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

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20 **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
25 species *B. pumilus* and two to *B. 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 to 15 $\mu\text{g ml}^{-1}$ (*B. pumilus*) or 20 to 30 $\mu\text{g ml}^{-1}$ (*B. licheniformis*). The toxic properties of the two mastitic *B. licheniformis* strains were similar to those of *B. licheniformis* strains known to produce the
30 lipopeptide lichenysin A and the synthetase genes *lchAA*, *lchAB* and *lchAC* for lichenysin were found in the mastitic strains 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.*
35 *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.

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Keywords: *Bacillus pumilus*; *Bacillus licheniformis*; mastitis; PCR; toxin; cereulide; lichenysin; surfactin

45 **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. 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).

50 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 *B. weihenstephanensis*. Members of the species *B. amyloliquefaciens*, *B. licheniformis* and *B. 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*
55 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 to 20 s) and whey evaporation at 50 to 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.

60 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).

65 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
70 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
75 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 *Bacillus 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-
80 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).

85

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.

90 For ribotyping the isolates were grown for 12 h at 28 °C on Trypticase soy broth agar (Becton Dickinson U.S.A). 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
95 amplified using the Microseq kit and the ABI PRISM 310 genetic analyzer (PE Applied Biosystems, USA). The obtained sequences were edited by Sequencher™ 3.0 or Chromas™ 1.6 software and deposited in the EMBL database (accession numbers AM493713, AM493714, AM493715 and AM493716)

100 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) according to the manufacturer's instructions. The boar spermatozoan mobility inhibition test was carried out as described by Suominen et al. (2001).

105 Functional stainings for the subcellular target identification of the toxins were executed with the fluorescent stains calcein-AM and propidium iodide for the plasma membrane permeability and with JC-1 for the mitochondrial depolarization as described by Suominen et al. (2001).

2.4 PCR detection and cloning of the peptide synthetase genes

110 DNA was extracted from liquid cultures of the target strains with DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions for Gram-positive bacteria. DNA was quantified

spectrophotometrically using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE USA).

The PCR primers used in this study are presented in Table 1. Dynazyme II DNA polymerase (Finnzymes, Espoo, Finland) in concentration of $0.02 \text{ U } \mu\text{l}^{-1}$ and $1 \times$ Dynazyme reaction buffer containing 1.5 mM MgCl_2 were used in all PCR reactions (total volume $50 \mu\text{l}$ per reaction). DNA template concentration in all PCR reactions was from 0.2 to $0.6 \text{ ng } \mu\text{l}^{-1}$. Surfactin synthetase gene targeted PCR was performed as described by Hsieh et al. (2004) using dNTP concentration of $250 \mu\text{M}$ and primer concentration of $0.4 \mu\text{M}$. For detecting the lichenysin synthetase genes, dNTP concentration of $200 \mu\text{M}$ and primer concentration of $1 \mu\text{M}$ were used with the following thermocycler program: initial denaturation at $94 \text{ }^\circ\text{C}$ for 2 min, 30 cycles of amplification ($94 \text{ }^\circ\text{C}$ for 15 s, annealing temperature for 30 s, $72 \text{ }^\circ\text{C}$ for 50 s) and final extension at $72 \text{ }^\circ\text{C}$ for 5 min. Annealing temperature for the primer pairs LicA1 and LicB2 was $60 \text{ }^\circ\text{C}$ and for the primer pair LicC $57 \text{ }^\circ\text{C}$. Cereulide synthetase gene targeted PCR was done using the primer pair EMI as described by Ehling-Schulz et al. (2004) and the primer pair CER1 / EMT1 as described by Horwood et al. (2004). PCR targeting DNA markers affiliated with cereulide synthesis was done using the primer pair BE as described by Toh et al. (2004) and the primer pair RE234 as described by Nakano et al. (2004).

