only for understanding the evolution of virulence in the *B. cereus* group, but also for rapidly and accurately characterizing these organisms, a concern which has become of increasing scientific and political importance in recent years.

B. thuringiensis is an environmentally common Gram-positive bacterium. As the other members of the *B. cereus* family, it can exist in two morphologies: the vegetative cell and a dormant spore. The most distinctive property of *B. thuringiensis*, is its entomopathogenicity and production of insecticidal Cry toxin proteins that accumulate in the mother cell as crystalline inclusions during sporulation of the bacterium. Due to specific activity against insects, formulations of *B. thuringiensis* spores has been exploited as pesticides, for more than 40 years, to control agriculturally and medically important pest. However, most of genetic determinants involved in *B. cereus*-associated food borne illness, like haemolysin BL (HBL), non haemolytic enterotoxin (NHE), cytotoxin K, and bc-D-ENT enterotoxin, have been found frequently also in *B. thuringiensis* strains. As the *B. thuringiensis* spores survive many of the currently applied food preservation treatments, they can persist in food as residues of biopesticides applied in the field (Frederiksen et al., 2006; Hendriksen and Hansen, 2006). Only rigorous methods have been shown to be capable of destroying all spores present in food. Hence, the presence of *B. thuringiensis* in foodstuff and its impact on food safety still needs further investigation.

The research in this thesis aim to describes the germination of *B. thuringiensis* spores in vegetable food. The first purpose of this study was the development of a vegetable based food model, that would allow to asses the behaviour of *B. thuringiensis* spores in food, after the simulation of an industrial processing treatment. Genome-wide microarray-based transcriptome analysis, was used to explore transcriptional changes and to understand the molecular mechanism, behind the process of *B. thuringiensis* spore germination, outgrowth and toxin production, in food model. RT-qPCR analysis were performed to quantify the expression, in food, of the major virulence genes involved in *B. cereus*-associated food borne disease. The production of the L2 component of HBL enterotoxin, involved in the diarrhoeal syndrome was assessed, in culture medium and in food model, to confirm that trend of HBL mRNAs, evaluated with RT-qPCR, and microarray analysis, leads to toxic

protein biosynthesis. Transcriptomic has been demonstrated to be not only a powerful tool to study the germination and outgrowth of *B. thuringiensis* spores, but also a suitable method to assess the environmental response to bacterial pathogens in food. Data obtained, provide new basic knowledge on *Bacillus cereus* group.

2 Materials and methods

2.1. Bacterial strain, and growth condition

Bacillus thuringiensis UC10070 var. *kurstaki* serotype H3a 3b, was employed for all the experiments. This strain was isolated from a commercial bio-insecticidal product in our laboratory, by plating on BCA selective medium agar plates, decimal dilution of the commercial powdered after incubation at 30°C for up to 72h (see section 2.1, chapter 2). The strain was then routinely cultured on Brain Heart Infusion (BHI, OXOID) broth at 37°C, on continuous shaking.

2.2 Spore generation and germination conditions

Spore of *B. thuringiensis* UC10070 were produced from cells cultured in BP medium (Bacillus Genetic Stock Center, Ohio State University). BP plates were inoculated with 500µl of *B. thuringiensis* UC10070 overnight cultures, and incubated for 4 days at 37°C. Spores were harvested and purified by extensive washing with MilliQ water at 4°C (Nicholson, et al. 1990). The spore crops, inspected by phase-contrast microscopy, were free (>99%) of vegetative cells, germinating spores, and debris (see section 3.3, chapter 2). Spore suspensions were stored at -20°C for use in subsequent analysis.

The CPM model described in the previous chapter was employed to monitor the germination process of *B. thuringiensis* spores developed in food matrix (see section 3.4, chapter 2). After spreading with 100 µl of *B. thuringiensis* spores suspension (10^7 cfu/ml) on the CPM model plates, inoculated vegetable mix were anaerobically stored with Anaerocult[®] A mini foil bags, and heat treated for 15 minutes at 70° C, to activate spores. Germination process was monitored by phase contrast microscope: observations were carried out on spores, outgrowing spores, vegetative and sporulating cells.

At the same time, 1 ml samples for RNA isolation were harvested with saline water at regular intervals. The samples were spun down in a microcentrifuge, and pellets were rapidly frozen in liquid nitrogen.

2.6. Microarray construction

Genome of *B. thuringiensis* 97-27 sv. *Konkukian* serotype *H34* (NCBI reference sequences: chromosome NC_005957, plasmid NC_006578 - IMG annotations, Doe Joint Genome Institute), was chosen for high homology with *B. thuringiensis* UC10070, to design probes corresponding to 5,197 genes spotted in duplicates onto ElectraSenseH 12K microarrays chip (CombiMatrix Corp. Mukilteo, WA).

The chip used contains 12,544 individually addressable electrodes linked by the semiconductor circuitry. Contact pads on the array allow for electrical connectivity with an external device to control custom synthesis of unique probes at each electrode, and electrochemical detection with the ElectraSenseH microarray reader (CombiMatrix Corp, WA). Each electrode has a distinct DNA probe above it, and each electrode can be read electronically or fluorescently to determine the level of hybridization for a specific DNA sequence. Probes were designed using the Combimatrix Automated Probe-Design Suite of programs (CombiMatrix Corp., WA) and were selected without secondary structure and with homogeneous melting temperature (Table 1). The program started the design on the 3' end until finding a good oligo probe. Maximum distance from 3' end is 1500 bp.

Probes corresponding to 5,197 genes of *B. thuringiensis* genome were synthesized in 2 replicates randomly distributed on the chip. Thirteen negative control probes designed on 3 *Arabidopsis* genes (Table 1) were synthesized in 30 replicates randomly distributed on the chip. 13 spike control probes (Ambion, USA) were synthesized in 30 replicates randomly distributed on the chip. Other negative control probes were designed on plant, phage and bacterial genome (Table 1).

Table 1. Oligo design program and negative controls designed on the chip

Oligo length 35-40
 Tm range 80-86
 %GC 35-60
 Tm Struct 65°C (Tm min -10)
 Tm X Hyb 65°C (Tm min -15)
 Nbr Oligo 1
 Dist 3'-1500
 Prohibited (NTP)5

negative controls:

Neg_lambda1_1283_35_S	Neg_lambda1	CTGATGCCGTTAACGATTTGCTGAACACACCAGTG
Neg_lambda1_1070_35_S	Neg_lambda1	AAAACAGCCGCATTATGGGGATCCTCAACTGTGAG
Neg_lambda1_847_35_S	Neg_lambda1	CTTGTTAACAGCACCGATGCGATCACCGTCATGCG
Neg_lambda1_587_35_S	Neg_lambda1	GTAATTACGGTGCTGCGCTGGAGAAACAGGGTGTG
Neg_lambda1_359_35_S	Neg_lambda1	ATCCGATGGTGGACGGCATTCTGCTCGATATGGAC
Neg_agro1_1065_35_S	Neg_agro1	CGATTTCTCAGGCATATAACCAGCTTCGTATGGCTG
Neg_agro1_614_35_S	Neg_agro1	AATGCGTGTACCCCTGGGATGTTTCGTGGAACAACG
Neg_agro1_407_35_S	Neg_agro1	GAAGACGCGAAGGCAATGGGGAAGACAGTCCTTTG
Neg_agro1_192_35_S	Neg_agro1	CATGATCGCCACGAACACCAAGCCATTTTCATCCGG
Neg_bant_349_38_S	Neg_bant	CCACAAGCATTAAACAAATGCAGTTGAGATTTTAGGTGG
Neg_lambda4_665_35_S	Neg_lambda4	GCGGCTTCCTTTCCATTAACAAACTTTTCGCAGTAA
Neg_lambda4_348_35_S	Neg_lambda4	GAACCTCGTCAACGGAACAGTTACGCCGATCCGG
Neg_lambda4_9_35_S	Neg_lambda4	TATCCGGCAGGAAACACTGAATGAATGCACCCGTG
Neg_lambda6_866_35_S	Neg_lambda6	CAAACACAGACTGGATTTACGGGGTGGATCTATGA
Neg_lambda6_647_35_S	Neg_lambda6	ATTTTGCTGGGTGGGCTAACGATATCCGCCTGATG
Neg_lambda6_290_38_S	Neg_lambda6	ATATTATCAAGCAGCAAGGCGGCATGTTTGACC AAAAT
Neg_lambda6_89_36_S	Neg_lambda6	CCAGACTATCAAATATGCTGCTTGAGGCTTATTCGG
NM_129257.2_3147_40_S	NM_129257.2	CCGATTCATAGCTTATTTCTGCATGGTTACATTCCAGAAG
NM_129257.2_2946_39_S	NM_129257.2	AGATAAAGAAAGCATGGTCCTTAAGTATCTCGGAAGCTT
NM_129257.2_2701_40_S	NM_129257.2	GCACATTTTATGGCTAAAGACACAATGGACCATTTAAACA
NM_129257.2_2500_40_S	NM_129257.2	GCGTTAGATTTTCTTAATATTATTAGTTGCGGGAGCTTGT
Neg_lambda2_992_35_S	Neg_lambda2	ACCCTGATGAGTTTCGTGTCCGTACAACCTGGCGTAA
Neg_lambda2_752_35_S	Neg_lambda2	TCGTGTATTCCGGACAGTACGTGGAACCGGCGTC
Neg_lambda2_528_35_S	Neg_lambda2	CACGTATGACCCGACCGACGATATCGAAGCCTACG
Neg_lambda2_234_35_S	Neg_lambda2	CAAGCCGAAGCATGAAGTGAATCCGCAGATGACCC
Neg_lambda2_34_39_S	Neg_lambda2	GCAAATGAGCAGAAATTTAAGTTTGATCCGCTGTTTCTG
Neg_agro2_848_35_S	Neg_agro2	ATGTGAAGCGCGTCTAAAGGAGGTGAGAGGATGA
Neg_agro2_648_40_S	Neg_agro2	TGTACGCATACCCTCCATCTACACGATAAATCCGGATGGA
Neg_agro2_448_35_S	Neg_agro2	GCACAAGAGAAGGCTGTTGCAGACCGTATACGTGC
Neg_agro2_248_35_S	Neg_agro2	ATTATCTTTTCTTCAAGGCTAGCAAGGTTCTCCCA
NM_105969.2_1475_40_S	NM_105969.2	TTTGAACCTAAAGCCGATGTGGTGCTAATACATAAAACAAT
NM_105969.2_1243_40_S	NM_105969.2	GCTGGAATCAGGGTACTCACTCATAAATGAACAGAACTT
NM_105969.2_1014_40_S	NM_105969.2	ACGAGAAAGATGGTATATCGCGGAAAGATGTTATAAGCAT
NM_105969.2_813_40_S	NM_105969.2	TGATGAATCTTGCGGATAATCCTACAAGGGTAAGTGTAGG
NM_105969.2_610_39_S	NM_105969.2	AGTTATAATGTCTGCAGGAGAGTTGGAATCTGATAGAGC
Neg_labmda3_656_35_S	Neg_labmda3	TTGCTGCAGGCAAGGTCAACATTCGGTTGTATCC
Neg_lambda5_558_35_S	Neg_lambda5	CAATGTTCTGCCTGTTCTGTACGGGAAATGCGCG
Neg_lambda5_260_35_S	Neg_lambda5	TAATTCATATTGTTCCAGAGTCGCCGGGGCCAAG
Neg_agro3_619_35_S	Neg_agro3	AAGGAGCATCAAAAGACTGCGGTCATCTTTGGGGC
Neg_agro3_400_35_S	Neg_agro3	GACAACCTGGAGACGATGGACAAACGCTTAACAGC
Neg_agro3_180_35_S	Neg_agro3	TTATGGCGTCACCGCCCTACTA ACTTCGCTCAACC
Neg_haedu_403_35_S	Neg_haedu	GAGATTTTAAATAACGCAATAAGACGCGCGCTACG
Neg_haedu_203_38_S	Neg_haedu	AGCGTTATAATAAAGGCTACACCGGCAACATATTACAG
NM_117721.1_3446_35_S	NM_117721.1	TCGTTATCGAACTCCCGGTTCTCTAATGATGATG
NM_117721.1_3246_35_S	NM_117721.1	CGGATCTCCGCTGTACGTTTGGAGTTCAGAATGG
NM_117721.1_3037_35_S	NM_117721.1	GTGGTAAGCCAAGTCATGTTGGTGGTGAGAGAGAG
NM_117721.1_2837_40_S	NM_117721.1	CATTGAGTGGATTGCGTTTCACAAATTCATTGCTGGAAGA

2.7. RNA isolation, cDNA synthesis, labelling and hybridization

After harvesting with saline water, cells grown in CPM model agar plates, were RNA extracted using RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. Samples lysis was carried out in a FastPrep instrument (MP Biomedicals) with different setting for spores, germinating spores and vegetative cells: spores and germinating spores were processed three times for 50 seconds each in the FastPrep machine at setting 6.5 m/s using zirconium beads; vegetative cells were treated only one time for 40 seconds at 6.0 m/s.

RNA samples were resuspended in 40 μ l of RNase-free water and rapidly frozen at -80°C . The quantity and quality were determined by NanoDrop® ND-1000 (NanoDrop Technologies, Inc.) and analysis on a RNA 6000 Nano LabChip (Agilent Technologies) using a 2100 bioanalyzer (Agilent Technologies).

RNA amplification was performed using the MessageAmp™ II-Bacteria Kit for prokaryotic RNA (Ambion, USA). The MessageAmp II-Bacteria Kit is a linear in vitro transcription based RNA amplification system (Van Gelder et al., 1990) to produce amplified RNA (aRNA also commonly called copied RNA or cRNA). Five hundred nanograms of template RNA were polyadenylated each poly(A) RNA sample was converted to cDNA using T7 oligo(dT) primers and amplification by in vitro transcription to synthesize amino allyl-modified aRNA. The purified aRNA, (20 μ g per sample), was labelled with Cy-5 dye; Cy5-aRNA was subsequently purified and its concentration was determined using NanoDrop® ND-1000 (NanoDrop Technologies, Inc.). During fragmentation reaction, Cy5-aRNA (6 μ g per sample) was fragmented to lengths of 50–200 bases. The fragmented Cy5-aRNA was then mixed with hybridization buffer (6 \times SSPE, 0.05% Tween-20, 20 mM EDTA, 25% deionised formamide, 0.1 mg/mL sheared salmon sperm DNA and 0.04% SDS). Hybridization and washing were performed as indicated by CombiMatrix. Slides were scanned with a Perkin Elmer Scanarray 4000 XL raw data was extracted with Scanarray Express 4.0 and Microarray Imager (CombiMatrix) software.

The experiment was carried out in triplicate (biological triplicate).

Each gene was present at least in duplicate on the slide, so each sample was hybridized in duplicate on the same microarray (technical duplicate).

2.8. Microarrays stripping for Re-hybridization

RNA targets labelled were stripped from CombiMatrix 12K microarrays in according manufacturer's instructions of the CustomArray Stripping Kit. Stripping and re-hybridization were repeated six time for each microarray used for a total of 12 hybridization.

2.9. Microarray data analysis

The fluorescence signal for Cy5 channel and background subtractions were determined with Microarray Imager software (CombiMatrix Corp.). The fluorescence signal of each spot was calculated as the difference between the mean of pixel intensities and the mean of background fluorescence signals, defined by surrounding pixel intensity (Heiskanen et al. 2000). Background level was defined as the average signal of the negative and degradation controls plus two times their standard deviation. The normalization between arrays was performed using the quantile normalization method using the R software. Normalized signals were Log₂ transformed and differentially expressed genes in the different conditions tested were identified with a one-way ANOVA test, for p-value < 0.05 and for induction or repression ratio equal or higher than 1-fold. Microsoft Excel was used for the elaboration of the data; a quality control of the data was estimated according to the principal component analysis (PCA).

Filtered data were then analysed using Microarray Expression Viewer software (MEV-TIGR; <http://www.tm4.org/mev.html>), (Saed et al., 2003); to identify groups of genes with similar transcription profiles, the significantly regulated genes were subdivided into clusters with different expression patterns by using K-means clustering (MacQueen, 1967). To calculate mRNA abundance in dormant spores, Significance Analysis of Microarrays (SAM) using a one class design was used (Tuscher et al., 2001).

For a functional interpretation of the transcriptional activity, the *B. thuringiensis* 97-27 sv. *Konkukian* genome annotations (provided at Integrated Microbial Genomes data management system (IMG), <http://img.jgi.doe.gov/>, U.S. Department of Energy Joint Genome Institute (DOE JGI)), were used. Six groups of functionally related genes were identified and ordered by HCL hierarchical clustering using Microarray Expression Viewer software MEV-TIGR (see above).

