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Dermatophagoides pteronyssinus lytFM encoding an NlpC/P60 endopeptidase is also present in mite-associated bacteria that express LytFM variants

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D,L-endopeptidase; *Dermatophagoides pteronyssinus*; host; LytFM; microbes; NlpC/P60

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The bodies and faecal pellets of the house dust mite (HDM), *Dermatophagoides pteronyssinus*, are the source of many allergenic and nonallergenic proteins. One of these, the 14-kDa bacteriolytic enzyme LytFM, originally isolated from the spent HDM growth medium, may contribute to bacteriolytic activity previously detected by zymography at 14 kDa in the culture supernatants of some bacterial species isolated from surface-sterilised HDM. Based on previously reported findings of lateral gene transfer between microbes and their eukaryotic hosts, we investigated the presence of *lytFM* in the genomes of nine Gram-positive bacteria from surface-sterilised HDM, and the expression by these isolates of LytFM and its variants LytFM1/LytFM2. The *lytFM* gene was detected by PCR in the genomes of three of the isolates: *Bacillus licheniformis* strain 1, *B. licheniformis* strain 2 and *Staphylococcus aureus*. Expression of the variant LytFM1 was detected in culture supernatants of these bacteria by mass spectrometry (MS) and ELISA, and the bacterial LytFM proteins were shown by zymography to be able to hydrolyse peptidoglycan. Our previous reports of LytFM homologues in other mite species and their phylogenetic analysis had suggested that they originated from a common mite ancestor. The phylogenetic analysis reported herein and the detection of other *D. pteronyssinus* proteins by MS in the culture supernatants of the three isolates that secreted LytFM1 further support the hypothesis of lateral gene transfer to the bacterial endosymbionts from their HDM host. The complete sequence homology observed between the genes amplified from the microbes and those in their eukaryotic host indicated that the lateral gene transfer was an event that occurred recently.

The house dust mite (HDM), *Dermatophagoides pteronyssinus*, and related species are associated with clinical allergy, and their bodies and faecal pellets are sources of many allergenic and nonallergenic proteins [1–3]. These include enzymes that function on the cuticle during the moulting process, regulate the turnover of structural components during morphogenesis and growth [4,5] and digestive enzymes produced by the salivary glands and the midgut [3,6]. One of the enzymes found in the faecal pellets is a 14-kDa bacteriolytic enzyme designated LytFM [7,8]. Sequence data indicate that LytFM is a member of the NlpC/P60 family, which belongs to the peptidase CO clan [9]. It is a single domain (P60 domain) protein with putative

Abbreviations
BSA, bovine serum albumin; CSL, Commonwealth Serum Laboratories; DAP, diaminopimelic acid; EIS, electrospray ionisation mass spectrometry; gDNA, genomic DNA; HDM, house dust mite; HEWL, hen egg white lysozyme; MS, mass spectrometry; TSB, tryptone soy broth.

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D,L-endopeptidase activity that cleaves the bacterial peptidoglycan bridging peptide between the second and third residues to release the peptide L-Ala-D-Glu and diaminopimelic acid or Lys [8].

As mites do not possess peptidoglycan, LytFM possibly plays a role in defence against potential pathogens, including activation of innate immunity, or in the utilisation of bacteria (endosymbionts or ingested) for food, or both [8]. This is based on the observation that bacteriolytic activity, originally classified as lysozyme, detected in the spent growth medium of *D. pteronyssinus* correlated positively with the increase in number of the HDM following rearing on a diet containing *Micrococcus lysodeikticus*, a Gram-positive bacterial species particularly susceptible to lysis. In contrast, the 'lysozyme' activity in whole mite extracts of the HDM was inversely correlated with that in their spent growth medium, suggesting a digestive function in the gut and a defensive function in other parts of the body such as the haemolymph and fat body [10].

Homology studies have indicated an evolutionary link between LytFM homologues of mites and bacteria [7,8,11], and it is possible that the mite genes originated from either endosymbionts or pathogens or from environmental species associated with food ingested by the mites. It is also possible that the LytFM enzyme is produced by mite-associated bacteria as zymographic studies have demonstrated the presence of bacteriolytic proteins at a molecular weight corresponding to the enzyme found in both whole mite extracts [7] and bacterial culture supernatants from HDM-associated isolates, as well as from house dust samples collected from homes of asthmatic children [12]. However, although a range of bacterial species found in association with mites have been described (reviewed by Ref. [13]) and some of these are known to possess genes encoding proteins containing an NlpC60/P60 domain, a single domain equivalent to the mite enzyme has not been reported.

We have examined some of the bacterial species previously isolated from mites [12] for the presence and expression of the mite gene, as it is possible that the gene encoding LytFM could have been transferred to particular strains, as the presence of a eukaryotic gene in bacterial species coexisting within their hosts has been reported previously [14–16]. We have used PCR with *lytFM*-specific primers, zymographic analyses, mass spectrometric analyses of secreted bacterial products and enzyme-linked immunosorbent assay (ELISA) using antisera raised against LytFM sequence-based peptides to assess this possibility. The results suggest that lateral gene transfer between HDM and its bacterial endosymbionts has recently occurred.

Materials and methods

The HotStarTaq Master Mix Kit and QIAQuick Gel Extraction Kit were from Qiagen (Hilden, Germany), all PCR primers were from Invitrogen (Mulgrave, Vic., Australia), the *D. pteronyssinus* cDNA library was kindly provided by Wayne Thomas from the Telethon Institute for Child Health Research (Perth, Western Australia, Australia), and *D. pteronyssinus* spent growth medium (SGM) was a kind gift from the Commonwealth Serum Laboratories (Vic., Australia). Other reagents used included tryptone soy broth (TSB) (Becton Dickinson, Franklin Lakes, NJ, USA), ammonium sulfate, Bradford reagent, hen egg white lysozyme (HEWL), Triton X-100 and TWEEN® 20, which were obtained from Sigma-Aldrich, 10 mL Bio-Gel®P-6DG gel desalting columns (BioRAD Laboratories Pty. Ltd., Gladesville, Australia), acetone (BDH, Poole, UK), biotinylated goat anti-rabbit IgG secondary antibody and horseradish peroxidase-conjugated streptavidin (KPL, Gaithersburg, MD, USA) and K Blue substrate (Elisa Systems, Windsor, Qld, Australia).

Preparation of pure genomic DNA for PCR

The genomic DNA of each HDM-associated bacterial isolate was purified as described previously [12], and its concentration was determined as described [17].

PCR screening of the genomes of the nine HDM-associated bacterial isolates

PCR was performed using the HotStarTaq Master Mix Kit as described previously [12] in a thermal cycler (Model PTC 200 Engine Version 4.0; MJ Research, Inc., Waltham, MA, USA) and every amplification was repeated twice. Results reported in this study were obtained with PCR using the primer set GSUTR1/GSR3 (5′-CTATTATGAA ATTCTTCTTCACT-3′ and 5′-TTACCAACATCGTGCA ACATTAGC-3′) [12].

Preparation of PCR products for DNA sequencing and analysis of sequencing data

Amplicons to be sequenced were prepared using the QIAQuick Gel Extraction Kit. DNA sequencing was performed by the Lotterywest State Biomedical Facility Genomics at the Royal Perth Hospital, Perth, Australia. Analyses of sequencing data were performed as described previously [8].

Dermatophagoides pteronyssinus SGM and bacterial culture supernatants

Soluble proteins were extracted from SGM as described previously [7]. The HDM-associated bacterial isolates, *Bacillus cereus*, *B. licheniformis* strain 1, *B. licheniformis*

strain 2, *B. licheniformis* strain 3, *B. licheniformis* strain 4, *Micrococcus luteus*, *S. aureus*, *Staphylococcus epidermidis* and *Staphylococcus capitis* were grown at 37 °C in TSB to either the stationary or death phase of the growth curve before their culture supernatants were obtained as described previously [12]. A LytFM-enriched preparation was prepared by batch cation-exchange chromatography and gel filtration chromatography of SGM.

Concentration of bacterial culture supernatants by ammonium sulfate precipitation

The bacterial culture supernatants obtained at stationary phase were concentrated by adding saturated ammonium sulfate to achieve an 80% saturation. The proteins were precipitated overnight at 4 °C and recovered by centrifugation at 4 °C for 10 min at 28 000 ***g***. The resultant pellet was resuspended in 0.01 M sodium phosphate buffer, pH 6.2 to 1/10 of the volume of the original supernatant, and the sample was desalted by gel filtration chromatography on a 10-mL Bio-Gel® P-6DG column (BioRAD) equilibrated with 10 mM sodium phosphate, pH 7.0, containing 10 mM NaCl. Fractions collected from the column were stored at −20 °C until analysed by zymography and MS.

Concentration of bacterial culture supernatants by acetone precipitation

The bacterial culture supernatants obtained at stationary and death phases were concentrated by adding nine volumes of acetone at −20 °C to one volume of culture supernatant and the proteins precipitated overnight at 4 °C. The proteins were subsequently recovered by centrifugation at 28 000 ***g*** for 30 min at 4 °C and the resultant pellet reconstituted in 0.01 M sodium phosphate buffer, pH 6.2 to 1/10 of the volume of the original supernatant. The samples were stored at −20 °C until analysed by ELISA and inhibition ELISA.

Determination of protein concentrations

Protein concentrations were determined using the Bradford reagent and bovine serum albumin (BSA) as a standard.

Zymographic analysis of bacteriolytic activity

Following desalting, fractions of the concentrated bacterial culture supernatants of the HDM-associated isolates obtained at stationary phase were analysed by zymography as described previously [17,18]. Following SDS/PAGE, gels were rinsed in deionised water before incubation in 25 mM Tris/HCl buffer, pH 6.2 containing 1% (v/v) Triton X-100 at 37 °C overnight. Assays were repeated with the addition of 3.2 mM DTT to 0.1 M phosphate buffer, pH 6.2, according to the method previously reported [7].

Silver staining and trypsin digestion

Following SDS/PAGE, the gels were silver-stained as described previously [19] before the targeted protein bands were excised and pooled for trypsin digestion by Proteomics International Pty Ltd as described [20].

Mass spectrometry (MS)

MS was performed as described in www.proteomics.com and Bringans *et al.*, [20] by Proteomics International Pty Ltd. Tryptic peptides were analysed by electrospray ionisation mass spectrometry (EIS) using the Ultimate 3000 nano HPLC system (Dionex, Sunnyvale, CA, USA) coupled to a 4000 Q TRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA). In the analysis, the first run of standard EIS identified the time point at which the peptide of interest was eluted during HPLC. Subsequently, a second run of EIS focusing on a selected number of most intense peaks obtained in the first run (with peaks known to be trypsin disregarded) generated the MS/MS spectrum. The MS/MS data were submitted to MASCOT sequence matching software (Matrix Science, Boston, MA, USA) for comparison of the sequence of the peptide analysed with theoretical sequences found in the Ludwig NR (http://www.matrixscience.com/help/seq_db_setup_nr.html).

Preparation of rabbit polyclonal anti-peptide antisera against LytFM1 peptides

Anti-peptide antisera were commercially generated using peptides synthesised by GL Biochem Ltd (Melbourne, Vic., Australia) as described previously [21]. Four potential peptide candidates were identified (Fig. S4) [22], namely MKFFFTLALFCTLAISQVYC (1–20), SWGGGGIHG KSRGIGEGANT (38–57), PHTGTKVREENIGGD (127–141) and GQYSDPKCHHVAYGSHQPGD (84–103). Peptides GQYSDPKCHHVAYGSHQPGD (84–103) and PHTGTKVREENIGGD (127–141) were chosen on the basis of minimal sequence identity with known NlpC/P60 proteins based on a 2010 BLAST search. Peptide GQYSDPK CHHVAYGSHQPGD (84–103) was conjugated without modification to BSA, whereas PHTGTKVREENIGGD (127–141) was conjugated using an additional N-terminal cysteine residue. The location of these two peptides (including the LytFM and LytFM2 variants, PHTGTNVREENIWSD (127–141) and PHTGTKVREENIWSD (127–141), respectively) was modelled using PHYRE2 [23], and CHIMERA [24] was used to superimpose the appropriate pairs of structures.

ELISA

The wells of a 96-well-flat-bottom high-binding ELISA plate (Greiner Bio-One, Frickenhausen, Germany) were

coated with 100 μL of *D. pteronyssinus* SGM or bacterial culture supernatants prepared in 0.1 M alkaline carbonate buffer, pH 9.6. Following incubation at 4 °C overnight, the wells were washed with 100 μL of wash buffer [1 × PBS containing 0.05% (v/v) TWEEN® 20] and blocked with 100 μL of blocking buffer [wash buffer containing 0.05% (v/v) BSA]. The wells were washed before 100 μL of the anti-peptide antisera diluted in blocking buffer was added. Subsequently, 100 μL of biotinylated goat anti-rabbit IgG antiserum diluted 1/5000 in blocking buffer was applied prior to 100 μL of horseradish peroxidase-conjugated streptavidin diluted 1/4000 in blocking buffer. Immunoreactivity was observed with the addition of 100 μL of K Blue Peroxidase substrate and the reaction terminated with 100 μL of 1.0 M HCl before the absorbance values were determined at 450 nm using a spectrophotometer (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA).

Inhibition ELISA

Serially diluted samples of *D. pteronyssinus* SGM or bacterial culture supernatants were prepared at 10 $\mu g \cdot mL^{-1}$ in blocking buffer. One hundred microlitre of the serially diluted samples was then mixed with 100 μL of anti-peptide antisera diluted 1/10 000 in blocking buffer and incubated for 1 h at room temperature. The samples were subsequently transferred into the wells of an ELISA plate that had been coated with 100 μL of *D. pteronyssinus* SGM diluted to 1 $\mu g \cdot mL^{-1}$ in 0.1 M alkaline carbonate buffer, pH 9.6 and blocked with blocking buffer. Goat anti-rabbit biotinylated IgG secondary antibody was applied prior to the addition of horseradish peroxidase-conjugated streptavidin. Following development with K Blue peroxidase substrate, the percentage inhibition was determined using the formula $[1-(A_{450}$ of sample$-A_{450}$ of blank$)/(A_{450}$ of negative control$-A_{450}$ of blank$)] \times 100\%$.

Phylogenetic analysis

The phylogenetic relationship of the bacterial and mite LytFM proteins with the NlpC/P60 proteins of firmicutes, actinomycetes, ascomycetes and other eukaryotes used in a previous phylogenetic study [8] was analysed as described previously [8].

