Toxin Production by *Bacillus cereus* Dairy Isolates in Milk at Low Temperatures

ANDERS CHRISTIANSSON,† A. SATYANARAYAN NAIDU,‡ INGRID NILSSON,§
TORKEL WADSTRÖM,∥ AND HANS-Erik PETTERSSON¶

Swedish Dairies' Association, Box 205, S-201 22 Malmö; Department of Medical Microbiology, Malmö General Hospital, University of Lund, S-214 01 Malmö; and Department of Medical Microbiology, University of Lund, S-223 62 Lund, Sweden

Received 6 February 1989/Accepted 1 June 1989

A total of 136 strains of *Bacillus cereus* isolated from milk and cream were evaluated for toxin production based on HeLa S3, Vero, and human embryonic lung (HEL) cell cytotoxicity in vitro. HEL cell monolayers were more susceptible than the other two cell lines. The percentage of isolates exhibiting HEL cytotoxicity was similar (43.0 and 48.4%) when the strains were grown in brain heart infusion broth containing 0.1% glucose (BHIG) at 7 and 24 h, respectively, at 30°C. In milk, only 21.8% of isolates showed HEL cytotoxicity at 7 h, and the number increased significantly to 73.2% at 24 h at 30°C. Further, 102 toxin-positive isolates were acclimatized to grow at 8°C in milk. Ninety-four (92.2%) of the strains produced HEL cytotoxicity of various degrees with no strict correlation to bacterial cell numbers and also elicited vascular permeability reaction in rabbit skin. Under aerated growth conditions (agitation, 200 rpm) *B. cereus* elicited cytotoxicity in BHIG and in milk at temperatures of 30, 15, and 8°C. However, in nonaerated (stagnant) cultures toxin production was diminished (BHIG) or completely lost (milk) at all temperatures. Toxin production at 8°C was evaluated in two different types of commercial cardboard milk packages by inoculation with a potent toxigenic dairy isolate. No detectable HEL cytotoxicity was observed in milk in any of the packages either at stagnant conditions or during mechanical shaking. However, the same strain produced cytotoxin in whipped cream at 8°C.

*B. cereus* is a widely distributed organism in the environment and undoubtedly the most important of the aerobic sporeforming species found in milk (1, 5). *B. cereus* is one of the organisms responsible for the spoilage of pasteurized milk and cream referred to as 'sweet curdling' (23) and 'bitty cream' (3). *B. cereus* is generally the organism that sets the limits for the keeping quality of Swedish consumer milk, particularly during the summer season. Despite low initial numbers of *B. cereus* in raw milk, the legal limit of 10³/ml on the day labeled “best before” is often easily reached due to the presence of psychrotrophic strains that grow rapidly at 8°C. This temperature is the highest legally permitted for the storage of milk, and 10⁴ *B. cereus* cells per ml is the ultimate limit of expiry. Thus, from public health and economic points of view, the monitoring and control of *B. cereus* are of high importance for the dairy industry.

*B. cereus* has been implicated in two distinct forms of food-borne illnesses: a delayed-onset (8 to 16 h) diarrheal syndrome characterized by symptoms of enterotoxiosis, abdominal cramps, and rectal tenesmus, usually resolving within 24 h, and a rapid-onset (1 to 5 h) emetic syndrome with nausea, vomiting, and occasionally diarrhea (8–10, 19, 20). Two different toxins were recognized with these illnesses. First is an unstable enterotoxin (30) with a molecular weight of approximately 50,000 and a pI of 4.9 (34). More recent studies have indicated that this toxin consists of several subunits slightly differing in molecular weights and pI (13, 32). Second, a heat-tolerant enterotoxin with a molecular weight of <5,000 has also been identified (18, 34). The pathologic role, if any, of cereolysin, protease(s), and phospholipase C complex in *B. cereus* is still not known (13).

Laboratory conditions for optimum production of *B. cereus* toxins have been defined (6). Toxin production is strongly dependent on culture medium and bacterial growth conditions (6, 29, 31). The production of emetic toxin seems to occur during sporulation, but the relation between sporulation and enterotoxin production is unclear (33).

