Food Microbiology xxx (2015) 1–13

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Transcriptome analysis of *Bacillus thuringiensis* spore life, germination and cell outgrowth in a vegetable-based food model

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ARTICLE INFO

Article history:
Received 23 April 2015
Received in revised form
3 November 2015
Accepted 10 November 2015
Available online xxx

Keywords:
Bacillus cereus sensu lato
Food model
Microarray
Spores
Gene expression

ABSTRACT

Toxigenic species belonging to *Bacillus cereus sensu lato*, including *Bacillus thuringiensis*, cause foodborne outbreaks thanks to their capacity to survive as spores and to grow in food matrixes. The goal of this work was to assess by means of a genome-wide transcriptional assay, in the food isolate *B. thuringiensis* UC10070, the gene expression behind the process of spore germination and consequent outgrowth in a vegetable-based food model. Scanning electron microscopy and Energy Dispersive X-ray microanalysis were applied to select the key steps of *B. thuringiensis* UC10070 cell cycle to be analyzed with DNA-microarrays. At only 40 min from heat activation, germination started rapidly and in less than two hours spores transformed in active growing cells. A total of 1646 genes were found to be differentially expressed and modulated during the entire *B. cereus* life cycle in the food model, with most of the significant genes belonging to transport, transcriptional regulation and protein synthesis, cell wall and motility and DNA repair groups. Gene expression studies revealed that toxin-coding genes *nheC*, *cytK* and *hblC* were found to be expressed in vegetative cells growing in the food model.

1. Introduction

The genus *Bacillus* comprises Gram-positive species that can be found ubiquitously in the environment and can survive and persist to starvation and adverse growth conditions thanks to their ability to form resistant and metabolically inert spores (Schmidt et al., 2011). Six different *Bacillus* species, namely *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus thuringiensis* and *Bacillus weihenstephanensis*, although exhibiting different biological characteristics, such as pathogenicity and toxin production, have been classified on the basis of 16S sequencing (Daffonchio et al., 2003), multilocus sequence typing (MLST) (Priest et al., 2004) and comparative genome sequencing (Rasko et al., 2005) as a single taxonomical unit, the *Bacillus cereus sensu lato* (Helgason et al., 2000; Schmidt et al., 2011). Moreover, the *B. cereus sensu lato* was recently extended to comprise two further species: *Bacillus toyonensis*, a strain employed in animal nutrition (Jiménez et al., 2013) and *Bacillus cytotoxicus*, which is a thermotolerant species associated with food poisonings (Guinebretière et al., 2013). All these species are associated by their genome structure, but they often demonstrate specific traits that are due to large plasmid determinants (Jensen et al., 2003). *Cry* genes located on a large plasmid in *B. thuringiensis* (Schnepf et al., 1998), cereulide gene on a mega virulence plasmid in *B. cereus* (Hoton et al., 2005) and capsule genes on pXO1 and pXO2 plasmids in *B. anthracis* (Ehling-Schulz et al., 2006) are only some examples. Moreover, the toxicological potential, the occurrence of *B. cereus*-related food-borne outbreaks (Bargabus et al., 2002), the possible food contamination of *B. thuringiensis* as a result of its wide biopesticide use (Hendriksen and Hansen, 2006), and the high level of genomic similarities between the species forming the group (Schmidt et al., 2011), indicate that the *B. cereus sensu lato* should be studied as a whole.

Bacillus spores are indeed often present in food matrices, where they can resist preservation treatments and germinate in the end-product if nutrients and proper conditions to return to vegetative life are available (Dworkin and Shah, 2010). Their presence is indeed responsible for serious food-borne illnesses (EFSA, 2015) and significant numbers of food spoilage cases (Drean et al., 2015;

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http://dx.doi.org/10.1016/j.fm.2015.11.006
0740-0020/

Markland et al., 2013). Factors triggering germination in food can be represented by amino acids, sugars and other molecules or conditions (pH, sub-lethal thermal treatments, a_w, storage conditions) that let the spore exit from its dormant state (Moir and Smith, 1990; Moir et al., 2002), the precondition for the subsequent growth and toxins production.

The spore inert state has been demonstrated with the evidence of a jelly core and a partial dehydration that forms an immobile matrix (Cowan et al., 2003; Driks, 2002; Nakashio and Gerhardt, 1985), but it's still disputed if the spore maintains some minimal metabolic activity. What has been already demonstrated is that spores of different *Bacillus* and *Clostridium* species contain RNA transcripts (Bassi et al., 2013; Bettegowda et al., 2006; Dembek et al., 2013; Keijser et al., 2007) and that the amount of RNA seems to be affected by the spore incubation temperature and spore age (Segev et al., 2012). The function of these transcripts is still largely unknown: they could represent preserved RNA molecules ready for a new cycle, or degraded molecules useful to be the starting point for a *de novo* synthesis. The RNA state appears to affect the exit from dormancy together with the effects of environmental signals (Segev et al., 2012). The *Bacillus* spores behaviour in a food matrix in terms of RNA state and gene expression during germination is also still unknown. Factors triggering germination and outgrowth have been largely studied in *Bacillus subtilis* (Keijser et al., 2007) and *B. cereus* (Abee et al., 2011), while germination studies of *B. cereus* in foodstuffs have been recently performed in milk (Bartoszewicz et al., 2013), cream, béchamel sauce and mixed vegetable soup (Samapundo et al., 2014) and as a probabilistic model of predictive microbiology in refrigerated processed foods of extended durability (Daelman et al., 2013). The germination process has been studied also in terms of gene expression by DNA-microarrays particularly in *B. cereus* for screening purposes (Sergeev et al., 2006), and to assess the microorganism' response to salt stress (Den Besten et al., 2009), preservatives such as sorbic acid (Van Melis et al., 2011), disinfectants (Ceragioli et al., 2010) and modified atmosphere (Passalacqua et al., 2009). DNA-microarrays were also applied to assess the germination of spores in *B. subtilis* (Berka et al., 2002; Keijser et al., 2007), *Clostridium novyi* (Bettegowda et al., 2006), *Clostridium sporogenes* (Bassi et al., 2013) and *Clostridium difficile* (Dembek et al., 2013).

