

Multi-locus sequence typing and virulence profile in *Bacillus cereus sensu lato* strains isolated from dairy products

Angelica Bianco, Loredana Capozzi, Angela Miccolupo, Simona Iannetti, Maria Luisa Danzetta, Laura Del Sambro, Marta Caruso, Gianfranco Santagada, Antonio Parisi
Experimental Zooprophyllactic Institute of Apulia and Basilicata, Foggia, Italy

Abstract

Members of *Bacillus cereus* group are important food contaminants and they are of relevant interest in food safety and public health due to their ability to cause two distinct forms of food poisoning, emetic and diarrhoeal syndrome. In the present study, 90 strains of *B. cereus* isolated from dairy products, have been typed using Multi-locus Sequence Typing (MLST) analysis and investigated for the occurrence of 10 enterotoxigenic genes (*hblA*, *hblC*, *hblD*, *nheA*, *nheB*, *nheC*, *cytK*, *entFM*, *entS* and *bceT*) and one emetogenic gene (*ces*), to determine their genetic diversity. A total of 58 sequence types were identified and among these 17 were signalled as new profiles. Among the virulence genes, the majority of our strains carried the *entS* (92%), *entFM* (86%), *nhe* (82%) and *cytK* (72%) genes. All remaining genes were identified in at least one strain with different prevalence, stressing the genetic diversity, how even the different grade of pathogenicity of *B. cereus* isolated from dairy products.

Introduction

Bacillus cereus (*B. cereus*) is Gram-positive, rod shaped, endospore-forming, facultative anaerobic organism of genus *Bacillus*. Several closely related *Bacillus* species share a significant genetic similarity with *B. cereus*, thus they are included in a heterogeneous *B. cereus* group (Chang *et al.*, 2003). *B. cereus* group is ubiquitous in the environment, particularly soil and water, because of the resistance of its spores to adverse environmental conditions (Brillard *et al.*, 2015). It has been isolated from a variety of foods, including meat, rice, pasta, condiments, raw fruits, spices and vegetables (Harmon and Kautter, 1991), chinese "take-out foods" (Beuchat Ma-Lin *et al.*,

1980), cooked chicken (López *et al.*, 2015), milk and dairy products (Christiansson, Bertilsson, and Svensson, 1999).

Some members of *B. cereus* group (*i.e.* *B. cereus* and *B. thuringiensis*) can cause human gastrointestinal diseases, the emetic and diarrhoeal syndromes. The emetic syndrome is characterised by vomiting, followed by diarrhoea (Beecher and Wong, 1994). This syndrome is caused by the ingestion of the cereulide toxin, preformed in the food (Agata *et al.*, 2002). The diarrhoeal syndrome, is characterized by watery diarrhoea associated with abdominal pain (Granum and Lund, 1997) and it is caused by the ingestion of vegetative cells of *B. cereus* present in food, that colonize the small intestine before starting the enterotoxins production (*i.e.* non-haemolytic enterotoxin NHE, haemolysin BL, cytotoxin K) (Granum and Lund, 1997). Additionally, enterotoxin FM (EntFM), enterotoxin T (BceT) and enterotoxin S (EntS) have been also described as potential diarrheal toxins, although it is not clear the role they play in the pathogenesis of the disease (Hendriksen *et al.*, 2006).

The aim of this study was to identify the virulence genes and to determine the sequence type of *B. cereus sensu lato* (*s.l.*) strains isolated from dairy products using Multi-locus Sequence Typing (MLST).

Materials and Methods

Multi-locus sequence typing

A total of 90 strains of *B. cereus s.l.* were isolated from dairy products: 64% (57/90) from caciocavallo, 25% (22/90) from mozzarella, 2% (2/90) from sheep's cheese; 2% (2/90) from butter; 2% (2/90) from milk; 1% (1/90) from Buffalo mozzarella; 1% (1/90) from caciocotta; 1% (1/90) from cream cheese; 1% (1/90) from caciotta; 1% (1/90) from yogurt. All the samples were collected from 2016 to 2018.

