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Persistence strategies of *Bacillus cereus* spores isolated from dairy silo tanks

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ABSTRACT

Survival of *Bacillus cereus* spores of dairy silo tank origin was investigated under conditions simulating those in operational dairy silos. Twenty-three strains were selected to represent all *B. cereus* isolates ($n = 457$) with genotypes (RAPD-PCR) that frequently colonised the silo tanks of at least two of the sampled eight dairies. The spores were studied for survival when immersed in liquids used for cleaning-in-place (1.0% sodium hydroxide at pH 13.1, 75 °C; 0.9% nitric acid at pH 0.8, 65 °C), for adhesion onto nonliving surfaces at 4 °C and for germination and biofilm formation in milk. Four groups with different strategies for survival were identified. First, high survival (log 15 min kill ≤1.5) in the hot-alkaline wash liquid. Second, efficient adherence of the spores to stainless steel from cold water. Third, a cereulide producing group with spores characterised by slow germination in rich medium and well preserved viability when exposed to heating at 90 °C. Fourth, spores capable of germinating at 8 °C and possessing the *cspA* gene. There were indications that spores highly resistant to hot 1% sodium hydroxide may be effectively inactivated by hot 0.9% nitric acid. Eight out of the 14 dairy silo tank isolates possessing hot-alkali resistant spores were capable of germinating and forming biofilm in whole milk, not previously reported for *B. cereus*.

1. Introduction

Bacillus cereus is a spore forming bacterium commonly contaminating raw milk and considered a major microbiological problem in the dairy industry (Andersson et al., 1995). Heat stable spores of *B. cereus* in milk are a source of contamination for milk derived products, such as milk powder, infant food formulas (Becker et al., 1994; Shaheen et al., 2006) and many food commodities (Wijnands et al., 2006).

It is known that *B. cereus* spores occur in low numbers (10^2–10^3 per liter) in farm collected milk (Banyko and Vyletelova, 2009; Bartoszewicz et al., 2008; Christiansson et al., 1999; Svensson et al., 2004, 2006; Vissers et al., 2007). Studies by global typing methods (fatty acid profiling, biochemical typing, RAPD (random polymorphic DNA)-PCR, rep-PCR fingerprinting) have shown that the distribution of genotypes in the dairy and its products differed from that in raw milk (Bartoszewicz et al., 2008; Lin et al., 1998; Svensson et al., 1999, 2004; Te Giffel et al., 1997, 2002). Thus the farms are not the sole source of *B. cereus* in dairy milk. Additional contamination of milk occurs after the arrival to the dairy plant.

A modern dairy plant is not an easy environment for *B. cereus* to colonise. The incoming milk is stored at cold temperature, heat treated, and the equipment is washed with hot, highly alkaline (pH > 13) and acid (pH < 1) liquids. It has been shown that certain genotypes of *B. cereus* recurr in dairy silo tanks (Svensson et al., 2004, 2006) but the phenotypic properties enabling the persistence under the dairy conditions are not understood. Spore adhesion to nonliving surfaces at cold temperature has rarely been studied.

The aim of this study was to identify phenotypic features of the recurrent *B. cereus* dairy silo genotypes to explain their frequent presence in the silo tanks. We report here on the spore survival properties of 23 *B. cereus* strains from dairy silo tanks, selected to represent the isolates ($n = 457$) with RAPD-PCR genotypes frequent in the silo tanks of several dairy plants. We exposed the spores to conditions simulating those in an operational dairy, including highly alkaline and acid liquids at high temperature applied during the cleaning-in-place (CIP) procedures. We also inspected the ability of the spores to adhere in cold environments to stainless steel and other nonliving materials, their germination at cold temperature and ability to form biofilm.

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2. Materials and methods

2.1. Bacterial strains and their characterisations

The dairy silo tank isolates of *B. cereus* (*sensu lato*) in this study originated from a study conducted in milk silos of eight different dairies over a period of two years (Svensson et al., 2004). The isolates indicated as psychrotrophs (marked P in tables/figures) grew (Svensson et al., 2004) on TSA at 8 °C in 7–10 days and were PCR positive (using the primers of Francis et al., 1998) for the *CspA* gene. The 23 strains selected for the present study represented all ($n = 453$) isolates that belonged to the frequently detected RAPD-PCR patterns (Nilsson et al., 1998; Svensson et al., 2004) and had been found from the silos of more than one dairy. Strains UM 218, GO 282, SU 160, SU 226, GR 177 were deposited to the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig DE).

Ribopattern analysis was done as described by Apetroaie et al. (2005). The reference libraries were the commercially available library of Qualicon (RiboExplorer, release 2008, software v.2.1.4216.0, Dupont, Wilmington NJ) and the in-house (Mirja Salkinoja-Salonen, Helsinki University) library of 120 well characterised strains of *B. cereus sensu lato*. Emetic toxin (cereulide) production was detected by the bioassay based on loss of sperm motility as described by Andersson et al. (2004) and confirmed by the chemical assay based on cereulide specific mass ions as described by Jääskeläinen et al. (2003).

Spores were prepared as described by Magnusson et al. (2006). The spore count was determined on plate count agar and the spores were diluted to a concentration of $1–5\times10^9$ cfu ml^{-1} and stored in sterile saline at 4 °C until use.

