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A multiparametric PCR-based tool for fast detection and identification of spore-forming bacteria in food

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ABSTRACT

The presence of psychrotrophic or highly thermoresistant spore-forming bacteria in food and feedstuff responsible for food poisoning and spoilage raises major safety and economical issues. The aim of this study was to evaluate the performances of a ready-to-use PCR assay (alternative method) in comparison with the standard microbiological plating method regarding spore-forming bacteria detection in food samples. An overnight sample enrichment was selected to increase sporeformer diversity recovery, spore germination, bacterial growth and favour DNA extraction. A total of 180 sporeformer isolates representing 38 different species and 8 genera were tested in the PCR assays. Inclusivity and exclusivity results ensured specific detection and identification of the majority of targeted genera and species. Validation studies carried on artificially contaminated food samples showed detection of the inoculated contaminants in most cases, with increased detection limit for the alternative method which enabled detection with up 1 spore of *B. cereus* in 25 g food sample. Using naturally contaminated food samples, standard method comforted the alternative method. In a number of cases, the alternative method was able to identify species not detected with the standard method. In addition, identification and discrimination between the *B. cereus* group members was possible. Thus, associated to a key element, i.e., the enrichment step, the developed multiparametric PCR-based assays reported in this study provide a fast, sensitive and reliable detection and identification tool for mostly encountered spore-forming food contaminants.

1. Introduction

In the food industry, unintended occurrence and growth of spoilage or pathogenic microorganisms in REPFEDs, RTE or processed food is a key issue. A number of species belonging mainly to *Clostridium*, *Bacillus* and related genera are able to form endospores. These structures allow the bacteria resisting harsh environmental conditions including heat, dehydration, UV light, biocides and other physical stresses for a long time even in the absence of nutrients, thus leading to survival and persistence of sporeformer contaminants along food processes. Their presence in raw materials, ingredients or during food processing and their extreme resistance to thermal treatments challenge the preservation processes. When triggered by specific environmental conditions, such as a heat shock or the presence of nutrients, the spores germinate (Moir, 2006) and initiate active vegetative growth. Foodborne sporeformer growth in food can be associated with the production of a wide range of enzymes yielding more or less visible spoilage with or without gas production. Many highly resistant spore-forming bacterial species responsible for food spoilage and outbreaks have been described (Carlier and Bedora-Faure, 2006; Le Bourhis et al., 2007; Scheldeman et al., 2006). Such an adaptation to temperature or extreme environmental conditions suggests that (re)emergence of foodborne isolates may partly result from a selection or adaptation by the food processes (Alcaraz et al., 2008; Guinebretiere et al., 2008; von Stetten et al., 1999). Therefore, the detection and identification of heat treatments resistant flora is of great importance to allow food industry to adequately handle associated microbiological risks. Despite the ubiquitous distribution of sporeformers in the environment, some strains require anaerobic or specific conditions that remain difficult to reproduce in laboratory yielding poor strain recovery on agar medium and thus poor strain characterization. Spore-forming bacteria include species with very diverse phenotypes and metabolic activities. Indeed several taxonomic rearrangements have been proposed for *Bacillus* related species with the creation of 18 new genera among which are *Alicyclobacillus*, *Brevibacillus*, *Geobacillus* and *Paenibacillus* (Xu and Cote, 2003). Closely related species belonging to the *Bacillus cereus* group still exhibit a wide range of

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genotypic and phenotypic features which makes their discrimination difficult. The absence of adapted detection, identification and tracking methods is currently the main hurdle to controlling these populations. Standard microbiological and biochemical methods can be used to target some of these specific features (Reva et al., 2001), such as API tests to identify members of the *Bacillus* genus or anaerobic bacteria, tryptose sulphite cycloserine (TSC) agar for identification of sulphite-reducing *Clostridia*, or Mossel agar for *Bacillus cereus*. Several methods have been developed to specifically enumerate spores of *Clostridium* (Cerf and Bergère, 1968), or *C. tyrobutyricum* (Abgrall and Bourgeois, 1985; Bergère et al., 1972) but were limited to particular applications such as dairy product analyses. More recently, immuno-diagnostic (ELISA) or molecular tools such as sequencing of 16S rDNA, DNA-DNA hybridization, PCR or quantitative PCR were employed to identify various spore-forming bacteria (Blake and Weimer, 1997; Hansen and Hendriksen, 2001; Herman et al., 1995; Herman et al., 1997; Huck et al., 2007; Oomes et al., 2007; Rueckert et al., 2005; Yoon et al., 2005). While some of these tests were fast, sensitive and accurate, none of them was satisfying for a simple, routine and universal use in the food industry. Indeed, they were either specific for a single species or a particular matrix, or were too laborious for non-specialist use. The development of PCR-based assays is a step forward and probably offers the greatest potential. However, despite the continuously growing knowledge about genomes and genomic tools, bacterial identification is dependent on the existing taxonomy and sequences available in databases. Recently, multiplex real time PCR assays have been developed and commercialized by Pall GeneSystems for the detection of food contaminants (Beutin et al., 2009; Yaradou et al., 2007). These assays consist in using disposable biochips (GeneDisc® plate) of 36 wells pre-loaded with primers and probes, and three central compartments to be filled with three independent DNA extracts to be analyzed. PCR are automatically run and analyzed on a specific thermocycler i.e. GeneDisc® cycler.