Genomic DNA was purified using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), according to manufacturer's protocol. The MLST analysis was performed as described elsewhere (<http://www.pubmlst.org/bcereus/>).

DNA sequencing was performed on both strands using ABI PRISM Big Dye™ Terminator Cycle 3.1 sequencing kit cycle (Life Technologies) according to the manufacturer's instruction and analyzed by ABI Prism 3130 Genetic Analyzer (Applied Biosystem/ThermoScientific inc.). The sequence of each allele was imported and assembled by Bionumerics v7.5 software (Applied Maths, Belgium), and then com-

Correspondence: Antonio Parisi, Experimental Zooprophyllactic Institute of Apulia and Basilicata, Foggia, Italy. Tel.: +39.080.4057858; Fax: +39.080.4057753. E-mail: antonio.paris@izspb.it

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pared to those available in the MLST database. New allele sequences were submitted to the *B. cereus* MLST database.

Virulence genes detection

The presence of potential virulence genes *hblA*, *hblC*, *hblD*, *nheA*, *nheB*, *nheC*, *cytK*, *entFM*, *entS*, *bceT* and *ces* was evaluated by PCR using primers listed in Table 1 (Asano *et al.*, 1997; Guinebretière *et al.*, 2002; Fagerlund *et al.*, 2004) and followed the procedure described in Ghelardi *et al.*, 2002 and Fricker *et al.*, 2007

Results

In our study we collected a total of 90 *B. cereus s.l.* strains isolated from different dairy products (Table 2). The MLST profiles of all isolates are shown in Table 2. A total of 58 STs were identified with ST 1986 as the most frequent. Additionally, 17 STs never described before were identified (Table 2). Thirteen STs belonged to 7 clonal complexes (CC). The major clonal complex was represented by the CC-142, that includes two STs, ST4 and ST2010 (Table 2). The CC-23 included three STs, ST197, ST2013 and ST2033; each CC-111, CC-142 and CC-365 included two STs: ST562 and ST800, ST4 and ST2010, ST1263 and ST2084, respectively. The CC-97 and CC-

205 included each only one ST, 1578 and 255, respectively. The ST1986 was the most frequent ST as it was detected only in caciocavallo. When we considered the distribution of STs among the samples collected in this study (including samples with two or more strains isolated), we identified 34 different STs in caciocavallo, 15 STs in mozzarella, 2 STs were identified in both butter and milk and sheep's cheese. Only two STs, ST92 and ST142, were identified in more than one dairy product.

All strains analyzed in this study were tested for the presence or absence of eleven virulence genes; the results were summarized on Table 2. Three genes encoding enterotoxin HBL were identified in 58% (53/90) of strains, with different prevalence: the *hblA*, *hblC* and *hblD* were identified in 67% (60/90), 64% (58/90) and 79% (71/90) of the strains, respectively. Similarly, the cluster of *nhe* genes was identified in 84% (76/90) of the strains with different prevalence between genes: the *nheA*, *nheB* and *nheC* genes were identified in 92% (83/90), 93% (84/90) and 90% (81/90) of the strains, respectively. The *cytK*, *entFM*, *bceT* and *entS* genes were identified in 72% (65/90), 86% (77/90), 64% (59/90) and 92% (83/90) of the strains, respectively. Only the 10% (9/90) of the strains carried the *ces* gene.

Discussion and Conclusions

B. cereus s.l. is a ubiquitous bacterium in the environment that can be present in a

wide range of foodstuffs. It is prevalent in soil from cattle grazing fields, raw milk and dairy products. In this study we describe the characterization and the pathogenic potential of a collection of 90 *B. cereus* strains isolated from dairy products. Based on MLST profiles our strains showed a high genetic diversity. Indeed, on a total of 90 isolates 58 MLST profiles were identified, of which 17 were new ST profiles and were submitted in available online database. The ST1986 was the most frequent ST detected and it was found only in the strains isolated from caciocavallo. Interestingly, only the ST92 and ST142 were identified in more than one dairy product; all the other 56 STs were detected in a particular dairy product variety. Among previously described STs, ten (ST4, ST12, ST15, ST24, ST26, ST33, ST164, ST205, ST1063 and ST1097) were identified in milk or milk products (Cardazzo *et al.*, 2008; Didelot, Barker, Falush, and Priest, 2009; Yang *et al.*, 2017). The remaining 31 STs were isolated in other foods or in the environment (Didelot *et al.*, 2009; Priest *et al.*, 2004).

