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Development of a Multiplex-PCR assay for the rapid identification of *Geobacillus stearothermophilus* and *Anoxybacillus flavithermus*

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ABSTRACT

The presence of thermophilic bacilli in dairy products is indicator of poor hygiene. Their rapid detection and identification is fundamental to improve the industrial reactivity in the implementation of corrective and preventive actions.

In this study a rapid and reliable identification of *Geobacillus stearothermophilus* and *Anoxybacillus flavithermus* was achieved by species-specific PCR assays. Two primer sets, targeting the ITS 16S-23S rRNA region and the *rpo*B gene sequence of the target species respectively, were employed. Species-specificity of both primer sets was evaluated by using 53 reference strains of DSMZ collection; among them, 13 species of the genus *Geobacillus* and 15 of the genus *Anoxybacillus* were represented. Moreover, 99 wild strains and 23 bulk cells collected from 24 infant formula powders gathered from several countries worldwide were included in the analyses. Both primer sets were highly specific and the expected PCR fragments were obtained only when DNA from *G. stearothermophilus* or *A. flavithermus* was used. After testing their specificity, they were combined in a Multiplex-PCR assay for the simultaneous identification of the two target species. The specificity of the Multiplex-PCR was evaluated by using both wild strains and bulk cells. Every analysis confirmed the reliable identification results provided by the single species-specific PCR methodology.

The easiness, the rapidity (about 4 h from DNA isolation to results) and the reliability of the PCR procedures developed in this study highlight the advantage of their application for the specific detection and identification of the thermophilic species *G. stearothermophilus* and *A. flavithermus*.

1. Introduction

Thermal processes based on high temperatures are commonly used in food industries to guarantee that food products remain stable for long period at ambient temperatures (Prevost et al., 2010). However, the thermal treatments are not always sufficient to inactivate all spore-forming bacteria, especially those that are highly heat-resistant, but non-pathogenic (Hornstra et al., 2009). Among them, the thermophilic bacilli were reported to be important contaminants in milk powders (Ronimus et al., 2003; Rueckert et al., 2004, Scott et al., 2007), canned foods and dairy products (Denny, 1981; Jay et al., 2005; Scott et al., 2007; Prevost et al., 2010).

In the dairy industry, the thermophilic bacilli can be divided in two main groups: facultative thermophiles (also known as thermotolerant microorganisms) and obligate thermophiles. The obligate thermophiles grow at elevated temperatures (approximately 48–60 °C) and mainly include the two species: *Geobacillus* (*G.*) *stearothermophilus* and *Anoxybacillus* (*A.*) *flavithermus* (Flint et al., 2001; Ronimus et al., 2003; Scott et al., 2007; Burgess et al., 2010).

Both species exhibit a fast growth rate and tend to form biofilm on the stainless steel surfaces of processing equipment (Scott et al., 2007).

The presence of the thermophilic bacilli in dairy products is indicator of poor hygiene; high counts are unacceptable, since they can lead to product defects caused by the production of heat-stable enzymes, such as proteinases and lipases, and acids capable to spoil the final product (Chopra and Mathur, 1984; Cosentino et al., 1997; Chen et al., 2004; Gundogan and Arik, 2004).

Identification of the bacteria capable to contaminate milk powders and possibly cause their spoilage can help in implementing corrective and preventive actions, in particular at level of the manufacturing process steps before heat treatment. Molecular

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Table 1
Strains of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) collection used in this study and evaluation of species-specificity of the PCR primers.

	Species	DSM strain	Species-specific PCR with primer pair:	
			Fits2/ Rits2	Anx-RpoB5f/ Anx-RpoB1bisr
1	*Anoxybacillus amylolyticus*	15939^T	–	–
2	*Anoxybacillus bogrovensis*	17956^T	–	–
3	*Anoxybacillus caldiproteoliticus*	15730^T	–	–
4	*Anoxybacillus contaminans*	15866^T	–	–
5	*Anoxybacillus eryuanensis*	23212^T	–	–
6	*Anoxybacillus flavithermus* subsp. *flavithermus*	2641^T	–	+
7	*Anoxybacillus flavithermus* subsp. *flavithermus*	21510	–	+
8	*Anoxybacillus flavithermus* subsp. *yunnanensis*	23293	–	–
9	*Anoxybacillus kamchatkensis*	14988^T	–	–
10	*Anoxybacillus mongoliensis*	19169^T	–	–
11	*Anoxybacillus pushchinoensis*	12423^T	–	–
12	*Anoxybacillus rupiensis*	17127^T	–	–
13	*Anoxybacillus salavatliensis*	22626^T	–	–
14	*Anoxybacillus tengchongensis*	23211^T	–	–
15	*Anoxybacillus tepidamans*	16325^T	–	–
16	*Anoxybacillus thermarum*	17141^T	–	–
17	*Anoxybacillus voinovskiensis*	17075^T	–	–
18	*Bacillus amyloliquefaciens* subsp. *amyloliquefaciens*	7^T	–	–
19	*Bacillus cereus*	31^T	–	–
20	*Bacillus coagulans*	1^T	–	–
21	*Bacillus firmus*	12^T	–	–
22	*Bacillus licheniformis*	13^T	–	–
23	*Bacillus pumilus*	27^T	–	–
24	*Bacillus sporothermodurans*	10599^T	–	–
25	*Bacillus subtilis subsp. subtilis*	10^T	–	–
26	*Clostridium novyi*	14992^T	–	–
27	*Clostridium saccharobutylicum*	13864^T	–	–
28	*Clostridium sporogenes*	795^T	–	–
29	*Clostridium thermobutyricum*	4928^T	–	–
30	*Geobacillus caldoxylosilyticus*	12041^T	–	–
31	*Geobacillus debilis*	16016^T	–	–
32	*Geobacillus galactosidasius*	18751^T	–	–
33	*Geobacillus kaustophilus*	7263^T	–	–
34	*Geobacillus stearothermophilus*	22^T	+	–
35	*Geobacillus stearothermophilus*	297	+	–
36	*Geobacillus stearothermophilus*	456	+	–
37	*Geobacillus stearothermophilus*	1550	+	–
38	*Geobacillus stearothermophilus*	2027	+	–
39	*Geobacillus subterraneus*	13552^T	–	–
40	*Geobacillus tepidamans*	16325^T	–	–
41	*Geobacillus thermocatenulatus*	730^T	–	–
42	*Geobacillus thermodenitrificans*	465^T	–	–
43	*Geobacillus thermoglucosidasicus*	2542^T	–	–
44	*Geobacillus thermoleovorans*	5366^T	–	–
45	*Geobacillus toebii*	14590^T	–	–
46	*Geobacillus uzenensis*	23175^T	–	–
47	*Moorella thermoacetica*	521^T	–	–
48	*Moorella thermoautotrophica*	1974^T	–	–
49	*Paenibacillus graminis*	15220^T	–	–
50	*Paenibacillus macerans*	24^T	–	–
51	*Paenibacillus turicensis*	14349^T	–	–
52	*Thermoanaerobacterium aciditolerans*	16487^T	–	–
53	*Thermoanaerobacterium saccharolyticum*	7060^T	–	–

