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# Toxinogenic *Bacillus pumilus* and *Bacillus licheniformis* from mastitic milk

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**Abstract**

To elucidate the occurrence of heat-stable toxin-producing strains among mastitic *Bacillus* isolates, 100 milk samples of mastitic cows from different parts of Finland were screened. *Bacillus* was identified as the major organism in 23 samples. Toxinogenic *Bacillus* isolates identified by sperm cell motility inhibition assay were isolated from six samples. Four isolates belonged to the species *Bacillus pumilus* and two to *Bacillus licheniformis*. The toxic substances were heat-stable and soluble to methanol thus being of non-protein nature. The methanol extracted substances disrupted the sperm cell plasma membrane permeability barrier at exposure concentrations of 1–15 μg $ml^{-1}$ (*B. pumilus*) or 20–30 μg $ml^{-1}$ (*B. licheniformis*). The toxic properties of the two mastitic *B. licheniformis* strains were similar to those of *B. licheniformis* strains known to produce the lipopeptide lichenysin A and the synthetase genes *lchAA*, *lchAB* and *lchAC* for lichenysin were found in the mastitic strains by PCR. Toxin synthetase genes for the syntheses of lichenysin or surfactin were searched but not found in the toxic *B. pumilus* strains. The ribopatterns of the mastitic *B. pumilus* and *B. licheniformis* isolates were similar to those of the toxinogenic strains described earlier from food poisoning incidents and contaminated indoor air. *B. licheniformis* and *B. pumilus* survive pasteurization and other heat treatments as spores. Toxin-producing strains of these species in the dairy production chain may thus be of food safety concern.


*Keywords:* *Bacillus pumilus*; *Bacillus licheniformis*; Mastitis; PCR; Toxin; Cereulide; Lichenysin; Surfactin

## 1. Introduction

*Bacillus cereus* is known to cause bovine mastitis, although at lower incidence level than the major udder pathogens *Staphylococcus* spp., *Streptococcus* spp. and *Escherichia coli* (Radostits et al., 2000). *Bacillus* spp.

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have also been found among normal flora of the teat skin (Woodward et al., 1988). *Bacillus licheniformis* has been connected to bovine abortion (Agerholm et al., 1995).

Some *Bacillus* species, connected to food poisoning cases, have been shown to produce heat-stable toxins, e.g. the emetic toxin, cereulide, of *B. cereus* and *Bacillus weihenstephanensis*. Members of the species *Bacillus amyloliquefaciens*, *B. licheniformis* and *Bacillus pumilus* of food poisoning origin have also been shown to produce substances toxic to mammalian cells (Mikkola et al., 2000; Salkinoja-Salonen et al., 1999; Suominen et al., 2001; Mikkola et al., 2004). Endospore-forming *Bacillus* strains producing heat-stable toxins introduce a potential safety risk to dairy products because both the endospores and the toxins can survive current dairy processes like pasteurization (typically 74 °C 15–20 s) and whey evaporation at 50–70 °C.

*Bacillus* strains producing heat-stable toxic substances have been isolated from the dairy production chain (Shaheen et al., 2006; Svensson et al., 2006) but their origin is usually unknown. One source of toxinogenic heat-resistant endospore-forming bacteria in raw milk may be cows with history of mastitis. *Bacillus* strains originating from milk of cows suffering from clinical mastitis should not occur in dairies as mastitic milk is not normally sent to dairies. However, toxinogenic *B. licheniformis* has been isolated from the udder of a clinically healthy cow recovered from mastitis (Salkinoja-Salonen et al., 1999).

In order to elucidate the occurrence of heat-stable toxin-producing strains among mastitic *Bacillus*, we screened 100 mastitic milk samples, isolated 23 *Bacillus* strains and tested these for the presence of heat-stable toxins using the boar sperm motility test previously shown useful for detecting the heat-stable toxin cereulide of *B. cereus* (Andersson et al., 2004, 2007). Biological activity of the detected heat-stable toxins was characterized and the strains were screened for known peptide toxin synthetase genes using PCR.

## 2. Materials and methods

### 2.1. Bacillus strains used in this study

The mastitic *Bacillus* strains used in this study were isolated from milk of 23 mastitic milking cows by Valio Ltd.'s regional laboratories in different parts of Finland in the year 2000.

The origins and the properties of the cereulide-producing strains F4810/72, NC 7401, IH 41385 and of the non-producing strains ATCC 4342 and ATCC 14579$^T$ of *B. cereus* are described by Carlin et al. (2006) and Apetroaie et al. (2005). The toxicity and the origins of the *B. licheniformis* strains DSM 13$^T$, 553/1, Hulta 52/97 and F2896/95 were described by Salkinoja-Salonen et al. (1999) and lichenysin production by Mikkola et al. (2000). The strain Hulta 51/97 was isolated from the same subclinical mastitic cow as Hulta 52/97. *B. pumilus* DSM 27$^T$ and ES20 were described by Suominen et al. (2001). The surfactin producing (Hsieh et al., 2004) *B. subtilis* strains DSM 1970 and DSM 10 were from the German Collection of Micro-organisms and Cell cultures (DSMZ).

### 2.2. Identification methods

The whole cell fatty acid compositions of the isolates were analyzed as described by Suominen et al. (2001). Version 5.0 of the commercial library TSBA50 (MIDI Inc., Newark, DE, USA) was used for identification.

For ribotyping the isolates were grown for 12 h at 28 °C on Trypticase soy broth agar (Becton Dickinson, USA). The ribopatterns were generated using an automated Riboprinter (Qualicon, Du Pont, Wilmington, DE) as described by Suominen et al. (2001) using the restriction enzyme EcoRI.

DNA for the partial sequence analysis of the 16S rRNA gene was extracted from cells grown overnight in Trypticase soy broth. A 500-bp fragment from the 5′-end of the 16S rRNA gene was amplified using the Microseq kit and the ABI PRISM 310 genetic analyzer (PE Applied Biosystems, USA). The obtained sequences were edited by Sequencher$^{TM}$ 3.0 or Chromas$^{TM}$ 1.6 software and deposited in the EMBL database (accession numbers AM493713, AM493714, AM493715 and AM493716)

### 2.3. Toxicity assays

*B. cereus* enterotoxin production was tested using an enterotoxin-reversed passive latex agglutination kit (BCET-RPLA TD950, Oxoid Ltd., Basingstoke, UK)