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Behavior of an Emetic *Bacillus cereus* Strain in Rice Food

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Abstract

Subject description: This article deals with the possible competitiveness of emetic *B. cereus* against non-emetics, likely to confer a development advantage to the first mentioned. The goal of this study was to evaluate the behavior of an emetic strain with respect to other *B. cereus* contaminants.

Method: A rifampicin-resistant mutant of the emetic strain k5975c was grown in Luria-Bertani. Broth overnight at 30°C, then inoculated in cooked rice or in naturally contaminated rice and incubated for 24 h at 23°C and 30°C. The emetic toxin and its toxicity were detected using the boar sperm motility inhibition bioassay and the liquid chromatography-ion trap mass spectrometry (LC-MS).

Results: With an inoculum level of ca. 6 log CFU g^{-1}, the emetic strain showed unhampered growth in rice contaminated by other *B. cereus* at both incubation temperatures, although it did not inhibit the growth of *B. cereus* contaminants. When the inoculum level of the emetic strain was reduced to ca. 3 log CFU g^{-1}, its development was unaffected when the background of resident *B. cereus* was below 5 log CFU g^{-1}. However, above this level of resident *B. cereus* strains, the emetic strain developed modestly at both 23°C and 30°C.

The presence of the emetic toxin activity was detected when the final concentration of the emetic strain reached ca. 5.6 log CFU g^{-1} and above this level.

Conclusion: Our study mimicked naturally occurring emetic food poisonings. It relates to the reported severe forms of rice food poisoning caused by emetic *B. cereus*.

Keywords *B. cereus* toxin; Emetic strains; Bacterial behavior; Food; LC-MS; Rice

Introduction

Bacillus cereus sensu stricto is a ubiquitous spore-forming Gram-positive microorganism. The capacity of this bacterium to grow in foodstuffs can cause serious problems to the food industry, not only as spoilage but also as the source of contaminations and/or intoxications. *B. cereus* is an important cause of foodborne disease worldwide [1-3], although it is probably under-reported in official lists of foodborne diseases.

This enteropathogenic bacterium is responsible for two types of foodborne illness in humans: diarrhea and emesis. The diarrheal syndromes are thought to be associated with three chromosomally encoded toxins: the Hemolysin BL (Hbl), the Nonhemolytic enterotoxin (Nhe) and/or the Cytotoxin K (CytK). The emetic syndrome is caused by the cereulide, a pH and heat stable cyclic peptide toxin [4,5]. The toxicity of cereulide has been tested extensively upon a variety of cell types, including rat liver and boar spermatozoa [6-8]. However, these studies were performed using enriched laboratory media and did not mimic naturally occurring emetic poisoning. Moreover, the number of *B. cereus* cells required to produce sufficient emetic toxin amounts to trigger disease is difficult to determine.

In most emetic food poisonings, the microbiology tests conducted on the incriminated food revealed a significant number of emetic bacteria. Levels of 103-109 CFU g^{-1} food have been found and, in most cases, at least 105 CFU g^{-1} food [9-11].

Among the food matrices associated with *B. cereus* emetic poisoning, rice is certainly one of the most important sources of contamination [12-14]. Not only is rice easily contaminated with soil-borne spores of *B. cereus* [15,16], but the way rice is prepared for human consumption can promote its survival, outgrowth and toxin production during vegetative growth [17-19]. Similarly, rice artificially inoculated with emetic strain was reported as the food containing the highest cereulide concentration [20].

In this work, because of its frequent involvement in emetic food poisoning, cooked rice was used to evaluate the behavior of an emetic strain with respect to other *B. cereus* contaminants.

Materials and Method

Preparation of bacterial inoculum

The cereulide-producing strain Kinrooi 5975c was isolated from the Belgian lethal food-poisoning outbreak case in 2003 [21]. A spontaneous rifampicin mutant (k5975cR) was used in the experimental setup to allow an easier recovery and discrimination from the resident B. cereus microflora. The strain was grown in Luria-Bertani broth (LB, Oxoid) overnight at 30℃. From appropriate dilution in peptone buffer water, PBW (3 g l^{-1} Peptone, 1.5 g l^{-1} NaCl, 1.5 g l^{-1} Na_2HPO_4, KH_2PO_4), 1 ml of culture was taken and used as inoculum for rice, providing inoculation levels of ca. 103 or ca.106 CFU g^{-1} depending on the experiment (Figure 1).

