The fate of bacteria of the Bacillus cereus group in the environment : interaction with amoebas

Haibo Chen

Thèse de doctorat

NNT : 2021UPASB044

université PARIS-SACLAY

Le devenir des bactéries du groupe *Bacillus cereus* dans l'environnement : interaction avec les amibes

The fate of bacteria of the *Bacillus cereus* group in the environment: interaction with amoebas

Thèse de doctorat de l'université Paris-Saclay

Ecole doctorale n°581 : agriculture, alimentation, biologie, environnement et santé (ABIES)
Spécialité de doctorat: Microbiologie
Unité de recherche : Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, 78350, Jouy-en-Josas, France.
Référent : AgroParisTech

Thèse présentée et soutenue à Paris-Saclay, le 28/09/2021, par

Haibo CHEN

Composition du Jury

Fabienne MISGUICH Maître de conférences, UVSQ	Présidente
Agnès FOUET Directrice de recherche, CNRS, Institut Cochin	Rapporteur & Examinatrice
Julien BRILLARD Chargé de recherche, INRAE Centre Occitanie-Montpellier	Rapporteur & Examinateur
Alice CHATEAU Maître de conférences, Université d´Avignon et des Pays du Vaucluse	Examinatrice
Jacques MAHILLON Professeur, Université catholique de Louvain	Examinateur
Fuping SONG Professeur, CAAS Institute of Plant Protection	Examinateur

Direction de la thèse

Didier LERECLUS Directeur de recherche, INRAE Centre IdF-Jouy-en-Josas-Antony	Directeur de thèse
Emilie VERPLAETSE Ingénieur d'Etudes, INRAE Centre IdF-Jouy-en-Josas-Antony	Co-encadrante & Examinatrice

Acknowledgement

This study is performed under the supervisor of Didier Lereclus and the co-supervisor of Emilie Verplaetse. I thank them very much for all the guidance in my study. Their profession, patience helped me finishing the study. I performed all my work in the GME team in the Micalis institute. All the team members are very kind and helpful; they gave me many supports, professionally or personally. Especially, I had many helpful discussions with Leyla Slamti, Christina Nielsen-Leroux. Natahalie Gilois, Agnès Réjasse, Michel Gohar, Stéphane Perchat, Hasna Toukabri, Nay El-Khoury have also helped me in some experiments. I also thank Ludovic Bridoux, he helped clean the lab many times. I like the team very much; they all are very good colleagues and best friends. I thank everyone for the cakes, drinks, laughs…

I also thank the help of Shuyuan GUO and Fuping Song. Shuyuan is the supervisor during my master study, she taught me about *B. thuringiensis* and molecular technics. Both of them helped me applying the doctor project.

I would thank my family. Especially my wife Linghui Song. She helped me to decide to have a doctoral study, and fully supported my study in France. Because of her, I am not worried about failure, and I decided to work hard to have a better future for us. I would thank my parents. They also supported my studies and care for me a lot. I love them all.

I would also thank the Chinese government. I received a scholarship from the China Scholarship Council for four years. Without this scholarship, I will not have the chance to study in France. I would also thank the French government because I received Aide au lodgement for so many months.

Merci tout le monde!

Thank you everyone!

感谢所有人！

List of figures and tables

List of abbreviation

AFLP: Amplified Fragment Length Polymorphism

ALP: GPI-anchored alkaline phosphatase

AMP: adenosine monophosphate

ANI: average nucleotide identity

APN: GPI-anchored aminopeptidase-N

ARB: amoeba-resisting bacteria

ASM: artificial soil microcosms

Ba: *Bacillus anthracis*

Bc group/ Bc sl: The *Bacillus cereus sensu lato* group

Bc/ Bc ss: *Bacillus cereus sensu stricto*

BCAA: branched chain amino acids

BLAST: Basic Local Alignment Search Tool

Bs: *Bacillus subtilis*

Bt: *Bacillus thuringiensis*

Bw: *Bacillus weihenstephanensis*

CaDPA: Ca2+-dipicolinic acid

CFU: colony-forming unit

CHO: hamster ovary cells

Cry: crystal proteins of *Bacillus thuringiensis*

C-terminal: carboxyl-terminal of a protein

Cyt: cytotoxin proteins of *Bacillus thuringiensis*

CytK: cytotoxin K

DNA: Deoxyribonucleic acid

DPA: dipicolinic acid

EF: edema factor of *B. anthracis*

FLA: free-living amoeba

GAE: granulomatous amoebic encephalitis

GBDP: genome BLAST Distance Phylogeny

GTP: guanosine-5'-triphosphate

h: hour

Hbl: haemolysin BL

hPMNs:	human neutrophils
InhA:	immune inhibitor A
kDa:	kilo Dalton
LB:	Luria-Bertani medium
LCV:	Legionella-containing vacuoles
LF:	lethal factor of *B. anthracis*
MEE:	multilocus enzyme electrophoresis
min:	minute
MLST:	multilocus sequence typing
mRNA:	messenger ribonucleic acid
Nhe:	nonhaemolytic enterotoxin
N-terminal:	amino-terminal of a protein
OD:	optical density
PA:	protective antigen of *B. anthracis*
PAM:	primary amoebic encephalitis
PDGA:	poly-d-γ-glutamic acid
RNA:	ribonucleic acid
rRNA:	ribosomal ribonucleic acid
SASPs:	small acid-soluble proteins
SESOM:	soil-extracted soluble organic matter
Sf-FGFR:	fibroblast growth factor receptor of *Spodoptera frugiperda*
Sf-SR-C:	scavenger receptor class C like protein of *Spodoptera frugiperda*
Sip:	secreted insecticidal protein
T4SS:	type IV secretion system
TEM:	transmission electron microscopy
UV:	ultraviolet
Vip:	vegetative insecticidal protein of *B. thuringiensis*

Table des matières

1. Introduction

The *Bacillus cereus sensu lato* group (Bc group) bacteria are ubiquitous in the environment and includes important mammalian and insect pathogens. The *Bacillus* spores are frequently found in soils while they cannot grow in this environment without nutrient input. The soil protozoa amoebas are predators of bacteria and a reservoir for some pathogenic species. During my thesis, I studied the interaction between amoebas and Bc group bacteria to explain the abundance of these bacteria in soils. The insects are another important niche for entomopathogenic *B. thuringiensis* strains. The role of the different *B. thuringiensis* virulence factors and toxins is not fully explained during the infection cycle. In the second part of my thesis, as an annex to the main project, I have initiated a study to explain the role of the **V**egetative **i**nsecticidal **p**rotein (Vip3Aa). As a preliminary approach, I studied the expression and regulation of the *vip3Aa* gene in *B. thuringiensis*. As *vip3A* is involved in a potential patent, this part was not presented in the context, but it was provided to the Thesis Jury directly.

1.1 General information about the *B. cereus* group bacteria

The *Bacillus cereus sensu lato* bacteria are rod-shaped spore-forming, facultative anaerobic bacteria. This bacterial group consists of several species including *B. anthracis* (Ba), *B. cereus* (Bc), *B. thuringiensis* (Bt), *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, *B. cytotoxicus*, and *B. toyonensis* (Liu et al. 2015). Phylogenetic analyses showed that these bacteria are closely related low-GC-content bacteria belonging to the phylum *Firmicutes* (Helgason et al. 2000; Liu et al. 2015; Rasko et al. 2005). The genomes of the Bc group species are highly conserved with a size of 5.2 to 5.9 Mb except the *B. cytotoxicus* with a smaller chromosome of 4.1 Mb, and they have very similar 16S rRNA gene sequences (Lapidus et al. 2008; Stevens et al. 2019). All the Bc group bacteria share about 3000 ± 200 common genes (Lapidus et al. 2008). The definition of these species is based on their phenotypes and pathogenicity, which are usually related to their plasmid content (Kolstø, Lereclus, and Mock 2002; Rasko et al. 2005). Ba harbors the pXO1 and pXO2 plasmids that carry genes for toxin synthesis and capsule formation, respectively (Moayeri et al. 2015). The emetic Bc strains harbor a large plasmid encoding a cluster of genes (nonribosomal peptide synthetase) responsible for the production of cereulide (Ehling-Schulz et al. 2006).This plasmid has a similar origin as the pXO1 plasmid of Ba (Rasko et al. 2007). Bt harbors plasmids encoding various toxins (Cry, ∂-enterotoxin) and cytotoxic proteins (Cyt), which form a parasporal crystal during sporulation (Schnepf et al. 1998).

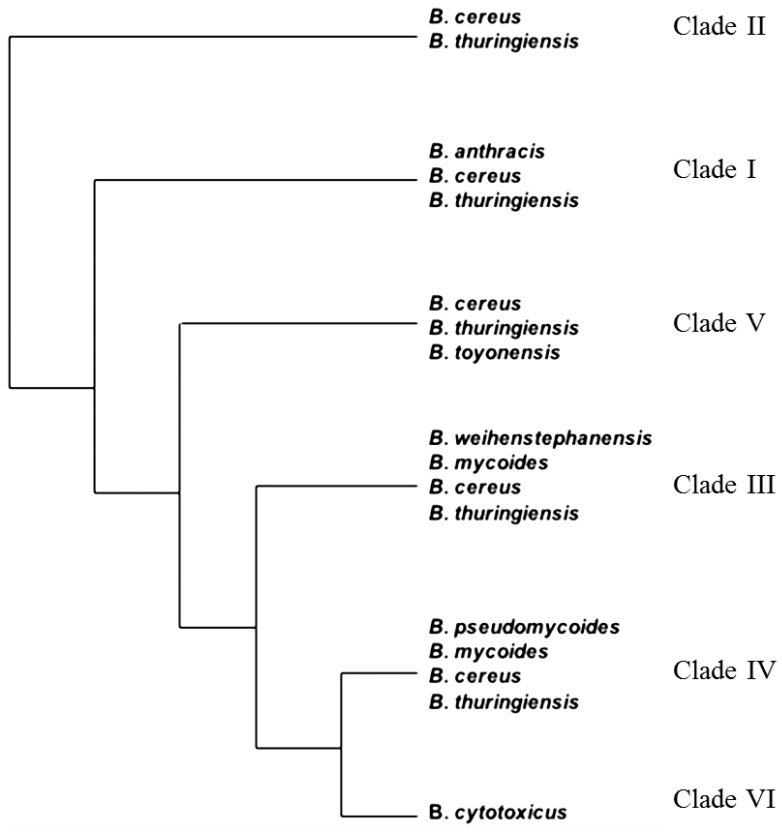


Figure 1 Six major phylogenetic clades of the *B. cereus* group.

Adapted from (Ehling-Schulz et al. 2019).

1.1.1 The diversity and taxonomy

The classification of the species of Bc group bacteria has evolved as technologies advanced. The species were originally classified according to their phenotypes. The Ba, Bc and Bt species were established as early as the 1900s (Ehling-Schulz, Lereclus, and Koehler 2019). Ba is pathogenic to mammals and causes anthrax, Bc is able to cause emetic or diarrheal food poisoning and Bt produces entomopathogenic Cry toxins and are toxic to different insect larvae.

DNA-DNA pairing was used to distinguish the Ba, Bt and Bc species in early times and no clear distinction is evident between those species (Somerville and Jones 1972). In the 1990s, the ribosomal RNA was used to distinguish the species and it was found that the 16S and 23S ribosomal RNA of Bt, Ba and Bc are almost identical with only a few bases difference (Ash et al. 1991; Ash and Collins 1992). New methods based on chromosome gene sequences were used for the classification of Bc group bacteria. Single gene analysis (Chen and Tsen 2002), amplification differences - the Amplified Fragment Length Polymorphism (AFLP) (Hill et al. 2004), multilocus enzyme electrophoresis (MEE) analysis of the 13 enzyme loci (Helgason et al. 2000), as well as multilocus sequence typing (MLST) of seven housekeeping genes (Helgason et al. 2004) were used to differentiate Bt, Bc and Ba. However, these methods can separate Ba but cannot distinguish Bc and Bt. Even comparative analysis based on chromosome genomes fails to discriminate members of the Bc group from each other (Rasko et al. 2005). Recent research using the whole-genome sequence-based Genome BLAST Distance Phylogeny (GBDP) approach has classified 224 genomes into 30 clusters (Liu et al. 2015). The separation between Bt and Bc remained unclear, and some Bt or Bc isolates were clustered with Ba.

As the Bc group bacteria are so close to each other, we can consider them as a super species with a common backbone but evolved to adapt to different environments. The horizontal transfer of plasmids could be a key that caused the differences between species either by the presence of plasmids in the strains or by introducing new genes to the chromosome. Here I present the commonly accepted classification of six clades of the Bc group (Fig. 1). The Clade I is the Ba clade but also contains some pathogenic Bt or Bc strains. The Clade II includes most of the common Bt or Bc commercial and type strains. The clade III is represented by the psychrotolerant isolate *B. weihenstephanensis*. Clade IV and V includes various Bc group species based on MLST, AFLP and whole-genome sequencing, and the thermotolerant *B. cytotoxicus* strains cluster separately (Clade VI).

1.1.2 Brief description of different *B. cereus* group species

B. anthracis was first identified as the pathogen of anthrax by Robert Koch in 1876 (Koch 2018) and then a vaccine was produced by Louis Pasteur using an attenuated strain. Their studies on Ba are the most important breakthroughs on microbiology and vaccination in the 19th century. Ba spores can germinate in the mammalian host after infection through inhalation, the vegetative cells will produce capsule and virulence factors and they can escape from the immune systems and disseminate through lymph nodes associated with the infection site (Moayeri et al. 2015). The cutaneous infection can cause edema and necrosis resulting in a black eschar, a representative feature of anthrax infection; while the infection through gastrointestinal and inhalation will eventually lead to sepsis and shock-like vascular collapse (Moayeri et al. 2015). This bacteria was developed as a bio-weapon since World War II (Wenner and Kenner 2004). During WWII, the Japanese bio-weapon unit 731 in the northeast of China developed bio-weapons based on anthrax and other diseases and subjected humans as guinea pigs to gruesome medical experiments, causing over 3 000 death in experiments and most of the victims exposed to aerosolized anthrax (Gold 2011). Ba spores obtained from the US military labs were used in a bioterrorism attack leading to 22 cases of anthrax infection and 5 dead after the anthrax mailing in the USA in 2001 (Jernigan et al. 2002).

The two plasmids pXO1 and pXO2 encodes the most important virulence factors. The pXO1 contains the *pag* (protective antigen, PA), *lef* (lethal factor, LF), and *cya* (edema factor, EF) genes and their regulators genes *atxA* and *pagR* (Okinaka et al. 1999). PA binds the cellular receptors and translocate LF or EF into cells to cause lethality or edema. The transcription of these toxin genes decreases in the *atxA* mutant suggesting that they are positively controlled by AtxA (Dai et al. 1995). The pXO2 plasmid contains the poly-d-γ-glutamic acid (PDGA) capsule synthesis genes, *capBCADE*, whose expression are also indirectly controlled by AtxA via the activation of the AcpA and AcpB regulators (Drysdale et al. 2004; Fouet and Mock 2006). The *pag* gene can affect the expression of regulators *acpA* and *acpB*, and then regulates capsule synthesis (Liang et al. 2017). The PDGA capsulation is synthesized through sequential isomerization, ligation, secretion and transpeptidation steps, and attached to cell wall peptidoglycan in the end (Chateau et al. 2018). The capsule protects Ba from macrophage phagocytosis (Ezzell and Welkos 1999).

B. thuringiensis strain was first isolated from infected silkworm larvae in Japan in 1901 and the bacteria is the causing agent of the Sotto disease. Berliner isolated the bacterium from the

cadavers of flour moth collected in a mill in Thuringia, Germany, and named the bacteria *Bacillus thuringiensis* (Berliner 1915). Bt is featured by the production of parasporal **Cry**stal (Cry) toxins which can account for about 25% of the dry weight of the sporulated bacteria (Agaisse and Lereclus 1995). The Cry toxins are the main virulence factors responsible for the insecticidal activity to the Lepidoptera, Diptera and Coleoptera insects, as well as to the other targets including aphids, mites, nematodes and cancer cells (Ehling-Schulz et al. 2019; Mendoza-Almanza et al. 2020). Bt based insecticide Sporine was primarily used to control flour moths in France in 1938, and Bt product was commercially produced and registered by the US Environmental Protection Agency in 1961. The Bt based pesticides are widely used in the world now. These bio-pesticides account for approximately 75% of the global bio-insecticide market, representing about 4% of global insecticides in 2005 (Sanchis 2011). The global bio-pesticides market increased to about 4 billion dollars in 2020 and expected strong growth in the following five years (Anon 2021). Besides the Cry toxins, Bt **cyt**otoxin (Cyt), **v**egetative **i**nsecticidal **p**rotein (Vip) and **s**ecreted **i**nsecticidal **p**rotein (Sip) are also important for their insecticidal activity (Donovan et al. 2006; Estruch et al. 1996). All the *cry*, *cyt* and *vip* toxin genes are located on large plasmids (Berry et al. 2002; Espinasse et al. 2003; Schnepf et al. 1998). The plasmids are conjugative and the *cry* genes are frequently found in the vicinity of mobile genetic elements such as transposon or insertion sequences (Ehling-Schulz et al. 2019), this may explain the prevalence and diversity of these genes in Bt.

Bacillus cereus sensu stricto bacteria was first isolated from the air of a cow shed and described as a highly motile bacterium (Ehling-Schulz et al. 2019). Bc strains are widely spread and frequently isolated from soils, they also adapt to the intestinal tract of insects and mammals (Stenfors Arnesen, Fagerlund, and Granum 2008). Some strains can cause emetic or diarrhoeal type food poisoning. Even though the symptoms of most of the cases are mild and self-limiting, some serious cases can lead to death (Veysseyre et al. 2015). Many kinds of food have been associated with Bc foodborne disease, including spices, meats, poultry, sprouts, rice and pasta (Stenfors Arnesen et al. 2008) and the Bc bacteria have drawn the attention of the food industrial world. The toxins that might be involved in the diarrhoeal includes haemolysin BL (Hbl), nonhaemolytic enterotoxin (Nhe) and cytotoxin K (CytK). Hbl and Nhe would be involved in the disruption of the plasma membrane of epithelial cells of the small intestine (Fox et al. 2020; Tausch et al. 2017), while the role of CytK remained questionable (Castiaux et al. 2015). Other virulence factors such as phospholipases or collagenase may also contribute to the pathogenicity (Beecher et al. 2000). Those genes are chromosome genes and are under the

control of the transcriptional regulator PlcR (Gohar et al. 2008). The emetic symptom is mainly caused by a small ring-formed nonribosomal peptide – the cereulide (Ehling-Schulz, Fricker, and Scherer 2004). The cereulide synthesis genes are located in a pXO1-like plasmid (Ehling-Schulz et al. 2006) which is found in few genetically closely related strains (Ehling-Schulz et al. 2005). The detection of the diarrhoeal strains was well established (Ehling-Schulz et al. 2004) and the emetic strains can be detected by the presence of cereulide synthesis genes or by detecting the cereulide using mass spectrum (Doellinger et al. 2020). It is important to develop easy and accurate methods to discriminate the pathogenic and non-pathogenic Bc strains.

B. weihenstephanensis are psychrotolerant strains that can be distinguished from the others based on their capability to grow at 7 °C but not at 43 °C (Lechner et al. 1998; Soufiane and Côté 2013). Some of the *B. weihenstephanensis* strains can produce the cereulide, like the emetic Bc strains (Guérin et al. 2017). The *B. weihenstephanensis* KBAB4 strain produces PlcR-controlled virulence factors and is pathogenic to insect *Galleria mellonella* in vivo or to mammalian cells in vitro at 15 °C but not at 30 °C (Réjasse et al. 2012).

B. cytotoxicus was recently described as a novel thermotolerant species that has been associated with severe food poisoning outbreak (Guinebretière et al. 2013). They can be isolated from various food such as potato and flour products (Contzen, Hailer, and Rau 2014; Kindle et al. 2019; Stevens and Johler 2020). Even though it forms a separate species, it still has at least 90% of the average nucleotide identity (ANI) with the other members of the Bc group (Stevens and Johler 2020).

B. mycoides was thought to be rhizosphere-associated bacteria isolated from soil, and some of the *B. mycoides* strains are used as biocontrol agents (Wu, Huang, and Deng 2020; Yi et al. 2018). The metabolites produced by *B. mycoides* may be responsible for probiotic effects, such as the styrene produced by *B. mycoides* kills rhizosphere nematodes efficiently (Luo et al. 2018). Some strains were reported to have a nitrogen-fixing effect (Singh et al. 2020). The exopolysaccharide of *B. mycoides* has antitumor activity (Farag et al. 2020). There is a rare report showing that a *B. mycoides* strain has infected a man and the strain was isolated from his blood (Heidt, Papaloukas, and Timmerman 2019).

B. pseudomycoides is a close relative of *B. mycoides* with high similarity (98%) in 16S RNA sequences (Nakamura 1998). These bacteria were used to produce fibrinolytic enzymes (Gu et al. 2009) and polysaccharides (Solmaz et al. 2018), or used to treat the oil waste (Li et al. 2016) and wastewater (Mehrotra et al. 2020).

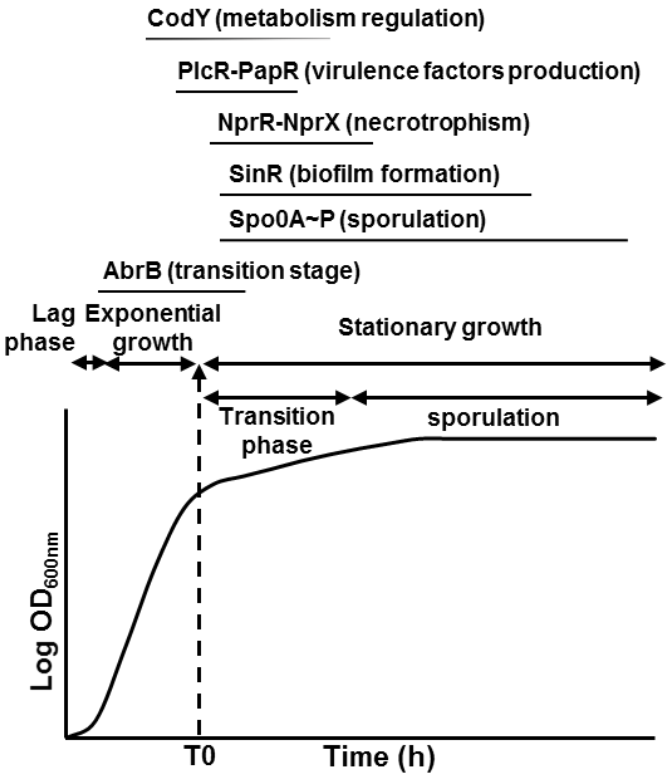


Figure 2 The growth stages of *B. cereus* bacteria in LB medium and the active periods of important regulators.

The growth stages are indicated using double arrows. The important regulators and processes they regulate are indicated in the figure. The bars below the names of regulators represent the expression or activation periods corresponding to bacteria growth. The active periods of each regulator were described in corresponding references: CodY (Ratnayake-Lecamwasam et al. 2001), PlcR-PapR, NprR-NprX, Spo0A~P (Verplaetse et al. 2015), SinR (Gaur, Cabane, and Smith 1988) and AbrB (Strauch et al. 1989).

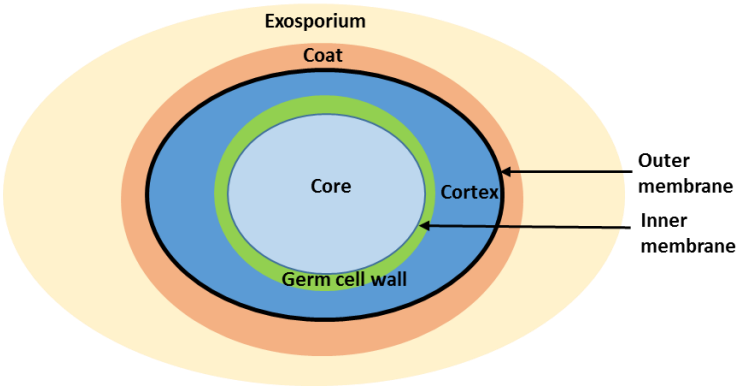


Figure 3 *B. cereus* group bacteria spore structures.

Adapted from Setlow (Setlow 2014b).

B. toyonensis is a newly defined Bc group species that have ANI values below 92% compared with the type Bc strains and ANI values below 94% compared with the other species in the Bc group (Jiménez et al. 2013). The *B. toyonensis* type strain BCT-7112^T has been commercially used as a probiotic in the name TOYOCERIN® from 1975. The spores of this strain have been used as a feed additive of pig, poultry, cattle, rabbits and aquaculture (Jiménez et al. 2013). The TOYOCERIN® is also the first microorganism based feed additive authorized in the European Union. This strain displayed no toxicity to various animals including humans (Trapecar et al. 2011; Williams et al. 2009). Recent studies even found that this species can improve the immune response of the mice and ewes to vaccines (Santos et al. 2018, 2021), and improve plant growth (Contreras-Pérez et al. 2019; Rojas-Solis et al. 2020).

1.2 The development of the *B. cereus* group

The Bc group bacteria have two distinct forms, the typical rod-shaped bacilli form corresponding to bacteria in metabolic active exponential or stationary phase, and the spore form, which is a stress-resistant dormant form. The growth stages of Bc group bacteria are shown on Fig. 2. First, the spores germinate when germinants are provided. Germination is followed by an exponentially growing phase where bacteria grow and divide actively. Bacteria will grow as single cells in rich media like LB, but they can form chain structures under certain conditions, such as in glucose medium during the late exponential and transition growth phases (Huillet et al. 2017), or in the presence of amoebas (Beeton, Atkinson, and Waterfield 2013). After the exponential growth, the bacteria will enter the stationary growth under the regulation of several transition state transcription regulators (Ehling-Schulz et al. 2019). The bacteria will sporulate in the late stationary growth when nutrients are deprived. They will sporulate faster when grown in the sporulation-specific medium (Purohit, Sassi-Gaha, and Rest 2010). Several important regulators are active during the growth and control different physiological events. Those regulators will be described in section 1.2.2.

1.2.1 Germination of spores

Bacillales bacteria such as all the Bc group bacteria and *Bacillus subtilis* can form spores when the environment is not conducive to growth (Setlow 2014b). The special structure of spores contribute to its high resistance. The Bc group spore structure from outside to inside consists of the exosporium, spore coat, outer membrane, cortex, germ cell wall, inner membrane, and core (Fig. 3). The *B. subtilis* spores lack the outermost exosporium layer. Most of studies on spore is based on *B. subtilis* and the spore properties of Bc group should be similar as they have

similar structures. The *B. subtilis* spores are highly resistant to physical pressures, including desiccation, freezing, thawing, wet or dry heat, UV light and γ-radiation, high pressures, and various kinds of chemical pressures, including oxidizing agents, alkylating agents, aldehydes, halogens, acids, and bases (Setlow 2014b). The factors that contribute to the *B. subtilis* spore resistance vary depending on the stresses. The spore coat helped the resistance to digestion, disinfectants, as well as oxidation agents. The spore DNA is saturated by α/β-type small acid-soluble proteins (SASPs) and dipicolinic acid (DPA) resulting in low water content in the core. The low water content in the spore core and the low permeability of the spore membrane can help spore resistance to heat, radiation desiccation, as well as genotoxic and oxidative chemicals (Setlow 2014b). The exosporium of Bc group bacteria is the primary interactive site with the outside world worked as a semi-permeable barrier that excludes antibodies and destructive enzymes, and it is the surface antigen of Ba spores (Stewart 2015).

The dormant spores will germinate in the presence of nutrient germinants and certain physical pressures. The amino acids such as L-alanine, L-cysteine, L-threonine, and L-glutamine or their combination (Hornstra et al. 2006), and inosine, glucose (Foerster and Foster 1966), Ca^{2+}-dipicolinic acid (CaDPA) and dodecylamine are nutrient germinants. The ammonia is not a germinant but it can work together with L-alanine and inosine to cause rapid germination (Preston and Douthit 1984). High-pressure treatment, lysozyme digestion, peptidoglycan fragments from other cells can also induce spore germination (Moir and Cooper 2015).

Knowledge of spore germination is mainly obtained from studies with *B. subtilis*. Considering the high similarity of germination proteins between Bc group and *B. subtilis* or other Bacillales species, the germination process of those bacteria should be similar. Such as both Bc group bacteria and *B. subtilis* have similar GerABCD germination receptors, muropeptides dependent germination protein PrkC, the SpoVA operon which regulates ions and Ca-DPA release during sporulation and rehydration of spore during germination, SleB and CwlJ responsible for cortex hydrolysis during spore germination (Paredes-Sabja, Setlow, and Sarker 2011). The *B. subtilis* spore germination includes several steps (Moir and Cooper 2015). Take germination under the action of nutrient germinants as an example: firstly, the germinants pass through the outer layers of the spore to reach the germinant receptors in the inner membrane, and activate the receptors. The spores will then commit to germinate. The loss of germination receptors and the presence of germinant degradation enzymes in the spore coat of Bc group bacteria or *B. subtilis* can decrease the germination efficiency (Moir and Cooper 2015). The monovalent cations like

H^+, Na^+ and K^+ are excreted from the spore in stage I of germination, and later the CaDPA stored in the core is released and replaced by water. Proteins of the *spoVA* operon mediate CaDPA uptake during sporulation and the release of CaDPA during germination in *B. subtilis* (Li et al. 2012; Tovar-Rojo et al. 2002). The heat resistance of the spores is lost at this stage of germination, but the metabolism has not started yet. The second stage of germination is the cortex hydrolysis by the lytic enzymes like CwlJ and SleB, and leads to full rehydration and expansion of the core. The SASPs associated with DNA are then degraded and the DNA is ready for transcription. DNA repair enzymes also start to repair the damages incurred during spore dormancy. In the third step, the germination signal is transduced to the surface and induces partial degradation of the spore coat. Lastly, the spores resume the normal metabolism and grow into vegetative cells.

The germination induced by nutrients is mainly dependent on the germination receptors GerA, GerB, and GerK, which form the 'Germinosome' connected by GerD (Moir and Cooper 2015). The germination receptors, SpoVA proteins and SleB-YpeB proteins are usually expressed during sporulation under the regulation of Sigma G factor (Bagyan, Hobot, and Cutting 1996; Boland et al. 2000; Feavers et al. 1990). A recent report showed the stable presence of some mRNA associated with Sigma G in a small portion of the spore population, which suggests these mRNA may play a role during germination (Korza et al. 2019).

The spores of Ba are able to infect the host through different routes indicating that germination happens in different host sites. The macrophages can phagocytose spores and transport them to the lymph nodes when infected through inhalation (Guidi-Rontani 2002) and spores may germinate within the macrophage (Guidi-Rontani et al. 1999). This suggests the macrophage intracellular environment favors Ba spore germination. However, Ba spores also germinate in the macrophage-conditioned media (Weiner and Hanna 2003). Ba can also cause skin and intestinal infections, which is different from in vivo environments.

Studies about Bt and Bc spore infection of different insect larvae showed that spores can germinate in the larvae midgut and the hemocoel. The larvae midgut fluid or simulation alkaline buffer stimulates germination (Wilson and Benoit 1990, 1993). A later study, performed with the strain Bt *israelensis*, has shown that plasmid genes encoding germination receptors are involved in the alkaline activation (Abdoarrahem et al. 2009). Nasal instillation experiments have shown that Bt and Bc spores can germinate in vivo in mice (Salamitou et al. 2000).

1.2.2 Exponential, transition and stationary growth

There is a lag phase when bacteria moved to a new environment. During that time, the bacteria do not multiply and will modify their physiology to adapt to the new environment. Such as the Bc strains activate the RNA helicase-encoding genes *cshA* during early adaptation of lag phase, and then start to express the master regulator *abrB* (Français et al. 2019).

The Bc group bacteria grow exponentially in rich medium after germination or after the lag phase. The generation time of Bc group bacteria is 20-30 min when grown at 37 or 30°C, respectively, in LB medium. Due to nutrient deprivation, the bacteria stop multiplying exponentially when OD_{600nm} reaches about 2.5 - 4.0 in LB medium; this decrease in growth rate corresponds to the onset of the stationary phase (Fig. 2). The time point from exponential growth to stationary growth was defined as T0 in bacilli studies. Different regulators control the transition phase, notably CodY, AbrB and Spo0A. CodY is a metabolism transcriptional regulator conserved in low G+C Gram-positive bacteria (Sonenshein 2005). In Bc group, CodY mainly represses various metabolism pathways from amino acid transport, ion transport to energy production (Lindbäck et al. 2012; Slamti et al. 2016). In Bs, the intracellular branched chain amino acids (BCAA) and GTP cause conformation changes in CodY to increase the binding of CodY to target genes. Under nutrient deprivation, the concentration of BCAA and GTP decreased, CodY binding to targets decreased and then the repression effect decreased. Stationary metabolism pathways are activated allowing the bacteria to enter the stationary growth (Sonenshein 2005). SinR is a constitutively expressed transcriptional regulator that represses the expression of sporulation regulator *spo0A* during exponential growth. SinI antagonist binding to SinR and releases repression to the expression of *spo0A* in early sporulation, and releases repression to other genes such as *spoIIA*, and possibly *spoIIG* and *spoIIE* in later sporulation stage (Bai, Mandic-Mulec, and Smith 1993; Mandic-Mulec et al. 1992; Mandic-Mulec, Doukhan, and Smith 1995). SinI-SinR also regulates biofilm formation in late stationary growth (Milton et al. 2020). *Spo0A* is expressed in growing cells and its expression increased rapidly under nutrient limitation conditions when repression from SinR was removed (Chastanet and Losick 2011). The nutrient limitation signal sensed by KinA and CodY results in the phosphorylation of Spo0A after several phosphate transfer steps (Fig. 4) (Burbulys, Trach, and Hoch 1991). AbrB is a master regulator that represses about 171 genes during exponential growth (Chumsakul et al. 2011). Activated Spo0A~P represses the expression of *abrB* (Perego, Spiegelman, and Hoch 1988) leading to bacteria metabolism changes and thus entering stationary growth stage.

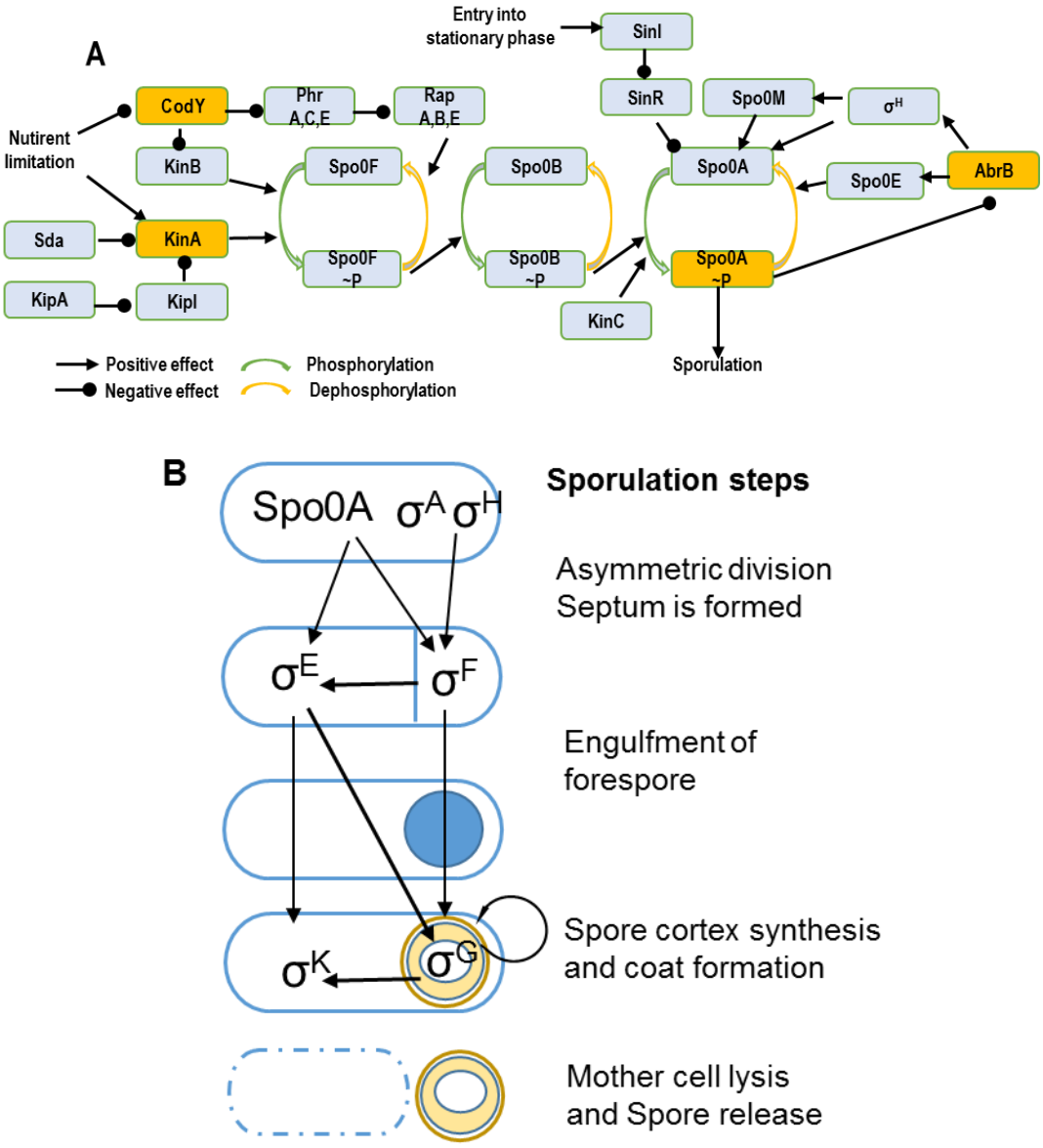


Figure 4 The schema of sporulation regulation and sporulation steps in Bacilli.

(A) The sporulation regulation networks of *B. subtilis*. The nutrient limitation signal sensed by CodY and KinA leads to the phosphorylation of Spo0F, Spo0B and finally Spo0A, then starts sporulation. Other regulators are also involved to form a complex regulation network (Vecchia et al. 2014). (B) The sporulation process and the activation of regulators during sporulation. The round structure inside the cell represents prespore. The arrows represent direct or indirectly activation by regulators. Details are described in the text.

In Bt and Bc, two quorum-sensing systems, PlcR-PapR and NprR-NprX, are activated during the transition stage, they play a major role in the pathogen properties and in the development of the bacteria (Slamti et al. 2014). They control the production of virulence factor and the saprophytic necrotrophic life style of Bt and Bc, respectively. I will describe these two systems in detail below.

1.2.3 Sporulation

Bacilli and Clostridia are spore formers. The sporulation has been extremely studied in *B. subtilis*, but all the bacilli and clostridia share similar core sporulation genes including *spo0A*, *sigE*, *sigF*, *sigK* and most bacilli possess *kinA* and *kinB* genes involved in the sporulation initiation (Galperin et al. 2012). Bc group species have a similar spore structure as *B. subtilis* except Bc strains have an exosporium, however, *B. subtilis* have more genes related to spore coat formation (Galperin et al. 2012). In general, the sporulation process of Bc group species could refer to the *B. subtilis*. The Clostridia strains lacks *spoIIB*, *sda*, *spoVID* and *safA* genes and have nonorthologous *spoIIQ* and *spoIVFA* genes indicating they are different from bacilli in engulfment and spore coat formation during sporulation (Galperin et al. 2012).

Spo0A is a master regulator that controls the initiation of sporulation in both *B. subtilis* and Bc group bacteria (Galperin et al. 2012; Green, Olmedo, and Youngman 1991; Molle et al. 2003). The activity of Spo0A is dependent on both the transcription of *spo0A* and the concentration level of active form Spo0A~P in the cells.

The Spo0A~P is produced by a series of phosphorylation transduction -called phosphorelay, which signifies the sporulation initiation (Fig. 4A) (Burbulys et al. 1991; Phillips and Strauch 2002; Vecchia et al. 2014). When nutrients are limited, CodY and KinA will sense the nutrient signal and then transfer the signal to Spo0F by phosphorylation, triggering the start of the phosphorelay. Spo0F~P transfer the phosphate to Spo0B, which transfer the phosphate to Spo0A to form Spo0A~P (Fig. 4A) (Burbulys et al. 1991; Phillips and Strauch 2002; Vecchia et al. 2014). Besides KinA and KinB, kinases KinC, KinD, KinE were discovered (Jiang et al. 2000). The activity analysis showed that KinC and KinD are responsible for the phosphorylation in the absence of KinA and KinB. The activity of Spo0A~P is negatively regulated by the Rap-Phr quorum sensing systems by dephosphorylating Spo0F~P - when the cell concentration increases, the Phr signal peptides are imported to the cells to antagonize Rap protein, thus decreases the dephosphatase activity (Cardoso et al. 2019).

In the end of exponential growth stage, the expression of *spo0A* and sporulation related genes increased, while the dephosphorylation genes decreased. The expression of *spo0F*, *spo0A* is positively regulated by Spo0A~P at low concentration, the sporulation related genes such as *spoIIG*, *spoIIE* and *sinI* - antagonist of SinR - are expressed at high Spo0A~P concentration; the expression of *abrB* decreased at low Spo0A~P concentration and the dephosphorylation gene *rapA* decreased at high Spo0A~P concentration (Fujita, González-Pastor, and Losick 2005). The increased expression of *spo0A* and phosphorelay result in the progressive increase of Spo0A~P and finally start sporulation.

Bc and Bs probably have the same progress and regulation during sporulation because key Sigma factors involved in sporulation were found in both species (Schmidt, Scott, and Dyer 2011). In *B. subtilis*, SigF, SigE, SigG and SigK were activated sequentially during sporulation (Fig. 4B) (Higgins and Dworkin 2012; Losick and Stragier 1992; Stragier and Losick 1990). Firstly, a septum is formed under the control of SigH. The asymmetric cell division of bacteria forms a smaller cell (forespore) and a larger cell (mother cell). Spo0A activates σ^F and σ^E; regulon of σ^F in the forespore activate σ^E in the mother cell. In the second step, the proteins controlled by σ^E and σ^F mediate the mother cell engulfment of the forespore. σ^G was then activated by products of σ^E and σ^F in the forespore. σ^K was activated by σ^G products in the mother cell after σ^G activation. In the next step, under the control of σ^G and σ^K, the mother cell produces spore cortex and spore coat. The mother cell is lysed in the last step and mature spores released. Only the cells activated σ^F and σ^E will commit to sporulation. The SpoIID, SpoIIM and SpoIIP controlled by σ^E worked in concert to regulate the polar division step (Eichenberger, Fawcett, and Losick 2001) and SpoIID worked as peptidoglycan degradation enzyme in this step in *B. subtilis* (Gutierrez, Smith, and Pogliano 2010).

1.3 The ecology of *B. cereus* spp and its pathogenic properties

1.3.1 Soil as ecological niche

Soil is the most important niche for Bc group bacteria. The spore-forming bacteria account for 15.7-46.0% of total bacteria in forest soil samples, and the *B. cereus* represents 6-14% of the total spore population (Siala, Hill, and Gray 1974). The spores can reach a concentration of approximately 10^6 spores/g of soil (Hong et al. 2009) and can persist in the environment for at least 4 years after spray (Van Cuyk et al. 2011). The *Bacillus* adapts to a wide range of temperature from 4 °C to 50 °C (Guinebretière et al. 2008). They can be isolated from various soils, such as permafrost, forest soil, mining soils and saline-alkali soil (Ayangbenro and

Babalola 2020; Han et al. 2020; Timofeev et al. 2019). The pH, moisture, nutrient availability and indigenous microorganisms of the soils will affect the survival of bacilli (West et al. 1985).

It is believed that Bc group bacteria exist in soil as spores, and that the vegetative cells cannot persist for a very long time. It was shown that the wild-type bacteria will form viable spores in the soil environment and maintain their viability until the end of a 20 days experiment, while asporogenous mutant strains were no longer recovered after 8 days of incubation (Vilas-Bôas et al. 2000). When the vegetative cells and nutrient broth inoculated simultaneously in the soil, the bacteria will grow and form spores, however, the spores that were formed *in situ* in the soil died and only the originally ungerminated spores survived after two weeks of incubation (Petras and Casida 1985). Even though it is believed that bacilli cannot grow in the soil without nutrient input, the spores can germinate and grow in the soil-extracted soluble organic matter (SESOM) and artificial soil microcosms (ASM) saturated with SESOM (Vilain et al. 2006).

1.3.2 Insect as ecological niche

Besides the soil, the Bc group bacteria is also adapted for growth in the intestinal tract of insects and mammals (Fakhry et al. 2008; Margulis et al. 1998).

The Bt strains are well adapted to the insect larva life (Nielsen-LeRoux et al. 2012; Raymond et al. 2010). Bt can effectively kill susceptible insects, multiply using the nutrients of the cadaver, and sporulate when nutrients are limited. The Bc and *B. weihenstephanensis* strains are virulent to the insect larvae when infected via oral route with the addition of Cry1C proteins or via haemocoel injection (Stenfors Arnesen et al. 2011). Bc have the same toxicity to *G. mellonella* larvae as Bt strain when Cry toxin is added to the bacteria during the infection due to the similar virulence factors profile of Bt and Bc (Salamitou et al. 2000). Bc SW7-1 strain can also cause masses of death to *Bombyx mori* as the Bt strains in the absence of Cry toxins (Li et al. 2015).