Results

The presence of *lytFM* in the genomes of the HDM-associated *B. licheniformis* and *S. aureus* strains

Following optimisation of PCR conditions using the primer set GSUTR1/GSR3, amplicons of the appropriate size were obtained from *B. licheniformis* strain 1 (GenBank: KU589271), *B. licheniformis* strain 2 (GenBank: KU589272) [12] and *S. aureus* (GenBank: KU589273), but not from *B. cereus*, *B. licheniformis* strain 3, *B. licheniformis* strain 4, *M. luteus*, *S. epidermidis* and *S. capitis* (Fig. 1A). The amplicons were sequenced and the deduced amino acid sequences are shown in Fig. 1B. Amplicons were not detected in the absence of template nor in the absence of primers (Fig. S1).

Detection of 14-kDa bacteriolytic activity in the culture supernatants of HDM-associated *B. licheniformis* and *S. aureus*

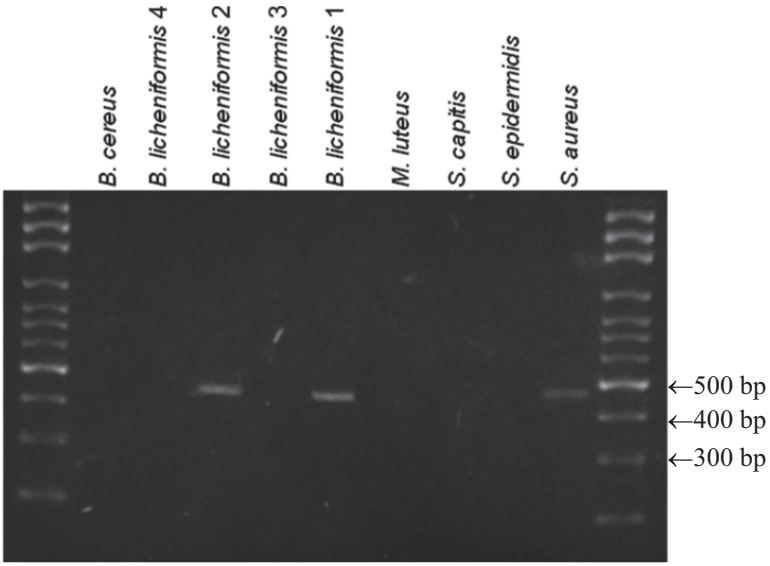
When bacteriolytic activity of the nine isolates was previously investigated with zymography, very faint lytic bands were observed at 14 kDa in the culture supernatants of *B. licheniformis* strain 1 and *B. licheniformis* strain 2 [12]. These studies were repeated herein. The bacterial culture supernatants of the nine isolates collected at stationary phase were concentrated by 80% saturated ammonium sulfate precipitation and desalted by gel filtration chromatography before the resultant first six or seven fractions were analysed. Using *D. pteronyssinus* SGM as a positive control and HEWL as the size marker, bacteriolytic activity in the region of 14 kDa was detected in the culture supernatants of *B. licheniformis* strain 1, *B. licheniformis* strain 2 and *S. aureus* (Fig. 2), but not in the other six isolates (Fig. S2). The activity was observed in the absence of DTT, not in its presence (data not shown).

Detection of the LytFM variants, LytFM1/LytFM2, in HDM-associated *B. licheniformis* and *S. aureus*

Whether LytFM was expressed and secreted by the three isolates described above was ascertained by subjecting concentrated and desalted samples of bacterial culture supernatants to MS after separation by SDS/PAGE. Bands in the region of 14 kDa as shown in a representative polyacrylamide gel of concentrated culture supernatant of *B. licheniformis* strain 2 were pooled and subjected to tryptic digestion (Fig. 3A). Table 1 shows the peptides detected in supernatants of *B. licheniformis* strain 1, *B. licheniformis* strain 2 and *S. aureus*, respectively. Two peptides, namely SQIGVPYSWGGGGIHGK (11–27) and MINAPHTGTK (103–112), were detected in the culture supernatants of all three strains (Fig. S3B–D), which corresponded to amino acid sequence positions 11–27 and 103–112, respectively, in LytFM1 (Fig. 3B).

MS analysis of the corresponding SDS/PAGE bands obtained from *D. pteronyssinus* SGM revealed peptides

A



B

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                                -20       -10       -1        10        20        30
LytFM                           MKFFFTLALFCTLAISQVYCNGAAIVSAARSQIGVPYSWGGGGIHGKSRGIG
LytFM1                          MKFFFTLALFCTLAISQVYCNGAAIVSAARSQIGVPYSWGGGGIHGKSRGIG
B. licheniformis strain 1       MKFFFTLALFCTLAISQVYCNGAAIVSAARSQIGVPYSWGGGGIHGKSRGIG
B. licheniformis strain 2       MKFFFTLALFCTLAISQVYCNGAAIVSAARSQIGVPYSWGGGGIHGKSRGIG
S. aureus                       MKFFFTLALFCTLAISQVYCNGAAIVSAARSQIGVPYSWGGGGIHGKSRGIG

                                       40        50        60        70        80
LytFM                           EGANTVGFDCSGLAQYSVYQGTHKVLARVASGQYSDPKCHHVAYGSHQPGDL
LytFM1                          EGANTVGFDCSGLAQYSVYQGTHKVLARVASGQYSDPKCHHVAYGSHQPGDL
B. licheniformis strain 1       EGANTVGFDCSGLAQYSVYQGTHKVLARVASGQYSDPKCHHVAYGSHQPGDL
B. licheniformis strain 2       EGANTVGFDCSGLAQYSVYQGTHKVLARVASGQYSDPKCHHVAYGSHQPGDL
S. aureus                       EGANTVGFDCSGLAQYSVYQGTHKVLARVASGQYSDPKCHHVAYGSHQPGDL

                                     90        100       110       120       130
LytFM                           VFFGNPIHHVGIVSAHGRMINAPHTGTNVREENIWSDHIANVARCW
LytFM1                          VFFGNPIHHVGIVSAHGRMINAPHTGTKVREENIGGDHIANVARCW
B. licheniformis strain 1       VFFGNPIHHVGIVSAHGRMINAPHTGTNVREENIWSDHIANVARCW
B. licheniformis strain 2       VFFGNPIHHVGIVSAHGRMINAPHTGTNVREENIWSDHIANVARCW
S. aureus                       VFFGNPIHHVGIVSAHGRMINAPHTGTNVREENIWSDHIANVARCW
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Fig. 1. Screening of the genomes of nine HDM-associated bacterial isolates for the presence of the *lytFM* gene. (A) PCR was performed with the primer set GSUTR1/GSR3, and an amplicon was only obtained from *Bacillus licheniformis* strain 1, *B. licheniformis* strain 2 and *Staphylococcus aureus*. (B) The amplicons were sequenced and their translated amino acid sequences aligned with those of LytFM (accession number KF113885) and LytFM1 (accession number AF409109). The amino acid residues underlined show the binding sites for the forward primer GSUTR1 (residues -20 to -15) and reverse primer GSR3 (residues 124–130). Nonhomologous residues are highlighted in boldface.

VASGQYSDPK (61–70) and MINAPHTGTK (103–112) (Table 1, Fig. S3A). A subsequent MS study using a LytFM-enriched SGM-derived sample revealed several peptides including VASGQYSDPK (61–70) and MINAPHTGTK (103–112) as described above, as well as SQIGVPYSWGGGGIHGK (11–27), MINAPHTGTNVR (103–114), EENIWSDHIANVAR (115–128) and MINAPHTGTNVREENIWSDHIANVAR (103–128) (Table 1). The sequences from the bacterial samples corresponded to either the LytFM1

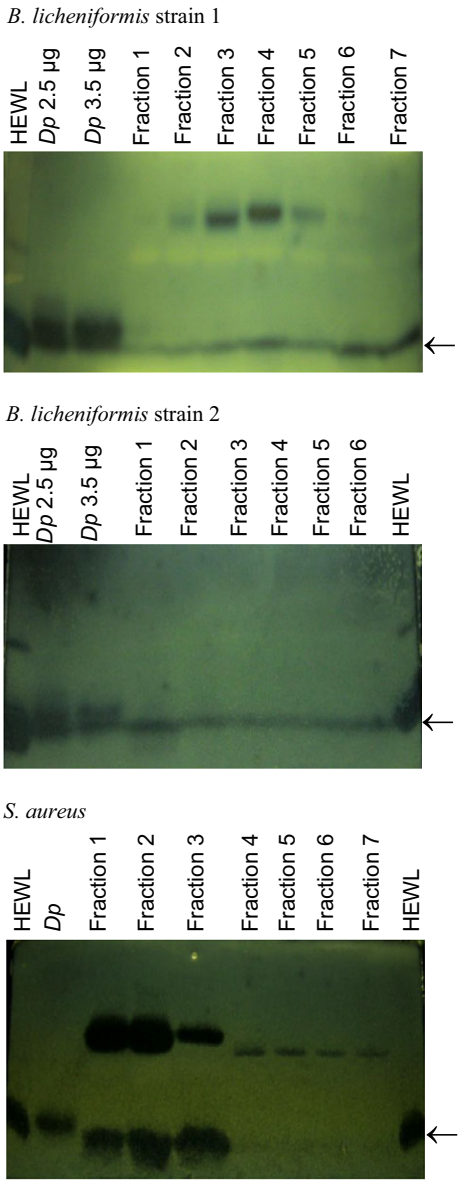


Fig. 2. Zymographic analysis of bacteriolytic activity in bacterial culture supernatants. Following concentration by 80% saturated ammonium sulfate precipitation and desalting of the supernatants of the HDM-associated *Bacillus licheniformis* strain 1, *B. licheniformis* strain 2 and *Staphylococcus aureus* by gel filtration chromatography, bacteriolytic activity was detected in the first six/seven fractions, including activity at 14 kDa as indicated by arrow. The bacteriolytic activity of HEWL and *Dermatophagoides pteronyssinus* SGM served as the size marker and positive control, respectively. *D. pteronyssinus* SGM (*Dp*).

or LytFM2 variants, whereas the mite-derived sequences corresponded to the LytFM and LytFM2 variants (Fig. 3B).

A summary of proteins other than LytFM1/2 detected in the culture supernatants is shown in Table S1: these included allergens and a protease of *D. pteronyssinus*, as well as proteins from a variety of bacterial species.

LytFM of HDM-associated *B. licheniformis* strain 1, *B. licheniformis* strain 2 and *S. aureus* verified by anti-peptide antisera Rb1001 and Rb999

Polyclonal antibodies raised against peptides derived from LytFM1 (Fig. 3B, Fig. S4) were used in ELISA to determine whether LytFM could be detected in culture supernatants from any of the three bacterial isolates. Figure S5 shows the likely locations of the two peptides on the surface of a model of LytFM generated using PHYRE2 [23]. The top ranked model (125 residues modelled, 100% confidence) was based on the rv1477 protein of *Mycobacterium tuberculosis* [25]. Anti-peptide Rb1001 (GQYSDPKCHHVAYGSHQ PGD (84–103)) and Rb999 (PHTGTKVREENIGGD (127–141)) antisera did not react with stationary-phase culture supernatants of all nine isolates (data not shown). However, LytFM1 was detected in the *D. pteronyssinus* SGM and the culture supernatants of *B. licheniformis* strain 1, *B. licheniformis* strain 2 and *S. aureus* collected from the death phase of the growth cycle (Fig. 4A). Similar results were obtained with anti-peptide antiserum Rb999 (Fig. 4B). Neither of the antisera reacted with death-phase culture supernatants of any of the other six HDM-associated isolates, namely *B. licheniformis* strain 4, *S. epidermidis*, *M. luteus*, *B. cereus*, *B. licheniformis* strain 3 and *S. capitis* (Fig. S6A,B). Inhibition ELISA studies showed that concentrated culture supernatants of the HDM-associated *B. licheniformis* strain 1, *B. licheniformis* strain 2 and *S. aureus* inhibited both antisera with 50% inhibition achieved using extracts in the region of 0.10–0.51 $\mu g \cdot mL^{-1}$ (Fig. 5).

Acquisition of *lytFM* and its variants by the three HDM-associated bacterial isolates

All the amino acid sequences of LytFM homologues included in a previous phylogenetic analysis [8] except that of *Ceratitis capitate* (XP 012159888) which has been withdrawn from GenBank were used. Phylogenetic analysis revealed clustering of LytFM homologues into distinct monophyletic groups similar to that observed in the previous analysis [8]. The mite homologues and LytFM obtained from *B. licheniformis* strain 1, *B. licheniformis* strain 2 and *S. aureus* formed a clade distinct from those of other microbes, fungi and other eukaryotes (Fig. 6), indicating a possible lateral gene transfer of *lytFM* and its variants from