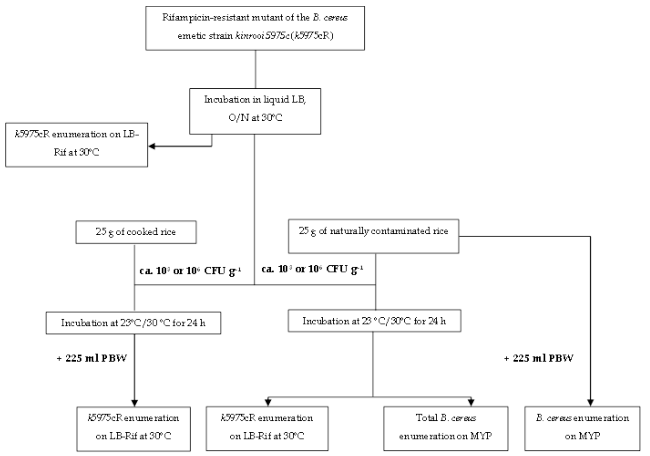

Figure 1: Experimental set-up to follow the growth behavior of the rifampicin-resistant *B. cereus* emetic strain k5975cR in cooked rice and in naturally contaminated rice. (A): Cooked rice was inoculated with 1 ml of k5975cR overnight culture to reach either ca. 103 or 106 CFU g^{-1} in rice and incubated at 23°C and 30°C for 24 h. After incubation, cooked rice was mixed with 225 ml BPW followed by an enumeration of k5975cR on LB-Rif plates. (B): 25 g of cooked rice left at room temperature during 48-72 h for natural contamination (reaching between 102 to 108 CFU ml^{-1}, see Table 1 were also inoculated with 1 ml of k5975cR culture to reach either 103 or 106 CFU g^{-1} in rice and incubated at 23°C and 30°C for 24 h. After incubation, rice was mixed with 225 ml PBW followed by enumerations of k5975cR on LB-Rif agar and total *B. cereus* on MYP. The original contamination of rice by resident *B. cereus* was also assessed on MYP after dilution in PBW.

Cooked rice and naturally contaminated rice

White rice was purchased from a supermarket at Louvain-la-Neuve (Belgium). 200 g of dry rice was added to 400 ml boiling tap water. The rice was boiled for 15 min in order to eliminate the vegetative bacterial background and then transferred into different bowls. One set of bowls was kept at low temperature (<4℃) to avoid germination of the *B. cereus* spores in rice and/or contamination by other bacteria. This boiled rice was used in the study as "cooked rice". The other set of bowls was incubated at room temperature for 24-72 h and used as "naturally contaminated" rice.

Bacterial inoculation and enumeration of cooked and naturally contaminated rice

25 g of cooked rice, or naturally contaminated rice, were weighed in stomacher bags 400 (Led Techno) and mixed with 1 ml of k5975cR culture to reach final concentration levels of ca. 103 or 106 CFU g-1, followed by incubation at 23°C and 30°C for 24 h. A negative control consisting in not inoculated rice sample was also added. Enumeration of the k5975cR emetic strain from the overnight culture was carried out in parallel (Figure 1) in order to determine its actual concentration.

After 24 h of incubation, 25 g of each rice samples (cooked or naturally contaminated) were added to 225 ml of sterile PBW in sterile stomacher bags. The samples were then dispersed by stomaching for 1 min at 230 rpm in a stomacher 400. A tenfold serial dilution was made, and from the appropriate dilution, 100 µl was spread plated onto *B. cereus* selective Mannitol-egg Yolk-Polymixin (MYP, BioRad) agar plates and on LB agar plates containing Rifampicin (LB-Rif). Following the incubation for 24 h at 30°C, CFU displaying a pink color with irregular edge surrounded by white area on the MYP medium were considered as positive (*B. cereus* sensu lato) and enumerated as described by Valero and coll [22].The emetic toxin was detected using the boar sperm motility inhibition bioassay and the liquid chromatography-ion trap mass spectrometry (LC-MS).

Boar sperm was supplied by the "Windey pig-breeding farm" (Pécrot, Belgium). The semen was stored at room temperature and used for the boar sperm bioassay [7,9] within the day of collection. Three grams of rice sample (cooked or naturally contaminated) were mixed with 6 ml of DMSO, in a 10 ml conical flask, placed in boiling water for 15 min and cooled. Ten µl of extract was applied into tubes containing boar semen. After 10 min of exposure at 37°C, the spermatozoid motility was estimated using a phase-contrast microscope with a heating stage at 37°C. Five microscopic fields with about 50 sperm cells in each were observed. The test was considered as positive when at least 90% exhibited loss of motility.

Liquid chromatography-mass spectrometry analysis

The extraction method described by Delbrassinne et al. was used [1]. Briefly, 3 g of the inoculated rice with k5975cR (either cooked or contaminated rice) was extracted with 6 ml of methanol and boiled for 15 min, followed by evaporation under N2 atmosphere. After redissolution of the residue in 3 ml of methanol and centrifugation, the supernatant was stored at -20℃ prior to analysis. Cereulide content of each extract was analyzed on a LCQ Deca-XP Plus ion trap mass analyzer using a modified LC-MS method inspired by Häggblom et al. [22,23]. A Symmetry C8 column was used for chromatographic separation using an isocratic method (mobile phase of 95% acetonitrile, 4.9% water, 0.1% trifluoroacetic acid). Valinomycin (Fluka, Germany) was used as external standard. For quantification of cereulide, the m/z values for adduct ions 1,170.5 (NH_4+ adduct) and 1,191.5 (K+ adduct) were monitored.