The bloodsucking or flesh-eating mosquitoes or flies are able to transmit *B. anthracis* (Blackburn et al. 2010; Bradarić and Punda-Polić 1992; Turell and Knudson 1987). The Ba protective antigen and elements from other bacteria like *Clostridium septicum* alpha toxin can trigger innate immune system of *Drosophila* flies and *Culex* mosquitoes protecting the vector species from Ba (Alameh et al. 2020). There are two studies about the toxicity of Ba to *G. mellonella*. The attenuated *B. anthracis* BIG19 strain showed no lethal activity and no synergize with a sublethal dose of Cry1C to *G. mellonella* 48 h after force-feeding with 10^6

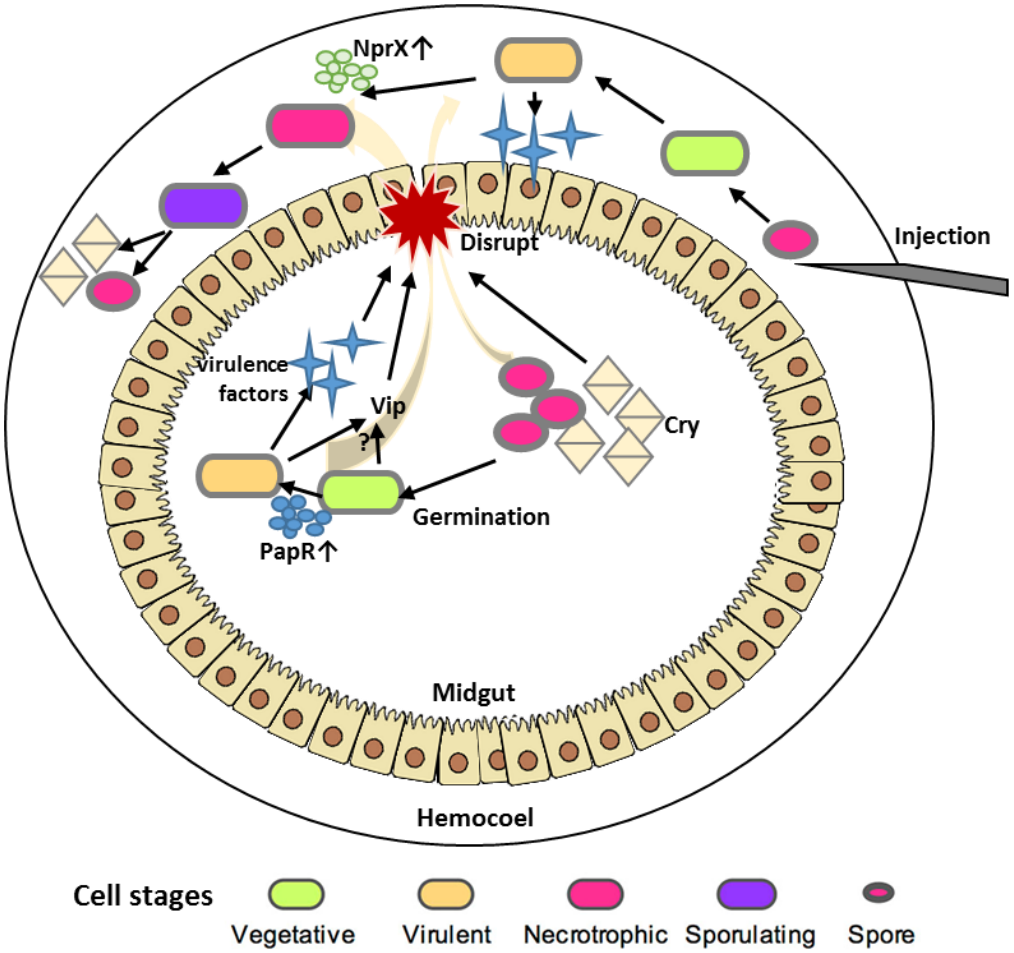


Figure 5 Schematic representing the different steps of the infection of larvae by *B. thuringiensis* through oral route or hemocoel injection.

The cross-sectional view of the larvae, adapted from (Raymond et al. 2010). The Bt cells in different stages are presented using different colors as indicated at the bottom. Cry toxins are presented as bipyramids, virulence factors are presented as stars, and quorum-sensing peptides are presented by small oval dots. PapR↑ and NprX↑ represent the concentration of peptides increased.

CFU/larvae of spores or vegetative cells (Fedhila et al. 2010). Another attenuated *B. anthracis* Sterne strain 34F2 killed over 70% of *G. mellonella* larvae at 48 h when 10^5 CFU/larvae of spores were injected via abdominal (Norris et al. 2020). The different results may be caused by the strains, or by the different mode of infection because larvae death could be induced by septicemia when the abdominal injection was used.

i. Bt infection cycle and its regulatory systems

The development of the bacteria population during the infection of a susceptible insect has been thoroughly studied in our lab. Key regulators and genes involved in the infectious process have been identified using fluorescent reporters; we could follow the activation of several transcriptional regulators at the cell level to determine the differentiation course of a Bt population as well as to establish the lineage between cells. We could determine that the life cycle of Bt inside an insect can be divided into 3 steps. Bt will experience successively the virulence, necrotrophism and sporulation stages (Fig. 5) (Ben Rejeb, Lereclus, and Slamti 2017; Slamti et al. 2014; Verplaetse et al. 2015). The infection process and activation of these stages was described in detail below.

The first step of infection – killing of the insect. After ingestion by susceptible larvae, the Cry toxins become activated by midgut juice, then the activated Cry toxins bind to receptors located on the surface of the epithelium cells and form oligomeric pores that finally insert into the cell membrane, which cause the lysis of cells (Bravo et al. 2011). The disruption of the midgut tissue is followed by a septicemia which kill the larvae (Raymond et al. 2010).

The alkaline midgut environment and the midgut fluid can trigger spore germination (Wilson and Benoit 1990, 1993). Then the vegetative Bt cells will grow in the midgut. With the proliferation of the bacteria, cell density increases. The concentration of the quorum sensing signal peptides PapR will increase in the environment at the same time and allows the activation of its cognate regulator PlcR, leading to the production of a series of virulence factors including enterotoxins Hbl and Nhe, neutral protease NprB, NprC, metalloprotease InhA2 (Agaisse et al. 1999; Fedhila, Nel, and Lereclus 2002; Gohar et al. 2008). Using a fluorescent transcriptional fusion between the PlcR-activated *plcA* promoter and a fluorescent gene, it was shown that all the Bt population in the larvae activates PlcR (Verplaetse et al. 2015).

Some other virulence factors like InhA1 and hemolysin HlyII not regulated by PlcR are also expressed at the same time as PlcR regulated virulence factors, they start to be expressed from

T0 in LB medium or reach highest activity 24 h post haemocoel injection infection (Guillemet et al. 2010; Tran et al. 2013). InhA1 is regulated by AbrB and is expressed in the stationary growth stage (Grandvalet, Gominet, and Lereclus 2001). Hemolysin HlyII is repressed by HlyIIR in glucose rich medium and is activated in stationary growth when glucose is consumed (Guillemet et al. 2013). Overall, these virulence factors contribute to the midgut disruption together with Cry toxins, and when infected through haemocoel injection, they can also exhibit full virulence to insects without Cry toxins (Fedhila et al. 2002; Guillemet et al. 2010; Salamitou et al. 2000; Tran et al. 2013). The regulator SigB is also involved in the adaptation of the gut environment and the pathogenicity of Bt by controlling Bt stress response when infecting insects through the oral route (Henry, Lereclus, and Slamti 2020).

The second step of infection – necrotrophic life. After the disruption of the larvae midgut, the Bt cells invade the hemocoel and provoke a septicemia. Then, the production of NprX by the high number of cells presented in the insect cadaver triggering the activation of the quorum-sensing regulator NprR in a part of the bacteria population (Dubois et al. 2016; Verplaetse et al. 2015). At least 41 genes are regulated by the NprR-NprX system, including genes encoding degradative enzymes that might be essential for the cadaver degradation, the major extracellular protease NprA, and a lipopeptide Kurstakin that is involved in swarming and biofilm formation (Dubois et al. 2016; Perchat et al. 2011). Together, those gene products are essential for the survival of the whole bacteria population in the cadaver (Dubois et al. 2016; Verplaetse et al. 2015). Using a transcriptional fusion between the NprR-controlled *nprA* promoter and a reporter gene, it was shown that only a part of the Bt population that colonized the insect cadaver activates NprR. This indicates that the benefits of the expression of the NprR regulon are shared with the whole population, allowing the survival of all the Bt cells in the cadaver (Verplaetse et al. 2015). The activation of NprR is not synchronized in the Bt population as the cells expressing NprR were detected 18 h post intrahemocoel injection and the population increases up to 72 h, demonstrating the continuous commitment of cells to the necrotrophic lifestyle.

The third step of infection – sporulation within the insect cadaver. After all the nutrients of the cadaver were used, the bacteria will sporulate as described in 1.2.3, and sporulation occurs concomitantly to necrotrophism, in a part of the population (Verplaetse et al. 2015). Survival in the cadaver and completion of the lifecycle is also achieved through sporulation. The sporulation deficient Bt strain can survive in the cadaver during a 96 h of infection (Dubois et

al. 2016), however, the asporogenous Bt may not survive for so long as the highly resistant spores, as it has shown in soil (Vilas-Bôas et al. 2000). Using a transcriptional fusion between the SigE controlled *spoIID* promoter and a flourecence gene, it was whown that some cells commit to sporulation 24 h post infection within the nectrotrophic population (Verplaetse et al. 2015). Similar to the activation of NprR, commitment to sporulation occurs asynchronously with the Bt population, with highest number of sporulating cells detected 48 h post infection.

It is notable that Rap-Phr systems regulate sporulation, they were found in all Bc group bacteria on chromosome or plasmids, and they are far more abundant in plasmids of Bt strains (Cardoso et al. 2019). Two plasmidic Rap-Phr systems were studied in Bt HD73, they have similar sporulation regulating activity as the other Rap-Phr systems, but they are specifically needed during insect infection (Cardoso et al. 2020; Fazion et al. 2018). This suggests that the Bt plasmids may confer advantages to colonize specific environments, and sporulation regulation may be different depending on the environment.

ii. The insecticidal proteins of Bt

Bt produces several types of pesticidal toxins including Cry, Cyt, Vip and Sip toxins. Cry and Cyt toxins are produced in the parasporin crystal, and Cyt toxin is named after its **cyt**otoxicity. Vip is the abbreviation of **V**egetative **i**nsecticidal **p**rotein because they are produced during the vegetative growth stage. Sip is the abbreviation of **S**ecreted **i**nsecticidal **p**rotein.

The toxins played important roles during insect infection. Cry toxins paralyze the midgut of insect, permitting the germination of spores, and the toxins disrupt the midgut membrane allowing bacteria to enter the hemocoel (Nielsen-LeRoux et al. 2012; Raymond et al. 2010). Cyt toxins may have similar role like Cry toxins. However, the involvement of Vip is to be determined.

In 1998, a nomenclature was proposed for the pesticidal crystal proteins of Bt, which is highly accepted and still in work until now (Crickmore et al. 1998). The toxin genes are named according to their degree of evolutionary divergence as estimated by phylogenetic tree algorithms of the gene sequences. There are four ranks discriminate with alphabets and numbers, such as *cry1Aa2*, determined by a sequence identity of 45%, 78%, and 95%, respectively. Cyt, Vip and Sip proteins are also named in a similar way.

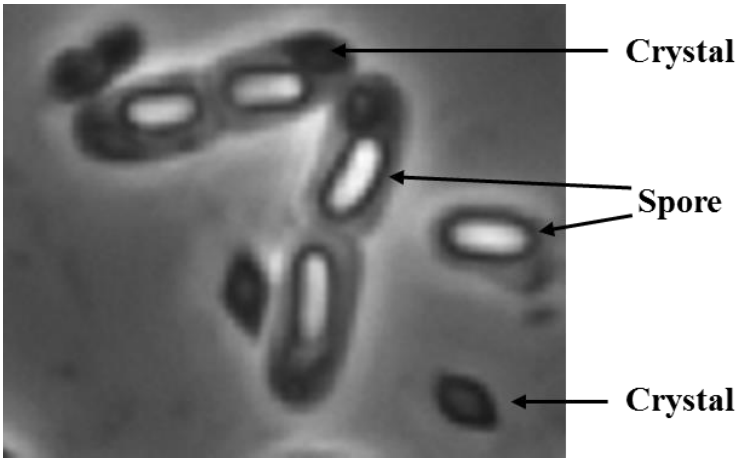


Figure 6 The spores and Crystals of *B. thuringiensis* HD73 strain.

The bipyramid crystal is formed in the mother cells and released with spore after the mother cell lysis.

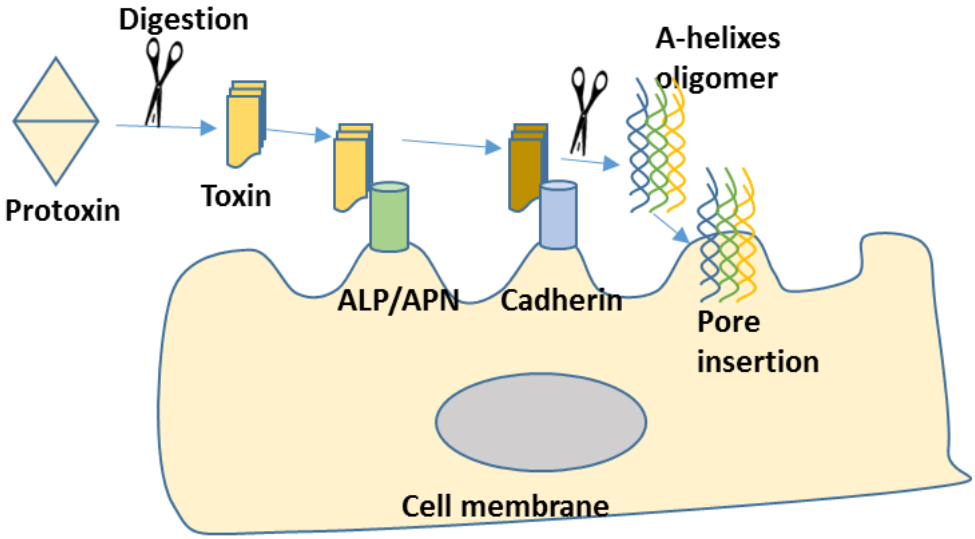


Figure 7 The sequential binding model of 3D-Cry toxin mode of action.

Adapted from (Bravo et al. 2011).

Cry toxins

Bt produces the Cry and Cyt toxins (delta-toxins) during sporulation, and those toxins form microscope visible crystals inside the mother cell and are released when the mother cell was lysed (Fig. 6) (Schnepf et al. 1998). The shape of Crystals varies depending on the strains, including bipyramid, cuboidal, flat rectangular, irregular, spherical and rhomboidal (Schnepf et al. 1998).

The Crystal toxins are the most abundant proteins in the sporulating cells. The protein composition of the crystal varies between strains. Cry toxin genes are located on large plasmids and one strain can have a serial of different *cry* genes. The Bt *kurstaki* HD1 strain is most widely used as biopesticide in the name of Dipel®. All the *cry* genes of Bt HD1 locates on a 299 kb plasmid pBMB299 (CP004876.1), and the plasmid encodes *cry1Aa*, *cry1Ia*, *cry2Aa* *cry2Ab* genes, and a *vip3Aa* gene.

The mode of action for 3D-Cry toxins is described as a sequential binding model (Fig. 7) (Bravo et al. 2011). Take Cry1A as an example. The 130 kDa protoxin Cry1A will dissolve in the midgut of insect larvae, and then the gut proteases partially digest the N- and C-terminal to expose the active three domain core (Bravo et al. 2011). Shorter toxins like Cry2Aa will lose only the N-terminal by proteolytical cleavages. The activated toxins sequentially bind to different membrane proteins and form pores in the end. Activated Cry1Ab binds to the abundant ALP (GPI-anchored alkaline phosphatase) and APN (GPI-anchored aminopeptidase-N) with low affinity via domain II and III, then the exposed domain II loops bind the cadherin receptor with high affinity; the domain I further cleaved in the N-terminal end and the alpha-helixes form a pre-pore oligomer, and finally the pore insert into the membrane. The binding receptors of different Lepidoptera, Diptera and Coleoptera species vary from ALP, APN, Cadherin, to glucosidase, metalloprotease (Bravo et al. 2011).

The insecticidal spectrum of Cry proteins including Lepidoptera, Diptera, Coleoptera, Hemiptera and nematodes (Krieg et al. 1983; Porcar et al. 2009; Wei et al. 2003). A study has summarized the results of around 1700 bioassays of the Cry and Cyt toxins (Frankenhuyzen 2009). Cry1 and Cry9 are mainly toxic to Lepidoptera and some of them are toxic to Diptera; Cry3, Cry8 and Cry35 are mainly toxic to Coleoptera; Cry5 and Cry6 are toxic to Rhabditida. The targets of the Cry toxins are mainly determined by receptors presented in insects and the alteration in any steps of the mode of activation, especially mutations in receptors, confer insect resistance to Cry toxins (Bravo et al. 2011; Peterson, Bezuidenhout, and Van den Berg 2017).

Cyt toxins

Cyt toxins encoded by *cyt* genes are another insecticidal protein family of Bt. Cyt proteins have a general hemolytic activity to cells according to its definition (Crickmore et al. 1998). The structure of Cyt proteins is composed of two outer layers of alpha-helix hairpins wrapped around a beta-sheet (Soberón, López-Díaz, and Bravo 2013). The protoxins are digested by midgut proteases to produce a 25 kDa active form and then interact with unsaturated lipids such as phosphatidyl choline, sphingomyelin and phosphatidyl ethanolamine (Thomas and Ellar 1983). It seems that Cyt toxins do not need binding receptors like the Cry proteins. Two modes of action were proposed for Cyt. In the pore-forming model, the beta-sheets oligomerize to form a pore of about 400 kDa that inserts in the membrane and kills the cell by osmotic (Chow, Singh, and Gill 1989; Promdonkoy and Ellar 2000). In the detergent effect model, the Cyt proteins aggregate on the surface of the lipid bilayer to cause the disassembly of membrane and cell death (Butko 2003). The toxicity of Cyt is relatively weak than Cry toxins, however, they can work in synergy with the Cry toxins probably working as membrane receptors for Cry toxin binding (Bravo, Gill, and Soberón 2007; Promdonkoy, Promdonkoy, and Panyim 2005). The Cyt toxin is a key component for the mosquito killing Bt *israelensis* strains (Lacey 2007; Valtierra-de-Luis et al. 2020).

Vip toxins

In a program to evaluate active insecticidal components from *Bacillus* isolates, researchers found that culture supernatants from Bc AB78 induce 100% mortality of *Diabrotica virgifera virgifera* and *Diabrotica longicornis barberi* larvae (Warren 1997). Two proteins of 80 and 45 kDa were responsible for the toxicity and they were named Vegetative insecticidal protein Vip1Aa and Vip2Aa, respectively. Another 88.5 kDa protein from Bt AB88 that is highly toxic to *A. ipsilon* and other lepidopteran larvae was name Vip3 (Estruch et al. 1996; Warren 1997).

Vip1 shared some similarity with *C. botulinum* toxin C2-II (29%) and Ib component of iota-toxin from *C. perfringens* (31%) (Chakroun et al. 2016). Vip2 is over 30% similar to Rho-ADP-ribosylating exotoxin C3 of *C. clostridial*. That similarity suggests Vip1 and Vip2 act as binary toxins - Vip1 act as binding and translocation component and form channels, and Vip2 act as toxins and enter the cells perhaps by endocytosis (Leuber et al. 2006). A study of Vip1Ad and Vip2Ag has confirmed the binary toxin mode of action (Geng et al. 2019).

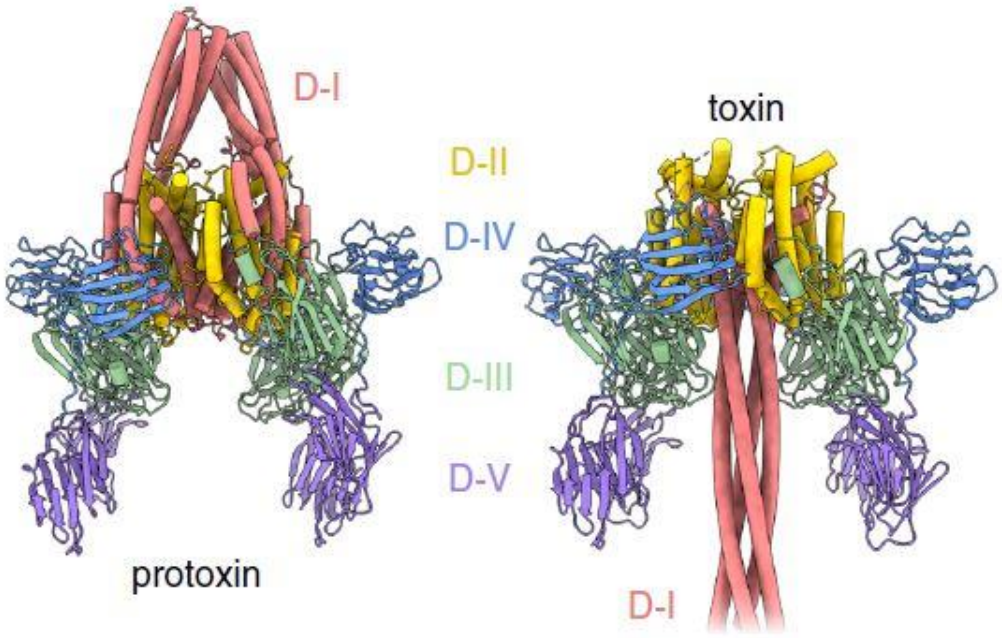


Figure 8 The structure of protoxin and activated toxin of Vip3Aa.

Adapted from (Núñez-Ramírez et al. 2020). Pink - domain I, yellow - domain II, green - domain III, blue - domain IV, violet - domain V. The proteases cleave between domain I and II resulting in the formation of 4 helixes needle-like structures.

The mode of action of Vip3 is similar to that of Cry toxins but is not clearly determined yet. Vip3 binds different receptors than those recognized by the Cry toxins (Chakroun and Ferré 2014; Gouffon et al. 2011; Kahn et al. 2018). The scavenger receptor class C like protein (Sf-SR-C) and Fibroblast Growth factor Receptor (Sf-FGFR) of *Spodoptera frugiperda* and a tenascins-like protein of *A. ipsilon* may be the Vip3 receptors (Jiang, Xiao-yue Hou, et al. 2018; Jiang, Xiaoyue Hou, et al. 2018; Osman et al. 2019).Vip3 proteins needs cleavage activation like the Cry toxins. They have a molecular weight of 89 kDa and form tetramers in solution, the cleaved 65 kDa and 21 kDa products associate closely (Palma et al. 2017). Recent structure studies of Vip3 revealed that the proteins form a tetramer structure with hitherto unreported topology (Byrne et al. 2021; Núñez-Ramírez et al. 2020; Zheng et al. 2020). The protease trypsin cleaves the residue between domain I and II to activate the toxin, causing big conformation change - the domain I helixes turned over to form a 4 helixes needle-like structure, which inserts into the target cell membrane (Fig. 8) (Byrne et al. 2021; Núñez-Ramírez et al. 2020). Byrne and colleagues have observed that activated Vip3A insert into the liposome membranes without the help of any receptor (Byrne et al. 2021). The death of cells may be caused by ion transporting through the needle-like structure, similar to the Rocker transporter or the influenza M2 channel (Lee et al. 2003; Núñez-Ramírez et al. 2020).

Vip3 proteins are believed to be secretory proteins even though they do not contain a signal peptide. Vip3A was produced and retained in the mother cell compartment when grown in sporulation medium, and it was secreted to the medium when grown in rich medium (Z. Wang et al. 2021). This result suggests Vip3A secretion is nutrient-dependent. Vip3A is produced from the mid-log growth until sporulation, unlike the Cry proteins, whose genes (except the *cry3* genes) are only expressed at the sporulation stage (Agaisse and Lereclus 1995; Estruch et al. 1996). The description of Vip3A production in Estruch's report is not very convincing, because the Bt growth condition and description of growth are different from those generally used in Bt studies. On the other hand, western blot cannot describe the expression of *vip3A* quantitatively. Thus, the expression of *vip3A* needs more detailed descriptions. Moreover, the regulation of *vip3A* expression has never been studied.

1.3.3 Mammals as ecological niche

Bacillus species such as Ba and Bc serovar *anthracis* are human/mammal pathogens. Ba uses mammals as a survival niche. Ba can infect humans through inhalation or wounded skin. The classical infection of Ba through the human respiratory system was described as the "Trojan

horse model" (Guidi-Rontani 2002). The Ba spores reach the lungs through inhalation, and the alveolar macrophages phagocytose the spores to eliminate the intruders. Once inside the macrophage, the Ba spores germinate and produce toxins, which disrupt the phagolysosome. The disruption of the phagolysosome allows nascent bacilli transit to the macrophage cytoplasm. The migration of macrophages transmits the bacteria to lymph nodes and blood, where the bacilli multiply and cause bacteremia and toxemia. The poly-γ-D-glutamic acid capsule of Ba cells helps bacteria resist phagocytosis (Ezzell and Welkos 1999), thus allows the bacilli to survive in the blood and lymph system.

Deadly Ba infection of wild animals favors the Ba persistence in the field. An *in situ* study of Ba infected carcasses has shown that Ba infects and kills the animals whose carcasses remain on the ground. After the death of the animal, the characteristic of the soil changed and favors the growth of grass in the site and attracts herbivores, which may result in the next cycle of infection (Turner et al. 2014). The concentration of spores on root and soil remained stable for 3 years, and the Ba found in the grass only decreased 3 years after the death of the animals (Turner et al. 2014). The high concentration and long persistence of spores in soil and grass increased the possibility of a new infection.

Some other *Bacillus* strains have a similar pathogenic property as Ba. The Bt subsp. *konkukian* strain was isolated from a wounded soldier and it can infect immunosuppressed mice (Hernandez et al. 1998). *B. thuringiensis* 407 Cry^- and *B. cereus* ATCC14579 resulted in 100% mortality of mice in nasal instillation experiments (Salamitou et al., 2000). The *B. cereus* species are not only involved in food poisoning in humans, but they may also cause anthrax-like progressive pneumonia, fulminant sepsis, and devastating central nervous system infections in immunosuppressed individuals (Bottone 2010). Nevertheless, it is not sure if the Bt and Bc species can use mammals as a survival niche like Ba and Bc serovar *anthracis*.

Both spore and vegetative forms of *Bacillus* can survive in the human gastrointestinal tract (Berthold-Pluta, Pluta, and Garbowska 2015). The Bt species can be isolated from feces of cows, sheep, human and chicken (Ammons et al. 2016; Barbosa et al. 2005; Jensen et al. 2002; Pinto et al. 2017; Sinott et al. 2012). However, the isolation of *Bacillus* from feces may mainly result from the food intake (Turnbull and Kramer 1985). The Bt and Bc spores can have a hemocoel life in insect suggests they can adapt to the aerobic environment. Those bacteria occasionally colonize human gut resulting severe food poisoning. The bacteria may be ingested with food. The highly resistant spore can protect them from the acid and enzymatic stomach and small

intestine environment. When the intestinal environment of patient changes, the pH rises to alkaline, the Bc spores may germinate (Wilson and Benoit 1993). Then the bacteria can adhere to the mucin of gut (Tsilia et al. 2016). The vegetative cells will produce virulence factors such as hemolysin Hbl, nonhemolytic enterotoxin Nhe, cytotoxin K, metalloproteases, sphingomyelinase, and phospholipases (Gohar et al. 2008). Those virulence factors may cause the damage on the gut or inflammation. The Cereulide produced by emetic Bc species may also cause cell damages and provoke diarrhea symptoms in human (Ehling-Schulz et al. 2004). The bacteria will also produce different antimicrobial peptides, such as thurincins for Bt strains and cereins for Bc strains, to compete for the intestinal environment with the other microbes (Abriouel et al. 2011). The combination of disruption in the intestine and the imbalance of gut microbiota will result in the food poisoning symptoms. However, as the condition of intestine is usually not favorable for *Bacillus* spore germination, their colonization in the gut of normal human is rare. The microbiota studies showed that the phylum *Firmicutes* bacteria are predominant in the gut, the spore-forming *Clostridium* spp is very rich whereas Bc group bacteria were rarely identified (Adak and Khan 2019; Holman and Gzyl 2019). Besides human gut, the Bc species can cause infection in other sites such as skins, respiratory tract, eyes and blood (Bottone 2010; Callegan et al. 2003; Veysseyre et al. 2015).

1.4 The phagocytic cells and their interaction with bacteria

The phagocytic cell is a general name describing cells that can engulf target particles such as bacteria. This name usually referred to the phagocytes of animals as well as the phagocyte-like protozoa amoebas. The Bc group bacteria may encounter macrophages, such as when Ba enters mammalian blood or Bt enters the hemocoel of insects, and they may encounter the amoebas as both *Bacillus* and amoebas are abundant in the soil.

Phagocytosis of bacteria by phagocytes includes the following steps: recognition of the microorganism through phagocyte receptors, internalize bacteria into a vacuole (the early phagosome), the vacuole fuse with endocytic vesicles and then fuse with lysosomes to form a mature phagosome and kill the bacteria (Uribe-Querol and Rosales 2017). After full digestion of the bacteria, the phagosome will disappear. The core mechanisms used by amoebas to ingest and kill bacteria are similar to that of human phagocytic cells (Bozzaro and Eichinger 2011; Cosson and Lima 2014). The digestion residues in amoebas will be excreted through the contractile vacuole.

Special receptors are involved in the recognition step for the mammalian macrophages and neutrophils, non-opsonic receptors mediate direct ingestion by phagocytes, and the opsonic receptors mark the targets then mediate ingestion (Uribe-Querol and Rosales 2017). Amoebas may have different receptors as macrophages or even different ways of recognition as they can phagocytose latex beads, bacteria, erythrocytes and yeast (Allen and Dawidowicz 1990). The *Dictyostelium* amoebas prefer gram-negative bacteria to gram-positive ones (Rashidi and Ostrowski 2019), and specific genes are required to phagocytose different bacteria (Lamrabet et al. 2020; Nasser et al. 2013). The *Acanthamoeba* seems to be a less picky eater, they can use both gram-positive or -negative bacteria for growth and delay cyst formation (De Moraes and Alfieri 2008)

1.4.1 General information about amoebas

The amoebas are protists that have pseudopodia-based shape-altering movements. The free-living amoebas (FLAs) are living in the environment without a need for a host. They are widely spread in the soil, fresh water, and some of them adapt to extreme environment such as sea water, hot spring, low or high pH environments (Samba-Louaka et al. 2019). The FLAs feed on environmental bacteria by phagocytosis or use other soluble nutrients and small fragment nutrients by pinocytosis (Bowers 1977). Researchers discovered amoebas over two hundred years before and regain interest in FLAs as they were thought to be potential reservoirs and 'training camps' for pathogenic bacteria (Samba-Louaka et al. 2019).

1.4.1.1 The classification of amoebas

The name amoeba is a generic name for many protists. The classification of amoebas is a complicated job for taxonomists. Cavalier-Smith emended the taxon Amoebozoa as a phylum (Cavalier- Smith 1998). The classification of Amoebozoa was revised based on both ultrastructural research and molecular phylogenetic studies in 2005, the researchers propose Amoebozoa to have several classes including Tubulinea, Flabellinea, Stereomyxida, Acanthamoebidae, Entamoebida, Mastigamoebidae, Pelomyxa, Eumycetozoa (Adl et al. 2005). However, the classification differs dependent on the choice of sites and taxa when analyzing the small subunit rDNA of amoebas. When presenting the amoebozoan phylogeny with a basal multifurcation that reflects better the uncertainties, six major clades can be distinguished:

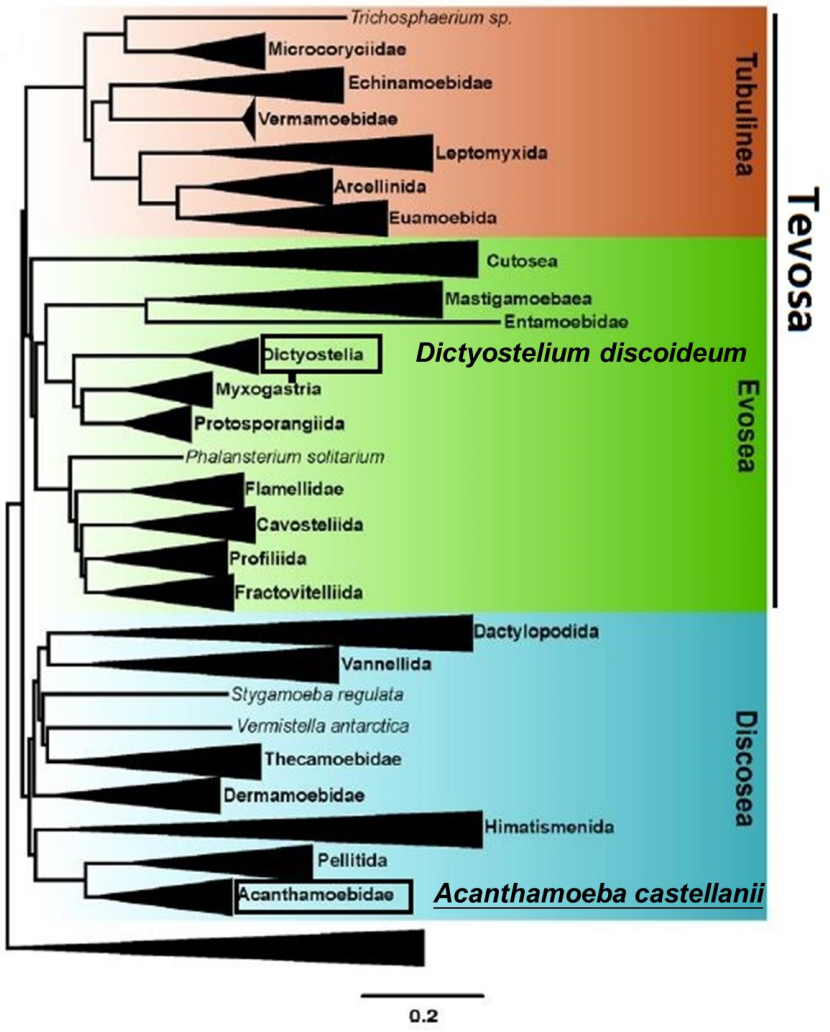


Figure 9 The phylogeny of Amoebozoa.

Adapted from (Kang et al. 2017; Samba-Louaka et al. 2019).

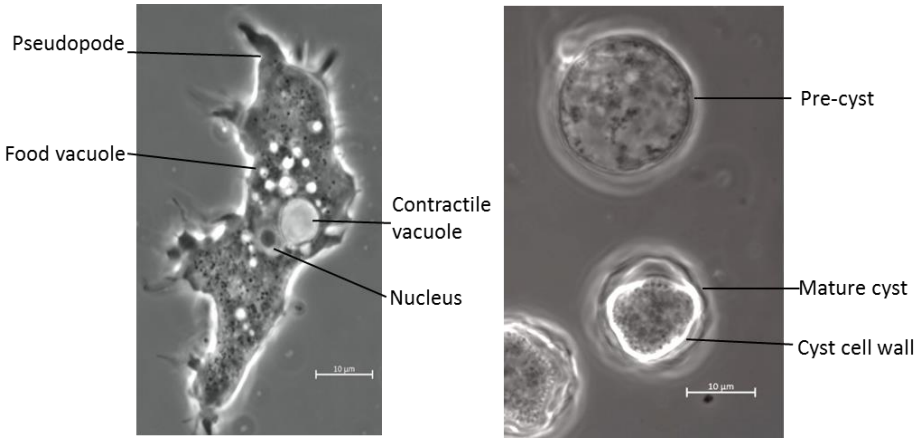


Figure 10 Phase contrast microscope images of *Acanthamoeba castellanii* 30010 trophozoite (left) and cyst (right).

Scale bar = 10 µm.

Tubulinea, Flabellinea, Conosea, Variosea, Thecamoebida, and Acanthopodida (Pawlowski and Burki 2009). A more recent study uses a phylogenomic framework (325 genes) analysis to present a robust phylogeny of amoebaozoa. The authors classified amoebaozoa into two major clades, Discosea, and Tevosa (Fig. 9) (Kang et al. 2017). The *Dictyostelium discoideum* and *Acanthamoeba castellanii* belonging to the Tevosa and Discosea clades, respectively, are used in my study.

1.4.1.2 Free-living *Acanthamoeba*

The *Acanthamoeba* belongs to the Discosea class and is represented by *Acanthamoeba castellanii* species. The *Acanthamoeba* can be distinguished at the genus level based on morphological characteristics but the differences between species are not apparent, the species can only be identified by genomic features such as 18S rDNA (Kong 2009). *Acanthamoeba* is the most well studied amoebas with classical amoeba-movement. They have two developmental forms: the trophozoite, an active form that has stretched pseudopodia, food vacuole, and a clear nucleus; the cyst form, a stress-resistant dormant form, usually round with doubled cell walls (Fig. 10).

The trophozoites form cysts when encountering unfavorable environments, such as starvation, hypoxia, change of osmolarity (Lloyd 2014). The encystment of amoebas including pre-encystment, cyst initiation and cyst wall synthesis steps (Khunkitti et al. 1998). In the pre-encystment step, corresponding to the occurrence of environmental stresses, the trophozoite stops moving and retracts the pseudopodia to become a compact cell. The amoeba can easily resume normal shape when stresses were removed. In the second step, the amoeba starts to synthesize the outer cell wall of the ectocyst and the internal wall of the endocyst. The ectocyst contains proteins and polysaccharides and the endocyst is mainly composed of cellulose (Lemgruber et al. 2010). The cysts become mature dormant cysts after cyst wall synthesis. The cysts are highly resistant to environmental conditions including physical or chemical factors (Lloyd 2014). They can be stored at 4°C for one year (Campbell et al. 2008) or even 50 years (Lloyd 2014). Cysts will resume growth when cultured in proper condition for about one week.

1.4.1.3 Social amoebas *Dictyostelium*

Dictyostelium discoideum are soil amoebae that feed on bacteria (Otto and Kessin 2001). *Aerobacter aerogenes* (*Klebsiella aerogenes*) and *Escherichia coli* B/R20 are usually used to grow *Dictyostelium* (Fey et al. 2007). The lab strains can grow axenically in protease peptone

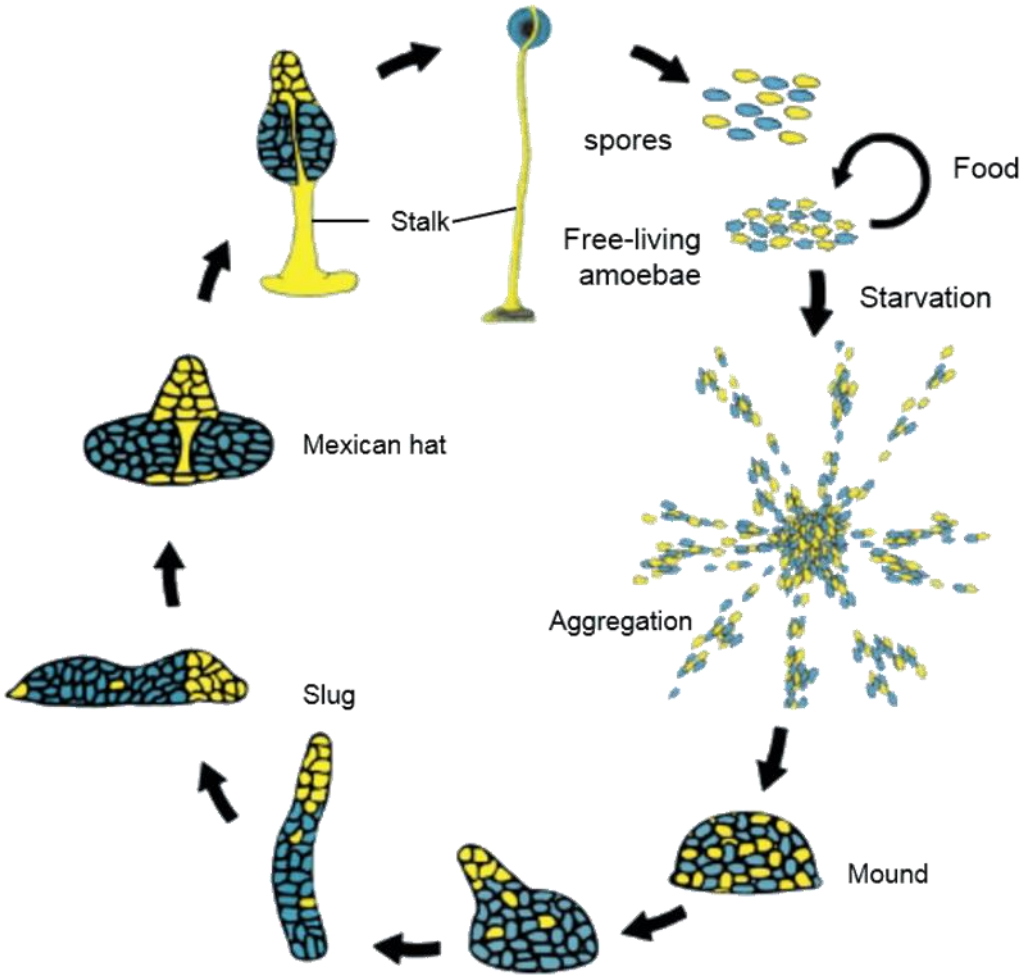


Figure 11 The life cycle of *Dictyostelium discoideum*.

From (Otto and Kessin 2001). Stalk cells (yellow) and spore cells (blue).

liquid medium like HL5 or FM medium containing mainly amino acids, or on plates (Fey et al. 2007). Their ability to grow on liquid media is mainly due to mutation in some genes resulted in enhanced liquid intake (Bloomfield et al. 2015).

D. discoideum is a model organism to study social evolution because of its unique life cycle from unicellular to multicellular stage (Fig. 11) (Bozzaro 2019; Otto and Kessin 2001). The amoeba spores germinate and grow under nutrient-rich conditions. The active amoebas develop into multicellular form under starvation. The chemoattractant cyclic AMP attracts single amoebas to an aggregation center forming a mound. The cells differentiate into the pre-stalk cells and pre-spore cells, and then the pre-stalk cells aggregate to form a tip on the top of the mound. Then the amoeba slug moves to lights and lower humidity regions. The accumulation of pre-stalk cells leads to form a Mexican hat form. Then amoebas form the stalk to rise the fruit body, which contains pre-spore cells. The spores mature in the fruit body and be released into the environment, while the stalk cells died in the end. The *Dictyostelium* spores are resistant cells similar to *Acanthamoeba* cysts, they are resistant to heating, freezing, or drying (Cotter and Raper 1968).

Dictyostelium was used to study cytokinesis, cell motility, phagocytosis, chemotaxis, signal transduction, and cell differentiation during development. It was also used for bacteria-amoeba interaction studies (Cosson and Lima 2014; Paquet and Charette 2016).

1.4.1.4 Pathogenicity of amoebas

Different species of free-living amoebas are associated with severe diseases - the Amoebic meningoencephalitis including primary amoebic encephalitis (PAM) and granulomatous amoebic encephalitis (GAE), and the *Acanthamoeba* keratitis (Gompf and Garcia 2019; Lorenzo-Morales, Khan, and Walochnik 2015). Amoebic meningoencephalitis is very rare but causes death with a high mortality rate, the rate is over 95% for *Naegleria fowleri* PAM , 44-85% for *Acanthamoeba* GAE and over 95% for *B. mandrillaris* GAE (Gompf and Garcia 2019). The anti-amoeba antibody present in a high percentage of the population, which suggests that usually the amoebas infection is a self-limited disease (Brindley, Matin, and Khan 2009; Gompf and Garcia 2019). The immunosuppressed patients are more likely to develop a severe disease after infection. The *Acanthamoeba* and occasionally *Dictyostelium polycephalum* amoebas are the causal agents of amoebal keratitis which may result in loss of vision (Lorenzo-Morales et al. 2015; Srirampur et al. 2018). All the amoeba infections are difficult to diagnosis because amoebas have a similar shape like the macrophages, and it is very hard to treat the

infection because of the highly resistant cysts (Gompf and Garcia 2019; Lorenzo-Morales et al. 2015).

1.4.2 The interaction of amoebas with bacteria

Because of their ubiquity, bacteria and amoebas are very likely to meet in the environment. Shi and colleagues described the interactions between amoebas and bacteria as three types, predation, parasitic interactions, and mutualistic interactions (Shi et al. 2021). The differences between parasitic interactions and mutualistic interactions are hard to define as they all can have a symbiosis interaction, and it is hard to determine if both organisms benefit or not.

1.4.2.1 Amoeba predation of bacteria

Predation is the dominant relationship between amoebas and bacteria. Amoebas consume bacteria through phagocytosis. Amoeba is one of the key factors that eliminates bacteria in the environment and restructures the bacteria community (Rosenberg et al. 2009). In rhizosphere bacteria predation experiments, amoebas preferentially digest *Betaproteobacteria* and *Firmicutes* bacteria; the number of amoeba disliked species increased in the same time, such as *Actinobacteria*, *Nitrospira*, *Verrucomicrobia* and *Planctomycetes*, and the abundance of some of the bacteria did not change even though the community composition shifted (Rosenberg et al. 2009).

