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Milk-originated *Bacillus cereus sensu lato* strains harbouring *Bacillus anthracis*-like plasmids are genetically and phenotypically diverse

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ABSTRACT

Bacillus cereus sensu lato is widely distributed in food products, including raw and processed milk. Plasmids often determine bacterial virulence and toxicity, but their role in the evolution of *B. cereus sensu lato* is only partly known. Here, we observed that nearly 8% of *B. cereus sensu lato* isolates were positive for pXO1-like plasmids and 12% for pXO2-like plasmids *in* raw and ultra-heat-treated (UHT) milk from one dairy plant. However, pXO1-like plasmids were significantly more frequent in raw milk, while pXO2-like plasmids were more frequent in processed milk. Strains from raw and UHT milk were enterotoxigenic, with up to one-fifth of the isolates being psychrotolerant. Phylogenetic assessment using multilocus sequence typing revealed a polyphyletic structure for these bacilli, with distinct groups of cold-adapted isolates and pathogenic strains (including emetic *B. cereus*). Populations corresponding to both sampling sites exhibited significant linkage disequilibrium and the presence of purifying selection. The far-from-clonal population structure indicated the presence of sequence types or ecotypes adapted to specific conditions in the dairy industry. A high recombination-to-mutation ratio suggested an important role for horizontal gene transfer among *B. cereus sensu lato* isolates in milk.

1. Introduction

Bacillus cereus sensu lato comprises at least nine gram-positive, facultative anaerobic rods, including *B. cereus*, which is sporadically associated with food poisoning and intestinal infections; *B. thuringiensis*, an entomopathogen; *B. anthracis*, an anthrax agent; *B. mycoides* and *B. pseudomycoides*, both of which are characterized by rhizoidal colonies on solid media; *B. weihenstephanensis*, a psychrotolerant bacterium; *B. toyonensis*, which exhibits both probiotic and haemolytic properties; psychrotolerant and cytotoxic *B. wiedmannii* (Miller et al., 2016); and, finally, thermotolerant *B. cytotoxicus*, which is responsible for occasional infections (Jensen et al., 2003; Guinebretiere et al., 2013). The common prevalence of these bacteria (e.g., in soil, water, plant material, digestive tracts and animals' bodies) results in their transfer into food. Specifically, milk contamination has been reported by several authors (Svensson et al., 2004; Bartoszewicz et al., 2008). The risk associated with *B. cereus* and its relatives is connected to their frequent cold adaptation, toxicity and spore-forming ability (Jensen et al., 2003; Stenfors Arensen et al., 2008; Glasset et al., 2016). All of these features may result in the spoilage of food products stored under unfavourable circumstances (e.g., too much time out of cold storage or when a chain of cold storage is broken). Moreover, severe cases of *B. cereus*-related food poisoning have also been described (Mahler et al., 1997; Dierick et al., 2005).

The characteristic properties of *B. cereus sensu lato* strains are often connected with their plasmids, e.g., the virulence of *B. anthracis*, cereulide synthesis by *B. cereus* strains, or the insecticidal activity of *B. thuringiensis*. On the basis of their nucleotide sequences, two important types of large and potentially conjugative plasmids have been distinguished in *B. cereus sensu lato*, namely, pXO1-like plasmids and pXO2-like plasmids. The pXO1-like plasmids include pXO1 of *B. anthracis*, which harbours genes for anthrax toxin synthesis (Mock and Fouet, 2001). Related plasmids, such as pCER270, are devoid of pathogenicity islands but may contain the *ces* operon, which is necessary for cereulide synthesis (Rasko et al., 2004; Ehling-Schulz et al., 2006). Their replication depends on a highly conserved protein encoded by the *repX* gene. Its presence is therefore a good indicator of the occurrence of a pXO1-like plasmid. The pXO2-like plasmids include pXO2, which harbours genes for anthrax capsule formation, and pBtoxis, which carries genes encoding the delta-endotoxins of *B. thuringiensis*

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subsp. *israelensis*; they can be detected by the presence of a specific *repA* gene, which plays an important role in their replication (Hu et al., 2009). Whether a common evolutionary origin (Rasko et al., 2007) or horizontal gene transfer (Van der Auwera et al., 2007) causes the distribution of these plasmids among non-*B. anthracis* isolates is unknown. The genetic relatedness among other *B. cereus sensu lato* strains with *B. anthracis*-like plasmids and their population structure in relation to the dairy industry environment remains unknown, especially with regard to food-related isolates.

To assess the phylogenetic relationships among *B. cereus sensu lato* strains harbouring *B. anthracis*-like plasmids and to evaluate the potential role of horizontal gene transfer (HGT) in their distribution and persistence among a food-borne population, we aimed to (1) establish the frequencies of *repX* and *repA* homologues in *B. cereus sensu lato* populations from raw and ultra-heat-treated (UHT) milk; (2) investigate their phylogeny and the milk population structure using multi-locus sequence typing (MLST); and (3) evaluate their psychrotolerance and toxicity.

2. Material and methods

2.1. Bacterial strains

In total, 251 *B. cereus sensu lato* isolates were isolated from raw milk (site 1) and UHT milk (site 2) in northeastern Poland using the membrane-filtration method described by Christiansson et al. (1997). All isolates were grown on mannitol-egg yolk-polymyxin agar (MYP agar, Oxoid, Basingstoke, UK) and underwent subsequent identification. All of the properties that were used to identify each species are given in Table 1S (Supplementary Materials). The procedure used for the present research enabled us to distinguish *B. cereus/B. toyonensis/B. wiedmannii*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides*, *B. cytotoxicus*, and *B. anthracis*.

