Minireview

Progress in Food-related Research Focussing on *Bacillus cereus*

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Bacillus cereus is a gram-positive, rod-shaped, endospore-forming bacterium that occurs ubiquitously and is frequently isolated from soil and food products. When *B. cereus* is present in foods, it can cause spoilage and poisoning. The work of our group is focussed on several properties of *B. cereus* that are of key importance in the food-industry, e. g. sporulation, spore properties, biofilm formation and stress response. This review describes our progress and current research with *B. cereus* isolates from natural and industrial sources that will utilize the fully sequenced and annotated genome of the model strain, *B. cereus* ATCC 14579.

Key words: *Bacillus cereus*, food, biofilm, stress sigma-factor, spore

Introduction: *Bacillus cereus* in gut microbiology

B. cereus is responsible for an increasing number of food-borne diseases in industrial countries. The exact number of food poisonings caused by *B. cereus* in different countries is not known because it is not a reportable illness and is not always diagnosed²¹; furthermore, reporting procedures vary between countries⁹. The numbers of *B. cereus* poisonings reported in Europe between 1973–1985 range from 17.8% of total food-borne diseases in Finland to 0.8% in Scotland⁹. Careful investigation of food-borne outbreaks in the German Federal Armed Forces showed that *B. cereus* was by far the most frequently isolated pathogen in the retained food samples⁹, being responsible for 42% of the outbreaks reported between 1985 and 2000¹⁹. In Japan, few food poisonings are attributed to *B. cereus*, but the incidence could be much higher than reported considering that two surveys revealed the presence of *B. cereus* in food pro-

cessing factories and whole and processed vegetables sold in shops in Tokyo^{17,18}.

There are two types of *B. cereus* food poisoning syndromes: the emetic type, characterized by nausea and vomiting, and the diarrheal type, which includes abdominal pain, diarrhea and nausea^{10,11}. The emetic type is caused by cereulide, a heat stable, circular peptide antibiotic that is toxic to mitochondria by acting as a potassium ion channel^{2,22}. Cereulide is preformed by *B. cereus* in the food, is not destroyed upon heating and induces emetic symptoms shortly after ingestion of the poisoned food. The diarrheal type is caused by entero-toxins, of which the *B. cereus* genome is known to harbour several^{10,11}. These entero-toxins are heat-labile, and formed by *B. cereus* cells in the digestive tract after ingestion of food containing *B. cereus* spores. In these cases, the spore form allows *B. cereus* to survive in the acid stomach environment.

Surprisingly, despite its ability to cause food poisoning, *B. cereus* is reportedly being used in probiotic spore preparations to promote gut health⁸. Interestingly, *B. cereus* strains isolated from several commercial spore-based probi-

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otics were able to produce enterotoxins and can be expected to cause diarrheal syndrome in humans⁸). Obviously, more research is needed to study the safety and eventual effectiveness of *B. cereus*-containing probiotic products.

In conclusion, *B. cereus* is an important organism in gut microbiology. In the following mini-review, we give an overview of our current work on this interesting bacterium.

Biofilm formation by *B. cereus*

Several *B. cereus* strains are capable of biofilm formation^{25,26}). Biofilms are sessile, multicellular communities, embedded in a matrix consisting mainly of exopolysaccharides, and they are ubiquitously found in aqueous environments^{27,37}). They can cause a number of problems in industry, such as equipment failure, inefficient heat transfer and corrosion. Moreover, in the food industry, the release of biofilm cells from processing equipment can cause continuous contamination and recontamination of foods³⁸). Therefore, biofilm formation by *B. cereus* is an important hazard in the food industry.

To study the biofilm forming capacity of *B. cereus*, we collected 57 *B. cereus* strains, mainly isolated from the dairy industry, foods and food poisoning events. We tested these strains, including the model strain *B. cereus* ATCC 14579, for their ability to form biofilms, using an assay in which the bacteria are grown in polystyrene 96-wells microtiter-plates⁷). After incubation, the planktonic cells are washed away and the attached biofilm cells are stained with crystal violet. The amount of crystal violet bound to these biofilm cells is measured at 595 nm, and is a direct measure for the amount of biofilm formed.

The biofilm-forming capacity of the strains tested was found to be highly variable. In rich culture conditions (Luria Bertani broth), the majority of strains, including the type strain, were unable to form a biofilm, while several natural isolates produced large amounts of biofilm. However, in poor culture conditions (chemically defined medium⁶), the ability to form biofilms was much higher in almost all strains tested.

