1 Introduction

Bacillus cereus sensu lato comprises closely related species that are of significant economic and clinical importance. With respect to human health, these bacteria can cause a wide range of diseases, including food poisoning, systemic infections, and highly lethal forms of anthrax (Didelot et al. 2009). *Bacillus cereus sensu stricto* is a frequent contaminant of food processing plants and is associated with food poisoning and spoilage. Hence, identification of *B. cereus sensu lato* is an important concern for the food industry.

Recent changes in the classification of the *B. cereus* group notably definition of phylogenetic groups (Guinebretière et al. 2008), modified to a large extent, identification within this group of bacteria. Indeed, the *B. cereus* group is actually composed by species that are not considered as separate genomic species but are distributed between seven phylogenetic groups (Guinebretière et al. 2008, 2010). These phylogenetic groups can be recovered in the Tourasse-helgason MLST database (http://mlstoslo.uio.no/). In addition to the six known species (*B. cereus sensu stricto*, *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, and *Bacillus weihenstephansis*), a potentially novel species, *Bacillus cytotoxicus* has been placed in the phylogenetic group VII, and characterized by its high toxicity, its particular thermotolerance (growth at 50 °C), and its relatively rare occurrence.

Strains of the *B. cereus* group are also recognized to be highly, weakly, or non-pathogenic, depending on their potential to produce or not produce toxins (Svensson et al. 2007; Guinebretière et al. 2008, 2010; De Jonghe et al. 2010). Moreover, the ability of strains to cause food poisoning was recently reported to vary according to their phylogenetic affiliation (groups I to VII) rather than species affiliation (Guinebretière et al. 2010). Consequently, in the food industry identification of bacterial strains at the phylogenetic group level is more pertinent than at species level to assess the food safety of the products.

In the dairy industry, *B. cereus* group spp., especially psychrothrophic strains, are recognized to limit the keeping quality of pasteurized milk (Svensson et al. 2004; Hanson et al. 2005; Barbano and Santos 2006; Aires et al. 2009). Contamination of pasteurized milk has been mainly traced to raw milk (Lin et al. 1998; Huck et al. 2007; Banykó and Vyletelová 2009) and/or equipment surfaces. The role of processing equipment as a reservoir for *B. cereus* milk recontamination is well documented (Te Giffel et al. 1997; Svensson et al. 1999, 2000, 2004; Schlegelova et al. 2010) notably post-pasteurization contamination (Eneroth et al. 2001; Sharma and Anand 2002; Salustiano et al. 2009). Currently, the persistence of this pathogen in various environments has been attributed to the formation of spores as well as biofilms. Thus it is important to accurately identify the sources of contamination and trace the spread of *B. cereus* in dairy plants. This may lead to a reduction in the level and incidence of *B. cereus* in pasteurized milk and improve the quality and shelf-life of the product.

Previous studies have reported a high incidence of *B. cereus* group strains in Algerian raw and pasteurized milk samples (Moussa Boudjemaa et al. 2004). High counts of these bacteria were also found on the process equipment surfaces of five dairy plants, which were suspected to be an important source of potential pasteurized milk recontamination (unpublished data). However, these strains have not been characterized further.

Rapid molecular typing methods such as sequence-based PCR or RAPD are widely used for typing *B. cereus* isolates (Guinebretière and Nguyen-The 2003; Svensson et al. 2004; Ehling-Schulz et al. 2005; Svensson et al. 2006; Thorensen et al. 2010). These genotypic methods are recognized to be good tools for tracing contaminations routes of *B. cereus* in pasteurized milk and have successfully been used for this purpose (Te Giffel et al. 1997; Svensson et al. 1999, 2000; Eneroth et al. 2001). Likewise the *panC* gene is a housekeeping gene described to provide a better opportunity to distinguish very closely related ecological populations, than ribosomal sequence data, and to rapidly assign bacterial isolates to groups I to VII of the phylogenetic classification (Guinebretière et al. 2008, 2010).

In the present study, isolates from one of these dairy plants, which had been investigated for several years for the presence of strains of *B. cereus*, were further characterized using the molecular typing M13-sequence-based polymerase chain reaction (M13-PCR). They were also assigned to phylogenetic groups (I to VII) based on *panC* sequence analysis. The main objectives were (1) to give more comprehensive data concerning the main sources of contamination for pasteurized milk, (2) to predict, in a first rapid step, their food poisoning potential using, as suggested by Guinebretière et al. (2010), a more accurate identification of representative strains (affiliation to phylogenetic groups within the *B. cereus* group).