16S rRNA gene targeted PCR as described by Leskelä et al. (2005) was used to screen the DNA extracts for the presence of PCR inhibitors. PCR amplicons obtained with the primer pair CER1/EMT were re-amplified using the same primer pair and cloned in *E. coli* using the TopoTA cloning kit (Invitrogen, Carlsbad CA, USA). The obtained sequences were deposited in the EMBL database (accession numbers AM493711 and AM493712)

135

3. Results

3.1 Heat-stable toxin production among mastitic *Bacillus* isolates

One hundred *Bacillus*-containing mastitic milk samples were collected from dairy farms in different parts of Finland. Twenty-three milk samples were found to contain *Bacillus* as the dominant bacterial genus. These were investigated for the presence of toxinogenic strains by the sperm motility inhibition assay and the BCET-RPLA enterotoxin test. Six isolates were positive in the sperm motility inhibition assay and three in the BCET-RPLA test. The isolates were characterized by whole cell fatty acid composition, partial sequencing of the 16S rRNA gene and by ribopattern analysis. The results showed that four of the toxic isolates belonged to the species *Bacillus pumilus* and two were *Bacillus licheniformis* (Table 2). The three BCET-RPLA positive isolates were *B. cereus*.

Results of the ribopattern analysis, where whole genomic DNA was restriction digested with *Eco*R1 and the resulting fragments hybridized with a DNA probe covering the complete rRNA operon, are shown in Figure 1. It shows that the sperm toxic isolates NR 5160 and NR 6768, tentatively identified as *B. licheniformis* based on the whole cell fatty acid composition, were highly similar and possibly identical in ribopatterns with the type strain DSM 13^T. They were also highly similar with the previously characterized *B. licheniformis* strains Hulta 52/97 isolated from mastitic milk and an infant food isolate 553/1 implicated in a fatal case of food poisoning (Salkinoja-Salonen et al., 1999). The isolates NR 5160 and NR 6768 were concluded to be *B. licheniformis*.

The ribopatterns of the toxinogenic putative *B. pumilus* isolates revealed (Fig. 1) that out of the seven to nine fragments obtained with *Eco*R1, covering a total length of c.a. 50 kb, four fragments were shared by the mastitic isolates. Differences between the strains occurred in the 3 to 4 fragments sizing from 8 to 16 kbp. Each of the four toxinogenic mastitic strains had an individually differing ribopattern, indicating the strains were not clonal. The isolate 19/5 had a ribopattern highly

160 similar with that of *B. pumilus* ES20, a toxinogenic strain originating from indoor air (Suominen et al. 2001).

The commercial whole cell fatty acid library also identified the four strains NR 19/5, NR 440, NR 13890 and NR 15703 as members of *B. pumilus*. The library indicated that the strains were affiliated to the GC subgroup B (other than type strain). This subgroup differs from the type strain
165 by containing more iso 15:0 (42 to 46%) and less 15:0 anteiso (26 to 29%) pentadecanoic acid than the type strain DSM 27^T (results not shown).

To confirm the identity of the toxinogenic strains as *B. pumilus*, 444 - 500 bp fragments of 16S rRNA gene (*E. coli* numbering 19 - 513) were sequenced for both strands. The obtained sequences were identical for the isolates NR 19/5, NR 440, NR 13890 and NR 15703. Comparison of the
170 strain NR 440 sequence with the other nucleotide sequences in the NCBI database using BLAST showed that only three nucleotides out of 500 were different from the *B. pumilus* type strain DSM 27^T. From the hundred sequences closest to those of the four mastitic *B. pumilus* strains all sequences possessing a species name (55 sequences) belonged to *B. pumilus* (Genbank). The strains NR 440, NR 19/5, NR 13890 and NR 15703 were thus confirmed to be *B. pumilus*.