Sporeforming and psychrotrophic properties enable *B. cereus* to survive pasteurization as well as to grow in milk at refrigerated storage conditions (5). Despite a high worldwide occurrence of *B. cereus* in milk, surprisingly few reports on food poisoning have been reported (11, 14, 17, 33, 36). The toxin-producing ability of *B. cereus* in milk under different dairy processing and storage conditions is not known. Therefore, we have investigated for the production of cytotoxins, hemolysins, and amylases by strains of *B. cereus* grown in milk under different conditions. Selected psychrotrophic strains were further tested for toxin production by vascular permeability reaction (VPR) tests.

MATERIALS AND METHODS

Bacterial strains. Samples of milk and cream stored at 8°C for 6 to 7 days were analyzed for the presence of *B. cereus*. Strains showing typical *B. cereus* colony morphology and hemolysis on agar plates containing 5% bovine blood and 0.001% polymyxin B sulfate (Sigma Chemical Co., St. Louis, Mo.) were isolated. Species identity was confirmed by mannnitol fermentation test and egg yolk reaction on mannnitol egg yolk phenol red agar (21) and by microscopic examination. Selected strains were further confirmed by API 50CH and API 20E (15). Finally, a total of 136 isolates were identified as *B. cereus*. These strains originated from milk and cream produced at 37 different Swedish dairy plants. Laboratory reference strains for enterotoxin production

* Corresponding author.
(443373, 4581B76, 409673, ATCC-14579, and NCDO-1771) and a reference strain for emetic toxin production (4810/72) were also included in this study. The origin of the reference strains is described elsewhere (18, 34, 35).

Chemicals and media. Blood agar base no. 2 was from Oxoid (London, England). Brain heart infusion (BHI) broth and Bacto-Peptone were purchased from Difco. Sodium caseinate was purchased from U.S. Biochemical Corporation, Cleveland, Ohio. BHI supplemented with 0.1% glucose (pH 8.0) (BHI C) was prepared according to Glatz and Goepfert (6). Milk (9.4% dry matter) was reconstituted from skim milk powder obtained from Scandmilk AB, Stockholm, Sweden, and was sterilized at 110°C for 15 min. Tissue cell culture medium was prepared using minimal essential medium with 10% fetal calf serum and was supplemented with 1% l-glutamine and 0.1% gentamicin, all from Flow Laboratories, Herts, England. All chemicals used in the preparation of buffer solutions were of analytical grade.

Media and growth conditions. Strains were grown static in skim milk overnight at 30 and 15°C or for 7 days at 8°C. A 1% inoculum of this culture was added to 10 to 15 ml of sterile skim milk in 150-ml Erlenmeyer flasks and cultivated at 30, 15, and 8°C with an agitation of 200 rpm. After centrifugation at 17,000 rpm for 15 min, the cell-free liquid between the fat (top phase) and casein-containing bacterial cell mass (bottom phase) was aspirated aseptically and tested for the presence of toxin. Toxin production was also tested in BHIG according to Glatz and Goepfert (6). In both culture conditions, cell-free supernatants were collected at different time intervals ranging from 5 to 96 h of growth and tested.

B. cereus BC-131 was further tested for toxin production in pasteurized milk in two different types of commercial cardboard packages (1-liter Purepak and 1-liter Tetra Brik). The strain was grown at 8°C in sterile skim milk as described above, and an inoculum giving a final concentration of 4 × 10⁴ to 6 × 10⁴ cells per ml was injected into the milk packages. The packages were stored at 8°C under three different conditions: (i) sealed and stagnant, (ii) sealed with shaking at 300 rpm, and (iii) opened (half emptied) and stagnant. Toxin production by the same strain was also tested in whipped cream at 8°C and in skim milk with aeration at the same temperature.

Hemolytic activity. The hemolytic activity of 50 B. cereus BHIG culture supernatants were tested by well diffusion technique on blood agar plates containing human, sheep, and rabbit washed erythrocytes. Based on the zone size, hemolysis results were scored as ++ +, + +, +, + + +, and negative.

