The peptide toxin amylosin of Bacillus amyloliquefaciens from moisture-damaged buildings is immunotoxic, induces potassium efflux from mammalian cells, and has antimicrobial activity.

Stiina Rasimus-Sahari, Vera V. Teplova, Maria A. Andersson, Raimo Mikkola, Päivi Kankkunen, Sampsa Matikainen, Carl G. Gahmberg, Leif C. Andersson, Mirja Salkinoja-Salonen

Department of Food and Environmental Sciences (Microbiology), University of Helsinki, Helsinki, Finland; Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow, Russia; Unit of Systems Toxicology, Finnish Institute of Occupational Health, Helsinki, Finland; Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, University of Helsinki, Helsinki, Finland; The Haartman Institute (Pathology), Helsinki, Finland.

Running title: Effects of amylosin on mammalian cells

#Address correspondence to Stiina Rasimus-Sahari, stiina.rasimus@helsinki.fi.
*Present address: Raimo Mikkola, Department of Building Technology, Aalto University, Espoo, Finland.
**Present address: Päivi Kankkunen, VITA Laboratorio, Helsinki, Finland.
ABSTRACT

Amylosin, a heat-stable channel-forming non-ribosomally synthesized peptide toxin produced by strains of *Bacillus amyloliquefaciens* isolated from moisture-damaged buildings, is shown in this paper to have immunotoxic and cytotoxic effects on human cells as well as antagonistic effects on microbes. Human macrophages exposed to 50 ng of amylosin ml⁻¹ secreted high levels of cytokines IL-1β and IL-18 within two hours, indicating activation of the NLRP3 inflammasome, an integral part of the innate immune system. At the same exposure level, expression of IL-1β and IL-18 mRNA increased. Amylosin caused dose-dependent potassium ion efflux from all tested mammalian cells (human monocytes and keratinocytes and porcine sperm cells) at 1 to 2 μM exposure. Amylosin also inhibited the motility of porcine sperm cells and depolarized the mitochondria of human keratinocytes. Amylosin may thus trigger the activation of the NLRP3 inflammasome and subsequently cytokine release by causing potassium efflux from the exposed cells. The results of this study indicate that exposure to amylosin activates the innate immune system which could offer an explanation for the inflammatory symptoms experienced by occupants of moisture-damaged buildings. In addition, the amylosin-producing *B. amyloliquefaciens* inhibited the growth of both prokaryotic and eukaryotic indoor microbes and purified amylosin also had an antimicrobial effect. These antimicrobial effects could make amylosin-producers dominant and therefore significant causal agents of health problems in some moisture-damaged sites.

Keywords: amylosin, *Bacillus amyloliquefaciens*, moisture-damaged building, NLRP3, inflammasome, cytokine, IL-1β, IL-18, potassium efflux
INTRODUCTION

Amylosin is a heat-stable 1197 Da peptide toxin originally found to be produced by strains of *Bacillus amyloliquefaciens* isolated from moisture-damaged buildings (1, 2). *B. amyloliquefaciens* belongs to the *Bacillus subtilis* group, members of which have been found in high numbers in moisture-damaged buildings where occupants have experienced building-related ill health symptoms (3, 4). Amylosin forms cation-permeant ion channels with high selectivity for K⁺ in lipid membranes (5) and thus depolarizes the plasma membrane and the mitochondria inside live mammalian cells, disrupting cellular ion homeostasis, mitochondrial functions and energy metabolism (1, 2). It has been suggested that amylosin causes health risks for people living or working in moisture-damaged buildings contaminated with amylosin-producing bacteria (1). Amylosin has also been shown to be produced by *B. subtilis* and *Bacillus mojavensis* strains connected to foodborne illness (6).

Moisture-damaged buildings harbor many kinds of fungi and bacteria including producers of bioactive compounds ((7, 8), reviewed in (9)). The symptoms experienced by people living or working in moisture-damaged buildings vary (10), but they could at least partly result from activation of the innate immune system (11, 12). Symptoms such as acute inflammatory responses and pseudo-allergic reactions may be a result of the activation of inflammasomes (12, 13). Inflammasomes are components of the innate immune system which activate inflammatory caspases leading to cytokine release (14). Activation of the NLRP3 inflammasome (also known as NALP3 and cryopyrin), which stimulates the secretion of active cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18) by activating caspase-1 (15), has been shown to be dependent on K⁺ efflux (16, 17) and can be triggered by bacterial toxins (reviewed in (17) and (18)). Both IL-1β and IL-18 act as pro-inflammatory cytokines, activating
signaling pathways in mammalian cells which lead to inflammation (reviewed in (19, 20) and (21)).

The purpose of this research was to study the detrimental effects of amylosin on cells of the human innate immune system in order to assess whether ill-health symptoms experienced by people exposed to amylosin (via, e.g., inhaled air in moisture-damaged buildings, contaminated food, or commercial products produced using possible amylosin-producing bacterial strains) could result from the activation of the innate immune system. Human macrophages and peripheral blood mononuclear cells (PBMC) from healthy donors as well as human keratinocytes (HaCaT) and porcine spermatozoa were used as test cells to record the outcome of amylosin exposure. As toxicity endpoints, cytokine secretion, cytokine mRNA transcription, potassium efflux and mitochondrial effects were measured. Effects of amylosin-producing *B. amyloliquefaciens* and purified amylosin on selected microorganisms isolated from moisture-damaged buildings were also assessed to see if the production of amylosin could offer a competitive advantage to producer strains.