We identified 180 *B. cereus* (without discrimination between *B. toyonensis* and *B. wiedmannii*) isolates, 45 *B. thuringiensis* isolates, 16 *B. weihenstephanensis* isolates, and 10 *B. mycoides* isolates, according to the properties listed in Table 1S (Supplementary Materials). *B. cereus* ATCC 14579 (American Type Culture Collection), *B. thuringiensis* HD-1 (Bacillus Genetic Stock Centre, BGSC), *B. weihenstephanensis* DSM 11821 (German Collection of Microorganisms and Cell Cultures), *B. mycoides* ATCC 6462, and *B. pseudomycoides* DSM 12442 were included as reference strains. For MLST comparisons, the allelic profile of the *B. anthracis* Ames strain was obtained from the PubMLST database (Jolley and Maiden, 2010).

2.2. DNA extraction

Genomic DNA was isolated from overnight bacterial cultures grown in Luria-Bertani broth (Oxoid, Basingstoke, UK), using the DNeasy Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) with the protocol for gram-positive bacteria. Pelleted cells were suspended in lysis buffer with 5 mg ml^{-1} lysozyme (Sigma, St Louis, USA) and used as the starting material with the Automated System for Nucleic Acid Isolation QIACube (QIAGEN). The final DNA quality, concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

2.3. Detection of repX and repA homologues and of genes necessary for psychrotolerance and toxin formation

PCR was used to detect the *repX* and *repA* homologues, as per Hu et al. (2009). The *nhe* (genes *nheA*, *nheB*, and *nheC*) and *hbl* (genes *hblA*, *hblC*, and *hblD*) operons, which encode tri-component enterotoxins, were detected as per Hansen and Hendriksen (2001), Melnick et al. (2012) and *cytK-1* and *cytK-2* were detected as per Guinebretiere et al. (2006). All strains were screened for the psychrotolerant variant of the *cspA* gene, as per Francis et al. (1998). All primers used are listed in the Supplementary Materials (Table 2S). The PCR products were analysed using the QIAxcell Capillary Electrophoresis System (QIAGEN).

2.4. Phenotypic assessment of cold adaptation and toxicity

To investigate cold adaptation, 100 μl of 18-h cultures incubated at 30 °C (diluted with a physiological solution to $OD_{590} = 1.0$) of each strain were used as inocula for both nutrient broth (Biocorp, Warsaw, Poland) and nutrient agar (Biocorp). Next, the cultures were incubated for 10 d at 7 °C. Visible growth in both cases confirmed psychrotolerance.

Strains that were positive by PCR screening for the *nhe* and/or *hbl* operons (see Section 2.3) were tested for production of the NHE or HBL enterotoxins using immunochromatographic rapid tests (Duopath Cereus Enterotoxins, Merck Millipore, Darmstadt, Germany). For this purpose, 200 μl of fresh 18-h cultures incubated at 30 °C in CGY (casein hydrolysate glucose yeast) broth with 1% glucose were diluted in 20 ml of CGY broth with 1% glucose in a 200-ml flask and incubated at 37 °C for 8 h before being visually checked and cooled to room temperature. Subsequent analyses were performed according to the manufacturer's protocol. To avoid any false-negative results in the case of psychrotolerant isolates, the presence of NHE and/or HBL toxins was also tested after 16 h of incubation at 30 °C.

2.5. Investigation of population structure and phylogeny

To probe the population structure, an MLST scheme including *glpF* (glycerol uptake facilitator protein), *gmk* (guanylate kinase, putative), *ilvD* (dihydroxy-acid dehydratase), *pur* (phosphoribosylaminoimidazole-carboxamide), *pycA* (pyruvate carboxylase), *pta* (phosphate acetyltransferase) and *tpi* (triosephosphate isomerase) gene sequencing was applied as per Drewnowska and Swiecicka (2013). Amplicons were purified away from remaining dNTPs, enzymes and oligonucleotides using a Clean-up Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol. Purified DNA in nuclease-free distilled water was then used as a template for a sequence-extension reaction. Sequencing PCRs were performed in a GeneAmp 9700 thermal cycler with 30 cycles of denaturation (95°C/15 s), annealing (50°C/30 s) and elongation (60°C/1 min). Excess dye terminators were removed using an ExTerminator Kit (A&A Biotechnology). The purified samples were sequenced in an ABI3500 automated DNA sequencer (Applied Biosystems). All sequences were examined using the Chromas Lite ver. 2.01 program and aligned with the BioEdit Sequence Alignment Editor ver. 7.0.1 (Hall, 1999). Phylogenetic relationships and the evolutionary history were inferred using the maximum likelihood (ML) method, based on the general time-reversible model with gamma and invariant site parameters (GTR+G+I) and with 1000 bootstrap repetitions, according to the results of tests for the best DNA model. The presented model is that for which the score of the Bayesian information criterion (BIC = 18,916.780) was lowest, indicating that the GTR+G+I model best describes the substitution patterns (Nei and Kumar, 2000). All tests and the construction of the phylogenetic tree were performed using Mega 6 software (Tamura et al., 2007). An index of association was calculated with START 2 software for two independent populations: (i) 25 isolates from raw milk and (ii) 24 isolates from UHT milk. The relative importance of HGT and mutation was estimated by calculating the *r/m* (recombination per mutation) ratio using