Different virulence genes have been associated to the pathogenic power of *B. cereus*, the Enterotoxins BL (HBL), Non-Hemolytic Enterotoxin (NHE), cytotoxin K, and enterotoxin FM are primarily associated to diarrheal disease (Madeira *et al.*, 2015; Sergeev *et al.*, 2006); evidence suggests that the enterotoxin EntS and BceT also contribute to the severity of diarrheal illness Agata *et al.*, 1995; Castiaux *et al.*, 2016; whereas the ability of *B. cereus s.l.* to synthesize the heat stable cereulide protein is

connected to the emetic disease (Ehling-Schulz *et al.*, 2005). In our study we found that the non-emetic strains (90%) were more numerous than the emetic strains (10%). This finding agreed with previous studies, which showed a low prevalence rate of emetic toxin gene *ces* in *B. cereus s.l.* isolated from milk, dairy product and other various food sources (Yim *et al.*, 2015; Owusu-Kwarteng *et al.*, 2017). All these data seem to suggest that emetic toxin gene is rare among *B. cereus s.l.* strains, but an infection supported by this specific toxin is considered to be the worst-case scenario (Wetterhall *et al.*, 2019). Generally, *B. cereus s.l.* strains related with emetic food poisoning were associated with ST26 (Ehling-Schulz *et al.*, 2005). Based on the genetic profile of our strains, we not only corroborated this thesis, but we identified additionally five STs (ST4, ST33, ST1986, ST2045 and ST2062) as potential emetic strains. Interestingly, among these strains, those belonging to ST26 did not carry HBL gene complexes, as previously reported (Ehling-Schulz *et al.*, 2005); among these, in the BC003 strain we identified only two virulence genes, *ces* and *entS*. Similarly, to ST26, both the strains belonged to ST2045 did not carry HBL gene complexes. Among the non-emetic *B. cereus s.l.* strains, we found a high prevalence rate ($\geq 60\%$) of all enterotoxin genes investigated. The higher prevalence rate was related to *entS* gene, identified in 93% of all strains, followed by *entFM* gene (83%), *nhe* gene complex (82%), *cytK* gene (72%), *bceT* gene (66%) and *hblACD* gene complex

Table 1. Sequence of PCR primers targeting eleven virulence genes used in this study.

Target	Primer name	Primer sequence (5'-3')	Reference
<i>hblA</i>	hblA_F HBLA_R	GTGCAGATGTTGATGCCGAT ATGCCACTGCGTGGACATAT	(Guinebretière <i>et al.</i> , 2002)
<i>hblC</i>	L2A_F L2B_R	AATGGTCATCGGAACCTAT CTCGCTGTTCTGCTGTTAAT	(Guinebretière <i>et al.</i> , 2002)
<i>hblD</i>	L1A_F L1B_R	AATCAAGAGCTGTCACGAAT CACCAATTGACCATGCTAAT	(Guinebretière <i>et al.</i> , 2002)
<i>nheA</i>	nheA_F nheA_R	TACGCTAAGGAGGGGCA GTTTTTATTGCTTCATCGGCT	(Guinebretière <i>et al.</i> , 2002)
<i>nheB</i>	nheB_F nheB_R	CTATCAGCACTTATGGCAG ACTCCTAGCGGTGTTCC	(Fagerlund <i>et al.</i> , 2004)
<i>nheC</i>	nheC_F nheC_R	CGGTAGTGATTGCTGGG CAGCAITTCGTACTTGCCAA	(Fagerlund <i>et al.</i> , 2004)
<i>cytK</i>	cytK_F cytK_R	ACAGATATCGGKCAAATGC TCCAACCCAGTTWSCAGTTC	(Ghelardi <i>et al.</i> , 2002)
<i>entFM</i>	entFM_F entFM_R	ATGAAAAAGTAATTTGCGAGG TTAGTATGCTTTTGTGTAACC	(Asano <i>et al.</i> , 1997)
<i>bceT</i>	entT_F entT_R	GCTACGCAAAAACCGAGTGGTG AATGCTCCGGACTATGCTGACG	(Agata <i>et al.</i> , 1995)
<i>Ces</i>	ces_F ces_R	GGTGACACATTATCATATAAGGTG GTAAGCGAACCTGTCTGTAACAACA	(Fricker <i>et al.</i> , 2007)

