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Toxigenic potential and heat survival of spore-forming bacteria isolated from bread and ingredients

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ABSTRACT

Fifty-four spore-forming bacterial strains isolated from bread ingredients and bread, mainly belonging to the genus *Bacillus* (including *Bacillus cereus*), together with 11 reference strains were investigated to evaluate their cytotoxic potential and heat survival in order to ascertain if they could represent a risk for consumer health. Therefore, we performed a screening test of cytotoxic activity on HT-29 cells using bacterial culture filtrates after growing bacterial cells in Brain Heart Infusion medium and in the bread-based medium Bread Extract Broth (BEB). Moreover, immunoassays and PCR analyses, specifically targeting already known toxins and related genes of *B. cereus*, as well as a heat spore inactivation assay were carried out. Despite of strain variability, the results clearly demonstrated a high cytotoxic activity of *B. cereus* strains, even if for most of them it was significantly lower in BEB medium. Cytotoxic activity was also detected in 30% of strains belonging to species different from *B. cereus,* although, with a few exceptions (e.g. *Bacillus simplex* N58.2), it was low or very low. PCR analyses detected the presence of genes involved in the production of NHE, HBL or CytK toxins in *B. cereus* strains, while genes responsible for cereulide production were not detected. Production of NHE and HBL toxins was also confirmed by specific immunoassays only for *B. cereus* strains even if PCR analyses revealed the presence of related toxin genes also in some strains of other species. Viable spore count was ascertained after a heat treatment simulating the bread cooking process. Results indicated that *B. amyloliquefaciens* strains almost completely survived the heat treatment showing less than 2 log-cycle reductions similarly to two strains of *B. cereus* group III and single strains belonging to *Bacillus subtilis, Bacillus mojavensis* and *Paenibacillus* spp. Importantly, spores from strains of the *B. cereus* group IV exhibited a thermal resistance markedly lower than *B. cereus* group III with high values of log-cycle reductions. In conclusion, our results indicate that spore-forming bacteria contaminating bread ingredients and bread could represent a source of concern for consumer health related to the presence of strains, such as strains of *B. cereus* group III and single strains of other species, showing the ability to produce toxic substances associated to a thermal resistance enough to survive the bread cooking conditions.

1. Introduction

Due to an almost ubiquitous occurrence and to the intrinsic high stress resistance of spores, spore-forming bacteria are widely distributed in the environment and their presence in foods and raw materials used for food production is very frequent (Postollec et al., 2012). For example aerobic spore-forming bacteria, classified in the genus *Bacillus* and other strictly related genera, have been found in many foods such as rice (Ankolekar et al., 2009), vegetable products (Guinebretière and Nguyen-The, 2003; King et al., 2007), spices (Sagoo et al., 2009), dairy products (Cosentino et al., 1997; Lücking et al., 2013), cereals (Park et al., 2009), bread and raw materials (Valerio et al., 2012). Spore-forming bacteria in foods can cause both food poisoning or spoilage problems. In particular, *Bacillus cereus* is well known to cause two types of foodborne illnesses named the diarrheal and the emetic syndromes. The former is due to the production of different enterotoxins such as the hemolytic enterotoxin hemolysin BL (HBL), the non-hemolytic enterotoxin (NHE) and the cytotoxin K (CytK) (Senesi and Ghelardi, 2010). HBL and NHE are different three-component protein complexes, although homology studies revealed some common structural characteristics (Fagerlund et al., 2008). HBL consists of the proteins B, L1 and L2 which are encoded by the *hblA*, *hblD*, and *hblC* genes, respectively, organized in a single operon (Ryan et al., 1997). NheA, NheB and NheC are the three protein components of NHE encoded by the *nheABC* operon (Granum et al., 1999). CytK is a single-component toxin showing dermonecrotic, cytotoxic and haemolytic activity which belongs to a family of beta-barrel channel-forming toxins (Lund et al., 2000). Two forms of this toxin were identified, CytK-1 and CytK-2, showing 89% amino acid sequence homology (Fagerlund et al., 2004). The emetic syndrome caused by *B. cereus* is due to the production of a cyclic dodecadepsipeptide named cereulide. This