2.2. Reagents and media

Reagents. The neutralizing reagent was 67 mM Sörensen Na–K phosphate (KH_2PO_4–Na_2HPO_4) buffer according to Sörensen, pH 7.0. Fixation reagent for electron microscopy was 2.5% (w/v) glutaraldehyde (J.T. Baker Chemical Co.) in 0.1 M Na–K–phosphate buffer pH 7.2. Acridine orange staining solution contained 100 μg of acridine orange ml^{-1} (Molecular Probes Europe, Leiden, The Netherlands) in water. The Live/Dead stain was Syto 9 (3.34 μM) with propidium iodide (PI, 1.5 mg ml^{-1}), Molecular Probes Europe (Leiden, The Netherlands) Media. Plate Count Agar (PCA) and R2 were prepared as described in Eaton et al. (2005), tryptic soy broth (TSB) and skim milk medium were from Difco (Detroit, MD, USA). The whole milk medium was pasteurized milk (3.5% fat) purchased from a local store and autoclaved (15 min, 121 °C). The electric conductances of the media used for the spore adhesion experiments were measured as follows (mS cm^{-1}): skim milk, 5.3, tryptic soy broth 13.6, R2 broth 0.87, drinking water 0.15.

2.3. Alkali and acid tolerance of the spores

To prepare the test suspension of spores, 100 μl of the stock ($1–5 \times 10^9$ cfu ml^{-1}) was diluted in 100 ml of sterile water and the spore count determined (in triplicate, concentration at time zero) following dilution 1:10 in the neutralizing reagent. For measurement of hot-alkaline resistance, 100 ml of 1% (w/v) aqueous NaOH (pH 13.1) in a 200 ml flask was heated at 77 °C in a water bath. When the temperature in a parallel flask containing 100 ml of water (measured with a thermocouple) reached 75 °C, 100 μl of the spore test suspension was added into the flask, kept under gentle mixing with a magnetic stirrer. This mixture (temperature measured was 74.2–75.2 °C) was sampled (1 ml) at intervals of 1 min up to 15 min. Each sample was diluted in 9 ml of the neutralizing reagent and the viable spores counted on Plate Count Agar (PCA) read after 24 h at 30 °C or 7–9 days at 8 °C. Resistance of the spores to hot acid was measured similarly, except that 0.9 %w/v aqueous HNO_3 (pH 0.8) was used instead of 1% NaOH, the temperatures of the water bath was 66.7 °C and that of the test flasks from 65.3 to 65.8 °C.

D-values were calculated by linear regression using SYSTAT® 9 (Systat Software Inc., Chicago, USA) from the log-transformed count of viable spores (cfu on PCA) versus heating time. The initial linear parts of the killing curves were used to calculate the reciprocals of the regression coefficients.

2.4. Assays of spore adherence and biofilm formation on nonliving surfaces

Adherence of the spores to polystyrene and to glass was measured using 96-well plates Nunclon™Δ (with hydrophobic optical bottom or with optical bottom with cover glass base), Nunc F96 MicroWell™ (with untreated or with hydrophilic cell culture treated polystyrene bottom). The media used to fill the wells (200 μl per well) were: sterilized drinking water, R2 medium, tryptic soy broth, skim milk medium and whole milk medium. The microplate wells were inoculated with 5×10^6–5×10^7 spores per well, covered with a lid and incubated on a rotary shaker (160 rpm, 4 °C) for 2 days. The wells were emptied, washed three times with drinking water to remove unadhered spores. The adhered spores were stained with 300 μl of aqueous acridine orange (100 μg ml^{-1}) for 3 min and then washed three times under running water. The cumulative fluorescence emission of the wells was measured using a scanning fluorometer (Fluoroskan Ascent, Thermofisher Scientific, Vantaa, Finland) with band pass filter of 450–480 nm for excitation and a long pass filter of 520 nm for emission.

Adhesion of the spores to stainless steel (AISI 304) was assayed with coupons of stainless steel (AISI304, ~1 cm^2), cleaned before use with 1% w/v detergent (Nelli soap, Farmos, Turku, Finland), disinfected with ethanol (96 vol %) and autoclaved. The coupons were aseptically mounted into the wells of a 6-well polystyrene plate (Nunc multidish) with 4 ml of sterilized drinking water per well. Spores, 5×10^7 (or 5×10^6, strain UM 169) cfu or none (background) per well were added, the plate covered with a lid and incubated with shaking (160 rpm) for 2 d at 4 °C. The coupons were then washed with drinking water, stained with acridine orange for 3 min, rinsed with water and the fluorescence emission from the whole surface area of each coupon was measured with the scanning fluorometer at 485 nm (excitation) and 520 nm (emission). Background fluorescence (wells with no added spores) was subtracted from each reading.

Capacity of the *B. cereus* spores to form biofilm in milk was measured as wall growth in 96-microplate wells. Polystyrene microplates were filled with 200 μl of full-fat milk or 1:10 diluted milk, inoculated with ~5×10^7 spores per well and incubated on a rotary shaker (160 rpm, 21 °C) for 2 days. The wells were emptied, washed three times with running water to remove the unadhered materials and then Live-Dead stained, 300 μl per well for 20–30 min, then washed three times under running water. The cumulative fluorescence of the biofilm was measured using the scanning fluorometer with the filter pairs 485/538 nm (Syto 9). Biofilms on the microplates were also observed using an epifluorescence light microscope.

2.5. Microscopy

Microscopy of the microplate biofilms and adhered spores on the steel coupons was done using epifluorescence microscope (Nikon Eclipse E800, Tokyo, Japan) with filters 485 nm (excitation) and 520 nm (emission).