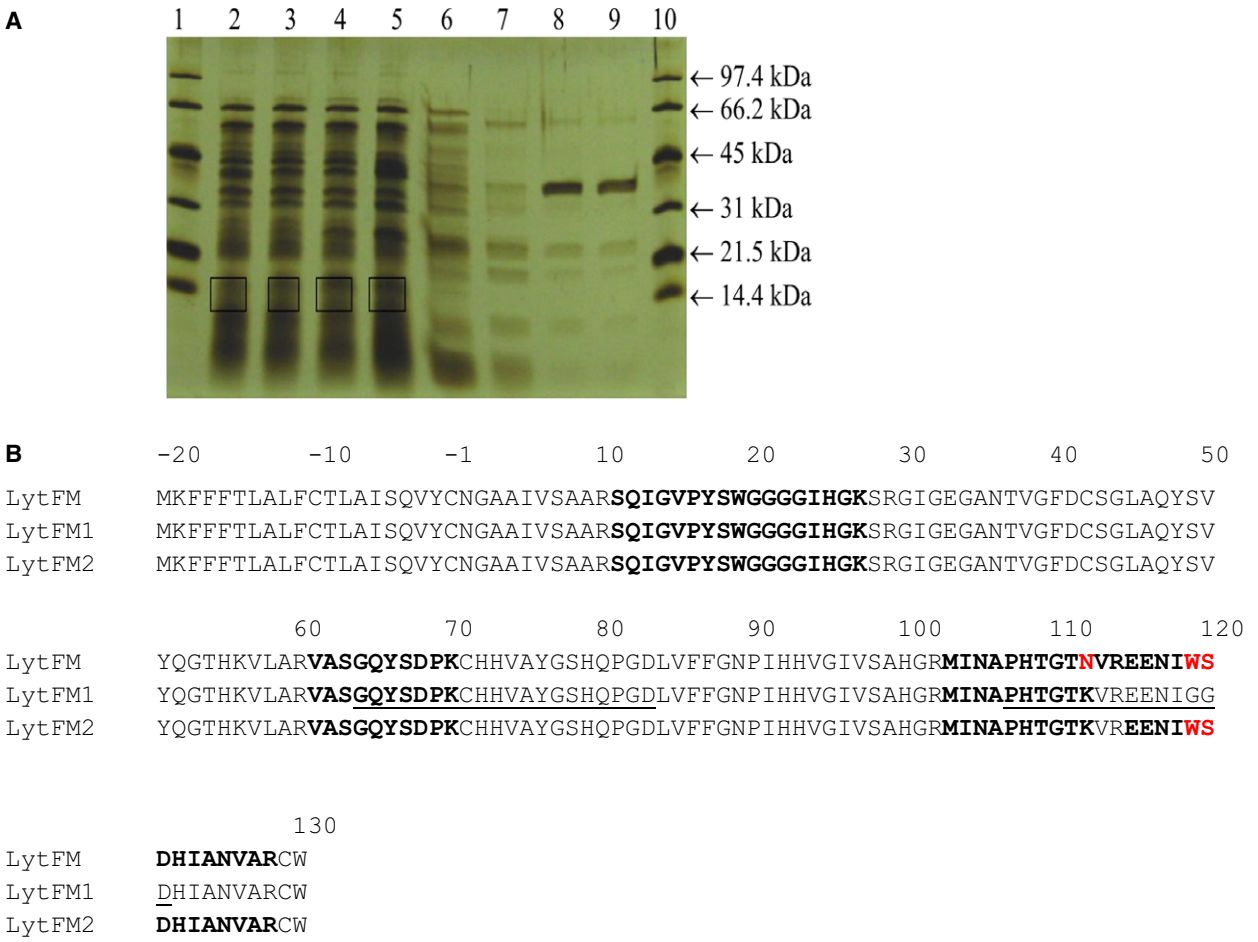


Fig. 3. Detection of LytFM in bacterial culture supernatants of *Bacillus licheniformis* and *Staphylococcus aureus*. (A) A representative gel of *B. licheniformis* strain 2 showing the fractions obtained from the concentrated and desalted supernatant and the regions (as marked by boxes) pooled for MS analysis. (B) Alignment of the amino acid sequences of LytFM, LytFM1, LytFM2 and the peptides detected in the culture supernatants of *B. licheniformis* strain 1, *B. licheniformis* strain 2, *S. aureus* and *Dermatophagoides pteronyssinus* SGM which are highlighted in boldface. Nonhomologous residues are highlighted in red. The peptides used for generating anti-peptide antisera Rb1001 and Rb999 are underlined.

D. pteronyssinus to its endosymbiotic microbes. In addition, consistent with the previous analysis [8], the mite LytFM homologues were most closely related to those of some of the actinomycetes and shared a common ancestor with both the actinomycetes and ascomycetes.

Discussion

To investigate the possibility that the *lytFM* gene in *D. pteronyssinus* was horizontally transferred to microorganisms cohabiting within the HDM, nine Gram-positive HDM-associated bacteria isolated from surface-sterilised *D. pteronyssinus* [12] were screened by PCR for the existence of *lytFM* or its variants in their genomes. The *lytFM* gene was amplified from genomic DNA of *B. licheniformis* strain 1, *B. licheniformis* strain 2 and *S. aureus*, but not from the other two *B. licheniformis* strains, or from the other bacterial isolates from *D. peteronyssinus* [12]. Similarly, the LytFM protein was found to be present in the culture supernatants from only these three bacterial species as judged by MS (stationary-phase samples) and by ELISA (death-phase samples). With regard to the PCR studies, only the originally described LytFM variant [7] was distinguished in the bacterial extracts, in contrast to the detection of this and the other two variants distinguished in other molecular studies [8]. However, MS detected peptides from all the three possible variants thus far described.

Table 1. LytFM and its variants detected by MS in the SGM of *Dermatophagoides pteronyssinus* and bacterial culture supernatants of *Bacillus licheniformis* strain 1, *B. licheniformis* strain 2 and *Staphylococcus aureus*.

Source of peptide sequence	Peptide sequence detected	Amino acid	LytFM variants with complete sequence homology to peptides
D. pteronyssinus SGM	MINAPHTGTK	103–112	LytFM and LytFM2
	VASGQYSDPK	61–70	
	SQIGVPYSWGGGGIHGK	11–27	
	MINAPHTGTNVR	103–114	
	EENIWSDHIANVAR	115–128	
	MINAPHTGTNVREENIWSDHIANVAR	103–128	
B. licheniformis strain 1	SQIGVPYSWGGGGIHGK	11–27	LytFM1 or LytFM2
	MINAPHTGTK	103–112	
B. licheniformis strain 2	SQIGVPYSWGGGGIHGK	11–27	LytFM1 or LytFM2
S. aureus	SQIGVPYSWGGGGIHGK	11–27	LytFM1 or LytFM2
	MINAPHTGTK	103–112	

The results obtained in this study verified the contribution of LytFM or its variants, LytFM1/LytFM2, to bacteriolytic activity in the *D. pteronyssinus* SGM as indicated previously by zymographic analysis at 14 kDa [7]. Faint lytic bands at 14 kDa were also previously reported in a zymographic analysis of culture supernatants of HDM-associated isolates *B. licheniformis* strain 1 and *B. licheniformis* strain 2 [12]. Concentration of the bacterial culture supernatants by 80% saturated ammonium sulfate precipitation resulted in enhanced bacteriolytic activity observed at about 14 kDa in the culture supernatants of *B. licheniformis* strain 1, *B. licheniformis* strain 2 and *S. aureus* in the present study in the absence of DTT but not its presence. These data are in contrast to those from previous findings that bacteriolytic activity in *D. pteronyssinus* SGM is enhanced by DTT as determined using a plate lytic assay [7] and that bacteriolytic activity of bacterial proteins from the NlpC/P60 superfamily is enhanced by thiols [7,27–29].

The differences between the two systems, that is plate lytic assay applied previously [7] and zymography, possibly accounted for the phenomenon that bacteriolytic activity at 14 kDa was not observed in the samples analysed if DTT was added to the zymographic buffer. Of the three cysteine residues in LytFM, that is C42, C71 and C129 [7], C42 is a highly conserved cysteine residue found in the C-terminal region of all members of the NlpC/P60 superfamily [30] and is therefore likely to be involved in the catalytic function of the bacteriolytic enzyme. In that regard, C71 likely forms a disulfide bond with C129 [7]. In a zymographic analysis, following SDS/PAGE during which proteins were separated in a denatured form, immersing the polyacrylamide gel in a zymographic buffer containing DTT might lead to protein renaturation that favoured the formation of disulfide bonds between C42 and C71 or C42 and C129, or both, thus inhibiting the bacteriolytic activity of LytFM.

The presence of LytFM was detected by ELISA using anti-peptide antisera against two epitopes, namely GQYSDPKCHHVAYGSHQPGD (84–103) and PHTGTKVREENIGGD (127–141), originally designed based on the LytFM1 sequence [7]. Whilst the anti-GQYSDPKCHHVAYGSHQPGD (84–103) antiserum should recognise all three of the LytFM variants, it is possible that the observed sequence differences in the C-terminal portions may have influenced immunoreactivity using the anti-PHTGT KVREENIGGD (127–141) antiserum, although unlikely given recent information about epitopes and antisera [31]. For example, the corresponding sequences of LytFM and LytFM2 are PHTGTNV REENIWSD (127–141) and PHTGTKVREENIWSD (127–141), respectively, highlighting variation in three residues (K versus N; and GG versus WS). Molecular superimposition modelling shows slight structural deviations with the 'WS' sequence compared with the 'GG' sequence (Fig. S5), but the N versus K substitution did not appear to markedly influence local structures. However, modelling indicates that the antisera recognise surface-located regions as well as possessing disordered secondary structure (Fig. S5), highly predictive indicators of B-cell epitopes [31].

The obvious question arising from the ELISA studies is whether the antisera cross-reacted with non-LytFM-related bacterial proteins belonging to the NlpC/P60 family, as they are ubiquitous and are known to be present in most bacterial species including *B. licheniformis* and *S. aureus* [9,11]. As the sequences chosen for the antisera production were based on a

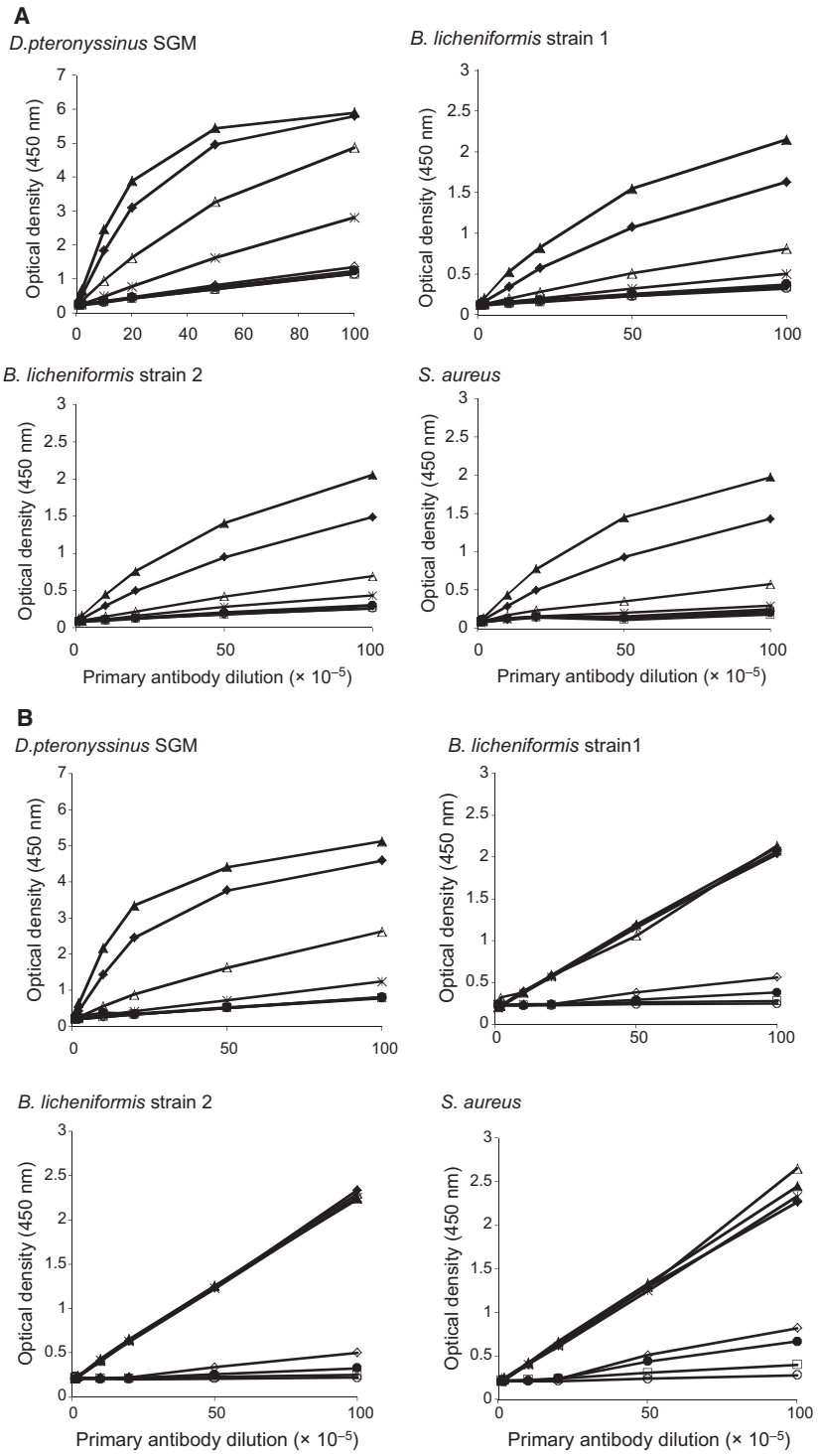


Fig. 4. Immunoreactivity of anti-peptide antisera Rb1001 (A) and Rb999 (B) with *Dermatophagoides pteronyssinus* SGM and bacterial culture supernatants of the HDM-associated *Bacillus licheniformis* strain 1, *B. licheniformis* strain 2 and *Staphylococcus aureus*. Both the polyclonal anti-peptide antisera reacted with 0.01 μg (*), 0.1 μg (△), 1 μg (♦) and 5 μg (▲) of protein found in the *D. pteronyssinus* SGM or bacterial culture supernatants. Preimmune sera Rb 1001 and Rb999 were used as the respective negative controls and were tested using 0.01 μg (○), 0.1 μg (□), 1 μg (●) and 5 μg (◊) of protein in the *D. pteronyssinus* SGM or bacterial culture supernatants. Assays were performed in duplicate, and mean optical density values are shown.