Starch and salcin hydrolysis. The ability of B. cereus strains to hydrolyze starch was tested on nutrient agar plates containing 0.95% soluble starch. After incubation at 30°C for 24 h, plates were flooded with 3% Lugol’s iodine solution. The test was considered positive when a clear zone appeared around the bacterial growth (24). Limited hydrolysis beneath the bacterial growth was scored as negative. The salcin fermentation test was performed on a basal ammonium salt medium containing yeast extract and 0.5% salcin with bromothymol blue as a pH indicator (24). Upon acid fermentation, yellow color was developed within 7 days at 30°C.

Cytotoxicity. The cytotoxicity of B. cereus culture supernatants from skim milk and BHIG was examined on HeLa S3, Vero, and human embryonic lung (HEL; also known as MRC-5) cell monolayers. Supernatants were diluted (twofold from 1:5 to 1:640) in incomplete tissue cell culture medium, and 100 µl of each dilution in duplicate was added to microtiter wells. Each well contained 1.4 × 10⁴ HeLa, 3.8 × 10⁴ Vero, or 1.5 × 10⁵ HEL cells. Microtiter plates containing HeLa, Vero, or HEL cells were placed in a carbon dioxide incubator (7% CO₂) at 37°C for 12 or 48 h. Plates were washed with 0.7 M phosphate-buffered saline (pH 7.2). HeLa cells were fixed with 2% Formalin for 1 min, and Vero and HEL cells were fixed with methanol for 10 min. Cells were stained with 0.13% crystal violet (Merck, Darmstadt, Federal Republic of Germany) solution for 20 min. After three washings with phosphate-buffered saline, the stained cells retained in the wells were dissolved in 200 µl of 1% sodium dodecyl sulfate solution. The absorbance was measured on a Titertek Multiscan (Flow Laboratories, Svenska AB, Solna, Sweden) at 595 nm. Sterile BHIG broth and skim milk were used as negative controls.

VPR. The vascular permeability reaction (VPR) test was performed according to Glatz et al. (7). Skim milk and BHIG cell-free culture supernatants (0.1 ml) were injected intradermally into rabbits weighing 2.5 to 3.0 kg. After 3 h, 4 ml of 2% Evans blue dye solution was injected intravenously. Measurements of perpendicular diameters of the zones of light and dark blue, and necrosis when present, were made after 1 h. Total reaction was calculated as the sum of the mean radii of the blue zones.

RESULTS

Hemolytic pattern and carbohydrate utilization. Fifty B. cereus dairy isolates were grown in BHIG according to standard procedures, and the supernatants were tested for hemolytic activity on human, sheep, and rabbit erythrocytes (Table 1). Hemolysin activity was found among 22 to 28% of the isolates. Human erythrocytes were more susceptible, with stronger lytic reactions than to sheep and rabbit. Hemolysin production showed no strict correlation with cell monolayer toxicity assays. In carbohydrate utilization tests, B. cereus reference strain F-4810/72, positive for emetic toxin, was unable to hydrolyze starch or ferment salcin; whereas the diarrheagenic reference strains were able to utilize these compounds. Of 136 dairy isolates tested, only 7 (5.1%) did not hydrolyze starch, whereas 39 (28.7%) did not ferment salcin.

Screening of toxin-producing strains of B. cereus isolated from milk. A total of 136 B. cereus dairy isolates and six reference strains (positive for toxin production) were initially screened for toxin production under optimal culture conditions as described in the literature (i.e., growth in BHIG at 30°C with aeration and a harvest time of 5 h). B. cereus NCDO-1771 produced high levels of toxin (HeLa

---

<table>
<thead>
<tr>
<th>TABLE 1. Hemolysin production and carbohydrate utilization by strains of B. cereus isolated from milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Determination</td>
</tr>
<tr>
<td>--------------------------------------------------------</td>
</tr>
<tr>
<td>Hemolysis</td>
</tr>
<tr>
<td>Human erythrocytes</td>
</tr>
<tr>
<td>Sheep erythrocytes</td>
</tr>
<tr>
<td>Rabbit erythrocytes</td>
</tr>
<tr>
<td>Carbohydrate utilization</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
</tr>
<tr>
<td>Salcin fermentation</td>
</tr>
</tbody>
</table>