2 Materials and methods

2.1 The processing plant

B. cereus isolates were collected in a dairy plant situated in northwest Algeria. Approximately 60,000 l of milk are handled per day in the plant. As local milk production is insufficient, in the 1970s the Algerian authorities began to import milk powder from various foreign countries. The milk powder is reconstituted and processed to obtain recombined pasteurized milk (Fig. 1).

2.2 Bacterial strains

Fifty strains belonging to the *B. cereus* group were isolated from monthly samplings of a pasteurized milk processing line over 4 years (in 2006 and 2010), and also from milk powder samples. All isolates were Gram-positive rods, spore producers, catalase positive, motile, and produced typical reactions on MYEP agar and hemolysis in blood agar. From these 50 isolates, 20 strains were selected for a more accurate molecular typing, based on their origin and morphological aspect of colonies. This resulted in a set of 20 strains with distinct origins and isolation periods (Table 1).

2.3 Molecular typing

2.3.1 M13-PCR

DNA preparation was performed according to the protocol described by Guinebretière and Nguyen-The (2003). The 20 isolates were typed by M13-PCR, using

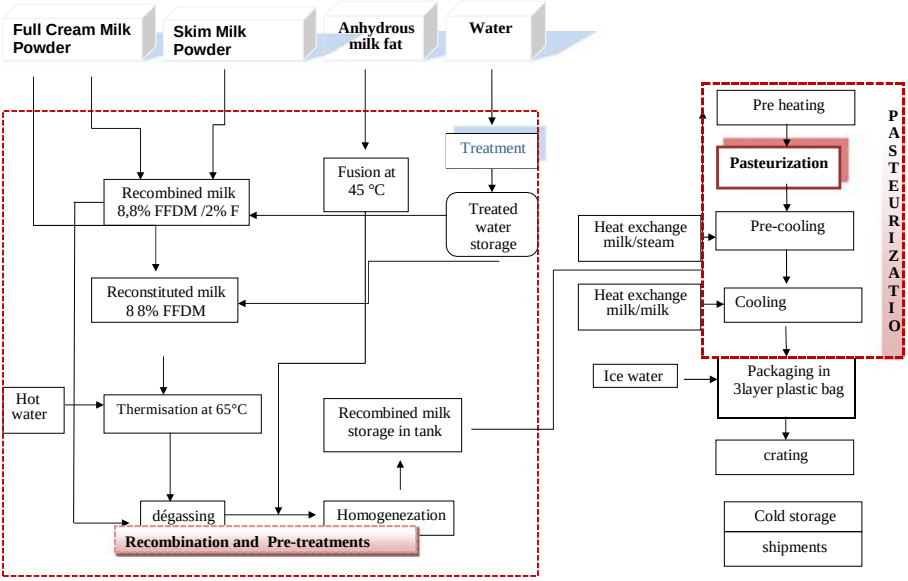

Fig. 1 Process flow diagram of recombined pasteurized milk at the dairy plant

Table 1 Molecular typing (M13-PCR) and phylogenetic affiliation of 20 *B. cereus* group strains isolated from different origins along the processing line

Strain origin	Year	M13 genotype	Representative strains	*Pan C* gene sequence analysis phylogenetic group—identity (%)[a]	
Milk powder					
BC1	2010	A			
BC5	2010	A			
BC9	2010	A			
BC12	2010	A			
BC19	2010	A	BC19	Group III	99.72
Pre-pasteurization					
Segments					
BC2	2010	A	BC2	Group III	99.72
BC6	2010	A			
BC7	2010	A			
BC8	2010	A			
BC13	2010	A			
BC18	2010	A			
BC20	2010	A			
BC10	2006	A			
BC11	2006	A			
BC17	2006	A			
Post-pasteurization					
Segments					
B15	2010	A	BC15	Group III	99.72
B16	2010	A			
Pre-pasteurization					
Segment					
BC3	2010	B	BC3	Group III	100
BC4	2006	B			
Pre-pasteurization					
Segment					
BC14	2010	C	BC14	Group IV	100

[a] According to phylogenetic groups defined in Guinebretière et al. (2008, 2010)

the PCR reaction and the thermal cycling previously described (Guinebretière and Nguyen-The 2003). PCR products from M13-PCR were separated on 1.5% agarose gel, and the molecular weight DNA marker SmartLadder SL (Eurogentec) was used as a reference. Gels were stained with ethidium bromide and digitized using a gel imager (Bioblock, III-kirch, France).