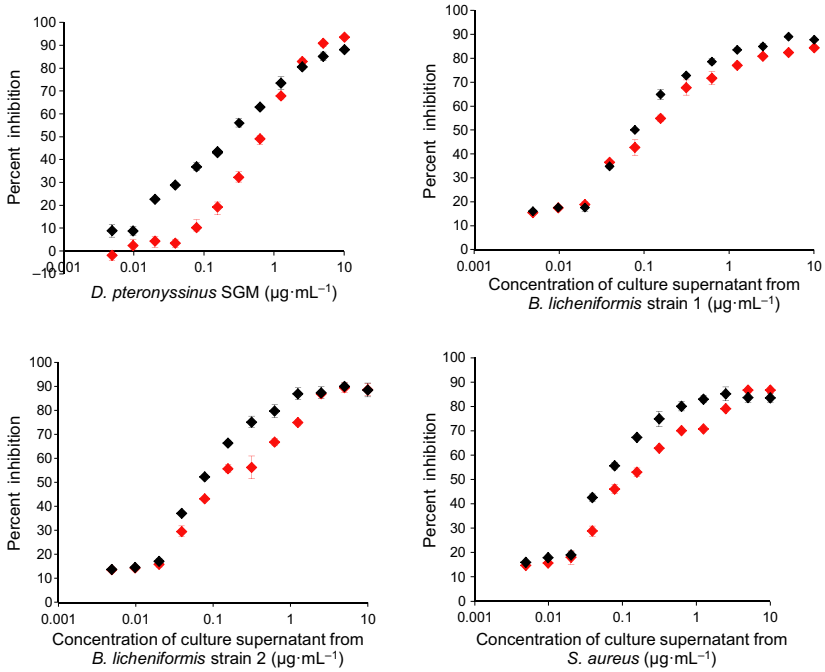
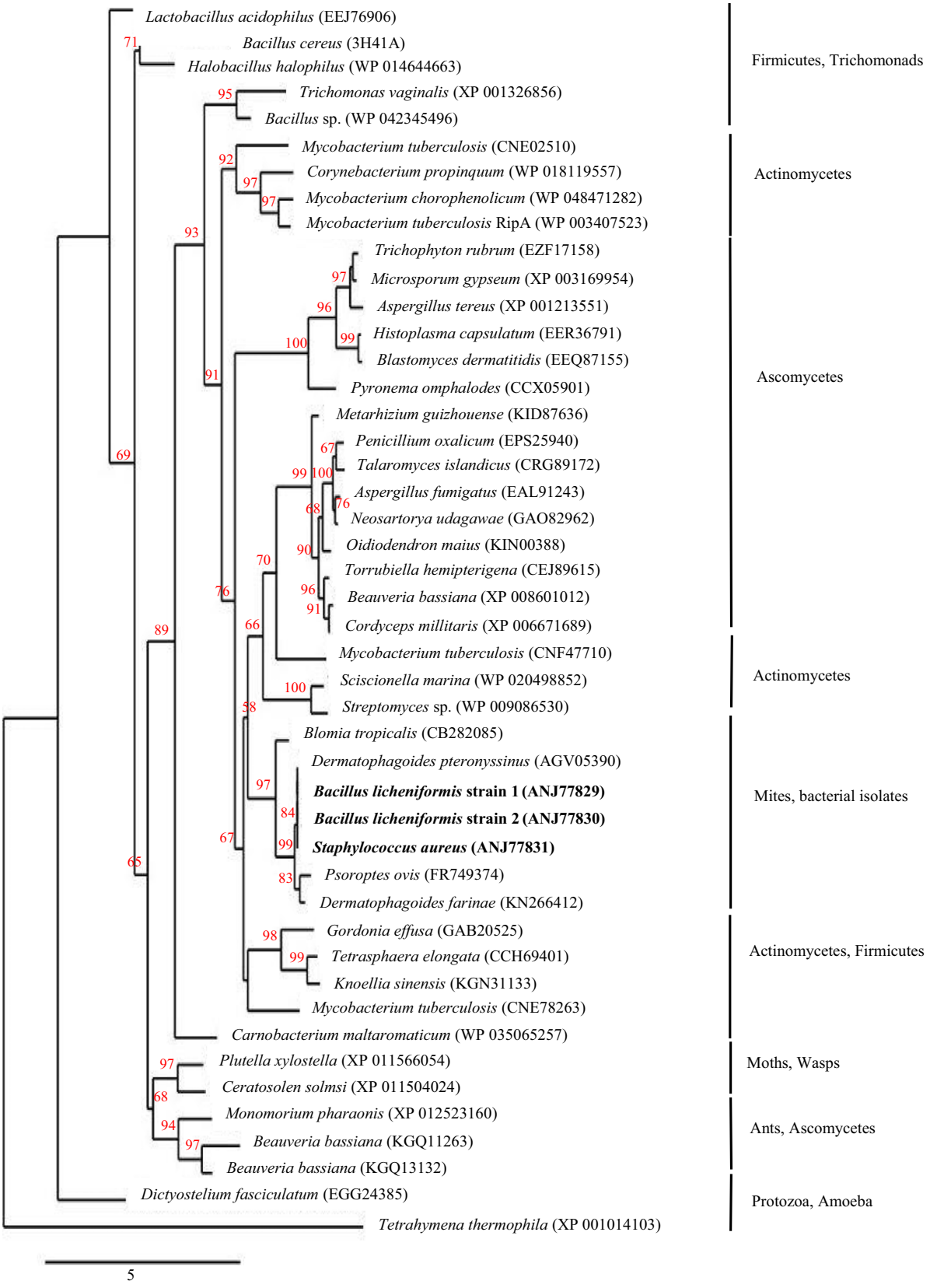


Fig. 5. Inhibition of anti-peptide antisera Rb1001 (in red) and Rb999 (in black) by *Dermatophagoides pteronyssinus* SGM and the bacterial culture supernatants of the HDM-associated *Bacillus licheniformis* strain 1, *B. licheniformis* strain 2 and *Staphylococcus aureus*. Assay for the positive control *D. pteronyssinus* SGM was performed in triplicate, and all the other assays were performed in duplicate. Mean percentage inhibition values are shown. The correlation coefficients (Rb1001/Rb999) for *D. pteronyssinus* SGM, *B. licheniformis* strain 2 and *S. aureus* are 0.93/0.99, 0.95/0.92, 0.97/0.91 and 0.97/0.90, respectively. The IC_{50}, that is concentrations of *D. pteronyssinus* SGM, bacterial culture supernatants of *B. licheniformis* strain 1, *B. licheniformis* strain 2 and *S. aureus* required to generate 50% inhibition of the immunoreactivity of the anti-peptide antisera (Rb1001/Rb999) are 0.51/0.23, 0.15/0.11, 0.17/0.1 and 0.17/0.1 $\mu g \cdot mL^{-1}$, respectively.

2010 BLAST search when limited sequence homology was detected, further sequence identity searches were undertaken (G. A. Stewart, unpublished observations) using nonspecies-restricted pSI-BLAST. In this regard, the peptide sequence GQYSDPKCHHVAYGSHQ PGD (84–103) matched a RipB-like protein (E = 0.002, 13/19 identities) from the American snowberry fruit fly (*Rhagoletis zephyria*). The complete sequences for *R. zephyria* LytFM appear to be the first reported for an insect species and are single domain proteins highly homologous to the mite sequences including those from *Dermatophagoides farinae*, *Psoroptes ovis* and *Blomia tropicalis*. From a phylogenetic perspective, the *R. zephyria* sequences are more similar to those of *B. tropicalis* than the other mite species (Fig. S7).

A search using the peptide sequence PHTGTK VREENIGGD (127–141) revealed homology with LytFM1 (E = 2e-06) and LytFM (E = 0.035) and with a single domain hypothetical NlpC/P60 protein from the ascomycete *Torrubiella hemipterigena* (E = 6.3, 9/15 identities, HTGTKVREQSI (132–142)). A species-specific search against the peptides did not reveal any perfect matches but short (4–5 contiguous amino acids) sequence stretches of homology were identified in various bacterial proteins, although none were related to members of the NlpC/P60 family. Whether such short homologous regions present in bacterial proteins confer immunoreactivity sufficient to account for the data reported in the studies here is unclear, but unlikely. For example, antibody paratopes of about 17 amino acid residues usually interact with sequences (linear or discontinuous) of about 15–22 residues within a B-cell epitope and the 2–5 residues required for optimal binding within this sequence will be dis-

Fig. 6. Phylogenetic relationship of mite and bacterial LytFM homologues and prokaryotic and eukaryotic NlpC/P60 proteins. According to the GenBank records, the accession numbers of *Aspergillus fumigatus* (XP 753281), *Neosartorya udagawae* (GAO84874), *Tetrahymena thermophila* (XM 001014103) and *Dictyostelium fasciculatum* (GL883007) are updated to EAL91243, GAO82962, XP 001014103 and EGG24385, respectively. Phylogenetic analysis was performed with Phylogeny.fr pipeline [26], approximate likelihood ratio test was used, and the confidence values are shown on the branches.


Lactobacillus acidophilus (EEJ76906)
Bacillus cereus (3H41A)
Halobacillus halophilus (WP 014644663)
Trichomonas vaginalis (XP 001326856)
Bacillus sp. (WP 042345496)
Mycobacterium tuberculosis (CNE02510)
Corynebacterium propinquum (WP 018119557)
Mycobacterium chorophenolicum (WP 048471282)
Mycobacterium tuberculosis RipA (WP 003407523)
Trichophyton rubrum (EZF17158)
Microsporum gypseum (XP 003169954)
Aspergillus tereus (XP 001213551)
Histoplasma capsulatum (EER36791)
Blastomyces dermatitidis (EEQ87155)
Pyronema omphalodes (CCX05901)
Metarhizium guizhouense (KID87636)
Penicillium oxalicum (EPS25940)
Talaromyces islandicus (CRG89172)
Aspergillus fumigatus (EAL91243)
Neosartorya udagawae (GAO82962)
Oidiodendron maius (KIN00388)
Torrubiella hemipterigena (CEJ89615)
Beauveria bassiana (XP 008601012)
Cordyceps millitaris (XP 006671689)
Mycobacterium tuberculosis (CNF47710)
Sciscionella marina (WP 020498852)
Streptomyces sp. (WP 009086530)
Blomia tropicalis (CB282085)
Dermatophagoides pteronyssinus (AGV05390)
Bacillus licheniformis strain 1 (ANJ77829)
Bacillus licheniformis strain 2 (ANJ77830)
Staphylococcus aureus (ANJ77831)
Psoroptes ovis (FR749374)
Dermatophagoides farinae (KN266412)
Gordonia effusa (GAB20525)
Tetrasphaera elongata (CCH69401)
Knoellia sinensis (KGN31133)
Mycobacterium tuberculosis (CNE78263)
Carnobacterium maltaromaticum (WP 035065257)
Plutella xylostella (XP 011566054)
Ceratosolen solmsi (XP 011504024)
Monomorium pharaonis (XP 012523160)
Beauveria bassiana (KGQ11263)
Beauveria bassiana (KGQ13132)
Dictyostelium fasciculatum (EGG24385)
Tetrahymena thermophila (XP 001014103)
Firmicutes, Trichomonads
Actinomycetes
Ascomycetes
Actinomycetes
Mites, bacterial isolates
Actinomycetes, Firmicutes
Moths, Wasps
Ants, Ascomycetes
Protozoa, Amoeba
5

tributed throughout the epitope rather than being contiguous [31]. In addition, the majority of antibodies tested against B-cell epitopes react very poorly with peptides of between 7–12 residues and less [31].

LytFM immunoreactivity was not detected with any bacterial sample by ELISA using culture supernatants grown to stationary phase in contrast to data obtained using MS, suggesting differences in sensitivity between the two techniques. LytFM immunoreactivity was, however, readily detected in culture supernatants obtained at the death phase, suggesting that expression of this gene at this time might offer some selective advantage to the organism. For example, peptidoglycan cleavage of the cell wall of dead or dying bacteria may help viable organisms survive when nutrients are fast depleting and waste and toxin levels are rapidly increasing. Expression at an earlier time point, such as log or stationary phase, would likely interfere with peptidoglycan biosynthesis and thus cell wall integrity. In this regard, bacteriolysis has been observed in both Gram-positive and Gram-negative bacteria under conditions of nutrient depletion [32,33]. In addition, starved *Bacillus subtilis* has been reported to secrete lytic enzymes during the early stages of sporulation prompting the suggestion that such enzymes enable a viable population to obtain nutrients from the lysed cells to delay or avoid sporulation [34,35].

In this study, genes *lytFM* and *lytFM1* were found associated with genomic DNA derived from two *B. licheniformis* strains and *S. aureus* isolated from surface-sterilised laboratory-reared *D. pteronyssinus*. However, whole-genome sequencing of *B. licheniformis* and *S. aureus* strains reported to date in public databases such as GenBank has so far failed to reveal any gene encoding the mite enzyme, suggesting that the *lytFM* sequences were not amplified from the bacterial chromosome. However, they may have been amplified from an additional replicon within the mite-associated isolates, such as a plasmid, but this remains to be determined. This is not without precedent for a protein belonging to the NlpC/P60 family as a multidomain hypothetical protein possessing a C-terminal NlpC/P60 domain with significant homology to LytFM and its variants (E = 1e-26) has been found in one of two plasmids [pKRAD01 (accession number CP000751.1)] of the Gram-positive bacterial species *Kineococcus radiotolerans*.

The possibility of a lateral gene transfer of a mite gene and its variants to microbial endosymbionts is consistent with our previous data [8] and the phylogenetic data presented in this study showing that mite LytFM homologues form a monophyletic group with an evolutionary link to one or more prokaryotic endosymbionts. Similar phenomena involving an arthropod [16] and humans [14,15] and their microbial endosymbionts have been reported. Transposons, direct or indirect repeats were not detected in the 5′ and 3′ flanking sequence of *lytFM* in a *D. pteronyssinus* genomic DNA library [8], suggesting no involvement of mobile genetic elements such as transposons [14–16] in the cross-kingdom gene transfer. However, lateral gene transfer between two microbes within the HDM might also be facilitated by plasmid transfer [36], which has been reported to occur between *Bacillus* strains [37] and between *Bacillus* species and *S. aureus* [37,38]. In either scenario, the gene acquired in a plasmid by a microbe could move by recombination events into its chromosome or be further transferred to another microbe [36].

Regardless of the exact paths through which *B. licheniformis* strain 1, *B. licheniformis* strain 2 and *S. aureus* acquired the genes, the presence of a gene in a microbe that showed complete homology with that of its eukaryotic source indicates that lateral gene transfer occurred recently [14]. The absence of bacterial homologues of LytFM/LytFM1/LytFM2 in any protein databases, which is in line with the findings reported previously [15], is further indication that the lateral gene transfer occurred recently. In addition, *lytFM* and its variants were not found in *B. cereus*, two other *B. licheniformis* strains and two other Staphylococcal species isolates included in this study, with similar phenomena reported previously [14,36]. In concert with data obtained from MS analysis revealing that several other *D. pteronyssinus* proteins were expressed by *B. licheniformis* strain 1, *B. licheniformis* strain 2 and *S. aureus* (Table S1), these results indicated for the first time, the possible acquisition of genes by mite-associated bacterial species from a HDM through lateral gene transfer.

Acknowledgements

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Author contributions

VHT designed, planned and performed the experiments, acquired and analysed the data and wrote the manuscript. GAS provided the experimental reagents and materials, advised in the design and planning of

experiments, contributed to data analysis and edited the manuscript. BJC contributed to the design and planning of experiments, oversaw the progress of the experimental work, contributed to data analysis and edited the manuscript.

References

1 Tovey ER, Chapman MD and Platts-Mills TA (1981) Mite faeces are a major source of house dust allergens. *Nature* **289**, 592–593.

2 Arlian LG, Bernstein IL, Geis DP, Vyszenski-Moher DL, Gallagher JS and Martin B (1987) Investigations of culture medium-free house dust mites. III. Antigens and allergens of body and fecal extract of *Dermatophagoides farinae*. *J Allergy Clin Immunol* **79**, 457–466.

3 Stewart GA, Lake FR and Thompson PJ (1991) Faecally derived hydrolytic enzymes from *Dermatophagoides pteronyssinus*: physicochemical characterisation of potential allergens. *Int Arch Allergy Appl Immunol* **95**, 248–256.

4 Arlian LG and Platts-Mills TA (2001) The biology of dust mites and the remediation of mite allergens in allergic disease. *J Allergy Clin Immunol* **107**, S406–S413.