The aim of this work was to simulate a *B. cereus* contamination in an experimental vegetable food model and to study the gene expression profile during the initial colonization of food as spore entities and during germination and outgrowth phases. To do this, we investigated the phenotypic behaviour of a strain isolated from spoilt food through OD measurements, scanning electron microscopy (SEM) and Energy Dispersive X-ray (EDAX) microanalysis that allowed us to determine the critical steps representing the entire time-course of *Bacillus* life cycle in a foodstuff. An array-based transcriptome analysis was then applied to study genes differentially expressed in these steps, particularly during the germination process.

2. Materials and methods

2.1. Bacterial strain isolation, growth conditions and spore production

The strain UC10070 was isolated from a biofilm on a spoilt vegetable-based puree using a *B. cereus* selective agar medium (Oxoid, Milan Italy). It was assigned to the *Bacillus cereus sensu lato* by means of 16S rRNA gene analysis, and specifically to *B. thuringiensis* on the basis of cry toxins identification. The strain was cultured in Brain Heart Infusion (BHI) broth at 37 °C in aerobic conditions on continuous shaking.

Spores were produced from cells cultured in BP medium (Bacillus Genetic Stock Center, Ohio State University, Columbus, OH, USA). BP plates were inoculated with 500 μl of *B. thuringiensis* UC10070 overnight cultures, and incubated for 4 days at 37 °C. Spores were harvested and purified by extensive washing with distilled water at 4 °C (Nicholson and Setlow, 1990). The spore crops, checked by phase-contrast microscopy, were free (<1%) of vegetative cells and germinating spores. Spore suspensions were maintained at 4 °C, and immediately used for the subsequent analyses.

2.2. Toxin assays

The presence of enterotoxin genes and their expression in *B. thuringiensis* UC10070 were assessed by means of PCR and RT-PCR analyses. Primer sets and conditions listed in Table 1 were used to detect *nheBC*, *hblCD*, *cytK*, *cry* and *ces* genes.

The ability of the strain to produce the diarrhoeal enterotoxin HBL (haemolytic fraction L2) was also tested by the reverse passive latex agglutination test using BCET-RPLA toxin detection kit (Oxoid). After 1% inoculum of the strain in BHI broth, and incubation at 37 °C for 18 h on shaking (250 cycles/min), the culture was centrifuged; supernatants were filter-sterilized and stored at −20 °C until the assay performance according to the manufacturers' instructions.

2.3. Food model and germination assays

Three commercial thermally treated vegetable creams based respectively on pepper, artichoke and spinach, and an experimental pasteurized soup, made with courgettes, potatoes and milk (CPM), were tested in triplicates. CPM food model was prepared by mixing homogeneously fresh courgettes and potatoes, previously washed, trimmed and peeled, to UHT milk with a ratio of 3:1:1. The mixture obtained was sterilized at 121 °C for 15 min. pH and a_w values of each soup were determined before proceeding with the experiments. The samples were inoculated with 10^5 CFU/ml and incubated at 20 °C and at 4 °C for seven days. Growth dynamics for *B. thuringiensis* UC10070 were analyzed by plate counts.

Germination assays were carried out at room temperature by inoculating 100 μl of *B. thuringiensis* UC10070 spore suspension (10^7 CFU/ml) in selected food products followed by anaerobic packaging with Anaerocult A packs (Merck, Darmstadt, Germany) and by a heat treatment for 15 min at 80 °C mimicking processing conditions.

The germination process in the CPM food model was monitored by optical and scanning electron microscopy (SEM) together with Energy Dispersive X-ray (EDAX) analyses as described below. Microscopy data were confirmed by CFU counts plating before and after pasteurization for 20 min at 80 °C respectively for total cells (vegetative and spores) and spore counts.

For expression studies, the time points T_0 (dormant spores), 40 min (GSP, germinating spores), 2 h (C2h, early-log phase) and 12 h (C12h, mid-log phase) after thermal treatment, were selected as the most representative of the *B. thuringiensis* UC10070 life cycle under the tested conditions. Samples used for microscopy and molecular analyses were taken from the inoculated CPM food model at the above time points and immediately stored at −80 °C for RNA extraction. All studies were carried out in three independent experiments.

2.4. Microscopy examinations

The transition from spores to vegetative cells was monitored by optical phase contrast microscopy, SEM and EDAX microanalyses.

Please cite this article in press as: Bassi, D., et al., Transcriptome analysis of *Bacillus thuringiensis* spore life, germination and cell outgrowth in a vegetable-based food model, Food Microbiology (2015), http://dx.doi.org/10.1016/j.fm.2015.11.006