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3.2 Extraction of toxic substances from the mastitic *Bacillus* isolates

To characterize the toxic heat-stable substances from the six mastitic *Bacillus* isolates, EC₅₀ values were measured for the substances extractable to boiling water or to methanol, respectively.
180 The EC₅₀ values for the mastitic isolates and the reference strains are shown in Table 3. The biomasses of the six mastitic isolates contained methanol soluble substances that inhibited sperm motility at exposure concentrations less than 0.03 mg ml⁻¹. The mastitic *B. pumilus* isolates had lower EC₅₀ values (1 to 15 µg ml⁻¹) than the *B. licheniformis* isolates (20 to 30 µg ml⁻¹). The hot water as well as the methanol extracts of the six mastitic isolates were more toxic than those

185 prepared from the *B. licheniformis* type strain DSM13^T but less toxic than those similarly prepared from the cereulide-producing reference strain *B. cereus* F4810/72. Toxicities of the mastitic isolates of *B. pumilus* were in the same order of magnitude as that of the type strain DSM 27^T (EC₅₀ 1 to 7 µg methanol soluble substance ml⁻¹).

190 3.3 Toxic effects provoked by the mastitic *Bacillus* extracts

The subcellular toxicity targets in the sperm cells provoked by exposure to boiled methanol extracts prepared from the mastitic *Bacillus* isolates were assessed by functional staining using calcein-AM and propidium-iodide. The sperm cells exposed to extracts prepared from the four *B. pumilus* isolates or from the type strain induced red fluorescence in sperm cells stained with
195 calcein-AM and propidium-iodide, whereas the vehicle-only exposed cells remained fluorescing green (Figure 2). This change occurred with exposure to 1 to 10 µg (dry wt) methanol soluble substance per ml, i.e. the same concentration at which the motility of boar sperm cells vanished (Table 3). A similar change from green to red occurred in sperm cells after an exposure to 30 µg ml⁻¹ of extracts prepared from the two *B. licheniformis* isolates. Since viable spermatozoa are
200 impermeable (and thus not stained by) propidium iodide, these results indicate damage to the permeability barrier of the spermatozoan plasma membrane by exposure to extracts of the mastitic *Bacillus* isolates.

To investigate if mitochondrial damage was involved, the sperm cells exposed to the mastitic *Bacillus* extracts were stained with JC-1. No change in the mitochondrial fluorescence was
205 observed with any of the mastitic *Bacillus* extracts when exposed to concentrations where the plasma membrane remained undamaged. Therefore the loss of sperm motility in response to exposure was more likely to be due to damaged plasma membranes than to mitochondrial damage.

210 3.4 Screening for known *Bacillus non-ribosomal peptide synthetase genes*

To assess the presence of known non-ribosomal peptide synthetase genes the mastitic *Bacillus* isolates were screened with PCR using the primers displayed in Table 1. Strains of *B. licheniformis*, *B. subtilis* and *B. cereus* known to produce lichenysin, surfactin or cereulide, respectively, were used as references. The results are shown in Table 4. Primers targeted to lichenysin synthetase genes *lchAA*, *lchAB* and *lchAC* yielded amplicons of the expected size from the seven tested *B. licheniformis* strains, including the toxic mastitic isolates NR5160, NR6768, but from none of the five tested *B. pumilus* strains. The possibility of false negatives results due to PCR inhibitors was excluded by amplifying 16S rRNA gene of each strain from the same DNA preparation.

With primers targeted to the surfactin synthetase gene, correctly-sized amplicons were obtained from the two positive reference strains of *B. subtilis* but none from the mastitic isolates. The PCR primer pairs targeted for detecting cereulide synthetases *cesA* and *cesB* (Table 1) produced, as expected, amplicons from the two cereulide-producing reference strains and none from the cereulide-deficient strains of *B. cereus*. Surprisingly, amplicons of the correct size were obtained from *B. licheniformis* NR 5160 and *B. pumilus* NR 19/5 with the *cesA* targeted primer pair CER1/EMT1. The amplicon yields were lower, visible as lower band intensities in the ethidium bromide-stained agarose gel compared to those obtained from the cereulide-producing *B. cereus* strains (Figure 3). No amplicon was obtained with the *cesB* targeted primer pair EM1. The products obtained with the primer pair CER1/EMT1 were therefore cloned and sequenced. It was found that the complete sequences of the 146 bp (priming regions not included) amplicons obtained from the strains *B. licheniformis* NR 5106 and *B. pumilus* NR 19/5 were identical with that obtained from the cereulide-producing strain *B. cereus* IH41385. Comparison of the obtained sequence to the EMBL database showed 100 % similarity with the corresponding fragment in the known *B. cereus cesA* gene (EMBL accession number DQ360825). DNA from the isolates NR 5160 and NR 19/5

produced no amplicon with primer pairs RE234 and BE, targeted to the DNA markers believed to
235 be associated with cereulide synthesis (Table 1).