---

* Cell-free culture supernatants of B. cereus grown in BHIG at 30°C for 5 h were tested for hemolytic activity on washed erythrocytes obtained from different sources.  
* Ability of the test isolate to hydrolyze soluble starch incorporated in agar medium at 30°C after 24 h.  
* Results were scored after 7 days of incubation at 30°C.

---

Downloaded from http://aem.asm.org/ on July 9, 2017 by guest http://aem.asm.org/
cells when grown in milk. In contrast, B. cereus strains showed no differences in cytotoxicity production when grown in BHIG for 7 or 24 h.

When grown in BHIG, enterotoxin-positive laboratory reference strains showed different cytotoxicity profiles. Strain 4096/73 demonstrated HEL cytotoxicity only at 7 h, and strain 4433/73 showed a decreased cytotoxicity at a 24-h harvest time, while strains NCDO-1771 and ATCC-14579 showed higher HEL cytotoxicity at 24 h compared to a 7-h harvest time. However, when grown in milk all the reference strains demonstrated a high HEL cytotoxicity at both harvest times. On the other hand, 4810/72, a reference strain for emetic toxin, failed to produce any detectable cytotoxicity in BHIG; however, the same strain demonstrated a high HEL cytotoxicity in milk at both harvest times.

**Toxin production at low temperatures with and without aeration.** B. cereus reference strain NCDO-1771 and one dairy isolate, BC-50, were tested for toxin production at 15°C in milk and BHIG during a time course with aeration by mechanical shaking at 200 rpm. Strain NCDO-1771 demonstrated a moderate HeLa S3 and a high Vero cell cytotoxicity during late stationary growth phase (68 to 92 h) in milk and BHIG. The cytotoxicity pattern of the dairy isolate BC-50 was similar to that of NCDO-1771 when grown in milk; however, in BHIG medium the BC-50 strain showed a low Vero cell cytotoxicity at 92.5 h and no HeLa S3 cell cytotoxicity. When grown in 92.5 h BHIG medium without mechanical shaking (i.e., aeration), neither of the two strains produced any detectable levels of cytotoxins.

We have further investigated toxin production at low temperatures, i.e., at 8°C. Strains NCDO-1771 and BC-50 did not acclimatize to this temperature. One strain, BC-179, was selected and studied in detail. BC-179 was grown with and without aeration in BHIG or milk, and the cells reached stationary phase during 72 to 82 h of growth under both culture conditions. Cytotoxic activity in the aerated BHIG culture supernatant appeared during this time and reached a maximum during 72 to 96 h. In the absence of aeration, cytotoxicity titers were low during the early 72 h, with some increase at 120 h. When grown in milk, cytotoxicity titers were low during late logarithmic phase and gradually increased to a titer of 1:128 during 72 to 96 h. BC-179 demonstrated no detectable cytotoxicity in nonaerated milk (Table 3).

A total of 102 strains positive for extracellular cytotoxin production at 30°C in milk and BHIG at 7 and 24 h were further tested for cytotoxin production at 8°C in milk after 72 h (Table 4). Of the three cell lines tested for cytotoxicity, HEL seemed more susceptible (7.8% negativity compared to 24.5 and 31.4% negativities of Vero and HeLa S3 cytotoxicity). Thus, in the HEL cytotoxicity assay, 75 (73.5%) strains demonstrated a strong toxicity while 19 (18.6%) strains showed a low toxicity.