Table 2. Source, clonal complex (CC), sequence type (ST) and enterotoxin and emetic toxin genes identified in *B. cereus* s.l. isolated from dairy products. The new STs identified for the first time were indicated with asterisks.

ID sample	Source	CC	ST	Virulence genes										
				hblA	hblC	hblD	nheA	nheB	nheC	cytK	entFM	bceT	entS	ces
BC001	Caciocavallo		1967*	+	+	+	+	+	+	+	+	+	+	-
BC002	Mozzarella		26	-	-	-	+	+	+	-	+	-	+	+
BC003	Mozzarella		26	-	-	-	-	-	-	-	-	-	+	+
BC004	Caciocavallo		26	-	-	-	+	+	+	-	+	-	+	+
BC005	Caciocavallo		34	-	-	-	+	+	-	+	+	+	+	-
BC006	Caciocavallo		33	+	+	+	+	+	+	+	+	+	+	+
BC007	Caciocavallo		509	-	-	+	+	+	+	+	-	+	+	-
BC026	Caciocavallo	ST-111 complex	800	+	+	+	+	+	+	+	+	+	+	-
BC009	Caciocavallo		34	-	-	-	+	+	+	+	+	+	+	-
BC0010	Caciocavallo	ST-8 complex	8	+	+	-	+	+	-	+	+	+	+	-
BC0011	Caciocavallo	ST-142 complex	2010*	+	+	+	+	+	+	+	+	+	+	-
BC0012	Caciocavallo		2002*	+	+	+	+	+	+	-	+	+	+	-
BC0013	Caciocavallo		500	+	+	+	+	+	+	+	+	-	+	-
BC0014	Caciocavallo	ST-23 complex	2013*	+	+	+	+	+	+	+	+	+	+	-
BC0015	Caciocavallo		2026*	+	+	+	+	+	+	-	+	+	-	-
BC0016	Caciocavallo		2028*	-	-	+	+	-	+	-	+	-	-	-
BC0017	Caciocavallo	ST-365 complex	1263	-	-	-	+	+	-	-	+	-	+	-
BC0018	Caciocavallo	ST-8 complex	15	+	+	+	+	+	+	+	+	+	+	-
BC0019	Caciocavallo	ST-142 complex	4	+	+	+	+	+	+	+	+	+	+	+
BC0020	Caciocavallo	ST-142 complex	4	+	+	+	+	+	+	+	+	+	+	-
BC0021	Caciocavallo		2031*	+	+	+	+	+	+	+	+	-	+	-
BC0022	Caciocavallo		34	-	-	-	+	+	+	+	+	+	+	-
BC0023	Caciocavallo	ST-23 complex	2033*	+	-	+	+	+	+	-	-	+	+	-
BC0025	Caciocavallo		2036*	+	+	+	+	+	+	-	-	+	+	-
BC0024	Cream cheese		996	-	-	-	+	+	-	-	+	+	+	-
BC0027	Caciocavallo	ST-142 complex	4	+	+	+	+	+	+	+	+	+	+	-
BC0028	Caciocavallo		59	+	-	+	+	+	+	+	+	+	+	-
BC0029	Caciocavallo		2041*	+	+	+	+	+	+	+	+	+	+	-
BC0030	Mozzarella		369	-	-	-	+	+	+	+	+	+	+	-
BC0031	Caciocavallo		2045*	-	-	-	+	+	+	-	-	-	+	+
BC0032	Caciocavallo		2045*	-	-	-	+	+	+	+	-	+	+	+
BC0033	Mozzarella		12	+	+	+	+	+	+	+	-	+	+	-
BC0034	Caciocavallo		1986*	-	+	+	+	+	+	+	+	-	+	+
BC0035	Mozzarella		1420	+	+	+	+	+	+	+	+	+	+	-
BC0036	Buffalo mozzarella		278	-	-	+	+	+	+	+	+	+	+	-
BC0037	Caciocavallo		1986*	+	+	+	+	+	+	+	+	-	+	-
BC0038	Caciocavallo		1986*	+	+	+	+	+	+	+	+	-	+	-
BC0040	Caciocavallo		1986*	+	+	+	+	+	+	+	-	-	+	-
BC0041	Caciocavallo	ST-23 complex	197	+	+	+	+	+	+	+	+	+	+	-
BC0042	Caciocavallo		2062*	-	-	+	-	+	+	-	+	-	+	+
BC0043	Caciocavallo		1986*	+	+	+	+	+	+	+	+	-	+	-
BC0044	Caciocavallo		262	+	+	+	+	+	+	+	+	+	+	-
BC0045	Caciocavallo		1986*	+	+	-	+	+	+	+	+	-	+	-
BC0046	Caciocavallo		2116	-	-	+	-	-	-	+	-	+	-	-
BC0047	Caciocavallo		1986*	+	+	+	+	+	+	+	+	-	+	-
BC0048	Caciocavallo	ST-205 complex	205	+	+	+	+	+	+	+	+	+	+	-
BC0049	Cacioricotta		164	-	-	+	+	+	+	+	+	+	+	-
BC0050	Mozzarella		818	-	-	+	+	+	+	+	+	+	+	-
BC0051	Mozzarella		1097	-	-	+	+	+	+	-	+	-	+	-
BC0052	Mozzarella		2038	+	+	+	+	+	+	+	+	+	+	-