To gain more insight into the mechanisms involved in biofilm formation, we use several approaches. A random transposon knockout library has been generated using a *mini-Tn10* transposon³²), and is being screened for mutants affected in biofilm formation. We are currently testing a number of mutants with increased biofilm formation, to find the genes in which the transposon has been inserted. Other lines of investigation are the role of surface proteins in cell hydrophobicity and attachment to different substrata, and

the identification of biofilm-induced proteins using 2-dimensional gel electrophoresis^{25,26}). With these experiments, we aim to identify genes involved in *B. cereus* biofilm formation. The genes of interest will be investigated in detail by establishing knockout and overexpression mutants. The effect of these mutations on biofilm formation, virulence and stress resistance will be determined.

Role of σ^B in the stress response of *B. cereus*

Bacteria have to continuously adapt to changes in their environment. This process of adaptation is termed the microbial stress response. This response allows the cell to react to changes in its environment, either leading to a rapid resumption of its growth or increasing its chances of survival before the cell is irreversibly damaged. The stress response generally confers cross-protection against a wide variety of other stresses to the cell. The phenomenon of microbial stress response is of considerable interest in the study of food pathogens as these organisms may survive or even grow under the unfavorable conditions occurring during food production or storage, when a stress response is triggered¹). The cross-protection of *B. cereus* by stress exposure has been studied in considerable detail. Several stresses, such as osmotic upshock, acid shock, mild heat treatment or H₂O₂ can lead to increased thermotolerance or resistance to acid^{3,4,29}).

One of the central regulators of the stress response in gram-positive bacteria is the alternative sigma factor, σ^B . This factor is a secondary subunit of RNA polymerase (RNAP) that is known to play an important role in regulating gene expression when there are major changes in the environment. Upon binding of σ^B to core RNAP, genes downstream of a promoter that can be recognized by the σ^B -RNAP complex are transcribed. The role of σ^B in the stress response and its regulation has been extensively studied in the gram-positive model-organism *Bacillus subtilis* (Reference 12 for a comprehensive review), but also in *B. cereus*. In *B. cereus*, σ^B is activated in several stress conditions, such as ethanol exposure and acid shock. However, the largest activating effect is observed with heat shock³⁴). Furthermore, detailed studies on the expression of key sigma factors in a tightly controlled growth experiment, showed that σ^B of *B. cereus* is activated during the first stages of the stationary growth phase⁶).

We identified 17 σ^B -dependent genes of *B. cereus* by the combination of two-dimensional gel electrophoresis, σ^B -dependent promoter consensus sequences and in vitro transcription assays. Only two proteins encoded by these σ^B -de-

pendent genes, i.e. a catalase and an intracellular protease, which can turnover mis-folded proteins, have an obvious role in stress response. A larger group of proteins seem to have a role in metabolic reprogramming of the cell. These proteins have diverse functions, including the synthesis of the vitamin thiamine, the uptake of Mg^{2+} and/or Co^{2+} -ions, and of amino acids or peptides by the OppA system. Furthermore, the function of a considerable portion of σ^B -dependent proteins in *B. cereus* is still unknown. The determination of the function of these proteins is a challenge for future research and may lead to the discovery of novel mechanisms that can contribute to stress resistance³⁵.

Future research will focus on the role of σ^B of *B. cereus* in cold adaptation and oxidative stress. Moreover, we will analyse the signaling pathway that leads to the activation of σ^B upon stress exposure. This pathway appears to be different from the signaling cascade leading to σ^B -activation in *B. subtilis* and may provide clues to the exact triggers leading to the activation of σ^B in *B. cereus*.

ECF sigma factors of *B. cereus*

In the genome of *B. cereus* ATCC 14579, ten Extra Cytoplasmic Function (ECF) sigma factors are annotated¹⁶. ECF sigma factors are expressed as a reaction to extracytoplasmic stimuli and are found in a large variety of gram-positive and gram-negative bacteria. Like other alternative sigma factors, ECF sigma factors can bind to core-RNAP and stimulate transcription of distinct subsets of genes. ECF sigma factors are involved in controlling cell envelope-related functions such as secretion, synthesis of exopolysaccharides, or ion transport^{13,23}, and play a role in stress tolerance¹⁴.