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heat-resistant toxin is produced by a nonribosomal peptide synthetase which is encoded by the cereulide synthetase (*ces*) gene cluster of 24-kb located on a megaplasmid (Ehling-Schulz et al., 2006a). Although generally the emetic syndrome is not severe, two lethal cases caused by cereulide and due to liver failure after consumption of pasta have been reported (Dierick et al., 2005; Mahler et al., 1997). The potential of *B. cereus* to produce the cereulide in different bakery products has also been studied by Jääskeläinen et al. (2003). Authors showed that products with a_w values of >0.953 and pHs of >5.6 were prone to cereulide production. Foodborne illnesses may be also caused by a number of *Bacillus* species other than *B. cereus* which are also known to produce toxic metabolites. For example, *Bacillus licheniformis, Bacillus subtilis* and *Bacillus mojavensis*, and *Bacillus pumilus* produce different toxins called lichenysin A (Mikkola et al., 2000), amylosin (Apertroaie-Constantin et al., 2009; Mikkola et al., 2007) and pumilacidin (Naruse et al., 1990), respectively and they have been implicated in food-associated illness (From et al., 2007; Salkinoja-Salonen et al., 1999). *Bacillus amyloliquefaciens* also produces amylosin (Mikkola et al., 2004), while *Bacillus simplex* produces a heat-stable toxin, which has been considered similar to cereulide after a partial purification and characterization (Taylor et al., 2005). Strains of the *Bacillus* genus are also known as causative agents of food spoilage. For example, *B. cereus* strains are considered important spoilage organisms of dairy products as reported by Lücking et al. (2013). *Bacillus* species including *B. subtilis*, *Bacillus megaterium*, *B. pumilus* and *B. cereus* group strains have been isolated from ingredients (mainly samples of durum wheat semolina) and bread and associated to the rope spoilage of bread (Pepe et al., 2003; Valerio et al., 2012, in press), together with *B. amyloliquefaciens* which was identified as the bacterial species mainly implicated in ropy spoilage for the first time by Valerio et al. (2012). Concerning products prepared from durum wheat semolina, more recently Ziane et al. (2014) studied heat resistance and growth potential of mesophilic aerobic spore-forming *Bacillus* strains isolated from commercial couscous: the strain-dependent heat resistance of spores was identified as a key factor in foodborne illness associated to the couscous consumption. Indeed, the number of spores inactivated after a heat treatment is fundamental to define the potential of a strain to cause successive spoilage or food poisoning problems. These evidences confirm the importance to assess the ability of spore-formers isolated from food and ingredients to survive a heat treatment and to grow giving high bacterial loads in food during storage. Moreover, medium composition could greatly influence the resistance of spores to a heat treatment: in particular, low pH values enhance inactivation of spores and also inhibit recovery of injured spores, while low water activity values protect spores from heat injury and prevent spores from growing out and indeed the net effect is mostly a lower recovery (Esteban et al., 2013; Smelt and Brul, 2014).

Since many of the above-mentioned bacterial species (Valerio et al., 2012) originating from bread and ingredients are potentially able to produce toxic metabolites, the aim of the present study was to investigate cytotoxic activity, the presence of genes involved in toxin production, the enterotoxin production, and the spore survival after a heat treatment of strains that could represent a health risk for consumers.

2. Materials and methods

2.1. Bacterial strains

Spore-forming bacterial strains used in this study are listed in Table 1; they were isolated from ingredients for bread production and bread samples and were characterized by rep-PCR and identified by gene sequencing and some appropriate differential phenotypic tests as previously described (Valerio et al., 2012). *B. subtilis* 168/DSM10, *B. amyloliquefaciens* DSM7, *B. licheniformis* DSM13, *B. cereus* DSM4312 and DSM4313, *Bacillus thuringiensis* DSM2046, *B. megaterium* DSM32, *B. pumilus* DSM27, *Bacillus firmus* DSM12 and *Bacillus clausii* DSM8716 (all obtained from DSMZ, Braunschweig, Germany) and *B. amyloliquefaciens* ATCC8473, formerly classified as *B. subtilis*, (ATCC-LGC Standards S.r.l., Sesto San Giovanni, MI, Italy) were also used as reference strains. All the strains are stored in the Microbial Collection of the Institute of Sciences of Food Production (National Research Council, Bari, Italy) at −80 °C under cryoprotection.