**MATERIALS AND METHODS**

**Microbial strains.** The following bacterial strains were used: *Bacillus amyloliquefaciens* strains 19b (HAMBI 2660, amylosin producer (1)) and IAM 1521 (HAMBI 2718), *Bacillus cereus* strains NS 58 (HAMBI 2450, cereulide producer (22)) and F4810/72 (DSM 4312, cereulide producer (23)), *Bacillus megaterium* Ne10 (DSM 17641), *Williamsia muralis* 140/96\(^T\) (DSM 44343\(^T\), type strain), *Mycobacterium murale* MA113\(^T\) (DSM 44340\(^T\), type strain), *Sphingomonas aurantiaca* MA101b\(^T\) (DSM 14748\(^T\), type strain), *Dietzia* sp. MA147 (HAMBI 3595) and *Bacillus* sp. OS16 (isolated from hay dust). *Chaetomium globosum* MTAV35 (HAMBI 3336) is
a toxigenic mold originating from a microbially damaged indoor building environment involved in a serious human case (fatal). Strains were from the DSMZ culture collection (Braunschweig, Germany) or the HAMBI culture collection (University of Helsinki, Helsinki, Finland) except for Bacillus sp. OS16, which was from our laboratory collection. The strains were grown on tryptic soy agar (TSA; Scharlab SL, Barcelona, Spain) plates at 25 ± 3°C, except for S. aurantiaca that was grown at 18°C.

**Preparation of amylosin-containing and amylosin-free bacterial extracts.** Biomass of strains *B. amyloliquefaciens* 19b and IAM 1521 was harvested from TSA plates (grown for 4 days to 4 weeks) into glass vials prewashed with methanol. The vials were exposed to three cycles of freeze-thaw. Methanol was then added (1 ml per 100 mg of wet wt biomass) after which the vials were sealed and held in a water bath at 100°C for 10 min. After cooling, the vials were agitated (200 rpm) overnight (20 - 22°C), then centrifuged (3800 rpm, 15 min), and the supernatants were transferred to pre-weighed glass ampoules. The methanol was then evaporated and the residue weighed and redissolved in methanol (10 mg dry wt ml⁻¹). The biological activity of the extracts was assessed using the boar sperm motility inhibition assay as described by Andersson et al. (24). The extracts were stored at −20°C.

**Purification and quantification of amylosin.** Amylosin was purified by reversed-phase high-performance liquid chromatography (RP-HPLC) from a methanol extract of *B. amyloliquefaciens* 19b as described previously (6), except for the eluent which was a gradient of A (0.1% formic acid) and B (methanol) from 70% B to 75% B in 10 min, to 85% B in one minute, and then continued for 20 min by isocratic elution with 85% B at a flow rate of 1 ml
Absorbance at wavelength 365 nm was used for detection. Quantification of amylosin was done by a HPLC-UV method using amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) as a reference compound as described by Mikkola et al. (2). HPLC mass spectrometry (HPLC-MS) analysis of amylosin was done as described previously (6) using the HPLC conditions presented above.

**Mammalian target cells.** Human macrophages were obtained by differentiating primary peripheral blood mononuclear cells isolated from the buffy coats of healthy blood donors. Cells were purified, differentiated and cultured at a density of 0.75 - 1.5 × 10^6 monocytes ml⁻¹ as described previously (13). The spontaneously immortalized human keratinocyte cell line HaCaT, originating from adult human skin and exhibiting normal differentiation (25), was cultivated in RPMI1640 as described by Hoornstra et al. (26). Human monocyte-enriched peripheral blood mononuclear cells (PBMC) were primary cells freshly isolated from buffy coats of healthy human blood donors (Finnish Red Blood Service, Helsinki, Finland) with ethical permission, maintained for experiments in RPMI1640 as described previously (26). Porcine spermatozoa were primary cells obtained from a commercial source (Figen Ltd, Tuomikylä Finland) as extended boar semen containing 3 ± 1 mM of extracellular K⁺ as described by Hoornstra et al. (26).

**Macrophage stimulation.** Prior to stimulation, human macrophages were primed for 3 h with lipopolysaccharide (LPS, *Escherichia coli* O111:B4; Sigma-Aldrich, St. Louis, MO, USA) diluted in Dulbecco’s Phosphate Buffered Saline (DPBS) without calcium and magnesium (Lonza, Basel, Switzerland) and used at a final concentration of 1 µg ml⁻¹. For stimulation,
predetermined amounts of cell-free bacterial extracts, purified toxins or control substances were added into the stimulation medium, which was Macrophage-SFM (Serum-Free Media; Invitrogen, Carlsbad, CA, USA) containing L-glutamine and supplemented with 10 ng ml$^{-1}$ GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor; ImmunoTools, Friesoythe, Germany), or Gibco® RPMI (Roswell Park Memorial Institute) 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM alanyl-L-glutamine (UltraGlutamine 1; Lonza, Basel, Switzerland) when cell culture supernatants were collected for Western blot analysis. Both stimulation media were supplemented with 50 U penicillin ml$^{-1}$ and 50 µg streptomycin ml$^{-1}$ (Pen Strep; Invitrogen, Carlsbad, CA, USA). After stimulation for 2 to 18 h, supernatants and cells were collected from the plates and stored at −70°C. Roridin A (Sigma-Aldrich, St. Louis, MO, USA), known to stimulate cytokine production in human macrophages (13), was used as a positive reference compound in macrophage stimulation. All stimulations were carried out in flat-bottomed sterile plastic cell culture microtiter plates.

**Cytokine detection methods.** Concentrations of cytokines in the supernatants collected from the stimulated macrophage cultures were determined with enzyme-linked immunosorbent assays (ELISA) according to the manufacturers’ instructions. IL-1β was analyzed using Human IL-1β Eli-Pair (Nordic BioSite, Täby, Sweden) and IL-18 using Human IL-18 Matched Antibody Pairs for ELISA (eBioscience, San Diego, CA, USA).

IL-1β and IL-18 secretion was analyzed by Western blotting from concentrated human macrophage culture supernatants collected after 4 h stimulation. Supernatants (4 ml per sample) were concentrated to ca. 100 µl using Amicon Ultra-15 tubes (Millipore, Billerica, MA, USA) according to the manufacturer’s instructions. Five µl of concentrated cell supernatants
were separated on 12% pre-cast sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Mini-Protean Precast Gels; Bio-Rad, Hercules, CA, USA) at 200 V for 30 min and transferred onto Immobilon-P Transfer Membranes (Millipore, Billerica, MA, USA) with the PowerPac 3000 (Bio-Rad, Hercules, CA, USA) at +4°C and 100 V for 1 h. Precision Plus Protein Dual Color Standard (Bio-Rad, Hercules, CA, USA) was used as a molecule weight marker. Blotted membranes were stained with Ponceau red to confirm transfer of protein samples, washed three times with Phosphate Buffered Saline (PBS) containing 0.05% Tween 20 for 10 min and blocked for 45 min in PBS containing 5% skimmed milk. Membranes were then stained overnight with previously described anti-IL-1β and anti-IL-18 primary antibodies (27, 28) at +4°C and subsequently washed three times with PBS containing 0.05% Tween 20 for 10 min. The membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako A/S, Glostrup, Denmark) for 1 h at 20 ± 2°C and then visualized using the Immobilon Western Chemiluminescent HRP Substrate kit (Millipore, Billerica, MA, USA) and the ImageQuant LAS 4000 imager (GE Healthcare Life Sciences, Piscataway, NJ, USA).