Acanthamoeba is a generalist predator that eats a variety of bacteria. They use different bacteria for growth delaying their encystment, and increasing their number, such as *E. coli*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *B. subtilis*, *Bacillus megaterium*, *Micrococcus luteus*, and *Staphylococcus aureus* (De Moraes and Alfieri 2008). *Dictyostelium* uses the *A. aerogenes* (*K. aerogenes*) and *E. coli* B/R20 as feed in the standard culture protocol. In a survey of *Dictyostelium*, one-third of the wild amoeba fruiting bodies contain bacteria, most of the bacteria are Proteobacteria and some Actinobacteria, Bacteriodetes, or Firmicutes (Brock et al. 2018). In addition, over 75% of those fruiting bodies associated bacteria support the growth of amoebas.

The preferential digestion of bacteria by amoebas is determined by many factors. Amoebas have special receptors that recognize different bacteria or organisms (Allen and Dawidowicz 1990; Declerck et al. 2007; Pan et al. 2018). *A. castellanii* mannose-binding receptor recognizes *L. pneumophila*, and *Dictyostelium* folic acid receptor 1 binds to lipopolysaccharide of *K. aerogenes*. *Dictyostelium* mutation in *phg1a* gene impairs its growth

on *K. pneumoniae* but not on *B. subtilis* (Benghezal et al. 2006). *Dictyostelium* cell-surface protein gp130 and putative lysozyme AlyL are involved in the discrimination of gram-positive and gram-negative bacteria, respectively (Nasser et al. 2013).

The efficiency of amoeba phagocytosis is affected by the sizes of the bacteria. Amoeba phagocytosis latex beads with a diameter of 1.3-2.8 μm individually, while phagocytosis the smaller ones in batch (Korn and Weisman 1967). The filamentous bacteria such as Bc group species can form very long chains in certain conditions, which is much longer than normal amoebas and resist the phagocytosis of amoebas (Beeton et al. 2013). The environment such as temperature can affect amoeba phagocytosis. Chilling of amoebas decreases the phagocytosis in stationary-phase cultures, but the amoebas will adapt to the lower temperature by increasing membrane fatty acid unsaturation (Avery, Lloyd, and Harwood 1995).

1.4.2.2 Amoebas-resistant bacteria use amoebas as a reservoir

Amoebas eat many bacteria, while some bacteria can resist amoeba predation or even infect amoebas. These bacteria are called amoeba-resisting bacteria (ARB). ARB can escape from phagocytosis or survive, replicate, and escape after internalization (Shi et al. 2021).

The pathogen *Legionella* was isolated from *Acanthamoeba* in 1983 (Rowbotham 1983). This explains the presence of this bacteria in a hospital water system (Helms et al. 1983). The *Legionella* was further isolated from amoebas of drinking-water/wastewater systems and natural pools, which further expanded its persistence (Magnet et al. 2015). This discovery drew scientists' attention to what role the amoebas played in the survival of pathogens in the environment. Since then, various other pathogens, such as *Mycobacterium*, *Salmonella*, *Vibrio*, *Listeria* were found to use amoebas as a survival reservoir (Gaze et al. 2003; Schuppler 2014; Van der Henst et al. 2016; Wheat et al. 2014). A systematical review of *Acanthamoeba* intracellular microorganism showed that the bacteria species differ depending on the environment, *E. coli*, *Mycobacterium* spp. and *P. aeruginosa* are observed in amoebas isolated from clinical samples, and *Legionella* is more likely to be isolated from environmental *Acanthamoeba* (Rayamajhee et al. 2021). *Legionella*, *Pseudomonas* and *Mycobacterium* can also be isolated from clinical samples (Iovieno et al. 2010). Recent analysis of *Acanthamoeba* intracellular microbiomes indicates that the orders of *Clostridiales* and *Bacteroidales* are the major population (Y.-J. Wang et al. 2021). The *Ruminococcus gnavus*, *Eubacterium dolichum*, *Roseburia faecis*, and *Blautia producta* were identified from keratitis amoeba isolates, in which *Blautia producta* is related to early-stage keratitis (Y.-J. Wang et al. 2021). The different

bacterial phyla found in the wastewater amoeba samples included Proteobacteria (40.25%), Planctomycetes (17.71%), Bacteroidetes (15.02%) and Firmicutes (10.79%), which represented 83.77% of the total bacterial FLA microbiome (Moreno-Mesonero, Ferrús, and Moreno 2020). The pathogen species including *Aeromonas*, *Arcobacter*, *Campylobacter*, *Helicobacter*, *Klebsiella*, *Legionella*, *Mycobacterium*, *Pseudomonas* and *Salmonella* were detected in the wastewater FLA microbiome (Moreno-Mesonero et al. 2020).

The ARB can also survive within the social amoeba *Dictyostelium* species. A screening survey found 45 bacterial isolates are resistant to amoeba, and seven of them can be packaged by the amoebas (Paquet and Charette 2016). The microbiomes isolated from the fruiting body of soil-dwelling *Dictyostelium* included Proteobacteria, Bacteroidetes, Actinobacteria, Chlamydiae, Firmicutes, and Acidobacteria (Sallinger, Robeson, and Haselkorn 2021).

The resistance of bacteria to predation could be the first step of pathogenicity and virulence to amoebas and mammals (Adiba et al. 2010). Experiments have shown that various virulence genes of *L. pneumophila* were kept (selected) after interaction with amoebas, and those genes promote the growth of the bacteria in macrophages (McDougald and Longford 2020). A similar result was observed with *E. coli* (Adiba et al. 2010). The interaction with amoebas promotes the development and evolution of mechanisms that allow the amoeba-resistant bacteria to survive in eukaryotic host cells (Molmeret et al. 2005). In other words, amoebas are training grounds for intracellular bacterial pathogens.

1.4.2.3 Bacterial strategies against amoebas

Bacteria survival in vacuoles

The survival of *Legionella* within amoebas as well as human phagocytes is well studied. The bacteria form *Legionella*-containing vacuoles (LCV) that are resistant to lysis and allow bacteria to multiply in the vacuole (Steiner, Weber, and Hilbi 2018). The LCV formation is controlled by the Icm/Dot type IV secretion system (T4SS), which translocates approximately 300 different "effector proteins" into host cells (Steiner et al. 2018). Those proteins regulate signal transduction, cytoskeleton dynamics and membrane trafficking during the LCV formation (Swart et al. 2018). After micropinocytosis of *Legionella* by macrophage or amoebas, the food vacuole acquires phosphatidylinositol PtdIns(3)P in the surface, which helps the vacuole to resist fusion with lysosomes. The surface molecules of the LCV shift to PtdIns(4)P;

the LCV recruits the endoplasmic reticulum as a membrane and expands; then the bacteria multiply in the vacuole.

The *Salmonella* spp take another way to escape from amoebas. A very small population (0.1-1%) of *Salmonella typhimurium* is able to enter the contractile vacuoles of *Acanthamoeba polyphaga*, and multiply in the vacuoles (Gaze et al. 2003). It was also reported that *Salmonella* can multiply in the normal food vacuoles and kill the *Acanthamoeba* possibly using the virulence factors of the bacteria (Bleasdale et al. 2009; Tezcan-Merdol et al. 2004). The studies of *Salmonella* survival in *D. discoideum* suggest these bacteria exploit a set of conserved molecular mechanisms to survive within protozoa and animal host cells (Riquelme et al. 2016; Valenzuela et al. 2021). The bacteria inject effector proteins into the host cell via the Type III secretion system or the twin-arginine translocation system to form the *Salmonella*-containing vacuole (Urrutia et al. 2018; Valenzuela et al. 2021). Effectors SopB and SifA help shaping the proteome of the vacuole (Valenzuela et al. 2021).

The pathogen bacterium *Vibrio cholera* is also reported to survive and replicate within the contractile vacuole of *A. castellanii* until encystment, then the bacteria enter into the cytosol of cysts and lyse the cyst to be released into the environment (Van der Henst et al. 2016). *V. cholera* uses extracellular lecithinases to lyse the amoeba cyst and uses the flagellum-based motility to escape the lysed cyst (Van der Henst et al. 2018).

Bacteria survival in the cyst

Except for vacuoles, some bacteria are retained and survive within the amoeba cysts. The *Mycobacterium leprae* can be retained in different amoebal cysts for up to 8 months, and the virulence of the bacteria remained high (Wheat et al. 2014). The *Streptococcus* spp can survive in the *Acanthamoeba* cysts, and can be recovered from amoeba cysts at a very high rate (Siddiqui et al. 2017). The cyst is a very resistant form of amoebas that allows them to survive adverse environmental conditions. The retaining of bacteria inside cysts can protect those bacteria from harsh environments. It was shown that foodborne pathogens *Salmonella enterica, E. coli, Yersinia enterocolitica*, and *Listeria monocytogenes* survived in cysts of *A. castellanii* when exposed to antibiotic treatment or highly acidic conditions (Lambrecht et al. 2015).

Bacteria grow at a cost of partial digestion

The *Listeria* cannot survive or multiply inside amoeba, they are usually digested by amoebas (Doyscher et al. 2013). The amoebas seem to use a backpack strategy that carry the aggregates

of *Listeria* in the posterior pole as a food storage. However, the bacteria can benefit from the interaction with amoebas (Schuppler 2014). The *Listeria* population increased significantly in the presence of amoeba (Fieseler et al. 2014). It seems the bacteria grow using metabolites released by amoebas (Schuppler 2014). The interactions between *Listeria* and amoebas indicates, even though amoebas can use the bacteria, the bacteria can benefit from their interaction.

The bacteria farmer – Dictyostelium

Many wild social amoebas *Dictyostelium* have a primitive farming symbiosis (Brock et al. 2011). The amoebas stop consuming bacteria early and incorporate some of the bacteria into their fruiting bodies. The amoeba spores carry the bacteria, disperse to a new environment, and seed the bacteria for eating after. This brings advantages to the amoebas to survive in new sites and gives a chance for the bacteria to survive and multiply.

Besides the above survival mechanisms, the amoebas and bacteria may secret small products that target each other (Iqbal, Siddiqui, and Khan 2014). Conditioned medium from *A. castellanii* is highly toxic to methicillin-resistant *S. aureus* exhibiting an almost 100% kill rate. In addition, the conditioned medium of *E. coli* K1 and *Enterobacter* sp. exhibits toxicity to *A. castellanii* depending on concentration.

The giant viruses take a parasitic life in amoebas. Mimiviruses, marseilleviruses, pandoraviruses, pithoviruses, faustoviruses and molliviruses were isolated from amoebas (Colson et al. 2017). Those viruses enter the amoeba through phagocytosis and then release the genome into the amoeba cytoplasm to multiply. The viruses lyse amoebas to release mature viruses after about 12 h of infection. Another virus virophages use the mimivirus factories for their multiplication (Colson et al. 2017).

1.4.2.4 Interactions between amoebas and bacilli

The Bc group consists of ubiquitous bacteria spread in soil, water, occupying a similar environment as the amoebas. The isolation of Bc group bacteria from amoebas was reported among long lists of other bacteria. The microbiome studies usually classified the bacteria at the phyla level. The most abundant genera of the bacterial microbiome of wastewater FLA were *Bacillus* (8.91%) (Moreno-Mesonero et al. 2020), even though we do not know if Bc group bacteria were identified. There are 2 *B. toyonensis* strains out of a total of 174 strains of bacteria isolated from soil *D. discoideum* fruiting bodies (Brock et al. 2018). A recent report identified

8 *Bacillus*, 15 *Paenibacillus* in the *D. discoideum* fruiting bodies (Sallinger et al. 2021). These results suggest it is possible for Bc group bacteria to interact with amoebas.

The interaction of Bc group bacteria with amoebas is not consistent in different studies. *Bacillus* resists phagocytosis in one study but been digested and support the growth of amoebas in another study. All of those studies did not give direct evidence of bacteria digestion, nor described the effect of amoebas on the development cycle of bacteria. We still do not know what effect the virulence factors of Bt and Bc have on amoebas, and whether the lysis of amoebas is limited to the Ba strains (Dey, Hoffman, and Glomski 2012).

Different Bt/Bc strains and the non-capsule Ba Sterne ASC1 have shown similar behavior when cocultured with *Acanthamoeba* (Beeton et al. 2013). The spores can sense the products excreted by amoebas and germinate. The vegetative cells will form micro-colonies on the surface of amoebas when the concentration of amoebas is low, and they will form long chains when the concentration of amoebas is high. The bacteria have a similar behavior when cultured with *Dictyostelium*. This kind of morphology changes in bacilli was thought to be a strategy to resist phagocytosis by amoebas. The virulence regulator PlcR seems to have no effect on the *Bacillus*-amoeba interactions.

Santos and colleagues found that the spores and vegetative cells of *B. cereus* ATCC 14579 can support the growth of *A. castellanii*, *T. pyriformis* and *Cercomonas* sp. (Santos et al. 2017). However, we do not know if the growth of protists is due to the digestion of endospores, or the digestion of germinated spores (vegetative cells). The bacteria can be detected inside the protists after two antibiotic treatments suggesting that amoebas protected bacilli from environmental stresses. The authors detect a high rate of spore internalization and a low rate of vegetative cell internalization by *A. castellanii*, which may suggest that spores are resistant to digestion, while vegetative cells are not (Santos et al. 2017).

The interaction of Ba with amoebas is quite similar to its interaction with macrophages (Dey et al. 2012). Ba spores can germinate and multiply inside the *A. castellanii*. Then, it lyses the amoeba in about 6 h of culture. The chromosomal gene *gerH* and other uncharacterized gene(s) on the pXO1 plasmid are essential for their multiplication in amoebas.

1.4.3 The macrophages and their interactions with bacilli

Macrophages are phagocytes of different animals that eliminate all the invader bacteria or viruses. They share similar core mechanisms of bacteria ingestion and killing as the amoebas

(Bozzaro and Eichinger 2011; Cosson and Lima 2014). Ba is a pathogen of humans and, as indicated above, they infect their hosts using macrophages as a Trojan horse (Guidi-Rontani 2002). Some other Bc species may also be involved in human infections and cause death (Hernandez et al. 1998; Veysseyre et al. 2015). The bacilli are potentially able to encounter macrophages during their infection and the resistance to macrophages is important for their virulence.

1.4.3.1 Ba infection of macrophage

The invading of alveolar macrophages is the first step of Ba infection. Time-lapse microscope observation showed that about 10% of Ba spores germinate inside the murine RAW264.7 macrophage, the germinated bacilli elongate by 4-5 h, replicate by 5-6 h, and bacteria destroy the macrophage by 7 h (Ruthel et al. 2004). Spores also germinate extracellularly. AtxA, the regulator of virulence factors, is essential for Ba to escape from macrophages (Dixon et al. 2000).

The transcriptome of Ba during the infection of macrophages showed that Ba adapts to the intracellular environment very quickly (Bergman et al. 2007). At the beginning of infection (1-2 h post spore infection), spores germinate and start to grow. The energy metabolism pathways increase steadily, while the sporulation- and germination-related pathways are downregulated; this is similar to the changes between early outgrowth and exponential growth in vitro (Bergman et al. 2007). When compared to growth in vitro, Ba grown in macrophage during the early infection stage have elevated expression of purine ribonucleotide biosynthesis and siderophore biosynthesis pathways (Bergman et al. 2007). Siderophores are responsible for acquiring iron from the environment. The increasing siderophore synthesis permits Ba cells to better utilize the nutrients in the host. The siderophore is also important for the growth of Bt/Bc in insect larvae as well as the virulence of Bt/Bc (Harvie et al. 2005; Segond et al. 2014). At a later stage of infection of macrophages, Ba made a more profound change in gene expression in order to fully adapt to the intracellular environment (Bergman et al. 2007). The expression of many virulence genes increased, including *lef*, *pagA* and *cya* and their regulator genes *atxA* and *pagR*, as well as genes encoding hemolysin, phospholipases, adhesion lipoproteins and catalase gene *katB* (Bergman et al. 2007).

1.4.3.2 Bt/Bc infection of macrophages and their cytotoxicity

The phagocytosis of three Bc strains by BALB/c mouse peritoneal macrophages and RAW264.7 macrophage was assessed (Rolny, Racedo, and Pérez 2017). Around 24 - 50% of macrophages contain or are associated with *Bacillus* after coculture. The efficiency of phagocytosis is dependent on the strains - the food poisoning Bc B10502 strain is phagocytosed at a higher rate than the milk contaminating Bc 2 and M2 strains. The bacteria survived intracellularly after gentamicin treatment. Over 50% of phagosomes containing Bc are acidic compartments indicating they recruited the lysosomes to the food vacuoles and the bacteria would be digested. In contrast, some Bt spores can escape the macrophage with the help of immune inhibitor InA1 (Ramarao and Lereclus 2005). However, the macrophage cell line J774 used in the later study does not reflect the response of primary macrophages (Andreu et al. 2017).

Various experiments have demonstrated the cytolytic activity of Bt/Bc virulence factors to macrophages. The virulence factors produced by Bt 407 are strongly cytolytic to *G. mellonella* plasmatocytes (Pl) and granular haemocytes 1 (Salamitou et al. 2000). Hemolysin HlyII of Bt 407 is sufficient to induce apoptosis of the macrophages J774 via the caspase3/8 pathway (Tran, Guillemet, et al. 2011). In addition, the other virulence factors controlled by PlcR are also involved in macrophage cytotoxicity. However, the deletion of PlcR-regulated toxins CytK and Hbl did not change the cytotoxicity. Different mammalian cell types (human fibroblasts (HT1080) and neutrophils (hPMNs), hamster ovary cells (CHO), and mouse macrophages (RAW264.7)) showed similar susceptibility to supernatant virulence factors of Bc strain ATCC 10876 (Sastalla et al. 2013) and the cytotoxicity was abolished in the PlcR deletion strain. The hemolysin BL was the major toxin that caused cell lysis in that research.

2. Objective – the interaction of *Bacillus* with amoebas

The previous studies have shown that *Bacillus* may be associated with amoebas in the environment. Beenton and collegues have shown that *Bacillus* spores can germinate in the amoeba environment, and the vegetative cells can form microcolonies or filaments to resist amoeba phagocytosis (Beeton et al. 2013). However, the *Bacillus* vegetative cells and spores were ingested by amoebas in another study (Santos et al. 2017). Contradicting to intact of cells in amoeba food vacuoles, the spores and vegetative cells promoted the growth of amoebas. They also cannot exclude that the growth of amoebas on spores is due to the digestion of germinated vegetative cells. No direct evidence showed the digestion of *Bacillus* vegetative cells or spores by amoebas in both studies. The interaction of human pathogen Ba with amoebas showed that Ba spores can germinate intracellularly and lyse amoebas in about 6 h (Dey et al. 2012), which is distinctly different from the other species of the Bc group.

Whether the *Bacillus cereus* spp can resist phagocytosis by amoebas or amoebas digest them? Can they support the growth of amoebas? Can the other bacilli lyse the amoebas like Ba and what effect the *Bacillus* virulence factors have to amoebas? Can the amoebas help the persistence of bacilli in the environment? To answer these questions, we studied the interaction between *Bacillus* and amoebas.

This study described the fate of representative strains of Bc group in the amoeba environment, the toxicity of Bt and Bc virulence factors to amoebas, and determined the growth of amoebas on vegetative cells or spores. The effect of amoebas on the developmental stages of the Bt is also studied.

The spores or vegetative cells of representative *Bacillus* strains were co-cultured with *Acanthamoeba castellanii*; then the germination and chain-formation were observed under microscope. The digestion of vegetative Bt 407 spores or vegetative cells in amoeba vacuoles was examined using time-lapse microscope observation. The effect of virulence factors on amoeba viability was examined using trypan blue exclusion after culturing amoebas in a medium containing virulence factors. The developmental stages of Bt 407 cells during co-culture were followed using stage-specific fluorescence reporters.

3. Article - The fate of bacteria of the *Bacillus cereus* group in the amoeba environment

Microbial Ecology
https://doi.org/10.1007/s00248-021-01828-2

HOST MICROBE INTERACTIONS

The Fate of Bacteria of the *Bacillus cereus* Group in the Amoeba Environment

Haibo Chen[1] · Emilie Verplaetse[1] · Tania Jauslin[2] · Pierre Cosson[2] · Leyla Slamti[1] · Didier Lereclus[1]

Received: 8 February 2021 / Accepted: 21 July 2021

Abstract

The *Bacillus cereus* sensu lato group consists of several closely related species, including *B. anthracis*, *B. cereus* sensu stricto, and *B. thuringiensis*. Spores of these pathogenic bacteria are commonly found in the soil but evidence suggests that they are unable to grow in such a natural environment in the absence of nutrient input. Amoebas have been reported to be an amplifier for several species of pathogenic bacteria and their potential involvement to explain the large amount of *B. thuringiensis* and *B. cereus* spores in soil has been frequently proposed. Here, we studied the fate of *Bacillus* and amoebas when cultured together. We show that the virulence factors produced by *B. thuringiensis* and *B. cereus* do not affect the amoeba *Acanthamoeba castellanii*, which, on the contrary, can phagocytose and effectively digest vegetative *Bacillus* cells to grow and prevent the formation of cysts. Bacterial spores can germinate in the amoeba environment and the vegetative cells can then form chains or aggregates that appear to be less efficiently phagocyted by the amoeba. The use of transcriptional fusions between fluorescent reporter genes and stationary phase- and sporulation-specific promoters showed that the sporulation process occurs more efficiently in the presence of amoebas than in their absence. Moreover, our results showed the amoeba environment to promote spore germination and allow the bacteria to complete their developmental cycle. Overall, this study suggests that the amoeba-*Bacillus* interaction creates a virtuous circle in which each protagonist helps the other to develop.

Keywords *Bacillus thuringiensis* · *Acanthamoeba* · Soil · Sporulation · Phagocytosis

Introduction

The *Bacillus cereus* sensu lato group (Bc sl) is comprised of a growing number of species, including *Bacillus cereus* sensu stricto (Bc ss), *Bacillus thuringiensis* (Bt), and *Bacillus anthracis* (Ba) [1]. Spores of the Bc sl group are frequently found in the soil, which is generally regarded as a reservoir rather than a favorable environment for the multiplication of these Bacilli [2–4]. The development of these bacteria in the soil has long been a subject of debate and there is currently no available evidence to definitively settle the issue. Studies have addressed the ecology of Bc ss in soil and showed it to be a saprophytic bacterium, with a life cycle in soil-extracted soluble organic matter and artificial soil microcosms [5]. Other studies that addressed the survival of Bt in the soil showed that the bacteria are maintained as spores that do not germinate or multiply [6, 7]. In accordance with these latter observations, genomic analysis revealed that the Bc strain ATCC14579 cannot efficiently utilize complex plant carbohydrates like *B. subtilis*, suggesting that the Bc ss species is better adapted to a protein diet [8]. Given the close relationship between Bt and Bc ss [9], this finding is also relevant to Bt and presumably all species of the Bc sl group. Moreover, the presence of numerous genes encoding proteolytic enzymes in bacteria of the Bc sl group is consistent with their pathogenic properties [10]. Bc ss and Ba are pathogenic for mammals [11, 12], and Bt is an entomopathogen bacterium that produces insecticidal crystal toxins (Cry and Cyt proteins) and various virulence factors that allow the bacteria to damage host tissues and resist immune defenses, ultimately causing septicemia [13].

All species of the Bc sl group share a similar genomic background and a set of core virulence factors [10, 14]. Most of these exported virulence factors are positively

✉ Didier Lereclus
Didier.Lereclus@inrae.fr

[1] Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, 78350 Jouy-en-Josas, France

[2] Cell Physiology and Metabolism Dpt, Faculty of Medicine, University of Geneva, 1 rue Michel Servet, CH-1211 Geneva 4, Switzerland

Published online: 03 August 2021

controlled by the regulator PlcR at the end of exponential growth and early stationary phase, except for Ba, in which a nonsense mutation abolishes the activity of PlcR [15, 16]. It has been shown that the PlcR regulon plays a major role in the virulence of Bt and Bc ss in various animal models [17–20]. Bt and Bc ss produce other virulence factors not regulated by PlcR, such as the metalloprotease InhA1 and the hemolysin HlyII, which are produced at the onset of the stationary phase [21–23]. In the context of macrophage infection, InhA1 gives Bt spores the ability to escape from macrophages [24] and HlyII provokes macrophage cell death by apoptosis through its pore-forming activity [25]. However, the macrophage cell line J774 used in these studies does not reflect the response of primary macrophages [26].

Amoeba is another phagocytic organism. Free-living protozoa, such as *Acanthamoeba*, live in soil and adopt two developmental forms depending on the encountered environmental growth conditions: trophozoites, a metabolically active feeding form, and cysts, a round, dormant, stress-resistant form. Major feeding mechanisms of free-living amoebas are pinocytosis and phagocytosis of soil microorganisms such as bacteria, fungi, or algae present in their environment [27]. Some bacteria are able to resist to amoebas, since they are able to escape predation, others are able to resist intracellular digestion and survive therefore intracellularly, or are able to survive, grow, and escape from amoebas after phagocytosis. About 20% of human pathogenic bacteria, among them are *Legionella*, *Mycobacterium*, or *Listeria*, are able to live and multiply inside free-living amoebas [28, 29]. Amoebas might represent a reservoir for bacteria, allowing the propagation of bacteria in the environment or provide a shelter to protect them from various physical and chemical stresses such as biocides [28].

Acanthamoeba has been reported to be an amplifier and reservoir for bacteria such as *Mycobacterium* and *Legionella* [30–32]. *Bacillus* and amoebas share the same ecological niches, such as soil and insects, and *Bacillus* has been identified as the most abundant genus in the bacterial microbiome of free-living amoebas [33]. The interaction between the two organisms has been investigated by several research groups. It has been demonstrated that after phagocytosis by *A. castellanii*, the spores of Ba are able to germinate inside amoebic phagosomes [34]. Vegetative cells subsequently grew as chains before lysing the host cell. Similar to mammalian macrophage infection, the pXO1 plasmid was required for the growth of Ba within amoebas. The main pXO1-encoded toxin genes (*pagA*, *lef*, and *cya*) appeared to be dispensable for the germination of Ba spores and the subsequent outgrowth process, suggesting that other pXO1 genes play a role in this process [34]. The relationship between the Bt *israelensis* 4Q7 strain and protozoa has also been studied. In the presence of *Acanthamoeba polyphaga* and *Dictyostelium discoideum*, Bt *israelensis* grew as long chains or formed micro-colonies, depending on the densities of the amoebas [35]. This observation suggested that these bacterial structures were resistant to the phagocytosis of the amoebas. Factors released by the amoebas were shown to promote Bt germination, allow growth, and induce filamentous development. Interactions between the Bc strain ATCC14579 and several protozoans were also studied [36]. It was demonstrated that *A. castellanii*, *Tetrahymena pyriformis*, and *Cercomonas* spp. can ingest and use Bc ss spores or vegetative cells for growth. They also observed survival and proliferation of ingested bacteria inside protist cells, suggesting that protists can act as an amplifier of Bc ss in soil.

Based on published data, it is still unclear whether amoebas allow all bacteria species of the Bc sl group to multiply, leaving open the question of the high abundance of Bc sl spores in soil. Ba uses amoebas to grow, leading to a decrease in the amoeba population [34], whereas Bc ss and Bt appear to be a prey for the amoebas, as no increase in the Bc ss or Bt population has been observed upon co-culture of the two organisms [35, 36]. In contrast, the amoeba population is able to grow on Bc ss and Bt. However, occasional germination of Bc ss and Bt and intracellular growth were reported in the last two cited studies. Overall, these observations raise the question of whether Ba represents an exception within the Bc sl group or whether other Bc sl bacteria are also able to use amoebas to amplify. They also raise the question of whether amoebas allow Bc sl bacteria spores to germinate and vegetative cells to grow and sporulate.

Here, we compared the fate of seven strains of the Bc sl group when they were co-cultured with *A. castellanii*. Reciprocal effects on the population of each organism were evaluated. The toxicity of the virulence factors secreted by Bt and Bc ss to amoebas was assessed. In addition, the effect of *A. castellanii* on the developmental process of Bt was analyzed. In order not to restrict our study to the Bc sl group–*A. castellanii* interaction, we also tested the impact of Bc sl bacteria on the growth of another soil amoeba known to feed on bacteria, *D. discoideum*.

Materials and Methods

Bacteria and Amoeba Strains, and General Culture Conditions

The following *Bacillus* species and subspecies were used in this study: *B. thuringiensis* 407, *B. thuringiensis kurstaki* HD73, *B. thuringiensis israelensis* IPS82, *B. thuringiensis konkukian* 97–27, *B. cereus* 569 (ATCC10876), *B. cereus* ATCC14579, *B. cereus* AH187, and *B. weihenstephanensis* KBAB4. These strains are described in Table 1. *Bacillus* strains were routinely grown in LB broth at 30 °C. Exponentially or stationary-growing

Table 1 *Bacillus cereus* strains used in this study

Strain	Features	Reference
Bt 407 Cry^+	A representative of the *Bacillus thuringiensis* subspecies, belongs to serotype 1, laboratory model strain. Produces Cry toxins active against lepidopteran insects	[39]
Bt 407 Cry^-	Acrystalliferous Bt 407 strain cured of its *cry* plasmid	[39]
Bt HD73	A Bt *kurstaki* strain, belongs to serotype 3abc, laboratory model strain	[40]
Bt *israelensis* (Bti) IPS82	A Bt *israelensis* strain, belongs to serotype H-14. Produces Cry toxins active against Diptera insects (especially mosquitoes)	[41]
Bt *konkukian* (Bt kon) 97–27	A Bt *konkukian* serotype strain, distantly related to all the pesticidal strains but close to Ba. This strain was originally isolated from a wounded soldier	[42]
Bc 569 (ATCC10876)	A representative of the *B. cereus* sensu stricto strain. Isolated by Northern Regional Research Laboratory, U.S	[43]
Bc ATCC14579	A representative of the *B. cereus* sensu stricto strain. It is a common reference strain	[8]
Bc AH187	A pathogenic Bc strain harboring the pCer270 plasmid, belonging to the pX01 family and encoding the genes involved in the production of the emetic toxin, the cereulide	[44]
B. weihenstephanensis (Bw) KBAB4	A psychrotolerant *B. weihenstephanensis* species strain. This strain was isolated from soil and produces virulence factors at 15 °C	[45, 46]
Bt 407 nprA	Bt 407 Cry^- carrying pHT304-18ΩP$_{nprA}$-*mCherry*, used to reflect the necrotrophism stage	[47]
Bt 407 spoIID	Bt 407 Cry^- carrying pHT304-18ΩP$_{spoIID}$-*yfp*, used to reflect the sporulation stage	[47]
Bt 407 P$_{apha3}$-*mCherry*	Bt 407 wt carrying pAT-P$_{apha3}$-*mCherry*-LGC, a strain constitutively expressing the mCherry	This work
Bt 407 P$_{sarA}$-*gfp*	Bt 407 wt carrying pHT304-18ΩP$_{sarA}$-*gfp*, a strain constitutively expressing the GFP	This work
Bt 407 pHT304	Bt 407 Cry^- carrying the pHT304 plasmid, used as a fluorescence negative control	[47]
Bt 407 Δ*plcR*	A *plcR* mutant Bt 407 Cry^- strain, defective for PlcR-regulated virulence factor production	[19]
Bc ATCC14579 Δ*plcR*	A *plcR* mutant Bc ATCC14579 strain, defective for PlcR-regulated virulence factor production	[19]

bacteria were harvested at an optical density of 600 nm (OD_{600nm}) = 1.0 or 4.0, respectively. *Bacillus* spores were prepared in sporulation-specific HCT medium [37]. *A. castellanii* (ATCC30010) was maintained in T75 tissue culture flasks at room temperature in PYG medium (ATCC medium 712) [38]. Heat inactivated *E. coli* K-12 strain TG1 was used as supplemental food for *Acanthamoeba* during co-culture experiments to delay cyst formation. *E. coli* was grown overnight in LB medium at 37 °C under vigorous shaking. *E. coli* cells were then resuspended in 1/10 of the initial culture volume in 10% glycerol and stored at − 80 °C. CFU counting was performed on an aliquot to determine the concentration of the *E. coli* suspension.

Plasmid Construction

The *gfp* coding sequence and P$_{sarA}$ promoter sequence were amplified from pCM12 [48] using primers SGFPFW and SGFPRV (5′-TTGGCGCGCCGCCAGTGCCAAGCT TCTG-3′ and 5′-GGGTAACCGCTTCCGGCTCGTATG TTGTG-3′, respectively). The amplified DNA fragment was cloned between the *Hin*dIII and *Bam*HI sites of plasmid pHT304-18 [49]. The resulting plasmid (pHT304-18ΩP$_{sarA}$-*gfp*) was used to transform the Bt 407 strain by electroporation [39].

Co-culture Assays

The protocol for *Bacillus*-amoeba co-culture was adapted from [35] and [34]. Briefly, confluent *A. castellanii* grown in PYG medium were harvested by centrifugation at 300 × *g* for 10 min and the pellet washed two times in Page's modified Neff's amoeba saline (PAS) buffer [50] supplemented with 0.5% glucose (PAS-Glu). The concentration of amoebas was adjusted to 1.25×10^5 cells/ml. A suspension of *Bacillus* spores or vegetative cells in PAS-Glu buffer was then added to reach a concentration of 2.5×10^7 CFU/ml and a multiplicity of infection (MOI) of 200. Physical interaction between the bacteria and amoebas was initiated in tubes by centrifugation at 500 × *g* for 5 min. The suspension was subsequently homogenized by gentle pipetting and dispatched into tissue culture flasks or 24-well plates (for microscopic analysis, each well contained a sterile round coverslip at the bottom), depending on the experiment. Amoebas were allowed to attach to the bottom of the substrates by stationary incubation at 30 °C for 1 h. Extracellular spores or vegetative cells were removed by washing with fresh PAS-Glu buffer two times. Then, an equal amount of PAS-Glu buffer containing 10^7 CFU/ml heat-inactivated (80 °C, 12 min) *E. coli* cells per ml (PAS-Glu-*E. coli*) was added. For experiments of longer than 24 h, 10^7 CFU/ml of heat-inactivated *E. coli* were added every 24 h to delay cyst formation [30]. The

co-culture assays were performed at 30 °C. T0 was defined as the time point after the washing step.

For co-culture with *D. discoideum*, *D. discoideum* DH1 cells were grown in the presence of different *Bacillus* strains as described previously [51]. *Bacillus* were grown overnight in LB medium at 30 °C. *Klebsiella aerogenes* (*Ka*) strain *KpGE* was used and grown overnight in LB medium at 37 °C. *Ka* is a commonly used food supply for *D. discoideum* (http://dictybase.org/techniques/media/dicty_growth.html). Fifty microliters of an overnight *Bacillus* culture (or 25 µL *Bacillus* and 25 µL *Ka*) was plated on 2 mL SM-Glucose-agar or HL5-agar (HL5 was diluted in water at 2, 5, 10, 20, 50, or 100% HL5). For growth on SB-agar (2 mM Na_2HPO_4 and 14.7 mM KH_2PO_4), 10 µL of concentrated *Bacillus* (3 mL overnight culture resuspend in 60 µL SB) was plated on SB-agar. After the bacteria dried, 10^4, 5×10^3, 10^3, 10^2, or 10 *D. discoideum* cells were deposited on top of the bacterial lawn. Growth of *D. discoideum* formed holes in the bacterial lawn after 2 to 6 days of incubation at 21 °C.

Enumeration of Amoebas and *Bacillus* in Co-cultures

The amoeba population during co-culture was counted as follows. A T25 tissue flask containing 10 mL of the *Bacillus*-amoeba suspension was first thoroughly scraped. The suspension was then centrifuged and the pellet resuspended in 2 mL PAS-Glu buffer. The amoebas contained in this five-fold concentrated suspension were enumerated using a hemocytometer. Due to the variation of the number of amoebas in different co-culture experiments, a growth index was calculated as (T24-T0)/T0 to show the relative growth of the amoebas. For amoebas grown with *Bacillus* collected in stationary phase, the bottom of the T25 culture flasks was observed directly under a reverse microscope and the number of amoebas present in areas of the same size was counted. The amoeba population was enumerated every 2 h during the first 6 h of contact and at 24 h. The time points were selected based on the co-culture of Ba with amoebas, as previously described [34]. The percentage of amoeba cysts during co-culture was measured as follows: arbitrarily chosen microscopy fields localized at the four angles and center of the T25 tissue flask (for vegetative cell-amoeba co-cultures) or coverslips (for spore-amoeba co-cultures) were imaged; a sufficient number of fields to represent at least 200 amoebas were imaged and the cysts identified on the images were counted. *Bacillus* present in the co-culture samples were quantified by collecting and concentrating the co-cultures of *Bacillus* spores or vegetative cells with amoebas in T25 tissue-culture flasks as described above. Amoebas were lysed by passing the sample five times through a 26-gauge needle to release intracellular bacteria. Serial dilutions of the suspension were performed. The diluted suspensions were plated on LB plates directly for vegetative cell quantification. Spores were quantified by heating the samples at 80 °C for 12 min before plating. The CFU were counted after an overnight incubation at 30 °C.

Lecithinase Activity Assay

Production of lecithinase by *Bacillus* in co-culture with amoebas or in LB was assessed on LB plates containing 5% egg yolk as described [52]. Briefly, the samples were collected by centrifugation, filtered on a 0.22-µm membrane, and then 100 µL loaded into wells dug into the plates. Plates were incubated at 30 °C for 24 h and scanned. Lecithinase activity was detected by the formation of rings around the wells.

Bacillus Growth in Amoeba-Conditioned Medium

Amoeba-conditioned medium was prepared extemporaneously as follows: amoebas were cultured in PAS-Glu-*E. coli* medium in tissue-culture flasks at 30 °C for 24 h at a concentration of 1.25×10^5 cell/ml. The residual amoeba and *E. coli* cells in the conditioned medium were removed by centrifugation at $300\times g$ for 10 min and filtration on a 0.22-µm membrane. Exponentially growing Bt cells at $OD_{600nm}=0.8$ were collected, washed in PAS-Glu, and resuspended in the amoeba-conditioned medium to reach approximately 2.5×10^7 CFU/ml, as estimated by the OD_{600nm}. Samples were dispatched in 24-well plates (1 ml/well) and the growth of Bt was assessed by measuring the OD_{600nm}. Samples were harvested at the indicated time points for microscopy and flow cytometry analysis and fixed as described above, except for the amoeba-lysis step.

Microscopy

Coverslips to which amoebas were attached were collected and inverted onto microscope slides for microscopic observation. The coverslips were fixed to the slides using wax. Cells were observed with a Zeiss AxioObserver.Z1 inverted fluorescence microscope equipped with a Zeiss AxioCam MRm digital camera and Zeiss fluorescence filters. The amoebas and *Bacillus* cells were observed using the ×40 or ×100 objective in phase-contrast mode. The filters 46 HE and 45 HE were used for the fluorescence imaging of YFP and mCherry, respectively. Wildtype cells harboring the pHT304 plasmid (Bt 407 pHT304) without fluorescence genes were analyzed to determine the background of YFP or mCherry and the optimal exposure time. For time-lapse observations, the co-culture procedure of P_{aphA3}-*mCherry* fluorescent Bt 407 cells with amoebas was the same as that described above, except for incubation in tissue-culture flasks for 30 min. The co-culture was scraped from the flask bottom after incubation and transferred into a 35-mm

cell culture dish with a glass bottom (Greiner Bio-One) for microscopy. Amoeba and Bt development were monitored by microscopy as described above, with the addition of a temperature-controlled chamber at 30 °C. The entire time-lapse observation lasted over 4 h. More than 15 amoebas that had phagocytosed Bt cells were observed. The observations started when bacteria were found inside the amoebas (0 min) and ended when the bacteria were no longer detected inside the amoebas, from several minutes to 2 h depending on the amoeba under observation. Images of the amoebas were captured every 3 min or less, depending on the speed of digestion or movement.

Flow Cytometry Analysis

For flow cytometry analysis, 500 µL of the amoeba-*Bacillus* co-culture were thoroughly scraped from the wells, resuspended, and the amoebas lysed by passing the sample through a 26-gauge needle five times. The suspension was centrifuged, the supernatant discarded, and the bacterial pellet fixed for 7 min in 4% formaldehyde-PBS and then washed in PBS. Fixed bacteria were resuspended in 30 µL GTE buffer [53] and maintained at 4 °C until use. Before the analysis, fixed samples were treated to disrupt any aggregates and obtain a homogeneous cell suspension of single bacteria by mild sonication with a Branson Sonifier 250. Samples were diluted in filtered PBS and the fluorescence measured using a CyFlow Space cytometer (Sysmex-Partec, France). FL1 and FL4 channels (with 527-nm and 585-nm band pass filters, respectively) were used to analyze the fluorescence signal of YFP and mCherry [47]. Data were collected using FlowMax software (Sysmex-Partec, France). The results were analyzed and overlays generated using Weasel version 3.4 software (WEHI, USA, and available from https://www.frankbattye.com.au/Weasel/). YFP- or mCherry-expressing cells were identified using histograms in which the cells showed a higher signal intensity than the reporterless cells used as controls.

Statistical Analyses

To determine whether the number of cysts formed when amoebas are grown in the presence of Bt 407 (spores or vegetative cells) are different from the control condition in the absence of Bt 407, a non-parametrical Mann–Whitney test was performed and the exact *P* value was calculated. Results were considered as statistically different when the *P* value < 0.05. To determine if the percentage of fluorescence-expressing cells grown with amoebas or grown in conditioned medium was different from the control condition (without amoebas or in non-conditioned medium, respectively), Mann–Whitney tests were performed. The exact *P* value were calculated and the differences were considered statistically relevant when the *P* value < 0.05. All the statistical analyses were done using Prism version 8.4.3 for macOS (GraphPad Software, La Jolla, CA).

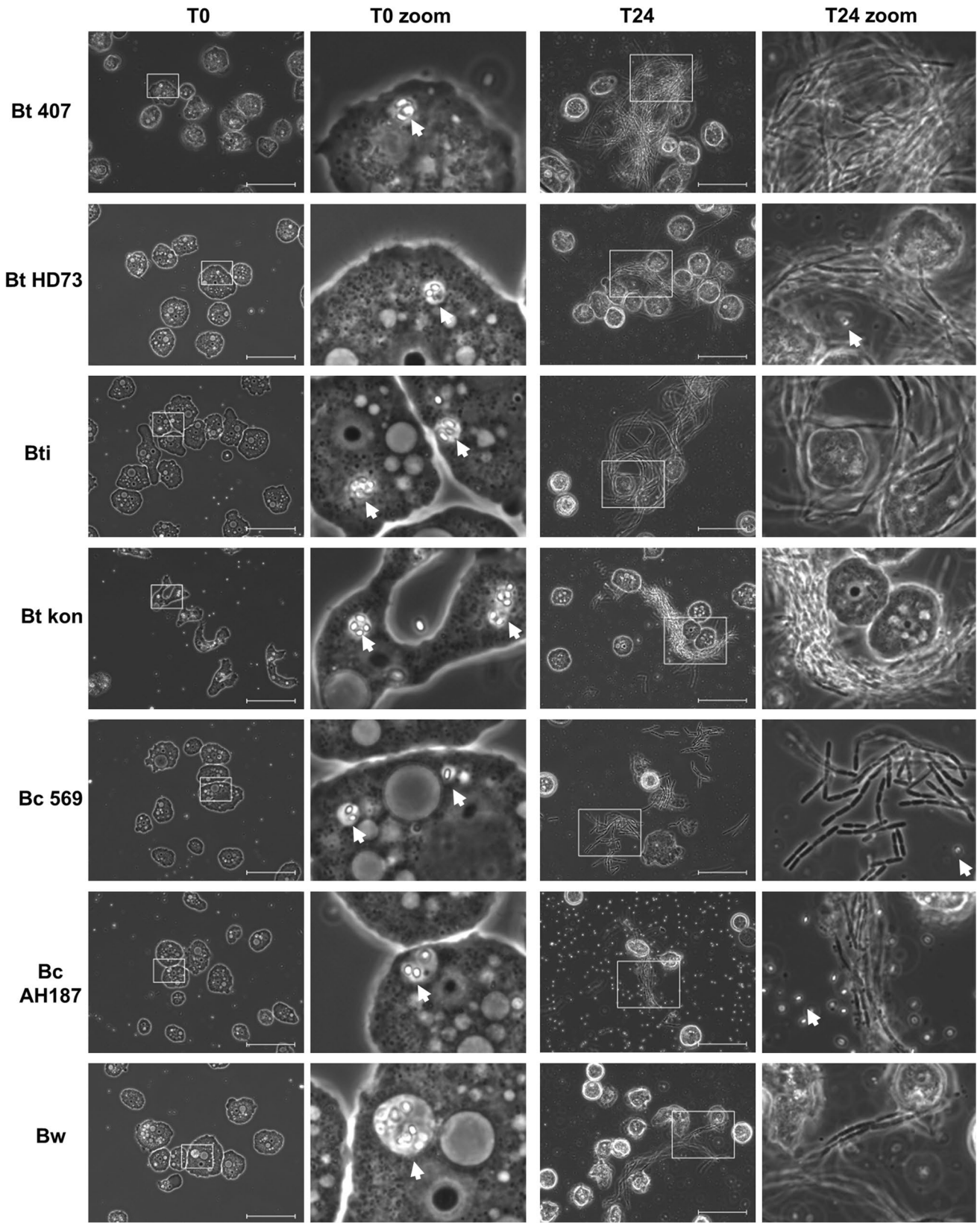
Results and Discussion

B. cereus spp. Spores Display Similar Germination and Aggregation Behavior in the Presence of Amoebas

We examined the development of seven representative strains of the Bc sl group to determine whether different species of the Bc sl group behave similarly when interacting with amoebas. The strains, described in Table 1, were the following: Bt 407, Bt *kurstaki* HD73, and Bt *israelensis* IPS82, selected as representatives of the insecticidal Bt strains; Bt *konkukian*, which is not pathogenic for insects but is close to *B. anthracis*; Bc 569 and the emetic Bc AH187 strains, chosen as representatives of Bc ss; and *B. weihenstephanensis* (Bw) KBAB4, selected as a representative of the psychrotolerant *Bacillus* species. *A. castellanii* and the *Bacillus* spores were statically co-cultured in PAS-Glu-*E. coli* medium and the development of the seven strains observed by microscopy. Micrographs taken at T0 showed spores of all strains present inside vacuole-type structures in the amoebas, indicating that they were efficiently phagocytosed by the amoebas (Fig. 1, left two columns). After 24 h of incubation (T24), we observed a heterogeneous bacterial population of spores and vegetative cells for all strains tested in co-culture with the amoebas (Fig. 1, right two columns). We also observed that vegetative cells formed chains or aggregates outside the amoebas. No spores of any strain germinated by 24 h in PAS-Glu medium in the absence of amoebas (Fig. S1). These results show that the seven Bt, Bw, and Bc ss strains behave similarly in the presence of amoebas. A portion of the spore population germinated and the vegetative cells developed subsequently as chains and aggregates in the amoeba environment for all strains. However, we did not observe germination of the spores or development of the bacteria in the amoebas. The same type of behavior was also previously observed with the Bt *israelensis* strain 4Q7 co-cultured with amoebas [35].