The results in Table 4 indicate that none of the *B. pumilus* strains possessed complete lichenysin
or surfactin genes or both. The results further indicated that one of the toxic *B. pumilus* strains and
one of the *B. licheniformis* strains possessed at least a 146 bp fragment identical to the *cesA* gene
but not to *cesB*. The substance observed to damage the permeability barrier of exposed sperm cells
240 may thus have been lichenysin in the strains *B. licheniformis* NR5160 and NR6768 but the identity
of similarly acting substance of the *B. pumilus* strains remains unknown.

4. Discussion

245 In this study we identified toxin producing *Bacillus* from mastitic milks. Three of these strains
were identified as *B. cereus*, two as *B. licheniformis* and four as *B. pumilus*. The toxic substances
extracted from the four *B. pumilus* and the two *B. licheniformis* isolates were heat stable. The
substances were detected by the boar spermatozoan motility inhibition test (Andersson et al., 2004,
2007) which is a sensitive indicator for ionophoric peptides such as cereulide (Hoornstra et al.,
250 2003), known for the high toxicity in humans (Dierick et al., 2005; Mahler et al., 1997).

Heat stability and solubility in methanol indicates that the toxic agents from the mastitic isolates
were not proteins. The toxins inhibited mobility of boar sperm cells and disturbed the plasma
membrane permeability barrier but did not affect the mitochondria. The bioactivities of the
toxinogenic mastitis *B. pumilus* and *B. licheniformis* isolates thus differed from that of the *B. cereus*
255 emetic toxin producers (Andersson et al., 1998). The mastitis isolates of *B. pumilus* and *B.*
licheniformis yielded no amplicon with PCR primers targeted to synthetases of cereulide and
surfactin confirming the view that the six toxic isolates produced neither of these substances.

The two mastitic *B. licheniformis* strains were similar in ribopattern to strains earlier reported from infant food and implicated in a fatal case of food poisoning (Salkinoja-Salonen et al., 1999).
260 Each of the seven *B. licheniformis* strains analyzed in present study contained the three lichenysin synthetase genes *lchAA*, *lchAB* and *lchAC* according to the PCR results. However, DNA from the non-toxic control strains DSM 13^T and F-2896/95 that are reported not to inhibit sperm cell motility (Salkinoja-Salonen et al., 1999) also contained lichenysin genes indicating that the synthetase genes are not limited to strains found toxic in the sperm test. The non-toxic strains may produce
265 lichenysin but not enough to see its toxic effect in the sperm test. Also the *B. cereus* strain IH41385, shown to possess the cereulide synthetase genes by PCR by three independent sets of primers in the present study, was reported as non-toxic in the spermatozoan motility assay (Pirttijärvi et al., 1999). Chemical assay showed that this strain did produce cereulide but the produced amount was only 1/1000 of that found in food poisoning isolates of *B. cereus* (Carlin et al., 2006).

270 The sperm toxic mastitic *B. pumilus* strains described in this study produced heat-stable membranolytic substances. Other *B. pumilus* strains have been reported to produce an antifungal lipopeptide, called pumilacidin (Naruse et al., 1990) and surfactin (Morikawa et al., 1992) that have similar bioactivity and solubility properties as the toxins detected in this study. Boar spermatozoan EC₅₀ value for pure surfactin was reported as 6 to 13 µg ml⁻¹ (Suominen et al., 2001). The crude
275 extracts used in this study were toxic to sperm cells at concentration of 1 to 15 µg ml⁻¹ indicating the responsible substances were more toxic than surfactin. Pumilacidin has structure similar to surfactin except for two amino acids that are different in the peptide ring consisting of seven amino acids (Kalinovskaya et al., 2002). The sperm toxicity of pumilacidin was not assessed as this substance is not commercially available.