Detection of cytokine mRNA transcription. IL-1β and IL-18 mRNA transcription was detected using a quantitative real-time reverse transcription PCR (qRT-PCR) assay as described previously (13).

Assay of potassium ion efflux from intact mammalian cells. Assays of potassium efflux from the human PBMC and HaCaT as well as the porcine spermatozoa were performed as described previously (26). Briefly, cells were pelleted by centrifugation, resuspended in isotonic, sodium
phosphate buffered K\textsuperscript{+}-free medium and placed in a measurement cuvette provided with
magnetic stirring, temperature control (24°C), and a potassium ion-selective electrode (NIKO-
ANALIT, Moscow, Russia) linked to PC recording software (Record 4; IBC, Pushchino,
Russia). The concentration of K\textsuperscript{+} in the extracellular medium was recorded once per second. The
electrode signal was calibrated by adding 100 µM of KCl into the cuvette at the end of each run
as done in a previous study (29). The isotonic (300 mOsm kg\textsuperscript{-1}) K\textsuperscript{+}-free medium contained 150
mM NaCl, 5 mM NaH\textsubscript{2}PO\textsubscript{4}, 1 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2}, 5 mM glucose and 10 mM HEPES (pH
adjusted to 7.2 with Trizma base). Alamethicin (Sigma-Aldrich, St. Louis, MO, USA) was used
as a positive reference compound.

Assessment of toxic effects on mammalian cells. The effects of toxins on boar sperm motility
were investigated using the boar sperm motility assay as described previously (24).

Testing for mitochondrial effects of amylosin inside live human cells was done
with HaCaT cells seeded to a density of 4 × 10\textsuperscript{4} cells ml\textsuperscript{-1} in RPMI1640 medium (Lonza,
Verviers, Belgium) supplemented with 2 mM glutamine, 10% FBS, 50 IU of penicillin and 50
µg of streptomycin (Sigma, St Louis, MO USA) ml\textsuperscript{-1}. The cells were grown for 48 h in 8-well,
flat-bottom glass chamber slides prior to toxin exposure. The assays were performed by adding
RP-HPLC purified amylosin to the wells and incubating up to 48 h. After exposure, the cells
were stained with the fluorogenic transmembrane potential (ΔΨ) responsive dye JC-1 (5,5',6,6'-
tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide, dissolved in dimethyl
sulfoxide; Invitrogen, Carlsbad, CA, USA) and propidium iodide (dead stain propidium iodide,
2.4 mg ml\textsuperscript{-1} in water; Invitrogen, Carlsbad, CA, USA) as described previously (26). All assays
were done in triplicate.
Assessment of antimicrobial growth inhibition. The growth inhibitory effects of amylosin-producing *B. amyloliquefaciens* 19b against bacteria and fungi were tested by co-culturing the strains on TSA plates for 10 d at 20 ± 2°C. Growth and inhibition were examined visually.

To assess the growth inhibitory effects of RP-HPLC purified amylosin, other lipopeptides known to be produced by *B. amyloliquefaciens* (surfactin, iturin and fengycin) (30) and purified cereulide on bacterial cultures, *B. megaterium* Ne10 and *Dietzia* sp. MA147 were exposed to the substances for 24 h in broth culture. Cereulide was purified from *Bacillus cereus* F4810/72 as described previously (31). Fengycin and iturin were from Aibi Lipopeptides (Gembloux, Belgium) and surfactin was from Sigma-Aldrich (St. Louis, MO, USA). The bacterial target strains were grown in tryptic soy broth (TSB; Scharlab SL, Barcelona, Spain) for 18 h (*B. megaterium* Ne10) or 40 h (*Dietzia* sp. MA147), after which the viable counts on TSA plates were $5 \times 10^7$ to $5 \times 10^8$ cfu ml$^{-1}$ and the turbidity of the cultures was 1000 (Klett-Summerson turbidometer Filter 520). For exposure, 10 μl of each target strain broth culture was used to inoculate 2 ml of TSB, leading to suspensions with viable counts and turbidity of $10^5$ to $10^6$ cfu ml$^{-1}$ and <50, respectively. These culture suspensions were then exposed to the test substances at concentrations from 0.001 μg to 100 μg ml$^{-1}$ TSB. Prior to being added to the culture suspensions, the pure substances were dissolved in methanol and heated in a water bath (80 °C) for 10 min and the bioactivity of the methanol solutions was verified using the boar sperm motility assay using a 30 min exposure time as described previously (24). TSB with the methanol extract of the test substances only was used as a sterility control of the test substances, and pure methanol, without a target strain, was used as an internal negative control (no bacterial growth = no increase in turbidity). Methanol only together with the target strains was used as a
positive control (no inhibition of bacterial growth as indicated by the maximal turbidity value after 24 h).