Bt and Bc Virulence Factors Do Not Affect the Viability of Amoebas

Bacteria of the Bc sl group (except *B. anthracis*) produce exported virulence factors, such as degradative enzymes, cytotoxins, and hemolysins during early stationary phase upon activation of the PlcR-PapR quorum sensing system [15, 16]. The cytotoxicity of these PlcR-regulated enzymes and toxins has been demonstrated for several eukaryotic


T0
T0 zoom
T24
T24 zoom
Bt 407
Bt HD73
Bti
Bt kon
Bc 569
Bc AH187
Bw

◂**Fig. 1** Micrographs of *A. castellanii* co-cultured with spores of seven *Bacillus* strains. Spores were mixed with amoebas at a MOI of 200 in PAS-Glu-*E. coli* medium. A centrifugation step was performed to increase the contact between the two organisms. The suspension was transferred into a flask. Amoebas were allowed to attach to the bottom for 1 h and washed to remove most of the unincorporated spores. T0 was defined after the washing step. Images were captured at T0 and T24. For each time point, the left column corresponds to a large field and the right column a focus on the area defined with a rectangle. Spores are indicated by white arrows. Magnification of ×40, scale bar represents 50 µm. The names of the strains, as described in Table 1, are indicated on the left of the panels

cell-types [19, 52, 54]. In addition to these virulence factors, the Bc AH187 strain produces the emetic toxin cereulide at the beginning of the stationary phase [11, 55] and the Bt 407 strain produces hemolysin II, which has been characterized for its ability to lyse J774 macrophages by apoptosis through a pore-forming activity [25].

We assessed the effect of the Bt and Bc ss virulence factors towards amoebas by co-culturing *A. castellanii* with *Bacillus* cells harvested at the beginning of the stationary phase. The Bt 407, Bc 569, and Bc AH187 strains were used for the assays. The avirulent strain Bt 407 Δ*plcR* was included as a control. The growth of the amoeba population in co-culture with the *Bacillus* strains was monitored for 24 h. The number of amoebas was stable during the first 6 h of co-culture and then increased by approximately1.5 fold by 24 h, regardless of the strain used, whereas the population did not grow in the absence of *Bacillus* (Fig. 2a). Microscopic observations of culture samples did not reveal any signs of amoeba lysis (not shown). These results suggest that the virulence factors produced by the Bt 407, Bc 569, and Bc AH187 strains have no detectable effect on amoebas. Alternatively, the Bt and Bc ss cells may have stopped the production of the virulence factors upon their transfer to the co-culture medium used in this study. Therefore, we used a lecithinase and a hemolytic assay to assess the presence of the PlcR-regulated lecithinase and hemolytic enzymes under such conditions [52]. The supernatants of the co-culture samples were collected after 2 h of contact between the amoebas and *Bacillus* to allow accumulation of the enzymes and the activity of the lecithinase (phosphatidylcholine-specific phospholipase C, PC-PLC) then assayed on egg-yolk plates (Fig. 2b). We did not observe the formation of a halo that would reflect activity of the lecithinase around the wells containing the supernatants of the co-culture samples. In contrast, lecithinase activity was detected in the supernatants of the Bt 407, Bc 569, and Bc AH187 early stationary phase cultures in LB medium (Fig. 2c). No lecithinase activity was detected in the fresh LB medium or the supernatant of the Bt 407 Δ*plcR* preculture. Overall, these results show that the *Bacillus* cells did not produce detectable lecithinase activity when co-cultured with amoebas, suggesting that PlcR activity was abolished upon the transfer of the cells from LB medium to the co-culture conditions. We confirmed this result by assaying the PlcR-dependent hemolytic activity of the Bt and Bc strains on 5% sheep blood plates (Fig. S2). The results were in accordance with those of the lecithinase assay; the supernatants of the *Bacillus*-amoeba co-cultures did not show any hemolytic activity. In contrast, we detected a hemolysis ring with the supernatants of Bt 407, Bc 569, and Bc AH187 stationary phase cultures and no hemolysis was detected with the Bt 407 Δ*plcR* sample and fresh LB. These results show the absence of PlcR-dependent activity in bacilli transferred into the amoebal environment but did not allow us to determine whether *A. castellanii* is able to resist the virulence factors produced by Bc and Bt.

We tested the sensitivity of the amoebas to the *Bacillus* virulence factors using the supernatants of the Bt 407, Bt 407 Δ*plcR*, Bc 569, and Bc AH187 LB cultures harvested at the beginning of the stationnary phase (*Bacillus*-conditioned medium). Fresh LB medium was used as a negative control. Viability of the amoebas was evaluated by trypan blue dye exclusion [56]. Most of the amoebas remained viable after 2 and 24 h of culture (Fig. S3). Less than 1% of the amoeba population was stained blue when incubated in the *Bacillus*-conditioned media, even after an extended duration of the incubation. A similar proportion of viable cells was obtained in the control sample when amoebas were incubated in fresh LB medium. This result suggests that the virulence factors produced by Bt 407, Bc 569, and Bc AH187 have no significant effect on amoeba viability.

Vegetative *Bacillus* Cells Support the Growth of Amoeba

It was previously shown that Ba spores can germinate and multiply within amoebas, causing their lysis in approximately 6 h and a twofold decrease in the amoeba population within 24 h of co-culture [34]. In contrast, our microscopic observations of *Bacillus* spore-amoeba co-cultures at T24 (Fig. 1) revealed no signs of amoebic lysis or suffering; most of the amoebas were present as trophozoites and were moving their pseudopodia. We confirmed this by quantifying the development of the amoeba population when grown alone or in the presence of *Bacillus* spores or vegetative cells. As all the *Bacillus* strains tested displayed a similar behavior when grown in the presence of *A. castellanii*, the Bt 407 strain was used for these assays as a representative of the Bc sl group. The amoeba cell number was quantified using a hemocytometer over 24 h and we determined a value (growth index) indicating the increase of the amoeba population between T0 and T24 (Fig. 3a). The total amoeba population was similar

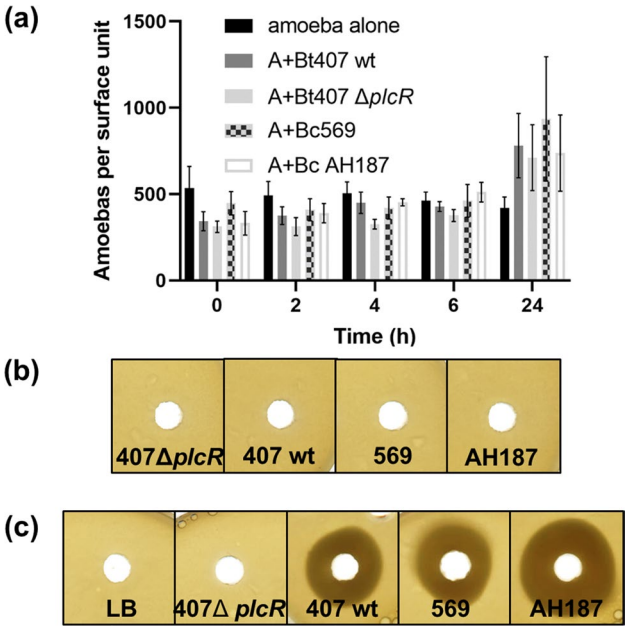


Fig. 2 Toxicity of the virulence factors produced by *Bacillus* on *A. castellanii*. Toxicity was assessed by measuring amoeba growth and lecithinase activity of the virulence factors. **a** The amoeba population in a defined area of the flask was enumerated when grown axenically or co-cultured with stationary phase Bt 407, Bt 407 Δ*plcR*, Bc 569, or Bc AH187 cells. Error bars represent the standard deviation from the mean, $n=3$. The lecithinase activity of the supernatants was assessed by depositing the suspension from wells onto 5% egg yolk-containing LB plates and checking for the formation of a clear ring after 24 h of incubation at 30 °C. **b** Lecithinase activity of supernatants of 2-h amoeba-*Bacillus* co-cultures. **c** Lecithinase activity of LB medium and supernatants of *Bacillus* cultures grown in LB and harvested at the beginning of the stationary phase. Images were captured after incubation at 30 °C for approximately 24 h

in the presence or absence of Bt 407 spores, with a growth index ranging from 0.4 to 0.5. The growth of the amoebas was also assessed when exponentially growing bacteria were provided. The growth index was 1.15 when the amoebas were co-cultured with vegetative Bt 407 cells versus 0.37 in the absence of bacilli (Fig. 3b). Experiments were also conducted in the absence of inactivated *E. coli* cells (Fig. S4). The growth index of amoebas cultured in the presence or in the absence of exponentially growing Bt 407 cells was 0.7 and 0.3, respectively. This result indicates that the growth of the amoebas was stimulated by the presence of vegetative *Bacillus* cells.

Amoebas form cysts when they are confronted with harsh conditions, such as starvation or the presence of biocides [57]. Cyst formation may thus indicate whether there is a negative effect of Bt on amoeba development. We thus evaluated the presence of cysts and their abundance during co-culture with Bt by microscopic observation and enumeration. The proportion of cysts reached approximately 25% of the total amoeba population after 24 h in the presence or absence of Bt 407 spores (Fig. 3c). In contrast, cysts accounted for less than 5% of the total amoeba population after 24 h of co-culture with vegetative Bt 407 cells, a proportion similar to that initially present at T0 (Fig. 3d). Same results were obtained in experiments performed in the absence of inactivated *E. coli* cells in the co-culture medium (Fig. S4). The increase in the number of amoebas and reduced transformation into cysts suggest that the amoebas consumed the vegetative *Bacillus* cells to sustain growth, whereas the spores were not used as food. However, a statistical analysis did not reveal a significant difference between the mean values of the percentage of cysts formed after 24 h of culture in the presence or in the absence of vegetative Bt 407 cells. In contrast, Santos et al. [36] observed that various species of amoebas (including *A. castellanii*) could grow on a medium containing spores of Bc (strain ATCC14579). However, under such conditions, the amoebas could consume the vegetative cells resulting from germination of the spores. The authors also observed the weak growth of the amoebas from spores that were inactivated by heat treatment. Such growth could have been the result of a lower resistance of heat-treated spores.

We determined whether *Bacillus* cells can be used to sustain the growth of another amoeba species by conducting experiments on *D. discoideum.* Vegetative *Bacillus* cells of 10 strains were provided as food as a layer on different media (Table 2). The culture of *D. discoideum* on a layer of Bt or Bc ss strains on SM-Glucose or HL5 agar medium did not lead to growth of the amoeba. However, *D. discoideum* grew well on a layer composed of any *Bacillus* strain tested mixed with 50% Ka on SM-Glucose medium. *D. discoideum* grew well with a *Bacillus* layer on SB-agar medium. A lower growth rate was nevertheless observed on Bt HD73 and Bt *israelensis* strains. Overall, these experiments suggest that *D. discoideum* as well as *A. castellanii* are able to use the vegetative cells of *Bacillus* as food.

The Fate of *Bacillus* in the Presence of Amoebas

The results presented above show that vegetative *Bacillus* cells can be used as food by amoebas (Fig. 3). However, some bacterial cells can escape this fate and multiply (Fig. 1). We determined the fate of the *Bacillus* in an amoeba environment by quantifying the development of the Bt 407 population co-cultured with amoebas and compared it to the development of bacteria grown alone under the same conditions. PAS-Glu-*E. coli* medium was inoculated with Bt spores in the presence or absence of amoebas. CFU-counting showed that the total number of spores remained stable over 48 h under both conditions (Fig. 4a). This observation

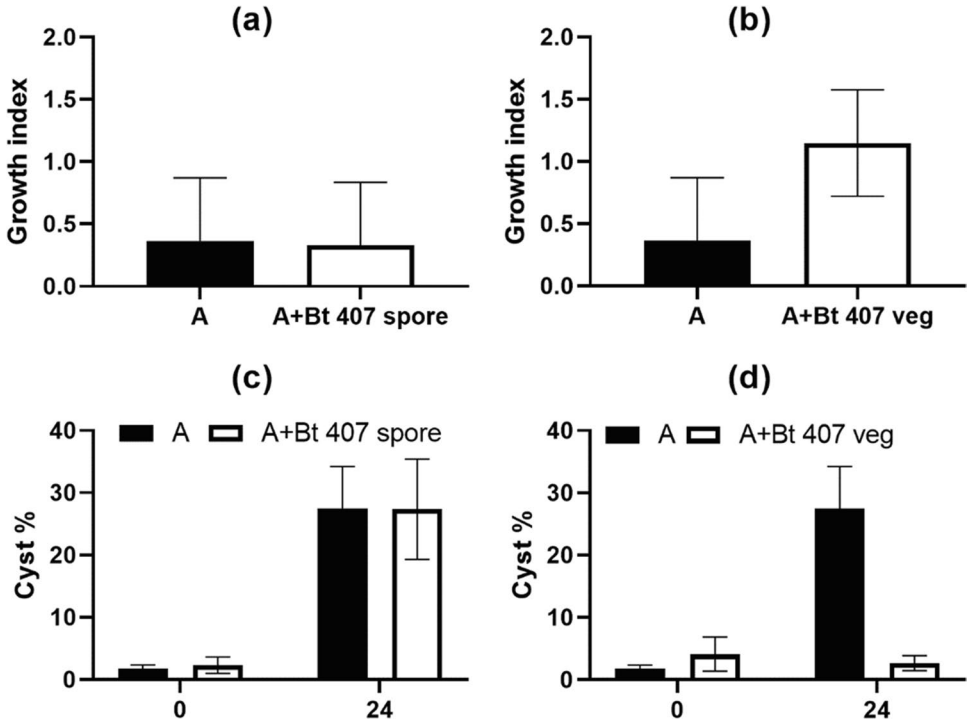


Fig. 3 Development of the amoeba population in co-culture with spores or vegetative Bt 407 cells. Amoeba growth index when they were cultured with or without Bt 407 spores (**a**), and with or without vegetative Bt 407 cells (**b**). Percentage of cysts in the amoeba population when they were cultured with or without Bt 407 spores (**c**), and with or without vegetative Bt 407 cells (**d**). Amoeba cultures were inoculated with spores or vegetative cells as described in Fig. 1. A, amoebas. Error bars represent the standard deviation from the mean, $n=3$. Statistical analysis performed using a Mann–Whitney test showed that the difference between the percentage of cysts when amoebas are grown with or without vegetative Bt 407 cells was not significant

Table 2 Growth of *D. discoideum* using *Bacillus* as food

Bacillus strains	Medium				
	SM-glucose agar	HL5-agar*	SM glucose agar**		SB-agar***
			50% Ka/50%Bt	1% Ka/99%Bt	
Bt 407 Cry$^+$	-	-	+	-	+
Bt 407 Cry$^-$	-	-	+	-	+
Bt 407 Cry$^-$ Δ*plcR*	-	-	+	-	+
Bt HD73 Cry$^+$	-	-	+	-	±
Bti	-	-	+	-	±
Bt *konkukian*	-	-	+	-	+
Bc 569	-	-	+	-	+
Bc ATCC14579	-	-	+	-	+
Bc ATCC14579 Δ*plcR*	-	-	+	-	+
Bw	-	-	+	-	+

SM-Glucose/HL5 agar medium is an axenic culture medium; SB-agar medium is a very poor medium; *K. aerogenes* (Ka) was routinely used as an appropriate food supply. The symbol "-" means that amoebas did not grow, "+" means that amoebas grow well, "±" means that amoebas grow slowly. Medium reference: http://dictybase.org/techniques/media/media.html

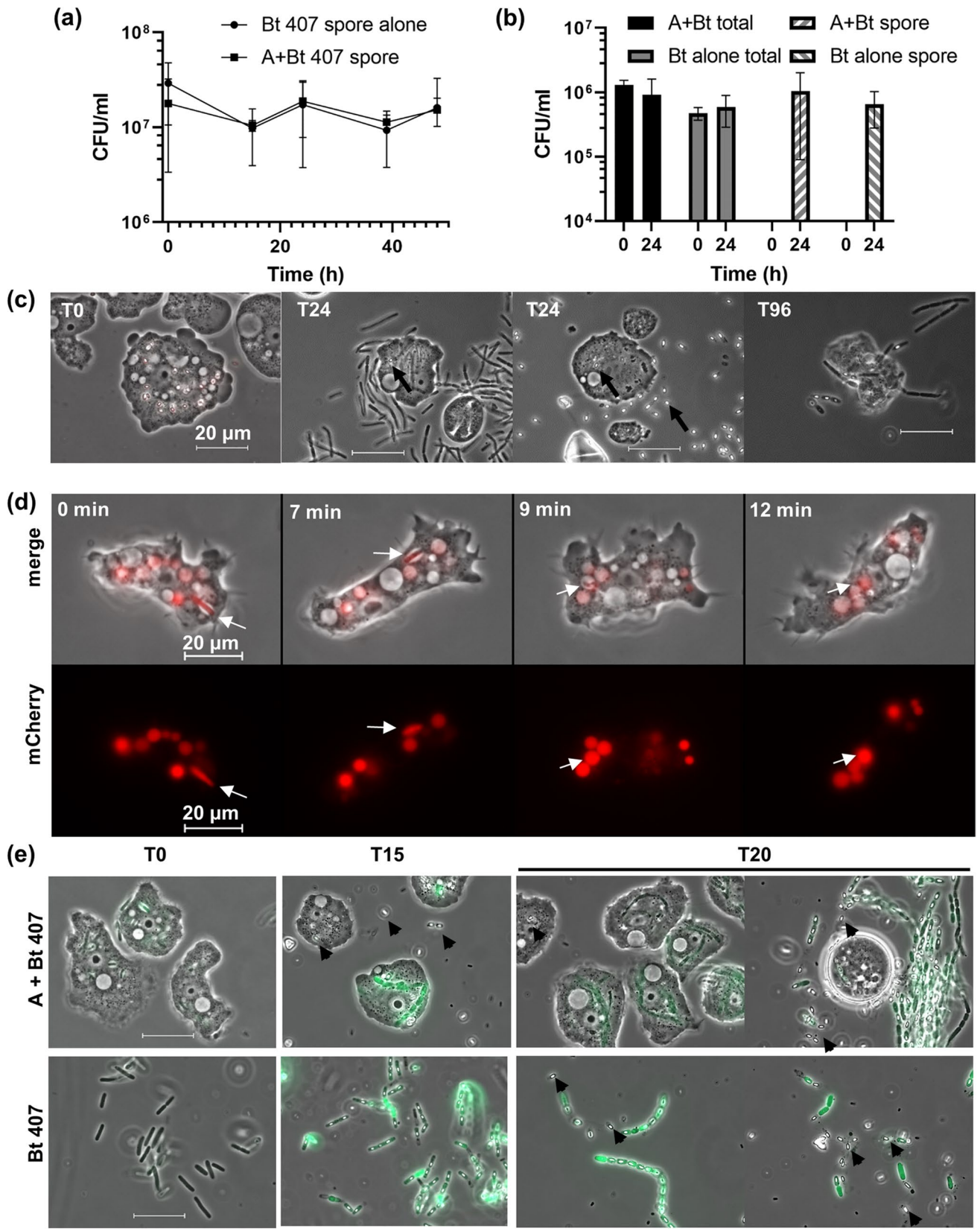
*We did not observe any amoeba growth whatever the concentration of HL5 (100, 50, 20, 10, 5, or 2%)

**SM glucose agar supplemented with an amount of Ka corresponding to 50% does not allow amoeba growth

***Amoebas do not grow in an axenic SB-agar medium

suggests that only a small portion of the spore population germinated and was at the origin of the vegetative population observed in the microscopy images (Fig. 1).

We also investigated the development of vegetative *Bacillus* cells in co-culture with amoebas (Fig. 4b). The total number of CFUs remained stable whether the


(a)
Bt 407 spore alone
A+Bt 407 spore
CFU/ml
Time (h)
(b)
A+Bt total
A+Bt spore
Bt alone total
Bt alone spore
CFU/ml
Time (h)
(c)
T0
T24
T24
T96
20 μm
(d)
merge
mCherry
0 min
7 min
9 min
12 min
20 μm
(e)
T0
T15
T20
A + Bt 407
Bt 407

◂**Fig. 4** The fate of Bt 407 spores or vegetative cells during co-culture with amoebas. **a** The development of the Bt 407 spore population cultured with or without amoebas was determined by counting the CFUs after heat treatment. **b** The development of vegetative Bt cells cultured with or without amoebas was determined by CFU counting. Spores present in the population were counted after heat treatment. **c** Microscopy images of amoeba-Bt spores co-cultured at the indicated time points (magnification × 100). Two distinct areas are shown for T24. **d** Time-lapse microscopy images showing Bt P_{apha3}-*mCherry* lysis in amoeba vacuoles (magnification × 100). The duration of observation is indicated in the images. The first image was arbitrarily designated as 0 min. Images are representative of four experiments. A vacuole identified as containing a bacterium at T0 and tracked for the duration of the experiment is indicated with an arrow. **e** Micrographs of Bt 407 P_{sarA}-*gfp* vegetative cells co-cultured with amoebas (top panels) or alone (bottom panels). The spores are indicated by the arrow heads. Images presented are representative of three experiments. Error bars represent the standard deviation from the mean ($n = 3$). The experiments in **a**, **b**, and **e** were performed in PAS-Glu-*E. coli* medium at 30 °C and those in c and d in PAS-Glu medium at 30 °C

vegetative cells were co-cultured with amoebas or cultured alone for 24 h. Most of the bacteria were detected as spores after culturing with or without amoebas at 24 h, with sporulation rates for Bt 407 of 96% and 99%, respectively. Thus, the entire bacterial population sporulated in PAS-Glu-*E. coli* medium, with or without amoeba.

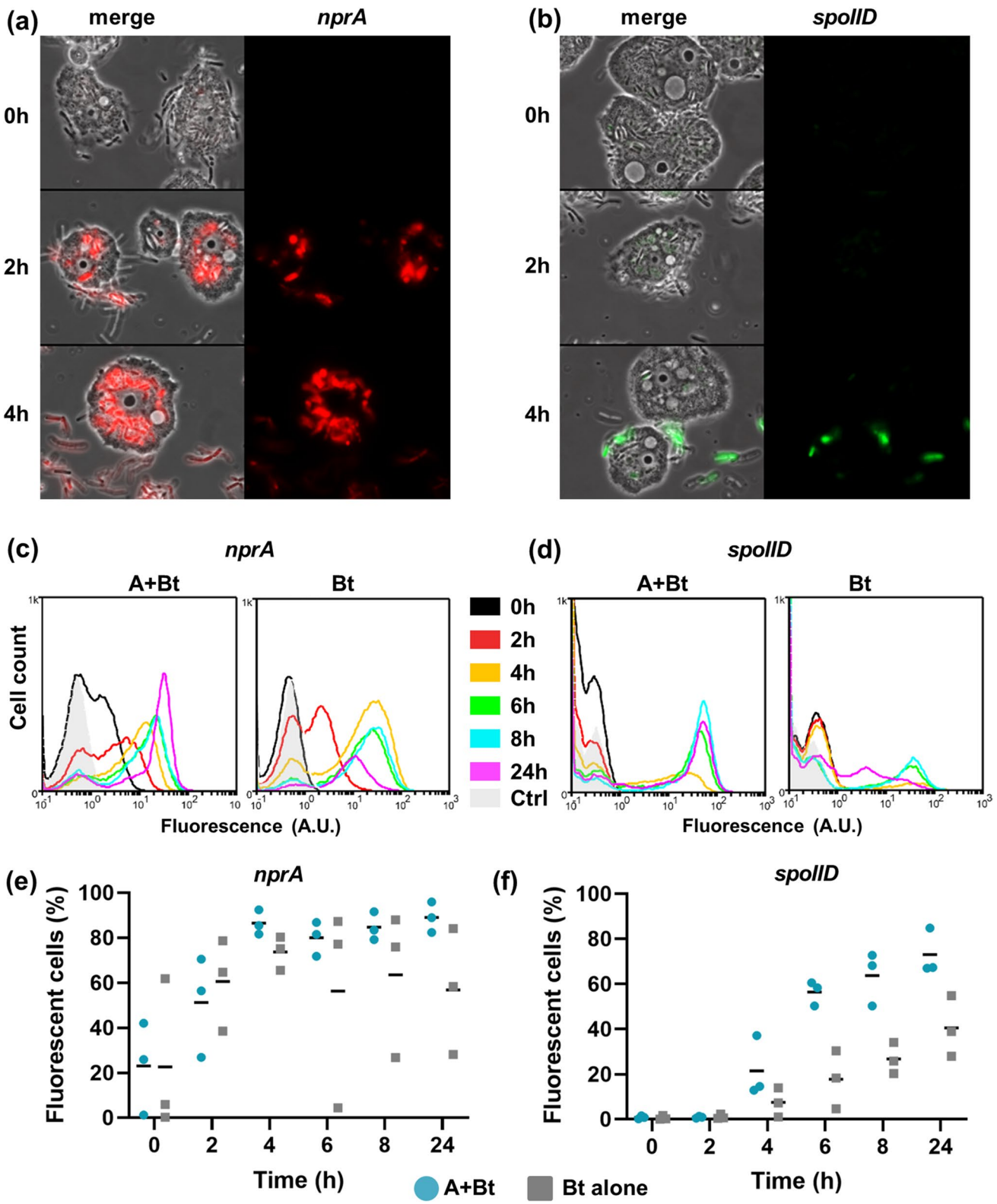
This quantitative approach was reinforced by microscopic observations over 96 h. The co-culture of spores with amoebas resulted in a heterogeneous bacterial population of spores and vegetative cells at 24 h (Fig. 4c). Some vegetative cells had formed small chains and aggregates. At T96, we observed a mixed population of vegetative cells, sporulating cells, and free spores. If Bt spores were inoculated alone in the same medium, the spores did not germinate and no vegetative cells were observed at T24 or T96 (Fig. S5). These results suggest that Bt 407 spores can complete an entire life-cycle (from spores to vegetative cells back to spores again) in the amoebic environment. However, these observations do not indicate whether Bt spores can germinate inside amoebas, as was shown for Ba [34].

We used time-lapse microscopy to follow the fate of vegetative Bt 407 cells phagocytosed by amoebas. Exponentially growing Bt 407 cells carrying pAT-P_{apha3}-*mCherry* were used. P_{apha3} is constitutively expressed and fused to the *mCherry* gene allows to follow the bacteria during exponential growth. Images from a representative experiment are presented (Fig. 4d). A rod-like bacterium was identified inside the amoeba selected for this time-lapse analysis (time arbitrarily set to T0 for the first observation). The shape of the fluorescence of the Bt cell became blurred after 7 min. The rod-like bacterium was broken down and the vacuole containing the bacterium became fluorescent at 9 min. The rod-shaped bacterium could not be identified after 12 min. Concomitantly, over the course of the observation, the content of the vacuole became fluorescent, indicating the expression of P_{apha3} and the release of the mCherry protein from the bacteria into the vacuole. The other red fluorescent vacuoles show that this amoeba had digested several Bt cells before the observation began.

The Effect of Amoebas on Bt Developmental Stages

We investigated the development of a bacterial population in the amoebic environment by microscopic observation of vegetative Bt 407 carrying a P_{sarA}-*gfp* transcriptional fusion (Table 1) co-cultured with amoebas over 20 h (Fig. 4e, top panels). P_{sarA} is constitutively expressed during the vegetative growth. A mixed population of vegetative cells and spores was present both inside and outside the amoebas after 15 h of co-culture. We also observed a heterogenous population of vegetative and sporulating cells when the vegetative cells were grown in PAS-Glu-*E. coli* medium in the absence of amoebas after 15 h (Fig. 4e, bottom line). However, the presence of free spores, i.e., spores that are released from the mother cells, was observed at T15 for bacteria grown in the amoebic environment, but was only identified from T20 when the bacteria were grown in the absence of amoebas. This observation suggests that the sporulation process was faster when the bacterial population grew in an amoebic environment.

We next monitored the physiological development of Bt in amoeba using transcriptional fusions between a fluorescent reporter gene and promoters that are activated during the stationary phase. Two promoters were selected: P_{nprA}, which is activated by the quorum-sensing regulator NprR and controls necrotrophism at the onset of the stationary phase [58, 59], and P_{spoIID}, which is activated by the sigma factor SigE during the early stage of the sporulation [60, 61]. Bt 407 nprA and spoIID strains harboring plasmid transcriptional fusions pP_{nprA}-*mCherry* or pP_{spoIID}-*yfp* (Table 1) were grown in the presence of amoebas or axenically in the same culture medium. Exponentially growing Bt cells (i.e., bacteria expressing no reporter genes) were collected and co-cultured with amoebas. Microscopic observations, as well as flow cytometry analysis, were performed (Fig. 5). Microscopic analyses showed that Bt cells were present inside and outside the amoebas and that the expression of P_{nprA}-*mCherry* and P_{spoIID}-*yfp* started before T2 and T4, respectively (Fig. 5a, b). We observed similar kinetics of expression for the two promoters when Bt grew in the absence of amoebas (Fig. S6). The P_{nprA}-expressing population was detected at T0 by flow cytometry (Fig. 5c, e). However, the level of fluorescence was low and may



explain why P_{nprA}-expressing cells were not detected at this time point by the less sensitive fluorescent microscopy. Two populations were present, indicating that P_{nprA} was expressed heterogeneously. The population of cells expressing P_{nprA} increased from 25% at T0 to over 80% at T4 and later in the presence of the amoebas (Fig. 5e). We observed similar kinetics of P_{nprA}*'-mCherry* expression and heterogeneity in the Bt 407 nprA population for

◂**Fig. 5** Expression of *nprA* and *spoIID* in vegetative Bt 407 cells co-cultured with amoebas. Fluorescence microscopy images of **a** Bt 407 nprA and **b** Bt 407 spoIID strains co-cultured with amoebas. Left panel, merge of phase-contrast images and fluorescence images; right panels, epifluorescence images. The fluorescent mCherry and YFP signals are false-colored red and green, respectively. Flow cytometry histograms showing the distribution of **c** Bt 407 nprA and **d** Bt 407 spoIID cells as a function of the fluorescence level. Each time point is indicated by a unique color. The gray area represents the fluorescence obtained with the reporterless Bt 407 pHT304 strain used as the control at T6. The *x*-axis represents the fluorescence intensity in arbitrary units (A.U.) and the *y*-axis the cell count. Percentage of Bt 407 cells expressing **e** P_{nprA} or **f** P_{spoIID} determined by flow cytometry. Blue dots, Bt cells co-cultured with amoebas; gray squares, Bt cells cultured in the in PAS-Glu-*E. coli* medium; black bars represent the means. The data are the results of 3 independent experiments, with each symbol representing one repetition. Statistical analysis performed using a Mann–Whitney test showed that the differences between fluorescence expressing cells grown with or without amoebas were not significant. T0 was defined as 1 h after the initial contact of Bt cells with amoebas

Bt cells grown in the absence of amoebas (Fig. 5c, e and S6). In co-culture with amoeba, P_{spoIID}*'-yfp* expression was detected from T4 in approximately 15% of the Bt 407 spoIID population and expression of the sporulation reporter gene increased over time to represent approximately 70% of cells by T8 (Fig. 5d, f). The percentage of P_{spoIID}*'-yfp*-expressing cells was lower when the Bt cells were grown in the absence of amoebas. Under axenic conditions, P_{spoIID}-expressing cells represented 7% of the population at T4 and 40% at T24. Flow cytometry analyses confirmed the microscopic observations (Fig. 5a, b); a higher percentage of bacteria became committed to sporulation when grown in the presence of amoebas than under axenic conditions. However, a statistical analysis did not reveal a significant difference between the mean values of the P_{spoIID}-expressing population in the two growth conditions.

We studied the development of the necrotrophic and sporulation reporter strains in 24 h amoebic-conditioned medium and non-conditioned medium to determine whether such stimulation of sporulation was due to direct contact of the bacteria with amoebas or the amoeba-released or -consumed components. The growth of vegetative cells was quantified by OD_{600nm} measurements (Fig. 6a). The growth profile of Bt was similar in the conditioned or non-conditioned medium, with the turbidity of the medium increasing from 0.3–0.4 at T0 to 0.5–0.8 at T10 under both conditions and showing no significant differences (Fig. 6a). We then determined the expression kinetics of the reporter genes under the two growth conditions by flow cytometry (Fig. 6b–e). P_{nprA} and P_{spoIID} expression started at T2 and T4, respectively. The expression of the P_{nprA}*'-mCherry* transcriptional fusion increased progressively in the bacterial population to reach 80% of the cells at T6 and later (Fig. 6b, d). The P_{nprA}-expressing population decreased to approximately 35% of the whole population at T24. Bt cells grown in conditioned and non-conditioned media showed similar P_{nprA} expression kinetics. During the first 10 h of culture, the necrotrophic population was slightly but not significantly smaller in the non-conditioned than conditioned medium (Fig. 6d). As for the sporulation reporter, P_{spoIID} expression increased faster in the amoebic-conditioned medium than in the non-conditioned medium; expression of the reporter gene started between T2 and T4 and increased to represent approximately 35% of the bacterial population (Fig. 6c, e). The first P_{spoIID}-expressing cells were detected at T4 in the non-conditioned medium, but they represented only 1% of the population (Fig. 6e). The sporulating population increased over time but remained below 20% until T10, finally representing 33% of cells by T24. The percentage of P_{spoIID}-expressing cells at T24 was similar in both conditioned and non-conditioned medium. Overall, these results suggest that the amoebic environment accelerates the commitment of Bt cells to sporulation.

Conclusions

This study of representative strains of three main species of the Bc sl group, except Ba, shows these bacteria to have a similar behavior when in the presence of amoebas, such as *A. castellanii* and *D. discoideum*. Spores of these *Bacilli* can use molecular signals and nutrients secreted by amoebas to germinate; the vegetative cells then develop as chains or aggregates. Although a portion of the vegetative cells are phagocytosed and digested by the amoebas, another portion of the bacterial population commits to sporulation and all the vegetative cells finally sporulate. Entry into this developmental pathway occurs faster in an amoebic environment than under axenic conditions. As a counterpart, amoebas can take advantage by preying on vegetative cells but not spores to grow and delay cyst formation. Consistent with this result, we show that amoebas are not affected by the numerous virulence factors produced by the tested Bt and Bc ss strains. This study also shows that the major virulence regulator PlcR is not expressed in the amoeba-Bacilli co-culture medium. This is consistent with the accelerated entry of the bacteria into the sporulation process, which involves a rapid increase in the intracellular concentration of the phosphorylated form of Spo0A, known to repress the transcription of the *plcR* gene [62]. Although Bt and Bc ss cannot replicate inside amoebas, unlike Ba, amoebas can nevertheless contribute to the spread of the *Bacillus* in the environment.

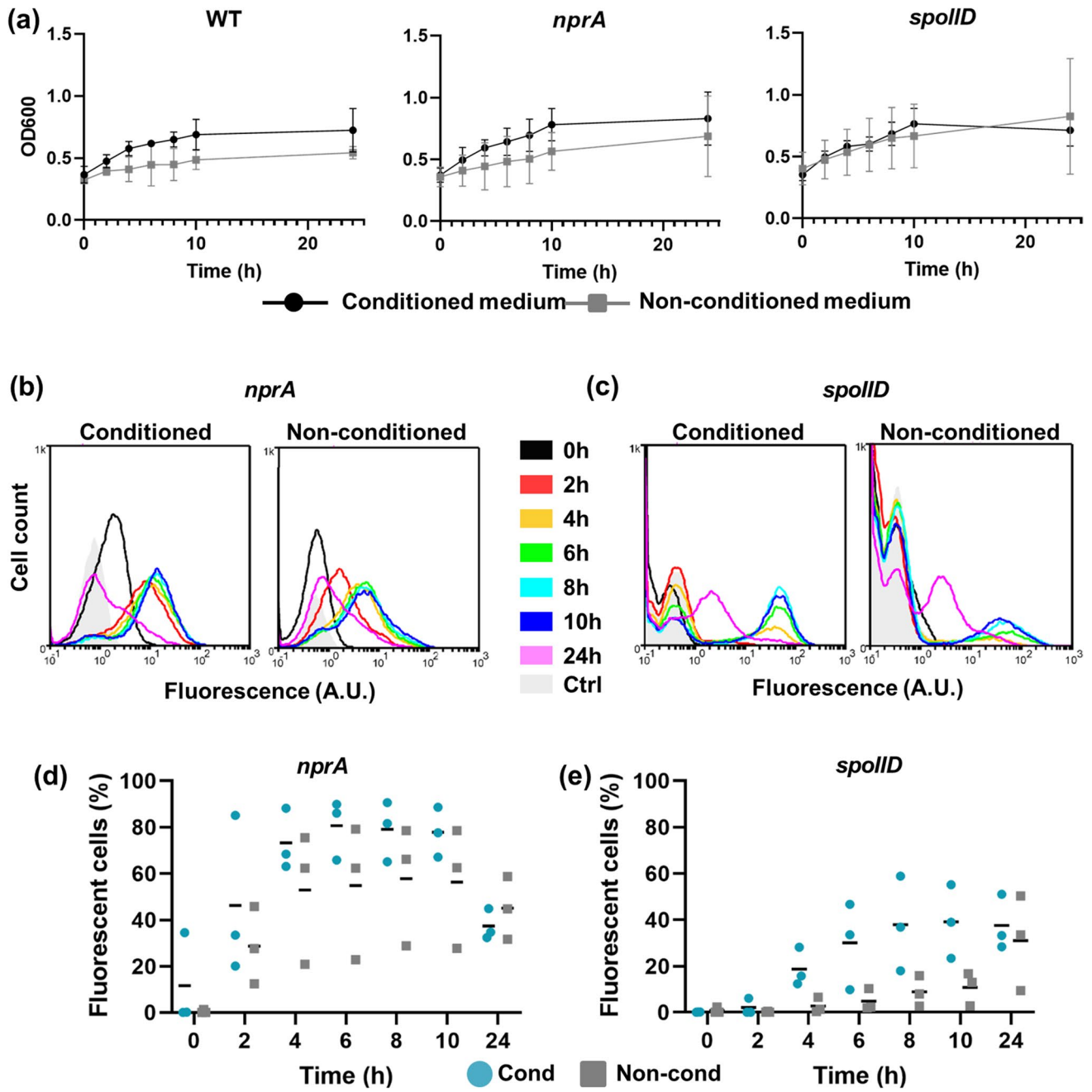


Fig. 6 Expression of *nprA* and *spoIID* in vegetative Bt 407 cells grown in conditioned or non-conditioned medium. **a** Growth of vegetative Bt 407 cells cultured in 24 h amoeba-conditioned medium or non-conditioned medium. Growth was determined by measuring the turbidity of the cultures at OD_{600nm}. **b** Flow cytometry histograms showing the distribution of Bt 407 nprA and **c** spoIID cells as a function of the fluorescence level at time points indicated by a unique color. The gray area represents the fluorescence obtained with the reporterless Bt 407 pHT304 strain used as control at T6. The *x*-axis represents the fluorescence intensity in arbitrary units (A.U.) and the *y*-axis the cell count. Percentage of Bt 407 cells expressing **d** P_{nprA} or **e** P_{spoIID} determined by flow cytometry. Blue dots, Bt cells cultured in amoeba-conditioned medium; gray squares, Bt cells cultured in fresh PAS-Glu-*E. coli* medium; black bars represent the means. The data are the results of 3 independent experiments, with each symbol representing one repetition. Statistical analysis performed using a Mann–Whitney test showed that the differences between fluorescence expressing cells grown in amoeba-conditioned or non-conditioned medium were not significant. T0 was defined as 1 h after the seeding of growth media

Indeed, *Acanthamoeba* may eventually compensate for the loss caused by the digestion of vegetative cells by providing a favorable environment for the growth of bacteria. Ultimately, our results suggest that the amoeba-*Bacillus* interaction could create a virtuous circle in which each protagonist helps the other to develop.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00248-021-01828-2.

Acknowledgements *Acanthamoeba castellanii* (ATCC 30010) was a kind gift from Fabienne Misguich (UVSQ). We are greatly indebted to Fabienne for the valuable advice she gave us on the amoeba world. We thank Michel Gohar for the gift of the plasmid PHT304-18ΩP$_{sar}$-*gfp*.

Author Contribution All authors contributed to the study conception, design, material preparation, data collection, and analysis. The first draft of the manuscript was written by Haibo Chen, Émilie Verplaetse, and Didier Lereclus, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This work was supported by the National Institute for Agriculture, Food and Environment (INRAE). HC was funded by the China Scholarship Council (CSC). The CyFlow space flow cytometer used in this study was funded by the DIM (Domaine d'intérêt majeur) Astrea (French regional program: Ast11 0137).

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval Principles of ethical and professional conduct have been followed.

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Consent for Publication Not applicable.

Financial Interests The authors have no relevant financial to disclose.

Conflicts of Interest The authors declare no competing interests.

