International Journal of Food Microbiology 127 (2008) 60–67

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Environment driven cereulide production by emetic strains of *Bacillus cereus*

Camelia Apetroaie-Constantin [a], Ranad Shaheen [a], Lars Andrup [b], Lasse Smidt [b], Hannu Rita [c], Mirja Salkinoja-Salonen [a,*]

[a] University of Helsinki, Department of Applied Chemistry and Microbiology, P.O. Box 56, Viikinkaari 9, FI-00014 Helsinki, Finland
[b] National Research Centre for the Working Environment, Lersø Parkallé 105, DK-2100, Copenhagen, Denmark
[c] University of Helsinki, Department of Forest Ecology, POB 27, Latokartanonkaari 7, FI 00014 Helsinki, Finland

ARTICLE INFO

Article history:
Received 20 August 2007
Received in revised form 9 June 2008
Accepted 9 June 2008

Keywords:
Cereulide
K^+ homeostasis
Glycine
Potassium
ces plasmid
Food additive

ABSTRACT

The impacts of growth media and temperature on production of cereulide, the emetic toxin of *Bacillus cereus*, were measured for seven well characterised strains selected for diversity of biochemical and genetic properties and sources of origin. All strains carried cereulide synthase gene, *ces*, on a megaplasmid of ca. 200 kb and all grew up to 48–50 °C, but produced cereulide only up to 39 °C. On tryptic soy agar five strains, originating from foods, food poisonings and environment, produced highest amounts of cereulide at 23 to 28 °C, whereas two strains, from human faeces, produced cereulide similarly from 23 to 39 °C, with no clear temperature trend. These two strains differed from the others also by producing more cereulide on tryptic soy agar if supplemented with 5 vol.% of blood, whereas the other five strains produced similarly, independent on the presence of blood. On oatmeal agar only one strain produced major amounts of cereulide. On skim milk agar, raw milk agar, and MacConkey agar most strains grew well but produced only low amounts of cereulide. Three media components, the ratio $[K^+]$:$[Na^+]$, contents of glycine and $[Na^+]$, appeared of significance for predicting cereulide production. Increase of $[K^+]$:$[Na^+]$ (focal variable) predicted ($P<0.001$) high cereulide provided that the contents of glycine and $[Na^+]$ (additional variables) were kept constant. The results show that growth medium and temperature up and downregulate cereulide production by emetic *B. cereus* in a complex manner. The relevance of the findings to production of cereulide in the gut and to the safety of amino acids as additives in foods containing live toxinogenic organisms is discussed.

1. Introduction

Bacillus cereus is a frequent contaminant in foods and increasingly reported as a major agent in cases of bacterially caused food related illness (Becker and Becker, 2005; Granum, 2007; Kleer et al., 2003; Niskanen et al., 2006; Reiche, 2004; Wijnands et al., 2006). Some strains produce a stable toxin, cereulide, that is not inactivated by any type of food processing including heating (Agata et al., 2002; Jay et al., 2005; Vilas-Bôas et al., 2007; Rajkovic et al. 2008). Eradication of the species *B. cereus* from foods and their raw materials is an unlikely task to succeed, considering the high thermal resistance and adherence of *B. cereus* spores (Hoornstra et al., 2006; Granum, 2007; Hornstra et al., 2007, Vilas-Bôas et al., 2007). Spores of cereulide producing *B. cereus* are several-folds more heat resistant than those of cereulide non-producing strains (Carlin et al., 2006).

Cereulide is a cyclic dodecadepsipeptide, a potassium ionophore and a mitochondriotoxin (Agata et al., 1995; Hoornstra et al., 2003; Mikkola et al., 1999; Sakurai et al., 1994; Shinagawa et al., 1995; Teplova et al., 2006). It is one of the most toxic substances among the known heat-stable toxins of microbial origin (Andersson et al., 2007) and has caused even fatalities in human (Dierick et al., 2005; Mahler et al., 1997). A single food may contain several different cereulide producing strains (Pirhonen et al., 2005). The amount of heat-stable *B. cereus* toxin accumulating in foods or media under different growth conditions may be very different also when the cfu of emetic *B. cereus* is similar (Jääskeläinen et al., 2004; Rajkovic et al., 2006b; Shaheen et al., 2006; Szabo et al., 1991), indicating that synthesis of cereulide depends on environmental factors and/or strain properties. The assessment of health risk posed by cereulide thus requires knowledge on the actual toxin content of the food and factors affecting it, as recently pointed out by Arnesen et al., (2008).