2.3.2 PanC gene sequence analysis

The *panC* gene was sequenced for representative strains of the three M13-PCR groups. Polymerase chain reaction amplification, purification, and sequencing were

carried out as previously described in Guinebretière et al. (2008). Affiliation to the phylogenetic groups using the resulting *panC* gene sequences was performed on the web site: http://www.tools.symprevius.org/Bcereus/english.php

3 Results

3.1 Clustering of isolates according to their M13-PCR patterns

After gel electrophoresis of the M13-PCR products for each of the 20 analyzed strains, a relatively simple profile of DNA fragments was obtained (Fig. 2). Thus, all the isolates were easily classified into three M13 genotypes, a major one (A) and two minor (B and C). A very low level of genetic diversity was identified between these strains. Indeed, most of the strains were represented in the major M13 genotype A, despite their various origins (Table 1). Seventeen strains isolated from the processing equipment and milk powder were similar (genotype A), suggesting that the milk powder is the source of contamination of the milk processing system. In contrast, genotypes B and C, represented respectively by strains BC3/BC4, and BC14, could not be related to a specific source of contamination and were not recovered in the pasteurized milk (only genotype A was recovered in the post-pasteurization segments).

Interestingly, strains originating from the 4-year-old culture collection were identical to recently isolated strains from the same processing line, suggesting the persistence of genotypes A and B. As the nearly unique source of contamination for the pasteurized milk, the milk powder seems to be a source of homogeneous contamination.

3.2 Identification to phylogenetic groups

Five strains representative of the M13 genotypess were identified by *panC* gene sequences analysis on the web site: https://tools.symprevius.org/Bcereus/english.php.

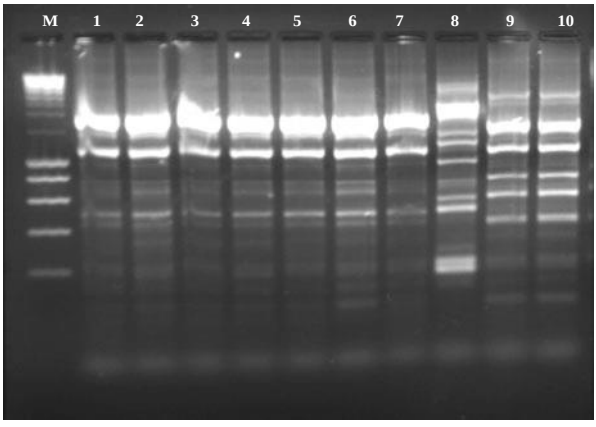

Fig. 2 M13-PCR patterns generated for *B. cereus* group strains. *Lanes 1 to 7* M13-PCR (genotype A): strains BC 19, 12, 13, 2, 5, 7, and 8. *Lane 8* M13-genotype C: strain BC 14. *Lanes 9 and 10* M13-genotype B: strains BC 4 and BC 3. *Lane M* DNA molecular mass marker (Ladder S L, Eurogentec)

The *B. cereus* group identity of isolates was thus confirmed and their assignation to phylogenetic groups, according to the Guinebretière et al. (2008) classification, was achieved (Table 1).

Results showed that genotypes A and B (i.e., 19 out of 20 analyzed strains) were affiliated to the mesophilic phylogenetic group III, whereas the last one (genotype C) to the mesophilic phylogenetic group IV (Table 1). Genotypes A and B are from the same phylogenetic group (group III), indicating a higher genetic relatedness than with genotype C. This finding corroborates the M13-PCR fingerprinting results. Therefore, the affiliation to phylogenetic groups III also indicates that these strains belong to one of the most cytotoxic groups (Guinebretière et al. 2010) comprising diarrheic as well as emetic strains, and that they probably represent a potential risk for consumers.

4 Discussion

Molecular methods used in this study allowed the genotyping and affiliation to phylogenetic groups of 20 *B. cereus* strains isolated from an investigated dairy plant. All strains were successfully characterized by the M13-PCR method used and their plant distribution accurately elucidated.

The fingerprints generated by M13-PCR revealed a low genetic diversity among the isolates. The 17 undistinguishable strains (genotype A) were found at different sampling points of the pasteurized milk processing line (pre- and post-pasteurization segments) and milk powder. This contrasts with data from the literature where a greater diversity of RAPD patterns among *B. cereus* strains isolated from dairy plants was recorded (Svensson et al. 1999, 2000, 2004). As an illustration, Eneroth et al. (2001) found that several *B. cereus* RAPD types originated from one sample only. The low genetic diversity identified by our results should be, first, ascribed to the nature of the raw material, i.e., milk powder which initially may harbor only a few genotypes. Indeed dehydrated products are assumed not to have favorable conditions for a wide diversity (Guinebretière and Nguyen-The 2003). Secondly, the pressure exerted by food processing conditions in factories is assumed to select some genetic groups (Carlin et al. 2010). Accordingly, restrictive processes such as heat treatment, often at abusive temperatures, both during milk powder production and milk powder processing may result in reducing the diversity of the processing plant microflora. This is consistent with the concept of the in-house microflora (Bagge-Ravn et al. 2003), which may be partly a reflection of the raw material used and partly a reflection of the particular warm climate that prevails in Algeria.