280 *Bacillus* species have been occasionally reported to cause mastitis (Miller et al., 1993). There are number of reported cases of mastitis caused by *Bacillus cereus* (Parkinson et al., 1999; Jones

and Turnbull, 1981) where symptoms included depression, fever and even death within 24 hours (Parkinson et al., 1999). The udders became swollen, painful and sometimes changed colour. The milk colour varied from brown to reddish. The toxic agents detected in this study may be connected
285 to some of the clinical symptoms of cows suffering mastitis caused by *Bacillus* species. The number of subclinical cases of mastitis caused by *Bacillus* is not known. The infection route for *Bacillus* spp. to udder was studied by Jones and Turnbull (1981). They isolated *B. cereus* from a cow's surroundings, the grass and the cow manure. In our study and other studies (in't Veld et al., 2001) enterotoxin-producing *B. cereus* were isolated from milk. Cereulide-producing *B. cereus* have been
290 isolated from dairy farms and milk silos (Svensson et al., 2006), but there appears to be no earlier report on heat-stable toxin producing *Bacillus pumilus* strains among mastitis isolates.

Our results show that heat-stable toxin producing *B. pumilus* and *B. licheniformis* occurred in milk of mastitis cows. The spores of these species are known for heat stability and resistance to hydrogen peroxide and may thus survive cleaning procedures and dairy processes like spray
295 evaporation. Milk from subclinical carriers of these bacteria may introduce a risk to safety of milk powder products.

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405 Figure captions

Figure 1. Ribopatterns of heat-stable toxin producing strains of *Bacillus licheniformis* and *B. pumilus* from mastitic milk. The type strains *B. licheniformis* DSM 13^T, *B. pumilus* DSM 27^T and previously known toxinogenic strains *B. licheniformis* 553/1, *B. pumilus* ES20 were used as reference. The genomic DNA was cut with *EcoRI* and hybridized with phosphorescently labelled
410 rRNA operon specific DNA probe.

Figure 2. The toxic effect of methanol soluble substances from mastitic strains of *B. pumilus* on boar spermatozoa. The sperm cells were stained with calcein-AM and propidium iodide. Green fluorescence indicates intact plasma membranes. Red fluorescence indicates permeability barrier
415 loss allowing uptake of propidium iodide by the cells. a) Sperm cells exposed to methanol (vehicle) only. The cells were mobile and the cell membranes intact. b) Sperm cells exposed to methanol extracts prepared from biomass of the strain *B. pumilus* NR 440. The toxins have damaged the cell membrane of the sperm cells. Extracts prepared from the strains NR 19/5, NR 13890 and NR 15703 had similar effect (not shown in the figure). Sperm head diameter is from 5 to 7 μm .

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Figure 3. Negative gel image of PCR products amplified with cereulide synthetase *cesA* targeted primer pair CER1 / EMR. Lanes: M. O'Generuler 100 bp DNA ladder plus (Fermentas); *Bacillus pumilus* (lanes 1-3): NR 19/5; NR 440; DSM 27^T; *B. licheniformis* (lanes 4-9): NR 5160; NR 6768; Hulta 51/97; 553/1; DSM 13^T (8 and 9); *B. cereus* (lanes 10-13): IH 41385; NC 7401; ATCC 4342;
425 ATCC 14579^T

