Available online at www.sciencedirect.com
ScienceDirect
International Journal of Food Microbiology 116 (2007) 292–296
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Short communication

Food poisoning potential of *Bacillus cereus* strains from Norwegian dairies

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Received 7 September 2006; accepted 29 December 2006

Abstract

Characteristics concerning diarrhoeal potential were investigated in *B. cereus* dairy strains. The thirty-nine strains, isolated from whipping cream, were tested for cytotoxicity after culturing at human body temperature as well as 25 °C and 32 °C. At 37 °C, none of the strains were highly cytotoxic. This observation suggests that those strains should be considered to pose a minor risk with regard to diarrhoeal food poisoning. However, some strains were moderately or highly cytotoxic when grown at 25 °C and 32 °C. While the majority of the strains were able to grow at refrigeration temperatures, only four *B. weihenstephanensis* strains were identified among them when subjected to discriminative PCR assays and growth temperatures which delimit this species.

Keywords: *Bacillus cereus*; *Bacillus weihenstephanensis*; Enterotoxin; Dairy

1. Introduction

Bacillus cereus is a species of Gram-positive bacteria inhabiting numerous environments including soil, plant materials and many foods. Due to their spore forming ability they can pose problems in the food industry (Meer et al., 1991; Andersson et al., 1995). In addition, production of toxins in foods (emetic) or in the human intestine can lead to emesis or diarrhoea, respectively. At least three enterotoxins are involved in pathogenesis of *B. cereus* diarrhoeal gastroenteritis (Lund and Granum, 1996; Granum and Lund, 1997; Lund et al., 2000). The genes encoding the enterotoxins are widespread among strains of the *B. cereus* group (Prüss et al., 1999a; Rivera et al., 2000; Stenfors and Granum, 2001; Ehling-Schulz et al., 2005). Unfortunately, there are presently no reliable, easy methods that can unambiguously discriminate pathogenic *B. cereus* group strains from harmless ones. The enterotoxin production of *B. cereus* strains can be evaluated by cell culture assays, and in the present study, an assay measuring cytotoxicity towards Vero cells is employed.

A new species in the *B. cereus* group, *B. weihenstephanensis*, was suggested in 1998 to comprise the psychrotrophic *B. cereus* group strains (Lechner et al., 1998). The criteria for discriminating strains of the new species from the other species of the *B. cereus* group include ability to grow below 7 °C, but not above 42 °C, and the presence of specific gene signature sequences in rRNA operons and in the major cold shock gene, *cspA*, detected by polymerase chain reaction assays (Francis et al., 1998; von Stetten et al., 1998). *B. weihenstephanensis* strains have previously been isolated from dairy products (Lechner et al., 1998) and soil samples from alpine and temperate climates (von Stetten et al., 1999). Psychrotrophic and mesophilic bacteria of the *B. cereus* group are some of the most important contaminators of milk and other dairy products (Phillips and Griffiths, 1986; Väisänen et al., 1991; Granum et al., 1993; Champagne et al., 1994; Crielly et al., 1994; Te Giffel et al., 1997; Holm et al., 2004). Despite this, milk is not a common vehicle for *B. cereus* gastroenteritis, and the reason for this has not yet been found (Johnson, 1984; Christiansson et al., 1989; Kramer and Gilbert, 1989; Drobniewski, 1993).

This study sought to determine aspects related to risk of diarrhoeal food poisoning in *B. cereus* dairy isolates from Norway. The cytotoxicity of 39 *B. cereus* dairy isolates at three different temperatures was investigated. As the strains had been isolated prior to the proposal of the psychrotrophic species *B. weihenstephanensis*, they were also tested for the properties defining this species.

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doi:10.1016/j.ijfoodmicro.2006.12.021

2. Materials and methods

2.1. Bacterial strains

The Department of Food Safety and Infection Biology at Norwegian School of Veterinary Science examined whipping cream samples from a number of dairies mainly from the Western parts of Norway for the presence of *B. cereus* in 1994. Before isolation of bacterial strains, the cream had been subjected to temperature abuse (room temperature for 12–24 h). A total of 118 *B. cereus* strains were isolated from the dairies, and 39 were investigated in this study (Table 1).