2.10 Relative quantification of enterotoxigenic gene expression

RNA used for all the quantification experiments, was the same prepared for microarray analysis as described above (section 2.7). An additional extraction step was performed 24 hours after heat activation of spores in food model. RT-qPCR was performed on a LightCycler Instrument and the FastStart DNA MasterPlus SYBR Green I kit (Roche). The mRNA level changes of each enterotoxigenic gene in the different conditions analyzed were normalized to the mRNA level of the unregulated P1-P4 gene encoding for 16S rRNA. The various oligonucleotide primer sets used for reference and target genes amplification, are shown in Table 2. Primer sets were designed against the complete nucleotide sequence, as deposited on GenBank, using Vector NTI 9.0.0 (InforMax, Frederick, MD). The optimum annealing temperature for each primer set was determined prior to the analysis of experimental samples. Reverse transcription was performed using 200 ng DNA-free RNA, random primers, and the Transcriptor First Strand cDNA Synthesis kit (Roche), following the supplier's recommendations. A sample volume of 20 μ l was used for all quantification assays, which contained a 1X final concentration of SYBR green PCR master mix, 0.5 μ M gene specific primers, and 1 or 2 μ l template. Lightcycler experimental run protocol was used for amplification. Samples were heated at 95 °C for 10 min before cycling for 45 cycles of 95°C for 10 s, 56°C or 57°C for 20 s, and 72°C for 25 s. In each step the temperature transition rate was 20°C/s. A melting curve plotted at the end of each run verified the specificity of the amplification product. All samples and standards were run in triplicate. Prior to quantitative analysis, standard curves were constructed for housekeeping and target genes using cDNA from late log *B. thuringiensis* cells grown in BHI medium: serial dilutions of the cDNA (1:1; 1:10; 1:100; 1:1000; 1:10,000) with nuclease-free water were used to generate standard curves for target genes as well as P1P4, which covered 3-5 orders of magnitude in the range of the samples in order to calculate the specific efficiency (E) using LightCycler Software 3.5. After completion of PCR, the LightCycler software calculates the copy number of target molecules by plotting logarithm of fluorescence versus cycle number and setting a baseline x-axis. The baseline identifies the cycle in which the log-linear signal can be distinguished from the background for each sample. The x-axis crossing point of each standard is measured and plotted against the logarithm of concentration to produce a standard curve. The concentrations of target sequence in the samples are extrapolated from the standard curve. The LightCycler Software 3.5. displays only the slope of a standard curve, which can be used to calculate the efficiency using the

equation $E=10^{-1/\text{slope}}$. Relative expression levels between samples were then calculated as fold changes, where each PCR cycle represents a two-fold change.

Table 2. Primer set of four enterotoxin target gene used for RT-qPCR

Genes	SEQ (5'-3')		Annealing temperatures	Amplification product
hblC real	Fw	5'-AAATTATTGAACCGGCTGCTC-3'	57	168pb
	Rv	5'-TCAATTGCTTCACGAGCTGC-3'		
nheC real	Fw	5'-GCTGGATTCCAAGATGTAATG-3'	57	149pb
	Rv	5'-TTGATGCTGAATCATATTTCCC-3'		
bceT real	Fw	5'-GAGGATAAAGAACATTTAGACG-3'	57	159pb
	Rv	5'-CTGCGTAATCGTGAATGTAG-3'		
cytK real	Fw	5'-CTGGCGCTAGTGCAACATTA-3'	56	171pb
	Rv	5'-GGCGTTGCAGAAGCTTTAAC-3'		

2.11 Enterotoxin assay in food model

Spores of *B. thuringiensis* UC10070 were added to CPM model at a concentration of 10^6 cfu/g.; inoculated samples were then anaerobically stored with Anaerocult[®] A mini foil bags, and heat treated for 15 minutes at 70 ° C, to activate spores. At defined time after heat activation (40', 2h, 12h, 24h) one volume of sodium chloride solution (0.85%) were added to 10g of vegetable mix samples. After homogenization, each sample were centrifuged (9,000 g) for 30 minutes at 4°C; supernatants were filtered and analyzed for the presence of the L2 component of the diarrheal enterotoxins HBL according to the BCET-RPLA protocol.

3. Results and discussion

The CPM model (see sections 2.10 and 3.5, chapter 2), inoculated with *B. thuringiensis* spores was used to analyse the molecular mechanism behind the process of spore germination, cell outgrow and toxins production and the possible role of *B. thuringiensis* in food intoxication.

B. thuringiensis UC10070 strain, was used in this study since, for its large use in biological agriculture to control a wide range of caterpillars in vegetables, tomatoes, vines, fruit trees, kiwifruit and tobacco, it is expected to possibly contaminate foods.

Spores of *B. thuringiensis* UC10070 were produced from cells cultured in BP medium agar plates; after incubation for 4 days at 37°C a spore suspension of 1.5×10^8 cfu/ml was obtained. The CPM model previously described, was chosen for its ability to support the development of the *B. thuringiensis* biological cycle, from the dormant spore state to the

vegetative cell production, through the germination process. It also provides a suitable model for simulating the industrial processing which foodstuffs are submitted, allowing an efficient system to assess the behaviour of a potential pathogen in food.

To identify the critical time for the extracting RNA from the outgrowing cells, the germination process was monitored by SEM and SEM-Xray techniques (see section 3.6, chapter 2) harvesting cells at various time points during growth in the CPM food model.

3.1 Genome-wide gene expression analysis

3.1.1. Microarray validation

Total RNA extractions were performed at different times to determine rate of *B. thuringiensis* UC10070 genes differentially expressed in food model, during spore germination, outgrowth and vegetative cells growth. Samples for RNA isolation were harvested from dormant spore (SP), after 40 minutes from heat activation (GSP), after 2 hours during outgrowing process (C2h), and from vegetative/sporulating cells (C12h), 12 hours from activation of spores. For each sampling, cells were rapidly harvested and frozen, to stabilize the nucleic acid in the different steps of the biological cycle; the bead-beating method performed in the FastPrep instruments, was an efficient system to extract RNA without enzymatic treatment: nucleic acids could be isolated efficiently from spores, germinating spores and vegetative cells. After isolations, RNA was quantified and RNA integrity was verified, (see figure 10); values of RNA integrity number (RIN) from 7 to 9 were obtained. Two prominent bands, corresponding to the 16S and 23S rRNA subunits, were observed in all samples on the bioanalyzer gel (Fig. 10); RNA from spores showed one additional band corresponding to an rRNA species (Fig.10-A; Fig. 11, sample 1-2-3), slightly smaller than the normal 23S rRNA, as already described in *Bacillus subtilis* and in *Clostridium novji* (Plomp et al. 2007).

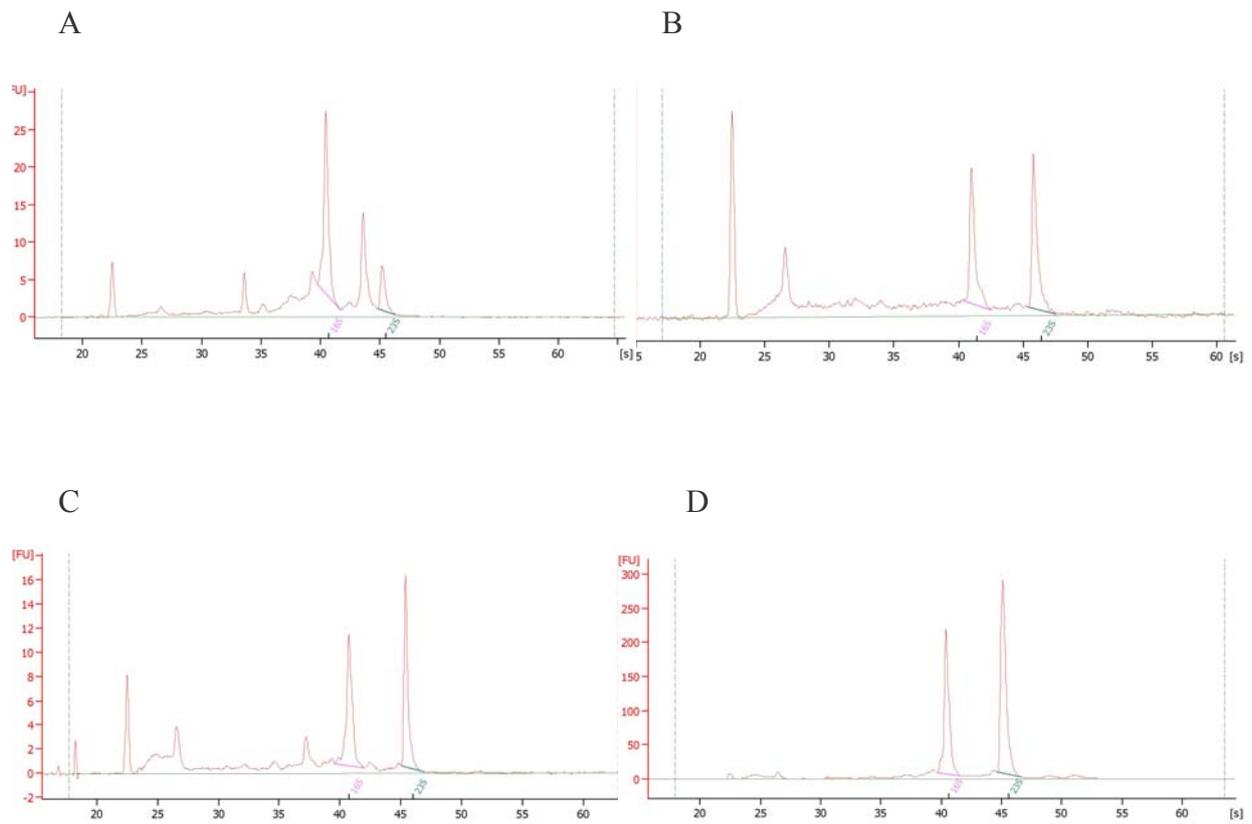


Fig. 10 Electropherogram Summary for RNA integrity at each time extraction: A= SP, B=GSP, C=C2h, D=C12h.

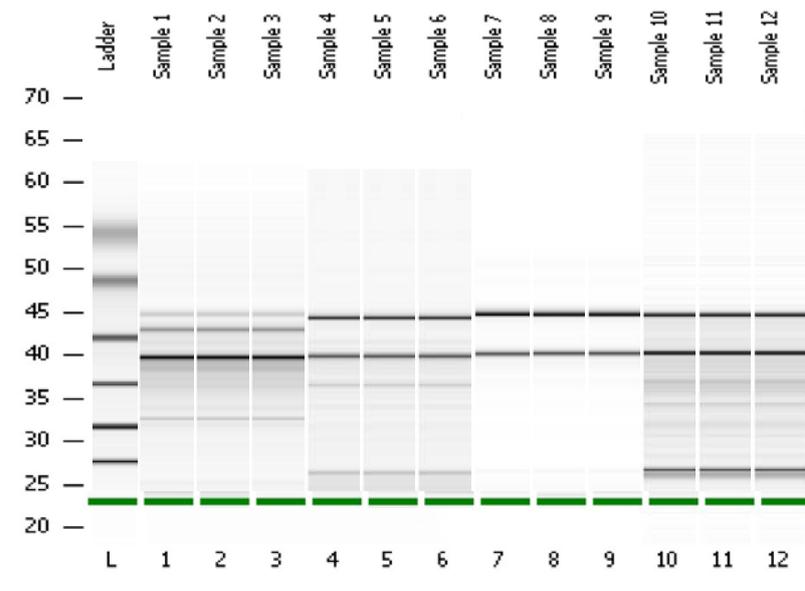
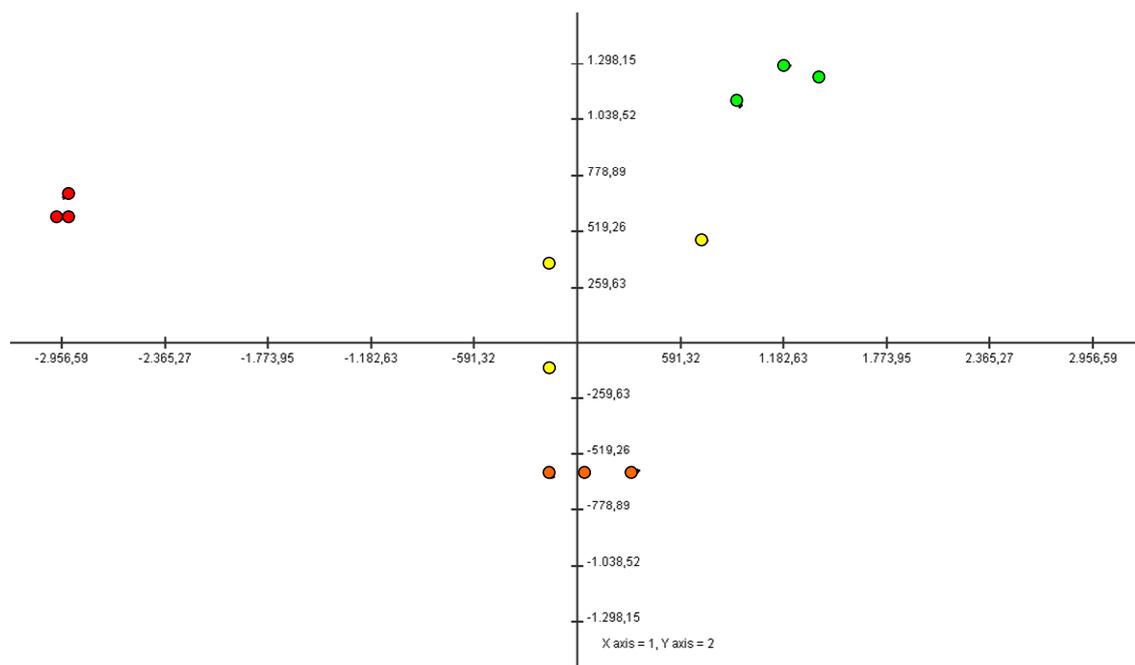


Fig. 11 RNA integrity Gel Image, measured by 2100 bioanalyzer (Agilent Technologies). In all samples analysed there are two major bands corresponding to the 16S and 23rRNA subunits. An extra band smaller than the 23S one, appears only in spore.

RNA isolated from each condition, was used to prepare fluorescently labelled cDNA which was hybridized onto synthesized 35-mer oligonucleotide slides. After washing and scanning, fluorescence intensity data were extracted and analyzed. Since total RNA was used for labelling, exogenous RNA negative controls from plant were used to define a background level and eliminate non-specific hybridization signals during data normalisation.

Principal Component Analysis (PCA) of the array data is showed in Graphic 1. The two main components explain 60,38 % and 13,26% of variance. Gene expression was found to be highly dynamic during the *B. thuringiensis* life cycle and was found to involve a large number of genes.



Graphic 1. Signal distributions for three biological replicates. In the two-dimensional principal component analysis (PCA) 1,646 differentially expressed genes were included and identified in a supervised way to distinguish between the 4 distinct conditions analyzed. The 12 spheres represent every replicates for each of the four condition of *B. thuringiensis* biological cycle. Spheres with the same color represent biological samples of the same condition (Red =SP, Orange=GSP, Yellow=C2h, Green=C12h).The first two principal components account for 73,64% of variation of the data (PC1 = 60,38 % , PC2 =13,26%).

3.1.2. Transcriptional analysis

Gene expression was found to be highly dynamic during germination and outgrowth of the germinated spore, and was found to involve a large number of genes, which is consistent with the relevant metabolic and morphologic changes that accompanies the *Bacillus* life cycle. Fold-change was employed as a measure of biological significance for gene selection (Chen et al., 2006). As shown in Table 3, four comparisons were done to identify genes regulated during different stages of *B. thuringiensis* life cycle. A total of 1,646 probe sets were found to be differentially expressed and modulated. In figure 12 the number of differentially expressed probe sets in the four comparisons done are graphically represented.

Table 3. Number of differentially expressed probe sets with a minimum 2-fold change is reported as number of up- and down-regulated genes referred to the first condition when compared to the second one. Comparisons are listed in the first column.