Toxin production in milk at 8°C (measured as HEL cytotoxicity) was further investigated in relation to the growth of B. cereus strains (Fig. 2). Slow-growing strains, i.e., <2 × 10^4 CFU/ml, demonstrated no HEL cytotoxicity. The cluster of toxin-producing strains showed values between 1 × 10^4 and 3 × 10^5 CFU/ml; however, the cytotoxicity titers widely varied between individual strains. Four of the dairy isolates (HEL cytotoxicity positive at 30°C), though acclimatized to grow in milk at 8°C with CFU values similar to those of toxigenic strains, failed to produce HEL cytotoxicity.

The nonaerated culture supernatants at a 72-h harvest time at 8°C had a pH of approximately 6.6, whereas the aerated

---

**TABLE 2. pH-dependent Vero cell cytotoxicity of B. cereus BHIG culture supernatants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Harvest time (h)</th>
<th>% Cytotoxicity at pH:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.5</td>
<td>7.0</td>
</tr>
<tr>
<td>BC-50</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>NCDO-1771</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

* Cytotoxicity was determined in microtiter plates as described in Materials and Methods. Each well contained 1.4 × 10^5 Vero cells in a monolayer. Percent cytotoxicity was calculated at 1:20 dilution of the supernatant. Complete lysis of the cell monolayer (at 1:20) was considered as 100% cytotoxicity, and the additional percentage at the next further dilution (i.e., 1:40) was summed up.

S3/Vero cell cytotoxicity titer, 1:80, indicating that this procedure was suitable for the reference strains. But when the same parameters were applied to the dairy isolates, only a low proportion (5/136; 3.7%) demonstrated HeLa S3 and Vero cell cytotoxicity. However, one dairy isolate, BC-50, produced cytotoxin at 7 h (Table 2), and thus the harvest time of 5 h did not seem suitable for the dairy isolates. Based upon this observation, further toxicity experiments with the dairy isolates were performed with harvest time at 7 h. Screening was repeated for toxin production, and 51 isolates (37.8%) showed HeLa S3, 50 (37.0%) showed Vero, and 58 (43.0%) showed HEL cell cytotoxicity at a 7-h growth phase.

**Toxin production in milk and BHIG at 7 and 24 h.** We have further investigated the ability of dairy isolates to produce toxin in milk during a time course (Fig. 1). Most of the strains positive for toxin production in BHIG were unable to produce detectable amounts of toxin in milk during the log phase of growth (i.e., at 7 h). The inoculum and the multiplication rate in BHIG and milk were similar. However, during the late stationary growth phase (i.e., 24 h), more than 50% of the strains demonstrated HeLa and Vero cell cytotoxicity, while 73.2% showed cytotoxicity against HEL

---

**FIG. 1.** Cytotoxin production by B. cereus dairy isolates in milk or BHIG during time course. A total of 136 dairy isolates were grown at 30°C in milk or BHIG, and cell-free supernatants were collected at 5, 7, or 24 h and tested for in vitro cytotoxicity on HeLa S3, Vero, and HEL (MRC-5) cell monolayers as described in Materials and Methods. Higher number of isolates produced toxins in milk than in BHIG, and the HEL cell line was found more susceptible to the extracellular cytotoxic factors.
culture supernatants demonstrated an increased pH of 7.4. The effect of pH on cytotoxin production in milk at 8°C was tested by growing strains BC-41, BC-65, and BC-131 in aerated or nonaerated conditions at pH 6.6 or 7.4. Cytotoxin production (titers) was similar at pH 6.6 and 7.4 for all three strains during aerated growth conditions. Stagnant growth conditions supported no cytotoxin production at either of the pH conditions. Sterile skim milk adjusted to pH 7.4 or 6.6 showed no cytotoxicity in a control experiment.

The ability of strain BC-131 to multiply and produce toxins under storage conditions (8°C) in cardboard packages containing pasteurized milk (3% fat content) and also in whipped cream (40% fat content) was examined (Table 5). Interestingly, no detectable cytotoxins were produced in pasteurized-milk packages although the cell numbers reached up to $1 \times 10^7$ cells per ml. The same strain, however, when grown in parallel in skim milk under aerated laboratory conditions at 8°C, demonstrated a high degree of HEL cytotoxicity (1/320 titer). The cytotoxin production was similar in pasteurized milk under aerated conditions. Cells multiplied more rapidly in whipped cream compared to pasteurized-milk packages at 8°C and reached $1.3 \times 10^8$ cells per g. In whipped cream the HEL cytotoxicity titer was 1/5 during day 3 and reached 1/40 by day 4. The cream was clearly unacceptable organoleptically at this stage.