Results and Discussion

Growth behavior of the *B. cereus* emetic strain k5975cR in cooked rice

When inoculum levels of 5.6 to 6.5 log CFU per gram of rice were used, k5975cR was able to reach counts of more than 9 log CFU g^{-1} within 24 h after inoculation, at both incubation temperatures of 23°C

and 30°C, with cell numbers slightly higher at 30°C than at 23°C (Table 2, experiments 1 to 8). When the inoculum concentration was reduced by ca. 3 log CFU g^{-1} (Table 2, experiments 9 to 16), the final counts after 24 h were almost similar at 30°C, but displayed an average 10-fold reduction at 23°C. No significant growth of k5975cR was observed after 24 h at 15°C, independently of the inoculation level. Similarly, Kranzler et al. [24] showed that temperatures below 21°C led to emetic strains strongly decelerated growth, with long pre-exponential and exponential phases.

Tests	B. cereus background flora in rice b	Inoculum level of k5975cR in rice c	k5975cR count in rice 24 h after inoculation d		Total B. cereus in rice 24 h after inoculation e	
			23°C	30°C	23°C	30°C
1	2.0	6.1	8.8	9.5	9.2	9.6
2	2.3	5.6	8.9	9.5	9.0	9.6
3	4.2	6.5	9.4	9.7	9.7	9.8
4	5.4	6.3	8.8	9.5	9.1	9.5
5	5.5	6.4	9.5	9.8	9.6	9.8
6	7.5	6.5	8.6	8.8	8.7	9.1
7	7.5	5.9	7.5	8.3	8.8	9.0
8	8.2	6.1	8.4	8.9	8.5	9.2
9	4.1	3.2	8.2	9.0	8.1	9.3
10	4.5	3.1	7.7	8.5	8.5	9.8
11	4.6	3.4	8.6	9.3	8.7	9.3
12	5.0	3.3	8.5	9.0	8.3	9.3
13	6.4	3.0	4.2	5.0	8.5	8.6
14	8.2	3.2	4.8	5.8	8.8	9.2
15	8.3	3.4	4.5	4.4	7.2	8.7
16	8.5	3.3	4.1	5.4	8.9	9.2

Numbers refer to independent tests carried out; b *B. cereus* background flora in rice, sorted by increasing order; c k5975cR level used as inoculum in rice; d k5975cR counts in rice after 24 h of incubation at 23°C or 30°C. e Total *B. cereus* counts in rice 24 h after inoculation at 23°C or 30°C (see text for details)

Table 1: Rifampicin-resistant *B. cereus* emetic strain k5975cR behavior in naturally contaminated rice.

The toxicity of the rice was then assessed using the boar sperm bioassay [7]. All the samples tested turned out positive (loss of spermatozoa motility within 10 min of exposure), indicating the presence of cereulide after 24 h incubation in cooked rice, independently of the incubation temperature (23°C or 30°C). Indeed, in a previous study, cereulide was found in 7.4% of randomly collected rice dishes from restaurants. The prevalence increased to 12.9% in samples subjected to temperature abuse during the storage. Moreover, the cereulide concentrations found in samples were approximately 4 ng/g of food [25,26].

Bauer et al., [27] developed a stable isotope dilution analysis and quantified cereulide in cooked rice for 96 h at 24°C. The toxin started to be produced only after 24h and constantly increased up to 6.12 g/g after 96 h. Moreover, similar high amount of cereulide in food have been reported by Muratovic et al [28]. According to these authors, the speed of the process of producing cereulide at concentrations well above doses causing diseases in humans, easily achievable in very small quantities of food stored at room temperature.

Growth behavior of the *B. cereus* emetic strain k5975cR in contaminated rice

Despite the presence of background flora in naturally contaminated rice, including vegetative *B. cereus*, k5975cR showed an unhampered growth 24 h after inoculation at 30°C, when its inoculum level was ca. 6.2 log CFU g^{-1} (Table 1, experiments 1 to 8). Moreover, when the inoculums concentration of the emetic strain was higher than the background of resident *B. cereus*, most of the final *B. cereus* counts consisted of k5975cR. Only when the inoculation level of k5975cR was lower than the resident *B. cereus* (7.5 log CFU g^{-1}) was the final proportion of k5975cR slightly lower (Table 1, experiments 6 to 8). The same contaminated rice samples were incubated in parallel at 23°C. In these conditions, the final total *B. cereus* counts were slightly lower than at 30°C, and the proportion of the emetic strain was slightly more variable than at 30°C. As a corollary, these observations also indicated that the k5975cR emetic strain did not significantly inhibit the growth of the *B. cereus* background flora. Although the cereulide seems to act as an effector of ecological competition against a range of gram-positive bacteria and certain fungi, according to some studies [29-31].