References

1. Bazinet AL (2017) Pan-genome and phylogeny of *Bacillus cereus sensu* lato. BMC Evol Biol 17:176. https://doi.org/10.1186/s12862-017-1020-1
2. Jensen GB, Hansen BM, Eilenberg J, Mahillon J (2003) The hidden lifestyles of *Bacillus cereus* and relatives. Environ Microbiol 5:631–640. https://doi.org/10.1046/j.1462-2920.2003.00461.x
3. Stenfors Arnesen LP, Fagerlund A, Granum PE (2008) From soil to gut: *Bacillus cereus* and its food poisoning toxins. FEMS Microbiol Rev 32:579–606. https://doi.org/10.1111/j.1574-6976.2008.00112.x
4. Vilas-Bôas GT, Peruca APS, Arantes OMN (2007) Biology and taxonomy of *Bacillus cereus*, *Bacillus anthracis*, and *Bacillus thuringiensis*. Can J Microbiol 53:673–687. https://doi.org/10.1139/W07-029
5. Vilain S, Luo Y, Hildreth MB, Brözel VS (2006) Analysis of the life cycle of the soil saprophyte *Bacillus cereus* in liquid soil extract and in soil. Appl Environ Microbiol 72:4970. https://doi.org/10.1128/AEM.03076-05
6. Van Cuyk S, Deshpande A, Hollander A et al (2011) Persistence of *Bacillus thuringiensis* subsp. *kurstaki* in urban environments following spraying. Appl Env Microbiol 77:7954–7961. https://doi.org/10.1128/AEM.05207-11
7. Vilas-Bôas LA, Vilas-Bôas GFLT, Saridakis HO et al (2000) Survival and conjugation of *Bacillus thuringiensis* in a soil microcosm. FEMS Microbiol Ecol 31:255–259. https://doi.org/10.1111/j.1574-6941.2000.tb00691.x
8. Ivanova N, Sorokin A, Anderson I et al (2003) Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. Nature 423:87. https://doi.org/10.1038/nature01582
9. Liu Y, Lai Q, Göker M et al (2015) Genomic insights into the taxonomic status of the *Bacillus cereus* group. Sci Rep 5:1–11. https://doi.org/10.1038/srep14082
10. Ehling-Schulz M, Lereclus D, Koehler TM (2019) The *Bacillus cereus* group: *Bacillus* species with pathogenic potential. Microbiol Spectr 7. https://doi.org/10.1128/microbiolspec.GPP3-0032-2018
11. Dommel MK, Lücking G, Scherer S, Ehling-Schulz M (2011) Transcriptional kinetic analyses of cereulide synthetase genes with respect to growth, sporulation and emetic toxin production in *Bacillus cereus*. Food Microbiol 28:284–290. https://doi.org/10.1016/j.fm.2010.07.001
12. Kolstø A-B, Tourasse NJ, Økstad OA (2009) What sets *Bacillus anthracis* apart from other *Bacillus* species? Annu Rev Microbiol 63:451–476. https://doi.org/10.1146/annurev.micro.091208.073255
13. Raymond B, Johnston PR, Nielsen-LeRoux C et al (2010) *Bacillus thuringiensis*: an impotent pathogen? Trends Microbiol 18:189–194. https://doi.org/10.1016/j.tim.2010.02.006
14. Rasko DA, Altherr MR, Han CS, Ravel J (2005) Genomics of the *Bacillus cereus* group of organisms. FEMS Microbiol Rev 29:303–329. https://doi.org/10.1016/j.fmrre.2004.12.005
15. Agaisse H, Gominet M, Økstad OA et al (1999) PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus thuringiensis*. Mol Microbiol 32:1043–1053. https://doi.org/10.1046/j.1365-2958.1999.01419.x
16. Gohar M, Faegri K, Perchat S et al (2008) The PlcR virulence regulon of *Bacillus cereus*. PLoS ONE 3:e2793. https://doi.org/10.1371/journal.pone.0002793
17. Callegan MC, Kane ST, Cochran DC et al (2003) Relationship of *plcR*-regulated factors to *Bacillus* endophthalmitis virulence. Infect Immun 71:3116–3124. https://doi.org/10.1128/IAI.71.6.3116-3124.2003
18. Doll VM, Ehling-Schulz M, Vogelmann R (2013) Concerted action of sphingomyelinase and non-hemolytic enterotoxin in pathogenic *Bacillus cereus*. PLoS ONE 8:e61404. https://doi.org/10.1371/journal.pone.0061404
19. Salamitou S, Ramisse F, Brehélin M et al (2000) The *plcR* regulon is involved in the opportunistic properties of *Bacillus thuringiensis* and *Bacillus cereus* in mice and insects. Microbiology 146:2825–2832. https://doi.org/10.1099/00221287-146-11-2825
20. Fedhila S, Gohar M, Slamti L et al (2003) The *Bacillus thuringiensis* PlcR-regulated gene *inhA2* is necessary, but not sufficient, for virulence. J Bacteriol 185:2820–2825. https://doi.org/10.1128/JB.185.9.2820-2825.2003
21. Grandvalet C, Gominet M, Lereclus D (2001) Identification of genes involved in the activation of the *Bacillus thuringiensis* inhA metalloprotease gene at the onset of sporulation. Microbiology 147:1805–1813. https://doi.org/10.1099/00221287-147-7-1805
22. Guillemet E, Tran S-L, Cadot C et al (2013) Glucose 6P binds and activates HlyIIR to repress *Bacillus cereus* haemolysin *hlyII* gene expression. PLoS ONE 8:e55085. https://doi.org/10.1371/journal.pone.0055085
23. Guillemet E, Cadot C, Tran S-L et al (2010) The InhA metalloproteases of *Bacillus cereus* contribute concomitantly to virulence. J Bacteriol 192:286–294. https://doi.org/10.1128/JB.00264-09

24. Ramarao N, Lereclus D (2005) The InhA1 metalloprotease allows spores of the *B. cereus* group to escape macrophages. Cell Microbiol 7:1357–1364. https://doi.org/10.1111/j.1462-5822.2005.00562.x
25. Tran S-L, Guillemet E, Ngo-Camus M et al (2011) Haemolysin II is a *Bacillus cereus* virulence factor that induces apoptosis of macrophages. Cell Microbiol 13:92–108. https://doi.org/10.1111/j.1462-5822.2010.01522.x
26. Andreu N, Phelan J, de Sessions PF et al (2017) Primary macrophages and J774 cells respond differently to infection with *Mycobacterium tuberculosis*. Sci Rep 7:1–12. https://doi.org/10.1038/srep42225
27. Bowers B (1977) Comparison of pinocytosis and phagocytosis in *Acanthamoeba castellanii*. Exp Cell Res 110:409–417. https://doi.org/10.1016/0014-4827(77)90307-X
28. Greub G, Raoult D (2004) Microorganisms resistant to free-living amoebae. Clin Microbiol Rev 17:413–433. https://doi.org/10.1128/CMR.17.2.413-433.2004
29. Thomas V, McDonnell G, Denyer SP, Maillard J-Y (2010) Free-living amoebae and their intracellular pathogenic microorganisms: risks for water quality. FEMS Microbiol Rev 34:231–259. https://doi.org/10.1111/j.1574-6976.2009.00190.x
30. Bakala N'Goma JC, Le Moigne V, Soismier N, et al (2015) *Mycobacterium abscessus* phospholipase C expression is induced during coculture within amoebae and enhances *M. abscessus* virulence in mice. Infect Immun 83:780. https://doi.org/10.1128/IAI.02032-14
31. Swart AL, Harrison CF, Eichinger L, et al (2018) *Acanthamoeba* and *Dictyostelium* as cellular models for *Legionella* infection. Front Cell Infect Microbiol 8. https://doi.org/10.3389/fcimb.2018.00061
32. Lambrecht E, Baré J, Chavatte N et al (2015) Protozoan cysts act as a survival niche and protective shelter for foodborne pathogenic bacteria. Appl Environ Microbiol 81:5604–5612. https://doi.org/10.1128/AEM.01031-15
33. Moreno-Mesonero L, Ferrús MA, Moreno Y (2020) Determination of the bacterial microbiome of free-living amoebae isolated from wastewater by 16S rRNA amplicon-based sequencing. Environ Res 190:109987. https://doi.org/10.1016/j.envres.2020.109987
34. Dey R, Hoffman PS, Glomski IJ (2012) Germination and amplification of anthrax spores by soil-dwelling amoebas. Appl Environ Microbiol 78:8075. https://doi.org/10.1128/AEM.02034-12
35. Beeton ML, Atkinson DJ, Waterfield NR (2013) An amoeba phagocytosis model reveals a novel developmental switch in the insect pathogen *Bacillus thuringiensis*. J Insect Physiol 59:223–231. https://doi.org/10.1016/j.jinsphys.2012.06.011
36. Santos SS, Hendriksen NB, Jakobsen HH, Winding A (2017) Effects of *Bacillus cereus* endospores on free-living protist growth. Microb Ecol 73:699–709. https://doi.org/10.1007/s00248-016-0905-7
37. Lereclus D, Lecadet M-M, Ribier J, Dedonder R (1982) Molecular relationships among plasmids of *Bacillus thuringiensis*: conserved sequences through 11 crystalliferous strains. Mol Gen Genet 186:391–398. https://doi.org/10.1007/BF00729459
38. Rowbotham TJ (1983) Isolation of *Legionella pneumophila* from clinical specimens via amoebae, and the interaction of those and other isolates with amoebae. J Clin Pathol 36:978. https://doi.org/10.1136/jcp.36.9.978
39. Lereclus D, Arantes O, Chaufaux J, Lecadet M (1989) Transformation and expression of a cloned delta-endotoxin gene in *Bacillus thuringiensis*. FEMS Microbiol Lett 51:211–217. https://doi.org/10.1016/0378-1097(89)90511-9
40. Wilcks A, Jayaswal N, Lereclus D, Andrup L (1998) Characterization of plasmid pAW63, a second self-transmissible plasmid in *Bacillus thuringiensis* subsp. *kurstaki* HD73. Microbiology 144:1263–1270. https://doi.org/10.1099/00221287-144-5-1263
41. Bourgouin C, Larget-Thiery I, de Barjac H (1984) Efficacy of dry powders from *Bacillus sphaericus*: RB 80, a potent reference preparation for biological titration. J Invertebr Pathol 44:146–150. https://doi.org/10.1016/0022-2011(84)90005-3
42. Hernandez E, Ramisse F, Ducoureau J-P et al (1998) *Bacillus thuringiensis* subsp.*konkukian* (Serotype H34) superinfection: Case report and experimental evidence of pathogenicity in immunosuppressed mice. J Clin Microbiol 36:2138–2139. https://doi.org/10.1128/JCM.36.7.2138-2139.1998
43. Clements MO, Moir A (1998) Role of the *gerI* operon of *Bacillus cereus* 569 in the response of spores to germinants. J Bacteriol 180:6729–6735. https://doi.org/10.1128/JB.180.24.6729-6735.1998
44. Turnbull PC, Kramer JM, Jørgensen K et al (1979) Properties and production characteristics of vomiting, diarrheal, and necrotizing toxins of *Bacillus cereus*. Am J Clin Nutr 32:219–228. https://doi.org/10.1093/ajcn/32.1.219
45. Lapidus A, Goltsman E, Auger S et al (2008) Extending the *Bacillus cereus* group genomics to putative food-borne pathogens of different toxicity. Chem Biol Interact 171:236–249. https://doi.org/10.1016/j.cbi.2007.03.003
46. Réjasse A, Gilois N, Barbosa I et al (2012) Temperature-dependent production of various PlcR-controlled virulence factors in *Bacillus weihenstephanensis* strain KBAB4. Appl Environ Microbiol 78:2553–2561. https://doi.org/10.1128/AEM.07446-11
47. Verplaetse E, Slamti L, Gohar M, Lereclus D (2015) Cell differentiation in a *Bacillus thuringiensis* population during planktonic growth, biofilm formation, and host infection. mBio 6:e00138-15. https://doi.org/10.1128/mBio.00138-15
48. Lauderdale KJ, Malone CL, Boles BR et al (2010) Biofilm dispersal of community-associated methicillin-resistant *Staphylococcus aureus* on orthopedic implant material. J Orthop Res 28:55–61. https://doi.org/10.1002/jor.20943
49. Agaisse H, Lereclus D (1994) Structural and functional analysis of the promoter region involved in full expression of the *cryIIIA* toxin gene of *Bacillus thuringiensis*. Mol Microbiol 13:97–107. https://doi.org/10.1111/j.1365-2958.1994.tb00405.x
50. Page FC (1967) Taxonomic criteria for limax amoebae, with descriptions of 3 new species of *Hartmannella* and 3 of *Vahlkampfia*. J Protozool 14:499–521. https://doi.org/10.1111/j.1550-7408.1967.tb02036.x
51. Froquet R, Lelong E, Marchetti A, Cosson P (2009) *Dictyostelium discoideum*: a model host to measure bacterial virulence. Nat Protoc 4:25–30. https://doi.org/10.1038/nprot.2008.212
52. Slamti L, Perchat S, Gominet M et al (2004) Distinct mutations in *plcR* explain why some strains of the *Bacillus cereus* group are nonhemolytic. J Bacteriol 186:3531. https://doi.org/10.1128/JB.186.11.3531-3538.2004
53. Vlamakis H, Aguilar C, Losick R, Kolter R (2008) Control of cell fate by the formation of an architecturally complex bacterial community. Genes Dev 22:945–953. https://doi.org/10.1101/gad.1645008
54. Jeßberger N, Dietrich R, Bock S et al (2014) *Bacillus cereus* enterotoxins act as major virulence factors and exhibit distinct cytotoxicity to different human cell lines. Toxicon 77:49–57. https://doi.org/10.1016/j.toxicon.2013.10.028
55. Ehling-Schulz M, Fricker M, Grallert H et al (2006) Cereulide synthetase gene cluster from emetic *Bacillus cereus*: structure and location on a mega virulence plasmid related to *Bacillus anthracis* toxin plasmid pXO1. BMC Microbiol 6:20. https://doi.org/10.1186/1471-2180-6-20
56. Zhou W, Warrilow AGS, Thomas CD et al (2018) Functional importance for developmental regulation of sterol biosynthesis in *Acanthamoeba castellanii*. Biochim Biophys Acta Mol Cell Biol Lipids 1863:1164–1178. https://doi.org/10.1016/j.bbalip.2018.07.004
57. Coulon C, Collignon A, McDonnell G, Thomas V (2010) Resistance of *Acanthamoeba* cysts to disinfection treatments used in health care

settings. J Clin Microbiol 48:2689–2697. https://doi.org/10.1128/JCM.00309-10

58. Perchat S, Dubois T, Zouhir S et al (2011) A cell–cell communication system regulates protease production during sporulation in bacteria of the *Bacillus cereus* group. Mol Microbiol 82:619–633. https://doi.org/10.1111/j.1365-2958.2011.07839.x
59. Dubois T, Faegri K, Gélis-Jeanvoine S et al (2016) Correction: Necrotrophism is a quorum-sensing-regulated lifestyle in *Bacillus thuringiensis*. PLOS Pathog 12:e1006049. https://doi.org/10.1371/journal.ppat.1006049
60. Bravo A, Agaisse H, Salamitou S, Lereclus D (1996) Analysis of *cryIAa* expression in *sigE* and *sigK* mutants of *Bacillus thuringiensis*. Mol Gen Genet 250:734–741. https://doi.org/10.1007/BF02172985
61. Rong S, Rosenkrantz MS, Sonenshein AL (1986) Transcriptional control of the *Bacillus subtilis spoIID* gene. J Bacteriol 165:771–779. https://doi.org/10.1128/jb.165.3.771-779.1986
62. Slamti L, Perchat S, Huillet E, Lereclus D (2014) Quorum sensing in *Bacillus thuringiensis* is required for completion of a full infectious cycle in the insect. Toxins 6:2239–2255. https://doi.org/10.3390/toxins6082239

The fate of bacteria of the *Bacillus cereus* group in the amoeba environment

Haibo Chen[1], Emilie Verplaetse[1], Tania Jauslin[2], Pierre Cosson[2], Leyla Slamti[1], and Didier Lereclus[1]

[1] Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, 78350, Jouy-en-Josas, France.

[2] Cell Physiology and Metabolism Dpt, Faculty of Medicine, University of Geneva, 1 rue Michel Servet, CH-1211, Geneva 4, Switzerland.

Corresponding author: Didier Lereclus: Didier.Lereclus@inrae.fr

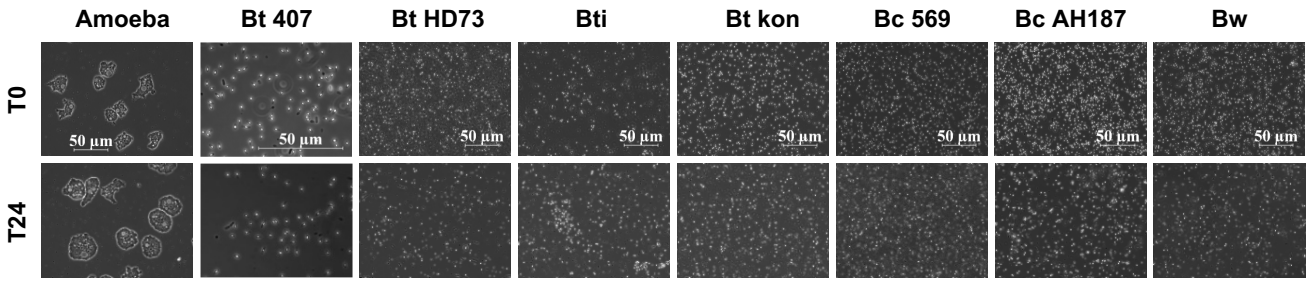


Figure S1. Micrographs of *Acanthamoeba castellanii* and the spores of seven *Bacillus* strains cultured axenically in the PAS-Glu-*E. coli* medium at T0 and T24. A magnification of 40× was used for all the strains except 100× for Bt 407.

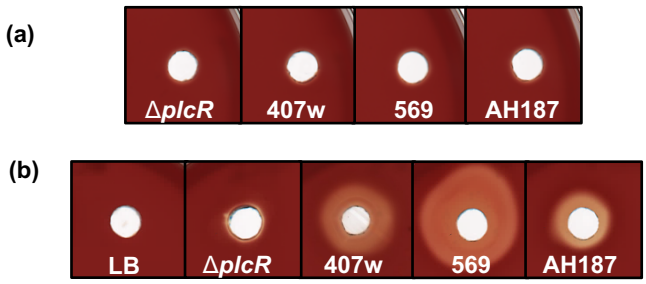


Figure S2. Hemolytic activity of the supernatants of the *Bacillus*-amoeba co-cultures and of the *Bacillus* axenic cultures harvested at the beginning of the stationary-phase assessed on sheep blood plates. Hemolytic activity was assessed by loading 100 µl of non-diluted supernatant in wells dug into the sheep blood plates. **a** Hemolytic activity of the supernatants of the amoeba-*Bacillus* co-cultures harvested at T2. T0 was defined as 1h after the initial contact of Bt cells with amoebas. **b** Hemolytic activity of fresh LB medium and the supernatants of the *Bacillus* stationary growth culture. Pictures were taken after 20 h of incubation at 30°C.

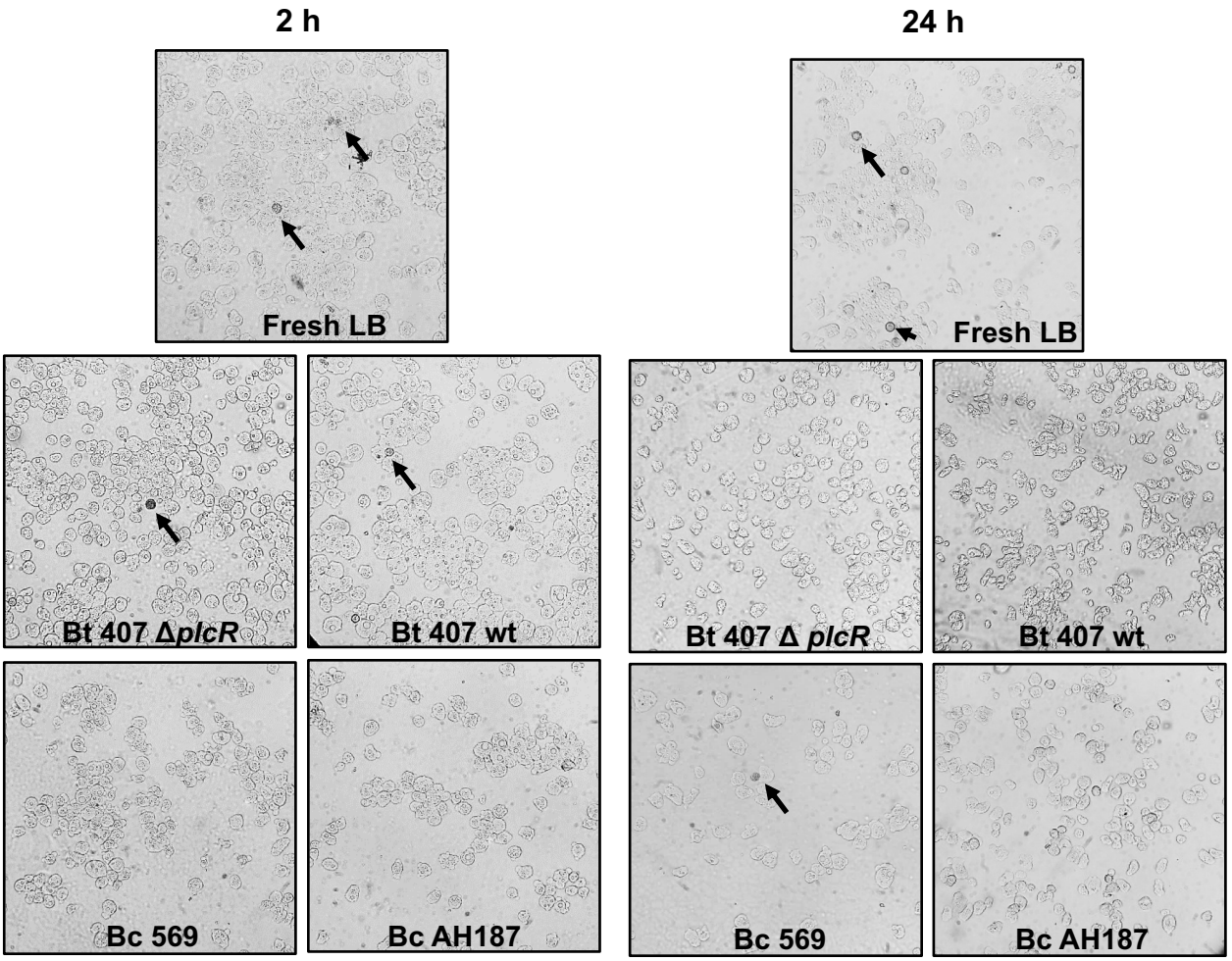


Figure S3. Viability of amoebas after cultured in the *Bacillus*-conditioned medium for 2h or 24h and assessed by trypan blue exclusion staining. The *Bacillus* supernatant was collected after growing the indicated strains to the beginning of the stationary phase (OD_{600nm} 3.0-4.0). 1.25×10^5 cell/ml of *A. castellanii* was cultured in the *Bacillus* supernatants for 2 h (left two columns) or 24h (right two columns) and stained by trypan blue. Representative microscopy images were shown. Cells stained in blue are indicated by arrows.

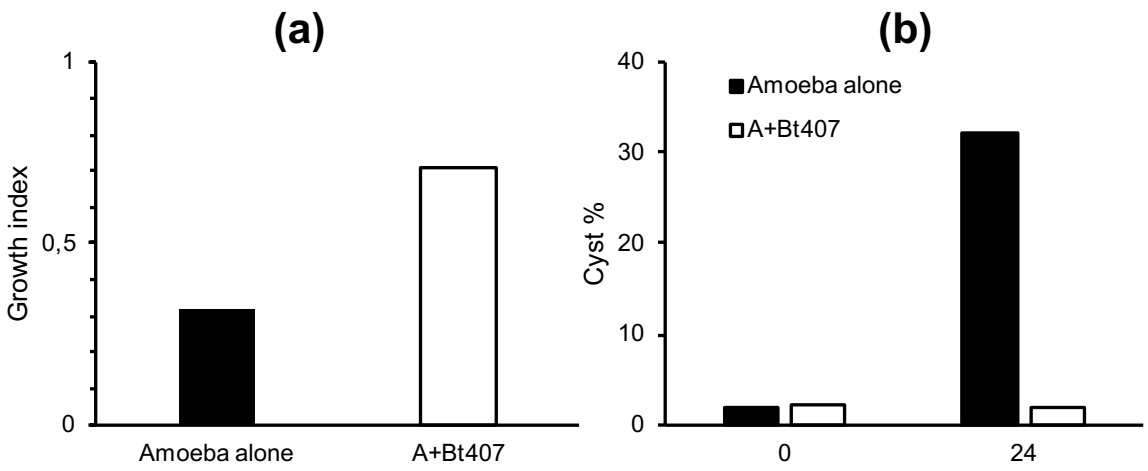


Figure S4. Development of the amoeba population in co-culture with spores or vegetative Bt 407 cells without *E. coli*. Amoeba growth index when they were cultured with or without Bt 407 Cry+ vegetative cells **(a)**. Percentage of cysts in the amoeba population when they were cultured with or without Bt 407 Cry+ vegetative cells **(b)**. The experiments were performed in PAS-Glu medium without *E. coli*.

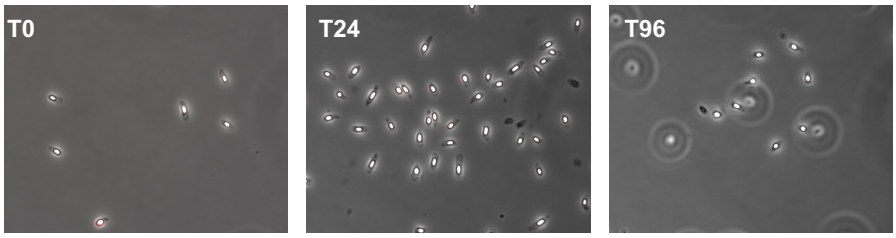


Figure S5. Micrographs of Bt 407 spores cultured axenically in the PAS-Glu-*E.coli* medium. Magnification of 100×, scale bar = 20 μm

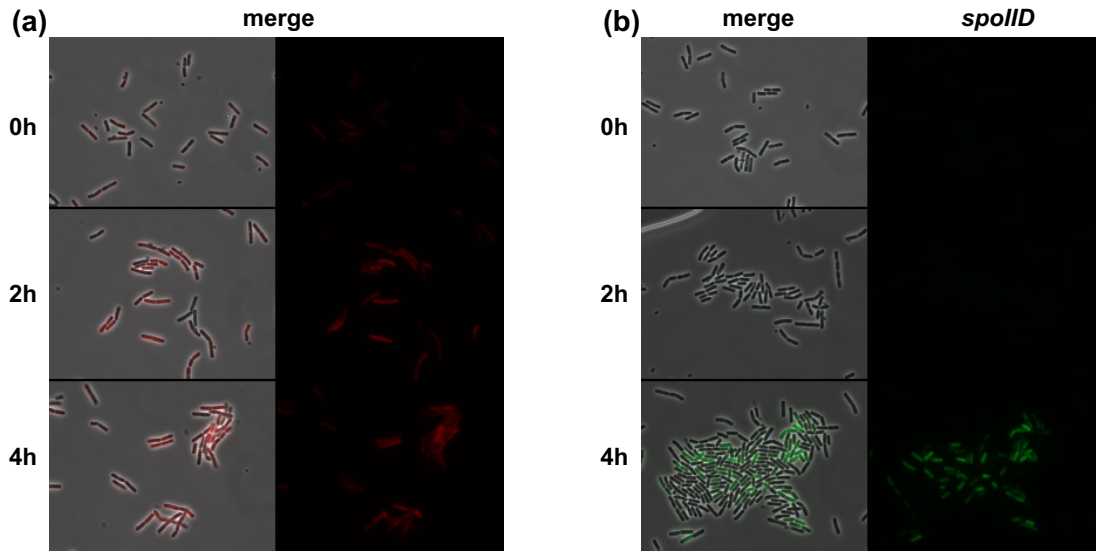


Figure S6. ***nprA*** **and** ***spoIID*** **promoter activities in vegetative Bt 407 cells in PAS-Glu-*E. coli* medium. a** Fluorescence microscopy images of Bt 407 nprA and **b** Bt 407 spoIID strains cultured in the medium for the indicated times. Left panel, merge of phase contrast and fluorescence images; right panels, epifluorescence images. The fluorescent mCherry and YFP signals were false-colored red and green, respectively. Pictures presented are representative pictures of 3 experiments

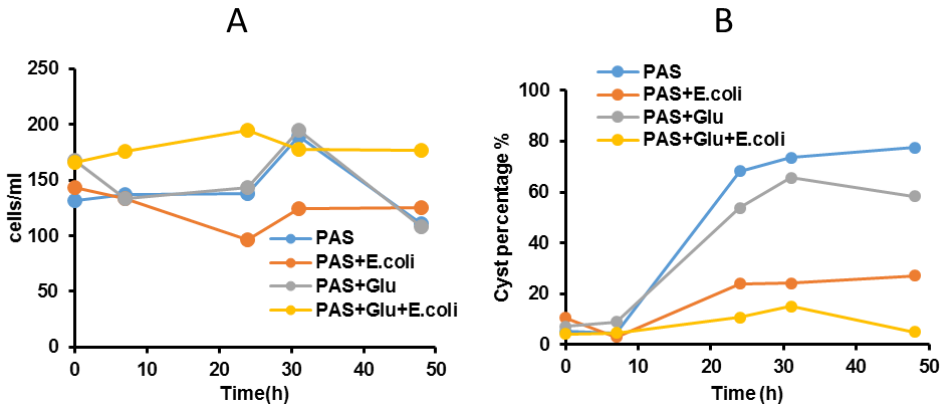


Figure 12 The effect of additional glucose and *E. coli* on amoebas growth and cyst formation.

The amoebas were cultured in PAS buffer with the addition of 0.1 M glucose or/and 10^7 *E. coli*/ (ml·24 h). The amoebas and cysts in the tissue culture flasks within the same size of field were counted. One experiment.

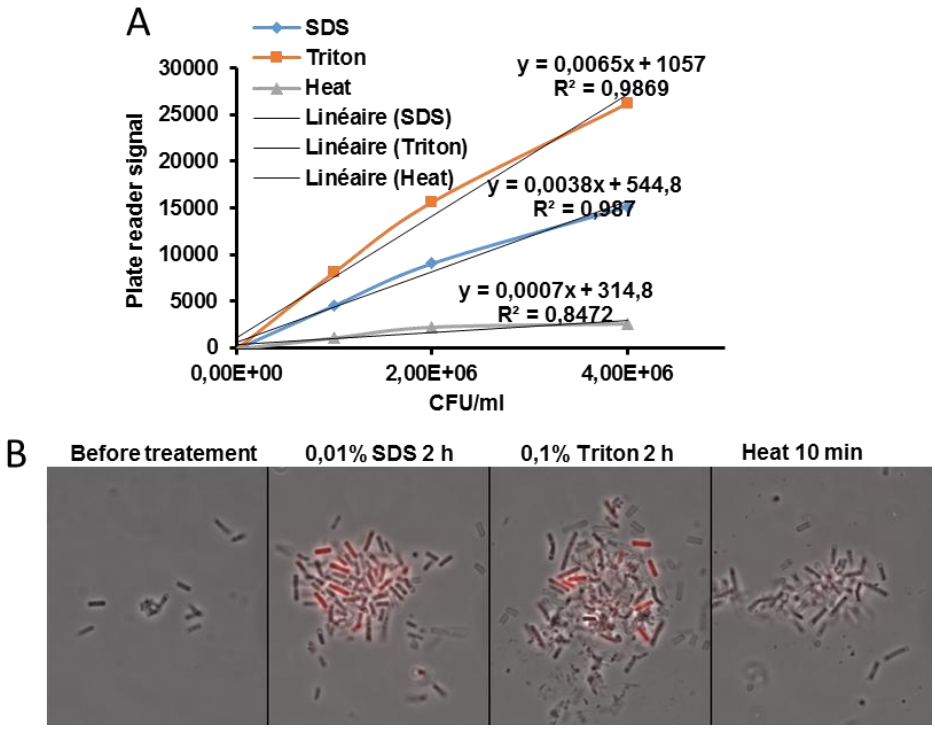


Figure 13 Plate reader signals intensities and microscope images of Bt cells after treatments.

(A) The correlation between Bt cell concentrations with plate reader signals. One experiment. The correlation equations were obtained by linear regression in excel. (B) Representative microscope images of bacteria before and after treatment.

The involvement of the PhD condidate in the work

The PhD candidate performed most of the experiments published in the article, except that the results about the growth of *Dictyostelium discoideum* on *Bacillus* were obtained by Tania Jauslin during collaboration with the lab of Pierre Cosson (Université de Genève).

The wildtype Bt strains are from the collection of the lab. The Bt strains harbouring plasmids or with *plcR* mutation are constructed previously by other lab members.

Additional results unincorporated in the published article

(1) I observed that the amoebas became cysts easily when cultured in the PAS buffer and this is not good to study the digestion of *Bacillus* because the cysts stop predation. Glucose and *E. coli* were included in co-culture experiments in some studies to delay cyst formation (Bakala N'Goma et al. 2015; Dey et al. 2012). To set up the bacteria-amoeba infection protocol, I first modified the PAS buffer. I have assessed the growth and cyst formation of amoebas in different PAS mediums with the addition of glucose or *E. coli* (Fig. 12). I found that the addition of 0.1 M glucose and 10^7 *E. coli*/ (ml·24 h) to the PAS buffer can help the amoebas to maintain a lower cyst formation compared to PAS buffer and amoebas have nearly no growth after 24 h in this modified buffer. This modified buffer was used in co-culture experiments of the article.

(2) To quantify the Bt population during co-culture, I tried to use a fluorescent probe to stain the Bt cells. I treated Bt 407 vegetative cells with SDS, Triton, or heat, then stained the cells with propidium iodide and assessed the fluorescence of cells.

Method: The Bt 407 cells were collected when bacteria OD_{600nm} reaches 1.0 and different volumes of culture were fixed in 50 % ethanol for 10 min. Then the bacteria pellets were treated with 250 µl of 1 mg/ml lysozyme at 37°C for 30 min. The bacteria were precipitated and resuspended in 250 µl of hybridization solution (0.01 % SDS) or Triton X100 (0.1 %) at room temperature for 2 h; or resuspended in hybridization solution without SDS and heated at 90°C for 10 min. Resuspend pellets in 100 µl PBS after 1 wash. Add 2 µl of propidium iodide to 20 µl of samples and incubate at room temperature for 10 min in dark. Then wash with PBS for 1 time and resuspend in 20 µl PBS. The fluorescence (excitation 535 nm, emission 617 nm) of the bacteria samples were assessed using a plate reader. The correlation between the concentration of bacteria and plate reader signals were shown in Fig. 13.

Different concentrations of Bt 407 vegetative cells were treated by 0.01 % SDS, 0.1 % Triton X100 or heat at 90°C for 10 min as described above. The Bt concentration and plate reader

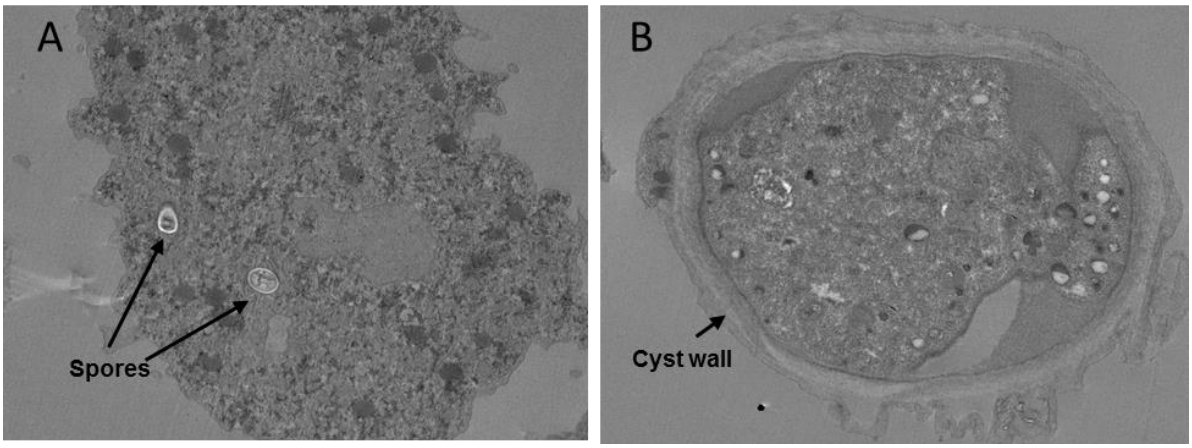


Figure 14 TEM images of amoebas co-cultured with Bt 407 spores for 48 h.

(A) Image of a trophozoite containing two spores. The spores are indicated by arrows. The gap between spores and amoeba indicates that the spores are located in vacuoles. (B) Image of an amoeba cyst. The cyst wall is indicated by arrow.

signals are ligne correlated (Fig. 13A). The signal of bacteria treated with 0.01 % SDS is the strongest, followed by Triton and heating. We observed the treated Bt cells under a fluorescent microscope and found that bacteria are lyzed efficiently in all treatments (Fig. 13B). The proportion of fluorescent bacteria population is higher when treated with 0.01 % SDS, the Triton and heating treated cells formed more ghost cells, which cannot retain the propidium iodide. This result indicates that the quantification of the Bt population by fluorescence is not a good method to use.

(3) Many bacteria can be retained between the cyst walls of amoebas, such as the *Mycobacterium*, *Streptococcus*, *Listeria* (Lambrecht et al. 2015; Siddiqui et al. 2017; Wheat et al. 2014). We tried to identify the presence of *Bacillus* in the amoeba cysts by Transmission electron microscopy (TEM).

Method: the amoebas were co-cultured with Bt 407 spores for 48 h. Ten milliliters of the coculture were harvested at 1200 rpm for 10 min, the pellets were washed with PAS buffer 2 times. The amoebas were resuspended in 50 µl of fixation solution (2 % glutaraldehyde in 0.1M cacodylate disodium buffer). Then the amoebas were kept at 4°C before sending to the TEM platform.

Results: After 48 h of co-culture, many amoebas became cysts; the spores in the coculture can germinate and form vegetative cells. We observed that the spores were presented in the vacuoles of amoebas (Fig. 14A), but no vegetative cells were observed, which are possibly digested by amoebas. We checked the cysts in all samples and found no spore nor vegetative cells in the cysts. Probably the *Bacillus* can not be retained inside amoeba cysts like the other bacteria.

5. Discussion and perspectives

The Bc group bacteria spread in different kinds of ecological niches. Ba uses animal carcasses (Turner et al. 2014), Bc infects humans causing food poisoning (Dietrich et al. 2021), Bt infects different species of arthropods (ie. Insects and nematodes) (Ehling-Schulz et al. 2019), and *B. mycoides* is frequently found in rhizosphere environment (Yi et al. 2018). Nevertheless, the soil is the original pool for all the *Bacillus* species. The stress-resistant *Bacillus* spores can persist in the soil for a very long time from several months to several years (Addison 1993; Van Cuyk et al. 2011). The *Bacillus* spores can reach a high concentration in the soil, about 10^6 spores/g (Hong et al. 2009; Siala et al. 1974) and the spores can be isolated from various types of soil in a wide range of temperatures (Guinebretière et al. 2008). However, *Bacillus* cannot grow effectively in the soil environment without nutrients input, except for some nutrient-rich sites with enough soluble organic matters (Vilain et al. 2006). How do the bacteria grow and reach such a high spore concentration in the soil is an important question. My thesis project was based on the hypothesis that the amoebas could favor the persistence of Bc group bacteria in the soil. The interaction of Bc group species with amoebas is studied in my thesis.

My results have shown that seven representative Bc group species have a similar behavior in the amoeba environment. A part of spores will germinate in the presence of amoeba or in the amoeba-conditioned medium, giving rise to vegetative cells that grow in the amoeba environment. The bacterial cells will then form chains or aggregates in the vicinity of the amoeba. Such an observation has been reported previously and it was suggested that these structures allow the *Bacillus* to resist the amoeba phagocytosis (Beeton et al. 2013). Our results obtained with representative species of Bt, Bc and psychrotolerant Bw suggest that all the Bc group bacteria have a similar germination and growth behavior in the amoeba environment.

Bacillus spores can germinate in the amoeba environment without direct contact with amoebas. Beeton and colleagues have shown the factors that caused spore germination are heat-resistant with small molecular weight (less than 5 kDa), they searched those factors in amoeba-conditioned PBS using mass spectrum but the spectrum profiles vary between experiments (Beeton et al. 2013). Our experiments used a similar medium - the amoeba-conditioned PAS-Glucose medium - and obtained similar results, a part of spores of different *Bacillus* species germinate in the amoeba-conditioned medium. The conditioned medium of macrophages has similar germination promotion effect on Ba spores (Weiner and Hanna 2003), suggesting germinants secreted in the medium may not be specific to amoebas. Weiner and Hanna have

found that the inosine and at least some amino acids secreted by macrophages triggered Ba germination. Small amino acids such as L-alanine, L-cysteine, L-threonine, and L-glutamine or their combination are nutrient germinants for spores (Hornstra et al. 2006). Amoebas produce a large amount of ammonia during vigorous growth (Weekers et al. 1996), which can work synergistically with L-alanine and inosine causing rapid germination, though ammonia alone is not an ion germinant (Preston and Douthit 1984). Amoebas possibly secrete some of these nutrient germinants and ammonia in the medium to trigger spore germination. To determine if it is the amino acids secreted by amoebas that have triggered spore germination and determine which amino acid is involved, we can use amino acid-specific germination receptor mutation *Bacillus* strains to test the amoeba-conditioned medium in the future, as has been shown (Weiner and Hanna 2003). Mass spectrum analysis of amoeba-conditioned medium focusing on detecting amino acids will also help to answer this question.

The proportion of spores that have germinated is low after cultured in amoeba-conditioned medium for 18 h ranging from 1.2 % to 22.4 %. The properties of spores and low concentration of germinants may explain the result. Some of the spores are superdormants, they have very low levels of germinant receptors and are hard to germinate (Setlow 2014a). The spores have variable mRNA contents. Only 6% of *Bacillus* mRNA has more than 1 copy per spore and these mRNA may be used as a ribonucleotides source for new RNA synthesis during germination (Byrd et al. 2021). The concentration of germinants in the environment affects spore germination efficacy. The germinants need to reach a certain concentration to trigger germination. The nutrient germinants L-alanine (100 mmol/L) and inosine (10 mmol/L) can trigger Bt and Ba spore germination in the soil, but they have no effect on spores when L-alanine concentration decreased to 5 mmol/L (Bishop 2014). The concentration of germinants in the amoeba-conditioned medium is not high due to the large volume of culture and low amino acids input from the PAS-Glu-*E. coli* buffer.

Some bacteria formed structures to resist phagocytosis, but amoebas may digest some vegetative cells. I used Bt 407 as a representative of the Bc group and observed that amoebas digested vegetative Bt cells to grow and delay encystment. However, the Bt spores were not used by amoebas. The Bt spores remained intact after 4 hours inside amoebas under time-lapse observation suggesting that spores can resist amoeba digestion. The spores do not promote amoeba growth or delay cyst formation. Santos and colleagues observed the growth of protists on both Bc spores and vegetative cells (Santos et al. 2017). The growth of *A. castellanii* on

vegetative cells and active spores is higher than growth on inactivated spores in that study, this may suggest that spore structure (inactivated spores) resists amoeba digestion (lower amoeba growth) and the amoebas may have grown on germinated spores rather than using active spores directly (high amoeba growth). The Ba spores not only resist amoeba digestion, but they can also germinate and multiply intracellularly and lyse amoebas to be released (Dey et al. 2012). Unlike Ba spores, I did not observe germination of Bt 407 spores when they were found inside amoebas. It seems Ba is an exception among the Bc species during interaction with protists. The chromosomally encoded germination receptor *gerH* and gene(s) located on pXO1 may be responsible for Ba germination intracellularly (Dey et al. 2012).

Most of the Bc group bacteria (except Ba) can produce a series of virulence factors during stationary growth, which are hemolytic to erythrocytes or epithelial cells, as well as mammalian macrophages (Lindbäck et al. 2004; Sastalla et al. 2013; Tran, Puhar, et al. 2011). We observed that the virulence factors produced by vegetative growth Bt or Bc strains do not affect the viability of amoebas. The virulence factors need to bind the target cells to form active toxins (Dietrich et al. 2021; Sastalla et al. 2013), they might not bind to amoeba membranes and thus showed no toxicity effect. In addition, amoebas have detoxification mechanisms that digest or export the toxic elements (Choi et al. 2005; Grechnikova et al. 2020; König et al. 2020). The social amoebas *Dictyostelium* protect themselves by the differentiated sentinel cells which engulf bacteria and sequester toxins (Chen, Zhuchenko, and Kuspa 2007). These mechanisms may protect the amoebas from Bt and Bc virulence factors. It will be interesting to determine the mechanisms amoebas use to resist *Bacillus* virulence factors in the future, and it is also interesting to study if those virulence factors can protect *Bacillus* from the other soil protists.

The Bt strain can also produce large amount of Cry and Cyt toxins during sporulation. I did not observe a side effect when Bt strains were cocultured with amoebas until sporulation. It will be interesting to study the toxicity of the Cry and Cyt toxins to amoebas in the future. The Cry and Cyt toxins played an important role during Bt infection of insects. Cry toxins paralyze and kill the insects, Cyt has a cytolytic activity and worked as receptors to enhance the toxicity of Cry (Cantón et al. 2011; Pérez et al. 2007). Cry toxins bind to specific receptors to lyse the target cells and they are toxic to many species of insect larvae and some cancer cells (Bravo et al. 2011; Mendoza-Almanza et al. 2020). Cyt toxins show non-specific toxicity to cells and are mainly toxic to Diptera insects (Palma et al. 2014). Besides the toxins with insecticidal, cancer killing, and nematocide activities, we do not know the targets of a large part of Cry toxins. It

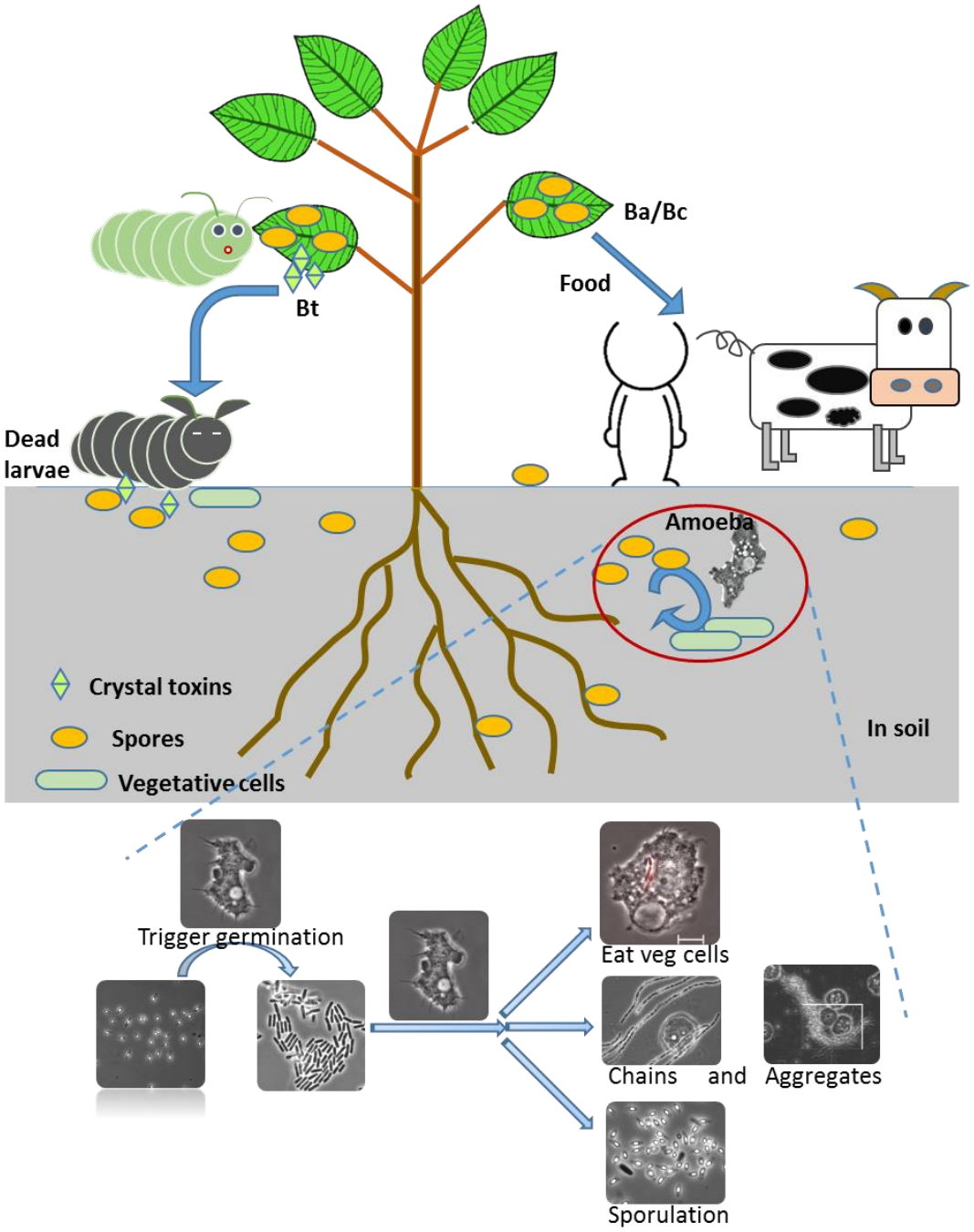


Figure 15 The ecology of the *B. cereus* group bacteria.