Table 1. The PCR primers

Primer pair	Sequence	Target sequence	Expected product size bp	Reference
LicA-f LicA-r	GTGCCTGATGTAACGAATG CACTTCCTGCCATATACC	Lichenysin synthetase <i>lchAA</i> ^a	735	This study
LicB2-f LicB2-r	TGATCAGCCGGCCGTTGTCT GGCGAATTGTCCGATCATGTCC	Lichenysin synthetase <i>lchAB</i> ^a	904	This study
LicC-f LicC-r	GCCTATCTGCCGATTGAC TATATGCATCCGGCACCA	Lichenysin synthetase <i>lchAC</i> ^a	1195	This study
sfp-f sfp-r	ATGAAGATTTACGGAATTTA TTATAAAAGCTCTTCGTACG	Surfactin synthetase <i>sfp</i> ^b	675	Hsieh et al., 2004
CER1 EMT1	ATCATAAAGGTGCGAACAAGA AAGATCAACCGAATGCAACTG	Cereulide synthetase <i>cesA</i> ^c	188	Horwood et al., 2004
EM1-f EM1-r	GACAAGAGAAATTTCTACGAGCAAGTACAAT GCAGCCTTCCAATTACTCCTTCTGCCACAGT	Cereulide synthetase <i>cesB</i> ^c	635	Ehling-Schulz et al., 2004
BE-f BE-r	ACTTAGATGATGCAAGACTG TTCATAGGATTGACGAATTTT	Hypothetical <i>B. cereus</i> NRPS gene ^d	850	Toh et al., 2004
RE234f RE234r	AACGTCGGTATGATTTTAGG CTCTTCTGCTCTCTATTTATGTC	SCAR-marker specific to emetic <i>B. cereus</i> ^d	234	Nakano et al., 2004

SCAR sequence-characterized amplified region

NRPS non-ribosomal peptide synthetase

^a According to lichenysin synthetase operon sequence by (Yakimov et al., 1998)

^b Primer pair targeting the *sfp* gene in the *srfA* operon responsible for surfactin synthetase in *B. subtilis*, *B. amyloliquefaciens* and *B. circulans* (Hsieh et al., 2004).

^c According to the cereulide synthetase gene cluster sequence published by (Ehling-Schulz et al., (2006).

^d No PCR product of the correct size should be generated from cereulide synthetase gene cluster based on the sequence published by Ehling-Schulz et al. (2006)

Table 2. Toxin production of *Bacillus* spp. isolates from mastitic milk. The isolate was considered toxin producing when it inhibited motility of boar sperm upon exposure to boiled bacterial suspension (≤ 4 mg wet wt. of bacteria per ml) within 3 d exposure.

Species and number (<i>n</i>) of mastitic isolates		Number of isolates inhibiting sperm motility
Species	<i>n</i>	
<i>B. cereus</i>	6	0
<i>B. licheniformis</i>	3	2
<i>B. pumilus</i>	5	4
<i>B. subtilis</i>	8	0
<i>Bacillus</i> spp.	1	0

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Table 3. Boar sperm motility inhibiting activities of aqueous and methanol extracts from the mastitic *Bacillus* isolates. ND, not determined.

	EC50 (mg ml ⁻¹) ^a for sperm motility inhibition		
	Hot water extract of bacteria ^b		Methanol soluble substance ^c
Exposure time →	1 d	3 d	3-4 d
<i>Bacillus</i> isolates from mastitic milk samples:			
<i>B. licheniformis</i> NR 5160	≤1	≤1	0.020
<i>B. licheniformis</i> NR 6768	≤1	≤1	0.026
<i>B. pumilus</i> NR 19/5	≤1	≤1	0.015
<i>B. pumilus</i> NR 440	2-4	≤1	0.0014
<i>B. pumilus</i> NR 13890	2-4	≤1	0.0068
<i>B. pumilus</i> NR 15703	≤1	≤1	0.011
Reference stains:			
<i>B. licheniformis</i> DSM 13 ^T	ND	>4 ^c	>0.1 ^d
<i>B. licheniformis</i> 553/1	ND	<2 ^c	0.004 – 0.008 ^d
<i>B. licheniformis</i> Hulta 51/97	ND	<2	ND
<i>B. pumilus</i> DSM 27 ^T	≤2 ^f	≤2 ^f	0.001-0.007 ^f
<i>B. cereus</i> F4810/72	≤1	≤1	0.00004
<i>B. cereus</i> ATCC 4342	>4	>4	>0.05

^a mg bacterial extract per ml of extended boar semen causing a loss of motility in ≥50% of the sperm cells.