**VPR with cytotoxins produced in milk at 8°C.** Dairy isolates positive for cytotoxicity in milk at 8°C under aerated conditions also showed a positive VPR. The reaction was always an intense bluing without any visible necrotic zone. The un aerated culture supernatants were negative both in cytotoxicity assays and in the VPR test.

### TABLE 4. Production of extracellular cytotoxins by *B. cereus* grown at 8°C in milk

<table>
<thead>
<tr>
<th>Cell monolayer</th>
<th>Positive</th>
<th>Weak</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>31 (12.7)</td>
<td>39 (38.2)</td>
<td>32 (31.4)</td>
</tr>
<tr>
<td>Vero</td>
<td>63 (61.8)</td>
<td>14 (13.7)</td>
<td>25 (24.5)</td>
</tr>
<tr>
<td>HEL</td>
<td>75 (73.5)</td>
<td>19 (18.6)</td>
<td>8 (7.8)</td>
</tr>
</tbody>
</table>

* A total of 102 strains positive for extracellular toxins at 30°C in milk were tested. The strains were acclimatized to 8°C before testing for cytotoxin production. All strains were inoculated at 1% inoculum in milk and incubated with constant shaking (200 rpm) for 72 h.

**DISCUSSION**

Milk and milk products serve as potential reservoirs for many bacterial pathogens. The role of staphylococci, *Listeria monocytogenes*, and *Salmonella* spp. in milk-borne infections and intoxications has been elucidated (16, 22, 26). These organisms are killed by heat treatment and generally are postpasteurization contaminants. The combined abilities of the spores to survive pasteurization and of certain strains to multiply at low temperatures make *B. cereus* unique compared to other milk-borne pathogens.

Cytotoxicity tests on various cell lines were the primary criterion for toxin production in the present study. Enterotoxins positive in VPR or ligated rabbit ileal loop tests or both also demonstrate in vitro cell cytotoxicity (13, 32). Kawano et al. have also suggested a quantitative correlation between VPR and cytotoxicity (12). In a previous study, Chopra et al. reported that 6 of 25 milk isolates of *B. cereus* were pathogenic in the rabbit ligated ileal loop test (4).

Hemolysin production in *B. cereus* did not correlate with cytotoxicity. Arribas et al. showed no strict relation between hemolysin titers and VPR (2). It has been proposed that *B. cereus* strains producing emetic toxin are unable to hydrolyze starch or ferment salicin (25, 28). Shinagawa et al. reported that all *B. cereus* strains associated with 36 food poisoning (emetic type) outbreaks were unable to hydrolyze starch (28). Dairy isolates belonging to this category were very few in the present study.

*B. cereus* enterotoxin is an unstable protein and susceptible to proteases (30, 34). Several workers have reported a decline in both toxin production and activity between early and late stationary phase (6, 7, 27, 31). However, for certain strains biologically active toxins were found at late stationary growth phase (35), where proteases also appear. Therefore, we have investigated toxin production in BHIG at 24 h. The frequency of toxin production among dairy isolates was similar in BHIG at both 7 and 24 h of growth at 30°C.

Since milk is a protein-rich medium stimulating protease production and is the natural source of the isolates in this study, the ability of *B. cereus* to produce toxin in milk was studied. The frequency of strains producing toxins at 7 h and 30°C in milk was significantly lower than that in BHIG; however, the reverse situation was observed at 24 h. All strains producing cytotoxins in BHIG at 24 h are toxin producers in milk; moreover, an additional number of strains produced cytotoxins in milk. This preliminary observation may suggest a milk-induced stimulatory effect on cytotoxin production.