Comparison	Probe sets >2	Probe sets >2	Total regulated
	Up regulated	Down regulated	
SP vs GSP	14	299	313
GSP vs C2h	24	18	42
2h vs 12h	60	1	61
C12h vs SP	463	150	613

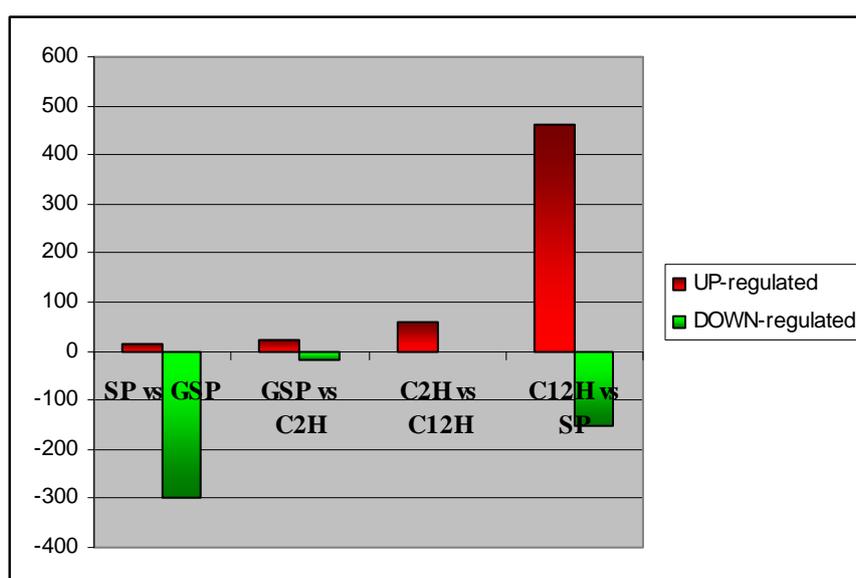


Fig. 12 Graphic representation of the number of differentially expressed probe sets in the 4 comparisons done during different stages of *B. thuringiensis* life cycle.

Spore is a metabolically dormant cell and any transcriptional response is inactivated. Therefore, the comparison between RNA content of spores and all the other conditions analyzed, indicates the difference in the mRNAs abundance. Ipotetically, transcripts having a role in germination already present in spores, are transcribed during previous phases.

The presence of 299 up-regulated genes in “GSP” vs “SP” reflect the transfer of the dormant spore to an active state, that initiates with a cascade of processes that gradually degrade the protective structures of the spore and resume cellular processes and its metabolism, ultimately leading to the vegetative cell. Only transcripts of 14 genes, present in the SP were less present in GSP. These transcripts, whose functions will be explained in more detail in the next section, are probably transcribed during sporulation phase to equip the spore with systems of adaptation against harsh environmental conditions and codes for transposase IS660, forespore specific protein, small acid soluble protein, PAP2 family protein, and other proteins with unknown functions.

The comparison between the two intermediate conditions such as “GSP” and “C2h” showed only 42 differentially expressed genes; this finding together with the small amount of total regulated genes in “C2h” vs “C12h”, gave evidence of the big transition in bacterial cell needs during germination process, and probably mean that resume of metabolic activity develops during first 40 minutes after spore activation.

3.1.2. QT-clustering

To identify potential co-regulated genes and to reveal patterns of temporal gene expression, the individual expression profiles of the 1,646 differentially expressed probe sets previously identified, were clustered into 5 groups of genes with similar expression profiles by K-means clustering (Fig. 14). Genes were subdivided in the optimal number of groups revealed by “Figure of merit” analysis (Fig.13). The groups were subsequently ordered by the timing of expression.

The first cluster (Group 1) groups the majority of genes, and consist of 824 genes, the transcripts of which were present in the dormant spores and disappeared rapidly during later stages of the biological cycle. In the second and third cluster (Group 2, and 3) are grouped approximately 407 genes, the expression of which occurred during the first 40 minutes of the germination process. According to previously published data on the

temporal gene expression during *Bacillus* spp. spores outgrowth (Keijsers et al, 2007), important house-keeping genes, encoding proteins such as translation initiation factor, ribosomal proteins, and elongation factors, were found to be transcribed in the first 40 minutes after activation of dormant spores, together with ample modulation of their expression levels during outgrowth and vegetative cycle. Group 4 consist of 210 genes that presented an over expression profile at the initiation of vegetative growth. Approximately 205 genes, clustered in Group 5, were strong positively modulated in the time occurring between vegetative growth and initiation of stationary phase.

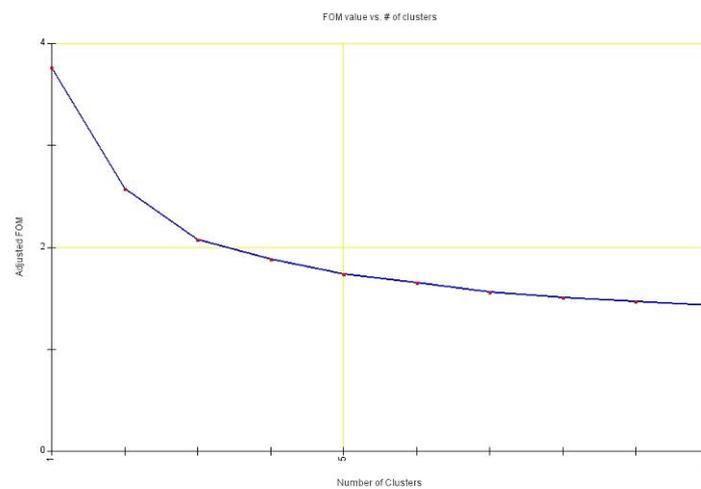


Fig.13 Figure of merit technique was used to identify the optimal number of cluster useful for microarray data analysis. Number 5 were selected as it corresponds to the point of inflection

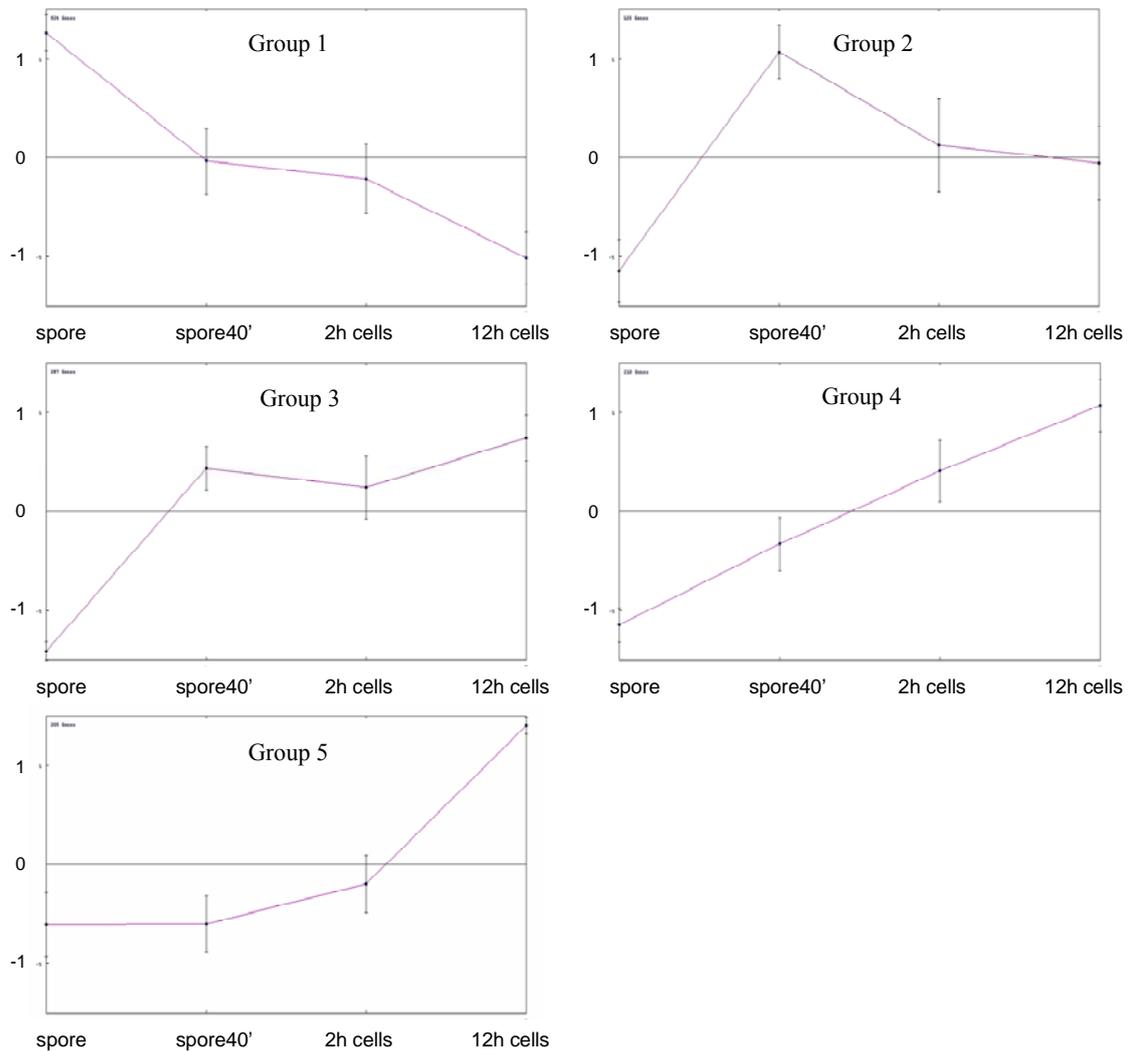


Fig.14 Gene expression profiles during spore germination and outgrowth. By K-means clustering. According to their gene expression pattern, genes were grouped in 5 clusters. The mean log₂ ratio of the individual genes in the 5 K-means clusters is plotted at the various time points (different time of life cycle in food model) over the average value. The bars indicate the standard deviations of the individual genes in the 12 K-means clusters

3.1.3. Transcriptome analysis of spores

In agreement with recent studies that confirm the presence of stable RNA in spores, transcripts of approximately 950 genes were found to be present in the dormant spore during analysis (Fig. 15). A 38% of these mRNAs had no known function; the second largest group (10%) were predicted to encode transport proteins for aminoacids, carbohydrates, lipids, cations and nucleotides. The 7% of transcripts was founded to consist of genes coding for enzyme transferase, mainly belonging to acetyltransferase (GNAT) family, involved in the regulation of cell growth for their important role in

transcription and DNA repair (Carrozza et al. 2003). Others mRNAs were predicted to encode for aminoacyl-tRNA synthetase (7%), energy production and conversion (5%), transcriptional regulator factors (4%), ribosomal proteins (30 and 50 S) together with 5S, 16S and 23S rRNA (3%), DNA repair (2%), and membrane protein (2,3%). Many transcripts identified in the dormant spores, belonged to genes expressed at a late stage of pre-spore formation; the late sporulation transcripts found, included genes encoding small acid soluble proteins (SASPs), transposase, forespore-specific protein, RNA polymerase σ K-factors, and sporulation proteins for stages 0, II, III, V (*spoA* gene family); the SASPs do protect the DNA during spore dormancy by complete saturation and tight binding, changing the structure to a more stable conformation (Setlow, 1995). Insertion sequences like ISs transposases, are small mobile units of DNA, which serve as the sites for recognition and cleavage by Tpsases in transposition reactions; they play an important role in internal genetic rearrangements in the genome, likely to support the mechanisms of adaptation to extreme environments, such as those with high or low pH, high or low temperature, high pressure, or high salinity (Takami et al. 2001). The RNA polymerase σ K-factor plays a key role in sporulation, as it is the first transcription factor whose activity is cell specific, and it sets into motion the entire cascade of compartmentalized gene expression. *Spo0A* gene acts as both activator and repressor of gene expression during the initial stage of sporulation process (Molle et al. 2003).

In addition, there were a kind of transcripts already present in the dormant spore, that seemed to be necessary to rapidly supply spores going to germinate: the mRNAs found for spore germination proteins (encoded from *GerA* operon), spore coat degradation proteins like cell wall endopeptidase, glycosyltransferase, involved in cell wall biogenesis, and rehydration systems (like aquaporin Z) should be essential to initiate a cascade of processes that gradually degrade the protective structures of the spore and resume cellular processes and its metabolism, ultimately leading to the vegetative cell.

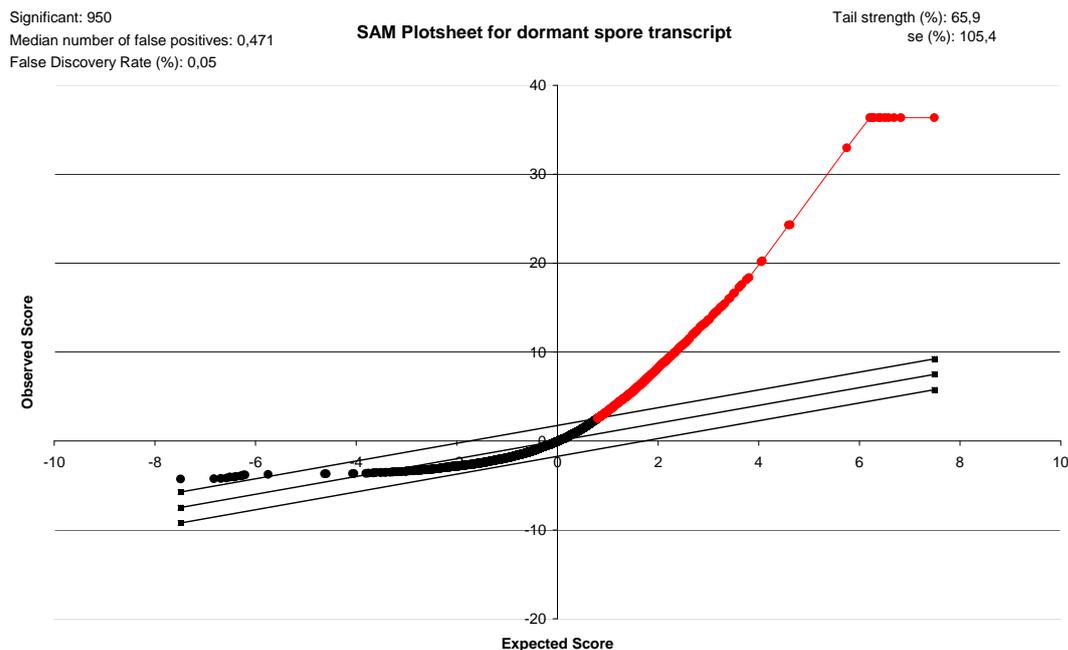


Fig. 15. Statistical analysis of mRNAs transcript present in *B. thuringiensis* dormant spore. SAM plot resulted from “one class” data analysis, shows the distribution of 950 genes found to be significant for $\Delta = 1,738$ obtained by array centered data normalisation.

3.1.4. Functional analysis

In order to have a functional interpretation of the transcriptional activity during germination process and outgrowth, six groups of functionally related genes were identified using *B. thuringiensis* 97-27 sv. *Konkukian* genome annotations and ordered by hierarchical clustering (Fig.16). Functional categories were then analyzed in relation to their expression pattern as shown in Table 4.

Table 4. Analysis of overrepresented genes with specific function during germination, outgrowth, and vegetative cell growth. Significantly overrepresented gene with specific functions are indicated in red; orange boxes indicate genes significantly represented, while the slightly modulated genes are shown in yellow.

	SPORE	SPORE40'	2H CELLS	12H CELLS
Transcription-regulation				
Transport				
Ribosomal activity				
Cell wall biosynthesis				
DNA repair				
Virulence				

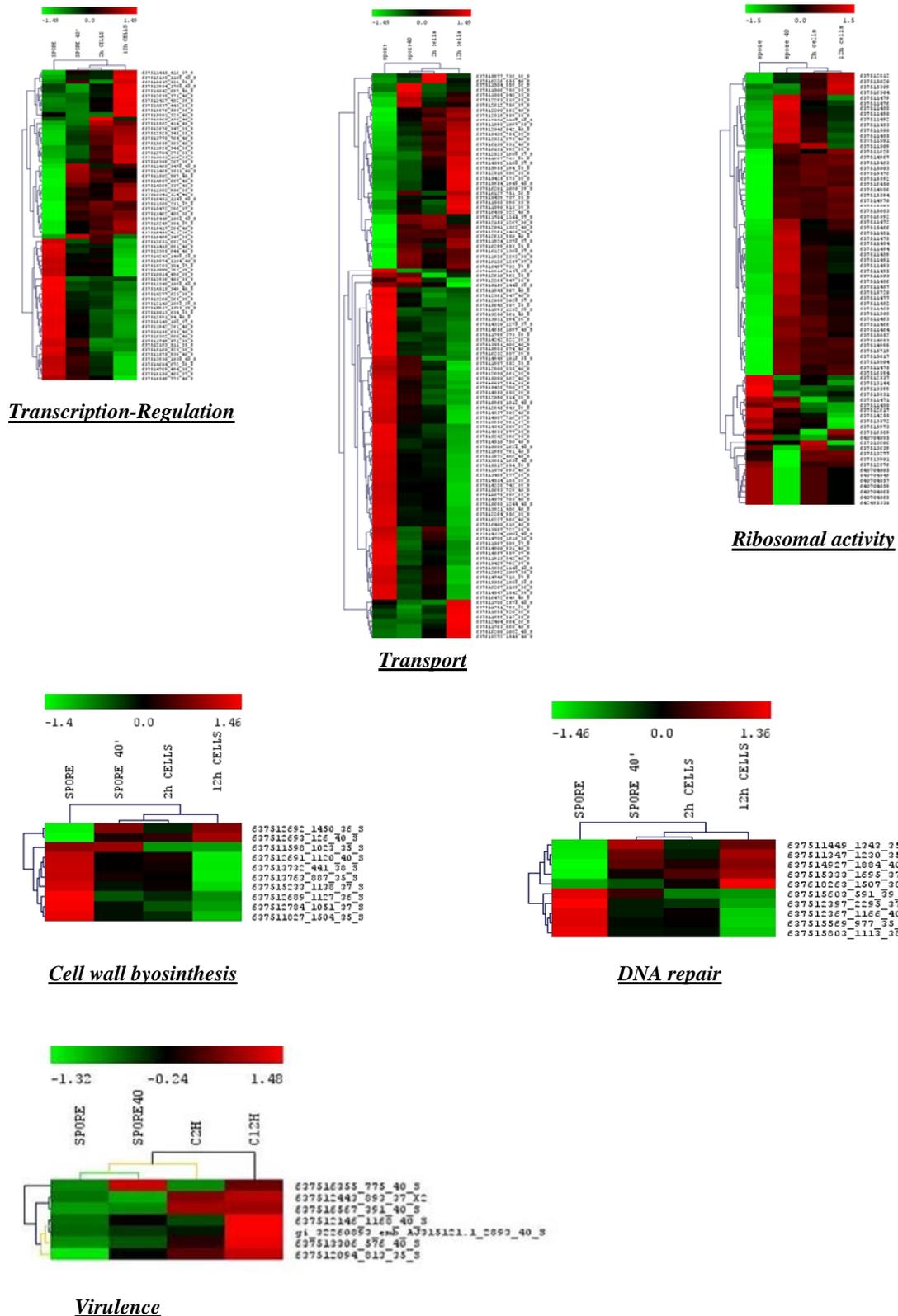


Fig.16. Hierarchic clustering of transcriptional profiles of genes associated with: transcription and regulation of transcription, transport, ribosomal activity, membrane biosynthesis, DNA repair, and virulence. Rows represent time points from spore to vegetative/sporulating cell. Red and green indicate genes that are induced and repressed, respectively.