This study was initiated in response to a clear industrial need for simple and fast diagnostic tools to track and identify various sporeformers along food chains. Based on a collection of 180 representative bacterial isolates, two GeneDisc plates were designed in order to identify commonly encountered spore-forming genera or species present during food processing. First, the GeneDisc plates were evaluated for their specificity and sensitivity to detect and identify pure cultures from the collection. Then, the method was evaluated using artificially contaminated food matrices belonging to various categories (dairy products, canned food and egg products). Co-inoculations were also performed in order to identify possible interactions. Finally, around 60 naturally contaminated matrices were used to evaluate the performances of the current method as compared to the reference method. These studies were conducted according to the NF EN ISO 16140 guidelines defining the methodology for validation of alternative methods in food microbiology.

2. Materials and methods

2.1. Strain collection

A total of 180 spore-forming strains was collected from industrial and scientific partners' existing collections (Table 1). Each strain was

Table 1
Spore-forming bacterial strains used for the development and validation of PCR 1 primers and probes. Number and origin of the isolates are mentioned for each species.

Genus and species	Origin [number of isolates]
Alicyclobacillus acidoterrestris	Ground (type strain) [1], dairy product [1]
Alicyclobacillus acidocaldarius	Dairy product [1]
Anoxybacillus flavithermus	Thermal spring (type strain) [1], dairy product [1]
Bacillus amyloliquefaciens	Spice [1]
Bacillus cereus	Type strain [1], egg product [6], dairy product [3], ground [2], vegetables [4]
Bacillus circulans	Ground (type strain) [1], dairy product [2], vegetables [3]
Bacillus coagulans	Type strain [1], dairy product [3]
Bacillus firmus	Dairy product [2]
Bacillus licheniformis	Egg product [4], dairy product [8], ground [1]
Bacillus macroides/simplex	Vegetables [4], egg product [1]
Bacillus megaterium	Type strain [1], dairy product [1], vegetables [1]
Bacillus mycoides	Ground (type strain) [1], egg product [3], dairy product [2], ground [1], vegetables [3]
Bacillus pumilus	Ready meal [2], dairy product [1], vegetables [5]
Bacillus pseudomycoides	Type strain [1], dairy product [1], vegetables [2]
Bacillus sonorensis	Dairy product [1]
Bacillus sporothermodurans	Milk (type strain) [1], dairy product [1]
Bacillus subtilis	Type strain [1], egg product [5], dairy product [1], vegetables [4]
Bacillus thuringiensis	Ground [5]
Bacillus weihenstephanensis	Egg product [5], dairy product [1], ready to eat meal [1], vegetables [3]
Brevibacillus laterosporus	Type strain [1], ground [10]
Brevibacillus agri	Water [1]
Brevibacillus parabrevis	Dairy product [1]
Clostridium baratii	Epithelium (type strain) [1], dairy product [1]
Clostridium beijerinckii	Type strain [1], dairy product [3]
Clostridium bifermentans	Dairy product [2], meat product [1]
Clostridium botulinum	Type strain [1]
Clostridium difficile	Type strain [1]
Clostridium tyrobutyricum	Type strain [1], dairy product [5], environment [1]
Clostridium perfringens	Dairy product [3], poultry [2]
Clostridium sordelli	Type strain [1], dairy product [1]
Clostridium sporogenes	Dairy product [5], poultry [1], vegetables [3]
Paenibacillus spp.	Dairy product [3], seafood [2]
Paenibacillus amylolyticus	Ground (type strain) [1]
Paenibacillus odorifer	Vegetables [2]
Paenibacillus polymyxa	Vegetables [1]
Paenibacillus macerans	Dairy product [2]
Moorella spp.	Canned food [3]
Moorella thermoacetica	Faeces (type strain) [1]
Moorella thermoautotrophica	Thermal spring (type strain) [1]
Geobacillus stearothermophilus	Canned food (type strain) [1], dairy product [1], seafood [4]