Toxin production by *B. cereus* at low temperatures has not been reported. Though 30°C has been shown to be optimum, enterotoxin production (10-fold decrease) has been demonstrated at 20°C in BHIG (6). The ability of *B. cereus* to produce toxins under Swedish milk-storage conditions, i.e., 8°C, was further investigated. A majority of the dairy isolates produced HEL cytotoxicity to various degrees. Our data suggest that the cell number does not strictly correlate to the amount of toxin production. Cytotoxicity-positive strains also elicited VPR in rabbit skin. Cytotoxicity and VPR positivity have been suggested to correlate with entero-toxicity (7, 12), and therefore the possibility of enterotoxin production by *B. cereus* at 8°C cannot be excluded.

Cytotoxicity was observed in aerated growth conditions, whereas in nonaerated growth conditions in milk (stagnant cultivation) dairy isolates failed to produce any detectable cytotoxicity. The low incidence of *B. cereus* milk-borne food poisonings may possibly be due to inadequate oxygen supply.
FIG. 2. Relation between bacterial cell number and toxin titer of *B. cereus* dairy isolates grown in milk at 8°C. A total of 102 strains were grown in milk at 8°C according to conditions described in Materials and Methods. After 72 h, bacterial cell numbers were determined by plate counts and the cell-free supernatants were tested for HEL cytotoxicity at different dilutions. Strains produced no detectable cytotoxicity when growth was below $2 \times 10^7$ CFU/ml. Variable degree of cytotoxicity was observed among strains showing cell counts between $1 \times 10^8$ and $2.5 \times 10^8$ CFU/ml.

in milk packages. This hypothesis was evaluated by tests for toxin production in milk packages with a potent toxigenic dairy isolate, BC-131. Despite a high inoculum, BC-131 failed to produce any cytotoxicity in shaken and stagnant milk packages. On the other hand, BC-131 produced a sizeable cytotoxicity in whipped cream. Therefore, aerated conditions allowing oxygen transfer seem important for toxin production in a milk environment as for BHIG, as suggested by Glatz et al. (7).

In conclusion, we have shown that the incidence of toxigenic *B. cereus* is high in milk and cream. Milk is a suitable medium for *B. cereus* to produce toxins even at 8°C. However, these toxins were only produced in strongly aerated conditions. The very low incidence of milk-borne food poisonings caused by *B. cereus* is probably due to insufficient aeration for toxin production under normal storage conditions. The toxin production by *B. cereus* at 8°C reported in this study raises questions on such implications in other well-aerated refrigerated foods. However, it remains to be established whether the toxins produced at 8°C are "true" enterotoxins.

TABLE 5. Growth and toxin production by *B. cereus* BC-131 at 8°C in commercial cardboard packages and in whipped creama

<table>
<thead>
<tr>
<th>Condition</th>
<th>Growth and toxin titera at time:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Package, sealed/</td>
<td>$4 \times 10^4$</td>
</tr>
<tr>
<td>stagnant</td>
<td></td>
</tr>
<tr>
<td>Package, sealed/</td>
<td>$6 \times 10^4$</td>
</tr>
<tr>
<td>shakenc</td>
<td></td>
</tr>
<tr>
<td>Package, open/</td>
<td>$6 \times 10^4$</td>
</tr>
<tr>
<td>stagnant</td>
<td></td>
</tr>
<tr>
<td>Skim milk, aerated</td>
<td>$2 \times 10^6$</td>
</tr>
<tr>
<td>Whipped creamd</td>
<td>$5 \times 10^4$</td>
</tr>
</tbody>
</table>

a *B. cereus* BC-131 was grown in skim milk at 8°C for 7 days. A 0.3% inoculum was injected into the packages with a sterile syringe.

b HEL cytotoxicity titer, standard toxin assay conditions.

c Mechanical shaking at 300 rpm.

d Inoculum was added to the cream before whipping.

ACKNOWLEDGMENTS

We thank Rangne Fondén for critical reading of the manuscript; Irena Öllermark, Kerstin Ekelund, and Lena Jönsson for excellent technical assistance; and Ingvar Frick for photography.

LITERATURE CITED