To manage cereulide risk in food, understanding is needed on the factors that up or downregulate the cereulide production in food matrix and the interactions between the producer strains and the environment. As a contribution towards this direction, we applied different growth environments to study cereulide production by strains selected for diversity of origin and biochemical properties, and analysed the dependence on *ces* gene carried on plasmids.

2. Materials and methods

2.1. Strains and media

The origins of the strains are shown in Table 1. The media used were Tryptic soy agar (TSA, Scharlau Chemie S. A., Barcelona, Spain),

* Corresponding author. Tel.: +358 40 573 9049; fax: +358 9 19159322.
E-mail address: mirja.salkinoja-salonen@helsinki.fi (M. Salkinoja-Salonen).

0168-1605/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.ijfoodmicro.2008.06.006

Table 1
The origins of the *B. cereus* strains used in this study

Strains	Origin	Source	Reference
AND508	Reference for large plasmids and containing the128 kb plasmid pBtoxis and the 350 kb plasmid pXO16	L. Andrup, NRCWE[a]	Andrup et al. (1993) Hoton et al. (2005)
AND1284	Isolate from pasta, also known as strain 10329	L. Andrup, NRCWE	Hoton et al. (2005)
AND 1403	Positive control for the pCEREO1 plasmid (Kinrooi 5975b)	L. Andrup, NRCWE	Hoton et al. (2005)
AND1421	Kinrooi 5975c plasmid cured derivative	L. Andrup, NRCWE	Hoton et al. (2005)
ATCC 14579[T]	*B. cereus* type strain	ATCC[b]	
B 308	Risotto, food poisoning, Finland	T. Pirhonen, EVIRA[c]	Apetroaie et al. (2005)
F 4810/72	Cooked rice, food poisoning, UK	A. Christiansson, SDA[d]	Andersson et al. (1998)
IH 41385	Dialysis fluid	A. Siitonen, NPHI[e]	Ehling-Schulz et al. (2006)
LKT1/1	Filler material from moisture damage building, Finland	Own collection	Apetroaie et al. (2005)
LMG 17604	Outbreak of food borne illness from Chinese pancake, Belgium	INRA[f]	Guinebretière et al. (2002)
NC 7401	Patient with emetic food poisoning	N. Agata, Nagoya City Public Health Institute, Japan	Agata et al. (1994)
NS 58	Live Norway spruce, Finland	own collection	Hoornstra et al. (2006)
RIVM BC00067	Faeces from a food poisoning patient, NL	RIVM[g]	Apetroaie et al. (2005)
RIVM BC00068	Faeces from a food poisoning patient, NL	RIVM	Apetroaie et al. (2005)
RIVM BC00075	Faeces from a food poisoning patient, NL	RIVM	Apetroaie et al. (2005)
UB 1020	Faeces from a 3 year old child with abdominal pain and diarrhoea. No *Salmonella*, *Shigella*, *Yersinia* or *Campylobacter* was detected, Finland	Own collection	Apetroaie et al. (2005)

[a] NRCWE — National Research Centre for the Working Environment, Copenhagen, Denmark.
[b] ATCC — American Type Culture Collection.
[c] EVIRA — The Finnish Food Safety Authority, Helsinki, Finland.
[d] SDA — Swedish Dairies Association, Lund, Sweden.
[e] NPHI — National Public Health Institute, Helsinki, Finland.
[f] INRA — French National Institute for Agricultural Research, Avignon (Paris Cedex), France.
[g] RIVM — National Institute of Public Health and the Environment, Bilthoven, The Netherlands.