The *B. thuringiensis* spores can infect insects and grow in cadavers. The *B. anthracis* and *B. cereus* strains can infect mammals including humans. In the soil environment, amoeba secreted germinants or environmental nutrients triggers *Bacillus* spore germination. Vegetative cells then grow as chains/aggregates resisting amoeba digestion and sporulate completing a life cycle. Amoebas promotes a faster sporulation.

will be interesting to know if some Cry or Cyt toxins are toxic to amoebas or other protists. This may further explain the role of these toxins in the ecological adaptation of Bt.

The survival and growth of Bc group in the amoeba environment is different from other pathogenic species. Some pathogenic bacteria can resist digestion by amoebas and form bacteria-containing vacuoles for survival and multiplication, such as *Legionella*-containing vacuole (Steiner et al. 2018) and *Salmonella*-containing vacuole (Valenzuela et al. 2021). However, amoebas can promote the growth of some bacteria without internalizing them such as *K. pneumoniae* (Maisonneuve et al. 2017), and *S. aureus* (Maisonneuve 2017). For the Bc group species, vegetative cells cannot survive in amoeba vacuoles (except Ba), they survive as spores or by forming chains or aggregate structures. The amoebas help the growth of Bc group bacteria in their vicinity by promoting spore germination and vegetative cell sporulation. Taking advantage of amoebas, it will be interesting to develop a pesticide containing both amoebas and Bt strains in the future. The amoebas may help the persistence of Bt in the field and thus increases the efficacy of pesticides.

In a general view of the ecology of Bc group bacteria, the bacteria persist mainly in the soil (Fig. 15). The *Bacillus* spores may contaminate plant leaves. For the Bt spores, they can be ingested by insects, then germinate in vivo and kill the insect starting necrotrophic growth, finally, the cells sporulate and be released to the environment. For the Ba and Bc cells, they can occasionally infect mammals including humans. Ba can kill the animal and multiply in the carcasses, and they will sporulate when exposed to the air. Ba spores in the soil and grass can cause a second round of infection. The Bc cells can be ingested and cause food poisoning in humans, and be released in feces. My work about amoebas complemented the persistence of *Bacillus* spores in the soil, showing that they can have a full lifecycle and growth in the amoeba environment.

In the soil environment, the amoeba population increases as the environmental nutrients increase, the chances of amoebas meet the *Bacillus* spores increase at the same time. The amoebas secrete germinants perhaps amino acids which triggers a small part of *Bacillus* spores to germinate. For most of the *Bacillus* cells except Ba, the vegetative cells multiply rapidly using the nutrients from the environment or nutrients secreted by amoebas, a part of cells form chains or aggregates to resist amoeba digestion, a part of single cells may be digested by amoebas. The vegetative cells sporulating again finishing a whole life. The presence of amoebas accelerates the sporulation process, which reduced the chances of *Bacillus* being

digested by protists. For Ba spores, they exploit amoebas to germinate, grow intracellularly, and are released after lysing amoebas. The presence of amoebas wakes up a part of *Bacillus* spores to exploit the environmental nutrients for multiplication, which may help *Bacillus* to maintain or increase the population.

References of Introduction and discussion

Abdoarrahem, M. M., K. Gammon, B. N. Dancer, and C. Berry. 2009. "Genetic Basis for Alkaline Activation of Germination in *Bacillus Thuringiensis* Subsp. Israelensis." *Applied and Environmental Microbiology* 75 (19): 6410–13. https://doi.org/10.1128/AEM.00962-09.

Abriouel, Hikmate, Charles M.A.P. Franz, Nabil Ben Omar, and Antonio Gálvez. 2011. "Diversity and Applications of *Bacillus* Bacteriocins." *FEMS Microbiology Reviews* 35 (1): 201–32. https://doi.org/10.1111/j.1574-6976.2010.00244.x.

Adak, Atanu, and Mojibur R. Khan. 2019. "An Insight into Gut Microbiota and Its Functionalities." *Cellular and Molecular Life Sciences* 76(3):473–93. doi: 10.1007/s00018-018-2943-4.

Addison, J.a. 1993. "Persistence and Nontarget Effects of *Bacillus thuringiensis* in Soil: A Review." *Canadian Journal of Forest Research* 23 (11): 2329–42. https://doi.org/10.1139/x93-287.

Adiba, Sandrine, Clément Nizak, Minus van Baalen, Erick Denamur, and Frantz Depaulis. 2010. "From Grazing Resistance to Pathogenesis: The Coincidental Evolution of Virulence Factors." *PLOS ONE* 5(8):e11882. doi: 10.1371/journal.pone.0011882.

Adl, Sina M., Alastair G. B. Simpson, Mark A. Farmer, Robert A. Andersen, O. Roger Anderson, John R. Barta, Samuel S. Bowser, Guy Brugerolle, Robert A. Fensome, Suzanne Fredericq, Timothy Y. James, Sergei Karpov, Paul Kugrens, John Krug, Christopher E. Lane, Louise A. Lewis, Jean Lodge, Denis H. Lynn, David G. Mann, Richard M. Mccourt, Leonel Mendoza, Øjvind Moestrup, Sharon E. Mozley-Standridge, Thomas A. Nerad, Carol A. Shearer, Alexey V. Smirnov, Frederick W. Spiegel, and Max F. J. R. Taylor. 2005. "The New Higher Level Classification of Eukaryotes with Emphasis on the Taxonomy of Protists." *Journal of Eukaryotic Microbiology* 52(5):399–451. doi: https://doi.org/10.1111/j.1550-7408.2005.00053.x.

Agaisse, H., and D. Lereclus. 1995. "How Does *Bacillus Thuringiensis* Produce so Much Insecticidal Crystal Protein?" *Journal of Bacteriology* 177 (21): 6027–32. https://doi.org/10.1128/jb.177.21.6027-6032.1995.

Agaisse, Hervé, Myriam Gominet, Ole Andreas Økstad, Anne-Brit Kolstø, and Didier Lereclus. 1999. "PlcR Is a Pleiotropic Regulator of Extracellular Virulence Factor Gene Expression in *Bacillus Thuringiensis*." *Molecular Microbiology* 32 (5): 1043–53. https://doi.org/10.1046/j.1365-2958.1999.01419.x.

Alameh, Saleem, Gloria Bartolo, Summer O'Brien, Elizabeth A. Henderson, Leandra O. Gonzalez, Stella Hartmann, Christopher P. Klimko, Jennifer L. Shoe, Christopher K. Cote, Laurence K. Grill, Anastasia Levitin, and Mikhail Martchenko Shilman. 2020. "Anthrax Toxin Component, Protective Antigen, Protects Insects from Bacterial Infections." *PLOS Pathogens* 16(8):e1008836. doi: 10.1371/journal.ppat.1008836.

Allen, Philip G., and Eliezar A. Dawidowicz. 1990. "Phagocytosis in *Acanthamoeba*: I. A Mannose Receptor Is Responsible for the Binding and Phagocytosis of Yeast." *Journal of Cellular Physiology* 145 (3): 508–13. https://doi.org/10.1002/jcp.1041450317.

Ammons, David R., John D. Short, Jeffery Bailey, Gabriela Hinojosa, Lourdes Tavarez, Martha Salazar, and Joanne N. Rampersad. 2016. "Anti-Cancer Parasporin Toxins Are Associated with Different Environments: Discovery of Two Novel Parasporin 5-like Genes." *Current Microbiology* 72(2):184–89. doi: 10.1007/s00284-015-0934-3.

Andreu, Nuria, Jody Phelan, Paola F. de Sessions, Jacqueline M. Cliff, Taane G. Clark, and Martin L. Hibberd. 2017. "Primary Macrophages and J774 Cells Respond Differently to Infection with *Mycobacterium Tuberculosis*." *Scientific Reports* 7 (1): 1–12. https://doi.org/10.1038/srep42225.

Anon. 2021. "Global Biopesticides Market Report 2021: Industry Trends, Share, Size, Growth, Opportunity and Forecast 2015-2026 - ResearchAndMarkets.Com." Retrieved May 24, 2021 (https://www.businesswire.com/news/home/20210219005412/en/Global-Biopesticides-Market-Report-2021-Industry-Trends-Share-Size-Growth-Opportunity-and-Forecast-2015-2026---ResearchAndMarkets.com).

Ash, C., and M. D. Collins. 1992. "Comparative Analysis of 23S Ribosomal RNA Gene Sequences of *Bacillus Anthracis* and Emetic *Bacillus Cereus* Determined by PCR-Direct Sequencing." *FEMS Microbiology Letters* 73 (1–2): 75–80. https://doi.org/10.1016/0378-1097(92)90586-d.

Ash, C., J. A. Farrow, M. Dorsch, E. Stackebrandt, and M. D. Collins. 1991. "Comparative Analysis of *Bacillus Anthracis*, *Bacillus Cereus*, and Related Species on the Basis of Reverse Transcriptase Sequencing of 16S RRNA." *International Journal of Systematic Bacteriology* 41 (3): 343–46. https://doi.org/10.1099/00207713-41-3-343.

Avery, S V, D Lloyd, and J L Harwood. 1995. "Temperature-Dependent Changes in Plasma-Membrane Lipid Order and the Phagocytotic Activity of the Amoeba *Acanthamoeba Castellanii* Are Closely Correlated." *Biochemical Journal* 312 (3): 811–16. https://doi.org/10.1042/bj3120811.

Ayangbenro, Ayansina Segun, and Olubukola Oluranti Babalola. 2020. "Genomic Analysis of *Bacillus Cereus* NWUAB01 and Its Heavy Metal Removal from Polluted Soil." *Scientific Reports* 10 (1): 19660. https://doi.org/10.1038/s41598-020-75170-x.

Bagyan, I., J. Hobot, and S. Cutting. 1996. "A Compartmentalized Regulator of Developmental Gene Expression in *Bacillus Subtilis*." *Journal of Bacteriology* 178 (15): 4500–4507. https://doi.org/10.1128/jb.178.15.4500-4507.1996.

Bai, U., I. Mandic-Mulec, and I. Smith. 1993. "SinI Modulates the Activity of SinR, a Developmental Switch Protein of *Bacillus Subtilis*, by Protein-Protein Interaction." *Genes & Development* 7 (1): 139–48. https://doi.org/10.1101/gad.7.1.139.

Bakala N'Goma, Jean Claude, Vincent Le Moigne, Nathalie Soismier, Laura Laencina, Fabien Le Chevalier, Anne-Laure Roux, Isabelle Poncin, Carole Serveau-Avesque, Martin Rottman, Jean-Louis Gaillard, Gilles Etienne, Roland Brosch, Jean-Louis Herrmann, Stéphane Canaan, and Fabienne Girard-Misguich. 2015. "Mycobacterium Abscessus Phospholipase C Expression Is Induced during Coculture within Amoebae and Enhances M. Abscessus Virulence in Mice" edited by C. R. Roy. *Infection and Immunity* 83(2):780. doi: 10.1128/IAI.02032-14.

Barbosa, Teresa M., Cláudia R. Serra, Roberto M. La Ragione, Martin J. Woodward, and Adriano O. Henriques. 2005. "Screening for Bacillus Isolates in the Broiler Gastrointestinal Tract." *Applied and Environmental Microbiology* 71(2):968–78. doi: 10.1128/AEM.71.2.968-978.2005.

Beecher, Douglas J., Timothy W. Olsen, Eileen B. Somers, and Amy C. L. Wong. 2000. "Evidence for Contribution of Tripartite Hemolysin BL, Phosphatidylcholine-Preferring Phospholipase C, and Collagenase to Virulence of *Bacillus Cereus* Endophthalmitis." *Infection and Immunity* 68 (9): 5269–76. https://doi.org/10.1128/IAI.68.9.5269-5276.2000.

Beeton, M. L., D. J. Atkinson, and N. R. Waterfield. 2013. "An Amoeba Phagocytosis Model Reveals a Novel Developmental Switch in the Insect Pathogen *Bacillus Thuringiensis*." *Journal of Insect Physiology*, Immune Interactions Between Insects and Their Natural Antagonists: a Workshop Honoring Professor Stuart E. Reynolds, 59 (2): 223–31. https://doi.org/10.1016/j.jinsphys.2012.06.011.

Ben Rejeb, Samia, Didier Lereclus, and Leyla Slamti. 2017. "Analysis of AbrB Expression during the Infectious Cycle of *Bacillus Thuringiensis* Reveals Population Heterogeneity." *Frontiers in Microbiology* 0. https://doi.org/10.3389/fmicb.2017.02471.

Benghezal, Mohammed, Marie-Odile Fauvarque, Régis Tournebize, Romain Froquet, Anna Marchetti, Evelyne Bergeret, Bernard Lardy, et al. 2006. "Specific Host Genes Required for the Killing of *Klebsiella* Bacteria by Phagocytes." *Cellular Microbiology* 8 (1): 139–48. https://doi.org/10.1111/j.1462-5822.2005.00607.x.

Bergman, Nicholas H., Erica C. Anderson, Ellen E. Swenson, Brian K. Janes, Nathan Fisher, Matthew M. Niemeyer, Amy D. Miyoshi, and Philip C. Hanna. 2007. "Transcriptional Profiling of *Bacillus Anthracis* during Infection of Host Macrophages." *Infection and Immunity* 75 (7): 3434–44. https://doi.org/10.1128/IAI.01345-06.

Berliner, Ernst. 1915. "Über Die Schlaffsucht Der Mehlmottenraupe (Ephestia Kühniella Zell.) Und Ihren Erreger *Bacillus Thuringiensis* n. Sp." *Zeitschrift Für Angewandte Entomologie* 2 (1): 29–56. https://doi.org/10.1111/j.1439-0418.1915.tb00334.x.

Berry, Colin, Susan O'Neil, Eitan Ben-Dov, Andrew F. Jones, Lee Murphy, Michael A. Quail, Mathew T. G. Holden, David Harris, Arieh Zaritsky, and Julian Parkhill. 2002. "Complete Sequence and Organization of PBtoxis, the Toxin-Coding Plasmid of *Bacillus Thuringiensis* Subsp. *Israelensis*." *Applied and Environmental Microbiology* 68 (10): 5082–95. https://doi.org/10.1128/AEM.68.10.5082-5095.2002.

Berthold-Pluta, Anna, Antoni Pluta, and Monika Garbowska. 2015. "The Effect of Selected Factors on the Survival of *Bacillus Cereus* in the Human Gastrointestinal Tract." *Microbial Pathogenesis* 82 (May): 7–14. https://doi.org/10.1016/j.micpath.2015.03.015.

Berthold-Pluta, Anna, Antoni Pluta, and Monika Garbowska. 2015. "The Effect of Selected Factors on the Survival of *Bacillus Cereus* in the Human Gastrointestinal Tract." *Microbial Pathogenesis* 82 (May): 7–14. https://doi.org/10.1016/j.micpath.2015.03.015.

Blackburn, Jason K., Andrew Curtis, Ted L. Hadfield, Bob O'Shea, Mark A. Mitchell, and Martin E. Hugh-Jones. 2010. "Confirmation of *Bacillus Anthracis* from Flesh-Eating Flies Collected during a West Texas Anthrax Season." *Journal of Wildlife Diseases* 46 (3): 918–22. https://doi.org/10.7589/0090-3558-46.3.918.

Bleasdale, Benjamin, Penelope J. Lott, Aparna Jagannathan, Mark P. Stevens, Richard J. Birtles, and Paul Wigley. 2009. "The *Salmonella* Pathogenicity Island 2-Encoded Type III Secretion System Is Essential for the Survival of *Salmonella Enterica* Serovar *Typhimurium* in Free-Living Amoebae." *Applied and Environmental Microbiology* 75 (6): 1793–95. https://doi.org/10.1128/AEM.02033-08.

Bloomfield, Gareth, David Traynor, Sophia P Sander, Douwe M Veltman, Justin A Pachebat, and Robert R Kay. 2015. "Neurofibromin Controls Macropinocytosis and Phagocytosis in *Dictyostelium*." Edited by W James Nelson. *ELife* 4 (March): e04940. https://doi.org/10.7554/eLife.04940.

Boland, Fiona M., Abdelmadjid Atrih, Haridasan Chirakkal, Simon J. Foster, and Anne Moir. 2000. "Complete Spore-Cortex Hydrolysis during Germination of *Bacillus Subtilis* 168 Requires SleB and YpeB." *Microbiology* 146 (Pt 1) (January): 57–64. https://doi.org/10.1099/00221287-146-1-57.

Bottone, Edward J. 2010. "*Bacillus Cereus*, a Volatile Human Pathogen." *Clinical Microbiology Reviews* 23 (2): 382–98. https://doi.org/10.1128/CMR.00073-09.

Bowers, B. 1977. "Comparison of Pinocytosis and Phagocytosis in *Acanthamoeba Castellanii*." *Experimental Cell Research* 110 (2): 409–17. https://doi.org/10.1016/0014-4827(77)90307-X.

Bozzaro, Salvatore. 2019. "The Past, Present and Future of *Dictyostelium* as a Model System." *The International Journal of Developmental Biology* 63 (8-9–10): 321–31. https://doi.org/10.1387/ijdb.190128sb.

Bozzaro, Salvatore, and Ludwig Eichinger. 2011. "The Professional Phagocyte *Dictyostelium Discoideum* as a Model Host for Bacterial Pathogens." *Current Drug Targets* 12 (7): 942–54. https://doi.org/10.2174/138945011795677782.

Bradarić, Nikola, and Volga Punda-Polić. 1992. "Cutaneous Anthrax Due to Penicillin-Resistant *Bacillus Anthracis* Transmitted by an Insect Bite." *The Lancet*, Originally published as Volume 2, Issue 8814, 340 (8814): 306–7. https://doi.org/10.1016/0140-6736(92)92395-V.

Bravo, Alejandra, Sarjeet S. Gill, and Mario Soberón. 2007. "Mode of Action of *Bacillus Thuringiensis* Cry and Cyt Toxins and Their Potential for Insect Control." *Toxicon : Official Journal of the International Society on Toxinology* 49 (4): 423–35. https://doi.org/10.1016/j.toxicon.2006.11.022.

Bravo, Alejandra, Supaporn Likitvivatanavong, Sarjeet S. Gill, and Mario Soberón. 2011. "*Bacillus Thuringiensis*: A Story of a Successful Bioinsecticide." *Insect Biochemistry and Molecular Biology*, Special Issue: Toxicology and Resistance, 41 (7): 423–31. https://doi.org/10.1016/j.ibmb.2011.02.006.

Brindley, Neil, Abdul Matin, and Naveed Ahmed Khan. 2009. "*Acanthamoeba Castellanii*: High Antibody Prevalence in Racially and Ethnically Diverse Populations." *Experimental Parasitology* 121 (3): 254–56. https://doi.org/10.1016/j.exppara.2008.11.009.

Brock, Debra A., Tracy E. Douglas, David C. Queller, and Joan E. Strassmann. 2011. "Primitive Agriculture in a Social Amoeba." *Nature* 469(7330):393–96. doi: 10.1038/nature09668.

Brock, Debra A., Tamara S. Haselkorn, Justine R. Garcia, Usman Bashir, Tracy E. Douglas, Jesse Galloway, Fisher Brodie, David C. Queller, and Joan E. Strassmann. 2018. "Diversity of Free-Living Environmental Bacteria and Their Interactions With a Bactivorous Amoeba." *Frontiers in Cellular and Infection Microbiology* 8. doi: 10.3389/fcimb.2018.00411.

Burbulys, David, Kathleen A. Trach, and James A. Hoch. 1991. "Initiation of Sporulation in *B. Subtilis* Is Controlled by a Multicomponent Phosphorelay." *Cell* 64 (3): 545–52. https://doi.org/10.1016/0092-8674(91)90238-T.

Butko, Peter. 2003. "Cytolytic Toxin Cyt1A and Its Mechanism of Membrane Damage: Data and Hypotheses." *Applied and Environmental Microbiology* 69(5):2415–22. doi: 10.1128/AEM.69.5.2415-2422.2003.

Byrd, Brandon, Emily Camilleri, George Korza, D. Levi Craft, Joshua Green, Maria Rocha Granados, Wendy W. K. Mok, Melissa J. Caimano, and Peter Setlow. 2021. "Levels and Characteristics of MRNAs in Spores of Firmicute Species." *Journal of Bacteriology*. doi: 10.1128/JB.00017-21.

Byrne, Matthew J., Matthew G. Iadanza, Marcos Arribas Perez, Daniel P. Maskell, Rachel M. George, Emma L. Hesketh, Paul A. Beales, Marc D. Zack, Colin Berry, and Rebecca F. Thompson. 2021. "Cryo-EM Structures of an Insecticidal Bt Toxin Reveal Its Mechanism of Action on the Membrane." *Nature Communications* 12(1):2791. doi: 10.1038/s41467-021-23146-4.

Callegan, Michelle C., Scott T. Kane, D. Clay Cochran, Michael S. Gilmore, Myriam Gominet, and Didier Lereclus. 2003. "Relationship of PlcR-Regulated Factors to *Bacillus* Endophthalmitis Virulence." *Infection and Immunity* 71 (6): 3116–24. https://doi.org/10.1128/IAI.71.6.3116-3124.2003.

Campbell, S. J., P. R. Ingram, C. W. Roberts, and F. L. Henriquez. 2008. "Induced Encystment Improves Resistance to Preservation and Storage of *Acanthamoeba Castellanii*." *Parasitology* 135 (12): 1401–5. https://doi.org/10.1017/S0031182008005003.

Cantón, Pablo Emiliano, Esmeralda Zanicthe Zanicthe Reyes, Iñigo Ruiz de Escudero, Alejandra Bravo, and Mario Soberón. 2011. "Binding of Bacillus Thuringiensis Subsp. Israelensis Cry4Ba to Cyt1Aa Has an Important Role in Synergism." *Peptides* 32(3):595–600. doi: 10.1016/j.peptides.2010.06.005.

Cardoso, Priscilla de F., Stéphane Perchat, Laurival A. Vilas-Boas, Didier Lereclus, and Gislayne T. Vilas-Bôas. 2019. "Diversity of the Rap-Phr Quorum-Sensing Systems in

the *Bacillus Cereus* Group." *Current Genetics* 65 (6): 1367–81. https://doi.org/10.1007/s00294-019-00993-9.

Cardoso, Priscilla, Fernanda Fazion, Stéphane Perchat, Christophe Buisson, Gislayne Vilas-Bôas, and Didier Lereclus. 2020. "Rap-Phr Systems from Plasmids PAW63 and PHT8-1 Act Together To Regulate Sporulation in the *Bacillus Thuringiensis* Serovar *Kurstaki* HD73 Strain." *Applied and Environmental Microbiology* 86 (18). https://doi.org/10.1128/AEM.01238-20.

Castiaux, Virginie, Xiaojin Liu, Laurence Delbrassinne, and Jacques Mahillon. 2015. "Is Cytotoxin K from *Bacillus Cereus* a Bona Fide Enterotoxin?" *International Journal of Food Microbiology* 211 (October): 79–85. https://doi.org/10.1016/j.ijfoodmicro.2015.06.020.

Cavalier-Smith, T. 1998. "A Revised Six-Kingdom System of Life." *Biological Reviews* 73(3):203–66. doi: https://doi.org/10.1111/j.1469-185X.1998.tb00030.x.

Chakroun, Maissa, Núria Banyuls, Yolanda Bel, Baltasar Escriche, and Juan Ferré. 2016. "Bacterial Vegetative Insecticidal Proteins (Vip) from Entomopathogenic Bacteria." *Microbiology and Molecular Biology Reviews* 80(2):329–50. doi: 10.1128/MMBR.00060-15.

Chakroun, Maissa, and Juan Ferré. 2014. "In Vivo and in Vitro Binding of Vip3Aa to *Spodoptera Frugiperda* Midgut and Characterization of Binding Sites by (125)I Radiolabeling." *Applied and Environmental Microbiology* 80 (20): 6258–65. https://doi.org/10.1128/AEM.01521-14.

Chastanet, Arnaud, and Richard Losick. 2011. "Just-in-Time Control of Spo0A Synthesis in *Bacillus Subtilis* by Multiple Regulatory Mechanisms." *Journal of Bacteriology* 193 (22): 6366–74. https://doi.org/10.1128/JB.06057-11.

Chateau, Alice, Justin Mark Lunderberg, So Young Oh, Teresa Abshire, Arthur Friedlander, Conrad P. Quinn, Dominique M. Missiakas, and Olaf Schneewind. 2018. "Galactosylation of the Secondary Cell Wall Polysaccharide of *Bacillus Anthracis* and Its Contribution to Anthrax Pathogenesis." *Journal of Bacteriology* 200 (5). https://doi.org/10.1128/JB.00562-17.

Chen, Guokai, Olga Zhuchenko, and Adam Kuspa. 2007. "Immune-like Phagocyte Activity in the Social Amoeba." *Science* 317(5838):678–81. doi: 10.1126/science.1143991.

Chen, M. L., and H. Y. Tsen. 2002. "Discrimination of *Bacillus Cereus* and *Bacillus Thuringiensis* with 16S RRNA and GyrB Gene Based PCR Primers and Sequencing of Their Annealing Sites." *Journal of Applied Microbiology* 92 (5): 912–19. https://doi.org/10.1046/j.1365-2672.2002.01606.x.

Choi, Min-Ho, Dana Sajed, Leslie Poole, Ken Hirata, Scott Herdman, Bruce E. Torian, and Sharon L. Reed. 2005. "An Unusual Surface Peroxiredoxin Protects Invasive Entamoeba Histolytica from Oxidant Attack." *Molecular and Biochemical Parasitology* 143(1):80–89. doi: 10.1016/j.molbiopara.2005.04.014.

Chow, E., G. J. Singh, and S. S. Gill. 1989. “Binding and Aggregation of the 25-Kilodalton Toxin of *Bacillus Thuringiensis* Subsp. *Israelensis* to Cell Membranes and Alteration by Monoclonal Antibodies and Amino Acid Modifiers.” *Applied and Environmental Microbiology* 55 (11): 2779–88. https://doi.org/10.1128/aem.55.11.2779-2788.1989.

Chumsakul, Onuma, Hiroki Takahashi, Taku Oshima, Takahiro Hishimoto, Shigehiko Kanaya, Naotake Ogasawara, and Shu Ishikawa. 2011. “Genome-Wide Binding Profiles of the *Bacillus Subtilis* Transition State Regulator AbrB and Its Homolog Abh Reveals Their Interactive Role in Transcriptional Regulation.” *Nucleic Acids Research* 39 (2): 414–28. https://doi.org/10.1093/nar/gkq780.

Colson, Philippe, Bernard La Scola, Anthony Levasseur, Gustavo Caetano-Anollés, and Didier Raoult. 2017. “Mimivirus: Leading the Way in the Discovery of Giant Viruses of Amoebae.” *Nature Reviews Microbiology* 15(4):243–54. doi: 10.1038/nrmicro.2016.197.

Contreras-Pérez, Miguel, Julie Hernández-Salmerón, Daniel Rojas-Solís, Carmen Rocha-Granados, Ma. del Carmen Orozco-Mosqueda, Fannie I. Parra-Cota, Sergio de los Santos-Villalobos, and Gustavo Santoyo. 2019. “Draft Genome Analysis of the Endophyte, *Bacillus Toyonensis* COPE52, a Blueberry (Vaccinium Spp. Var. Biloxi) Growth-Promoting Bacterium.” *3 Biotech* 9 (10): 370. https://doi.org/10.1007/s13205-019-1911-5.

Contzen, Matthias, Mandy Hailer, and Jörg Rau. 2014. “Isolation of *Bacillus Cytotoxicus* from Various Commercial Potato Products.” *International Journal of Food Microbiology* 174 (March): 19–22. https://doi.org/10.1016/j.ijfoodmicro.2013.12.024.

Cosson, Pierre, and Wanessa C. Lima. 2014. “Intracellular Killing of Bacteria: Is *Dictyostelium* a Model Macrophage or an Alien?” *Cellular Microbiology* 16 (6): 816–23. https://doi.org/10.1111/cmi.12291.

Cotter, David A., and Kenneth B. Raper. 1968. “Properties of Germinating Spores of *Dictyostelium Discoideum*.” *Journal of Bacteriology* 96 (5): 1680–89.

Crickmore, N., D. R. Zeigler, J. Feitelson, E. Schnepf, J. Van Rie, D. Lereclus, J. Baum, and D. H. Dean. 1998. “Revision of the Nomenclature for the *Bacillus Thuringiensis* Pesticidal Crystal Proteins.” *Microbiology and Molecular Biology Reviews* 62 (3): 807–13. https://doi.org/10.1128/MMBR.62.3.807-813.1998.

Dai, Zhihao, Jean-Claude Sirard, Michèle Mock, and Theresa M. Koehler. 1995. “The AtxA Gene Product Activates Transcription of the Anthrax Toxin Genes and Is Essential for Virulence.” *Molecular Microbiology* 16(6):1171–81. doi: 10.1111/j.1365-2958.1995.tb02340.x.

De Moraes, Josué, and Silvia C. Alfieri. 2008. “Growth, Encystment and Survival of *Acanthamoeba Castellanii* Grazing on Different Bacteria.” *FEMS Microbiology Ecology* 66 (2): 221–29. https://doi.org/10.1111/j.1574-6941.2008.00594.x.

Declerck, P., J. Behets, B. De Keersmaecker, and F. Ollevier. 2007. “Receptor-Mediated Uptake of *Legionella Pneumophila* by *Acanthamoeba Castellanii* and *Naegleria*

Lovaniensis." *Journal of Applied Microbiology* 103 (6): 2697–2703. https://doi.org/10.1111/j.1365-2672.2007.03530.x.

Dey, Rafik, Paul S. Hoffman, and Ian J. Glomski. 2012. "Germination and Amplification of Anthrax Spores by Soil-Dwelling Amoebas." *Applied and Environmental Microbiology* 78(22):8075. doi: 10.1128/AEM.02034-12.

Dietrich, Richard, Nadja Jessberger, Monika Ehling-Schulz, Erwin Märtlbauer, and Per Einar Granum. 2021. "The Food Poisoning Toxins of *Bacillus Cereus*." *Toxins* 13 (2): 98. https://doi.org/10.3390/toxins13020098.

Dixon, Terry C., Amin A. Fadl, Theresa M. Koehler, Joel A. Swanson, and Philip C. Hanna. 2000. "Early *Bacillus Anthracis*–Macrophage Interactions: Intracellular Survival and Escape." *Cellular Microbiology* 2 (6): 453–63. https://doi.org/10.1046/j.1462-5822.2000.00067.x.

Doellinger, Joerg, Andy Schneider, Timo D. Stark, Monika Ehling-Schulz, and Peter Lasch. 2020. "Evaluation of MALDI-ToF Mass Spectrometry for Rapid Detection of Cereulide From *Bacillus Cereus* Cultures." *Frontiers in Microbiology* 11. https://doi.org/10.3389/fmicb.2020.511674.

Donovan, William P., James T. Engleman, Judith C. Donovan, James A. Baum, Greg J. Bunkers, David J. Chi, William P. Clinton, et al. 2006. "Discovery and Characterization of Sip1A: A Novel Secreted Protein from *Bacillus Thuringiensis* with Activity against Coleopteran Larvae." *Applied Microbiology and Biotechnology* 72 (4): 713–19. https://doi.org/10.1007/s00253-006-0332-7.

Doyscher, Dominik, Lars Fieseler, Lone Dons, Martin J. Loessner, and Markus Schuppler. 2013. "*Acanthamoeba* Feature a Unique Backpacking Strategy to Trap and Feed on *Listeria Monocytogenes* and Other Motile Bacteria." *Environmental Microbiology* 15 (2): 433–46. https://doi.org/10.1111/j.1462-2920.2012.02858.x.

Drysdale, Melissa, Agathe Bourgogne, Susan G. Hilsenbeck, and Theresa M. Koehler. 2004. "AtxA Controls *Bacillus Anthracis* Capsule Synthesis via AcpA and a Newly Discovered Regulator, AcpB." *Journal of Bacteriology* 186 (2): 307–15. https://doi.org/10.1128/JB.186.2.307-315.2004.

Dubois, Thomas, Karoline Faegri, Sébastien Gélis-Jeanvoine, Stéphane Perchat, Christelle Lemy, Christophe Buisson, Christina Nielsen-LeRoux, et al. 2016. "Correction: Necrotrophism Is a Quorum-Sensing-Regulated Lifestyle in *Bacillus Thuringiensis*." *PLOS Pathogens* 12 (11): e1006049. https://doi.org/10.1371/journal.ppat.1006049.

Ehling-Schulz, Monika, Martina Fricker, Harald Grallert, Petra Rieck, Martin Wagner, and Siegfried Scherer. 2006. "Cereulide Synthetase Gene Cluster from Emetic *Bacillus Cereus*: Structure and Location on a Mega Virulence Plasmid Related to *Bacillus Anthracis* Toxin Plasmid PXO1." *BMC Microbiology* 6 (March): 20. https://doi.org/10.1186/1471-2180-6-20.

Ehling-Schulz, Monika, Martina Fricker, and Siegfried Scherer. 2004. "*Bacillus Cereus*, the Causative Agent of an Emetic Type of Food-Borne Illness." *Molecular Nutrition & Food Research* 48 (7): 479–87. https://doi.org/10.1002/mnfr.200400055.

Ehling-Schulz, Monika, Didier Lereclus, and Theresa M. Koehler. 2019. "The *Bacillus Cereus* Group: *Bacillus* Species with Pathogenic Potential." *Microbiology Spectrum* 7 (3). https://doi.org/10.1128/microbiolspec.GPP3-0032-2018.

Ehling-Schulz, Monika, Birgitta Svensson, Marie-Helene Guinebretiere, Toril Lindbäck, Maria Andersson, Anja Schulz, Martina Fricker, et al. 2005. "Emetic Toxin Formation of *Bacillus Cereus* Is Restricted to a Single Evolutionary Lineage of Closely Related Strains." *Microbiology (Reading, England)* 151 (Pt 1): 183–97. https://doi.org/10.1099/mic.0.27607-0.

Eichenberger, Patrick, Paul Fawcett, and Richard Losick. 2001. "A Three-Protein Inhibitor of Polar Septation during Sporulation in *Bacillus Subtilis*." *Molecular Microbiology* 42 (5): 1147–62. https://doi.org/10.1046/j.1365-2958.2001.02660.x.

Espinasse, Sylvain, Josette Chaufaux, Christophe Buisson, Stéphane Perchat, Michel Gohar, Denis Bourguet, and Vincent Sanchis. 2003. "Occurrence and Linkage Between Secreted Insecticidal Toxins in Natural Isolates of *Bacillus Thuringiensis*." *Current Microbiology* 47 (6): 501–7. https://doi.org/10.1007/s00284-003-4097-2.

Estruch, J. J., G. W. Warren, M. A. Mullins, G. J. Nye, J. A. Craig, and M. G. Koziel. 1996. "Vip3A, a Novel *Bacillus Thuringiensis* Vegetative Insecticidal Protein with a Wide Spectrum of Activities against Lepidopteran Insects." *Proceedings of the National Academy of Sciences* 93 (11): 5389–94. https://doi.org/10.1073/pnas.93.11.5389.

Ezzell, J. W., and S. L. Welkos. 1999. "The Capsule of Bacillus Anthracis, a Review." *Journal of Applied Microbiology* 87(2):250–250. doi: 10.1046/j.1365-2672.1999.00881.x.

Fakhry, S., I. Sorrentini, E. Ricca, M. De Felice, and L. Baccigalupi. 2008. "Characterization of Spore Forming Bacilli Isolated from the Human Gastrointestinal Tract." *Journal of Applied Microbiology* 105(6):2178–86. doi: https://doi.org/10.1111/j.1365-2672.2008.03934.x.

Farag, Mohamed M. S., Saad A. M. Moghannem, Amr M. Shehabeldine, and Mohamed S. Azab. 2020. "Antitumor Effect of Exopolysaccharide Produced by *Bacillus Mycoides*." *Microbial Pathogenesis* 140 (March): 103947. https://doi.org/10.1016/j.micpath.2019.103947.

Fazion, Fernanda, Stéphane Perchat, Christophe Buisson, Gislayne Vilas-Bôas, and Didier Lereclus. 2018. "A Plasmid-Borne Rap-Phr System Regulates Sporulation of *Bacillus Thuringiensis* in Insect Larvae." *Environmental Microbiology* 20 (1): 145–55. https://doi.org/10.1111/1462-2920.13946.

Feavers, I. M., J. Foulkes, B. Setlow, D. Sun, W. Nicholson, P. Setlow, and A. Moir. 1990. "The Regulation of Transcription of the GerA Spore Germination Operon of *Bacillus Subtilis*." *Molecular Microbiology* 4 (2): 275–82. https://doi.org/10.1111/j.1365-2958.1990.tb00594.x.

Fedhila, S., C. Buisson, O. Dussurget, P. Serror, I. J. Glomski, P. Liehl, D. Lereclus, and C. Nielsen-LeRoux. 2010. "Comparative Analysis of the Virulence of Invertebrate and Mammalian Pathogenic Bacteria in the Oral Insect Infection Model *Galleria*

Mellonella." *Journal of Invertebrate Pathology* 103 (1): 24–29. https://doi.org/10.1016/j.jip.2009.09.005.

Fedhila, Sinda, Patricia Nel, and Didier Lereclus. 2002. "The InhA2 Metalloprotease of *Bacillus Thuringiensis* Strain 407 Is Required for Pathogenicity in Insects Infected via the Oral Route." *Journal of Bacteriology* 184 (12): 3296. https://doi.org/10.1128/JB.184.12.3296-3304.2002.

Fey, Petra, Anthony S. Kowal, Pascale Gaudet, Karen E. Pilcher, and Rex L. Chisholm. 2007. "Protocols for Growth and Development of *Dictyostelium Discoideum*." *Nature Protocols* 2 (6): 1307–16. https://doi.org/10.1038/nprot.2007.178.

Fieseler, Lars, Dominik Doyscher, Martin J. Loessner, and Markus Schuppler. 2014. "Acanthamoeba Release Compounds Which Promote Growth of *Listeria Monocytogenes* and Other Bacteria." *Applied Microbiology and Biotechnology* 98 (7): 3091–97. https://doi.org/10.1007/s00253-014-5534-9.

Foerster, Harold F., and J. W. Foster. 1966. "Response of *Bacillus* Spores to Combinations of Germinative Compounds." *Journal of Bacteriology* 91 (3): 1168–77.

Fouet, Agnès, and Michèle Mock. 2006. "Regulatory Networks for Virulence and Persistence of *Bacillus Anthracis*." *Current Opinion in Microbiology*, Cell Regulation / Edited by Werner Goebel and Stephen Lory, 9 (2): 160–66. https://doi.org/10.1016/j.mib.2006.02.009.

Fox, Daniel, Anukriti Mathur, Yansong Xue, Yunqi Liu, Wei Hong Tan, Shouya Feng, Abhimanu Pandey, et al. 2020. "*Bacillus Cereus* Non-Haemolytic Enterotoxin Activates the NLRP3 Inflammasome." *Nature Communications* 11 (1): 760. https://doi.org/10.1038/s41467-020-14534-3.

Français, Marina, Frédéric Carlin, Véronique Broussolle, and Christophe Nguyen-Thé. 2019. "*Bacillus Cereus CshA* Is Expressed during the Lag Phase of Growth and Serves as a Potential Marker of Early Adaptation to Low Temperature and PH." *Applied and Environmental Microbiology* 85 (14). https://doi.org/10.1128/AEM.00486-19.

Frankenhuyzen, Kees van. 2009. "Insecticidal Activity of *Bacillus Thuringiensis* Crystal Proteins." *Journal of Invertebrate Pathology* 101 (1): 1–16. https://doi.org/10.1016/j.jip.2009.02.009.

Fujita, Masaya, José Eduardo González-Pastor, and Richard Losick. 2005. "High- and Low-Threshold Genes in the Spo0A Regulon of *Bacillus Subtilis*." *Journal of Bacteriology* 187 (4): 1357–68. https://doi.org/10.1128/JB.187.4.1357-1368.2005.

Galperin, Michael Y., Sergei L. Mekhedov, Pere Puigbo, Sergey Smirnov, Yuri I. Wolf, and Daniel J. Rigden. 2012. "Genomic Determinants of Sporulation in Bacilli and Clostridia: Towards the Minimal Set of Sporulation-Specific Genes." *Environmental Microbiology* 14(11):2870–90. doi: 10.1111/j.1462-2920.2012.02841.x.

Gaur, N. K., K. Cabane, and I. Smith. 1988. "Structure and Expression of the *Bacillus Subtilis* Sin Operon." *Journal of Bacteriology* 170 (3): 1046–53. https://doi.org/10.1128/jb.170.3.1046-1053.1988.

Gaze, W. H., N. Burroughs, M. P. Gallagher, and E. M. H. Wellington. 2003. “Interactions between *Salmonella Typhimurium* and *Acanthamoeba Polyphaga*, and Observation of a New Mode of Intracellular Growth within Contractile Vacuoles.” *Microbial Ecology* 46 (3): 358–69. https://doi.org/10.1007/s00248-003-1001-3.

Geng, Jianxun, Jian Jiang, Changlong Shu, Zeyu Wang, Fuping Song, Lili Geng, Jiangyan Duan, and Jie Zhang. 2019. “*Bacillus Thuringiensis* Vip1 Functions as a Receptor of Vip2 Toxin for Binary Insecticidal Activity against Holotrichia Parallela.” *Toxins* 11 (8). https://doi.org/10.3390/toxins11080440.

Gohar, Michel, Karoline Faegri, Stéphane Perchat, Solveig Ravnum, Ole Andreas Økstad, Myriam Gominet, Anne-Brit Kolstø, and Didier Lereclus. 2008. “The PlcR Virulence Regulon of *Bacillus Cereus*.” *PLOS ONE* 3 (7): e2793. https://doi.org/10.1371/journal.pone.0002793.

Gold, Hal. 2011. *Unit 731: Testimony*. Tuttle Publishing.

Gompf, Sandra G., and Cristina Garcia. 2019. “Lethal Encounters: The Evolving Spectrum of Amoebic Meningoencephalitis.” *IDCases* 15:e00524. doi: 10.1016/j.idcr.2019.e00524.

Gouffon, C., A. Van Vliet, J. Van Rie, S. Jansens, and J. L. Jurat-Fuentes. 2011. “Binding Sites for *Bacillus Thuringiensis* Cry2Ae Toxin on Heliothine Brush Border Membrane Vesicles Are Not Shared with Cry1A, Cry1F, or Vip3A Toxin.” *Applied and Environmental Microbiology* 77 (10): 3182–88. https://doi.org/10.1128/AEM.02791-10.

Grandvalet, Cosette, Myriam Gominet, and Didier Lereclus. 2001. “Identification of Genes Involved in the Activation of the *Bacillus Thuringiensis* InhA Metalloprotease Gene at the Onset of Sporulation.” *Microbiology* 147 (7): 1805–13. https://doi.org/10.1099/00221287-147-7-1805.

Grechnikova, Maria, Kateřina Ženíšková, Ronald Malych, Jan Mach, and Robert Sutak. 2020. “Copper Detoxification Machinery of the Brain-Eating Amoeba *Naegleria Fowleri* Involves Copper-Translocating ATPase and the Antioxidant System.” *International Journal for Parasitology: Drugs and Drug Resistance* 14 (December): 126–35. https://doi.org/10.1016/j.ijpddr.2020.10.001.

Green, B. D., G. Olmedo, and P. Youngman. 1991. “A Genetic Analysis of SpoOA Structure and Function.” *Research in Microbiology* 142(7):825–30. doi: 10.1016/0923-2508(91)90061-E.

Gu, Changling, Xiaojun Guo, Jia Li, Xiaoyu Zhao, and Baocheng Zhu. 2009. “[Identification of a fibrinolytic enzyme producing *Bacillus pseudomycoides* and purification and characterization of the enzyme].” *Wei Sheng Wu Xue Bao = Acta Microbiologica Sinica* 49 (4): 492–97.

Guérin, Alizée, Helene Thorsen Rønning, Claire Dargaignaratz, Thierry Clavel, Véronique Broussolle, Jacques Mahillon, Per Einar Granum, and Christophe Nguyen-The. 2017. “Cereulide Production by *Bacillus Weihenstephanensis* Strains during Growth at Different PH Values and Temperatures.” *Food Microbiology* 65 (August): 130–35. https://doi.org/10.1016/j.fm.2017.02.006.

Guidi-Rontani, Chantal. 2002. "The Alveolar Macrophage: The Trojan Horse of *Bacillus Anthracis*." *Trends in Microbiology* 10 (9): 405–9. https://doi.org/10.1016/S0966-842X(02)02422-8.