After 24 h incubation at 21 ± 2°C and 120 rpm, the turbidity of the exposed target strain cultures was measured (Klett-Summerson turbidimeter Filter 520). To verify the identity and purity of the exposed strains and to obtain post-exposure viable counts, aliquots from the exposed broth cultures were cultured on TSA plates. The identity of the target strains was concluded from the characteristic color of the colonies: deep red colonies indicated Dietzia sp. MA147 and white colonies of extremely big (3 × 2 µm) endospore-forming cells indicated B. megaterium Ne10. The turbidity and viable counts of the exposed cultures were compared to the turbidity and viable counts measured before exposure started. Inhibition of growth was detected as a decrease in the turbidity and viable count compared to those of the respective methanol controls. Inhibition of bacterial growth caused by a certain concentration of the test substance was calculated using the equation \[ I = 100\% - \left( \frac{G_i - G_0}{G_{\text{max}} - G_0} \right) \times 100 \], where \( G_i \) is turbidity or cfu ml\(^{-1} \) after exposure and incubation of 24 h, \( G_0 \) turbidity or cfu ml\(^{-1} \) prior to exposure and incubation, and \( G_{\text{max}} \) turbidity or cfu ml\(^{-1} \) of the positive control after incubation of 24 h. EC\(_{50}\) was calculated as the amount of the test substances in µg ml\(^{-1} \) TSB causing ≥50% inhibition of the growth of the target strains.

RESULTS

Amylosin-containing extract of \textit{B. amyloliquefaciens} stimulates secretion of cytokines IL-1β and IL-18 in human macrophages. To test if an amylosin-containing extract of \textit{B. amyloliquefaciens} can trigger cytokine secretion in human macrophages, LPS-primed and non-primed macrophages were exposed to extracts prepared from biomass of \textit{B. amyloliquefaciens}...
strains 19b (amylosin producer) and IAM 1521 (amylosin non-producer). After exposure, the concentrations of cytokines IL-1ß and IL-18 in the macrophage culture supernatants were analyzed by ELISA. Figure 1 shows that when macrophages primed with LPS were exposed to the extract of the amylosin-producing strain 19b containing ≥50 ng amylosin ml⁻¹, the secretion of IL-1ß was activated. The same amylosin concentration increased the secretion of IL-18 from both non-primed and LPS-primed macrophages, with the level of secretion being higher from the LPS-primed cells. The extract of _B. amyloliquefaciens_ IAM 1521, a strain that does not produce amylosin, stimulated no secretion of IL-1ß in the LPS-primed or the non-primed macrophages and had only a slight effect on the secretion of IL-18 in the macrophages primed with LPS. These observations indicate that amylosin, contained in the extract of strain 19b, was the cause of the increased cytokine secretion. The results also show that exposure to the amylosin-containing bacterial extract of _B. amyloliquefaciens_ 19b dose-dependently stimulated cytokine secretion in the human macrophages.

**Activation kinetics of cytokine secretion caused by the amylosin-containing bacterial extract.** To determine the activation kinetics of the cytokine secretion induced by the amylosin-containing extract from _B. amyloliquefaciens_ 19b, human macrophages were exposed to a set concentration (17 µg dry wt methanol-soluble compounds containing 50 ng amylosin ml⁻¹) of the extract for 2 to 18 h. After exposure, the concentrations of secreted IL-1ß and IL-18 in the macrophage culture supernatants were determined by ELISA and total RNA was extracted from the macrophages to determine the amounts of IL-1ß and IL-18 mRNA by real-time qRT-PCR. The results in figure 2 show that exposure to the amylosin-containing bacterial extract from strain 19b stimulated the secretion of cytokines IL-1ß and IL-18, as well as the expression of IL-
1β and IL-18 mRNA, in LPS-primed human macrophages within two hours. Maximal transcription of IL-1β and IL-18 mRNA was reached within four to six hours and maximal secretion of these cytokines within six hours. Western blot analysis of concentrated macrophage culture supernatants collected after four hours of exposure confirmed that processing and secretion of active IL-1β and IL-18 had occurred in the exposed cells (results not shown). It is concluded that the amylosin-containing extract of *B. amyloliquefaciens* 19b triggered high levels of both transcription and secretion of cytokines IL-1β and IL-18 within two hours of exposure.

**Preparation of purified amylosin.** Amylosin was purified and quantified from methanol extracts of *B. amyloliquefaciens* 19b using HPLC and identified using HPLC-MS. Figure 3A shows the HPLC-UV chromatogram of purified amylosin at 365 nm and figure 3B single and double charged sodium adducts [M+Na]⁺ and [M+2Na]²⁺ of amylosin (17.5 min) with m/z values of 1219.6 and m/z 621.4, respectively, corresponding to the mass of amylosin, 1196 Da. The thus purified amylosin was used for further experiments.

**Amylosin stimulates secretion of IL-1β and IL-18 in human macrophages.** LPS-primed and non-primed human macrophages were exposed to amylosin purified from biomass extracts of *B. amyloliquefaciens* 19b (purity of used amylosin preparation shown in Fig. 3). The amounts of secreted IL-1β and IL-18 were measured from the macrophage culture supernatants by ELISA. Figure 4 shows that secretion of IL-1β from macrophages primed with LPS was activated by exposure to 100 ng amylosin ml⁻¹. The same concentration of amylosin activated secretion of IL-18 in both non-primed and LPS-primed macrophages. The level of IL-1β secretion from the macrophages was similar to that induced by exposure to 100 ng ml⁻¹ of the mycotoxin roridin A,
a known activator of cytokine secretion (13). The results show that cytokine secretion by human
macrophages was stimulated by exposure to purified amylosin in a dose-dependent manner.

**Amylosin causes dose-dependent potassium ion efflux from mammalian cells.** To assess if
amylosin causes potassium efflux from live cells, leakage of K⁺ ions was measured from three
types of mammalian cells: PBMC, HaCaT, and porcine spermatozoa. The cells were placed in
potassium-free isotonic medium in a measurement cuvette and the change of extracellular [K⁺] in
response to the addition of 0 to 5 µg ml⁻¹ amylosin (purity of used amylosin preparation shown
in Fig. 3) was recorded in real time with a K⁺ selective electrode. The K⁺ efflux induced by
purified amylosin was compared to that induced by 0 – 60 µg ml⁻¹ of the pore-forming antibiotic
alamethicin. Amylosin caused the efflux of potassium ions in all three cell types (Fig. 5A, 5C
and 5E) at exposure concentrations approximately one-tenth of those required for alamethicin to
produce similar magnitudes of K⁺ efflux (Fig. 5B and 5D). The results show that low
concentrations (≤5 µg ml⁻¹) of purified amylosin caused dose-dependent efflux of K⁺ in human
somatic cells (PBMC and HaCaT) as well as porcine spermatozoa.