- ***Transport***

One of the biggest significantly overrepresented group of functional genes identified, encodes for proteins involved in transport of various molecules; ABC transporters specific for ions, sugars, and other organic compounds like drug/metabolite, were founded already in spores transcript.

For efficient outgrowth and to rapidly supply the germinated spore with the essential metabolite, the immediate initiation of transport functions is likely to be necessary .

The expression of the glycine betaine transport protein OpuAB, a widely diffuse bacterial system for cell protection against high solute concentration, may suggest that osmotic defence is important in the earliest stages of outgrowth, in agreement with previous studies on germination process (Keijser et al, 2007). Genes coding for aquaporin proteins was found to be significantly represented in this category: since these pore-forming integral membrane proteins, allow bacterial cells to control and efficiently regulate water homeostasis, essential for many biological processes, microbial aquaporins are likely to be involved in the first step of germination, which require a rapid re-hydration of the cytoplasm as proposed by Kruse et al. (2006). A large number of transporter genes encoded putative multidrug transporters, such as *SMR* and *Bcr/CflA* family, which were upregulated during the first 40 min of outgrowth, may also provide the germinated spore with a transient resistance against antimicrobial complexes. Active export during early stages of outgrowth may also provide the germinated spore with a transient resistance against antimicrobial complexes, which render cells resistant to different antimicrobial compounds.

- ***Transcription and regulation***

Many genes involved in transcriptional regulation for rapid recover of cell function were found to be already present among the spore transcript pool. A large number, includes genes coding for protein involved in the regulation of different biological processes in response to external stimuli; examples are members of TetR, MarR and MerR (expecially found in C12h) transcription factors families (TFs), that were identified as common families to *Bacillales*, *Lactobacillales* and *Clostridia* (Samadhi Moreno-Campuzano et al, 2006), which are responsible for rapid adaptation of the bacteria to changing environmental conditions, including resistance to different antimicrobial compounds and oxidative stress agents, controlling the expression of drug efflux pumps. Members of GntR family proteins,

also found in dormant spore, respond to environmental changes affecting the carbohydrate metabolism of the cell and may provide bacteria the ability to grow in the presence of several carbon sources and to rapidly adapt their gene expression to changing nutrient conditions (Reizer 1991). Transcript coding for the fumarate and nitrate reduction regulator CRP-Fnr, member of the cyclic AMP receptor proteins, was slightly up-regulated in spore; it is known to play an important role in modulating the expression of many metabolic genes in several facultative or strictly anaerobic bacteria; their functions also include the control of virulence factors (Baltes et al 2005, Bartolini et al 2006). In addition, transcript for RNA polymerase σ -factor, ECF type, that control genes involved in cell envelope functions (protein transport and secretion processes), in responses to extra-cytoplasmic stress in *B. subtilis* and *B. licheniformis* (Tina Wecke et al. 2006), and RNA polymerase σ_K factor, responsible for the expression of sporulation specific genes in the mother cell, were found in dormant spore. During outgrowth and vegetative cells growth, genes regulating complex involved in DNA replication, transcription (DNA polymerase III, DNA-directed RNA polymerase delta subunit) and RNA translation, like transcription elongation antitermination factor protein *NusG*, which function was recently assessed in *B. subtilis* (Yakhnin et al. 2008), were overrepresented. During late stages of outgrowth, the cell appeared to prepare for septation, as indicated by the overexpression of septation ring formation regulator *EzrA* (Jeff Errington, 2001), giving evidence that transition of the dormant spore to an actively growing vegetative cell, appears to be completed. Transcript for the transcriptional regulator PlcR (Phospholipase C Regulator) were found to be up-regulated in the late stage of cell vegetative growth. It takes part in the control of most known virulence factors in *B. cereus* (enterotoxin, haemolysins, phospholipases and proteases), (Michel et al 2008) acting as a quorum-sensing system. To be active PlcR needs the PapR peptide, transcript of which was also found to be overrepresented in vegetative/sporulating cells. It is thought that PlcR can monitor cell growth state through Spo0A sporulation protein, and self cell density through PapR peptide.

Transcript coding for a two-component sensor histidine kinase response regulator, was found to be up-regulated in vegetative cell. Several study focused on the importance of two-component signal transduction systems in controlling both metabolism and virulence factors in *B. cereus* (Duport et al. 2006).

- ***Ribosomal activity***

The germination process took place through a sequence of time-ordered events. The distribution of transcript coding for rRNA shows that dormant spores contained populations of ribosomes or ribosomal precursors: genes coding for 16 S, 5 S, and 23 S ribosomal RNA were overrepresented, but analysis showed a low amount of transcripts for both small and large rRNA subunit (30 and 50S). Spore seems to be also defective of transcripts for ribosomal proteins (L1-L34, S1-S21), synthesis of which started during the first 40 minutes after spore activation when rate of both rRNA and ribosomal proteins synthesis strongly increases. Transcripts level involved in the cellular process of translation remain stable for subsequent step of analysis.

- ***Cell wall biosynthesis***

Expression pattern analysis of genes coding for proteins involved in cell wall biosynthesis, reveals an abundant distribution in dormant spore of mRNAs for transpeptidase enzyme (D-alanyl-D-alanine carboxypeptidase family protein) that cross-links the peptidoglycan chains to form rigid cell walls and glycosyltransferase, involved in cell wall biogenesis; much effort has been put forward to the identification of glycosyltransferases because of their importance for synthesis of cell wall matrix polysaccharides: enzymes involved in nucleotide sugar transport are also important because of the potential to manipulate the composition of cell walls through substrate level control (David M. Gibeaut et al. 2000).

- ***DNA repair***

A number of general DNA repair genes are expressed actively during vegetative/sporulating cells (12H cells). These specific transcripts are still abundant in the dormant spore where are likely to exert their function upon spore germination. At the same time transcript of gene coding for protein involved in more specific repair activity can be found in dormant spore. Nucleotide excision repair, and DNA repair protein *RadC*, that was supposed to be involved in the protection of spores against harsh environmental conditions and damaging following X- and UV-irradiation (Felzenszwalb et al. 1986). DNA of dormant spores is believed to be in a supercoiled state, providing protection against damage. The helicase activity of Holliday junction *RuvB*, and methionine gamma-lyase, showed during early stages of germination, may be necessary to relax rapidly the

supercoiled DNA allowing an efficient reactivation of transcription, likely after the partial degradation of the SASPs that coat and protect DNA during spore dormant state.

- **Virulence**

B. thuringiensis was initially characterized as an insect pathogen, and its insecticidal activity was attributed to the parasporal crystals encoded by the known *cry* gene.

In the previous chapter our results indicate that *B. thuringiensis* shows a set of genes coding for virulence factors common to the opportunistic pathogen *B. cereus*. This set of genes includes the *hbl* operon (*hblCDBA*), that codes for the hemolytic enterotoxin, and the three non hemolytic enterotoxin genes *nheABC*, which are considered as the primary factors in diarrheal *B. cereus* food poisoning. Moreover the *bceT* gene coding for enterotoxinT, function of which is not well understood and the *cytK* gene coding for the haemolytic cytotoxin K. This last was firstly identified in a *B. cereus* strain from a cases of food-borne disease and responsible for the death of three people (Lund et al., 2000). Also one channel forming type III hemolysin and other defence systems harmless to humans, like phospholypase, were found in both *B. cereus* and *B. thuringiensis*.

In the microarray experiment the intensity values of probes specific for both *hbl* and *nhe* operons, and for *bceT* gene, were not considered significant by statistical data analysis. Genes coding for delta-endotoxin (*cryIIa*), cytotoxin K, haemolysin type III, and possible phospholipases, were found to be modulated during *B. thuringiensis* biological cycle; transcript for enterotoxin cell wall-binding protein, were also found to be significantly modulated. From the analysis of transcriptional profiles it was suggested that all the associated virulence genes, were upregulated especially during the late stage of cell growth. However RT-qPCR analysis were performed to validate this data and to clarify the real expression pattern of the major virulence genes in *B. thuringiensis*.

As shown in figure 17, transcripts encoding for insecticidal crystalline toxin (*CryIIA* gene) active against insect species of the order Lepidoptera, were found to strongly increase in the time frame between 2 and 12 hours after spore activation. A common characteristic of the *cry* genes is their expression during the stationary phase; in most cases, δ -endotoxin synthesis and sporulation are closely coupled. These δ -endotoxins, synthesized as protoxins, are produced in large quantities during sporulation and are packaged into intracellular inclusions. The transcription of many *cry* genes (e.g., *cry4Aa*, *cry4Ba*, *cry11Aa*, *cry15Aa*, etc.) is likely to be σ_K -dependent and the expression of all these *cry*

genes is therefore considered to be sporulation dependent (Schnepf et al 1998). However, in our study, transcription of *cryIIA* genes in *B. thuringiensis* were detected during the transition phase, even in the absence of abundant σ_K RNA polymerase transcript. Studies reported that low-level transcription of the *cry4A*, *cry4B*, and *cryIIA* genes in *B. thuringiensis*, probably due to other σ factor RNA polymerase, has been detected during the transition phase, lasting until the onset of sporulation (Poncet et al. 1997, Yoshisue et al. 1995).

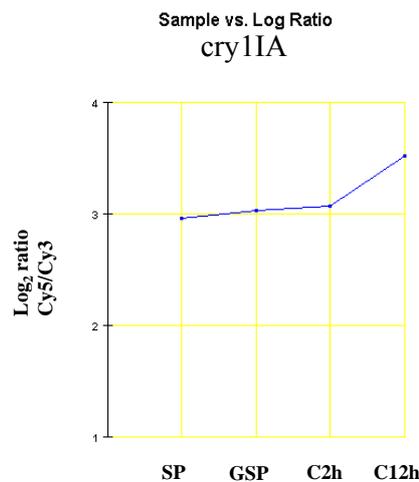


Fig.17 Gene graph for expression ratio from spore (SP) to vegetative cell (C12h) of *CryIIA* gene, coding for δ -endotoxin in *B.thuringiensis*

Our results reveal that transcriptional activation of *cytK* gene, encoding for cytotoxin K, starts quite early during the *B. thuringiensis* cell cycle: only after 40 minutes after spore activation (Fig. 18), the overexpression of *cytK* gene were observed. Unexpectedly, in the time frame between SP and GSP, *cytK* transcript seems to be slightly modulated, probably due to gene transcription during sporulation phase, resulting in slight accumulation of *cytK* transcript in spore. Cytotoxin K was first characterized in *B. cereus* strain 391-98, a strain isolated from cases of food-borne disease and responsible for the death of three people (Lund et al., 2000); interestingly, none of the other, commonly described enterotoxin genes (for example *hbl* and *nhe*) was detected in this strain, further implicating CytK as a major virulence factor. *In vitro* production of *B. cereus*-like enterotoxins, were observed to start when the total count is about 10^7 cfu/ml; such counts are normally encountered in the (late) exponential growth phase (Lucas Maria Wijnands, 2008). Considering the strong increase of *CytK* transcript found after 40 minutes in food model, one could assume that the

production of enterotoxins occurs soon after. Moreover, the strong up-regulation of genes involved in the recover of metabolic activity founded in GSP condition, and the rapids morphological changes, observed with SEM technique during the first two hours after spore activation (section 3.6, chapter 2), could indicate that *B.thuringiensis* UC10070 biological cycle total, evolves faster in food model, than laboratory medium.

Bacillus cereus is known to produce several extracellular hemolysins, including the hemolysin type III, considered as potential factors of virulence of the opportunistic pathogen. Studies on the *B. cereus* hemolysin III activity suggested that it acts as a pore-forming hemolysin that show an in vitro activity on human erythrocyte. During our analysis *B. thuringiensis* transcript for hemolysin type III, were found to be overrepresented expecially in the late stage of cell growth (Fig 18); a milder but still significant increase of the transcription level, were observed immediately after the first 40 minutes during germination.

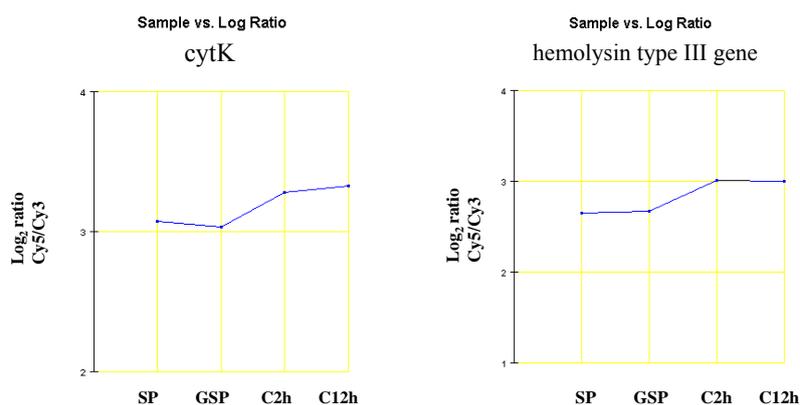


Fig.18 Gene graph for expression ratio from spore (SP) to vegetative cell (C12h) of the CytK gene, coding for cytotoxinK, and the homologous in *B.cereus* gene coding for hemolysin type III.

Transcript coding for possible enterotoxin cell wall-binding protein, were found to be overexpressed at the late stage of growth (from 2 to 12 hours); this could suggests the possibility that the cell is activated to produce components of the enterotoxic proteins complex at the later sporulation stage.

The significant expression level of *CytK* gene and other associated virulence factor gene (hemolysin type III, enterotoxin cell wall-binding protein), observed during the biological cycle of *B. thuringiensis*, gave evidence of the potential human pathogenicity of this

microorganism, which may possess the ability to produce toxins known to be involved in food borne disease.

3.2 Enterotoxin gene expression profiles

The use of microarray technology to assess gene expression levels is now widespread; however, to evaluate the performance of expression of specific genes and validate the microarray results, independent mRNA quantisation techniques, remains a desirable element. Given the extensive use of the *B.thuringiensis* UC10070, in forestry agriculture as pesticide, it seemed important to deepen investigate in food model the expression level of genes coding for virulence factors involved in food poisoning by using relative quantification of enterotoxin gene expression (RT-qPCR) technique. The total RNA, from the same samples used for microarray transcriptome analyses, was used for relative quantification to evaluate *hblC*, *nheC*, *bceT* and *cytK* mRNA levels, during the *B. thuringiensis* life cycle in food model. In table 5 are reported as normalized ratio, the relative expression of target genes in the test samples, which was determined using mRNA level of the unregulated P1-P4 gene (encoding for 16S rRNA) as the normaliser. In food model, toxin mRNAs were detected, in variable amounts, at all investigated growth stages of *B. thuringiensis* UC10070, including the first 40 minutes after thermal activation of spores. We could observe that toxin expression start quite early during *B. thuringiensis* biological cycle in food model; however the level of mRNA, for all the four genes analyzed, was found to particularly increase in the time corresponding to the 2 hours after spore activation (C2h) with the exception of *cytK*. From our previous observation, C2h corresponds to the early log phase of *B. thuringiensis* growth in food model: cell outgrowth is completed and cell division is at the maximum level (see section 3.6 chapter 2). Thus, at this stage, most of the genes involved in the metabolic activity are overrepresented in microarray analysis.