Guidi-Rontani, Chantal, Martine Weber-Levy, Elisabeth Labruyère, and Michèle Mock. 1999. "Germination of *Bacillus Anthracis* Spores within Alveolar Macrophages." *Molecular Microbiology* 31 (1): 9–17. https://doi.org/10.1046/j.1365-2958.1999.01137.x.

Guillemet, Elisabeth, Céline Cadot, Seav-Ly Tran, Marie-Hélène Guinebretière, Didier Lereclus, and Nalini Ramarao. 2010. "The InhA Metalloproteases of *Bacillus Cereus* Contribute Concomitantly to Virulence." *Journal of Bacteriology* 192 (1): 286–94. https://doi.org/10.1128/JB.00264-09.

Guillemet, Elisabeth, Seav-Ly Tran, Céline Cadot, Didier Rognan, Didier Lereclus, and Nalini Ramarao. 2013. "Glucose 6P Binds and Activates HlyIIR to Repress *Bacillus Cereus* Haemolysin HlyII Gene Expression." *PLOS ONE* 8 (2): e55085. https://doi.org/10.1371/journal.pone.0055085.

Guinebretière, Marie-Hélène, Sandrine Auger, Nathalie Galleron, Matthias Contzen, Benoit De Sarrau, Marie-Laure De Buyser, Gilles Lamberet, et al. 2013. "*Bacillus Cytotoxicus* Sp. Nov. Is a Novel Thermotolerant Species of the *Bacillus Cereus* Group Occasionally Associated with Food Poisoning." *International Journal of Systematic and Evolutionary Microbiology* 63 (Pt 1): 31–40. https://doi.org/10.1099/ijs.0.030627-0.

Guinebretière, Marie-Hélène, Fabiano L. Thompson, Alexei Sorokin, Philippe Normand, Peter Dawyndt, Monika Ehling-Schulz, Birgitta Svensson, et al. 2008. "Ecological Diversification in the *Bacillus Cereus* Group." *Environmental Microbiology* 10 (4): 851–65. https://doi.org/10.1111/j.1462-2920.2007.01495.x.

Gutierrez, Jennifer, Rachelle Smith, and Kit Pogliano. 2010. "SpoIID-Mediated Peptidoglycan Degradation Is Required throughout Engulfment during *Bacillus Subtilis* Sporulation." *Journal of Bacteriology* 192 (12): 3174–86. https://doi.org/10.1128/JB.00127-10.

Han, Qin, Qun Ma, Yong Chen, Bing Tian, Lanxi Xu, Yang Bai, Wenfeng Chen, and Xia Li. 2020. "Variation in Rhizosphere Microbial Communities and Its Association with the Symbiotic Efficiency of Rhizobia in Soybean." *The ISME Journal* 14(8):1915–28. doi: 10.1038/s41396-020-0648-9.

Harvie, Duncan R., Susana Vílchez, James R. Steggles, and David J. Ellar. 2005. "*Bacillus Cereus* Fur Regulates Iron Metabolism and Is Required for Full Virulence." *Microbiology (Reading, England)* 151 (Pt 2): 569–77. https://doi.org/10.1099/mic.0.27744-0.

Heidt, J., N. Papaloukas, and C. P. Timmerman. 2019. "A Rare Bloodstream Infection: *Bacillus Mycoides*." *The Netherlands Journal of Medicine* 77 (6): 227–30.

Helgason, Erlendur, Ole Andreas Økstad, Dominique A. Caugant, Henning A. Johansen, Agnes Fouet, Michéle Mock, Ida Hegna, and Anne-Brit Kolstø. 2000. "*Bacillus Anthracis*, *Bacillus Cereus*, and *Bacillus Thuringiensis*—One Species on the Basis of Genetic Evidence." *Applied and Environmental Microbiology* 66(6):2627–30. doi: 10.1128/AEM.66.6.2627-2630.2000.

Helgason, Erlendur, Nicolas J. Tourasse, Roger Meisal, Dominique A. Caugant, and Anne-Brit Kolstø. 2004. "Multilocus Sequence Typing Scheme for Bacteria of the *Bacillus Cereus* Group." *Applied and Environmental Microbiology* 70(1):191–201. doi: 10.1128/AEM.70.1.191-201.2004.

Helms, Charles M., R. Michael Massanari, Rodney Zeitler, Stephen Streed, Mary J. R. Gilchrist, Nancy Hall, William J. Hausler, J. Sywassink, William Johnson, Laverne Wintermeyer, and Walter J. Hierholzer. 1983. "Legionnaires' Disease Associated with a Hospital Water System: A Cluster of 24 Nosocomial Cases." *Annals of Internal Medicine* 99(2):172–78. doi: 10.7326/0003-4819-99-2-172.

Henry, Stéphanie, Didier Lereclus, and Leyla Slamti. 2020. "The Alternative Sigma Factor SigB Is Required for the Pathogenicity of *Bacillus Thuringiensis*." *Journal of Bacteriology* 202(21):e00265-20. doi: 10.1128/JB.00265-20.

Hernandez, Eric, Francoise Ramisse, Jean-Pierre Ducoureau, Thierry Cruel, and Jean-Didier Cavallo. 1998. "*Bacillus Thuringiensis* Subsp.*Konkukian* (Serotype H34) Superinfection: Case Report and Experimental Evidence of Pathogenicity in Immunosuppressed Mice." *Journal of Clinical Microbiology* 36(7):2138–39. doi: 10.1128/JCM.36.7.2138-2139.1998.

Higgins, Douglas, and Jonathan Dworkin. 2012. "Recent Progress in *Bacillus Subtilis* Sporulation." *FEMS Microbiology Reviews* 36(1):131–48. doi: 10.1111/j.1574-6976.2011.00310.x.

Hill, Karen K., Lawrence O. Ticknor, Richard T. Okinaka, Michelle Asay, Heather Blair, Katherine A. Bliss, Mariam Laker, Paige E. Pardington, Amber P. Richardson, Melinda Tonks, Douglas J. Beecher, John D. Kemp, Anne-Brit Kolstø, Amy C. Lee Wong, Paul Keim, and Paul J. Jackson. 2004. "Fluorescent Amplified Fragment Length Polymorphism Analysis of *Bacillus Anthracis*, *Bacillus Cereus*, and *Bacillus Thuringiensis* Isolates." *Applied and Environmental Microbiology* 70(2):1068–80. doi: 10.1128/AEM.70.2.1068-1080.2004.

Holman, Devin B., and Katherine E. Gzyl. 2019. "A Meta-Analysis of the Bovine Gastrointestinal Tract Microbiota." *FEMS Microbiology Ecology* 95(6). doi: 10.1093/femsec/fiz072.

Hong, Huynh A., Ellen To, Saad Fakhry, Loredana Baccigalupi, Ezio Ricca, and Simon M. Cutting. 2009. "Defining the Natural Habitat of *Bacillus* Spore-Formers." *Research in Microbiology* 160(6):375–79. doi: 10.1016/j.resmic.2009.06.006.

Hornstra, Luc M., Ynte P. de Vries, Marjon H. J. Wells-Bennik, Willem M. de Vos, and Tjakko Abee. 2006. "Characterization of Germination Receptors of *Bacillus Cereus* ATCC 14579." *Applied and Environmental Microbiology* 72(1):44–53. doi: 10.1128/AEM.72.1.44-53.2006.

Huillet, Eugénie, Ludovic Bridoux, Pagakrong Wanapaisan, Agnès Rejasse, Qi Peng, Watanalai Panbangred, and Didier Lereclus. 2017. "The CodY-Dependent ClhAB2 Operon Is Involved in Cell Shape, Chaining and Autolysis in *Bacillus Cereus* ATCC 14579." *PLOS ONE* 12(10):e0184975. doi: 10.1371/journal.pone.0184975.

Iovieno, Alfonso, Dolena R. Ledee, Darlene Miller, and Eduardo C. Alfonso. 2010. "Detection of Bacterial Endosymbionts in Clinical *Acanthamoeba* Isolates." *Ophthalmology* 117(3):445-452.e3. doi: 10.1016/j.ophtha.2009.08.033.

Iqbal, Junaid, Ruqaiyyah Siddiqui, and Naveed Ahmed Khan. 2014. "*Acanthamoeba* and Bacteria Produce Antimicrobials to Target Their Counterpart." *Parasites & Vectors* 7(1):56. doi: 10.1186/1756-3305-7-56.

Jensen, Gert B., Preben Larsen, Bodil L. Jacobsen, Bodil Madsen, Lasse Smidt, and Lars Andrup. 2002. "*Bacillus Thuringiensis* in Fecal Samples from Greenhouse Workers after Exposure to *B. Thuringiensis*-Based Pesticides." *Applied and Environmental Microbiology* 68(10):4900–4905. doi: 10.1128/AEM.68.10.4900-4905.2002.

Jernigan, Daniel B., Pratima L. Raghunathan, Beth P. Bell, Ross Brechner, Eddy A. Bresnitz, Jay C. Butler, Marty Cetron, Mitch Cohen, Timothy Doyle, Marc Fischer, Carolyn Greene, Kevin S. Griffith, Jeannette Guarner, James L. Hadler, James A. Hayslett, Richard Meyer, Lyle R. Petersen, Michael Phillips, Robert Pinner, Tanja Popovic, Conrad P. Quinn, Jennita Reefhuis, Dori Reissman, Nancy Rosenstein, Anne Schuchat, Wun-Ju Shieh, Larry Siegal, David L. Swerdlow, Fred C. Tenover, Marc Traeger, John W. Ward, Isaac Weisfuse, Steven Wiersma, Kevin Yeskey, Sherif Zaki, David A. Ashford, Bradley A. Perkins, Steve Ostroff, James Hughes, David Fleming, Jeffrey P. Koplan, and Julie L. Gerberding. 2002. "Investigation of Bioterrorism-Related Anthrax, United States, 2001: Epidemiologic Findings." *Emerging Infectious Diseases* 8(10):1019–28. doi: 10.3201/eid0810.020353.

Jiang, Kun, Xiaoyue Hou, Lu Han, Tongtong Tan, Zhanglei Cao, and Jun Cai. 2018. "Fibroblast Growth Factor Receptor, a Novel Receptor for Vegetative Insecticidal Protein Vip3Aa." *Toxins* 10(12):546. doi: 10.3390/toxins10120546.

Jiang, Kun, Xiao-yue Hou, Tong-tong Tan, Zhang-lei Cao, Si-qi Mei, Bing Yan, Jin Chang, Lu Han, Dan Zhao, and Jun Cai. 2018. "Scavenger Receptor-C Acts as a Receptor for *Bacillus Thuringiensis* Vegetative Insecticidal Protein Vip3Aa and Mediates the Internalization of Vip3Aa via Endocytosis." *PLOS Pathogens* 14(10):e1007347. doi: 10.1371/journal.ppat.1007347.

Jiang, Min, Weilan Shao, Marta Perego, and James A. Hoch. 2000. "Multiple Histidine Kinases Regulate Entry into Stationary Phase and Sporulation in *Bacillus Subtilis*." *Molecular Microbiology* 38(3):535–42. doi: https://doi.org/10.1046/j.1365-2958.2000.02148.x.

Jiménez, Guillermo, Mercedes Urdiain, Ana Cifuentes, Aránzazu López-López, Anicet R. Blanch, Javier Tamames, Peter Kämpfer, Anne-Brit Kolstø, Daniel Ramón, Juan F. Martínez, Francisco M. Codoñer, and Ramon Rosselló-Móra. 2013. "Description of *Bacillus Toyonensis* Sp. Nov., a Novel Species of the *Bacillus Cereus* Group, and Pairwise Genome Comparisons of the Species of the Group by Means of ANI Calculations." *Systematic and Applied Microbiology* 36(6):383–91. doi: 10.1016/j.syapm.2013.04.008.

Kahn, Theodore W., Maissa Chakroun, Jayme Williams, Tom Walsh, Bill James, Jessica Monserrate, and Juan Ferré. 2018. "Efficacy and Resistance Management Potential of a Modified Vip3C Protein for Control of *Spodoptera Frugiperda* in Maize." *Scientific Reports* 8. doi: 10.1038/s41598-018-34214-z.

Kang, Seungho, Alexander K. Tice, Frederick W. Spiegel, Jeffrey D. Silberman, Tomáš Pánek, Ivan Čepička, Martin Kostka, Anush Kosakyan, Daniel M. C. Alcântara, Andrew J. Roger, Lora L. Shadwick, Alexey Smirnov, Alexander Kudryavtsev, Daniel J. G. Lahr, and Matthew W. Brown. 2017. "Between a Pod and a Hard Test: The Deep Evolution of Amoebae." *Molecular Biology and Evolution* 34(9):2258–70. doi: 10.1093/molbev/msx162.

Khunkitti, W., D. Lloyd, J. R. Furr, and A. D. Russell. 1998. "*Acanthamoeba Castellanii*: Growth, Encystment, Excystment and Biocide Susceptibility." *Journal of Infection* 36(1):43–48. doi: 10.1016/S0163-4453(98)93054-7.

Kindle, Patrick, Danai Etter, Roger Stephan, and Sophia Johler. 2019. "Population Structure and Toxin Gene Profiles of *Bacillus Cereus* Sensu Lato Isolated from Flour Products." *FEMS Microbiology Letters* 366(20). doi: 10.1093/femsle/fnz240.

Koch, Robert. 2018. "Die Ätiologie der Milzbrand-Krankheit, begründet auf die Entwicklungsgeschichte des *Bacillus Anthracis* (1876)." Pp. 19–43 in *Robert Koch : Zentrale Texte, Klassische Texte der Wissenschaft*, edited by C. Gradmann. Berlin, Heidelberg: Springer.

Kolstø, A. B., D. Lereclus, and M. Mock. 2002. "Genome Structure and Evolution of the *Bacillus Cereus* Group." *Current Topics in Microbiology and Immunology* 264(2):95–108.

Kong, Hyun Hee. 2009. "Molecular Phylogeny of *Acanthamoeba*." *The Korean Journal of Parasitology* 47(Suppl):S21–28. doi: 10.3347/kjp.2009.47.S.S21.

König, Constantin, Martin Meyer, Corinna Lender, Sarah Nehls, Tina Wallaschkowski, Tobias Holm, Thorben Matthies, Dirk Lercher, Jenny Matthiesen, Helena Fehling, Thomas Roeder, Sophia Reindl, Maria Rosenthal, Nahla Galal Metwally, Hannelore Lotter, and Iris Bruchhaus. 2020. "An Alcohol Dehydrogenase 3 (ADH3) from Entamoeba Histolytica Is Involved in the Detoxification of Toxic Aldehydes." *Microorganisms* 8(10):1608. doi: 10.3390/microorganisms8101608.

Korn, E. D., and R. A. Weisman. 1967. "Phagocytosis of Latex Beads by *Acanthamoeba*. II. Electron Microscopic Study of the Initial Events." *The Journal of Cell Biology* 34(1):219–27. doi: 10.1083/jcb.34.1.219.

Korza, George, Emily Camilleri, Joshua Green, Janelle Robinson, Katja Nagler, Ralf Moeller, Melissa J. Caimano, and Peter Setlow. 2019. "Analysis of the MRNAs in Spores of *Bacillus Subtilis*." *Journal of Bacteriology* 201(9). doi: 10.1128/JB.00007-19.

Krieg, A., A. M. Huger, G. A. Langenbruch, and W. Schnetter. 1983. "*Bacillus Thuringiensis* Var. *Tenebrionis*: Ein Neuer, Gegenüber Larven von Coleopteren Wirksamer Pathotyp." *Zeitschrift Für Angewandte Entomologie* 96(1–5):500–508. doi: https://doi.org/10.1111/j.1439-0418.1983.tb03704.x.

Lacey, Lawrence A. 2007. "*Bacillus Thuringiensis* Serovariety *Israelensis* and *Bacillus Sphaericus* for Mosquito Control." *Journal of the American Mosquito Control Association* 23(2 Suppl):133–63. doi: 10.2987/8756-971X(2007)23[133:BTSIAB]2.0.CO;2.

Lambrecht, Ellen, Julie Baré, Natascha Chavatte, Wim Bert, Koen Sabbe, and Kurt Houf. 2015. "Protozoan Cysts Act as a Survival Niche and Protective Shelter for Foodborne Pathogenic Bacteria." *Applied and Environmental Microbiology* 81(16):5604–12. doi: 10.1128/AEM.01031-15.

Lamrabet, Otmane, Astrid Melotti, Frédéric Burdet, Nabil Hanna, Jackie Perrin, Jahn Nitschke, Marco Pagni, Hubert Hilbi, Thierry Soldati, and Pierre Cosson. 2020. "Transcriptional Responses of *Dictyostelium Discoideum* Exposed to Different Classes of Bacteria." *Frontiers in Microbiology* 11. doi: 10.3389/fmicb.2020.00410.

Lapidus, Alla, Eugene Goltsman, Sandrine Auger, Nathalie Galleron, Béatrice Ségurens, Carole Dossat, Miriam L. Land, Veronique Broussolle, Julien Brillard, Marie-Helene Guinebretiere, Vincent Sanchis, Christophe Nguen-the, Didier Lereclus, Paul Richardson, Patrick Wincker, Jean Weissenbach, S. Dusko Ehrlich, and Alexei Sorokin. 2008. "Extending the *Bacillus Cereus* Group Genomics to Putative Food-Borne Pathogens of Different Toxicity." *Chemico-Biological Interactions* 171(2):236–49. doi: 10.1016/j.cbi.2007.03.003.

Lechner, S., R. Mayr, K. P. Francis, B. M. Prüss, T. Kaplan, E. Wiessner-Gunkel, G. S. Stewart, and S. Scherer. 1998. "*Bacillus Weihenstephanensis* Sp. Nov. Is a New Psychrotolerant Species of the *Bacillus Cereus* Group." *International Journal of Systematic Bacteriology* 48 Pt 4:1373–82. doi: 10.1099/00207713-48-4-1373.

Lee, Mi Kyong, Frederick S. Walters, Hope Hart, Narendra Palekar, and Jeng-Shong Chen. 2003. "The Mode of Action of the *Bacillus Thuringiensis* Vegetative Insecticidal Protein Vip3A Differs from That of Cry1Ab Delta-Endotoxin." *Applied and Environmental Microbiology* 69(8):4648–57. doi: 10.1128/AEM.69.8.4648-4657.2003.

Lemgruber, Leandro, Pietro Lupetti, Wanderley De Souza, Rossiane C. Vommaro, and Bruno Da Rocha-Azevedo. 2010. "The Fine Structure of the *Acanthamoeba Polyphaga* Cyst Wall." *FEMS Microbiology Letters* 305(2):170–76. doi: 10.1111/j.1574-6968.2010.01925.x.

Leuber, Michael, Frank Orlik, Bettina Schiffler, Albert Sickmann, and Roland Benz. 2006. "Vegetative Insecticidal Protein (Vip1Ac) of *Bacillus Thuringiensis* HD201: Evidence for Oligomer and Channel Formation." *Biochemistry* 45(1):283–88. doi: 10.1021/bi051351z.

Li, Guan-Nan, Xue-Juan Xia, Huan-Huan Zhao, Parfait Sendegeya, and Yong Zhu. 2015. "Identification and Characterization of *Bacillus Cereus* SW7-1 in Bombyx Mori (Lepidoptera: Bombycidae)." *Journal of Insect Science* 15(1):136. doi: 10.1093/jisesa/iev121.

Li, Jing, Maocheng Deng, Yao Wang, and Weixin Chen. 2016. "Production and Characteristics of Biosurfactant Produced by *Bacillus Pseudomycoides* BS6 Utilizing Soybean Oil Waste." *International Biodeterioration & Biodegradation* 112:72–79. doi: 10.1016/j.ibiod.2016.05.002.

Li, Yunfeng, Andrew Davis, George Korza, Pengfei Zhang, Yong-qing Li, Barbara Setlow, Peter Setlow, and Bing Hao. 2012. "Role of a SpoVA Protein in Dipicolinic Acid

Uptake into Developing Spores of *Bacillus Subtilis*." *Journal of Bacteriology* 194(8):1875–84. doi: 10.1128/JB.00062-12.

Liang, Xudong, Jin Zhu, Zhongzhi Zhao, Feng Zheng, Huijuan Zhang, Jianchun Wei, Yon Ji, and Yinduo Ji. 2017. "The Pag Gene of PXO1 Is Involved in Capsule Biosynthesis of *Bacillus Anthracis* Pasteur II Strain." *Frontiers in Cellular and Infection Microbiology* 7. doi: 10.3389/fcimb.2017.00203.

Lindbäck, Toril, Annette Fagerlund, Marianne Skeie Rødland, and Per EinarYR 2004 Granum. 2004. "Characterization of the *Bacillus Cereus* Nhe Enterotoxin." *Microbiology* 150(12):3959–67. doi: 10.1099/mic.0.27359-0.

Lindbäck, Toril, Maarten Mols, Coraline Basset, Per Einar Granum, Oscar P. Kuipers, and Ákos T. Kovács. 2012. "CodY, a Pleiotropic Regulator, Influences Multicellular Behaviour and Efficient Production of Virulence Factors in *Bacillus Cereus*." *Environmental Microbiology* 14(8):2233–46. doi: 10.1111/j.1462-2920.2012.02766.x.

Liu, Yang, Qiliang Lai, Markus Göker, Jan P. Meier-Kolthoff, Meng Wang, Yamin Sun, Lei Wang, and Zongze Shao. 2015. "Genomic Insights into the Taxonomic Status of the *Bacillus Cereus* Group." *Scientific Reports* 5(1):1–11. doi: 10.1038/srep14082.

Lloyd, David. 2014. "Encystment in *Acanthamoeba Castellanii*: A Review." *Experimental Parasitology* 145:S20–27. doi: 10.1016/j.exppara.2014.03.026.

Lorenzo-Morales, Jacob, Naveed A. Khan, and Julia Walochnik. 2015. "An Update on *Acanthamoeba* Keratitis: Diagnosis, Pathogenesis and Treatment." *Parasite (Paris, France)* 22:10. doi: 10.1051/parasite/2015010.

Losick, Richard, and Patrick Stragier. 1992. "Crisscross Regulation of Cell-Type-Specific Gene Expression during Development in *B. Subtilis*." *Nature* 355(6361):601–4. doi: 10.1038/355601a0.

Luo, Tian, Shanshan Hou, Li Yang, Gaofu Qi, and Xiuyun Zhao. 2018. "Nematodes Avoid and Are Killed by *Bacillus Mycoides*-Produced Styrene." *Journal of Invertebrate Pathology* 159:129–36. doi: 10.1016/j.jip.2018.09.006.

Magnet, A., R. H. S. Peralta, T. S. Gomes, F. Izquierdo, C. Fernandez-Vadillo, A. L. Galvan, M. J. Pozuelo, C. Pelaz, S. Fenoy, and C. Del Águila. 2015. "Vectorial Role of *Acanthamoeba* in *Legionella* Propagation in Water for Human Use." *Science of The Total Environment* 505:889–95. doi: 10.1016/j.scitotenv.2014.10.064.

Maisonneuve, Elodie. 2017. "Interactions amibes libres / micro-organismes : préférence trophique et étude comparative avec les macrophages." phdthesis, Université de Poitiers.

Maisonneuve, Elodie, Estelle Cateau, Marion Delouche, Nathalie Quellard, and Marie-Helene Rodier. 2017. "An Observational Study of Phagocytes and *Klebsiella Pneumoniae* Relationships: Different Behaviors." *Microbes and Infection* 19(4):259–66. doi: 10.1016/j.micinf.2016.12.005.

Mandic-Mulec, I., L. Doukhan, and I. Smith. 1995. "The *Bacillus Subtilis* SinR Protein Is a Repressor of the Key Sporulation Gene Spo0A." *Journal of Bacteriology* 177(16):4619–27. doi: 10.1128/jb.177.16.4619-4627.1995.

Mandic-Mulec, I., N. Gaur, U. Bai, and I. Smith. 1992. "Sin, a Stage-Specific Repressor of Cellular Differentiation." *Journal of Bacteriology* 174(11):3561–69. doi: 10.1128/jb.174.11.3561-3569.1992.

Margulis, Lynn, Jeremy Z. Jorgensen, Sona Dolan, Rita Kolchinsky, Frederick A. Rainey, and Shyh-Ching Lo. 1998. "The Arthromitus Stage of *Bacillus Cereus*: Intestinal Symbionts of Animals." *Proceedings of the National Academy of Sciences* 95(3):1236–41. doi: 10.1073/pnas.95.3.1236.

McDougald, Diane, and Sharon R. Longford. 2020. "Protozoa Hosts Lead to Virulence." *Nature Microbiology* 5(4):535–535. doi: 10.1038/s41564-020-0699-8.

Mehrotra, Tithi, Mohammad Nawaid Zaman, Bhim Bali Prasad, Anuradha Shukla, Srijan Aggarwal, and Rachana Singh. 2020. "Rapid Immobilization of Viable *Bacillus Pseudomycoides* in Polyvinyl Alcohol/Glutaraldehyde Hydrogel for Biological Treatment of Municipal Wastewater." *Environmental Science and Pollution Research* 27(9):9167–80. doi: 10.1007/s11356-019-07296-z.

Mendoza-Almanza, Gretel, Edgar L. Esparza-Ibarra, Jorge L. Ayala-Luján, Marisa Mercado-Reyes, Susana Godina-González, Marisa Hernández-Barrales, and Jorge Olmos-Soto. 2020. "The Cytocidal Spectrum of *Bacillus Thuringiensis* Toxins: From Insects to Human Cancer Cells." *Toxins* 12(5):301. doi: 10.3390/toxins12050301.

Milton, Morgan E., G. Logan Draughn, Benjamin G. Bobay, Sean D. Stowe, Andrew L. Olson, Erik A. Feldmann, Richele J. Thompson, Katherine H. Myers, Michael T. Santoro, Daniel B. Kearns, and John Cavanagh. 2020. "The Solution Structures and Interaction of SinR and SinI: Elucidating the Mechanism of Action of the Master Regulator Switch for Biofilm Formation in *Bacillus Subtilis*." *Journal of Molecular Biology* 432(2):343–57. doi: 10.1016/j.jmb.2019.08.019.

Moayeri, Mahtab, Stephen H. Leppla, Catherine Vrentas, Andrei P. Pomerantsev, and Shihui Liu. 2015. "Anthrax Pathogenesis." *Annual Review of Microbiology* 69(1):185–208. doi: 10.1146/annurev-micro-091014-104523.

Moir, Anne, and Gareth Cooper. 2015. "Spore Germination." *Microbiology Spectrum* 3(6). doi: 10.1128/microbiolspec.TBS-0014-2012.

Molle, Virginie, Masaya Fujita, Shane T. Jensen, Patrick Eichenberger, José E. González-Pastor, Jun S. Liu, and Richard Losick. 2003. "The Spo0A Regulon of *Bacillus Subtilis*." *Molecular Microbiology* 50(5):1683–1701. doi: https://doi.org/10.1046/j.1365-2958.2003.03818.x.

Molmeret, Maëlle, Matthias Horn, Michael Wagner, Marina Santic, and Yousef Abu Kwaik. 2005. "Amoebae as Training Grounds for Intracellular Bacterial Pathogens." *Applied and Environmental Microbiology* 71(1):20–28. doi: 10.1128/AEM.71.1.20-28.2005.

Moreno-Mesonero, Laura, María Antonia Ferrús, and Yolanda Moreno. 2020. "Determination of the Bacterial Microbiome of Free-Living Amoebae Isolated from Wastewater by 16S RRNA Amplicon-Based Sequencing." *Environmental Research* 190:109987. doi: 10.1016/j.envres.2020.109987.

Nakamura, L. K. 1998. "*Bacillus Pseudomycoides* Sp. Nov." *International Journal of Systematic Bacteriology* 48 Pt 3:1031–35. doi: 10.1099/00207713-48-3-1031.

Nasser, Waleed, Balaji Santhanam, Edward Roshan Miranda, Anup Parikh, Kavina Juneja, Gregor Rot, Chris Dinh, Rui Chen, Blaz Zupan, Gad Shaulsky, and Adam Kuspa. 2013. "Bacterial Discrimination by Dictyostelid Amoebae Reveals the Complexity of Ancient Interspecies Interactions." *Current Biology: CB* 23(10):862–72. doi: 10.1016/j.cub.2013.04.034.

Nielsen-LeRoux, Christina, Sophie Gaudriault, Nalini Ramarao, Didier Lereclus, and Alain Givaudan. 2012. "How the Insect Pathogen Bacteria *Bacillus Thuringiensis* and Xenorhabdus/Photorhabdus Occupy Their Hosts." *Current Opinion in Microbiology* 15(3):220–31. doi: 10.1016/j.mib.2012.04.006.

Norris, Michael H., Alexander Kirpich, Andrew P. Bluhm, Diansy Zincke, Ted Hadfield, Jose Miguel Ponciano, and Jason K. Blackburn. 2020. "Convergent Evolution of Diverse *Bacillus Anthracis* Outbreak Strains toward Altered Surface Oligosaccharides That Modulate Anthrax Pathogenesis." *PLOS Biology* 18(12):e3001052. doi: 10.1371/journal.pbio.3001052.

Núñez-Ramírez, Rafael, Juanjo Huesa, Yolanda Bel, Juan Ferré, Patricia Casino, and Ernesto Arias-Palomo. 2020. "Molecular Architecture and Activation of the Insecticidal Protein Vip3Aa from *Bacillus Thuringiensis*." *Nature Communications* 11(1):3974. doi: 10.1038/s41467-020-17758-5.

Okinaka, R. T., K. Cloud, O. Hampton, A. R. Hoffmaster, K. K. Hill, P. Keim, T. M. Koehler, G. Lamke, S. Kumano, J. Mahillon, D. Manter, Y. Martinez, D. Ricke, ǂ R. Svensson, and P. J. Jackson. 1999. "Sequence and Organization of PXO1, the Large *Bacillus Anthracis* Plasmid Harboring the Anthrax Toxin Genes." *Journal of Bacteriology* 181(20):6509–15.

Osman, Gamal H., Raya Soltane, Ibrahim Saleh, Hussein H. Abulreesh, Khaled S. Gazi, Ibrahim A. Arif, Ahmed M. Ramadan, Hussien F. Alameldin, Yehia A. Osman, and Mamdouh Idriss. 2019. "Isolation, Characterization, Cloning and Bioinformatics Analysis of a Novel Receptor from Black Cut Worm (Agrotis Ipsilon) of *Bacillus Thuringiensis* Vip 3Aa Toxins." *Saudi Journal of Biological Sciences* 26(5):1078–83. doi: 10.1016/j.sjbs.2018.06.002.

Otto, Grant P., and Richard H. Kessin. 2001. "The Intriguing Biology of *Dictyostelium Discoideum* Meeting Report: International *Dictyostelium* Conference 2001." *Protist* 152(4):243–48. doi: 10.1078/1434-4610-00064.

Palma, Leopoldo, Delia Muñoz, Colin Berry, Jesús Murillo, and Primitivo Caballero. 2014. "*Bacillus Thuringiensis* Toxins: An Overview of Their Biocidal Activity." *Toxins* 6(12):3296–3325. doi: 10.3390/toxins6123296.

Palma, Leopoldo, David J. Scott, Gemma Harris, Salah-Ud Din, Thomas L. Williams, Oliver J. Roberts, Mark T. Young, Primitivo Caballero, and Colin Berry. 2017. "The Vip3Ag4 Insecticidal Protoxin from *Bacillus Thuringiensis* Adopts A Tetrameric Configuration That Is Maintained on Proteolysis." *Toxins* 9(5):165. doi: 10.3390/toxins9050165.

Pan, Miao, Matthew P. Neilson, Alexander M. Grunfeld, Phillip Cruz, Xi Wen, Robert H. Insall, and Tian Jin. 2018. "A G-Protein-Coupled Chemoattractant Receptor Recognizes Lipopolysaccharide for Bacterial Phagocytosis." *PLOS Biology* 16(5):e2005754. doi: 10.1371/journal.pbio.2005754.

Paquet, Valérie E., and Steve J. Charette. 2016. "Amoeba-Resisting Bacteria Found in Multilamellar Bodies Secreted by *Dictyostelium Discoideum*: Social Amoebae Can Also Package Bacteria." *FEMS Microbiology Ecology* 92(3). doi: 10.1093/femsec/fiw025.

Paredes-Sabja, Daniel, Peter Setlow, and Mahfuzur R. Sarker. 2011. "Germination of Spores of Bacillales and Clostridiales Species: Mechanisms and Proteins Involved." *Trends in Microbiology* 19(2):85–94. doi: 10.1016/j.tim.2010.10.004.

Pawlowski, Jan, and Fabien Burki. 2009. "Untangling the Phylogeny of Amoeboid Protists1." *Journal of Eukaryotic Microbiology* 56(1):16–25. doi: https://doi.org/10.1111/j.1550-7408.2008.00379.x.

Perchat, Stéphane, Thomas Dubois, Samira Zouhir, Myriam Gominet, Sandrine Poncet, Christelle Lemy, Magali Aumont‐Nicaise, Josef Deutscher, Michel Gohar, Sylvie Nessler, and Didier Lereclus. 2011. "A Cell–Cell Communication System Regulates Protease Production during Sporulation in Bacteria of the *Bacillus Cereus* Group." *Molecular Microbiology* 82(3):619–33. doi: 10.1111/j.1365-2958.2011.07839.x.

Perego, M., G. B. Spiegelman, and J. A. Hoch. 1988. "Structure of the Gene for the Transition State Regulator, AbrB: Regulator Synthesis Is Controlled by the Spo0A Sporulation Gene in *Bacillus Subtilis*." *Molecular Microbiology* 2(6):689–99. doi: https://doi.org/10.1111/j.1365-2958.1988.tb00079.x.

Pérez, Claudia, Carlos Muñoz-Garay, Leivi C. Portugal, Jorge Sánchez, Sarjeet S. Gill, Mario Soberón, and Alejandra Bravo. 2007. "Bacillus Thuringiensis Ssp. Israelensis Cyt1Aa Enhances Activity of Cry11Aa Toxin by Facilitating the Formation of a Pre-Pore Oligomeric Structure." *Cellular Microbiology* 9(12):2931–37. doi: 10.1111/j.1462-5822.2007.01007.x.

Peterson, B., C. C. Bezuidenhout, and J. Van den Berg. 2017. "An Overview of Mechanisms of Cry Toxin Resistance in Lepidopteran Insects." *Journal of Economic Entomology* 110(2):362–77. doi: 10.1093/jee/tow310.

Petras, Stephen F., and L. E. Casida. 1985. "Survival of *Bacillus Thuringiensis* Spores in Soil." *Applied and Environmental Microbiology* 50(6):1496–1501.

Phillips, Z. E. V., and M. A. Strauch. 2002. "*Bacillus Subtilis* Sporulation and Stationary Phase Gene Expression." *Cellular and Molecular Life Sciences: CMLS* 59(3):392–402. doi: 10.1007/s00018-002-8431-9.

Pinto, Natália Berne, Leonardo Mortagua de Castro, Gabriela de Almeida Capella, Tairan Ourique Motta, Ana Paula de Souza Stori de Lara, Micaele Quintana de Moura, Maria Elisabeth Aires Berne, and Fábio Pereira Leivas Leite. 2017. "Controlling Gastrointestinal Nematodes in Cattle by *Bacillus* Species." *Veterinary Parasitology* 245:1–4. doi: 10.1016/j.vetpar.2017.08.004.

Porcar, Manuel, Anne-Marie Grenier, Brian Federici, and Yvan Rahbé. 2009. "Effects of *Bacillus Thuringiensis* Delta-Endotoxins on the Pea Aphid (Acyrthosiphon Pisum)." *Applied and Environmental Microbiology* 75(14):4897–4900. doi: 10.1128/AEM.00686-09.

Preston, R. A., and H. A. Douthit. 1984. "Stimulation of Germination of Unactivated *Bacillus Cereus* Spores by Ammonia." *Journal of General Microbiology* 130(5):1041–50. doi: 10.1099/00221287-130-5-1041.

Promdonkoy, B., and D. J. Ellar. 2000. "Membrane Pore Architecture of a Cytolytic Toxin from *Bacillus Thuringiensis*." *Biochemical Journal* 350(Pt 1):275–82.

Promdonkoy, Boonhiang, Patcharee Promdonkoy, and Sakol Panyim. 2005. "Co-Expression of *Bacillus Thuringiensis* Cry4Ba and Cyt2Aa2 in *Escherichia Coli* Revealed High Synergism against *Aedes Aegypti* and *Culex Quinquefasciatus* Larvae." *FEMS Microbiology Letters* 252(1):121–26. doi: 10.1016/j.femsle.2005.08.038.

Purohit, Mitali, Sihem Sassi-Gaha, and Richard F. Rest. 2010. "Rapid Sporulation of *Bacillus Anthracis* in a High Iron, Glucose-Free Medium." *Journal of Microbiological Methods* 82(3):282–87. doi: 10.1016/j.mimet.2010.07.004.

Ramarao, Nalini, and Didier Lereclus. 2005. "The InhA1 Metalloprotease Allows Spores of the *B. Cereus* Group to Escape Macrophages." *Cellular Microbiology* 7(9):1357–64. doi: 10.1111/j.1462-5822.2005.00562.x.

Rashidi, Ghazal, and Elizabeth A. Ostrowski. 2019. "Phagocyte Chase Behaviours: Discrimination between Gram-Negative and Gram-Positive Bacteria by Amoebae." *Biology Letters* 15(1):20180607. doi: 10.1098/rsbl.2018.0607.

Rasko, David A., Michael R. Altherr, Cliff S. Han, and Jacques Ravel. 2005. "Genomics of the *Bacillus Cereus* Group of Organisms." *FEMS Microbiology Reviews* 29(2):303–29. doi: 10.1016/j.fmrre.2004.12.005.

Rasko, David A., M. J. Rosovitz, Ole Andreas Økstad, Derrick E. Fouts, Lingxia Jiang, Regina Z. Cer, Anne-Brit Kolstø, Steven R. Gill, and Jacques Ravel. 2007. "Complete Sequence Analysis of Novel Plasmids from Emetic and Periodontal *Bacillus Cereus* Isolates Reveals a Common Evolutionary History among the *B. Cereus*-Group Plasmids, Including *Bacillus Anthracis* PXO1." *Journal of Bacteriology* 189(1):52–64. doi: 10.1128/JB.01313-06.

Ratnayake-Lecamwasam, Manoja, Pascale Serror, Ka-Wing Wong, and Abraham L. Sonenshein. 2001. "*Bacillus Subtilis* CodY Represses Early-Stationary-Phase Genes by Sensing GTP Levels." *Genes & Development* 15(9):1093–1103. doi: 10.1101/gad.874201.

Rayamajhee, Binod, Dinesh Subedi, Hari Kumar Peguda, Mark Duncan Willcox, Fiona L. Henriquez, and Nicole Carnt. 2021. "A Systematic Review of Intracellular Microorganisms within *Acanthamoeba* to Understand Potential Impact for Infection." *Pathogens* 10(2):225. doi: 10.3390/pathogens10020225.

Raymond, Ben, Paul R. Johnston, Christina Nielsen-LeRoux, Didier Lereclus, and Neil Crickmore. 2010. "*Bacillus Thuringiensis*: An Impotent Pathogen?" *Trends in Microbiology* 18(5):189–94. doi: 10.1016/j.tim.2010.02.006.

Réjasse, A., N. Gilois, I. Barbosa, E. Huillet, C. Bevilacqua, S. Tran, N. Ramarao, L. P. Stenfors Arnesen, and V. Sanchis. 2012. "Temperature-Dependent Production of Various PlcR-Controlled Virulence Factors in *Bacillus Weihenstephanensis* Strain KBAB4." *Applied and Environmental Microbiology* 78(8):2553–61. doi: 10.1128/AEM.07446-11.

Riquelme, Sebastián, Macarena Varas, Camila Valenzuela, Paula Velozo, Nicolás Chahin, Paulina Aguilera, Andrea Sabag, Bayron Labra, Sergio A. Álvarez, Francisco P. Chávez, and Carlos A. Santiviago. 2016. "Relevant Genes Linked to Virulence Are Required for *Salmonella Typhimurium* to Survive Intracellularly in the Social Amoeba *Dictyostelium Discoideum*." *Frontiers in Microbiology* 7. doi: 10.3389/fmicb.2016.01305.

Rojas-Solis, Daniel, Miguel A. Vences-Guzmán, Christian Sohlenkamp, and Gustavo Santoyo. 2020. "*Bacillus Toyonensis* COPE52 Modifies Lipid and Fatty Acid Composition, Exhibits Antifungal Activity, and Stimulates Growth of Tomato Plants Under Saline Conditions." *Current Microbiology* 77(10):2735–44. doi: 10.1007/s00284-020-02069-1.

Rolny, Ivanna S., Silvia M. Racedo, and Pablo F. Pérez. 2017. "Fate of *Bacillus Cereus* within Phagocytic Cells." *International Microbiology: The Official Journal of the Spanish Society for Microbiology* 20(4):170–77. doi: 10.2436/20.1501.01.300.

Rosenberg, Katja, Joanne Bertaux, Kristin Krome, Anton Hartmann, Stefan Scheu, and Michael Bonkowski. 2009. "Soil Amoebae Rapidly Change Bacterial Community Composition in the Rhizosphere of Arabidopsis Thaliana." *The ISME Journal* 3(6):675–84. doi: 10.1038/ismej.2009.11.

Rowbotham, T. J. 1983. "Isolation of *Legionella Pneumophila* from Clinical Specimens via Amoebae, and the Interaction of Those and Other Isolates with Amoebae." *Journal of Clinical Pathology* 36(9):978. doi: 10.1136/jcp.36.9.978.

Ruthel, Gordon, Wilson J. Ribot, Sina Bavari, and Timothy A. Hoover. 2004. "Time-Lapse Confocal Imaging of Development of *Bacillus Anthracis* in Macrophages." *The Journal of Infectious Diseases* 189(7):1313–16. doi: 10.1086/382656.

Salamitou, Sylvie, Françoise Ramisse, Michel Brehélin, Denis Bourguet, Nathalie Gilois, Myriam Gominet, Eric Hernandez, and Didier Lereclus. 2000. "The PlcR Regulon Is Involved in the Opportunistic Properties of *Bacillus Thuringiensis* and *Bacillus Cereus* in Mice and Insects." *Microbiology* 146(11):2825–32.

Sallinger, Eleni, Michael S. Robeson, and Tamara S. Haselkorn. 2021. "Characterization of the Bacterial Microbiomes of Social Amoebae and Exploration of the Roles of Host and Environment on Microbiome Composition." *Environmental Microbiology* 23(1):126–42. doi: 10.1111/1462-2920.15279.

Samba-Louaka, Ascel, Vincent Delafont, Marie-Hélène Rodier, Estelle Cateau, and Yann Héchard. 2019. "Free-Living Amoebae and Squatters in the Wild: Ecological and Molecular Features." *FEMS Microbiology Reviews* 43(4):415–34. doi: 10.1093/femsre/fuz011.

Sanchis, Vincent. 2011. "From Microbial Sprays to Insect-Resistant Transgenic Plants: History of the Biospesticide *Bacillus Thuringiensis*. A Review." *Agronomy for Sustainable Development* 31(1):217–31. doi: 10.1051/agro/2010027.

Santos, F. D. S., M. R. A. Ferreira, L. R. Maubrigades, V. S. Gonçalves, A. P. S. de Lara, C. Moreira, F. M. Salvarani, F. R. Conceição, and F. P. Leivas Leite. 2021. "*Bacillus Toyonensis* BCT-7112T Transient Supplementation Improves Vaccine Efficacy in Ewes Vaccinated against *Clostridium Perfringens* Epsilon Toxin." *Journal of Applied Microbiology* 130(3):699–706. doi: https://doi.org/10.1111/jam.14814.

Santos, F. d. s., Y. a. Menegon, R. e. a. Piraine, P. r. c. Rodrigues, R. c. Cunha, and F. P. Leivas Leite. 2018. "*Bacillus Toyonensis* Improves Immune Response in the Mice Vaccinated with Recombinant Antigen of Bovine Herpesvirus Type 5." *Beneficial Microbes* 9(1):133–42. doi: 10.3920/BM2017.0021.

Santos, Susana S., Niels Bohse Hendriksen, Hans Henrik Jakobsen, and Anne Winding. 2017. "Effects of *Bacillus Cereus* Endospores on Free-Living Protist Growth." *Microbial Ecology* 73(3):699–709. doi: 10.1007/s00248-016-0905-7.

Sastalla, Inka, Rasem Fattah, Nicole Coppage, Poulomi Nandy, Devorah Crown, Andrei P. Pomerantsev, and Stephen H. Leppla. 2013. "The *Bacillus Cereus* Hbl and Nhe Tripartite Enterotoxin Components Assemble Sequentially on the Surface of Target Cells and Are Not Interchangeable." *PloS One* 8(10):e76955. doi: 10.1371/journal.pone.0076955.

Schmidt, Timothy R., Edgar J. Scott, and David W. Dyer. 2011. "Whole-Genome Phylogenies of the Family Bacillaceae and Expansion of the Sigma Factor Gene Family in the *Bacillus Cereus* Species-Group." *BMC Genomics* 12(1):430. doi: 10.1186/1471-2164-12-430.

Schnepf, E., N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D. R. Zeigler, and D. H. Dean. 1998. "*Bacillus Thuringiensis* and Its Pesticidal Crystal Proteins." *Microbiology and Molecular Biology Reviews* 62(3):775–806.