The measurement results summarized in figure 5 allow the calculation of the extent
and kinetics of the amylosin-induced efflux of K⁺ ions from the human cells (PBMC, HaCaT)
and porcine spermatozoa. The measurement chamber (1 ml) contained 20 × 10⁶ PBMC cells (cell
diameter 7 µm) or 6 × 10⁶ HaCaT cells (cell diameter 30 µm). The summed cell volumes in the
cuvette were thus ~4 µl (PBMC) or ~80 µl (HaCaT). Assuming the intracellular [K⁺] to be 150
mM (32), the summed potassium stores of the cells in the measurement chamber were 0.5 µmol
(PBMC) or 12 µmol (HaCaT). Dosing 5 µg of amylosin into the PBMC holding chamber (1 ml)
provoked an efflux Δ[K⁺] of 200 nmol within 900 seconds, i.e., 10 nmol of K⁺ discharged per
10^6 PBMC cells (Fig. 5A). Dosing 3.2 µg of amylosin into the HaCaT cell holding chamber (1 ml) provoked an efflux Δ[K⁺] of 80 nmol within 900 seconds, i.e., 13 nmol discharged per 10^6 HaCaT cells (Fig. 5C). The amounts discharged within 15 min of exposure to amylosin represented 40% and 1% of the cellular K⁺ stores of the PBMC and HaCaT cells, respectively.

As shown in figure 5E, the porcine spermatozoa responded to amylosin exposure by an instant, massive efflux of K⁺. The measurement chamber contained 54 × 10^6 sperm cells ml⁻¹, with a summed cell volume of ~0.6 µl (11 µm³ per sperm cell, (33)) and summed intracellular store of 90 nmol K⁺. Addition of 0.5 µg of amylosin into the chamber (1 ml) provoked an efflux Δ[K⁺] of 85 nmol already within 300 seconds (Fig. 5E), depleting the spermatozoan intracellular K⁺ stores. Exposure to 0.3 µg amylosin ml⁻¹ also depleted the K⁺ stores of the spermatozoa but less rapidly. The experimental evidence thus strongly indicates that exposure to low concentrations (0.3 to 5 µg ml⁻¹, i.e., 0.3 to 5 µM) of purified amylosin instantly (within seconds) caused a major efflux of cytoplasmic K⁺ into the extracellular fluid from primary and cultured human and porcine cells.

Exposure to low concentration (≤ 2 µM) of amylosin depolarizes human and porcine cellular membrane potentials (ΔΨ) and impairs motility of porcine spermatozoa. Amylosin provoked leakage of K⁺ ions from primary human PBMC and cultured keratinocytes (HaCaT), as shown in Fig. 5, in a medium which was isotonic (300 mOsm kg⁻¹) but potassium-free to avoid overloading the potassium electrodes used to measure the K⁺ efflux. Blood plasma contains 3.5 – 4 mM K⁺ (32). Since the intracellular [K⁺] is high (150 mM) (32), any major leakage of K⁺ from the cells should affect the cellular transmembrane potentials, ΔΨ. To observe this, monolayers of human keratinocytes (HaCaT) were exposed to purified amylosin in tissue culture medium with...
close to physiological [K+] (RPMI1640, 5 mM K+). To visualize changes in membrane potential, the cells were stained with membrane-permeant ΔΨ-responsive dye JC-1. JC-1 emits orange fluorescence in membranes with ΔΨ of >140 mV and green fluorescence when ΔΨ ≤100 mV. As a second indicator, the membrane non-permeant dye propidium iodide (death stain) was used to reveal any damage to the cell membrane permeability barrier. Figure 6 shows examples of the outcome of amylosin exposure in physiological [K+] medium. Epifluorescence microscopic views of the double-stained HaCaT cell monolayers show that exposure to 0.8 μg of amylosin ml⁻¹ for 24 h completely depolarized all cellular membranes, including the mitochondria. This occurred while the cell membrane permeability barrier, preventing the influx of propidium iodide, was preserved (no purple-red fluorescent cells visible in Fig. 6). Therefore, exposure to amylosin depolarized the cells without damaging the cell membrane and making it permeable to propidium iodide. This result matches well the results shown in figure 5, where measurable K⁺ efflux from the HaCaT cells was observed within 20 minutes (1200 s, Fig. 5C) upon exposure to 1.6 μg of amylosin ml⁻¹. Combining these results, it is concluded that exposure to 1 – 2 μg of amylosin ml⁻¹ (corresponding to 1 – 2 μM) conferred the HaCaT cells leaky to K⁺ ions under physiological ionic conditions. Similar results were obtained with the human primary PBMC (data not shown).

The motility of spermatozoa is known to be a highly sensitive indicator of damage to energized mitochondria (34). We therefore tested the effect of the amylosin preparation, used for the cytokine emission results shown in figures 1 and 2, and purified amylosin on porcine spermatozoa. It was found that exposure to 0.0022 μg of purified amylosin ml⁻¹ inhibited the motility of 27 × 10⁶ porcine spermatozoa within 2 days. Exposure to a biomass extract of B. *amylo liquefaciens* 19b at a concentration of 0.0024 μg of amylosin ml⁻¹ along with 0.8 μg of
methanol soluble biomass (dry wt) ml$^{-1}$ gave a similar result, whereas a similarly prepared extract of *B. amyloliquefaciens* IAM1521 (amylosin non-producer) did not affect sperm motility even at exposure concentrations of 50 μg of methanol soluble biomass (dry wt) ml$^{-1}$. These results show that amylosin blocked sperm motility at very low exposure concentrations (0.002 μg ml$^{-1}$) both in a crude bacterial extract and as a purified substance.