Overall, two different expression kinetics were observed (Fig. 19): the tripartite toxin transcripts *hblC* and *nheC* present the maximum expression at C2h, with values respectively threefold and twofold greater than in germinating spore (GSP). When the cells were entering the late log/early stationary phase (C12h) the level of mRNA decrease for these two genes. On the contrary, transcripts corresponding to *cytK* and *bceT* genes, raises more gradually reaching the maximum expression at C12h (*bceT*) and C24h (*cytK*).

One might hypothesize that a great and early production of *hblC* and *nheC* is due to time required for the HBL and NHE three-component proteins assembly, compared to *CytK* and *bc-D-ENT*, two single component toxins. Although no information on the *B. cereus* toxin expression in food is available, studies on *B. cereus* enterotoxins production in broth, report that the highest toxin level is achieved during the late log/early stationary phase for cultures grown, and that no significant increase in toxin production occurs during full and late stationary phase (John L. McKillip 2000). Our analysis in food, shows a higher level of transcripts during the log phase not reported for laboratory medium. According to previous finding, no significant increase in toxin expression occurred during full and late stationary phase except for *cytK* mRNAs. Thus, *cytK* expression pattern differs from the other trends showing in stationary phase a transcript level greater than the late log/early stationary phase. The relative quantification expression of *cytK*, confirmed the trend observed during microarray analysis.

The amount of toxin expression in *B. cereus* is dependent on the culture medium, as well as extrinsic factors, such as pH, aeration, and the presence and concentration of certain carbohydrates (John L. McKillip 2000): our finding confirm that the presence of starch, as ingredient in the food model described in this study, tends to support enterotoxin production as previously described in *B. cereus* by Garcia-Arribas & Kramer (1990).

Table 5: Relative quantification of virulence gene expression during *B.thuringiensis* biological cycle in food model. m: medium value of triplicate experiments and sd: standard deviation.

Gene(s)	Quantification of virulence genes expression during <i>B. thuringiensis</i> biological cycle							
	GSP		C2h		C12h		C24h	
	m	sd	m	sd	m	sd	m	
<i>hblC</i>	0.69 ±	1.85	2.70 ±	0.72	1.65 ±	1.85	1.24 ±	4.28
<i>nheC</i>	1.05 ±	0.83	2.17 ±	1.88	2.01 ±	1.06	1.22 ±	2.00
<i>cytK</i>	0.69 ±	2.26	0,93 ±	2.27	1,42 ±	0.56	1,72 ±	1.61
<i>bceT</i>	0.93 ±	0.72	1.37 ±	0.81	1.52 ±	0.65	1.38 ±	1.44

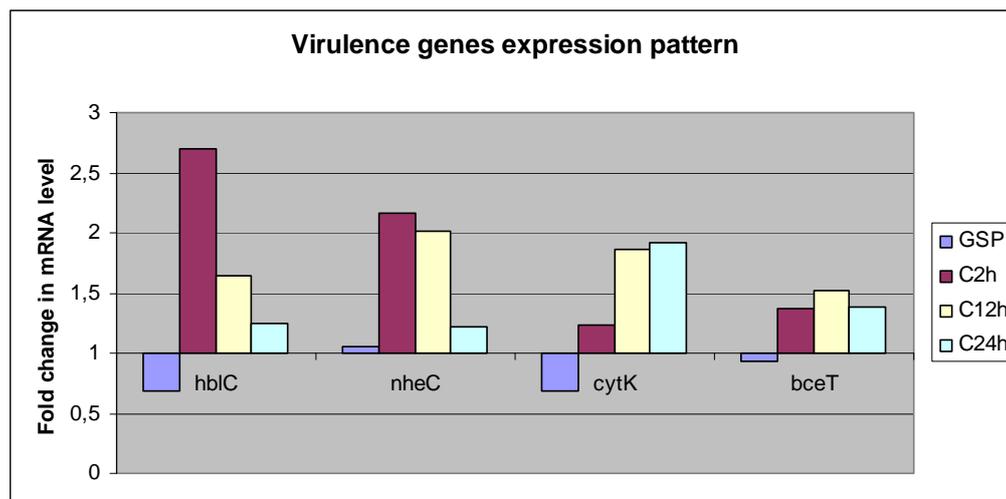
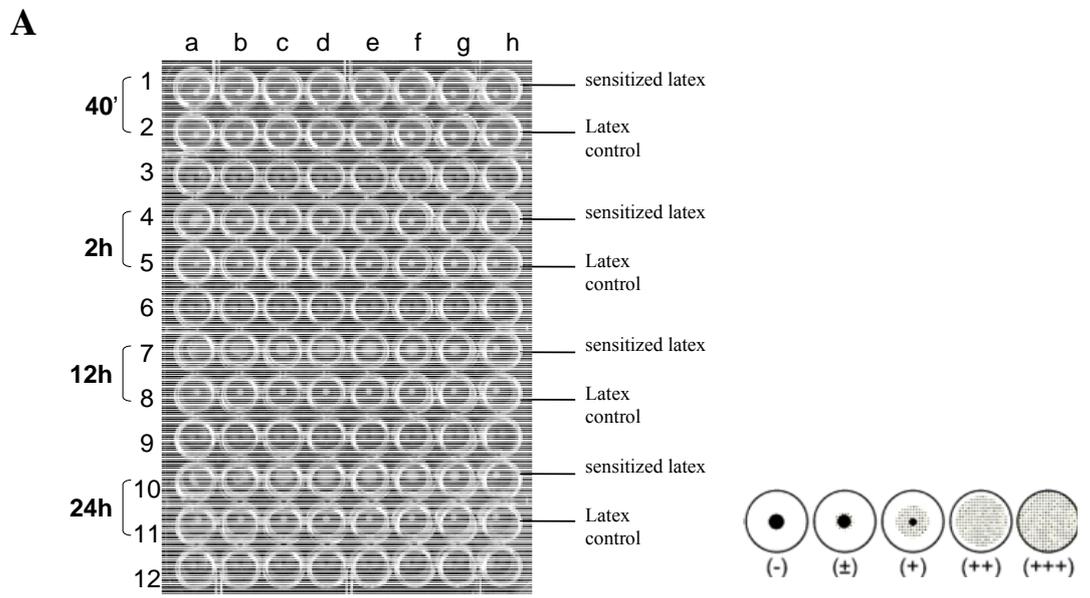


Fig 19. Histograms represent the expression pattern of virulence gene, in the different condition analyzed during *B. thuringiensis* biological cycle. Changes in the mRNA level for *hbl*, *nhe*, *cytK* and *bceT* genes under anaerobic growth condition in food model, were calculated in relation to the mRNA level for each target gene under aerobic growth condition in BHI medium.

3.3 Enterotoxin assay in CPM model

The production of the L2 component of HBL enterotoxin, involved in the diarrhoeal syndrome, was assessed in CPM model inoculated with spores of *B. thuringiensis* UC10070. Analysis was performed at 40', 2h, 12h and 24h from spore activation, to confirm that trend of HBL mRNAs, evaluated with RT-qPCR, leads to toxic protein biosynthesis. Time corresponding to spore condition, was not assessed because considered as a metabolically dormant state. Enterotoxin activities observed are reported in figure 20, A-B. No latex agglutination reactions were found up to 40 minutes after germination induction by thermal treatment (first row). The detection of the L2 component from hemolysin BL, gave positive result at the first dilution of the sampling carried out 2 hours after spore activation (fourth row). Up to 24 hours after the beginning of the *B. thuringiensis* biological cycle, the toxin component has been detected. These observations, in agreement with the transcriptional pattern reported in the previous section, demonstrate as the strong increase of *hblC* gene expression, found in the time corresponding to the two hours after heat activation of spores, leads to immediate toxin production. In studies on hemolysin BL characterisation, a growth period of 5 to 6 hours was used for *in vitro* routine production of enterotoxins (Beecher and Lee Wong, 1994; Dietrich et al. 1999). Our data suggest that in food model the translation process occur only two hours after

spore activation. Given this finding above we could assume that *B. thuringiensis* can complete an entire life cycle in food systems and produce enterotoxins as already demonstrated in laboratory cultures.



B

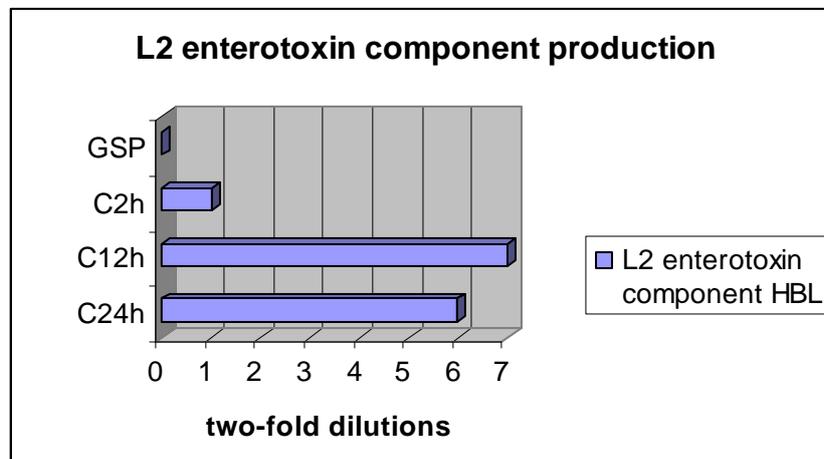


Fig.20A.Enterotoxin-Reversed Passive Latex Agglutination reaction. Wells from “a” to “g” contain two-fold serial dilutions of the samples; well “h” is the negative control. Agglutination reaction were performed on food model artificially contaminated with *B. thuringiensis* UC10070 spores, for sampling generated 40' (row 4), 2h (row 7), 12h (row 10), 24h (row 12) after spore activation. Results classified as (+), (++) were considered to be positive.**B.** Horizontal istograms reported the L2 component enterotoxin production during *B. thuringiensis* biological cycle. On x ass are reported supernatant dilution **1**: not diluted, **2**. dilution 1:2, **3**.dilution 1:4, **4**. dilution 1:6, **5**.dilution 1:8, **6**.dilution 1:8, **7**.dilution 1:16.

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RESEARCH AND INACTIVATION OF VIRULENCE
REGULATING SYSTEMS IN *BACILLUS SPP.*



1. Introduction

In pathogenic bacteria, the production of virulence factors employ various types of regulatory processes and is often regulated in response to changes in the bacterial cell environment. In Gram-positive bacteria, these processes may involve two-component systems, alternative sigma factors, or stand-alone transcription regulators. In some cases, the three regulatory mechanisms act together, each controlling a part in the production of virulence factors. This situation for instance is found 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 pathway involving the transcriptional regulator *SarA*, the two component regulator *Agr* and the general stress response regulator *SigB* (Novick 2003). In some other species, most of the virulence factors are controlled by a master regulator, which are therefore members of the same regulon. Virulence regulons may include a large number of genes: for example, the *PrfA* regulon of the foodborne pathogen *Listeria monocytogenes* includes 73 genes located on the chromosome (Milohanic et al 2003).

Identification of *Bacillus cereus* strains able to cause gastro-intestinal disorders, is complicated by the fact that mechanisms leading to infection are different and complex. Characterization of genes coding for diarrhogenic toxins, does not seem sufficient for the risk management associated to the different species. Not all the strains in which genes are identified, show the same ability to cause the disease. It was assumed that other genetic determinants, such as regulator systems of extracellular virulence factors, may be basic to guide pathogenic activity in *Bacillus cereus*, and other *Bacillus spp.*. Aim of a part of the work was the acquirement of more knowledge in regulating mechanisms of virulence in *Bacillus* genus.

ResDE two-component system is known as one of the major controlling factors of catabolic gene expression in fermentative (Nakano et al 1997, Cruz Ramos et al 2000), microaerobic, and aerobic growth (Hartig et al 2004) in *Bacillus subtilis*; moreover it regulates virulence in *Staphylococcus aureus* under low-oxygen conditions (Yarwood et al 2001). Homologs of the *B. subtilis* ResDE was found in *B. cereus* and was also demonstrated to play a role in the regulation of enterotoxin expression in *B. cereus*: the *resE* mutation abolished the production of enterotoxins under all of the conditions examined (Duport et al 2006). This finding led to the conclusion that enterotoxin

expression and fermentative metabolism may be controlled coordinately at the transcription level. However, the ResDE system, although important, is not essential for both fermentative metabolism and enterotoxin expression; another redox regulator that may act in synergy with ResDE to control the expression of fermentation and enterotoxin genes is the member of the cyclic AMP receptor protein (CRP)-Fnr (fumarate and nitrate reduction regulator) family (Korner et al 2003). The CRP-Fnr regulators play an important role in modulating the expression of numerous metabolic genes in many facultative and strictly anaerobic bacteria. Their functions also include the control of virulence factors (Baltes et al 2005, Bartolini et al 2006, Schmiel et al 2000). A recent study led by Esbelin and colleagues (2009) clarified some aspects of the *B. cereus* virulence regulation; a strict interaction between the two-component signal transduction systems ResDE and the one-component CRP-Fnr regulators, was described as a basic mechanism for controlling the production of major virulence factors hemolysin BL (Hbl) and nonhemolytic enterotoxin (Nhe) in the food-borne pathogen *B. cereus*.

Genome wide transcriptome analysis of *B. thuringiensis* UC10070 performed in the previous chapter, revealed the expression of gene coding for the one-component CRP-Fnr regulators. Transcript for a histidine sensor kinase with high homology with that described for *B. cereus* two-component regulatory system ResDE, was also found. In this section, amplification of ResDE and Fnr loci were carried out to assess gene functionality of both transcriptional regulators in *B. thuringiensis* UC10070. Inactivation of these general regulating systems, through null mutants construction, were then considered to evaluate changes in growth performance, cellular metabolism and toxins expression, in *B. thuringiensis* UC10070, and to lay scientific basis for management of potential risks associated with *B. thuringiensis*, and other *Bacillus* spp. in food intoxication.

1.2 Site-specific chromosomal mutagenesis

Integration of plasmid DNA has been successfully used to elucidate at least parts of bacterial chromosomes, by generating mutations (Niaudet et al. 1982, Shortle et al. 1982, Stahl et al. 1984), by mapping unselectable genes (Haldenwang et al. 1980, Wilson et al. 1986, Vosman et al 1982), and by cloning genes (Niaudet et al 1982, Vosman et al. 1987). The development of a useful integration strategy based on the use

of plasmids could provide an important tool for exploring the chromosome. For several organisms it has been shown that a plasmid that is unable to replicate in the recipient strain can integrate efficiently into the host chromosome when it carries chromosomal sequences. In bacteria, the most straightforward approach to integration relies on a non-replicating plasmid which carries a homologous chromosomal DNA segment and an antibiotic-resistance marker (Gutterson & Koshland, 1983; Leenhouts *et al.*, 1989). The region of homology provided usually stimulates the integration of the plasmid DNA by a Campbell-like mechanism, which leads to duplication of the homologous chromosomal insert as a consequence of this mode of integration as shown in figure 1. This Campbell-type integration event was first demonstrated in *B. subtilis* by Young, M. (1983).

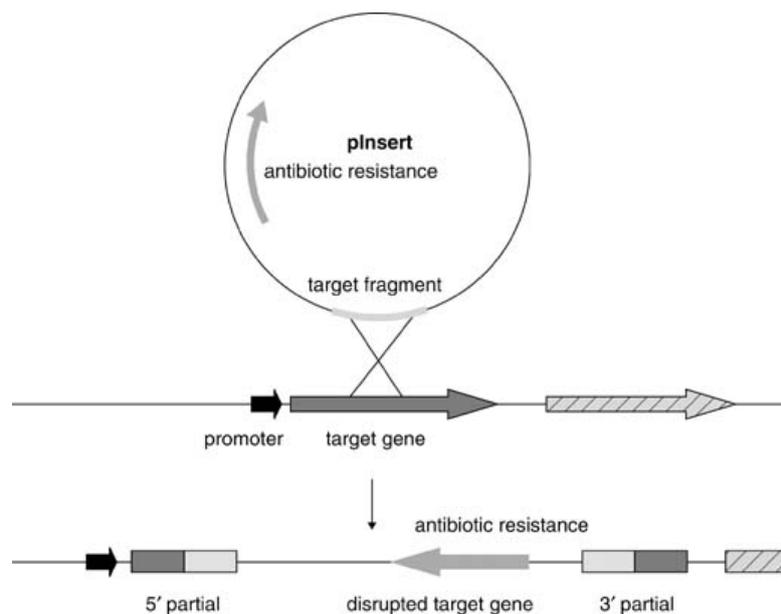


Fig.1 Schematic representation of plasmid integration in chromosome via Campbell Like mechanism. (Young, M. 1983).