Continued on the next page.

(60%). Similar results of high prevalence of *cytK*, *entFM* and *hbl* gene complexes in *B. cereus* isolated from milk and dairy products (Reis *et al.*, 2013) and of *nhe* gene complex, *entS* and *bceT* genes complexes in *B. cereus* isolated from several food and environmental source (*i.e.* soil) have been described (Yang *et al.*, 2005; Chaves *et al.*, 2012; Yim *et al.*, 2015). When we considered the virulence genes profile and the source analysed, none correlation was found. Whereas when we correlated the ST profiles to virulence genes detected we found nine STs (ST8, ST509, ST996, ST1097, ST1263, ST2028, ST2033,

ST2084 and ST2116) that were associated to an exclusive virulence genes combination. Nevertheless, this genetic profile correlation is questionable, considering that we identified one strain for each of nine STs previously mentioned. Thus, further studies will be necessary for demonstrated really correlation between MLST and virulence genes profiles.

Based on the results and considering the genetic diversity of *B. cereus s.l.*, the MLST approach has proven to be an accurate method for strain genetic typing. Interestingly, our analysis demonstrated the presence of toxin genes in all strains of the

B. cereus s.l., stressing the their potentially pathogenic power. Taken together, these data appear to be of significant interest for public health, since highlight potential risk connected to ready-to-eat foods, generally consumed without cooking. In order to prevent contamination by *B. cereus s.l.*, it is very important observe the correct hygiene practices along food chain. In addition, it would be advisable to implement scrupulous control measures of food preservation, to prevent the multiplication of the microorganism and the consequent production of toxins.

Table 2. Continued from previous page.