**Amylosin-producing *B. amyloliquefaciens* and purified amylosin inhibit microbial growth.**

The effect of amylosin-producing *B. amyloliquefaciens* 19b and purified amylosin on the growth of selected bacterial and fungal strains was investigated by co-culturing on TSA plates and by measuring the change in turbidity and viable counts of broth cultures after exposure to amylosin. Figures 7A and 7B show that *B. amyloliquefaciens* 19b, when inoculated on a TSA plate seeded with a spore suspension of *Chaetomium globosum* MTAV 35, a toxigenic fungal isolate from a moisture-damaged indoor space, prevented the growth of the mold up to a distance of 12 mm. Bacterial isolates from similarly troubled indoor spaces were inoculated across a streak of *B. amyloliquefaciens* 19b on a TSA plate. Figure 7D shows that the growth of the test bacteria was inhibited compared to a plate with no *B. amyloliquefaciens* 19b (Fig. 7C). In figure 7E, the sensitivities of same test bacteria were assayed on a plate streaked with *B. cereus* NS58, a strain which emits cereulide which is known to cause efflux of K$^+$ ions from live bacteria (22). As can be seen in figure 7E, *B. cereus* NS58 caused no or only minor growth inhibition of the test bacteria.

*B. amyloliquefaciens* is also a known producer of bioactive lipopeptide toxins. The results in table 1 show that amylosin was 100-fold more inhibitory towards the growth of *B. megaterium* Ne10 and *Dietzia* sp. MA147 in broth culture than the other tested lipopeptides.
produced by *B. amyloquefaciens* or cereulide produced by *Bacillus cereus*. The motility of porcine spermatozoa was inhibited by amylosin also at concentrations 100 to 1000-fold lower than the other lipopeptides. Based on the results in figure 7 and table 1, amylosin thus appears to be active towards both prokaryotic and eukaryotic microbial cells.

**DISCUSSION**

The results of our research show that the K⁺ ionophoric bacterial toxin amylosin causes many types of adverse effects in human cells and therefore could potentially explain symptoms of potassium homeostasis disruption (35, 36) in people exposed to it even at low concentrations in moisture-damaged indoor spaces. Exposure to a concentration down to 300 ng amylosin ml⁻¹ was in this paper shown to be sufficient to generate a major potassium efflux from live cells, similar to that shown previously for isolated mitochondria (2). Therefore, exposure to extremely low concentrations of amylosin may initiate *in vivo* potassium leakage from human cells (intracellular concentration of potassium 150 mM (32)) down the concentration gradient towards blood plasma (potassium concentration 3.5 to 5.0 mM (32)). Important cells of the human innate immune system, namely freshly isolated peripheral blood mononuclear cells (PBMC), representing the precursors of macrophages, and keratinocytes (HaCaT), responded within minutes (Fig. 5) to μM exposure to amylosin. Based on the results in the present paper (Fig. 1, 2 and 4), exposure to even lower amounts of amylosin may be expected to activate the NLRP3 inflammasome, leading to cytokine release from macrophages, and consequently activate cellular innate immunity responses. Although microbial pore-forming toxins have been previously reported as activators of the NLRP3 inflammasome (18), this is the first report of a channel-forming bacterial toxin stimulating cytokine release from human primary macrophages. To our
knowledge, amylosin is the first bacterial toxin causing activation of the NLRP3 pathway possibly solely via potassium efflux, thus potentially leading to human illness. Interestingly, reported cases of \textit{B. amyloliquefaciens} causing human illness have been environmental exposures, namely contaminated indoor air (1, 2) or food (6), whereas human infection by \textit{B. amyloliquefaciens} has not been reported.

In recent years, there has been increased focus on inflammasomes, cytoplasmic components of the innate immune system which are responsible for activating many types of inflammatory responses. Inflammasomes were first described only a decade ago (14) but have since been shown to have a significant role in the activation of inflammation and inflammatory diseases (37). The NLRP3 inflammasome has been most widely studied and is linked to several autoinflammatory and autoimmune diseases (17, 38, 39), and several bacterial toxins have been reported to trigger activation of this inflammasome (reviewed in (17) and (18)). Recently, NLRP3 inflammasome activation has also been connected to lung diseases including asthma (40). Microbial growth in moisture-damaged buildings and the consequent exposure of occupants to microbes and microbe-associated biological agents (e.g. cell components and microbial toxins) is accepted as a cause of various adverse health effects including asthma, allergies, and other symptoms connected with the functioning of the immune system (41). For example, exposure to moisture-damaged indoor environments is reportedly associated with a 30 to 50% increase in various respiratory and asthma-related symptoms (42). The common trigger in these illnesses could be the activation of the NLRP3 inflammasome and the consequent secretion of the pro-inflammatory cytokines IL-1\(\beta\) and IL-18, driven by potassium ion efflux (16). As our results show that amylosin causes potassium efflux from both primary and cultured mammalian cells, it appears likely that the secretion of active forms of IL-1\(\beta\) and IL-18, stimulated by...
exposure to amylosin, is a result of the activation of the NLRP3 inflammasome. Amylosin is a moderately lipophilic molecule with a log $K_{ow}$ between 3 and 4. This estimate is based on its RP-HPLC retention, where surfactin-containing iso-C12 fatty acid (log $K_{ow}$ 4.05) and amphotericin B (log $K_{ow}$ 2.46) were used as references (43). Therefore, inhaled amylosin could induce activation of alveolar macrophages. Secreted IL-1β and IL-18 affect many immune functions and are significant in both local and systemic inflammation (21). Therefore, the release of active cytokines caused by exposure of cells of the innate immune system to amylosin could explain some of the inflammatory symptoms occupants of moisture-damaged buildings display.

Our results also show that amylosin-producing *B. amyloliquefaciens* 19b can inhibit the growth of several bacteria with very different cell wall compositions as well as indoor fungi. In addition to amylosin, *B. amyloliquefaciens* 19b produces surfactin and antifungal fengycin (2, 43). Strains of *B. amyloliquefaciens* producing several antibacterial and antifungal linear and cyclic lipopeptides (e.g. fengycins, iturins and surfactins) have also been isolated from, e.g., marine environments, mangrove forests and plant rhizospheres (30, 44, 45). These reports together with our results suggest that the simultaneous production of several antimicrobial substances by *B. amyloliquefaciens* strains is widespread in nature and offers a competitive advantage to the producer microbe. In addition, our results show that amylosin inhibited the growth of several taxonomically unrelated bacteria at concentrations lower than those described for any of the other antibacterial compounds produced by strains of *B. amyloliquefaciens*, which could enhance the competitive advantage of amylosin-producing strains. Strains of *B. amyloliquefaciens* producing antibacterial and antifungal compounds may have many potential commercial and therapeutic applications. Our results suggest that moisture-damaged buildings and dust may contain microbes producing bioactive compounds suitable for...
commercial development. The symptoms of damp building related illness are most likely due to
the combined effect of exposure to many different microbes and bioactive compounds (41).
However, the suggested competitive advantage could make amylosin-producers dominant in
moisture-damaged materials when the surrounding conditions are suitable for their growth,
possibly making these strains a significant cause of ill health symptoms experienced by
occupants of moisture-damaged buildings.