Besides homologous recombination performed for initial experiments, a novel approach based on Group II intron property, was considered in this study for generation of site-specific chromosomal mutagenesis.

Mobile group II introns, can be found in bacterial and organellar genomes. They use a mobility mechanism termed retrohoming, mediated by a ribonucleoprotein complex

(RT + intron lariat), to insert themselves into any desired DNA target and to generate highly specific chromosomal gene disruption (Fig 2). They are both catalytic RNAs and retrotransposable elements and use an extraordinary mobility mechanism in which the excised intron RNA reverse splices directly into a DNA target site and is then reverse transcribed by the intron-encoded protein. After DNA insertion, the introns remove themselves by protein-assisted, autocatalytic RNA splicing, thereby minimizing host damage (Jin Zhong et al 2003).

The *Lactococcus lactis* L1.LtrB intron has been developed into a “targetron” that can be retargeted to inactivate specific genes of interest (Frazier et al 2003, Mohr et al 2001, Karberg et al 2000). Basal in this system the homing of the ribonucleoprotein complex (RNP) that consists of the group II intron RNA molecule (L1.LtrB) and the associated LtrA protein. The specificity of the subsequent integration event is conferred by the basepairing between exon binding site 1 (EBS1), EBS2, and δ of the RNA molecule, with intron binding site 1 (IBS1), IBS2, and δ' within the target gene (Fig. 1). Hence, the intron can be targeted to specific genes by replacing EBS1 and EBS2 with sequences complimentary to the insertion site within the gene of interest. L1.LtrB and LtrA are expressed from a donor plasmid, from which the L1.LtrB intron splices out of its flanking 5' and 3' exons and forms an RNA lariat that associates with LtrA (Karberg et al 2000, Long et al 2003). The LtrA protein facilitates formation of the intron's catalytic structure and recognition of the target gene. L1.LtrB reverse splices into the insertion site, while LtrA synthesizes an antisense copy of the RNA, catalyzing the reverse transcription of the intron RNA sequence.

Several studies have shown that this optimized targetron system works at high efficiency in different *Clostridia* spp. (Heap et al. 2007) and Gram negative bacteria (Rodriguez et al. 2008, Jun Yao et al. 2007), but no studies are reported in literature for the genus *Bacillus*. In the present study, we have adapted the TargeTron group II intron mutagenesis system for *B. thuringiensis*.

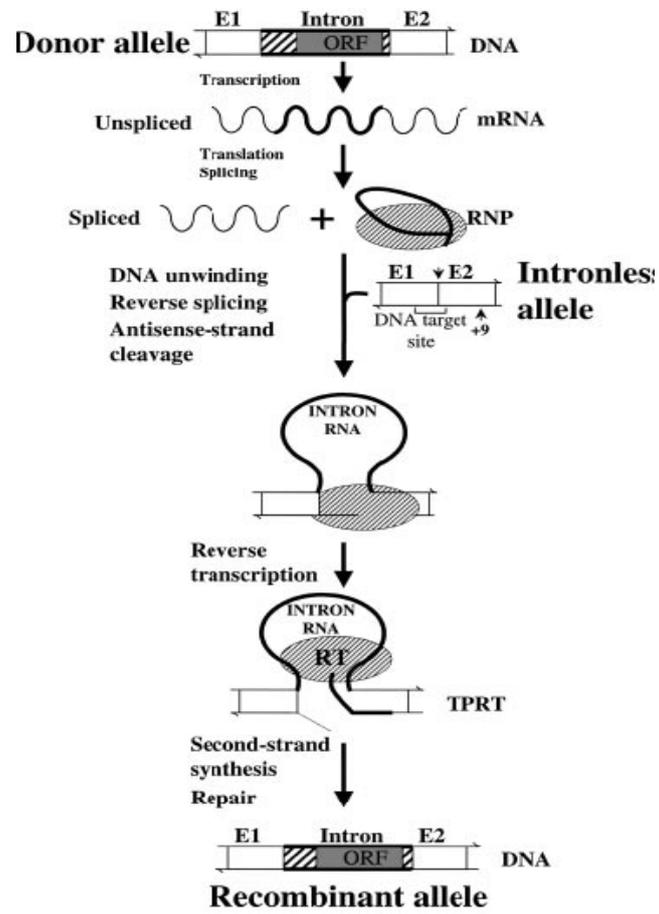


Fig. 2 Schematically representation of intron retrohoming mechanism. Martinez-Abarca et al. 2000

2. Materials And Methods

2.1 Bacterial strains and growth condition

Bacillus thuringiensis UC10070 var. *kurstaki* serotype H3a3b was used to perform insertional inactivation in this part of the study. Strain isolation was carried out from a commercial bio-insecticidal product in our laboratory, by plating on BCA selective medium agar plates, decimal dilution of the commercial powdered after incubation at 30°C for up to 72h of (see section 2.1, chapter 2). For the mutagenesis experiment, *B. thuringiensis* UC10070 were routinely grown on Brain Heart Infusion broth (BHI, Oxoid) at 37°C on continuous shaking at 250 rpm, or in Luria-Bertani (LB) agar plates at the same temperature. *Escherichia coli* TB1 strains (genotype: F- *ara* Δ (*lac-proAB*) [Φ 80*dlac* Δ (*lacZ*)M15] *rpsL*(*StrR*) *thi* *hsdR*) were propagated in LB medium. When required, antibiotics were used at the following concentrations: 100 μ g of ampicillin ml⁻¹, or 15 μ g chloramphenicol ml⁻¹ for *E. coli* and 5 μ g of erythromycin ml⁻¹, or 15 μ g chloramphenicol ml⁻¹ for *B. thuringiensis*.

2.2 DNA extraction and manipulation

Bacillus thuringiensis UC10070 genomic DNA, was extracted from 1.0-ml aliquots of the broth cultures by the use of FTA Starter Pack (WHATMAN), in accordance with the manufacturer's instructions. Restriction enzymes were used as recommended by the supplier (Promega or BioLabs, NEW ENGLAND). Plasmid DNA was isolated from *E. coli* using the Wizard Plus SV Minipreps DNA Purification System kit (Promega). For plasmid DNA isolation from *B. thuringiensis* UC10070 PAL miniprep procedure was used (Voskuil et al 1993).

2.3 Optimization of *B. thuringiensis* electroporation system

Transformation experiments were carried out using *pPSC10* plasmid to optimize electroporation system in *B. thuringiensis* UC10070. To prepare competent cells, *B. thuringiensis* UC10070 was grown in brain heart infusion medium (BHI, OXOID) containing 0.5 M sucrose (BHIS) at 37°C with shaking until the culture reached an optical density at 600 nm of 0.2. The cells were then harvested by centrifugation for 15 min at 4°C and 2,500 RPM. Pellet were washed once in 1 volume and thrice in 1/10th volume of 5 mM HEPES (N-2-hydroxyethylpiperazine- N9-2-ethanesulfonic acid)

0.5M sucrose (Sue Kalaman et al. 1995). Electroporation of 100 μ l of the cell suspension was performed in a 0.2-cm electrode gap cuvette with a Bio-Rad Gene Pulser set at: 2.500 V/cm, 2.5 μ F, 200 Ω . Following electroporation, the cells were transferred to 3 ml of BHIS and grown for 3 h at 37°C and 250 rpm before being plated on Luria broth medium (LB) containing 250 μ g/ml of erythromycin ml⁻¹.

2.4 Detection of *B. cereus* transcriptional regulators homolog in *B. thuringiensis* UC10070

Nucleotide sequences of *resDE* (1,776 kbp) and *fnr* (1,405 kbp) loci, are available in the database EMBL-Ebi with entry ID:DQ402422 and DQ681074 respectively. Primer set previously reported by Duport et al (2006, 2007) for *B. cereus* F4430/73, was used to amplify both the 2.91-kbp *ResDE* and 1,405-bp *fnr* *B. cereus* loci, from *B. thuringiensis* UC10070. Polymerase chain reactions (PCR) were carried out in a total volume of 25 μ l, with 0.5 μ M of each primers, MgCl₂ 15mM, and 0.5U of Taq polymerase (Promega) and 1X Buffer provided with Taq enzyme. Amplifications was performed in a Mastercycler Ep Gradient S Eppendorf PCR as follow: 1cycle at 94°C for 4 min and 35 cycles at 94°C for 30 s, 50°C for 1 min, and 72°C for 5 min. A final elongation step was performed at 72°C for 5 min. PCR reaction mix were electrophoresed on a 0.8% agarose gel in TAE1X buffer with SYBR® Safe DNA gel stain (Invitrogen).

2.5 Construction of *resE* and *fnr* mutants with homologous recombination system

To construct insertional inactivation mutants, internal fragments of the *resE* (324 bp) and *fnr* (288 bp) genes were first amplified by using the primer pair 5'-CGTCTTGAAAAGATCCGTCA-3' and 5'-AAATCAACCGTTAACGCAAC-3' and the primer pair 5'-GCAAACGAAGTTCCGAGATT-3' and 5'-GCAGAGCAATCTTCACAAGC -3', respectively, and cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's instructions. Recombinant plasmids were digested with *EcoRI* and *NotI* in parallel with the pMUTIN4 vector (*Bacillus Genetic Stock Center*, USA,) as described by Duport et al (2006). After heat inactivation of the enzymes, fragments and plasmid (3:1) were mixed for ligation. The ligation mixtures were used to transform competent cells of *E. coli* TB1. Recombinant plasmids named respectively pMresE and pMfnr, were extracted and then introduced

into *B. thuringiensis* UC10070 by two different electroporation experiments as previously described. Prior to strain transformations, primer set previously described were used to amplify fragments cloned in both vectors. Amplification products were sent to a commercial sequencing facility (BMR Cribi Padova Italy) for sequencing. The homologous recombination was expected in a single crossover event. The integrants were selected on LB plates by using erythromycin resistance.

2.6 Detection of genes disruption

To verify the successful insertion of the inactivation vectors pMresE and pMfmr in the desired target sites, PCR screening on the Erm^R clones was performed. Colonies were grown in the presence of antibiotic at increasing concentrations from 5 up to 64 µg per ml⁻¹. Gene disruption was verified by sizing of the PCR fragments generated from the target chromosomal region. The oligodeoxynucleotide primer *pspacF* 5'-CCTTGCCTACCTAGCTTCC-3', complementary to a region of the pMUTIN4 vector promoter, was designed using Vector NTI 9.0.0 (InforMax, Frederick, MD); primers complementary with genes to be knocked out, were used paired with *pspacF*: respectively *resE* 5'-ACGCCGCTTTACAATCAAAC-3', and *fmr* 5'-TCGTACAACAATTGGCCCTT-3'.

PCR amplification reaction was carried out in a total volume of 25 µl that contained 5ng of DNA, 0.5µM of each primer and the GoTaq Green Master Mix (Promega). Reaction was performed in a Mastercycler Ep Gradient S (Eppendorf) with an initial denaturation of 4min at 95°C, followed by 30 cycles PCR each comprising 15 s at 94°C, 45s at annealing temperatures (annealing temperature from 50 to 55°C were tested to increase reaction efficiencies) and 2 min at 72°C, final extension was 7 min at 72°C. PCR reaction mix was electrophoresed on a 1% agarose gel in TAE1X buffer with SYBR® Safe DNA gel stain (Invitrogen).

2.7 Construction of *B. thuringiensis resE* and *fmr* mutants with TargeTron system

The target sites for *resE* and *fmr* genes inactivation was found sending the sequence to www.sigma-aldrich.com/targetronaccess. The “TargeTron algorithm design site”, identifies all potential insertion sites for intron insertion along gene sequences: (1kb normally presents from 5 to 11) and a set of 3 primer (IBS, ebs1, ebs2) for each gene, suitable for intron re-targeting. In table 1 are listed primer sets for intron re-targeting.

Table 1. Primer sets corresponding to chosen design options in target genes identified by the TargetTron algorithm. The target sites are named by their insertion point within the DNA sequences and are noted as sense (s) or anti-sense(a).

genes	Primer name/ position	SEQ (5'-3')
<i>resE</i>	IBS 563/564a	5'-AAAAAAGCTTATAATTATCCTTAAACGCCAAGAAAGTGCGCCCAGATAGGGTG- 3'
	EBS1d 563/564a	5'- CAGATTGTACAAATGTGGTGATAACAGATAAGTCAAGAAAGTTAACTTACCTTTCTTTGT -3'
	EBS2 563/564a	5'TGAACGCAAGTTTCTAATTTTCGATTGCGTTTCGATAGAGGAAAGTGTC 3'
<i>fnr</i>	IBS 1062/1063s	5'-AAAAAAGCTTATAATTATCCTTAGATTGCTCTGCTGTGCGCCAGATAGGGTG- 3'
	EBS1d 1062/1063s	5'-CAGA TTGT ACAA T GT GGTGATAACAGATAAGTCTCTGCTTCTAACTTAC CTTTCTTTGT- 3'
	EBS2 1062/1063s	5'-TGAACGCAAGTTTCTAATTTTCGATTCAATCTCGATAGAGGAAAGTGCT- 3

Once target site and the three primer were identified, intron retargeting PCR was performed in Mastercycler Ep Gradient S (Eppendorf), in a final volume of 50 μ l, thus composed: 1 μ l of the "Four primer master mix" (IBS, EBS1,EBS2 and the EBS universal primer provided by the kit), 1 μ l of Intron template, Red Taq (Sigma) 25 μ l, nuclease free water 23 μ l. Samples was subjected to an amplification cycle with pre-denaturation at 94°C for 30'', annealing for 30'' at 55°C, extension at 72°C for 30'', and one cycle at 72°C for 2'.

The PCR products of 350 and 288 bp, were electrophoresed on a 2% agarose gel and purified with GenElute PCR Clean-up Kit (Sigma).

Intron fragments (about 20-25ng/ μ l), were ligated in pJIR750ai vector (10,262 kpb-Sigma), after in parallel HindIII/BsrGI digestion, so assembled: 2 μ l B10X, BsrGI 1 μ l, HindIII 1 μ l, DNA 10 μ l, H₂O 6 μ l: 20 μ l reaction incubated for 30' at 37°C, 30' at 60°C, 10' at 80°C (for enzymes inactivation).

Each digestion reaction was purified by Microclean protocol (Labogen). Linearized vector was submitted to an alkaline phosphatase treatment (CIAP-calf Drive-promega) following the manufacturer's instructions to prevent recircularization during ligation.

The ligation reactions was assembled as in Quik-Link T4 DnA Ligation Kit protocol (Sigma) then transformed in *B. thuringiensis* UC10070 with Bio-Rad Gene Pulser electroporator set to the following parameters: 2.500 v/cm, 2.5uf, 200 Ω . Competent cells were prepared as previously described (section 2.3). For mutants selection, electroporated culture, after 3 hours recovery, was spread on LB plates with 5 μ g/ml chloramphenicol, and incubated o.n. at 37°C. Plasmid DNA from transformed colonies was isolated with previously described (Voskuil et al 1993), and analysed by electrophoresis on 0, 8% agarose gel. PCR reactions were carried out to amplify the intron regions from transformed colonies (on plasmid DNA), and from intron template before targeted mutagenesis experiments. The primer sets and condition described for intron re-targeting PCR were used. Products obtained were purified and sent to a commercial sequencing facility (BMR Cribi Padova Italy) for sequencing.

2.8 Reverse transcription PCR to evaluate intron expression in *B. thuringiensis* UC10070

RNA from processed colonies was isolated in reason of 1 μ g/ul, after lysis in TE buffer (10mM Tris-Cl, 1mM EDTA), containing lysozyme 10mg/ml, with column extraction technique, following manufacture's instruction of the total RNA purification Protocol, of "RNeasy mini Kit" (Qiagen). Subsequently, RNA was treated with DNasi (Ambion) and stored at -80°C in aliquots of 10ul in order to preserve RNA stability.

To assess intron expression, RNA was analysed by RT-PCR following the procedure of "One-Step" (AB-gene) protocol, which provides a single amplification cycle in 50ul reaction volume, assembled as follows: 1 μ g RNA template, 50U retro-transcriptase enzyme (Reverse-it RTase Blend), primer-sense and anti-sense from TargeTron algorithm design (specific for *resE* and *fnr* genes) 0.2 μ M, Master-Mix 2X RT-PCR composed by: Thermoprime Plus Dna Polymerase (final concentration 1.25U/50ul), Buffer for reaction optimisation, dNTPs mix (final concentration 0.2mM for each nucleotide), MgCl₂ (final concentration 1.5mm).