Two of the ten ECF sigma factors of *B. cereus* ATCC 14579, σ^M (a homolog of *B. subtilis* σ^M , encoded by ORF RZC07902) and an unknown sigma factor encoded by ORF RZC03920, were examined in detail (gene designations are according to the *B. cereus* genome database^{16,28}). We followed their expression using RT-PCR and found that both are differentially expressed with different stress exposures: high salt, low pH, high pH, heat-shock, ethanol or oxidative stress. *In silico* analysis using the ERGO genome and discovery system²⁸ showed that RZC03920 has 26% homology with *B. subtilis* ECF sigma factors. In the case of σ^M the complete operon, consisting of two genes encoding the sigma factor and the anti-sigma factor, shows some homology with the σ^M operon of *B. subtilis*, 34% and 27% respectively. σ^M is one of the 7 ECF sigma factors of *B. subtilis* and has been reported to be essential for growth at high salt

concentrations¹⁴. Furthermore, antibiotics that inhibit cell wall biosynthesis induce the expression of the σ^M regulon in *B. subtilis*⁵.

Our future research aims to identify the role of ECF sigma factors in *B. cereus*, in particular of σ^M and the one encoded by ORF RZC03920. We constructed deletion mutants of RZC03920 and RZC07902 (encoding putative σ^M). These mutants will be used for phenotypic characterization. Furthermore, overexpression of these two sigma factors was realized in *B. cereus* using the NICE system²⁰. Analyzing both deletion mutants and the overexpression mutants of the ECF sigma factors with an amplicon-based micro-array of *B. cereus* ATCC 14579 will identify the regulons of these sigma factors.

B. cereus sporulation, spore resistance and germination

A key property of *B. cereus* is its ability to form dormant endospores. The spore form allows *B. cereus* to survive conditions that kill vegetative cells, such as starvation, desiccation, radiation, heat, low pH and toxic chemicals as well as antibiotics³⁰. In favourable conditions, spores can germinate and out-grow to form vegetative cells that can grow and proliferate (for recent reviews see References 24 and 31). Because of their resistance to cleaning, disinfection and food-processing spores are a major cause of food spoilage and poisoning, and intense world-wide research efforts are focussed on the elucidation of spore resistance and germination properties.

Our research focuses on sporulation in defined conditions, the state of the spore core as monitored by Electron Spin Resonance (ESR) and germination kinetics as well as genetics. Briefly, for sporulation studies a fermentation system was used in combination with a newly developed defined medium that supports growth and sporulation of *B. cereus*. Parameters such as carbon source, nitrogen source, pH, temperature and aeration can be manipulated precisely with this approach. We measured the expression of several key sigma factors involved in growth, stress response and sporulation, and determined key spore properties (such as heat resistance, germinability, dipicolinic acid content, hydrophobicity and (core) density) of spores produced in different conditions⁶ (de Vries *et al.*, presented at the European Spores Conference, Smolenice, Slovakia, 17 to 20 June 2004).

Spores have a very special structure, which is the basis for their extreme resistance properties^{33,36}. To study the native structure of spores, nondestructive techniques are re-

quired. We therefore used ESR, a non-destructive technique that allowed us to monitor the structure of the spore core during dormancy and after heat activation and germination (de Vries *et al.*, presented at the 9th meeting of the International Society for Microbial Ecology, Amsterdam, the Netherlands, 26–31 August, 2001).

A key spore property is the ability to germinate. Spores are equipped with special receptors that constantly monitor the environment for nutrients. When nutrients are sensed, the receptors are thought to transduce a signal that causes the spore to germinate^{24,31}). In the genome of *B. cereus*, 7 operons encoding such germination receptors have been identified, and knockout mutants have been constructed (Hornstra *et al.*, presented at Functional Genomics of Gram-Positive Microorganisms, 12th International Conference on Bacilli, Baveno, Italy, 22–27 June 2003; Hornstra *et al.*, presented at the European Spores Conference, Smolenice, Slovakia, 17–20 June 2004). Future work will focus on the function of these receptors, as well as on germination kinetics for which a high-throughput microplate method in combination with flow-cytometry and computer modelling will be deployed.

Conclusions and perspectives

In this mini-review, we have presented our research on *Bacillus cereus*, an important bacterium from both a scientific and applied perspective. Advances are being made to understand biofilm formation by identifying genes involved in the biofilmforming capacities of *B. cereus*. Better understanding of the activation of the alternative sigma factor σ^B , one of the central regulators of the stress response in gram-positive bacteria has recently been obtained for *B. cereus* and 17 σ^B -dependent genes have been identified. Moreover, two ECF sigma factors belonging to the *B. cereus* transcriptional activator were shown to be differentially expressed as a reaction to different extracytoplasmic stimuli. Finally, the conditions under which sporulation occurs have been defined and the structure of the spore core during dormancy and germination has been determined. The receptors involved in the induction of spore germination have been identified and their role in germination is being investigated. The anticipated availability of DNA micro-arrays, combined with proteomics and bio-informatics will further stimulate research and extend our knowledge of the important food-borne human pathogen *Bacillus cereus*.

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