methods able to rapidly detect and identify thermophilic contaminants are fundamental to improve the industrial reactivity (Prevost et al., 2010).

In the last few years several efforts were directed toward the development of PCR-based methods for the investigation of the thermophilic bacilli contamination in milk powders, but most of the studies were focused on their total enumeration by quantitative real-time PCR, without distinguishing among the different species. Moreover, some identification methods were too expensive or required a lot of expertise to be easily implemented in the routine analysis scheme of the industrial laboratories (Flint et al., 2001; Ronimus et al., 2003; Rueckert et al., 2005a,b; Rueckert et al., 2006; Prevost et al., 2010; Postollec et al., 2012).

The main objective of this study was to develop rapid and easy PCR-based assays for the detection and identification of the thermophilic species *G. stearothermophilus* and *A. flavithermus*. Both species detection was based on activation, germination and outgrowth of the spores on agar plates followed by PCR. This step was preferred to a direct PCR detection from milk powder samples to prevent false-negative reactions due to inhibitory substances present in the milks like calcium ions (Bickely et al., 1996) or proteinases (Powell et al., 1994) as well as false-positive reactions due to the free DNA of dead bacteria.

The *G. stearothermophilus* species-specific PCR assay described by Prevost et al. (2010) was deeply modified. A new primer set for the species-specific identification of *A. flavithermus* was designed by the alignment and comparison of the *rpo*B gene sequences of *Anoxybacillus* species. Specificity of both PCR assays was validated by testing reference strains and by analyzing the 16S rRNA sequence of a representative number of wild strains isolated from naturally contaminated milk powder samples collected worldwide. The species-specific PCR assays were also tested to detect and identify the two target species in the bulk cells collected from the same milk powder samples.

After fulfilling the validation process, the two PCR assays were combined in a unique reliable Multiplex-PCR assay, capable to further halve the analysis time and cost.

2. Materials and methods

2.1. Bacterial reference strains and growth conditions

This study involved 53 reference strains from DSMZ collection (Table 1), representing 15 different species of the genus *Anoxybacillus*, eight different species of the genus *Bacillus*, four different species of the genus *Clostridium*, 13 different species of the genus *Geobacillus*, two different species of the genus *Moorella*, three different species of the genus *Paenibacillus* and two different species of the genus *Thermoanaerobacterium*.

With the exception of *G. stearothermophilus* strains 297, 456, 1550 and 2027, all the reference strains were purchased as ready-to-use DNA from DSMZ.

Working cultures of *G. stearothermophilus* strains were purchased as lyophilized cultures and were grown on Nutrient Agar (NA; Oxoid Ltd., Basingstoke, Hampshire, UK) at 60 °C in aerobic conditions for 48 h.

2.2. Isolation of wild strains from infant formula (IF) milk powders

24 IF milk powder samples (Table 2) were collected worldwide from different factories and used to isolate wild strains, naturally contaminating IF powders and resistant to high temperature.

Ten grams of each sample were transferred into a sterile Stomacher bag, reconstituted with 90 ml of Tryptone Sodium Chloride broth + antifoam (TS + broth; Tryptone, Oxoid; Sodium Chloride, Merck; Silicon antifoam, Sigma) with the addition of 2 g/l of soluble starch (Merck) and treated with a Stomacher machine for 30 s. To induce spore outgrowth, milk suspensions were heat-treated at 100 °C for 30 min in an oil bath and then incubated at 60 °C for 48 h to promote the growth of thermophilic bacilli. After the incubation, serial decimal dilutions were prepared by using