2.9 Confirmation of knockout by colonies PCR

Two strategy were used to verify by PCR the intron insertions. Specific primers for *resE* and *fnr* fragments, that flank the insertion site, were designed (Table 2) to amplify

the entire inserted intron. Gene specific primers and an intron specific primer were used to amplify across gene-intron junctions.

Table 2 Primer set used to amplify the entire inserted intron, in PCR reaction for knockout confirmation.

Genes	Primer name	SEQ (5'-3')	Annealing temperatures	Amplification product
<i>resE</i>	TTresEF	5'-GTCGGAAAACCACTGCAATC-3'	52	216pb
	TTresER	5'-TCCCACGTGACACTTCAAAA-3'		
<i>fnr</i>	fnrF	5'-GCAAACGAAGTCCGAGATT-3'	51	288pb
	fnrR	5- GCAGAGCAATCTTCACAAGC -3		

3. Results

The role of both ResDE two component system, and the one-component CRP-Fnr regulators, as sentinels capable of sensing redox changes, and coordinating responses that modulates *B. cereus* virulence, was clearly defined in recent studies (Esbelin et al 2009). Genome-wide transcriptomic analysis (see section 3.1.4, Cap.3), revealed the expression of genes coding for Fnr regulator, and the probably sensor kinase of the two component system ResDE, during *B. thuringiensis* UC10070 biological cycle.

In this part of the work, the detection by PCR amplification of *B. thuringiensis* UC10070 chromosomal regions containing entire *resDE* (fig. 3A) and *fnr* loci (Fig. 3B), indicated that *resDE* and *fnr* genes, could compose transcriptional units included into a larger operon, supporting the transcriptional activity already observed in the previous chapter.

To generate site-specific mutations in *B. thuringiensis* UC10070, the construction of 4 vectors were performed. In order to construct insertional inactivation mutants, we made several tests to optimize electroporation system in *B. thuringiensis* strains. A new protocol for preparing competent cells, and specific parameters of electroporation were developed to achieve a transformation frequency of 5×10^5 cfu per μg of pPSC10 vector DNA, in *B. thuringiensis* UC10070.

Chromosomal fragments of *B. thuringiensis* UC10070, corresponding to internal region of *resE* and *fnr* transcriptional regulators, were cloned into pMUTIN4 vector (Fig. 4) in *E. coli* TB1. These fragments were of 325 and 288 bb respectively, and the resulting plasmids were designated pMresE and pMfnr.

Both pMresE and pMfnr plasmids carries an origin of replication for *E. coli* (colE1 replication origin) and selection of ampicillin resistance. Vectors fails to replicates in *Bacillus* but an Erm^{R} gene is present to select *Bacillus* colonies if the plasmid integrates into the chromosome. We examined whether pMresE and pMfnr could be used as a vehicle to insert DNA sequences into the chromosome of *B.thuringiensis* UC10070. After cloning of pMresE and pMfnr in *E. coli* TB1, PCR amplification and sequencing of *resE* and *fnr* fragments confirmed that both cloned fragments presented a 100% identity with chromosomal sequences of *B. thuringiensis* UC10070. *E. coli* TB1 plasmid DNA were isolated and EcoRI/NotI digested: electrophoretic analysis showed both pMresE and pMfnr vectors linearized and the respectively fragments corresponding to 325 and 288 bp (Fig. 5 A-B) giving evidence of the correct construction of the inactivation vector. As a control in each electroporation experiment, the pPSC10 cloning vector, which replicates in *B. thuringiensis*, was used to transform competent cells to Erm^{R} . The transformation frequency of pPSC10 was constant (of 5×10^5 transformants per μg of pPSC10 vector DNA). No transformant was obtained with vector pMUTIN4 without a chromosomal insert. Colonies were obtained with both pMresE and pMfnr. To assess the stability of the Erm^{R} phenotype of transformants, colonies were grown at increasing concentration of antibiotic; in some cases there growth were observed up to a concentration of $15 \mu\text{g per ml}^{-1}$. Total DNA was extracted from each resistant colony for PCR analysis. In Figure 6 (A and B) are schematically shown the desirable Campbell-like integration of both inactivation vectors, generated by a single crossover event. PCR reactions were performed with specific primers designed on the target genes and *pspac* promoter region of both vectors. Fragment lengths expected was 525bp and 438 bp, for *resE* and *fnr* genes respectively. In each transformation experiment the PCR analysis on resistant colonies gave products different as expected (Fig.7). Data obtained did not support the Campbell-like integration.

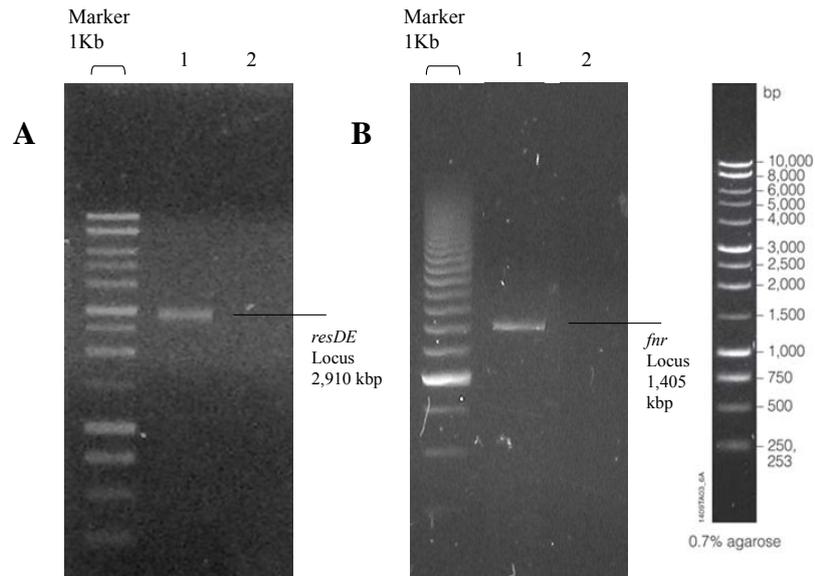


Fig. 3 Detection by PCR amplification of *resDE* (lane 1, A) and *fnr* (lane 1, B) Loci in *B. thuringiensis* UC 10070.

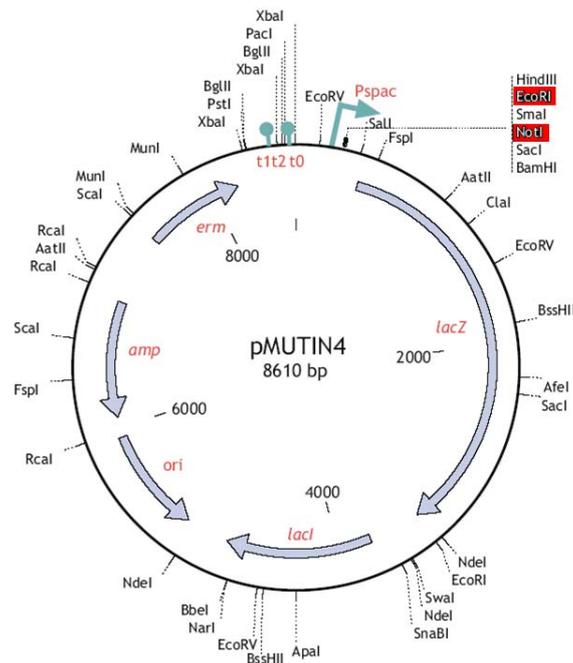


Fig. 4 Map of the pMUTIN4 plasmid transformed in *B. thuringiensis* UC10070. The vector replicates in *E. coli* via the *colE1* replication origin, and selection of ampicillin resistance. It fails to replicate in *Bacillus* but an *ErmR* gene is present to select *Bacillus* colonies if the plasmid integrates into the chromosome. In the multiple cloning site, red blocks indicate the restriction enzymes used for cloning of *resE* and *fnr* fragments. *Bacillus Genetic Stock Center* image.

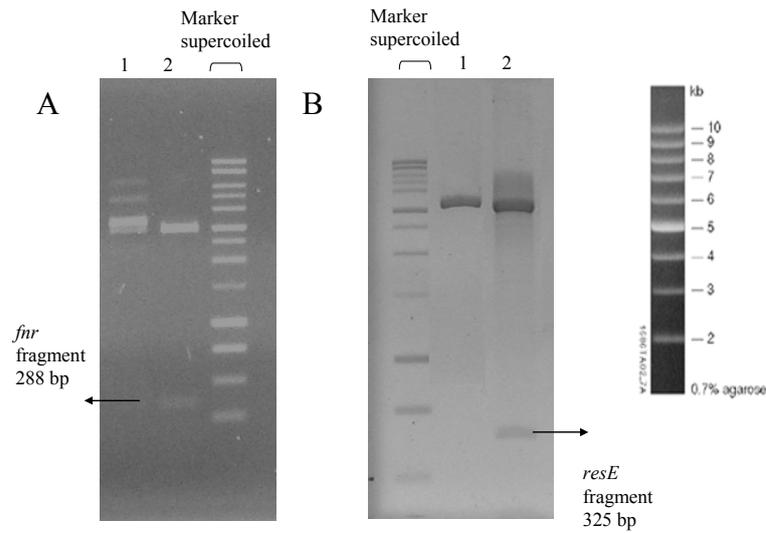
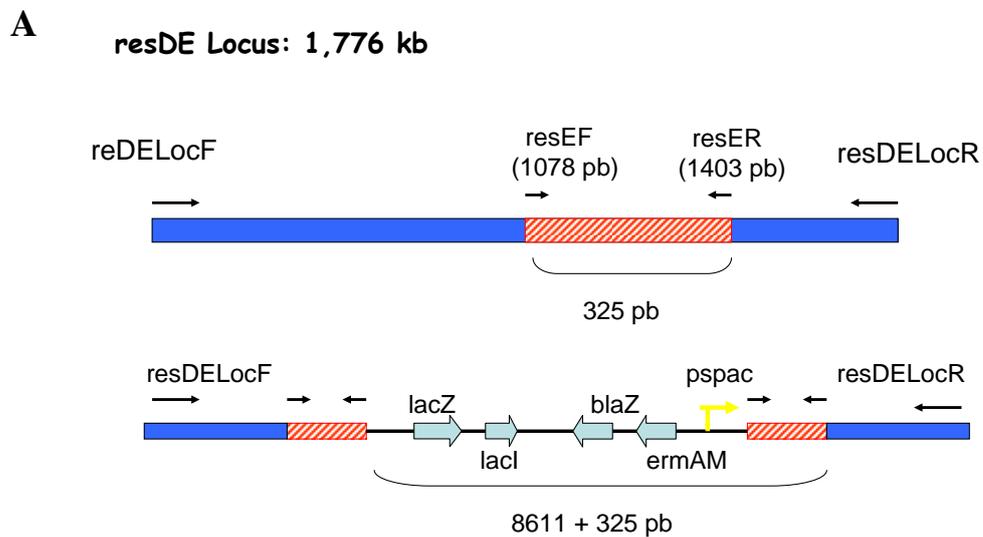


Fig. 5 Electrophoretic profiles of digested pMfnr (A) and pMresE (B) insertional vectors. pMUTIN4 was used as control (lane 1 A, lane 1 B). NotI/EcoRI treatment, generated linearized vectors and the corresponding inserted fragment of 288 bp (*fmr* gene) and 325bp (*resE* gene).



B

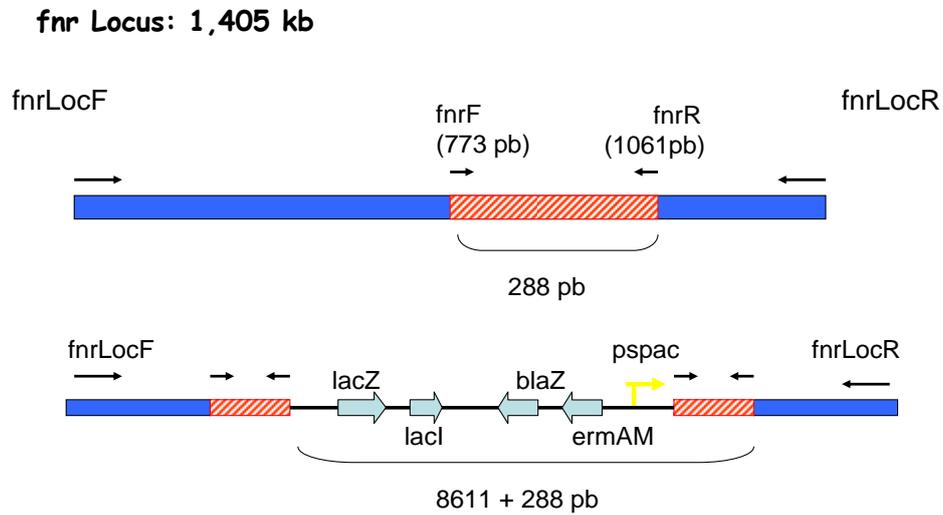


Fig. 6 Schematic representations of the relevant parts of the chromosomes of strain *B. thuringiensis* UC10070 before and after Campbell-like integration of plasmid pMresE (A) and pMfnr (B). The directions of each gene harboured by plasmids are shown by arrows. Yellow arrow indicates pspac gene vector promoter.

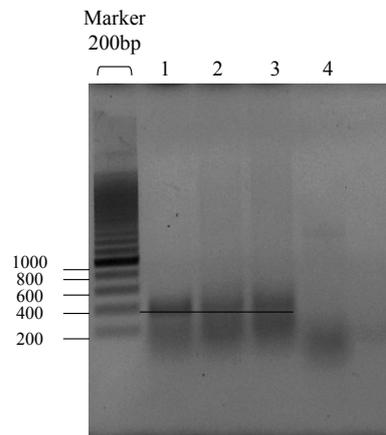


Fig. 7 PCR products obtained after *B. thuringiensis* UC10070 electroporation with pTGRE2 inactivation vector. Not specific bands are shown in lane 1-2-3. No amplification products were detected for one colony tested.

The TargeTron gene knock-out system was optimized for use in *B. thuringiensis*. Two inactivation vectors were constructed to achieve specific insertional inactivation in *B. thuringiensis* UC10070. TargeTron expression vector pJIR750ai (10,262 bp) was used for this experiment (Fig. 8). This circularized vector was constructed for targeted gene knockouts in gram-positive bacteria. The expression of the group II intron RNA is under the control of the b-2 toxin gene promoter, *cpb2* specific for *Clostridium perfringens* type A; moreover it present the following attributes: a plasmid origin for replication in *E.coli* to facilitate cloning (pIP404 ori), a plasmid origin for replication in *Firmicutes* (pMB1ori); a chloramphenicol antibiotic resistance gene and the RNP (L1.LtrB and LtrA) ORF. The basic principle of this system is that basepairing between the two loops (EBS1 and EBS2) and δ site within the L1.LtrB RNA structure and the target DNA sequence, guide the insertion event. Thus, changing the sequences of EBS1 and EBS2 to allow basepairing to the target site within the gene (IBS1 and IBS2), through a “retargeting PCR”, facilitates insertion of the intron into this specific sequence. Sequence elements that flank the IBS1 and IBS2 sites within the targeted gene are also recognized by the RNP complex. The TargeTron computer algorithm identifies potential insertion sites within any DNA sequence (Perutka et al. 2004). For each *B. thuringiensis* gene targeted for inactivation, the TargeTron algorithm (Sigma-Aldrich) was used to identify potential insertion sites and to design primers necessary to retarget the L1.LtrB intronA PCR product that encompasses EBS1, EBS2, and δ in L1.LtrB is amplified with specific primers. The PCR fragment was cloned between the HindIII and BsrGI sites in pJIR750ai vector to retarget the group II intron.

The TargeTron algorithm identified eight potential insertion sites within *resE* and nine in *fnr*. We chose targets located between nucleotides 563 and 564 (563/564a) and between nucleotides 1062 and 1063 (1062/1063s) respectively for *resE* and *fnr* target genes. The RNP recognizes in one case the targets on the antisense strand (designation “a”), while the other on the sense strand (designation “s”). These target sites were chosen based on the e-value derived from the algorithm (563/564a e-value = 0.114; 1062/1063s e-value = 0.045). The two targetron plasmids with targeting positions 563/564a (pTGRE2) and 1062/1063 (pTGOX), were transformed into *B. thuringiensis* UC10070. Two sets of primer were employed for each targetron plasmid for PCR screening to amplify in one case the entire inserted intron regions of (more or less)

2,000 kbp (Fig. 9A) , and another smaller regions in which intron insertions were expected (Fig. 9 B). Transformants were screened for the Cm^R. Several independent electroporation experiments were performed. The isolation of resistant colonies harbouring targetron plasmids (Fig. 10) after each transformation experiment, gave evidence of the efficient electroporation system developed for *B. thuringiensis* UC10070 (section 2.3). However, the PCR screening on Cm^R colonies, did not allow the detection of group II intron insertion at 563/564 and 1062/1063 target sites, respectively in *resE* and *fnr* genes. Sequence alignments between intron template and retarget introns from both pTGRE2 and pTGOX, showed single nucleotide variations replacing EBS1 and EBS2 regions in targetron plasmids. These data confirmed intron retargeting in pTGRE2 and pTGOX vectors. The activity of *cpb2* promoter was assessed in *B. thuringiensis* UC10070. The reverse transcription assay performed on total RNA isolated from resistant colonies, confirmed the expression of re-targeted intron (Fig.11). No fragments amplification were observed in control reaction performed without reverse transcriptase enzyme.