Our results show that amylosin was more effective at inducing potassium efflux
from human cells and porcine spermatozoa than alamethicin (Fig. 5), which is commonly used as
a model compound for ion channel formation. In addition, amylosin caused depolarization of
mitochondria in human keratinocytes (HaCaT) in a similar way as reported for cereulide (26).
Cereulide has a positive charge and is highly lipophilic (log $K_{ow} = 6$ (46)). The log $K_{ow}$ of
amylosin is estimated to be 3 to 4 (43), making it less lipophilic than cereulide or paenilide, a
toxin with higher hydrophobicity than cereulide which induces potassium efflux from
mammalian cells and mitochondria, produced by *Paenibacillus tundrae* (29). This could explain
why amylosin acts as a potassium efflux channel (2), leading to high levels of potassium ion
release down the concentration gradient, instead of acting as a potassium ionophore and passing
through the mitochondrial membrane as cereulide and paenilide do (29, 31). Interestingly,
cereulide has been reported to have no effect on the IL-1β production of monocytes (47),
whereas amylosin was shown in the current study to have a strong activating effect on human
macrophages, indicating that the potassium efflux-related activation of the NLRP3
inflammasome and subsequent cytokine secretion requires a channel-based flow of potassium
out of the cell.
Strains of *B. amyloliquefaciens* are of commercial interest due to the many bioactive compounds they produce. The U.S. Food and Drug Administration lists *B. amyloliquefaciens* as a food additive producer with a “generally recognized as safe” (GRAS) status (48), and the European Food Safety Authority (EFSA) has proposed *B. amyloliquefaciens* for “Qualified Presumption of Safety” (QPS) status (49). Several patents have been approved covering strains of *B. amyloliquefaciens* to be used for the production of antifungal and antibacterial substances for use in the food and feed industries (e.g. patents US 20100143316 A1 and US 20110274673 A1). However, it appears that the possible co-production of substances with adverse effects towards human cells has not been fully considered. Human consumption of L-tryptophan commercially produced by a genetically engineered strain of *B. amyloliquefaciens* causing an epidemic of eosinophilia-myalgia syndrome has been reported (50), but the causative agent has not been identified with certainty (research reviewed in (51)). Based on our results, the use of *B. amyloliquefaciens* to commercially produce enzymes and antimicrobial compounds could pose a risk to human health if the purity of the produced substances is not absolute.

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REFERENCES


connected to food poisoning produce the heat stable toxin amylosin. J. Appl. Microbiol.

7. Peitzsch, M, Sulyok, M, Täubel, M, Vishwanath, V, Krop, E, Borras-Santos, A,

school buildings inspected for moisture damage in Finland, The Netherlands and Spain. J.


8. Täubel, M, Sulyok, M, Vishwanath, V, Bloom, E, Turunen, M, Järvi, K, Kauhanen, E,

and fungal secondary metabolites in moisture-damaged indoor environments. Indoor Air.


9. Thrasher, JD, Crawley, S. 2009. The biocontaminants and complexity of damp indoor
spaces: more than what meets the eyes. Toxicol Ind Health. 25:583-615. doi:

10.1177/0748233709348386.


health effects, EUROEXPO: a multidisciplinary review of the literature (1998-2000) on
dampness and mite exposure in buildings and health effects. Indoor Air. 14:243-257. doi:


are highly potent inducers of inflammatory and cytotoxic effects. Indoor Air. 15 Suppl 9:65-70.


**Figure 1.** Amylosin-containing extract of *B. amyloliquefaciens* 19b stimulates dose-dependent secretion of cytokines IL-1ß and IL-18 in LPS-primed human macrophages. Macrophages obtained by differentiation from pooled monocytes extracted from human blood (three separate healthy donors for each experiment; total of six donors) were exposed for 18 h to extracts prepared from biomass of amylosin-producing *B. amyloliquefaciens* 19b and from amylosin non-producing *B. amyloliquefaciens* IAM 1521. The macrophages were exposed to methanol-soluble biomass as well as amylosin in the extract of *B. amyloliquefaciens* 19b at amounts indicated in the figure. The label “LPS” indicates that the macrophages were primed with 1 µg ml⁻¹ of LPS for 3 h prior to exposure to the bacterial extracts. IL-1ß and IL-18 were analyzed using ELISA. Results are expressed as means ± minimum and maximum values. The results obtained with 19b were marked with asterisks to indicate statistical difference from the corresponding IAM result (unpaired two-tailed Student’s *t* test; *, *P* ≤0.05; **, *P* ≤0.01).

**Figure 2.** Time course of the onset of the secretion of cytokines IL-1ß and IL-18 and the expression of IL-1ß and IL-18 mRNA in LPS-primed human macrophages in response to amylosin-containing extract from *B. amyloliquefaciens* 19b. Macrophages obtained by differentiation from pooled monocytes extracted from human blood (as for Fig. 1) were exposed for 2 to 18 h to an amylosin-containing extract of *B. amyloliquefaciens* 19b. After exposure, secreted IL-1ß and IL-18 in the supernatants was measured using ELISA and total RNA was extracted from the macrophages and used to synthesize cDNA, which was analyzed by real-time qRT-PCR to detect IL-1ß and IL-18 mRNA. Panels A and B show the concentrations of secreted IL-1ß and IL-18, respectively, expressed as mean ± minimum and maximum values, and panels C and D show the amounts of IL-1ß mRNA and IL-18 mRNA, respectively (representing the
results of two independent experiments). The measured relative units (RU) represent the fold
change in gene expression normalized to a reference gene (18S rRNA) and in relation to a no-
template-control calibrator. In all figures, “LPS” indicates macrophages primed with 1 µg ml⁻¹
of LPS for 3 h prior to exposure. Roridin A was used as a positive reference compound. The
cytokine measurement results obtained with 19b are marked with asterisks to indicate statistical
difference from the LPS control result (unpaired two-tailed Student’s t test; *, P ≤0.05; **, P
≤0.01).