5 Colloff MJ (2009) Dust mites. In *Identification and Taxonomy, Classification and Phylogeny* (Colloff MJ, ed.), pp. 1–44. CSIRO Publishing, Melbourne, Vic., Australia.

6 Stewart GA, Bird CH, Krska KD, Colloff MJ and Thompson PJ (1992) A comparative study of allergenic and potentially allergenic enzymes from *Dermatophagoides pteronyssinus*, *D. farinae* and *Euroglyphus maynei*. *Exp Appl Acarol* **16**, 165–180.

7 Mathaba LT, Pope CH, Lenzo J, Hartofillis M, Peake H, Moritz RL, Simpson RJ, Bubert A, Thompson PJ and Stewart GA (2002) Isolation and characterisation of a 13.8-kDa bacteriolytic enzyme from house dust mite extracts: homology with prokaryotic proteins suggests that the enzyme could be bacterially derived. *FEMS Immunol Med Microbiol* **33**, 77–88.

8 Tang VH, Stewart GA and Chang BJ (2015) House dust mites possess a polymorphic, single domain putative peptidoglycan d, l endopeptidase belonging to the NlpC/P60 Superfamily. *FEBS Open Bio* **5**, 813–823.

9 Rawlings ND, Barrett AJ and Finn R (2016) Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res* **44**, D343–D350.

10 Erban T and Hubert J (2008) Digestive function of lysozyme in synanthropic acaridid mites enables utilization of bacteria as a food source. *Exp Appl Acarol* **44**, 199–212.

11 Anantharaman V and Aravind L (2003) Evolutionary history, structural features and biochemical diversity of the NlpC/P60 superfamily of enzymes. *Genome Biol* **4**, R11.

12 Tang VH, Chang BJ, Srinivasan A, Mathaba LT, Harnett GB and Stewart GA (2013) Skin-associated Bacillus, Staphylococcal and Micrococcal species from the house dust mite, *Dermatophagoides pteronyssinus* and bacteriolytic enzymes. *Exp Appl Acarol* **61**, 431–447.

13 Chaisiri K, Mcgarry JW, Morand S and Makepeace BL (2015) Symbiosis in an overlooked microcosm: a systematic review of the bacterial flora of mites. *Parasitology* **142**, 1152–1162.

14 Anderson MT and Seifert HS (2011) Opportunity and means: horizontal gene transfer from the human host to a bacterial pathogen. *MBio* **2**, e00005–e00011.

15 Patrick S, Jobling KL, O'Connor D, Thacker Z, Dryden DT and Blakely GW (2011) A unique homologue of the eukaryotic protein-modifier ubiquitin present in the bacterium *Bacteroides fragilis*, a predominant resident of the human gastrointestinal tract. *Microbiology* **157**, 3071–3078.

16 Duplouy A, Iturbe-Ormaetxe I, Beatson SA, Szubert JM, Brownlie JC, McMeniman CJ, McGraw EA, Hurst GD, Charlat S, O'Neill SL *et al*. (2013) Draft genome sequence of the male-killing Wolbachia strain wBol1 reveals recent horizontal gene transfers from diverse sources. *BMC Genom* **14**, 20.

17 Sambrook J and Russell DW (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

18 Audy P, Grenier J and Asselin A (1989) Lysozyme activity in animal extracts after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Comp Biochem Physiol* **92**, 523–527.

19 Blum H, Beier H and Gross HJ (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**, 93–99.

20 Bringans S, Eriksen S, Kendrick T, Gopalakrishnakone P, Livk A, Lock R and Lipscombe R (2008) Proteomic analysis of the venom of *Heterometrus longimanus* (Asian black scorpion). *Proteomics* **8**, 1081–1096.

21 Modrow S and Wolf H (1986) Characterization of two related *Epstein-Barr* virus-encoded membrane proteins that are differentially expressed in Burkitt lymphoma and in vitro-transformed cell lines. *Proc Natl Acad Sci USA* **83**, 5703–5707.

22 Jameson BA and Wolf H (1988) The antigenic index: a novel algorithm for predicting antigenic determinants. *Comput Appl Biosci* **4**, 181–186.

23 Kelley LA and Sternberg MJ (2009) Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc* **4**, 363–371.

24 Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC and Ferrin TE (2004) UCSF Chimera – a visualization system for exploratory research and analysis. *J Comput Chem* **25**, 1605–1612.

25 Böth D, Schneider G and Schnell R (2011) Peptidoglycan remodeling in *Mycobacterium tuberculosis*: comparison of structures and catalytic activities of RipA and RipB. *J Mol Bio* **413**, 247–260.
26 Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, Guindon S, Lefort V, Lescot M *et al*. (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res* **36**, W465–W469.
27 Wuenscher MD, Kohler S, Bubert A, Gerike U and Goebel W (1993) The *iap* gene of *Listeria monocytogenes* is essential for cell viability, and its gene product, p60, has bacteriolytic activity. *J Bacteriol* **175**, 3491–3501.
28 Bourgogne T, Vacheron MJ, Guinand M and Michel G (1992) Purification and partial characterization of the gamma-D-glutamyl-L-di-amino acid endopeptidase II from *Bacillus sphaericus*. *Int J Biochem* **24**, 471–476.
29 Margot P, Pagni M and Karamata D (1999) *Bacillus subtilis* 168 gene *lytF* encodes a gamma-D-glutamate-meso-diaminopimelate muropeptidase expressed by the alternative vegetative sigma factor, sigmaD. *Microbiology* **145** (Pt 1), 57–65.
30 Bateman A, Birney E, Durbin R, Eddy SR, Howe KL and Sonnhammer EL (2000) The Pfam protein families database. *Nucleic Acids Res* **28**, 263–266.
31 Rahman KHS, Chowdhury EU, Sachse K and Kaltenboeck B (2016) Inadequate reference datasets biased toward short non-epitopes confound B-cell epitope prediction. *J Biol Chem* **291**, 14585–14599.
32 Rosenbluh A and Rosenberg E (1993) Developmental lysis and auticides. In *Myxobacteria II* (Dworkin M and Kaiser D, eds), pp. 213–233. American Society for Microbiology, Washington, DC.
33 Vollmer W (2008) Structural variation in the glycan strands of bacterial peptidoglycan. *FEMS Microbiol Rev* **32**, 287–306.
34 Gonzalez-Pastor JE, Hobbs EC and Losick R (2003) Cannibalism by sporulating bacteria. *Science* **301**, 510–513.
35 Ellermeier CD and Losick R (2006) Evidence for a novel protease governing regulated intramembrane proteolysis and resistance to antimicrobial peptides in *Bacillus subtilis*. *Genes Dev* **20**, 1911–1922.
36 Asteri IA, Boutou E, Anastasiou R, Pot B, Vorgias CE, Tsakalidou E and Papadimitriou K (2011) In silico evidence for the horizontal transfer of *gsiB*, a sigma(B)-regulated gene in gram-positive bacteria, to lactic acid bacteria. *Appl Environ Microbiol* **77**, 3526–3531.
37 Koehler TM and Thorne CB (1987) *Bacillus subtilis* (natto) plasmid pLS20 mediates interspecies plasmid transfer. *J Bacteriol* **169**, 5271–5278.
38 Polak J and Novick RP (1982) Closely related plasmids from *Staphylococcus aureus* and soil bacilli. *Plasmid* **7**, 152–162.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Amplification of *lytFM* from the HDM-associated *B. licheniformis* 1, *B. licheniformis* 2 (upper panel) and *S. aureus* (lower panel) using the primer set GSUTR1/GSR3 was validated by the absence of any amplicon when the PCR was repeated without any primers (-) or when a negative control PCR was performed with the primers in the absence of any template.

Fig. S2. Zymographic analysis of bacteriolytic activity in bacterial culture supernatants of *B. licheniformis* strain 4, *S. epidermidis*, *M. luteus*, *B. cereus*, *B. licheniformis* strain 3 and *S. capitis*.

Fig. S3. MS detection of the two peptides M_{103}INAPHTGTK$_{112}$ and V_{61}ASGQYSDPK$_{70}$ in the *D. pteronyssinus* SGM (A), S_{11}QIGVPYSWGGGGIHGK$_{27}$ and M_{103}INAPHTGTK$_{112}$ in the culture supernatant of *B. licheniformis* 1 (B), S_{11}QIGVPYSWGGGGIHGK$_{27}$ in the culture supernatant of *B. licheniformis* 2 (C) and S_{11}QIGVPYSWGGGGIHGK$_{27}$ and M_{103}INAPHTGTK$_{112}$ in the culture supernatant of *S. aureus* (D).

Fig. S4. The antigenic indices for all the potential epitopes of LytFM1 were calculated as described previously [22] using the programs PeptideStructure and PlotStructure [21] (upper panel).

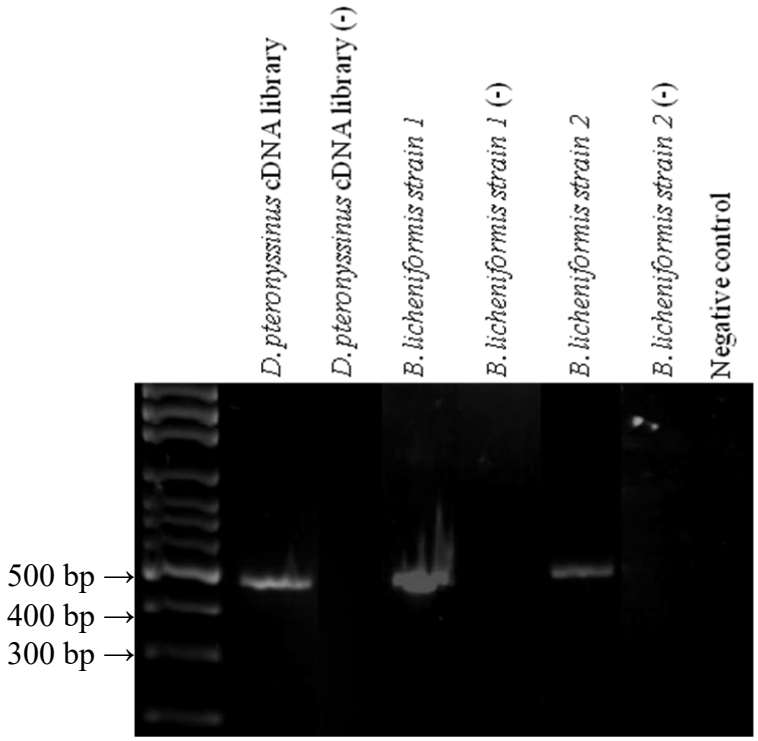
Fig. S5. Relative location of the two regions of LytFM used in the design of the anti-LytFM1 peptide antisera described in this study.

Fig. S6. Immunoreactivity of anti-peptide antisera Rb1001 (A) and Rb999 (B) with the bacterial culture supernatants of the HDM-associated *B. licheniformis* strain 4, *S. epidermidis*, *M. luteus*, *B. cereus*, *B. licheniformis* strain 3 and *S. capitis*.

Fig. S7. Alignment of the amino acid sequences of *Rhagoletis zephyria* peptidoglycan endopeptidase RipB-like protein, *D. pteronyssinus* LytFM and LytFM1 and the LytFM homologues of *B. tropicalis*, *D. farinae* and *P. ovis*.

Table S1. Summary of proteins other than LytFM1 present in the bacterial culture supernatants of the HDM-associated *B. licheniformis* strain 1, *B. licheniformis* strain 2 and *S. aureus* following analysis by MS.

Fig. S1


D. pteronyssinus cDNA library
D. pteronyssinus cDNA library (-)
B. licheniformis strain 1
B. licheniformis strain 1 (-)
B. licheniformis strain 2
B. licheniformis strain 2 (-)
Negative control
500 bp →
400 bp →
300 bp →

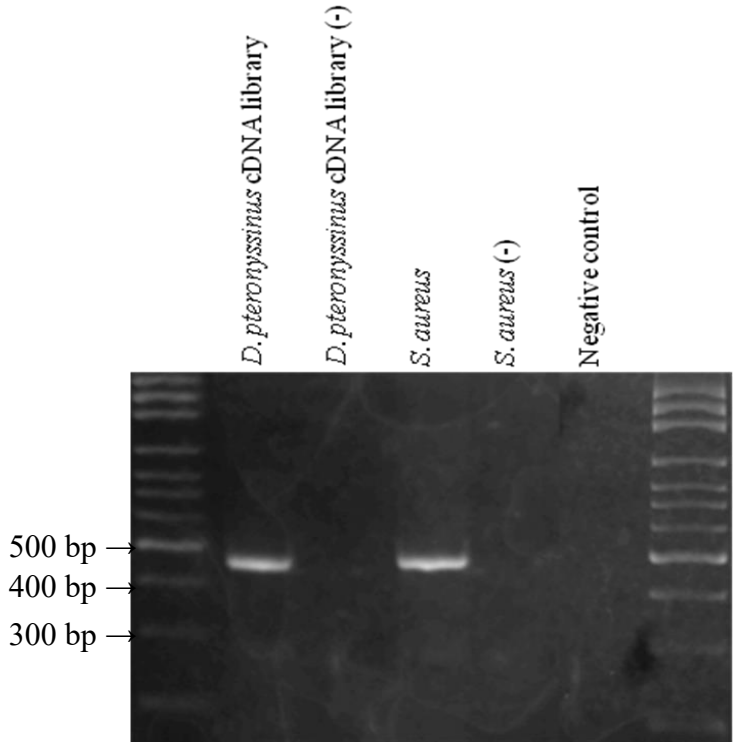
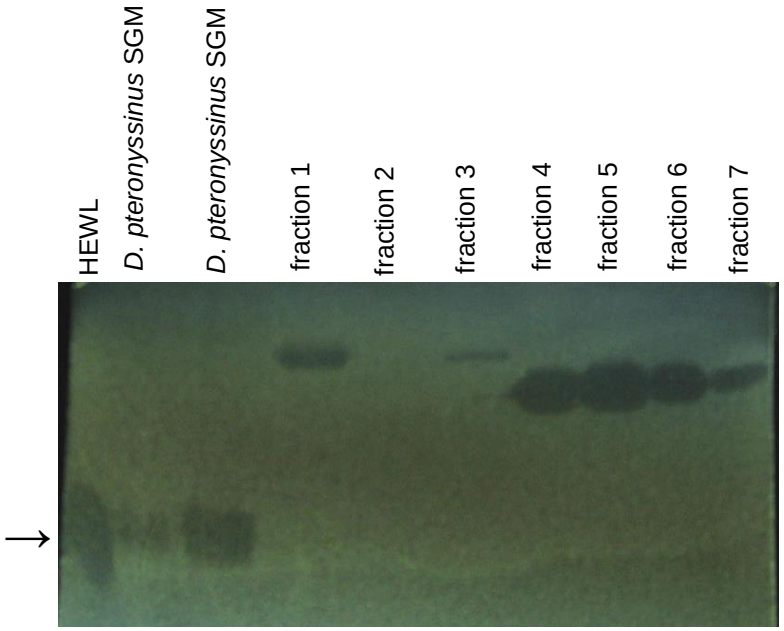
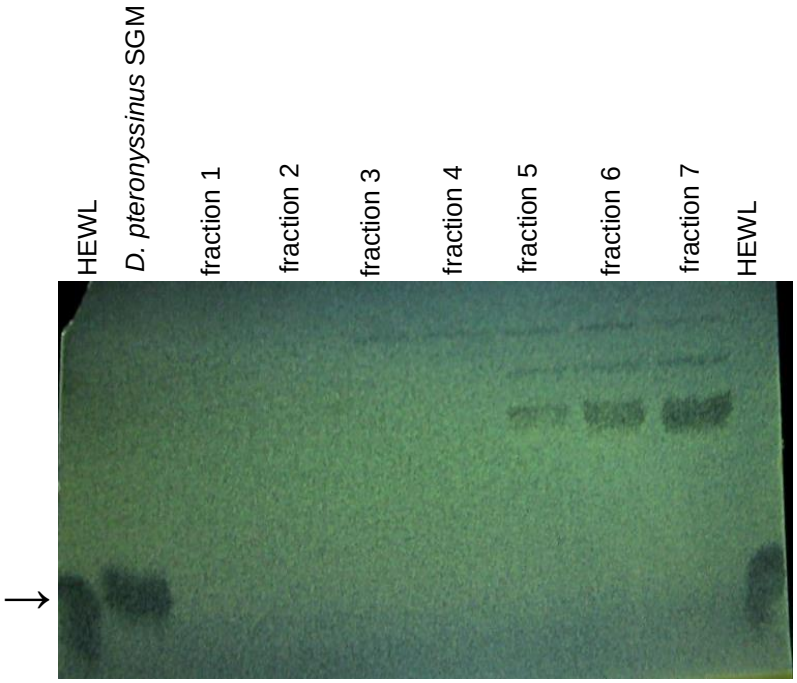