ID sample	Source	CC	ST	Virulence genes										
				<i>hblA</i>	<i>hblC</i>	<i>hblD</i>	<i>nheA</i>	<i>nheB</i>	<i>nheC</i>	<i>cytK</i>	<i>entFM</i>	<i>bceT</i>	<i>entS</i>	<i>ces</i>
BC0053	Caciocavallo		1986*	+	+	+	+	+	+	+	+	-	+	-
BC0054	Caciocavallo		26	+	+	+	+	+	+	+	+	+	+	-
BC0055	Caciocavallo		1986*	+	+	+	+	+	+	+	+	-	+	-
BC0056	Butter		120	+	-	+	+	+	+	+	+	+	+	-
BC0057	Mozzarella		1665	+	-	+	+	+	+	+	+	-	+	-
BC0058	Caciocavallo		1986*	+	+	+	+	+	+	+	+	-	+	-
BC0059	Caciocavallo	ST-142 complex	4	+	+	+	+	+	+	+	+	-	+	-
BC0060	Mozzarella	ST-97 complex	1578	+	+	+	+	+	+	+	+	-	+	-
BC0061	Caciocavallo		1986*	+	+	+	+	+	+	+	+	-	+	-
BC0062	Caciocavallo		500	+	+	+	+	+	+	+	+	-	+	-
BC0063	Mozzarella		1097	-	+	-	+	+	+	-	+	-	+	-
BC0064	Sheep's cheese		1063	-	-	+	+	+	+	-	+	+	+	-
BC0065	Caciotta		12	-	-	+	+	+	+	-	+	+	+	-
BC0066	Mozzarella		12	-	-	+	+	-	-	+	-	-	+	-
BC0067	Caciocavallo		371	+	+	+	+	+	+	+	+	+	+	-
BC0068	Mozzarella		1007	-	-	-	+	+	+	+	+	+	+	-
BC0069	Caciocavallo		92	+	+	+	+	+	+	+	+	+	+	-
BC0070	Caciocavallo		1986*	+	+	+	+	+	+	+	+	-	+	-
BC0071	Mozzarella		92	+	+	+	+	+	+	+	-	+	+	-
BC0072	Mozzarella		2261	+	+	+	+	+	+	+	+	+	+	-
BC008	Caciocavallo		632	+	+	+	+	+	+	-	+	+	-	-
BC0073	Mozzarella	ST-111 complex	562	-	-	-	+	+	+	-	+	-	+	-
BC0074	Mozzarella		92	+	+	+	-	+	-	+	+	+	-	-
BC0075	Sheep's cheese		1223	-	-	-	+	+	+	-	+	-	-	-
BC0076	Mozzarella		24	-	-	+	+	+	+	-	-	+	+	-
BC0077	Mozzarella		92	+	+	+	-	+	+	+	-	+	+	-
BC0078	Caciocavallo		2083*	+	+	+	+	+	+	+	+	+	+	-
BC0079	Caciocavallo	ST-365 complex	2084*	-	+	+	+	-	+	-	+	-	+	-
BC0080	Milk		56	+	+	+	+	+	+	+	+	+	+	-
BC0081	Caciocavallo		1833*	+	+	+	+	+	+	-	+	+	+	-
BC0082	Butter		1355	+	+	+	+	+	+	+	+	+	+	-
BC0083	Caciocavallo		1967*	+	+	+	+	+	+	+	+	+	+	-
BC0084	Mozzarella		414	-	-	-	+	+	+	+	+	+	+	-
BC0085	Mozzarella		142	+	+	+	+	+	+	+	+	+	+	-
BC0086	Mozzarella		24	+	+	+	+	+	+	+	+	+	+	-
BC0087	Caciocavallo		1967*	+	+	+	+	+	+	+	+	+	+	-
BC0088	Milk		1810	+	+	+	+	+	+	-	+	+	+	-
BC0089	Yogurt		142	+	-	-	-	+	+	-	+	+	+	-
BC0090	Caciocavallo		2095*	+	+	+	+	+	+	+	+	+	+	-
BC0094	Caciocavallo		2036*	+	+	+	-	-	-	-	+	+	-	-

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