blood agar (TSA with 5 vol.% sheep blood agar, BioMérieux, SA 69280 Marcy l'Etoile, France), skim milk agar (Difco, Becton Dickinson and Company, Sparks, MD, USA and agar), raw milk agar (pasteurized raw milk, Ingman Ltd, Finland, and agar), oatmeal agar (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), and MacConkey agar (Oxoid Ltd, Basingstoke, Hampshire, England). For rice water agar, 100 g rice was boiled in 1 L of water for 25 min (white rice, Rainbow, Inex Partners Ltd, Finland), or 8 min (brown rice, Risella, Herba Ricemills S.L.U., Spain). The water was collected by suction and solidified with agar. All media contained 15 g agar L^{-1} and were sterilized 15 min at 121 °C.

The compositions of the commercial media were analysed as purchased powders. The raw milk and the blood agar plates were lyophilised and the rice was finely ground. Amino acids and alkali metals were analysed according to the protocol EU Dir 98/64/EC, Annex A (EU, 1999) and NMKL standard No 139/1991 (NMKL (Nordic Committee on Food Analysis), 1991) respectively (AnalyCen Nordic AB, Lindköping, Sweden).

The incubators (Certomat HK, and Memmert BE 500) had a precision of ±0.1 °C and the incubators and the room temperature were on-line monitored with Tinytag Data Logger (Gemini Data Loggers,West Sussex, UK) with an accuracy of ±0.2 °C.

2.2. Toxin analysis

Cereulide was measured from 48 h plate-grown biomass by the liquid chromatography–ion trap mass spectrometry (LC–MS) method using four cereulide specific molecular ions for quantitation, 1153.8 ($M+H^+$), 1171.0 ($M+NH_4^+$), 1176.0 ($M+Na^+$), and 1191.7 ($M+K^+$), as described by Jääskeläinen et al. (2003). The detection limit for cereulide was 0.2 ng mg^{-1} bacterial biomass wet weight. Toxicity of the extracts was tested by the rapid sperm microassay as described by Andersson et al. (2004).

2.3. Biochemical tests

Tyrosine decomposition, lecithinase and haemolytic activity were recorded as described (Apetroaie et al., 2005). Caseinase activity was read from plates with skim milk (Difco Becton Dickinson and

Table 2
Cereulide content of *B. cereus* biomass grown on different agar media for 48 h at room temperature (20–23 °C) and at 37 °C

Culture medium	NS 58	F 4810/72	NC 7401	LMG 17604	RIVM BC00067	RIVM BC00075	UB 1020
Cereulide content of the biomass of the strains grown at 20–23 °C (ng mg^{-1} fresh weight)							
Tryptic soy agar	560–750	310–920	170–570	200–520	14–130	43–76	160–510
Blood agar	520–530	110–260	150–340	120–330	170–200	270–380	150–470
Skim milk agar	32–92	15–78	95–130	47–78	16–18	9–14	110–180
Raw milk agar	120–170	97–110	130–140	60–130	30–31	32–36	150–160
MacConkey agar	110–110	19–110	45–90	120–220	18–19	37–110	68–180
Oatmeal agar	23–48	27–74	30–48	110–490	5–6	11–41	39–95
Cereulide content of the biomass of the strains grown at 37 °C (ng mg^{-1} fresh weight)							
Tryptic soy agar	84–96	70–77		120–190	3–18		
Blood agar	73–140	50–120		130–240	50–87		
Skim milk agar	2–11	1–2		4–6	2–5		
Raw milk agar	1–50	2–42		4–42	1–2		
MacConkey agar	3–15	89–92		2–6	0–3		
Oatmeal agar	200–250	130–130		110–110	23–120		
White rice water agar	1–2	1–2		2–3	1–2		
Brown rice water agar	40–100	60–107		4–20	2–3		

The average values obtained for biomasses from two independent cultivations are given. The S.D. between individual measurements from the each lot of biomass was ≤20%. Cereulide content was measured by the LC–MS method. The detection limit was 0.2 ng of cereulide mg^{-1} biomass fresh wt. All extracts were found toxic in the boar sperm microassay.