D. pteronyssinus cDNA library
D. pteronyssinus cDNA library (-)
S. aureus
S. aureus (-)
Negative control
500 bp →
400 bp →
300 bp →

Fig. S2

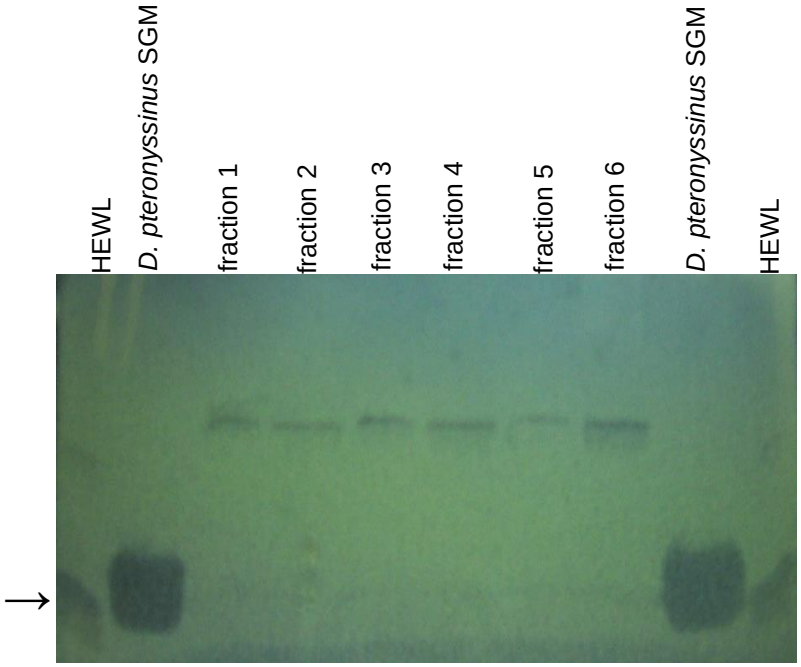
B. licheniformis strain 4



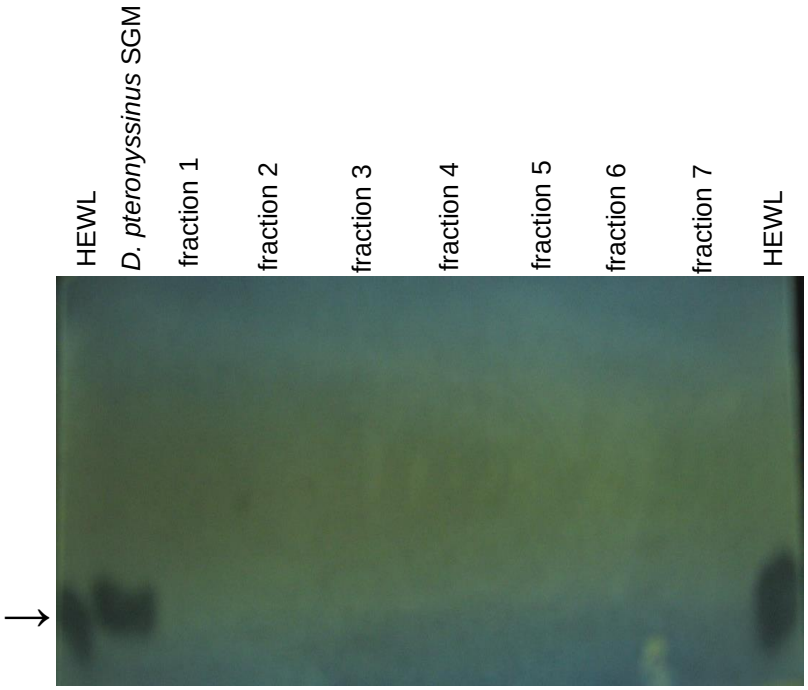
S. epidermidis



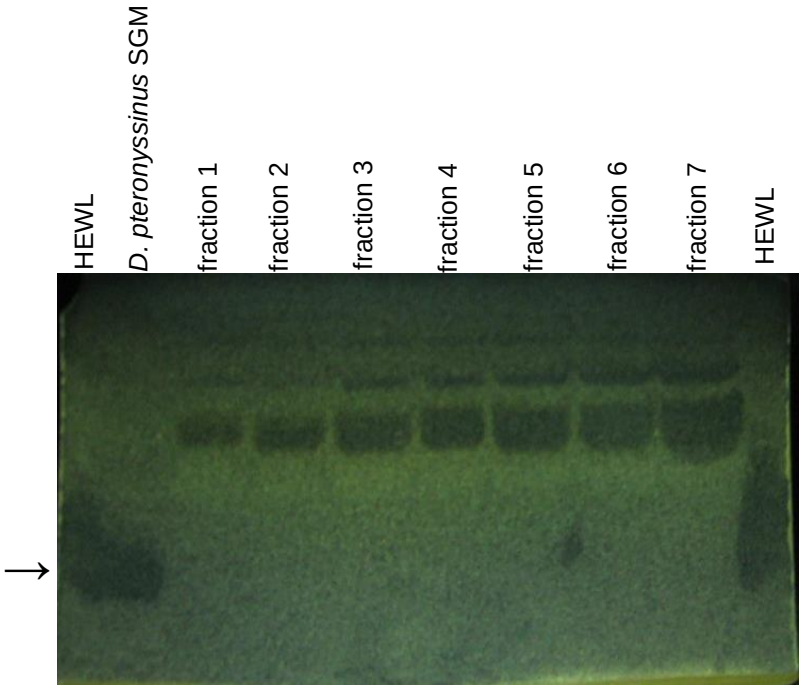
M. luteus



B. cereus



B. licheniformis strain 3



S. capitis

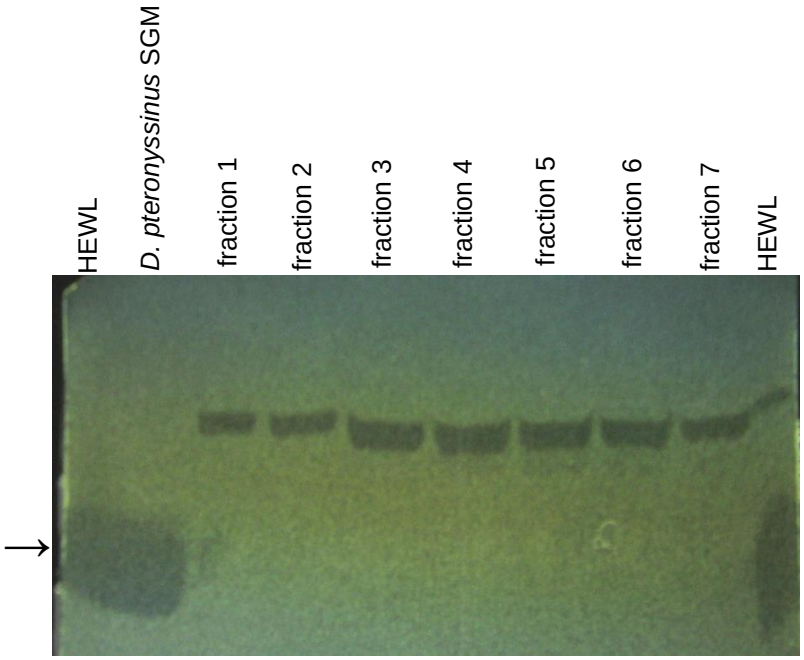
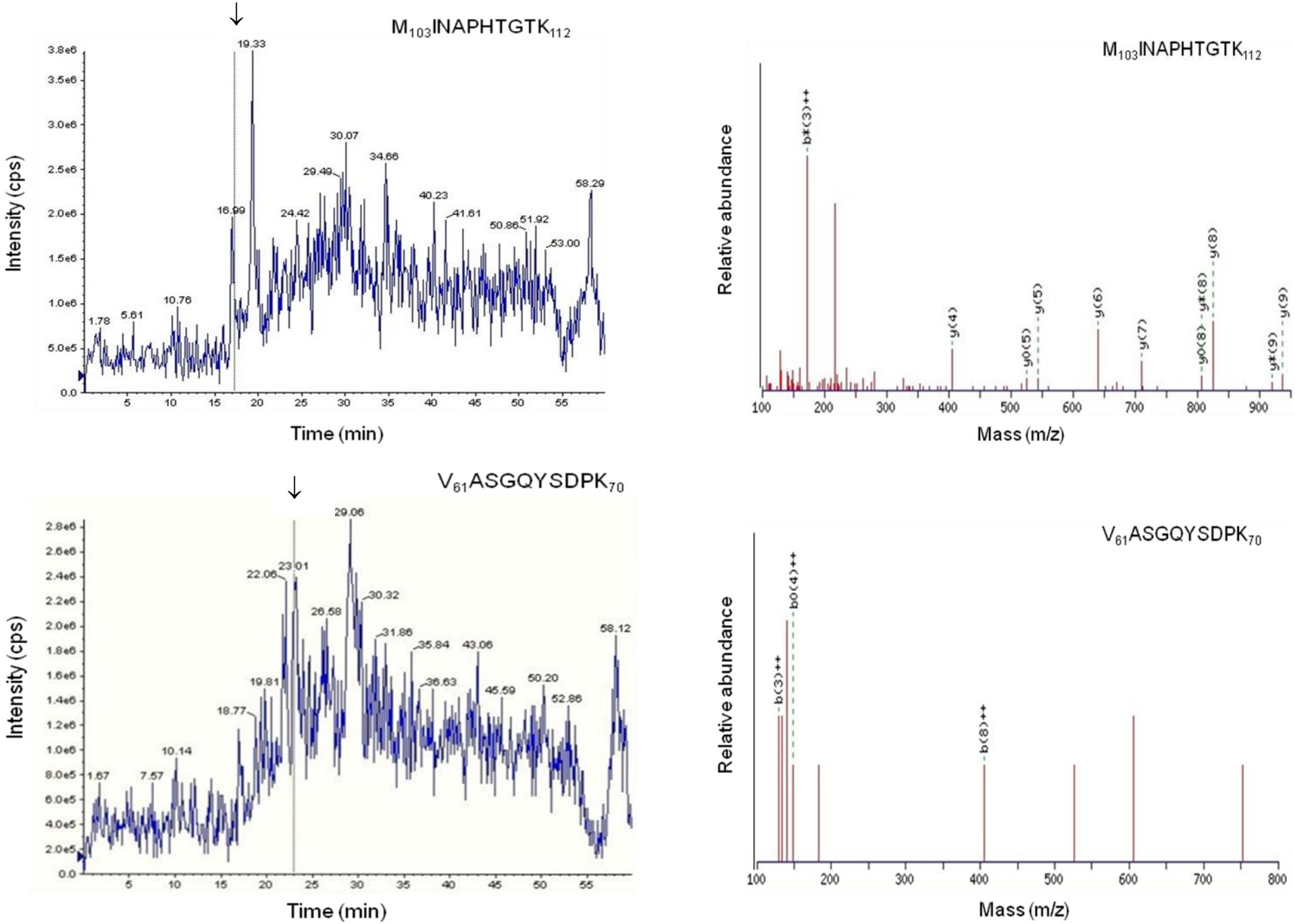
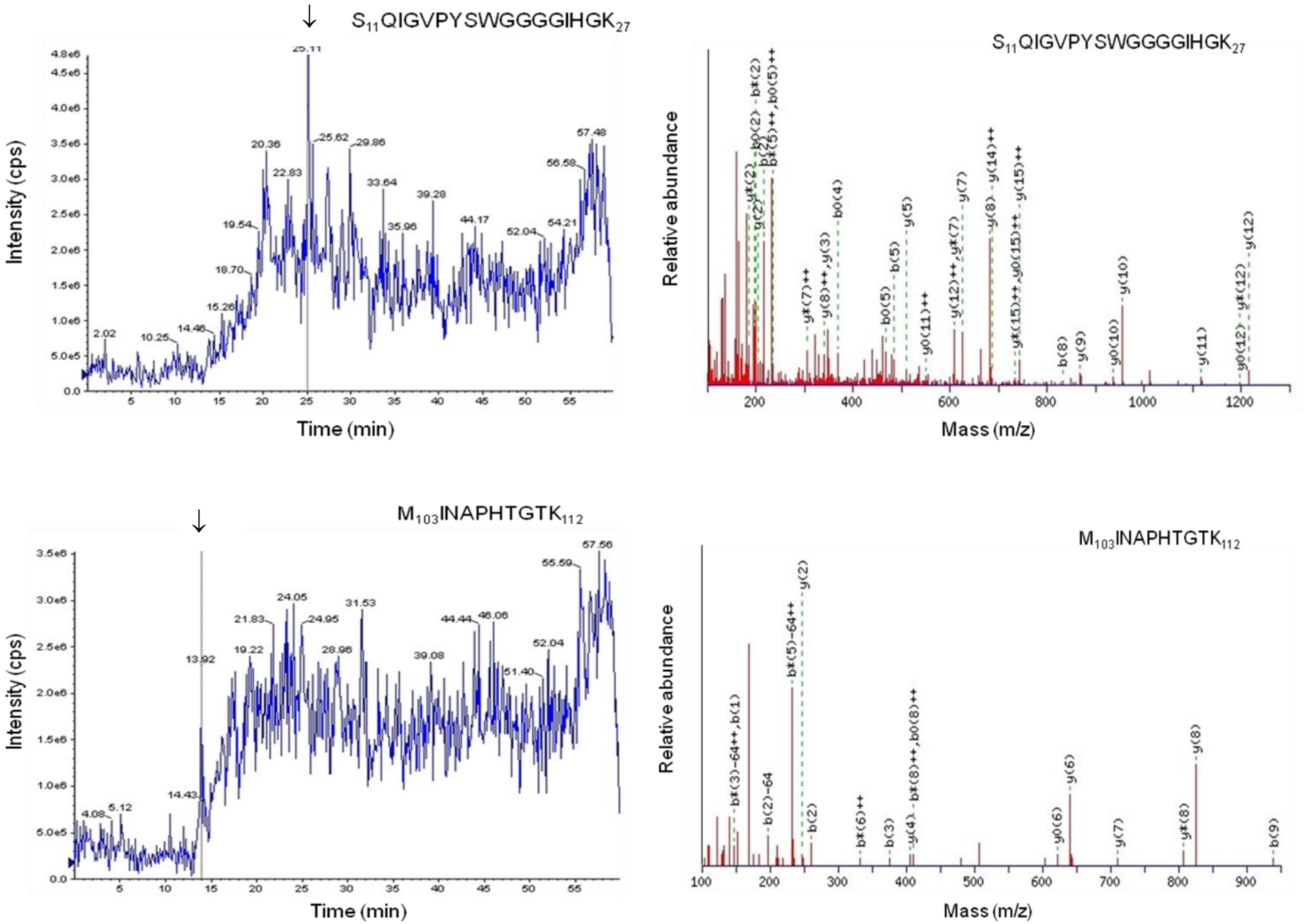