Cereulide production in rice

In order to more accurately assess the cereulide produced in the different growth conditions, the toxin was detected and estimated using the boar sperm motility inhibition bioassay and LC-MS. A good correlation between cereulide production and cereulide activity was noticed. As shown in Table 3, the highest concentration of cereulide in the cooked rice was observed at 23°C, when the primary inoculum was reached 6 log CFU g^{-1}. For lower inoculum, the amount of cereulide per gram was greater at 30°C than at 23°C. Pre-contamination of the rice did not drastically influence the final cereulide concentration in the case of an inoculum of 6 log of the k5975cR emetic strain.

The resident microflora, however, interferes with both the growth and cereulide production of k5975cR when lower inocula of the emetic strain were used (Table 3).

Tests a	Inoculum level of k5975cR in rice b	k5975cR count in rice, 24 h after inoculation c	
		23°C	30°C
1	5.6	9.0	9.3
2	5.9	9.1	9.6
3	6.1	9.4	9.7
4	6.1	9.4	9.8
5	6.3	9.4	9.6
6	6.3	9.3	9.6
7	6.4	9.1	9.8
8	6.5	9.2	9.8

9	3.0	8.6	9.5
10	3.1	8.5	9.2
11	3.2	8.8	9.3
12	3.2	8.1	9.4
13	3.3	8.4	9.1
14	3.3	8.2	9.2
15	3.4	8.6	9.4
16	3.4	8.0	9.6

aNumbers refer to independent tests carried out; b k5975cR level used as inoculum in rice, sorted in increasing order. c k5975cR counts in rice after 24 h of incubation at 23°C or 30°C (see text for details). The bacterial concentrations are expressed as log CFU g^{-1} of rice

Table 2: Rifampicin-resistant *B. cereus* emetic strain k5975cR behavior in cooked rice.

	Inoculum level of k5975cR log CFU g^{-1} of rice	**Cereulide (ng of valinomycin equivalent/g of rice) and k5975cR counts (log CFU g^{-1} of rice) 24 h after inoculation**			
		23°C		**30°C**	
Cooked rice	3.0	146 a	8.7 b	569 a	9.2 b
	6.0	4685	9.4	1153	9.6
Contaminated rice	3.0	<1	4.5	ca.1-5	5.6
	6.0	4279	8.4	2035	8.5

Cereulide LC-MS analysis was performed on cooked and naturally contaminated rice, inoculated with either ca. 3 or 6 log CFU g^{-1} of the rifampicin-resistant cereulide-producing strain k5975cR. *B. cereus* background flora in naturally contaminated rice before k5975cR inoculation was estimated as 7.4 log CFU g^{-1}. The tests were performed at two different temperatures (23°C and 30°C). A Cereulide concentration, b k5975cR counts.

Table 3: Cereulide quantification by LC-MS.

Taken together, all these observations indicated that the k5975cR emetic strain can grow from 103 to more than 108 CFU g^{-1} and produced emetic toxin within 24 h, both in cooked and naturally contaminated rice, unless the resident *B. cereus* flora had already reached levels above 5 log CFU g^{-1}.

The amounts of cereulide produced reached toxin levels previously shown to induce emesis (0.01-1.6 µg cereulide g^{-1} food) in humans (28,32,33). Interestingly, there is no strict correlation between the amount of cereulide produced and the number of *B. cereus* CFU. This indicates that cautions should be taken in assessing the toxicity of a food product by enumerating its *B. cereus* content, as reported by another study [29]. Furthermore, the k5975cR did not clearly inhibit the growth of the *B. cereus* background flora. Our study mimicked naturally occurring emetic food poisonings. It relates to the reported severe forms of rice food poisoning caused by emetic *B. cereus*, involving hospitalization or even death [13,34]. Although the cereulide production was not quantified in the foodstuffs, the toxin was determined as the causative agent of the illness caused by rice foods prepared the day before and left at room temperature [35-39].

Conclusion

In conclusion, our study showed that emetic strains can grow and produce the cereulide in 24 hours without affecting the development of other contaminants. In addition, amounts of cereulide at concentrations much higher than the doses causing disease in humans is easily reached in rice stored at room temperature. Furthermore, the inhibitory effect of the emetic strain on other *B. cereus* could not be demonstrated. Our study mimicked naturally occurring emetic food poisonings. It relates to the reported severe forms of rice food poisoning caused by emetic *B. cereus*.

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