Schuppler, Markus. 2014. "How the Interaction of *Listeria Monocytogenes* and *Acanthamoeba* Spp. Affects Growth and Distribution of the Food Borne Pathogen." *Applied Microbiology and Biotechnology* 98(7):2907–16. doi: 10.1007/s00253-014-5546-5.

Segond, Diego, Elise Abi Khalil, Christophe Buisson, Nadine Daou, Mireille Kallassy, Didier Lereclus, Paolo Arosio, Fadi Bou-Abdallah, and Christina Nielsen Le Roux. 2014. "Iron Acquisition in *Bacillus Cereus*: The Roles of IlsA and Bacillibactin in Exogenous

Ferritin Iron Mobilization." *PLOS Pathogens* 10(2):e1003935. doi: 10.1371/journal.ppat.1003935.

Setlow, Peter. 2014a. "Germination of Spores of *Bacillus* Species: What We Know and Do Not Know." *Journal of Bacteriology* 196(7):1297–1305. doi: 10.1128/JB.01455-13.

Setlow, Peter. 2014b. "Spore Resistance Properties." *Microbiology Spectrum* 2(5). doi: 10.1128/microbiolspec.TBS-0003-2012.

Shi, Yijing, David C. Queller, Yuehui Tian, Siyi Zhang, Qingyun Yan, Zhili He, Zhenzhen He, Chenyuan Wu, Cheng Wang, and Longfei Shu. 2021. "The Ecology and Evolution of Amoeba-Bacterium Interactions." *Applied and Environmental Microbiology* 87(2). doi: 10.1128/AEM.01866-20.

Siala, A., I. R. Hill, and T. R. G. Gray. 1974. "Populations of Spore-Forming Bacteria in an Acid Forest Soil, with Special Reference to *Bacillus Subtilis*." *Microbiology*, 81(1):183–90. doi: 10.1099/00221287-81-1-183.

Siddiqui, Ruqaiyyah, Timothy Yu Yee Ong, Suk Yul Jung, and Naveed Ahmed Khan. 2017. "*Acanthamoeba Castellanii* Interactions with *Streptococcus Pneumoniae* and *Streptococcus Pyogenes*." *Experimental Parasitology* 183:128–32. doi: 10.1016/j.exppara.2017.08.005.

Singh, Rajesh Kumar, Pratiksha Singh, Hai-Bi Li, Qi-Qi Song, Dao-Jun Guo, Manoj K. Solanki, Krishan K. Verma, Mukesh K. Malviya, Xiu-Peng Song, Prakash Lakshmanan, Li-Tao Yang, and Yang-Rui Li. 2020. "Diversity of Nitrogen-Fixing Rhizobacteria Associated with Sugarcane: A Comprehensive Study of Plant-Microbe Interactions for Growth Enhancement in *Saccharum* Spp." *BMC Plant Biology* 20(1):220. doi: 10.1186/s12870-020-02400-9.

Sinott, M. C., N. A. Cunha Filho, L. L. D. Castro, L. B. Lorenzon, N. B. Pinto, G. A. Capella, and F. P. L. Leite. 2012. "*Bacillus* Spp. Toxicity against *Haemonchus Contortus* Larvae in Sheep Fecal Cultures." *Experimental Parasitology* 132(2):103–8. doi: 10.1016/j.exppara.2012.05.015.

Slamti, Leyla, Christelle Lemy, Céline Henry, Alain Guillot, Eugénie Huillet, and Didier Lereclus. 2016. "CodY Regulates the Activity of the Virulence Quorum Sensor PlcR by Controlling the Import of the Signaling Peptide PapR in *Bacillus Thuringiensis*." *Frontiers in Microbiology* 6. doi: 10.3389/fmicb.2015.01501.

Slamti, Leyla, Stéphane Perchat, Eugénie Huillet, and Didier Lereclus. 2014. "Quorum Sensing in *Bacillus Thuringiensis* Is Required for Completion of a Full Infectious Cycle in the Insect." *Toxins* 6(8):2239–55. doi: 10.3390/toxins6082239.

Soberón, Mario, Jazmin A. López-Díaz, and Alejandra Bravo. 2013. "Cyt Toxins Produced by *Bacillus Thuringiensis*: A Protein Fold Conserved in Several Pathogenic Microorganisms." *Peptides* 41:87–93. doi: 10.1016/j.peptides.2012.05.023.

Solmaz, Kubra Betul, Yusuf Ozcan, Nazime Mercan Dogan, Omer Bozkaya, and Semra Ide. 2018. "Characterization and Production of Extracellular Polysaccharides (EPS) by

Bacillus Pseudomycoides U10." *Environments* 5(6):63. doi: 10.3390/environments5060063.

Somerville, H. J., and M. L. Jones. 1972. "DNA Competition Studies within the *Bacillus Cereus* Group of Bacilli." *Journal of General Microbiology* 73(2):257–65. doi: 10.1099/00221287-73-2-257.

Sonenshein, Abraham L. 2005. "CodY, a Global Regulator of Stationary Phase and Virulence in Gram-Positive Bacteria." *Current Opinion in Microbiology* 8(2):203–7. doi: 10.1016/j.mib.2005.01.001.

Soufiane, Brahim, and Jean-Charles Côté. 2013. "*Bacillus Weihenstephanensis* Characteristics Are Present in *Bacillus Cereus* and *Bacillus Mycoides* Strains." *FEMS Microbiology Letters* 341(2):127–37. doi: 10.1111/1574-6968.12106.

Srirampur, Arjun, Ashok Kumar Reddy, Swathi Siva, and Anupama Kalwad. 2018. "Keratitis Due to an Unusual Pathogenic Social Amoeba, *Dictyostelium Polycephalum*." *Open Forum Infectious Diseases* 5(2). doi: 10.1093/ofid/ofy009.

Steiner, Bernhard, Stephen Weber, and Hubert Hilbi. 2018. "Formation of the *Legionella*-Containing Vacuole: Phosphoinositide Conversion, GTPase Modulation and ER Dynamics." *International Journal of Medical Microbiology* 308(1):49–57. doi: 10.1016/j.ijmm.2017.08.004.

Stenfors Arnesen, Lotte, Per Einar Granum, Christophe Buisson, Jon Bohlin, and Christina Nielsen-LeRoux. 2011. "Using an Insect Model to Assess Correlation between Temperature and Virulence in *Bacillus Weihenstephanensis* and *Bacillus Cereus*." *FEMS Microbiology Letters* 317(2):196–202. doi: 10.1111/j.1574-6968.2011.02229.x.

Stenfors Arnesen, Lotte P., Annette Fagerlund, and Per Einar Granum. 2008. "From Soil to Gut: *Bacillus Cereus* and Its Food Poisoning Toxins." *FEMS Microbiology Reviews* 32(4):579–606. doi: 10.1111/j.1574-6976.2008.00112.x.

Stevens, Marc J. A., and Sophia Johler. 2020. "Draft Genome Sequence of CH_213, a Highly Cytotoxic *Bacillus Cytotoxicus* Strain Isolated from Mashed Potatoes." *Microbiology Resource Announcements* 9(35). doi: 10.1128/MRA.00836-20.

Stevens, Marc J. A., Taurai Tasara, Jochen Klumpp, Roger Stephan, Monika Ehling-Schulz, and Sophia Johler. 2019. "Whole-Genome-Based Phylogeny of *Bacillus Cytotoxicus* Reveals Different Clades within the Species and Provides Clues on Ecology and Evolution." *Scientific Reports* 9(1):1984. doi: 10.1038/s41598-018-36254-x.

Stewart, George C. 2015. "The Exosporium Layer of Bacterial Spores: A Connection to the Environment and the Infected Host." *Microbiology and Molecular Biology Reviews : MMBR* 79(4):437–57. doi: 10.1128/MMBR.00050-15.

Stragier, P., and R. Losick. 1990. "MicroReview Cascades of Sigma Factors Revisited." *Molecular Microbiology* 4(11):1801–6. doi: 10.1111/j.1365-2958.1990.tb02028.x.

Strauch, M. A., M. Perego, D. Burbulys, and J. A. Hoch. 1989. "The Transition State Transcription Regulator AbrB of *Bacillus Subtilis* Is Autoregulated during Vegetative

Growth." *Molecular Microbiology* 3(9):1203–9. doi: https://doi.org/10.1111/j.1365-2958.1989.tb00270.x.

Tausch, Franziska, Richard Dietrich, Kristina Schauer, Robert Janowski, Dierk Niessing, Erwin Märtlbauer, and Nadja Jessberger. 2017. "Evidence for Complex Formation of the *Bacillus Cereus* Haemolysin BL Components in Solution." *Toxins* 9(9):288. doi: 10.3390/toxins9090288.

Tezcan-Merdol, Dilek, Marianne Ljungström, Jadwiga Winiecka-Krusnell, Ewert Linder, Lars Engstrand, and Mikael Rhen. 2004. "Uptake and Replication of *Salmonella Enterica* in *Acanthamoeba Rhysodes*." *Applied and Environmental Microbiology* 70(6):3706–14. doi: 10.1128/AEM.70.6.3706-3714.2004.

Thomas, W. E., and D. J. Ellar. 1983. "Mechanism of Action of *Bacillus Thuringiensis* Var *Israelensis* Insecticidal δ-Endotoxin." *FEBS Letters* 154(2):362–68. doi: https://doi.org/10.1016/0014-5793(83)80183-5.

Timofeev, Vitalii, Irina Bahtejeva, Raisa Mironova, Galina Titareva, Igor Lev, David Christiany, Alexander Borzilov, Alexander Bogun, and Gilles Vergnaud. 2019. "Insights from *Bacillus Anthracis* Strains Isolated from Permafrost in the Tundra Zone of Russia." *PLOS ONE* 14(5):e0209140. doi: 10.1371/journal.pone.0209140.

Tovar-Rojo, Federico, Monica Chander, Barbara Setlow, and Peter Setlow. 2002. "The Products of the SpoVA Operon Are Involved in Dipicolinic Acid Uptake into Developing Spores of *Bacillus Subtilis*." *Journal of Bacteriology* 184(2):584–87. doi: 10.1128/JB.184.2.584-587.2002.

Tran, Seav-Ly, Elisabeth Guillemet, Didier Lereclus, and Nalini Ramarao. 2013. "Iron Regulates *Bacillus Thuringiensis* Haemolysin HlyII Gene Expression during Insect Infection." *Journal of Invertebrate Pathology* 113(3):205–8. doi: 10.1016/j.jip.2013.04.001.

Tran, Seav-Ly, Elisabeth Guillemet, Maud Ngo-Camus, Cyril Clybouw, Andrea Puhar, Arnaud Moris, Michel Gohar, Didier Lereclus, and Nalini Ramarao. 2011. "Haemolysin II Is a *Bacillus Cereus* Virulence Factor That Induces Apoptosis of Macrophages." *Cellular Microbiology* 13(1):92–108. doi: 10.1111/j.1462-5822.2010.01522.x.

Tran, Seav-Ly, Andrea Puhar, Maud Ngo-Camus, and Nalini Ramarao. 2011. "Trypan Blue Dye Enters Viable Cells Incubated with the Pore-Forming Toxin HlyII of *Bacillus Cereus*." *PLOS ONE* 6(9):e22876. doi: 10.1371/journal.pone.0022876.

Trapecar, Martin, Thomas Leouffre, Morgane Faure, Henrik E. Jensen, Per E. Granum, Avrelija Cencic, and Simon P. Hardy. 2011. "The Use of a Porcine Intestinal Cell Model System for Evaluating the Food Safety Risk of *Bacillus Cereus* Probiotics and the Implications for Assessing Enterotoxigenicity." *APMIS* 119(12):877–84. doi: https://doi.org/10.1111/j.1600-0463.2011.02797.x.

Tsilia, Varvara, Frederiek-Maarten Kerckhof, Andreja Rajkovic, Marc Heyndrickx, and Tom Van de Wiele. 2016. "*Bacillus Cereus* NVH 0500/00 Can Adhere to Mucin but Cannot Produce Enterotoxins during Gastrointestinal Simulation." *Applied and Environmental Microbiology* 82(1):289–96. doi: 10.1128/AEM.02940-15.

Turell, M. J., and G. B. Knudson. 1987. “Mechanical Transmission of *Bacillus Anthracis* by Stable Flies (Stomoxys Calcitrans) and Mosquitoes (Aedes Aegypti and Aedes Taeniorhynchus).” *Infection and Immunity* 55(8):1859–61.

Turnbull, P. C., and J. M. Kramer. 1985. “Intestinal Carriage of *Bacillus Cereus*: Faecal Isolation Studies in Three Population Groups.” *The Journal of Hygiene* 95(3):629–38.

Turner, Wendy C., Kyrre L. Kausrud, Yathin S. Krishnappa, Joris P. G. M. Cromsigt, Holly H. Ganz, Isaac Mapaure, Claudine C. Cloete, Zepee Havarua, Martina Küsters, Wayne M. Getz, and Nils Chr. Stenseth. 2014. “Fatal Attraction: Vegetation Responses to Nutrient Inputs Attract Herbivores to Infectious Anthrax Carcass Sites.” *Proceedings of the Royal Society B: Biological Sciences* 281(1795):20141785. doi: 10.1098/rspb.2014.1785.

Uribe-Querol, Eileen, and Carlos Rosales. 2017. “Control of Phagocytosis by Microbial Pathogens.” *Frontiers in Immunology* 8. doi: 10.3389/fimmu.2017.01368.

Urrutia, Ítalo M., Andrea Sabag, Camila Valenzuela, Bayron Labra, Sergio A. Álvarez, and Carlos A. Santiviago. 2018. “Contribution of the Twin-Arginine Translocation System to the Intracellular Survival of *Salmonella Typhimurium* in *Dictyostelium Discoideum*.” *Frontiers in Microbiology* 9. doi: 10.3389/fmicb.2018.03001.

Valenzuela, Camila, Magdalena Gil, Ítalo M. Urrutia, Andrea Sabag, Jost Enninga, and Carlos A. Santiviago. 2021. “SopB- and SifA-Dependent Shaping of the *Salmonella*-Containing Vacuole Proteome in the Social Amoeba *Dictyostelium Discoideum*.” *Cellular Microbiology* 23(1):e13263. doi: 10.1111/cmi.13263.

Valtierra-de-Luis, Daniel, Maite Villanueva, Liliana Lai, Trevor Williams, and Primitivo Caballero. 2020. “Potential of Cry10Aa and Cyt2Ba, Two Minority δ-Endotoxins Produced by *Bacillus Thuringiensis* Ser. *Israelensis*, for the Control of *Aedes Aegypti* Larvae.” *Toxins* 12(6):E355. doi: 10.3390/toxins12060355.

Van Cuyk, Sheila, Alina Deshpande, Attelia Hollander, Nathan Duval, Lawrence Ticknor, Julie Layshock, LaVerne Gallegos-Graves, and Kristin M. Omberg. 2011. “Persistence of *Bacillus Thuringiensis* Subsp. *Kurstaki* in Urban Environments Following Spraying.” *Appl. Environ. Microbiol.* 77(22):7954–61. doi: 10.1128/AEM.05207-11.

Van der Henst, Charles, Tiziana Scrignari, Catherine Maclachlan, and Melanie Blokesch. 2016. “An Intracellular Replication Niche for *Vibrio Cholerae* in the Amoeba *Acanthamoeba Castellanii*.” *The ISME Journal* 10(4):897–910. doi: 10.1038/ismej.2015.165.

Van der Henst, Charles, Audrey Sophie Vanhove, Natália Carolina Drebes Dörr, Sandrine Stutzmann, Candice Stoudmann, Stéphanie Clerc, Tiziana Scrignari, Catherine Maclachlan, Graham Knott, and Melanie Blokesch. 2018. “Molecular Insights into *Vibrio Cholerae* ’s Intra-Amoebal Host-Pathogen Interactions.” *Nature Communications* 9(1):3460. doi: 10.1038/s41467-018-05976-x.

Vecchia, Elena Dalla, Michael Visser, Alfons J. M. Stams, and Rizlan Bernier-Latmani. 2014. “Investigation of Sporulation in the Desulfotomaculum Genus: A Genomic Comparison with the Genera *Bacillus* and *Clostridium*.” *Environmental Microbiology Reports* 6(6):756–66. doi: 10.1111/1758-2229.12200.

Verplaetse, Emilie, Leyla Slamti, Michel Gohar, and Didier Lereclus. 2015. "Cell Differentiation in a *Bacillus Thuringiensis* Population during Planktonic Growth, Biofilm Formation, and Host Infection" edited by F. M. Ausubel. *MBio* 6(3):e00138-15. doi: 10.1128/mBio.00138-15.

Veysseyre, F., C. Fourcade, J. P. Lavigne, and A. Sotto. 2015. "*Bacillus Cereus* Infection: 57 Case Patients and a Literature Review." *Médecine et Maladies Infectieuses* 45(11):436–40. doi: 10.1016/j.medmal.2015.09.011.

Vilain, Sébastien, Yun Luo, Michael B. Hildreth, and Volker S. Brözel. 2006. "Analysis of the Life Cycle of the Soil Saprophyte *Bacillus Cereus* in Liquid Soil Extract and in Soil." *Applied and Environmental Microbiology* 72(7):4970. doi: 10.1128/AEM.03076-05.

Vilas-Bôas, Laurival A., Gislayne F. L. T. Vilas-Bôas, Halha O. Saridakis, Manoel Victor F. Lemos, Didier Lereclu, and Olivia M. N. Arantes. 2000. "Survival and Conjugation of *Bacillus Thuringiensis* in a Soil Microcosm." *FEMS Microbiology Ecology* 31(3):255–59. doi: 10.1111/j.1574-6941.2000.tb00691.x.

Wang, Yu-Jen, Sung-Chou Li, Wei-Chen Lin, and Fu-Chin Huang. 2021. "Intracellular Microbiome Profiling of the *Acanthamoeba* Clinical Isolates from Lens Associated Keratitis." *Pathogens* 10(3):266. doi: 10.3390/pathogens10030266.

Wang, Zeyu, Chunxia Gan, Jian Wang, Alejandra Bravo, Mario Soberón, Qing Yang, and Jie Zhang. 2021. "Nutrient Conditions Determine the Localization of *Bacillus Thuringiensis* Vip3Aa Protein in the Mother Cell Compartment." *Microbial Biotechnology* 14(2):551–60. doi: 10.1111/1751-7915.13719.

Warren, G. 1997. "Vegetative Insecticidal Proteins: Novel Proteins for Control of Corn Pests." *Undefined*.

Weekers, Peter H. H., John P. H. Wijen, Bart P. Lomans, and Godfried D. Vogels. 1996. "Axenic Mass Cultivation of the Free-Living Soil Amoeba, *Acanthamoeba Castellanii* in a Laboratory Fermentor." *Antonie van Leeuwenhoek* 69(4):317–22. doi: 10.1007/BF00399620.

Wei, Jun-Zhi, Kristina Hale, Lynn Carta, Edward Platzer, Cynthie Wong, Su-Chiung Fang, and Raffi V. Aroian. 2003. "*Bacillus Thuringiensis* Crystal Proteins That Target Nematodes." *Proceedings of the National Academy of Sciences* 100(5):2760–65. doi: 10.1073/pnas.0538072100.

Weiner, Matthew A., and Philip C. Hanna. 2003. "Macrophage-Mediated Germination of *Bacillus Anthracis* Endospores Requires the GerH Operon." *Infection and Immunity* 71(7):3954–59. doi: 10.1128/IAI.71.7.3954-3959.2003.

Wenner, Kimberly A., and Julie R. Kenner. 2004. "Anthrax." *Dermatologic Clinics* 22(3):247–56. doi: 10.1016/j.det.2004.03.001.

West, A. W., H. D. Burges, T. J. Dixon, and C. H. Wyborn. 1985. "Survival of *Bacillus Thuringiensis* and *Bacillus Cereus* Spore Inocula in Soil: Effects of PH, Moisture, Nutrient Availability and Indigenous Microorganisms." *Soil Biology and Biochemistry* 17(5):657–65. doi: 10.1016/0038-0717(85)90043-4.

Wheat, William H., Amy L. Casali, Vincent Thomas, John S. Spencer, Ramanuj Lahiri, Diana L. Williams, Gerald E. McDonnell, Mercedes Gonzalez-Juarrero, Patrick J. Brennan, and Mary Jackson. 2014. "Long-Term Survival and Virulence of *Mycobacterium Leprae* in Amoebal Cysts." *PLOS Neglected Tropical Diseases* 8(12):e3405. doi: 10.1371/journal.pntd.0003405.

Williams, Lonnie D., George A. Burdock, Guillermo Jiménez, and Marisol Castillo. 2009. "Literature Review on the Safety of Toyocerin®, a Non-Toxigenic and Non-Pathogenic *Bacillus Cereus* Var. Toyoi Preparation." *Regulatory Toxicology and Pharmacology* 55(2):236–46. doi: 10.1016/j.yrtph.2009.07.009.

Wilson, Gary R., and Thomas G. Benoit. 1990. "Activation and Germination of *Bacillus Thuringienis* Spores in *Manduca Sexta* Larval Gut Fluid." *Journal of Invertebrate Pathology* 56(2):233–36. doi: 10.1016/0022-2011(90)90105-F.

Wilson, Gary R., and Thomas G. Benoit. 1993. "Alkaline PH Activates *Bacillus Thuringiensis* Spores." *Journal of Invertebrate Pathology* 62(1):87–89. doi: 10.1006/jipa.1993.1079.

Wu, Je-Jia, Jenn-Wen Huang, and Wen-Ling Deng. 2020. "Phenylacetic Acid and Methylphenyl Acetate From the Biocontrol Bacterium *Bacillus Mycoides* BM02 Suppress Spore Germination in *Fusarium Oxysporum* f. Sp. *Lycopersici*." *Frontiers in Microbiology* 11:569263. doi: 10.3389/fmicb.2020.569263.

Yi, Yanglei, Elrike Frenzel, Jan Spoelder, J. Theo M. Elzenga, Jan Dirk van Elsas, and Oscar P. Kuipers. 2018. "Optimized Fluorescent Proteins for the Rhizosphere-Associated Bacterium *Bacillus Mycoides* with Endophytic and Biocontrol Agent Potential." *Environmental Microbiology Reports* 10(1):57–74. doi: https://doi.org/10.1111/1758-2229.12607.

Zheng, Meiying, Artem G. Evdokimov, Farhad Moshiri, Casey Lowder, and Jeff Haas. 2020. "Crystal Structure of a Vip3B Family Insecticidal Protein Reveals a New Fold and a Unique Tetrameric Assembly." *Protein Science* 29(4):824–29. doi: https://doi.org/10.1002/pro.3803.

Résumé substantiel en français

Le groupe *Bacillus cereus sensu lato* (Bc sl) comprend un nombre croissant d'espèces, dont *Bacillus cereus sensu stricto* (Bc ss), *Bacillus thuringiensis* (Bt) et *Bacillus anthracis* (Ba). Les spores de ces bactéries pathogènes sont couramment trouvées dans le sol, qui est généralement considéré comme un réservoir plutôt que comme un environnement favorable à la multiplication de ces bacilles. Le développement de ces bactéries dans le sol a longtemps fait l'objet de débats et il n'existe actuellement aucune preuve permettant de trancher définitivement la question. Des études ont abordé l'écologie de Bc ss dans le sol et ont montré qu'il s'agit d'une bactérie saprophyte, avec un cycle de vie dans la matière organique soluble extraite du sol et dans un microcosme artificiel du sol. D'autres études portant sur la survie de Bt dans le sol ont montré que la bactérie se maintient sous forme de spores qui ne peuvent pas germer, ni se multiplier. Conformément à ces dernières observations, une analyse génomique a révélé que la souche Bc ATCC14579 ne peut pas utiliser efficacement les glucides végétaux complexes comme peut le faire *B. subtilis*, ce qui suggère que l'espèce Bc ss est mieux adaptée à un régime protéique. Étant donné la relation étroite entre Bt et Bc ss, cette information est également pertinente pour Bt et probablement pour toutes les espèces du groupe Bc sl. De plus, la présence de nombreux gènes codant pour des enzymes protéolytiques chez les bactéries du groupe Bc sl est cohérente avec leurs propriétés pathogènes. Bc ss et Ba sont pathogènes pour les mammifères et responsables respectivement de toxi-infections alimentaires et de la maladie du charbon. Bt est une bactérie entomopathogène qui produit des toxines cristallines insecticides (protéines Cry et Cyt) et divers facteurs de virulence qui permettent à la bactérie d'endommager les tissus de l'hôte et de résister aux défenses immunitaires, provoquant finalement une septicémie. Toutes les espèces du groupe Bc sl partagent un fond génomique commun et un ensemble de facteurs de virulence. La plupart de ces facteurs de virulence exportés sont contrôlés positivement par le régulateur PlcR à la fin de la croissance exponentielle et au début de la phase stationnaire, sauf pour Ba, chez qui une mutation non-sens abolit l'activité de PlcR. Il a été démontré que le régulon PlcR joue un rôle majeur dans la virulence de Bt et de Bc ss dans divers modèles animaux. Bt et Bc ss produisent aussi d'autres facteurs de virulence non régulés par PlcR, tels que la métalloprotéase InhA1 et l'hémolysine HlyII, qui sont produits au début de la phase stationnaire. Dans un contexte d'infection des macrophages, InhA1 confère aux spores de Bt la capacité de s'échapper des cellules et HlyII provoque la mort cellulaire des macrophages par apoptose grâce à son activité de formation de pores. Cependant, la lignée

cellulaire de macrophages J774 utilisée dans ces études ne reflète pas la réponse des macrophages primaires.

Les protozoaires libres, comme *Acanthamoeba*, sont d'autres organismes présents dans le sol. En fonction des conditions de croissance environnementales rencontrées, ils peuvent adopter deux formes de développement : le trophozoïte, une forme de vie végétative métaboliquement active, et le kyste, de forme ronde, dormante et résistante au stress. Les principaux mécanismes d'alimentation des amibes libres sont la pinocytose et la phagocytose des micro-organismes du sol tels que les bactéries, les champignons ou les algues présents dans leur environnement. Certaines bactéries sont capables de résister aux amibes, car elles sont capables d'échapper à la prédation, d'autres sont capables de résister à la digestion intracellulaire et survivent donc de manière intracellulaire, ou encore sont capables de survivre, de se développer et de s'échapper des amibes après phagocytose. Environ 20 % des bactéries pathogènes humaines, parmi lesquelles *Legionella*, *Mycobacterium* ou *Listeria*, sont capables de vivre et de se multiplier à l'intérieur d'amibes libres. Les amibes pourraient donc représenter un réservoir pour les bactéries, permettant la propagation des bactéries dans l'environnement ou fournir un abri pour les protéger de divers stress physiques et chimiques tels que les biocides. *Acanthamoeba* a été signalé comme un amplificateur et un réservoir de bactéries telles que *Mycobacterium* et *Legionella*. Par ailleurs, *Bacillus* et les amibes partagent les mêmes niches écologiques, comme le sol et les insectes, et *Bacillus* a été identifié comme le genre le plus abondant dans le microbiome bactérien des amibes libres. L'interaction entre les deux organismes a été étudiée par plusieurs groupes de recherche. Il a été démontré qu'après phagocytose par *Acanthamoeba castellanii*, les spores de Ba sont capables de germer à l'intérieur des phagosomes des amibes. Les cellules végétatives se développent ensuite en chaînes avant de lyser la cellule hôte. Comme pour l'infection par les macrophages des mammifères, le plasmide pXO1 est nécessaire à la croissance de Ba dans les amibes. Les principaux gènes de toxine codés par pXO1 (*pagA*, *lef* et *cya*) semblent être inutiles pour la germination des spores de Ba et le processus de croissance externe qui s'ensuit, ce qui suggère que d'autres gènes pXO1 jouent un rôle dans ce processus. Les relations entre la souche Bt *israelensis* 4Q7 et les protozoaires a également été étudiée. En présence *d'A. polyphaga* et de *Dictyostelium discoideum*, Bt *israelensis* s'est développé sous forme de longues chaînes ou a formé des micro-colonies, en fonction de la densité des amibes. Ces observations suggèrent que ces structures bactériennes sont résistantes à la phagocytose par les amibes. Il a été démontré que les facteurs libérés par les amibes favorisaient la germination de Bt, permettaient sa croissance et induisaient un développement

filamenteux. Les interactions entre la souche Bc ATCC14579 et plusieurs protozoaires ont également été étudiées. Il a été démontré que *A. castellanii*, *Tetrahymena pyriformis*, et *Cercomonas* spp. peuvent ingérer et utiliser les spores ou les cellules végétatives de Bc ss pour leur croissance. Ils ont également observé la survie et la prolifération des bactéries ingérées à l'intérieur des cellules de protistes, ce qui suggère que les protistes peuvent agir comme un amplificateur de Bc ss dans le sol. Toutefois, sur la base des données publiées, on ne sait toujours pas si les amibes permettent à toutes les espèces de bactéries du groupe Bc sl de se multiplier, ce qui laisse ouverte la question de la forte abondance des spores de Bc sl dans le sol. Ba utilise les amibes pour se développer, ce qui entraîne une diminution de la population d'amibes, tandis que Bc ss et Bt semblent être des proies pour les amibes, car aucune augmentation de la population de Bc ss ou de Bt n'a été observée lors de la co-culture des deux organismes. En revanche, la population d'amibes est capable de se développer sur Bc ss et Bt. Cependant, une germination occasionnelle de Bc ss et Bt et une croissance intracellulaire ont été rapportées dans les deux dernières études citées. Globalement, ces observations soulèvent la question de savoir si Ba représente une exception au sein du groupe Bc sl ou si d'autres bactéries Bc sl sont également capables d'utiliser des amibes pour s'amplifier. Elles soulèvent également la question de savoir si les amibes permettent aux spores des bactéries Bc sl de germer et aux cellules végétatives de se développer et de sporuler.

Nous avons formulé l'hypothèse que les amibes peuvent être également un amplificateur pour Bc sl et étudié le devenir de ces bacilles et des amibes lorsque ces deux micro-organismes sont cultivés ensemble. Nous avons étudié le comportement de 7 souches appartenant au groupe Bc sl en interaction avec l'amibe libre *A. castellanii.* Les souches de *Bacillus* sélectionnées sont les suivantes : Bt 407, Bt *kurstaki* HD73, et Bt *israelensis* IPS82, en tant que représentantes des souches Bt insecticides ; Bt *konkukian*, qui n'est pas pathogène pour les insectes mais est proche de Ba; Bc 569 et la souche émétique Bc AH187, choisies comme représentatives de l'espèce Bc ss; et B. *weihenstephanensis* (Bw) KBAB4, sélectionnée comme représentante des espèces psychrotrophes. Les observations microscopiques de ces deux organismes cultivés de façon statique dans le milieu de co-culture PAS-Glucose-*E. coli* que nous avons mis au point, ont montré que les spores de ces sept souches étaient localisées dans les amibes, dans des structures de type vacuoles, indiquant qu'elles avaient été efficacement phagocytées après leur mise en contact. Après 24h de co-culture, une population bactérienne mixte, de spores et de cellules végétatives, a été observée. Nous avons également remarqué que les cellules végétatives avaient formé des structures de type chaines et des agrégats dans le milieu de

culture à l'extérieur des amibes. En absence d'amibes, aucune spore d'aucune souche n'avait germé sur une période de 24h dans le milieu de co-culture. Nos résultats montrent que les sept souches Bt, Bw et Bc ss étudiées se comportent de la même manière en présence d'*A. castellanii*, un comportement qui avait également déjà été observé pour la souche Bt *israelensis* 4Q7 lorsqu'elle était cultivée en présence *d'A. polyphaga* par Beeton et collaborateurs en 2013. Les bactéries du groupe Bc sl (à l'exception de Ba) produisent des facteurs de virulence exportés, tels que des enzymes de dégradation, des cytotoxines et des hémolysines, au début de la phase stationnaire lors de l'activation du système de détection du quorum sensing PlcR-PapR. La souche Bc AH187 produit également la toxine émétique, le céréulide, en début de phase stationnaire et la souche Bt 407 produit l'hémolysine II (HlyII), qui a été caractérisée pour sa capacité à lyser les macrophages J774 par apoptose grâce à une activité de formation de pores. Nous avons étudié les effets de ces facteurs de virulence et évalué la viabilité des amibes incubées dans un milieu de culture dans lequel les souches Bt 407, Bt 407 Δ*plcR*, Bc 569, et Bc AH187 ont poussé jusqu'en début en phase stationnaire par comparaison avec la viabilité des amibes cultivées dans le milieu de culture frais. La viabilité des amibes a été déterminée par exclusion du colorant bleu trypan. Nous avons observé que la plupart des amibes restent viables après 2h ou 24h d'incubation. Moins de 1% de la population d'amibes étant colorée en bleu lorsqu'elle était incubée dans le milieu conditionné par *Bacillus*. Une proportion similaire de cellules viables a été obtenue dans l'échantillon témoin lorsque les amibes ont été incubées dans un milieu de culture frais. Ce résultat suggère que les facteurs de virulence produits par Bt 407, Bc 569 et Bc AH187 n'ont pas d'effet significatif sur la viabilité des amibes. Au contraire, nous avons pu observer par des observations microscopiques en time-lapse qu'*A. castellanii* peut phagocyter et digérer efficacement les cellules végétatives de Bt 407 pour se développer et empêcher la formation de kystes. En collaboration avec le laboratoire de P. Cosson en Suisse, nous avons déterminé si les cellules de *Bacillus* peuvent être utilisées pour soutenir la croissance d'une autre espèce d'amibe en réalisant des expériences sur *D. discoideum* et montré que celle-ci est également capable d'utiliser les cellules végétatives de *Bacillus* comme nourriture. Cependant, certaines cellules bactériennes peuvent échapper à ce destin et se multiplier. Nous avons déterminé le sort des bacilles dans l'environnement amibien en quantifiant le développement de la population de Bt 407 co-cultivée avec des amibes et en le comparant au développement de bactéries cultivées seules dans les mêmes conditions. Le milieu de co-culture a été inoculé avec des spores de Bt en présence ou non d'amibes. Le comptage des UFC a montré que le nombre total de spores est resté stable pendant 48 h dans

les deux conditions. Cette observation suggère que seule une petite partie de la population de spores a germé et est à l'origine de la population végétative observée précédemment en microscopie. Nous avons également étudié le développement des cellules végétatives de *Bacillus* en co-culture avec des amibes. Le nombre total d'UFC est resté stable, que les cellules végétatives aient été mises en co-culture avec des amibes ou cultivées seules pendant 24 h. La plupart des bactéries ont été détectées sous forme de spores après 24 h de culture avec ou sans amibes, avec des taux de sporulation pour Bt 407 de 96 % et 99 %, respectivement. Ainsi, la totalité de la population bactérienne a sporulé dans le milieu co-culture, avec ou sans amibe. Nous avons renforcé ces données qualitatives par des observations microscopiques sur 96h. La co-culture de spores avec des amibes a donné lieu à une population bactérienne hétérogène de spores et de cellules végétatives à 24 h. Certaines cellules végétatives avaient formé de petites chaînes et des agrégats. Au bout de 96 h de co-culture, nous avons observé une population mixte de cellules végétatives, de cellules sporulantes et de spores libres. Lorsque les spores de Bt ont été inoculées seules dans le même milieu, nous n'avons pas observé de germination et en cohérence, aucune cellule végétative n'a été observée au bout de 24 h ou de 96 h de culture. Ces résultats suggèrent que les spores de Bt 407 peuvent accomplir un cycle de vie complet (des spores aux cellules végétatives puis à nouveau devenir des spores) dans l'environnement amibien. Cependant, nos observations ne nous ont pas permis d'établir si les spores de Bt peuvent germer à l'intérieur des amibes, comme cela a été montré pour Ba par Dey et ses collaborateurs en 2012. Cependant, nos observations microscopiques ont permis de remarquer que le processus de sporulation semblait se dérouler plus rapidement lorsque la population bactérienne se développait en présence d'amibes. En effet, la présence de spores libres, c'est-à-dire des spores libérées des cellules mères, a été observée au bout de 15 h de co-culture pour les bactéries cultivées dans l'environnement amibien, mais n'a été identifiée qu'à partir de 20h lorsque les bactéries étaient cultivées en l'absence d'amibes. Nous avons alors souhaité suivre le développement physiologique et le déroulement de la phase stationnaire chez Bt lorsque les bactéries se développent dans un environnement amibien à l'aide de fusions transcriptionnelles entre un gène rapporteur fluorescent et des promoteurs qui sont activés pendant la phase stationnaire. Deux promoteurs ont été sélectionnés : P_{nprA}, qui est activé par le régulateur de quorum sensing NprR qui contrôle le nécrotrophisme en début de la phase stationnaire et P_{spoIID} qui est activé par le facteur sigma SigE pendant le stade précoce de la sporulation. Des observations microscopiques, ainsi qu'une analyse par cytométrie de flux, ont été réalisées sur des cellules de Bt en co-culture avec des amibes sur une période de 24 h afin de quantifier le

nombre de cellules exprimant les rapporteurs. Les analyses microscopiques ont montré que les cellules de Bt étaient présentes à l'intérieur et à l'extérieur des amibes et que l'expression du promoteur P_{nprA} et P_{spoIID} a commencé avant T2 et T4, respectivement. Nous avons observé des cinétiques d'expression similaires pour les deux promoteurs lorsque le Bt s'est développé en l'absence d'amibes. La quantification des bactéries fluorescentes exprimant P_{nprA} par cytométrie en flux a montré que deux populations étaient présentes, indiquant que P_{nprA} était exprimé de manière hétérogène, et que les proportions de bactéries exprimant *nprA*, ou non, étaient identiques que Bt pousse en présence d'amibes ou en leur absence. La quantification des bactéries exprimant P_{spoIID} en co-culture avec des amibes, a montré que le rapporteur était exprimé de façon hétérogène dans environ 15 % de la population à T4 et augmente au cours du temps pour atteindre environ 70 % des cellules à T8. Le pourcentage de cellules exprimant P_{spoIID} était plus faible lorsque les cellules Bt étaient cultivées en l'absence d'amibes. Dans des conditions axéniques, les cellules exprimant le rapporteur de sporulation représentaient 7 % de la population à T4 et un maximum de 40 % à T24. La cinétique d'expression et la quantification des bactéries exprimant les deux rapporteurs ont également été étudié dans un milieu conditionné par les amibes pour déterminer si la stimulation de la sporulation était due au contact direct des bactéries avec les amibes ou aux composants libérés ou consommés par les amibes. Nous avons pu montrer que la croissance des bactéries était identique dans un milieu conditionné par les amibes, ou non. Nous avons également pu observer que les cellules Bt cultivées dans des milieux conditionnés et non conditionnés ont montré une cinétique d'expression de P_{nprA} similaire. L'expression du rapporteur de nécrotrophisme démarre à T2 dans une petite fraction de la population pour atteindre approximativement 80 % des cellules à T6 avant de diminuer pour ne concerner que 35 % de la population à T24. En ce qui concerne le rapporteur de sporulation, l'expression de P_{spoIID} a augmenté plus rapidement dans le milieu conditionné par les amibes que dans le milieu non conditionné ; l'expression du gène rapporteur a commencé entre T2 et T4 et a augmenté pour représenter environ 35% de la population bactérienne. Les premières cellules exprimant P_{spoIID} ont été détectées à T4 dans le milieu non conditionné, mais elles ne représentaient que 1 % de la population. La population sporulante a augmenté avec le temps mais est restée inférieure à 20 % jusqu'à T10, pour finalement représenter 33 % des cellules à T24. Le pourcentage de cellules exprimant P_{spoIID} à T24 était similaire dans le milieu conditionné et non conditionné. Globalement, ces résultats suggèrent que l'environnement amibien accélère l'engagement des cellules Bt dans la sporulation.

En conclusion, cette étude utilisant des souches représentatives des trois principales espèces du groupe Bc sl, à l'exception de Ba, montre que ces bactéries ont un comportement similaire en présence d'amibes, telles que *A. castellanii* et *D. discoideum*. Les spores de ces bacilles peuvent utiliser les signaux moléculaires et les nutriments sécrétés par les amibes pour germer; les cellules végétatives se développent ensuite sous forme de chaînes ou d'agrégats. Bien qu'une partie des cellules végétatives soit phagocytée et digérée par l'amibe, une autre partie de la population bactérienne s'engage dans la sporulation et toutes les cellules végétatives finissent par sporuler. L'entrée dans cette voie de développement se produit plus rapidement dans un environnement amibien que dans des conditions axéniques. En contrepartie, les amibes peuvent également tirer un avantage de cette interaction en se nourrissant des cellules végétatives, mais pas de spores, pour se développer et retarder la formation de kystes. En cohérence avec cette affirmation, nous montrons que les amibes ne sont pas affectées par les nombreux facteurs de virulence produits par les souches de Bt et Bc ss testées. Bien que les Bt et Bc ss ne puissent pas se répliquer à l'intérieur des amibes, contrairement aux Ba, les amibes peuvent néanmoins contribuer à la propagation des *Bacillus* dans l'environnement. En effet, *Acanthamoeba* peut éventuellement compenser la perte causée par la digestion des cellules végétatives en fournissant un environnement favorable à la croissance des bactéries. De plus, nos résultats ont montré que l'environnement des amibes favorise la germination des spores et permet aux bactéries de compléter leur cycle de développement. Globalement, cette étude suggère que l'interaction amibe-*Bacillus* crée un cercle vertueux dans lequel chaque protagoniste aide l'autre à se développer.

université PARIS-SACLAY

ÉCOLE DOCTORALE
Agriculture, alimentation, biologie, environnement, santé (ABIES)

Titre : Le devenir des bactéries du groupe *Bacillus cereus* dans l'environnement : interaction avec les amibes

Mots clés : *Bacillus thuringiensis*, Amibe, sol, sporulation, phagocytose, écologie

Résumé : Le groupe *Bacillus cereus sensu lato* comprend plusieurs espèces bactériennes très proches, incluant *B. anthracis* (Ba), *B. cereus sensu stricto* (Bc), et *B. thuringiensis* (Bt). Ba est l'agent responsable de la maladie du charbon, Bc est impliqué dans des toxi-infections alimentaires et Bt est un pathogène d'insectes. Les spores de ces bactéries pathogènes sont présentes abondamment dans le sol. Cependant, ces bactéries ne sont pas capables de se multiplier dans ce type d'environnement naturel sans apport nutritionnel. Il a été montré que les amibes peuvent être considérées comme des amplificateurs de plusieurs espèces de bactéries pathogènes et leur implication potentielle pour expliquer la grande quantité de spores de Bc et de Bt dans le sol a été fréquemment proposée. Nous avons formulé l'hypothèse que les amibes peuvent être également un amplificateur pour *Bacillus* et étudié le devenir des *Bacillus* et des amibes lorsque ces deux microorganismes sont cultivés ensemble. Mes résultats montrent que les facteurs de virulence produits par Bt et Bc n'affectent pas la viabilité de l'amibe *Acanthamoeba castellanii* qui, au contraire, parvient à phagocyter et digérer les bactéries sous leur forme végétative pour soutenir sa croissance et empêcher sa transformation sous forme de kyste. Nous avons également observé que les spores bactériennes peuvent germer dans l'environnement amibien et que les cellules végétatives produites forment ensuite des chainettes et des agrégats qui apparaissent moins efficacement phagocytés par les amibes. En utilisant des fusions transcriptionnelles entre des gènes codant pour des protéines fluorescentes et des promoteurs spécifiques de la phase stationnaire et de la sporulation, nous avons montré que le processus de sporulation se déroule plus efficacement en présence d'amibes qu'en leur absence. De plus, mes résultats montrent que l'environnement amibien favorise la germination des spores et permet ainsi aux bactéries d'accomplir leur cycle développemental. En conclusion, mes travaux suggèrent que les interactions entre les amibes et *Bacillus* créent un cercle vertueux dans lequel chaque protagoniste aide l'autre à se développer.

Title : The fate of bacteria of the *Bacillus cereus* group in the environment: interaction with amoeba

Keywords : *Bacillus thuringiensis*, *Acanthamoeba*, soil, sporulation, phagocytosis, ecology

Abstract : The *Bacillus cereus sensu lato* group consists of several closely related species, including *B. anthracis* (Ba), *B. cereus sensu stricto* (Bc), and *B. thuringiensis* (Bt). Ba is the causal agent of anthrax, Bc is involved in some food poisoning events and Bt strains are pathogens to insects. Spores of these pathogenic bacteria are commonly found in the soil. However, evidence suggests that they are unable to grow in such a natural environment without nutrient input. Amoebas have been reported to be an amplifier for several species of pathogenic bacteria and their potential involvement to explain the large amount of Bt and Bc spores in soil has been frequently proposed. We speculated that amoebas are an amplifier for *Bacillus* and studied the fate of *Bacillus* and amoebas when cultured together. My results show that the virulence factors produced by Bt and Bc do not affect the amoeba *Acanthamoeba castellanii*, which, on the contrary, can phagocytose and effectively digest vegetative *Bacillus* cells to grow and prevent the formation of cysts. Bacterial spores can germinate in the amoeba environment and the vegetative cells can then form chains or aggregates that appear to be less efficiently phagocytosed by the amoeba. The use of transcriptional fusions between fluorescent reporter genes and stationary phase- and sporulation-specific promoters showed that the sporulation process occurs more efficiently in the presence of amoebas than in their absence. Moreover, my results showed that the amoeba environment promotes spore germination and allows the bacteria to complete their developmental cycle. Overall, my study suggests that the amoeba-*Bacillus* interaction creates a virtuous circle in which each protagonist helps the other to develop.

Maison du doctorat de l'Université Paris-Saclay
2ème étage aile ouest, Ecole normale supérieure Paris-Saclay
4 avenue des Sciences,
91190 Gif sur Yvette, France