**Figure 3.** HPLC-UV chromatogram and the mass spectrum of amylosin purified from the
methanol extract of strain *B. amyloliquefaciens* 19b. Panel A. HPLC-UV elution profile (365
nm) of purified amylosin (17.5 min). Panel B. Single and double charged sodium adducts
[M+Na]⁺ and [M+2Na]²⁺ of amylosin (17.5 min) at m/z 1219.6 and m/z 621.4, respectively.

**Figure 4.** Purified amylosin stimulates dose-dependent secretion of cytokines IL-1β and IL-18 in
LPS-primed human macrophages. Macrophages obtained by differentiation from pooled
monocytes extracted from human blood (three separate healthy donors) were exposed for 18 h to
10 or 100 ng ml⁻¹ of purified amylosin or roridin A (used as reference compound). “LPS”
indicates priming of cells with 1 µg LPS ml⁻¹ for 3 h prior to toxin exposure and “MeOH”
indicates the vehicle (methanol) control. IL-1β and IL-18 were analyzed using ELISA. The
measurement results obtained are marked with asterisks to indicate statistical difference from the
LPS + MeOH control result (unpaired two-tailed Student’s t test; **, P ≤0.01). Results are
expressed as mean ± minimum and maximum values and are representative of two independent
experiments.
Figure 5. Purified amylosin causes dose-dependent potassium ion efflux from human primary PBMC, cultured keratinocytes (HaCaT) and porcine spermatozoa. Washed PBMC (20 × 10^6 cells ml\(^{-1}\), panels A and B), HaCaT (6 × 10^6 cells ml\(^{-1}\), panel C and D) or spermatozoa (54 × 10^6 cells ml\(^{-1}\); panel E) were placed in isotonic potassium-free medium in a measurement chamber. The traces show the extracellular [K\(^+\)] before and during exposure to the indicated concentrations of amylosin (panels A, C, and E) or alamethicin (panels B and D), recorded with a K\(^+\) selective electrode (one reading per second). The time points of amylosin and alamethicin additions are indicated by arrows (↓).

Figure 6. Purified amylosin causes dose-dependent loss of cellular transmembrane potential (∆Ψ) in human keratinocytes (HaCaT). Monolayers of HaCaT cells were exposed to 0, 0.2, 0.4 or 0.8 µg of purified amylosin ml\(^{-1}\) for 24 h and then double-stained with the membrane-permeant ∆Ψ-responsive dye JC-1 and the membrane non-permeant dye propidium iodide. The orange fluorescence in JC-1-stained cells indicates a high membrane potential (∆Ψ > 140 mV) and green fluorescence indicates a dissipated membrane potential (∆Ψ < 100 mV). 0 µg ml\(^{-1}\) indicates vehicle only (≤ 1 vol % methanol). None of the cells displayed purple-red fluorescence following propidium iodide staining (death staining). The images are representative of three independent microscopic views. Scale bar, 30 µm.

Figure 7. Amylosin-producing *B. amyloliquefaciens* 19b, isolated from a microbially damaged indoor space, inhibited the growth of fungi and bacteria, isolated from similarly damaged indoor spaces, on TSA plates.
Top row: Two TSA plates (A, B) were inoculated with a spore suspension of Chaetomium globosum MTAV 35 (indoor fungus) and grown for 1 d, after which a horizontal streak of B. amyloliquefaciens 19b was drawn across one of the plates (B). Upon further culturing (10 d), the spores of Ch. globosum MTAV 35 failed to germinate in the vicinity of the streaked B. amyloliquefaciens 19b (B). The inhibition zone is 12 mm wide.

Bottom row: Six bacterial strains were horizontally streaked on three TSA plates: 1) Williamsia muralis MA140/96T, 2) Mycobacterium murale MA113T, 3) Dietzia sp. MA147, 4) Sphingomonas aurantiaca MA101T, 5) Bacillus sp. OS16, and 6) Bacillus megaterium Ne10. Vertical streaks of B. amyloliquefaciens 19b (strain 8, amylosin producer) or Bacillus cereus NS58 (strain 7, cereulide producer) were drawn vertically across plates D and E, respectively.
Table 1. Inhibitory effects of purified amylosin, other known products of *B. amyloliquefaciens*, and purified cereulide on the growth of *Bacillus megaterium* and *Dietzia* sp. as well as the motility of porcine spermatozoa. The effect on *B. megaterium* Ne10 and *Dietzia* sp. MA147 is expressed as the substance concentration (EC₅₀) that blocked growth in broth culture (TSB), measured by turbidometry and viable count. The effect on porcine sperm cells is indicated as the concentration needed to block motility of >50% of the cells in the sperm extender. Negative control was vehicle only (1 vol % methanol).

<table>
<thead>
<tr>
<th>Tested substance and EC₅₀ (µg ml⁻¹)</th>
<th>Target cells and the measured parameter</th>
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<tbody>
<tr>
<td></td>
<td>Tested substance and EC₅₀ (µg ml⁻¹)</td>
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<tr>
<td></td>
<td>Amylosin</td>
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<td>Inhibition of bacterial growth, 24 h exposure</td>
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<tr>
<td><em>Bacillus megaterium</em> Ne10</td>
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<tr>
<td><em>Dietzia</em> sp. MA147</td>
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<td>Motility loss, 30 min exposure</td>
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<td>Porcine sperm cells</td>
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Min and max

- 8.5 µg dry matter ml⁻¹ (25 ng amylosin ml⁻¹ in 19b)
- 17 µg dry matter ml⁻¹ (50 ng amylosin ml⁻¹ in 19b)
- 25.5 µg dry matter ml⁻¹ (75 ng amylosin ml⁻¹ in 19b)
- 34 µg dry matter ml⁻¹ (100 ng amylosin ml⁻¹ in 19b)