Fig. S3

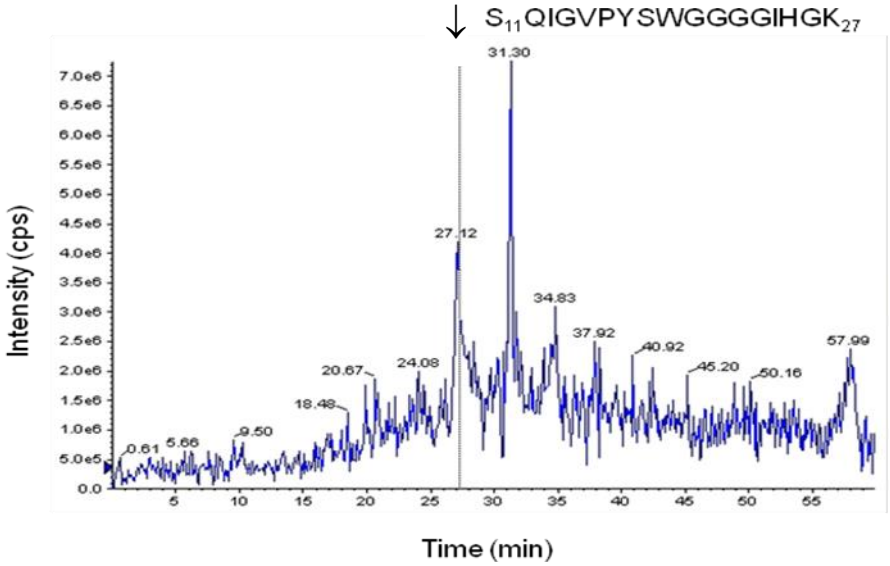
A

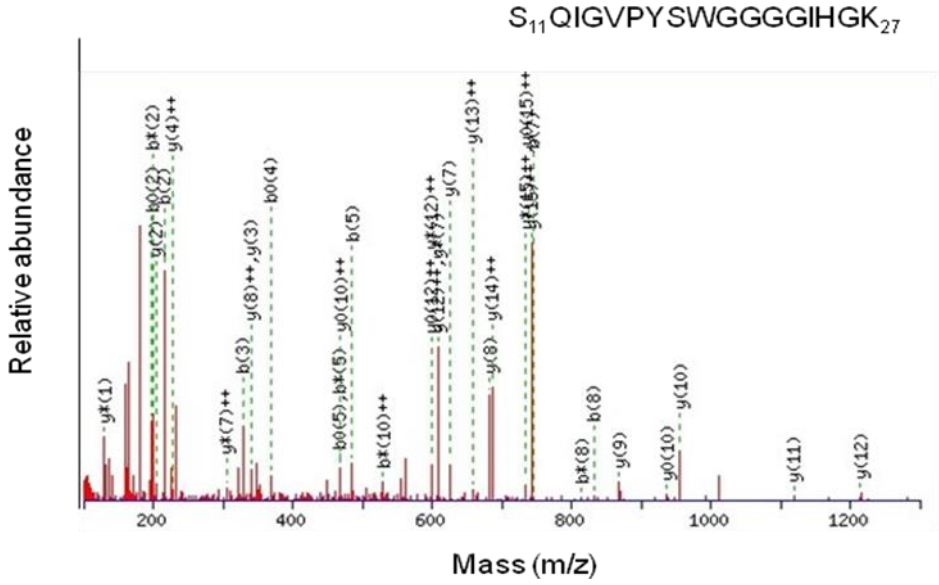

M103INAPHTGTK112
Intensity (cps)
Time (min)
19.33
30.07
34.66
29.49
40.23
41.61
58.29
16.99
24.42
50.86
51.92
53.00
10.76
5.61
1.78
M103INAPHTGTK112
Relative abundance
Mass (m/z)
b*(3)++
y(4)
y0(5)
y(5)
y(6)
y(7)
y0(8)
y*(8)
y(8)
y*(9)
y(9)
V61ASGQYSDPK70
Intensity (cps)
Time (min)
29.06
23.01
22.06
30.32
26.58
31.86
35.84
43.06
58.12
19.81
36.63
45.59
50.20
52.86
18.77
10.14
7.57
1.67
V61ASGQYSDPK70
Relative abundance
Mass (m/z)
b0(4)++
b(3)++
b(8)++

B


S11QIGVPYSWGGGGIHGK27
Intensity (cps)
Time (min)
Relative abundance
Mass (m/z)
M103INAPHTGTK112

C


S11QIGVPYSWGGGGIHGK27
Intensity (cps)
Time (min)
31.30
27.12
34.83
37.92
40.92
45.20
50.16
57.99
24.08
20.67
18.48
9.50
5.66
0.61


S11QIGVPYSWGGGGIHGK27
Relative abundance
Mass (m/z)

D

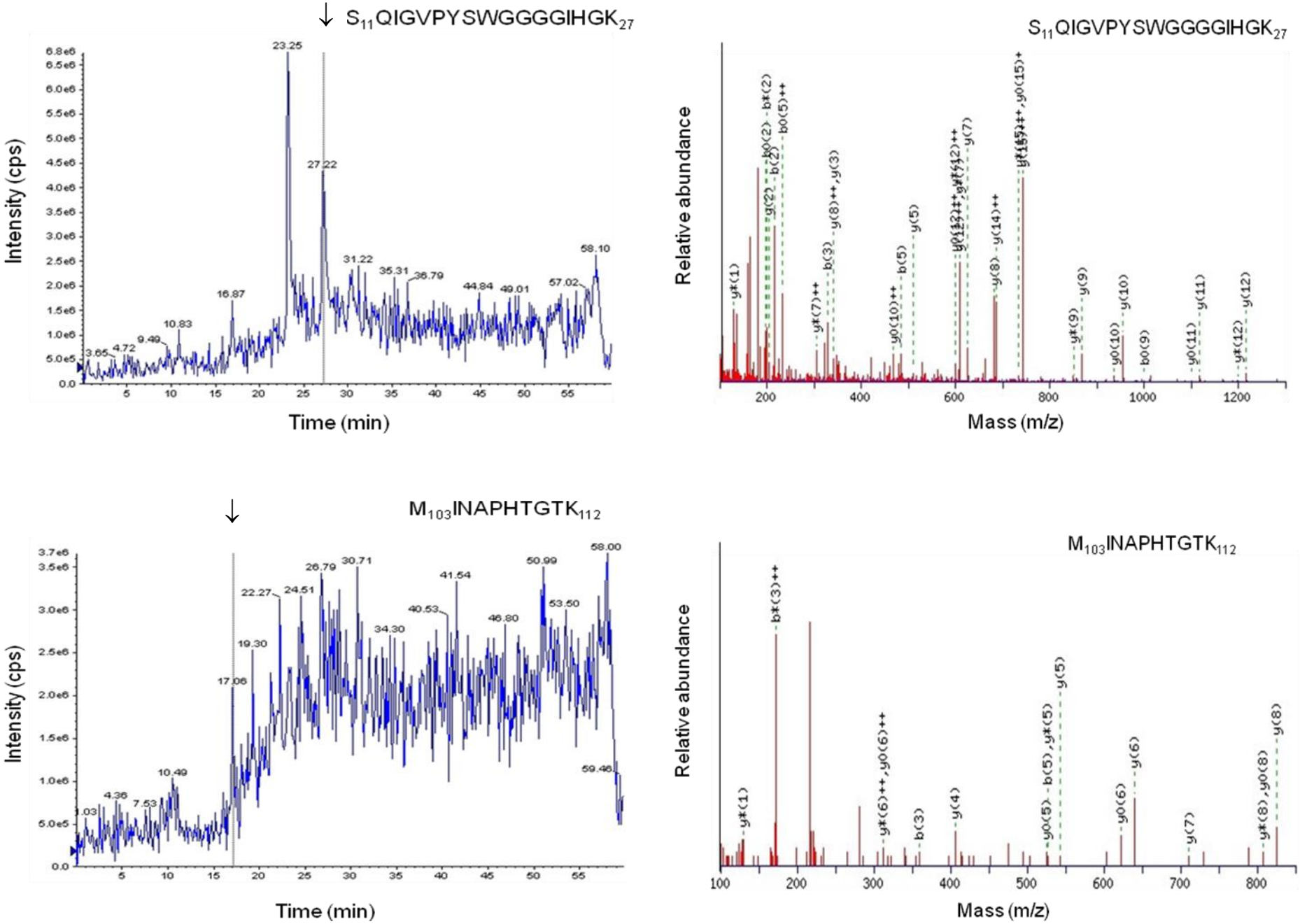
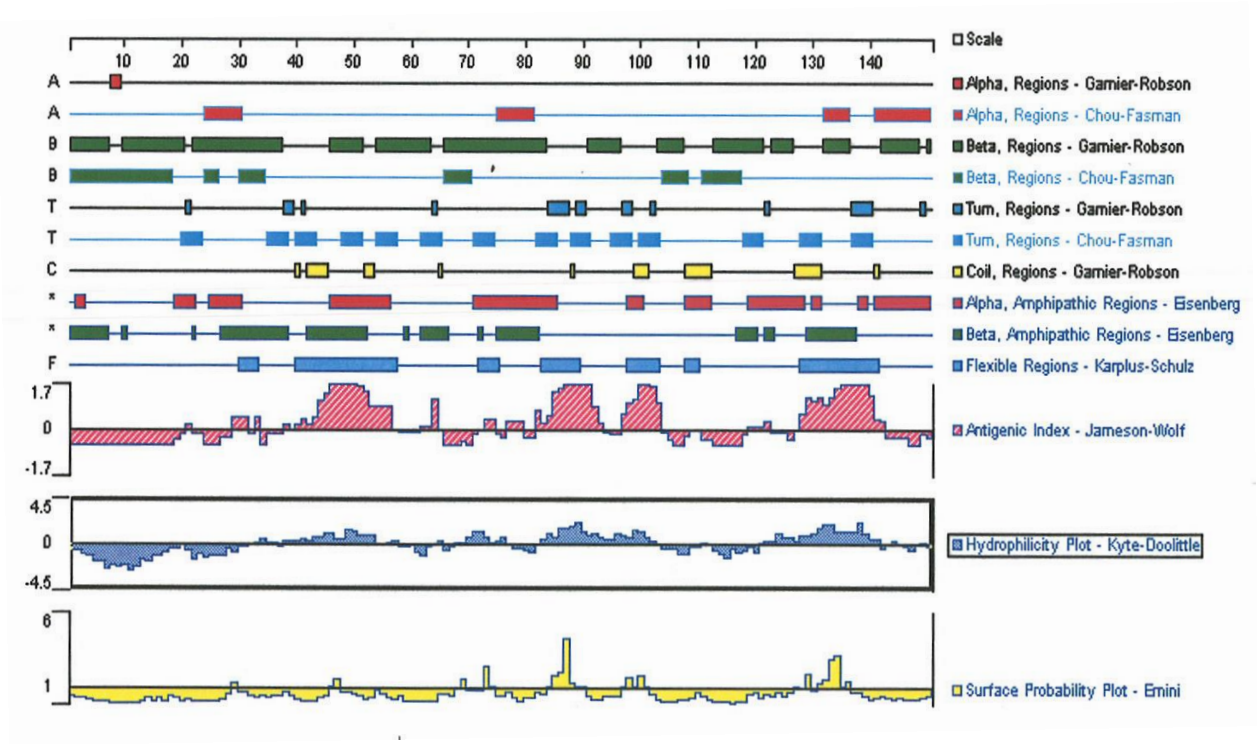

↓ S11QIGVPYSWGGGGIHGK27
Intensity (cps)
Time (min)
S11QIGVPYSWGGGGIHGK27
Relative abundance
Mass (m/z)
↓ M103INAPHTGTK112
Intensity (cps)
Time (min)
M103INAPHTGTK112
Relative abundance
Mass (m/z)

Fig. S4



MKFFFTLALFCTLAISQVYCNGAAIVSAARSQIGVPYSWGGGGIHGKSRGIGEGANTVGFDCSGLAQYS
VYQGTHKVLARVASGQYSDPKCHHVAYGSHQPGDLVFFGNPIHHVGIVSAHGRMINAPHTGTKVREENI
GGDHIANVARCW