All the strains were grown in two different media, i.e. in Brain Heart Infusion medium (Oxoid, Ltd.) supplemented with 0.1% glucose (BHIG) and in a bread-based medium (Bread Extract Broth, BEB) obtained from commercial bread with the following composition: 5.2% fat, 50.5% carbohydrates, 4% fibers, 7.5% proteins, 1.25% salt, expressed as g/100 g of bread. In detail, the BEB medium was prepared as described by Pepe et al. (2003), by stomaching 350 ml of distilled water and 100 g of commercial sliced white bread for 2 min; the suspension was then filtered through filter paper (Whatman no. 1), and the pH was adjusted to 6.8 with 1 M NaOH. The medium was sterilized at 121 °C for 15 min. Twenty milliliters of BHIG medium and BEB medium were 2% inoculated, incubated at 32 °C and shaken at 115 rpm for 18 h. The growth of each strain was monitored by measuring the OD_{600} and plate counts, each yielding at least about 10^8 CFU/ml. Cell-free supernatants were obtained by centrifugation (9000 × *g*, 4 °C, 10 min) and filtration through 0.22 μm filters (International PBI, Milan, Italy).

2.2. Cytotoxicity assays using HT-29 cells

HT-29 cell line was purchased from the European Collection of Cell Cultures (ECACC, UK). This human intestinal cell line was chosen because the intestine is the target organ more significantly exposed to cytotoxic substances of food origin. Cell line was routinely grown in Mc Coy's 5A medium containing 10% fetal bovine serum, 1% L-glutamine and 1% antibiotic–antimycotic solution (all purchased from Sigma) in 75 cm^2 plastic flasks (Corning, Milan, Italy) at 37 °C in a 5% CO_2 humidified atmosphere up to 90% confluence. Cell density and viability were determined by Scepter automated cell counter (Millipore, Milan, Italy). Then, aliquots of 100 μl of cell suspension (at cellular density of 3×10^5 cells/ml) were seeded in 96 multiwell plates (Corning, Milan, Italy) and treated with 100 μl of twofold serially diluted (from 1:2 to 1:128) cell-free bacterial supernatants. BHIG and BEB media, diluted 1:2 in medium, were used as negative controls. Both negative controls (BHIG and BEB media) and each dilution of cell-free bacterial supernatants were tested in six wells. The plates were further incubated for 24 h at 37 °C in a 5% CO_2 humidified atmosphere. The cell proliferation was measured using the MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] test as previously described (Minervini et al., 2004). The test is based on the cleavage of MTT to a blue colored product (formazan), which is indicative of mitochondrial succinate-dehydrogenase activity in viable cells. Absorbance was measured at 580 nm in a plate reader (ELISA Reader Multiskan MS Plus MK II Labsysten, Finland). Cytotoxic activity of bacterial supernatants was expressed as the highest dilution of cell-free bacterial supernatant for which a significant cytotoxic activity respect to negative control (BHIG or BEB medium) is measured.

Moreover, in order to measure the toxicity induced by strains on HT-29 cell proliferation, 1:2 dilutions of cell-free bacterial supernatants were used and, cytotoxic activity was expressed in terms of percentage of cell proliferation caused by cell-free supernatants considering 100% the proliferation caused by the respective controls (BHIG or BEB medium). Both negative controls (BHIG and BEB media) and 1:2 dilution of each cell-free bacterial supernatant were tested in six wells. Cytotoxic activity was evaluated in three independent experiments.

2.3. PCR assay to examine the presence of the yeaC indel in B. cereus group strains

The presence of a 72 bp deletion in the *yeaC* gene can be used as marker to distinguish *Bacillus anthracis* from the other closely related bacterial species of the *B. cereus* group (Ahmod et al., 2011). In order to assess the presence of such deletion in 12 *B. cereus* group strains,