Table 1
Cytotoxicity, growth and PCR results

Strain no.	Growth at 6.5 °C	Thermal type PCR			Vero cell assay [a] (%)		
		16S M [b]	16S P [b]	*cspA*	25 °C	32 °C	37 °C
3	−	+	+	−	65	59	–
4	+	+	+	+	35	–	–
5	−	+	+	−	58	V [c]	22
10	+	+	+	−	–	–	–
11	+	+	+	−	–	–	–
16	+	+	+	−	–	46	–
24	+	−	+	+	–	–	–
25	+	+	+	+	–	–	–
38	−	+	+	+	96	100	–
40	−	+	+	+	–	–	–
45	+	+	+	−	100	100	37
46	+	+	+	−	100	100	30
48	+	−	+	+	–	–	–
53	+	−	+	−	42	55	38
54	−	+	+	−	42	76	–
58	−	−	+	+	–	–	–
62	+	−	+	+	–	–	–
64	+	−	+	+	–	–	–
66	+	+	+	+	–	–	–
70	+	+	+	+	–	–	–
73	+	−	+	+	–	–	–
75	+	−	+	+	–	–	–
78	+	−	+	+	–	–	–
81	−	+	+	+	–	–	–
83	+	+	+	−	75	100	22
84	−	+	+	+	–	–	–
85	+	+	+	+	–	–	–
90	+	+	+	+	–	–	–
91	+	+	+	+	74	87	–
92	+	+	+	−	92	92	–
96	+	+	+	−	–	–	–
97	+	+	+	+	75	V [c]	–
99	+	−	+	+	–	V [c]	–
105	−	+	+	−	–	63	–
106	−	+	+	−	100	100	46
108	+	+	+	−	20	80	38
109	+	+	+	+	93	V [c]	22
111	−	+	+	−	82	90	39
112	−	+	+	−	100	95	–

[a] Percentage inhibition of protein synthesis. High cytotoxicity: 80–100% (food poisoning strains tested at the Norwegian Reference Laboratory for *B. cereus* are consistently cytotoxic to this level), moderate: 50–80%, low: 20–50%.

[b] Positive reaction is the correct sized "mesophilic" (16S M), respectively "psychrotrophic" (16S P) fragment in 16S rDNA-directed PCR (Table 2).

[c] Varying cytotoxicity in repeated assays, see Results.

2.2. Growth conditions and harvesting of culture supernatants

For cytotoxicity testing, the strains were grown at 25 °C, 32 °C and 37 °C. Overnight cultures of 5 ml BHIG (Brain Heart Infusion broth, Difco, with 1% w/v glucose), grown at 32 °C, were diluted 1:100 in BHIG and grown in a shaking water bath, at 100 movements/min. Optical density (OD_{600}) was measured in the cultures diluted 1:10 in BHIG to within the linear range of the spectrophotometer (Shimadzu UV-160A, cuvette path length 1 cm), and used as a reference point for harvesting samples in the same phase of growth. When culturing at 32 °C, a standard time of 6 h growth was used. These are the standard conditions used for *B. cereus* enterotoxin detection at our laboratory, the national reference laboratory for this method. After 6 h at 32 °C, corresponding to cultures entering late logarithmic phase of growth and reaching an OD_{600} of 2.5–3.0, the level of enterotoxins reaches high levels (Glatz and Goepfert, 1976; Garcia-Arribas and Kramer, 1990). For 25 °C and 37 °C cultures, samples were harvested at an OD_{600} of approximately 2.5–3.0. Samples were obtained by centrifugation of 1.5 ml of liquid culture at 20,000 ×*g* for 3 min. Supernatants were frozen immediately at −20 °C until performance of cytotoxicity assay. Five strains that were cytotoxic at 37 °C were additionally grown at this temperature under anaerobic conditions, as well as in BHIG with and without bile salts (0.5% w/v of Bile Salts, Oxoid, consisting mainly of sodium glycocholate and sodium taurocholate, used in the concentration generally recommended by the manufacturer). Growth at 6.5 °C was investigated by streaking on bovine blood agar plates (Oxoid, 5% bovine blood). Visible growth within two weeks was recorded as a positive result. Nine strains yielded PCR results typical of the species *B. weihenstephanensis*, and were tested for growth at 43 °C for species determination. Growth at 43 °C was followed on blood agar plates for one week; visible growth within this time was considered a positive result. Growth testing and culturing for cytotoxicity assays were performed at least twice.

2.3. Vero cell cytotoxicity assay

Cytotoxicity of culture supernatants was tested on monolayers of Vero C1008 cells (African green monkey kidney cells, ECACC no. 85020206). The assay measures cellular damage as the inhibition of protein synthesis in the Vero cells (Sandvig and Olsnes, 1982). Confluent monolayers of Vero cells were first incubated for 2 h with duplicates of 100 µl bacterial culture supernatant, diluted in 1 ml of leucine-free cell culture medium, before replacing this with cell culture medium containing ^{14}C-leucine for 1 h. The incorporation of ^{14}C-leucine into the Vero cells was measured by scintillation counting. Culture supernatant from a well-characterized, food poisoning-associated strain was used as positive control, while leucine-free cell culture medium was the negative control. The percentage of inhibition was calculated using the average scintillation count for the duplicates of each sample (performed twice), relative to the values obtained for the negative and positive controls of each tested cell tray.