However, the in-house microflora investigated in this study was almost restricted to a single lineage with a persistence phenomenon across 4 years. This suggests a strong adaptation pattern of this lineage towards the ecological niche represented by the processing unit. Indeed, certain dairy plants are assumed to harbor particular plant-specific *B. cereus* which constantly contributes to post-pasteurization contamination (Schraft et al. 1999). Similarly, Salustiano et al. (2009) found that most of the *B. cereus* strains isolated from post-pasteurization equipment surfaces belonged to the same ribogroup. Once it is recovered from post-pasteurization segments, temperature appears to be the major factor that selects and maintains genotype A which best

survives the various thermal stresses undergone in the unit and before that. Finally, phylogenetic classification previously revealed a genetic structure of *B. cereus* strains corresponding to groups with different abilities to adapt to temperature. Thus, *B. cereus* group III contains mesophilic strains characterized with a comparatively high heat resistance of their spores (in Carlin et al. (2010)).

Protection of strains within biofilms formed inside pipe milking system may also be a survival strategy for the selected genotype. This phenomenon is well known in the dairy industry (Tauveron et al. 2006; Wijman et al. 2007; Salustiano et al. 2009; Schlegelova et al. 2010; Shaheen et al. 2010), where the predominant type that could be detected is best capable of contaminating the milk processing system. This is particularly true for *B. cereus* strains which have frequently been described as efficient biofilm formers. Furthermore, spores of *B. thuringiensis* and not only *B. cereus sensu stricto* were recently found to have high potential to adhere to inert surfaces (Ankolekar and Labbé 2010; Auger et al. 2009).

In conclusion, the common origin associated to both the selective pressure of temperature and persistence into biofilms should be responsible for this plant-specific distribution of group III *B. cereus* specific genotype.

A particular interest of the genetic groups III and IV is that they contain strains which carry various genes encoding toxins, representing a high risk for food poisoning (Guinebretière et al. 2010). The enterotoxigenic genes, hbl, cytK-2, and nhe, are present in strains of the two genetic groups, while the emetic gene *ces* is present in some members of the phylogenetic group III. As previously reported (Svensson et al. 2006), milk powder represents an important source of emetic bacteria. Overall, the occurrence of both pathogenic and non-pathogenic *B. cereus* strains in the dairy environment is well-established. Numerous studies have shown that this organism originates from the raw milk and contaminates the process equipment and may contaminate the processed products. Such dairy products, notably pasteurized milk products, should be low at-risk or high at-risk foods depending on the toxins production potential of contaminating strains. This illustrates the need for techniques that rapidly and accurately provide discriminative and informative data for the food-poisoning risk associated with *B. cereus* strains, as expressed in several reports (Thorensen et al. 2010; Chaves et al. 2011; Oh et al. 2012). Associating the phylogenetic groups with the different virulence potentials of *B. cereus* strains, first observed in Guinebretière et al. (2010), meets this requirement.

Algerian pasteurized milk is consumed by a large segment of the population including infants of less than 2 years. With regard to the food poisoning potential of identified strains, it may represent a health risk for consumers. However, to the best of our knowledge, no episodes of food poisoning have been reported after the consumption of pasteurized milk in Algeria. The reason for this may be the fact that the product is boiled by consumers before consumption. Unfortunately, this additional heating compromises once again the nutritional attributes of the product previously submitted to several heat treatments. Processors have then to particularly be aware that high temperature not only negatively affects the nutritional value of pasteurized milk but may also result in increasing *B. cereus* count in this product as shown by several studies (Hanson et al. 2005; Aires et al. 2009; Ranieri et al. 2009). Moreover, more attention must be paid to the quality and safety of Algerian pasteurized milk as an infant food, taking into account the particularities of this group of consumers.

To our knowledge, this is the first report on molecular typing and phylogenetic affiliation of *B. cereus* strains isolated in Algeria. It is also the first practical application of this classification to the study of an individual plant. M13-PCR analysis successfully traced the spread of *B. cereus* strains in the dairy plant, indicating that contamination of pasteurized milk could be traced also to milk powder, and not as previously expected, only to the process equipment. The two molecular methods revealed the persistence of specific and potentially toxin-producing *B. cereus* genotypes. The toxigenic profiles of the corresponding strains have to be further determined in order to accurately assess pasteurized milk safety.

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