^b Aqueous extracts were prepared from 1, 2 or 4 mg (wet wt) of plate grown bacterial mass

^c Dry wt. of methanol extractable substance from the bacterial mass

^d Cited from Mikkola et al. (2000)

^e Cited from Salkinoja-Salonen et al. (1999)

^f Cited from Suominen et al. (2001).

Table 4. Amplification of DNA from mastitis *Bacillus* isolates with PCR primer pairs targeting synthetase genes of lichenysin (primer pairs LicA1, LicB2, LicC), cereulide (primer pairs CER1/EMT, EMI) and surfactin (primer pair sfp). Targets of the primer pairs BE and RE234 are associated to *B. cereus* cereulide synthesis but not parts of the synthetase genes.

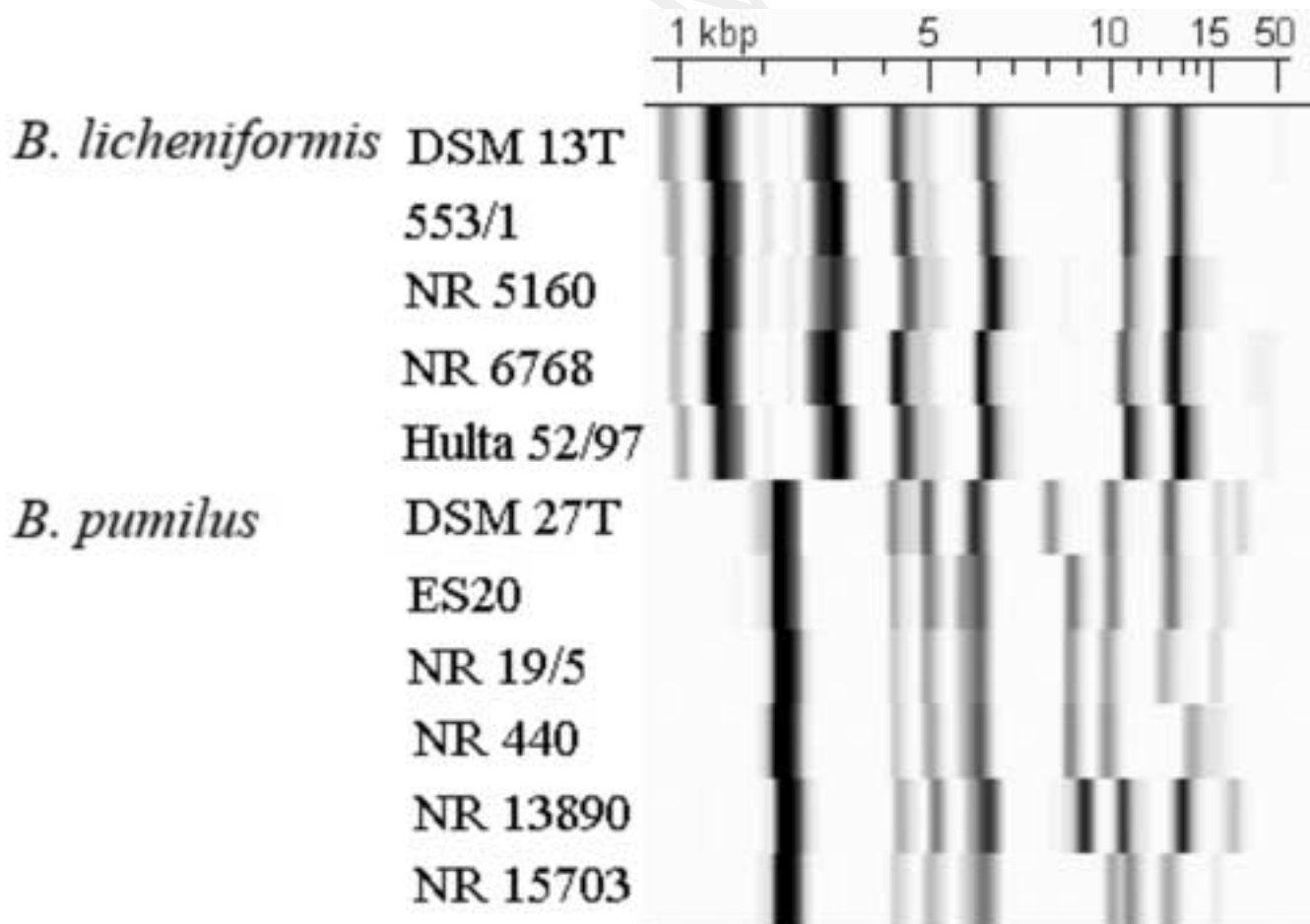
Primers targeted for	PCR product of the expected size obtained with primers ^a							
	Lichenysin synthetase			Cereulide synthetase		Cereulide associated		Surfactin synthetase
Tested strains ^b	LicA1 (f+r)	LicB2 (f+r)	LicC (f+r)	EMI (f+r)	CER1 / EMT1	BE (f+r)	RE234 (f+r)	Sfp (f+r)
<i>B. licheniformis</i> , lichenysin producing strain:								
553/1	+	+	+	-	-	-	-	-
<i>B. licheniformis</i> strains toxic in sperm assay:								
NR 5160	+	+	+	-	+	-	-	-
NR 6768, Hulta 51/97, Hulta 52/97	+	+	+	-	-	-	-	-
<i>B. licheniformis</i> strains non-toxic in sperm assay:								
F-2896/95 ^b , DSM 13 ^T	+	+	+	-	-	-	-	-
<i>B. pumilus</i> strains toxic in sperm assay:								
NR 19/5	-	-	-	-	+	-	-	-
NR 440, NR 13890, NR 15703, DSM 27 ^T	-	-	-	-	-	-	-	-
<i>B. subtilis</i> , surfactin producing strains:								
DSM 1970, DSM 10	-	-	-	ND	-	ND	ND	+
<i>B. cereus</i> , cereulide producing strains:								
IH 41385, NC 7401	-	-	-	+	+	+	+	-
<i>B. cereus</i> , cereulide non-producing strains:								
ATCC 4342, ATCC 14579 ^T	-	-	-	-	-	-	-	-