Fig. S5

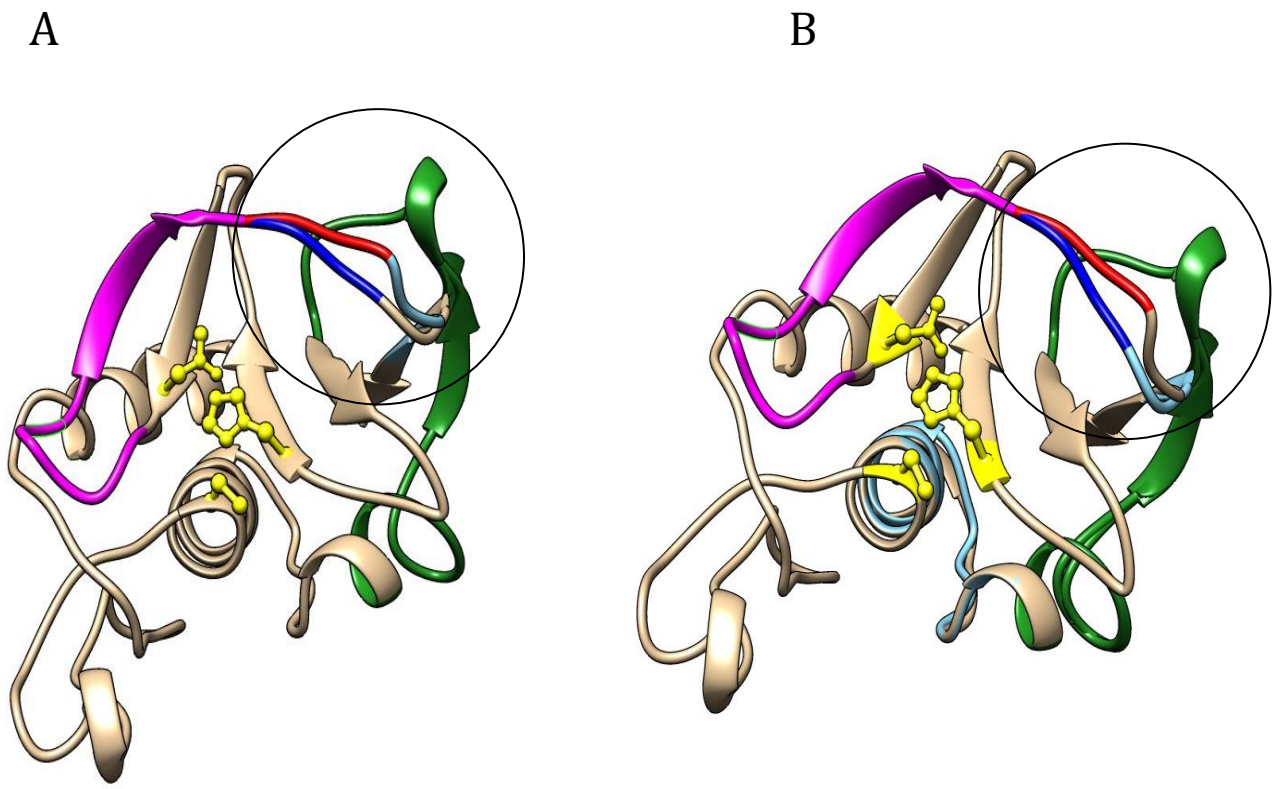
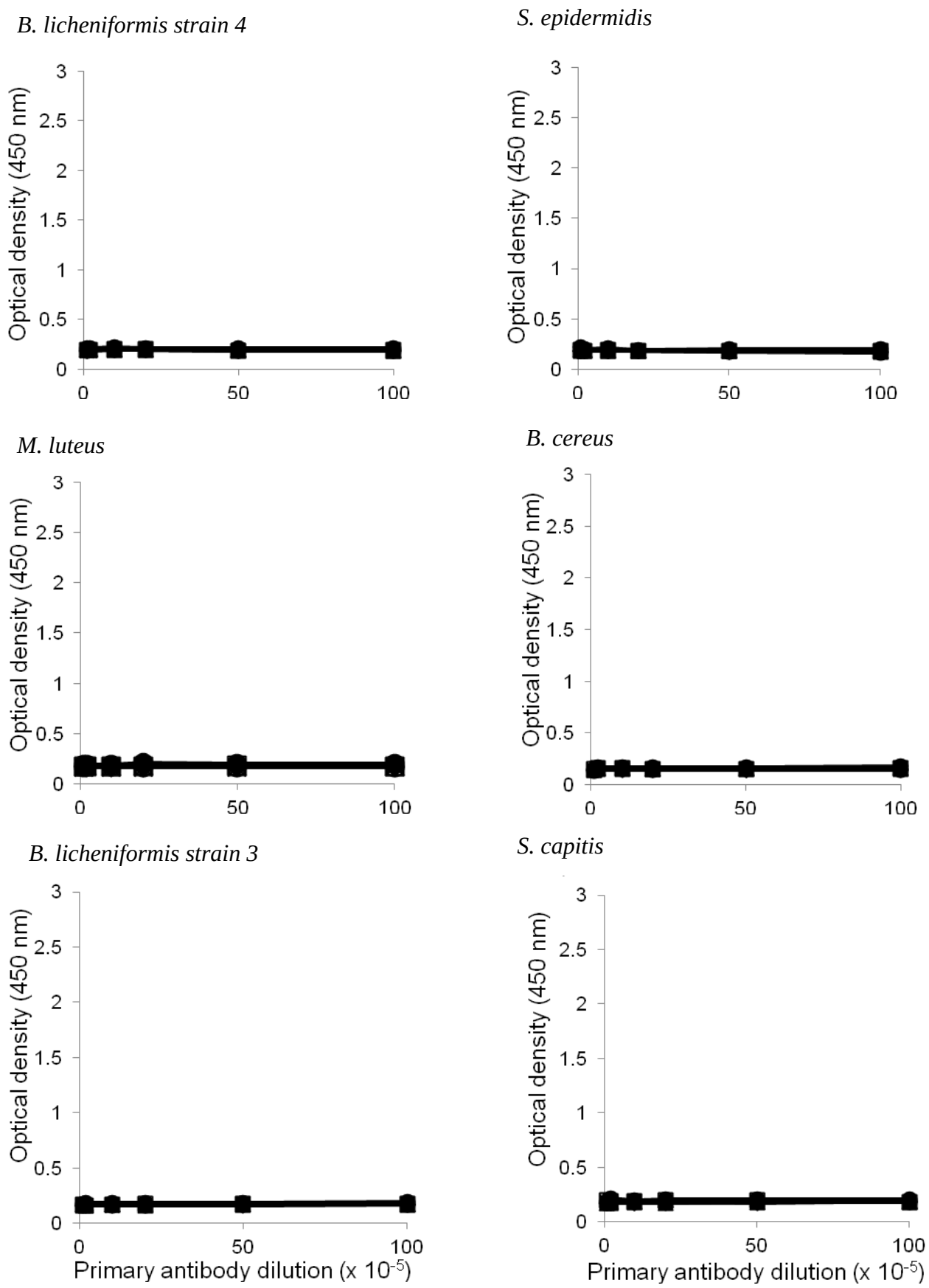


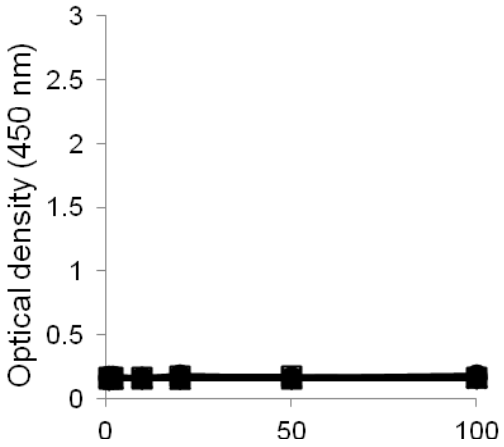
Fig. S6

A

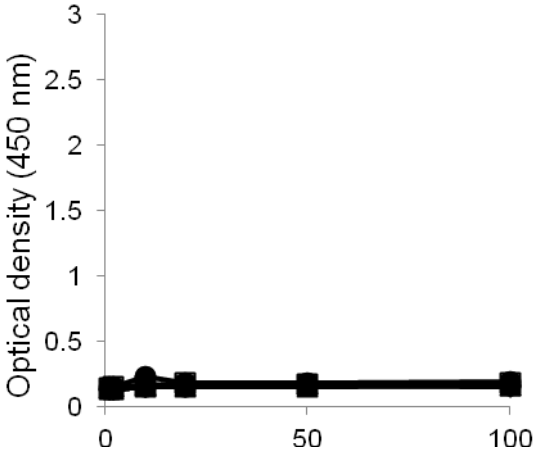

B. licheniformis strain 4
S. epidermidis
M. luteus
B. cereus
B. licheniformis strain 3
S. capitis
Optical density (450 nm)
0
0.5
1
1.5
2
2.5
3
0
50
100
Primary antibody dilution (x 10-5)

B

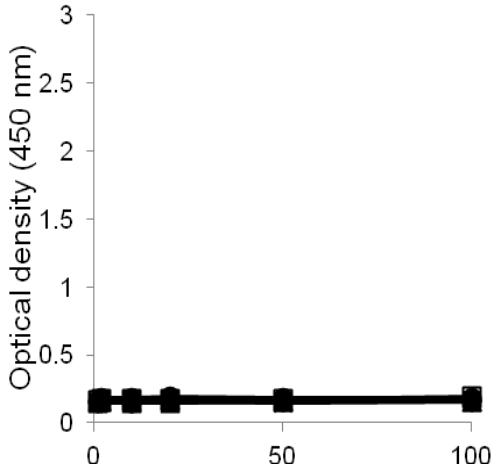
B. licheniformis strain 4



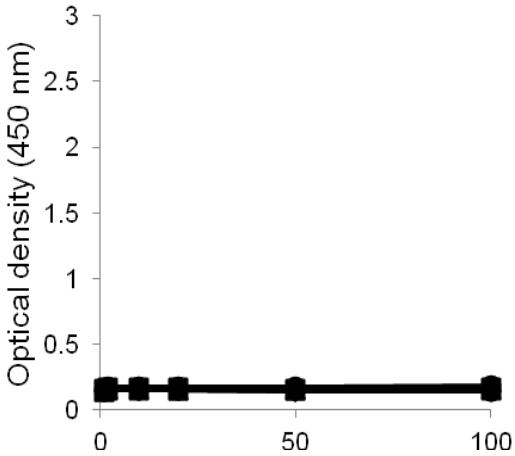
S. epidermidis



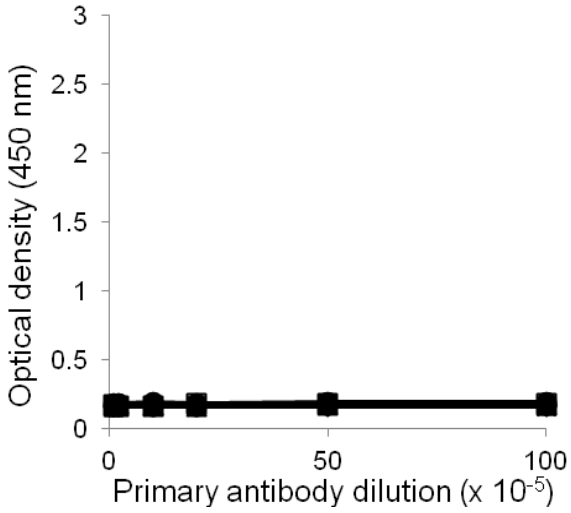
M. luteus



B. cereus



B. licheniformis strain 3



S. capitis

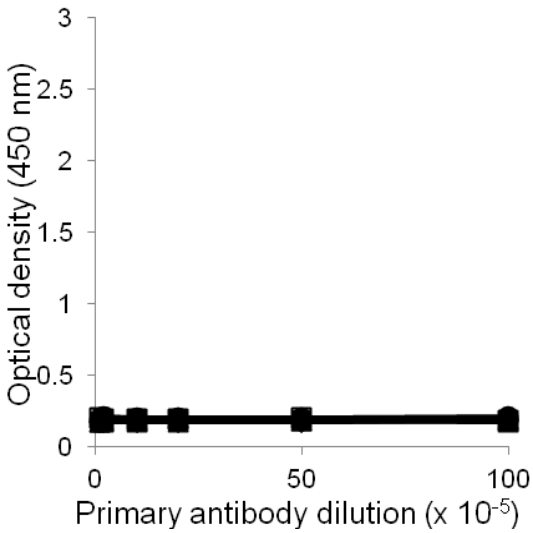


Fig. S7

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Table S1: Summary of proteins other than LytFM1 present in the bacterial culture supernatants of the HDM-associated *B. licheniformis strain 1*, *B. licheniformis strain 2* and *S. aureus* following analysis by MS.

Microorganism (source of secreted proteins)	Protein identity and accession number	Size of protein	Microorganism possessing protein	Peptide sequence	Probability-based score*	Rank
B. licheniformis 1	Trypsin-like serine protease (Q7Z163)	29 kDa	*Dermatophagoides pteronyssinus*	YPTIYSNVANLR	0.00100	1
B. licheniformis 1	17 kDa surface antigen (NC014117.1)	17 kDa	*Burkholderia spp.*	MGTVDSVR	0.05500	1
B. licheniformis 1	Spore coat protein E (A0RHE2)	20 kDa	*Bacillus thuringiensis*	VNYTDEVSIGYR	0.00095	1
B. licheniformis 2	Der p 1 allergen (Q3HWZ5)	34 kDa	*Dermatophagoides pteronyssinus*	QIEYIQHNGVVQESYYR	0.00024	1
B. licheniformis 2	Der p 2 allergen (B0FRE2)	15 kDa	*Dermatophagoides pteronyssinus*	SENVVVTVK	0.05600	1
S. aureus	Der p 1 allergen (Q3HWZ5)	34 kDa	*Dermatophagoides pteronyssinus*	GIEYIQHNGVVQESYYR	0.016	1
S. aureus	Putative uncharacterised protein (Q72X58)	16 kDa	*Bacillus cereus*	GSSLGTQSYTGIIEAAGR QATVVMTYER	5.1e-005 0.00027	1

*****Denotes the score used to determine if a positive hit is above the significance threshold which is generally $p < 0.05$, where p is the probability that the positive hit occurred by chance. The rank "1" was assigned to a peptide that gave rise to one of the fifteen most intense peaks based on calculations performed with Mascot.