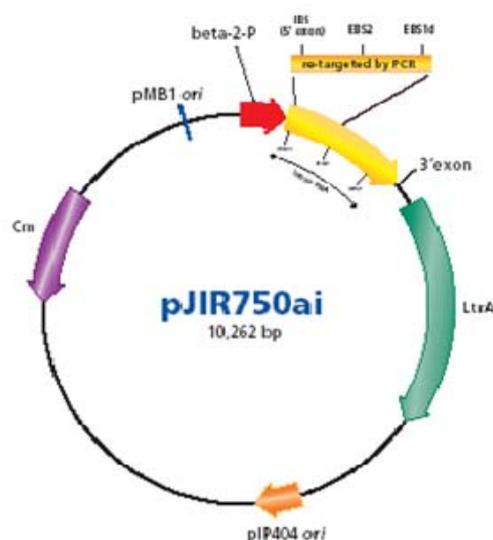
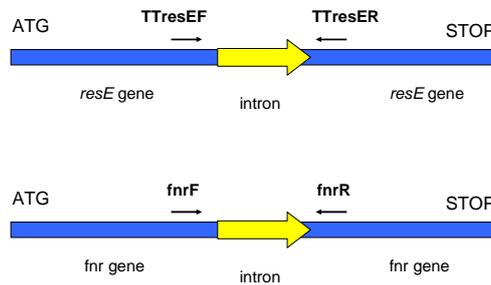


Fig. 8 Map of the pJIR750ai plasmid used to transform *B. thuringiensis* UC10070. The vector specific for inactivation of retargeted genes in gram positive microorganism, contains remaining portion of Intron group II, a promoter upstairs intron sequence, and a chloramphenicol resistance to select mutants. www.sigmaaldrich.com image.

A



B

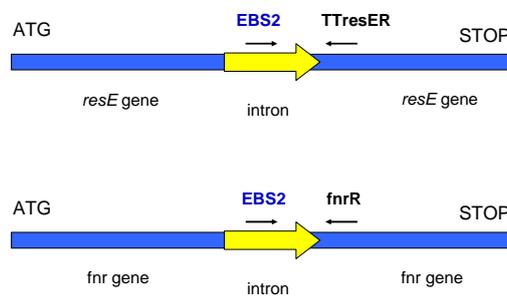


Fig. 9 Primer design for detection of intron insertions. Arrows indicates each primer used. **A.** Amplification of the entire inserted intron regions. **B.** The re-target intron primer sets for the initial PCR step (IBS, EBS1d, EBS2) could be used paired with genes specific primers to amplify regions in which intron insertions were expected.

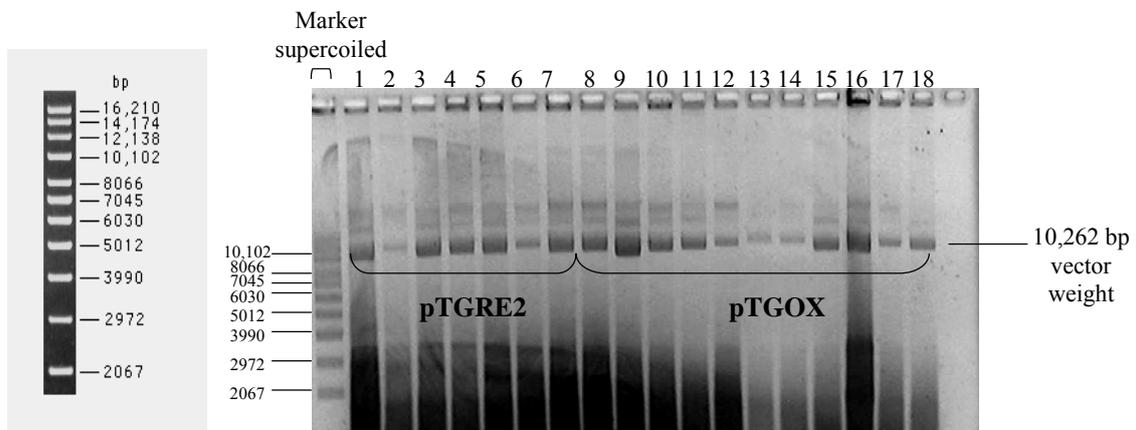


Fig.10 Plasmid DNA from *B.thuringiensis* UC10070 Cm^R colonies after transformation with pTGRE2 (lanes 1-2-3-4-5-6-7) and pTGOX (lanes 8-9-10-11-12-13-14-15-16-17-18) vector.

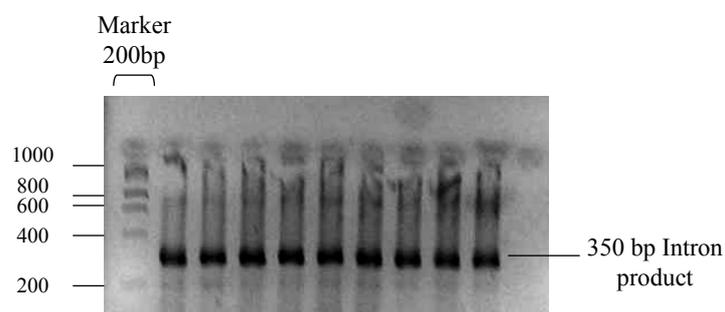


Fig. 11 Reverse transcription assay for retargeted intron expression in *B. thuringiensis* UC10070. Total RNA extracted from colonies transformed with pTGRE, was analysed by RT-PCR to assess the intron expression.

4.Discussion

B. thuringiensis belong to the *B. cereus* group. Since *B. thuringiensis* harbour and express genes coding for *B. cereus*-virulence factors, it could consider potentially dangerous for humans. However very little is known about how it can cause disease. To deeper investigate on important features, such as bacterial virulence mechanisms, the availability of molecular tools for creating knockout mutations of relevant genes is required. Gene knockout strategies routinely involve the introduction of plasmid or other extrachromosomal DNA into recipient strains to generate null mutant strains. Various protocols have been developed for electroporation and gene inactivation of gram-positive bacteria: cell weakening agents, variable washing buffer compositions and a variety of electric pulses, aim at the improvement of transformation efficiency; on the other hand, several non-replicative plasmids, useful for homologous recombination, have been constructed and employed to achieve ever more efficient chromosomal integration in Gram positive microorganism; an example is the one-step gene inactivation procedure developed for *Bacillus subtilis* by using the non replicative vector pMutin. Not last, the targetron system has been demonstrated to be useful for genetic analysis in different bacterial species. However these protocols were not successful in our hands for *B. thuringiensis* UC10070. Although Erm^{R} clones were achieved in several transformation experiments with pMUTIN derivative vectors constructed in this work, none of them was identified as null mutants.

It was supposed that colonies containing both mutated and not mutated cells resulted from transformation experiments. This could explain growth of some colonies in erythromycin BHI broth ($15 \mu\text{g per ml}^{-1}$).

The presence of restriction modification (RM) systems in recalcitrant *Bacillus* isolates, were reported (Groot et al 2008). The suggested function of RM systems in microorganisms is protection against invasion of foreign DNA. It was supposed that DNA modification occurred on inactivation vectors after transformation in *B. thuringiensis*, could explain at least part of the low frequency of recombination observed. However both plasmids pPSC10, used as control in electroporation experiments, and pJIR750ai were transformed at quite high frequency and stably maintained in *B. thuringiensis* UC10070. Hence, a low frequency of *B. thuringiensis* recombination and the presence of chromosomal rearrangements could explain the failure of these experiments.

To use a different approach a group II intron strategy was adopted. One of the advantages of group II introns is that, once expressed, the RNP is relatively host factor-independent. Moreover, high levels of DNA transformation are not necessary, as required for efficient homologous recombination procedures, because a single targetron plasmid can express multiple copies of the group II intron. However, intron integration frequencies vary widely between target sites, and can make the screening effort required to isolate a mutant prohibitively laborious, particularly if no simple phenotypic screen for gene inactivation is available. This approach did not lead to any null mutants for both *resDE* and *fnr* genetic loci. Although pJIR750ai were transformed at quite high frequency and stably maintained in *B. thuringiensis* UC10070, the transcription experiments revealed that the re-targeted intron was expressed in *B. thuringiensis* UC10070, and non mutants harbouring the desired insertion were isolated.

For future attempts could be considered the ingenious solution founded from Heap and co-worker (2007) for *Clostridium* spp. The introduction of an antibiotic resistance gene into the group II intron, which is itself interrupted by a self-splicing group I intron, was proposed using pMTL007 vector, derivative of pJIR750ai, to facilitate mutants screening. The three elements are arranged such that only after successful insertion of the group II intron into its target, when the nested group I intron will have spliced out, the integrity of the antibiotic resistance gene is restored. Acquisition of antibiotic

resistance is thereby strictly coupled to integration, and can be used to positively select for integration events. Moreover, the use of a T7 promoter (inducible with IPTG), for controlled production of the group II intron RNA, could lead to a strong increase in mutants selection efficiency.

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GENERAL CONCLUSION

General conclusions

B. thuringiensis was first characterized for its ability to produce a parasporal crystal active against several insect species, especially of the order *Lepidoptera*, *Diptera*, and *Coleoptera*. Due to its insect activities it is worldwide used in forestry and agriculture to control pests. Recent studies showed that most of the genetic determinants for *B. cereus* virulence, such as haemolysin BL (HBL), non haemolytic enterotoxin (NHE), cytotoxin K, and bc-D-ENT enterotoxin, are harboured by *B. thuringiensis* strains. Phylogenetic studies based on the analysis of chromosomal genes bring controversial results, and it is unclear whether *B. cereus* and *B. thuringiensis* are varieties of the same species or different species (Ivanova et al. 2003). Hence, what may seem to be a minor problem of taxonomy may therefore have serious implications for virulence and pathogenicity.

Since *B. thuringiensis* can contaminate food, being residual in spore form after treatment in the fields, it is ever more urgent to deepen investigate the potential risks arising from the presence of *B. thuringiensis* in food industry. This work of thesis was aimed to achieve a deeper scientific information on the food-associated bacilli, taking the advantage of new genome based molecular approaches, focusing the attention on *B. thuringiensis* strains used as commercial biopesticides.

The *in vitro* pathogenic profile of ten commercial *B. thuringiensis* strains, was characterised by the high distribution of the *nhe*, *hbl*, *bceT* and *cytK* genes. Enterotoxin genes were detected by PCR in all the strains analyzed. RT-PCR analysis confirmed the enterotoxin genes expression. Toxin productions was detected by RPLA test in the strains belonging to the widely used subsp. *kurstaki*.

These features and the difficult discrimination between *B. thuringiensis* and *B. cereus*, suggested that the role of *B. thuringiensis* in outbreaks of foodborne disease may have been underestimated. The development of a vegetable based food model, that would allow to asses the behaviour of *B. thuringiensis* spores, after the simulation of an industrial processing treatment, was an important point in this study. The analysis of *Bacillus* spore envelope, and its ability to interact with food environment, have been performed using SEM and SEM X-ray microanalysis applied to the food model proposed. In more detail, particular attention was devoted to morphological and chemical changes of *B. thuringiensis* spores during germination process in food. We observed a rapid evolution of the *B. thuringiensis* biological cycle compared to that of other spore forming

bacteria like *Clostridium* spp. (Bassi et al. 2008, personal communication). Interesting was that only two hours after spore activation, cell outgrowth was completed and cell division was at the maximum level.

In order to assess and to lay basis for manage risks associated with *B. thuringiensis* outgrowth in foodstuffs, we need to gain more information on its life cycle; and this is what we have done through transcriptome analysis of *B. thuringiensis* in four different stages of the biological cycle, from dormant spore to vegetative/sporulating cells. We could emphasized that mRNA is a component of bacterial spores. We discovered that spores are equipped with a large amount of transcripts probably useful to front the next steps of outgrowth. Dormant spores contained populations of ribosomes; during the first 40 minutes after spore activation, rate of both rRNA and ribosomal proteins synthesis strongly increased. A basic and strong activation of polyfunctional genes seemed to begin in germinant spores: most of the genes involved in the metabolic activity (house-keeping genes, translation initiation factor, ribosomal proteins, and elongation factors) were overrepresented at this time in microarray analysis. A large number of transcripts for protein involved in the regulation of different biological process, including resistance to different antimicrobial compounds and oxidative stress agents, were found to be present in *B. thuringiensis* vegetative cells. We hypothesized that *B. thuringiensis* cells may activate these systems in response to external stimuli for cell defence and adaptation to changing environmental conditions in food model. The transcripts for germination proteins (*ger* type) found in spore, are an index of the expression of this genes in previous sporulation stage and suggested the importance during dormancy, to monitor the environment for proper outgrowth conditions. This finding could explain the ability of *B. cereus*-like microorganism to occupy and complete a full life cycle within several different environmental niches. According to literature data, all the associated virulence genes, represented in microarray analysis, were up-regulated especially during the late stage of cell growth. RT-qPCR analysis were performed to quantify the expression, in food, of the major virulence genes involved in *B. cereus*-associated food borne disease. Toxin mRNAs were detected, in variable amounts, at all investigated growth stages of *B. thuringiensis*, with a strong increase during the log phase of microorganism growth. Although no information on the *B. cereus* toxin expression in food are available, previous *in vitro* studies on *B. cereus* enterotoxins production, reported that the highest toxin level is achieved during the late log/early stationary phase. The production of the L2

component of HBL enterotoxin, involved in the diarrhoeal syndrome was detected in food model, even in low amount, during the early log phase. We concluded that *B. thuringiensis* can complete an entire life cycle in food systems after an industrial processing simulation, producing enterotoxins as observed in broth cultures. Transcriptomic has been demonstrated to be not only a powerful tool to study the germination and outgrowth of *B. thuringiensis* spores, but also a suitable method to assess the environmental response to bacterial pathogens in food. Data obtained, provide new basic knowledge on *Bacillus cereus* group.

These data extends our knowledge on the metabolic versatility of *B. thuringiensis* and also added to our view of virulence traits of this potential food-pathogen.

Given this finding, the need to identify systems for manage the risks associated with *B. thuringiensis* in industrial fields has became clear. An experimental approach was described in the last part of this work of thesis. Identification and inactivation of general systems for regulating virulence, through null mutants construction, were considered to evaluate changes in growth performance, cellular metabolism and toxins expression, in the studied microorganism. Besides homologous recombination, the mobility mechanism of group II introns were assessed to generate highly specific chromosomal gene disruption in *B. thuringiensis*.

A novel approach and several experiments were performed to achieve the desired chromosomal inactivation, however no attempts gave the expected results.

Recent studies has been showed difficulties encountered screening mutants obtained with intron group II inactivation, in particular when, as in this case, it is not possible using phenotypic assay to recognise mutated cells: most common inconvenience is the presence of mixed colonies from which is difficult to separate mutated from wild type cells.

Literature reports how the introduction of an antibiotic resistance into Group II intron sequence, and use of a T7 promoter (inducible with IPTG), could led to a strong increasing in mutants selection efficiency. Since high frequency of chromosomal integration is reported for *B. subtilis* by using the non-replicative vector pMUTIN, this one-step gene inactivation procedure was not useful for *B. thuringiensis* UC10070 in this study. It was supposed that industrial *B. thuringiensis* strains are recalcitrant to genetic manipulation. Restriction modification (RM) systems in recalcitrant *Bacillus* isolates, were reported (Groot et al 2008). The presence of RM systems like protection against invasion of foreign DNA was not verified in these microorganisms, but it could explain

the low frequency of recombination observed despite the good efficiency of transformation.

Since *B. thuringiensis* is widely used and popular in biological farming, a careful monitoring of the strains used should be justified. Literature reports widespread the risks associated with the food-pathogen *B. cereus*, but those related to *B. thuringiensis* are often underestimated. From data obtained in this study we could assume that *B. thuringiensis* could actually be responsible for many of the food borne outbreaks previously attributed to *B. cereus*; taking this enterotoxigenic potential into account, as well as the fact that *B. thuringiensis* cannot be separated from *B. cereus* at the chromosomal level, food producers and food authorities, responsible for food safety, should consider the risk of *B. thuringiensis* insecticide residue in the food chain.