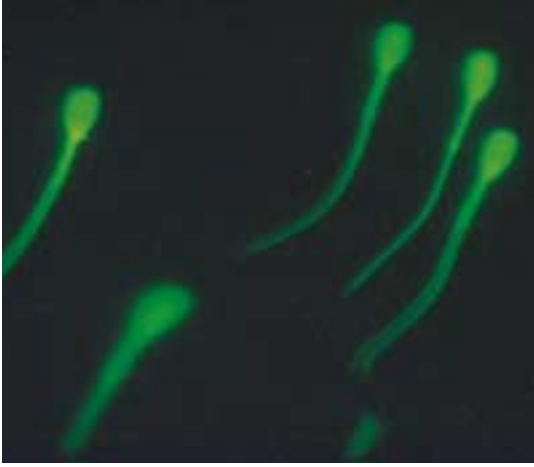
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ND no data

^a The primer sequences and targeted genes are explained in Table 1^b Toxin production of the strains, see materials and methods

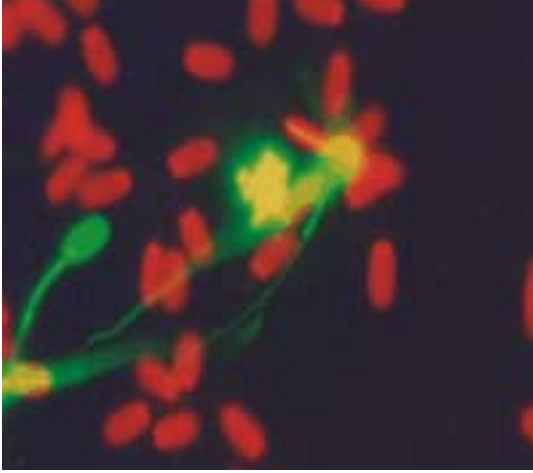
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Nieminen et al., Fig. 2b

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